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Characterization of Proteins Associated with the LFA-1 Mediated Cytoskeletal/Signaling Complex in Locomotory T Lymphocytes

A Thesis Submitted for the Degree of
Doctor of Philosophy (Ph.D.)

by

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January 2004
Declaration

I declare that this thesis is my own work, and has not been submitted previously for a Ph.D. degree at this or any other University. I agree that the library may lend or copy this thesis on request.

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Basma Salah
I dedicate this thesis to my Mother Salwa, my late father
My husband Sherif
And sons Ahmed and Mohamed
Summary

LFA-1 plays a crucial role in all aspects of T cell locomotion in both immune physiological functions and pathological conditions. T cell locomotion is associated with translocation of protein kinase C isoenzymes $\beta$ and $\delta$ to the MTOC and microtubules.

To identify key players in LFA-1 signaling in the locomotory T cells, the *in situ* immunoprecipitation technique was deployed in this study. This technique is based on single-ligand triggering of T cell locomotion via immobilized motility inducing antibodies to LFA-1, which mimics the effect of natural ligands.

A number of protein kinases (PKCe, PKC$\delta$, PKAI$a$) as well as key cytoskeletal components ($\alpha$ and $\gamma$-tubulin) and the motor protein dynein, all of which play critical roles in the T cell locomotion, have been studied in this work and were found to physically associate with the LFA-1 induced cytoskeletal/signaling complex. Moreover, a potential association between LFA-1 and members of the transcription factor NFkB (IxB$a$, P65) was also elucidated in this thesis.

Intracellular localization and function of a number of protein kinases and phosphatases has been recently shown to depend on the scaffolding protein CG-NAP. Expression of CG-NAP in peripheral blood lymphocytes and in the T lymphoma cell line HUT-78 was established in this thesis. CG-NAP localizes at the region of the MTOC and the Golgi complex. LFA-1-triggered T cell locomotion induces re-distribution of CG-NAP along the microtubules in the trailing cell extensions. Furthermore, CG-NAP physically associates with LFA-1 in a multi-molecular signaling complex which includes also tubulin and protein kinase C $\beta$ isoenzymes and $\delta$. The functional significance of CG-NAP for LFA-1 induced motility was studied using cell transfectants and microinjection techniques. Over expression of the C-terminal domain of CG-NAP impairs polarization and cell migration. Thus, CG-NAP may be critically required for the MTOC dependent cell functions such as active locomotory behavior. These findings highlight a new role for the scaffolding protein CG-NAP as a potential mediator of the LFA-1 induced T cell motility.
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By learning the language that cells use to speak to one another and to their internal "workers", we will be able to listen in on their conversations and, ideally, find ways to intervene when communications go awry and cause disease. We may yet reduce "body language" to a precise science (Scott and Pawson, Sci Am 282:72)

The body's adhesion molecules both stick cells together and transmit signals, playing a critical role in everything from wound healing to diseases such as multiple sclerosis. Integrins, a subset of these sticky molecules, are like fasteners that connect the cell's internal scaffolding to the protein matrix outside (Glue of life. Science 288:223).
CHAPTER I

GENERAL INTRODUCTION

1.1 Cell Migration

Cell migration is a fundamental feature of most physiological and pathological processes in the body including inflammation and cancer metastasis. Cell migration is vital for embryonic development, immune reaction, inflammatory response, wound repair, tissue regeneration, atherosclerosis, arthritis, osteoporosis, congenital brain defects, and tumor formation (Ford 1975, Mackay et al., 1992, Hourihan et al., 1993, Andrian and Mackay 2000, Lauffenburger and Horwitz 1996, Howe et al., 1998, Webb 2002, Bailly 2003). Furthermore, tumor invasion is commonly associated with increased cell migration (Kassis et al., 2001). Hence, considerable attention has enthralled scientists seeking to identify the molecular basis of cell migration in order to develop therapeutic strategies for controlling these pathological phenomena. Yet, this has proved to be challenging since cell migration requires the spatial and temporal integration and coordination of multiple cellular processes that occur in different locations in the cell.

1.1.1 T Cell Migration in Health and Disease

T cell migration is a complex process that requires continuous, coordinated receptor ligand interactions, assembly and disassembly of adhesion complexes (Webb et al., 2002). This includes whole cell locomotion, the regulation of the cell shape and extracellular attachment. The attraction of leukocytes particularly T cells to tissues is crucial in inflammatory phenomenon and host response to infection. As lymphocytes patrol the body for infectious or foreign agents (Fig. 1.1, Fig. 1.2), they circulate as non-adherent cells in the blood and lymph but migrate as adherent cells through tissues. Lymphocytes accumulate in lymphoid organs, cross endothelial and basement membrane barriers thus congregating at sites of infection or inflammation and adhere to cells bearing foreign antigen (Parrot and Wilkinson 1981).
Figure 1.1: Scanning electron micrograph of T cell attacking a cancer cell.
Adapted from "The lymphatic system", SigBio’s, Virtual Anatomy Textbook.
www.acm.uiuc.edu/sigbio/project
Two distinct adhesive modalities are required for leukocyte accumulation at inflammatory sites and lymphocyte homing. Tethering of rolling leukocytes to the vessel wall permitting the leukocyte to roll in the direction of flow, increasing the time a cell spends in a post-capillary venules thus enabling surveillance of endothelium for activating signals such as chemoattractants (Springer 1994). This is followed by firm adhesion allowing the arrest of the leukocyte in the postcapillary venules, with subsequent diapedesis. Lymphocytes possess displays of multiple surface receptors capable of mediating adhesion to a wide range of cell types and ECM (extracellular matrix) proteins. Since T cell locomotion plays a central role during cell migration to inflammatory organs, understanding how this process is regulated will be a key target in biomedical research.

1.1.2 T Cell Receptor Complex (TCR)
Physiological T cell activation requires appropriate recognition by the T cell antigen receptor (TCR)/CD3 complex (Marrack and Kappler 1987), T cells express on their surfaces multiple receptors essential for appropriate antigen recognition. The antigen-binding site is a heterodimer composed of α and β subunits. A subpopulation of T lymphocytes has an antigen-binding site made up of γ and δ subunits. The T-cell receptor is noncovalently coupled to the CD3 protein complex that is composed of γ, δ, ε, and two ζ subunits (Fig. 1.3). These proteins have a variety of functional activities involved in signal transduction including tyrosine kinase and phosphorylase activities. The second messenger activity, which occurs following stimulation of the T-cell antigen receptor, is important in T-cell activation.

1.1.3 Cell Attachment and Movement
Cells form contacts with each other and with the ECM around them. Adhesion holds tissues together and therefore is essential for survival, especially during eradication of infection, wound healing, blood clotting, and embryonic development. Prior to migration, leukocytes undergo polarization, with the formation of a lamellipodia at the leading edge and a uropod at the trailing edge; the resultant cell shape allows them to convert cytoskeletal forces into net cell-body displacement (Sanchez-Madrid and del Pozo 1999). Movement of the leukocyte is a multistep crawling type resembling amoeboid movement with extension of a protrusion at the leading edge, formation of
Figure 1.2: Immune surveillance by T cells patrolling the body visualized by electron microscopy. Note receptor-bearing microvilli on the surface of the T cells with a special focus on one cell on the left image (reproduced from Cinamon et al 2001).

Figure 1.3: Schematic representation of the T Cell Receptor Complex. From RobinsonE.D.www.ucihs.uci.edu/com/pathology/faculty/robinson_corenotes/TCell_Receptor_Complex.jpeg
stable attachments near the leading edge of the protrusion, translocation of the cell body forward, release of adhesion and retraction at the cell rear (Wilkinson 1986, Friedl and Brocker 2000, Lauffenburger and Horwitz 1996).

1.2 Cell Shape and T Cell Migration

T cell motility resembles amoeboid motility, which is characterized by a polarized yet simple shape allowing high speed, rapid directional oscillations, and low affinity interactions to the substrate (Friedl and Brocker 2000).

1.2.1 Polarization and T Cell Migration

T cell polarization involves the conversion from a spherical to an elongated morphology; during which important changes with reorganization of the cytoskeleton take place resulting in the polar redistribution of cell surface receptors and cytoskeletal elements. A dramatic reconfiguration of the tubulin cytoskeleton, and distribution of F-actin (filamentous actin) from a radial symmetry around the cell to its concentration at a particular region, resulting in the switch from a spherical to a polarized shape (Coates et al., 1992). The movement of polarized cell is accompanied by protrusions of the cytoplasm called pseudopodia and extension of the cytoplasm in the form of lamellipodia (extension of thin sheets of cytoplasm) and filopodia (thin and pointed protrusions). The protrusions are driven by the polymerization of actin and actin binding proteins and are stabilized through the formation of adhesive complexes. The microtubule-organizing center (MTOC) acquires a different orientation in the polarized migrating cells (Dustin et al., 1997, Ratner et al., 1997).

Cell polarity is innate to crawling lymphocytes prior to contact with antigen-presenting cell (APC) or target cells; it also plays a key role in immune cell-cell interactions. The polarity of T cells following contact with an APC has been well characterized on the basis of plasma membrane protein clustering, cytoskeletal and organellar reorganization, and cytokine secretion (Kupfer and Singer 1989). Crawling T cells recognize and bind APCs through their leading edge showing an enhanced sensitivity to antigen in this area (Negulescu et al., 1996). T cell polarization accompanies multiple cell-cell interactions, such as antigen presentation, target-cell recognition, or binding to endothelial cells (Campanero et al., 1993, Kupfer et al., 1994, del Pozo et al., 1995, Helander et al., 1997).
Polarization also involves protein cycling either to the leading edge, such as chemokine receptors, and focal adhesion kinase, or to a central polarizing compartment, such as MTOC (microtubule organizing centre) and protein kinases, or into the uropod, i.e. ICAM (intercellular adhesion molecule), and other integrin receptors. The function of such compartment formation may be important for chemotactic response, scanning of encountered cells, and also for flexible and adaptive interaction with the ECM.

1.2.2 T Cell Locomotion

The process of T lymphocyte rolling and locomotion proceeds as follows: lymphocytes make initial contacts with the endothelial cells by tethering and rolling along the vessel wall in the post capillary high endothelial venules of lymphoid or inflamed tissues. Firm adhesions to the vessel wall are achieved through binding of activated LFA-1 on the lymphocyte and its ligand ICAM expressed on the endothelial cells at inflammatory sites, ultimately leading to cell spreading, polarization with consequent lymphocyte migration and extravasation (Fig. 1.4) (Springer 1994). The interaction of the integrins with the extracellular matrix stabilizes the adhesions by recruiting signaling and cytoskeletal proteins (Yamada and Miyamoto 1995, Burridge and Chrzanowska-Wodniaka 1996). In a moving cell, actin is distributed at the trailing edge stretching from the tail of the cell to the front; at the front there are stress fibres, which are contractile bundles of actin (Becker et al., 1996).

A complex system of signal transduction molecules, including tyrosine kinases, lipid kinases, second messengers and members of the Rho family of small GTPases (guanosine triphosphatases) is thought to regulate the cytoskeletal rearrangements underlying leukocyte polarization and migration. New sites of attachment must be formed at the front of the cell, and contacts at the rear must be broken. Attachment sites between the cell and the substrate are complex involving interaction of proteins in the plasma membrane to the proteins on both the outside and the inside of the cell, this bidirectional interaction is fulfilled primarily by integrins (Becker et al., 1996).

Scientists from the University of California labelled T cells with fluorescent dye to study their motile behaviour in intact lymph nodes. After labelling the cells, they injected them back into mice followed by isolation and analysing the cells using three
Figure 1.4: T cell migration. Cell migration is regulated by a combination of different processes: forces generated by contraction of actin and myosin, G protein signaling, and microtubule dynamics. Migration is initiated by polymerization of an actin network at the cell’s leading edge. The growth of new focal complexes is determined by a Rho-dependent process. Contractile forces behind the leading edge drive movement of the cell body. The turnover of adhesive complexes is regulated by the combined activity of microtubules and regulators that reside in these complexes. The forces on the substratum and cell body are shown by solid and dotted arrows respectively. In motile cells, traction on the substrate results in net forward movement. From Horwitz and Parsons 1999.
dimensional time lapse, two photon imaging (Miller et al., 2002). T cells were found to move in short bursts, stopping and starting repeatedly, pausing frequently to make contacts with other cells. T cells in this experiment were examined in a steady state in absence of antigenic stimuli. The average velocity of T cells in steady state was 0.8 \( \mu \text{m/min} \) with a top speed of more than 25 \( \mu \text{m/min} \).

1.3 Co-ordination of Adhesion and Adhesion Complexes

As mentioned earlier, lymphocytes communicate with each other and with other cells of the immune system via cell surface receptors known as the adhesion molecules. Adhesion molecules are key players in cell/cell communication, migration, locomotion and recirculation. Adherent cells are subjected to tension forces during spreading. The mediators of tension are the integrin family of transmembrane receptors that link the extracellular matrix components on the outside of the cell with the cell’s intracellular cytoskeleton in complexes called adhesion complexes (Lauffenburger and Horwitz 1996, Burridge et al., 1997). Cell adhesion complexes contain multiple cytoskeletal and signal transduction molecules that bind to other proteins and control a number of essential functions in the cell including cell motility. Adhesive complexes are regions of the plasma membrane where integrin receptors, actin filaments, and associated proteins cluster together (Borisy and Svitkina 2000, Horwitz and Parsons 1999). Cross talk between these complexes plays a central role in lymphocyte migration. As the cells migrate, adhesive complexes at the front of the cell grow and strengthen into larger, more organized adhesive complexes that serve as points of traction over which the body of the cell moves. Following this, de-adhesions at the rear result in a net displacement of the cell (Horwitz and Parsons 1999).

1.3.1 The Integrins

Adhesion is vital in antigen-dependent functions associated with activation of the TCR, migration through the endothelium and extracellular matrix. This is fulfilled by specialized adhesion receptors of the integrin family. Integrins are major cell surface receptors that bind to various extracellular matrix proteins and to other components on the cell surface (Albelda and Buck 1990). Integrins play a crucial role in lymphocyte migration, homing and circulation (Salmi and Jalkanen 1997, Girard and Springer 1995). These adhesion molecules are expressed on the lymphocytes as well as the
endothelial cells (Dransfield and Hogg 1989), and act as crucial signal transducers between the cell exterior and the subcellar cytoskeletal scaffold (Clark and Brugge 1995). The name integrin was essentially given to denote its role as an integral membrane complex involved in the transmembrane association between the extracellular matrix and the cytoskeleton (Tamkun et al., 1986).

1.3.2 Integrin Structure
Integrins are a family of large heterodimeric, transmembrane glycoproteins that are formed by the noncovalent association of various α and β subunits (Fig. 1.5, Fig. 1.6, Table 1.1), expressed in wide range of cells and can be subdivided into broad groups based on their α and β subunits (Hynes 1992, Ruoslahti and Pierschbacher 1987). The α subunits are 25-65% identical in amino acid sequence; the β subunits are 37-45% identical (De Franco 1992). This unique structural feature of integrins enables them to serve as highly dynamic receptors capable of upregulation upon activation and bidirectional signal transduction between the cell interior and the extracellular ECM components.

The cytoplasmic domains of the integrin α subunits are diverse in both length and amino acid composition. The only generally conserved feature within the α subunit cytoplasmic domains is the KXGFFKR motif, which immediately follows the membrane-spanning domain (Dedhar 1990, Williams et al., 1994). The N terminus of all integrin α subunits including αL possess ligand-binding sites and is composed of seven 60-aa repeats predicted to fold into a β-propeller structure (Springer 1997).

1.3.3 Integrin Signaling and Cell Functions
Signaling denotes targeted involvement of participating molecules in regulation of specific intracellular processes such as cytoskeletal assembly. Most of the intracellular signaling processes are largely based on integrin signaling. Both integrin signaling and assembly of the cytoskeleton are intimately linked. As integrins bind to ECM, they become clustered in the plane of the cell membrane and associate with cytoskeletal and signaling complexes that promotes the assembly of actin filaments. The reorganization of actin filaments into larger stress fibers, in turn, causes more integrin clustering, thus enhancing matrix binding and organization by integrins in a positive feedback system.
**Figure 1.5:** The integrin family. Adapted from Kunicki 1989.

**Figure 1.6:** Schematic diagram of integrin receptor. A globular head projects more than 20nm from the lipid bilayer. By binding to a matrix protein outside the cell and the actin cytoskeleton (via talin and α-actinin) inside the cell, the protein serves as a transmembrane linker. The α and β chains are both glycosylated (not shown) and are held together by noncovalent bonds. In the receptor shown, α-chain is made initially as a single 140,000-dalton polypeptide chain, which is cleaved into one small transmembrane chain and one large extracellular chain that remain held together by disulfide bond (S-S), this extracellular chain is folded into 4 divalent cation binding domains. The extracellular part of the beta chain contains a repeating cysteine rich region, where intrachain disulfide bonding occurs; β chain has a mass of 100,000 daltons. Adapted from www.ntri.tamuk.edu/homepage-ntri/lectures/protein/junction1.htm
<table>
<thead>
<tr>
<th>Molecule</th>
<th>Other Names</th>
<th>Ligand</th>
<th>Lymphocyte expression</th>
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<tr>
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<tr>
<td>α1β1</td>
<td>CD49a/VLA-1</td>
<td>Collagen, laminin</td>
<td>Activated T cells</td>
</tr>
<tr>
<td>α2β1</td>
<td>CD49b/VLA-2</td>
<td>Collagen</td>
<td>Activated T cells</td>
</tr>
<tr>
<td>α3β1</td>
<td>CD49c/VLA-3</td>
<td>Collagen, laminin, fibronectin</td>
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</tr>
<tr>
<td>α4β1</td>
<td>CD49d/VLA-4</td>
<td>VCAM-1, fibronectin</td>
<td>Resting, activated T and B cells</td>
</tr>
<tr>
<td>α5β1</td>
<td>CD49e/VLA-5</td>
<td>Fibronectin</td>
<td>Activated T cells</td>
</tr>
<tr>
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<td>CD49f/VLA-6</td>
<td>Laminin</td>
<td>Activated T cells</td>
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<tr>
<td><strong>β2 integrins (β chain=CD18)</strong></td>
<td></td>
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<tr>
<td>αLβ2</td>
<td>CD11a/LFA-1</td>
<td>ICAM-1,2,3,..</td>
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</tr>
<tr>
<td>αMβ2</td>
<td>CD11b/MAC-1</td>
<td>C3bi/fibrinogen, ICAM-1</td>
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<tr>
<td>αxβ2</td>
<td>CD11c/p150-95</td>
<td>C3bi/fibrinogen</td>
<td>Activated T cells, B cells</td>
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<td>CD41/GPlIb</td>
<td>Fibrinogen, fibronectin, vitronectin, Von Willebrand’s factor</td>
<td>Not T or B cells</td>
</tr>
<tr>
<td>αxβ3</td>
<td></td>
<td>Vitronectin</td>
<td>Not T or B cells</td>
</tr>
<tr>
<td><strong>B7 integrins (β7 human, βp mouse)</strong></td>
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<tr>
<td>A4β7,α4βp</td>
<td>VCAM-1(Peyer’s patch)</td>
<td>T cells, B cells</td>
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</table>
Integrins transduce signals inside cells on binding to their ligands. In return, cytoskeletal and biochemical events within the cell can modify integrin functions (Coppolino et al., 1995, Smilenov et al., 1999, Burridge et al., 1997). The cytoplasmic domains of integrins are required for bidirectional transduction of information across the plasma membrane, interacting directly with different cytoskeletal and signaling molecules (Fig. 1.9).

Integrins deliver 'outside-in' and 'inside-out' signals (1.4.4, 1.4.5) that control important physiological functions and pathological processes (Fig. 1.10) including survival, gene induction, cell adhesion, migration, cell proliferation, differentiation, inflammatory disorders, atherosclerosis and metastasis (Clarke and Brugge 1995, Miyamoto et al., 1995). The adhesive properties of integrins can be controlled in multiple ways either by altering the activation state of the integrin, through conformational change or receptor clustering, all of which are regulated by intracellular proteins (Fernandez et al., 1998).

1.3.4 Aberrant Integrin Signaling
Aberrant cell adhesion has been implicated in the pathogenesis of several diseases, including inflammatory disorders such as rheumatoid arthritis (RA), inflammatory bowel disease (IBD), asthma, coronary heart disease as well as cancer (Curley et al., 1999).

1.3.5 Role of Integrins in Gene Expression
Integrins not only mediate numerous cell-cell and cell-matrix interactions, but are involved in biological processes such as tissue morphogenesis (growth of all anchorage dependent cells), gene expression, leukocyte recirculation and migration, wound healing, blood clotting, platelet activation and immune response (Akiyama 1996, Lauffenburger and Horwitz 1996). Adhesion of cells to the ECM result in the expression of specific genes (Dike and Farmer 1988). In addition, expression of collagen genes was found to be sensitive to adhesion by integrins (Werb et al., 1996). In certain cells such as monocytes, cell contact with fibronectin triggers the immediate expression of specific genes including interleukin-1 β and NFκB suppressor gene (Yurochko et al., 1992). In some studies adhesion or inhibition of adhesion by antibodies was shown to affect gene expression, for example: alkaline phosphatase gene...
Figure 1.7: Diagram of integrin clustering. Adapted from Giancotti and Ruoslahti 1999.

Figure 1.8: Schematic diagram showing integrin clustering in response to activation. Picture by Cress AE, Arizona cancer center.
www.ahsc.arizona.edu
**Figure 1.9:** Integrins signaling pathways. Reproduced from Clarke and Brugge 1995.

**Figure 1.10:** Signal transduction pathways emanating from integrins (blue) regulate numerous cellular processes, including actin organization (orange) and adhesion complex remodeling (green), which together influence cell movement (red), as well as gene expression and cell cycle regulation (purple) and cell survival (gray). From Martin et al 2002.

1.3.6 Intracellular Transport of Integrins
Several studies showed evidence that integrin containing vesicles can move from the rear of the cell to the perinuclear region (Laukaitis et al., 2001, Palecek et al., 1996). In addition, integrins containing complexes move from the perinuclear region to the base of the lamellipodium in some cells (Laukaitis et al., 2001).

1.4 LFA-1, from Structure to Function

1.4.1 Structural Features of LFA-1
One of the major integrins expressed on the T cell surface is LFA-1 (Stewart et al., 1998). LFA-1 (CD11a/CD18) is a member of the β2 group of integrins (αβ2), which includes also MAC-1 (CD11B/CD18) and p150, 95. All are exclusively expressed by leukocytes (Lub et al., 1995). LFA-1 is a transmembrane heterodimer composed of a unique α (α/CD11a) and β2 subunit (CD18) that is common to a subset of leukocyte integrins. Both subunits are composed of a large extracellular amino-terminal domain for the recognition of ligands, a transmembrane domain, and a small cytoplasmic carboxyl-terminal domain, which binds cytoskeletal and signaling components (Harris et al., 2000, Rodriguez-Femandez et al., 2001). LFA-1 is expressed on the cell surface of most leukocytes, plays a crucial immunoregulatory role by mediating adhesion between leukocytes, leukocytes and ECM, leukocytes and other cells (Springer et al., 1987, Martz 1987). The α chain genes are clustered on chromosome 16 while the β chain CD18 is on chromosome 21 (Marlin et al., 1986). LFA-1 is not constitutively adhesive, but become able to engage to its ligand after stimuli received through other cell membrane receptors or activators (Brown and Hogg 1996). Dynamic regulation of ligand-binding activity by αβ2 of LFA-1 in response to signals transmitted from inside the cell (inside-out signaling) activates LFA-1 adhesiveness in response to engagement of the antigen receptor on T lymphocytes in immune responses, and in response to chemoattractant binding to G-protein-coupled receptors in leukocyte adhesion to endothelium (Lawrence and Springer 1991, Von Andrian et al., 1991, Constantin et al., 2000).
1.4.2 Importance of LFA-1 in Immune/Inflammatory Responses

LFA-1 is essential for normal immune responses, T cell activation and effector functions (Shier et al., 1999, Lu et al., 1997). LAD-1 (leukocyte adhesion deficiency-1), a rare autosomal-recessive immunodeficiency was discovered in 1979 characterized by recurring life-threatening bacterial infections and persistent leukocytosis (Hogg and Bates 2000, Bunting et al., 2002). The genetic basis for LAD-1 lies in germline mutations in the gene encoding the β2 integrin subunit impairing cell-surface expression of LFA-1. Furthermore, LFA-1 is involved in lymphocyte recirculation and leukocyte extravasation (Fig. 1.11) to sites of inflammation (Carlos and Harlan 1994). LFA-1 is also important for effective T-cell activation by antigen-presenting cells upon ICAM-1 binding; in addition, LFA-1 provides a potent costimulatory signal for TCR-activated T cells (Van Seventer et al., 1990).

1.4.3 Role of Phosphorylation and LFA-1 Activation

Protein phosphorylation is vitally important in LFA-1 activation. PMA (phorbol 12-myristate 13-acetate) mediated activation of PKC (Protein kinase C) enhances LFA-1 adhesion. PMA is a potent activator of the intracellular phosphorylation enzymes of the PKC family. PMA treatment of lymphocytes was found to be important for the development of motile phenotype in PBTLs (Kelleher et al., 1995, Thorp et al., 1996, Volkov et al., 1998, Pyszniak et al., 1997) and represents an essential step in LFA-1 mediated T cell lymphocyte adhesion. In addition, using the serine/threonine phosphatase inhibitor okadaic acid, strong threonine phosphorylation of CD18 was found after stimulation with PMA (Valmu and Gahmberg 1995).

1.4.4 Inside-Out Signaling

Dynamic regulation of ligand-binding activity by LFA-1 and other integrins in response to signals transmitted from inside the cell, i.e. inside-out signaling activates β2 integrin adhesiveness in response to engagement of the antigen receptor on T lymphocytes in immune, inflammatory responses, and in response to chemoattractant binding to G-protein-coupled receptors in leukocyte adhesion to endothelium (Lawrence and Springer 1991, Constantin et al., 2000). Intracellular signaling via LFA-1 is initiated only after activation of lymphocyte through PMA, T cell receptor TCR/CD3 complex, G proteins or protein tyrosine kinases PTKs (Dustin and Springer 1989, Schwartz and Ginsberg
Figure 1.11: LFA-1 mediated T cell locomotion in immune responses: A) T cells adhere to the high endothelial venule, B) L-selectin binds to its ligand GlyCAM, C) LFA-1 is activated by chemokines bound to the ECM, D) Activated LFA-1 binds tightly to ICAM, E) T cells leaves the blood and enters the lymph node. Reproduced from www.montana.edu/wwwami/523/Reading6.htm
The binding of ligand by LFA-1 is activated through inside-out signaling (Van Kooyk and Figdor 2000, Schwartz and Ginsberg 2002). This "inside-out" mechanism of LFA-1 activation brings about a change in the conformation of the extracellular region of LFA-1, which is functionally reflected by the ability of the cells to adhere to LFA-1 ligands.

Chemokines and cytokines lead to the generation of second messengers with subsequent regulation of LFA-1. This inside-out signaling to LFA-1 is an essential effector response. LFA-1 mediates a tyrosine kinase-dependent activation of phospholipase Cγ1 (Kanner et al., 1993) with consequent activation of protein kinase C, which results in phosphorylation of target proteins. Activation of PKC leads to increase in intracellular calcium levels (Ca^{2+}). Calcium is an important signal in T cell activation, influx of extracellular Ca^{2+} in turn leads to LFA-1 activation (Van Kooyk et al., 1993, Hermanowski-Vosatka et al., 1992). Pharmacological agents such as calcium ionophores can mimic the TCR and induce T cell activation and IL-2 gene expression (Berry et al., 1989).

**1.4.5 Outside-In Signaling**

LFA-1 mediates bidirectional signal transduction across the plasma membrane. Subsequent binding to ligands on the cell surface results in signal transduction to the cytoplasm, i.e. outside in signaling (Diamond and Springer 1994, Hughes and Pfaff 1998, Schwartz et al., 1995). Thus, activation of LFA-1 molecules can also be induced directly from outside the cells by altering the extracellular divalent cation conditions, i.e. by addition of micromolar concentrations of Mn^{2+} or by the presence of millimolar levels of Mg^{2+} when Ca^{2+} is removed with EGTA bypassing the requirement for intracellular signaling (Dransfield et al., 1992, Stewart et al., 1996, Tominaga et al., 1998). Direct activation of LFA-1 from outside the cells can also be achieved with specific activating mAbs (monoclonal antibodies). Crosslinking of LFA-1 at the cell surface by these antibodies triggers intracellular signaling (Kanner et al., 1993, Arroyo et al., 1994). These types of antibodies recognize and bind to the α/β subunits of LFA-1, altering the conformation of LFA-1 to a state of increased affinity for ICAM (van Kooyk et al., 1991, Stephens et al., 1995, Ortlepp et al., 1995). Phorbol esters such as phorbol-12, 13-dibutyrate (PDBu), have also been used extensively as another means of inducing LFA-1-mediated adhesion to ICAM (Rothlein and Springer 1986, Stewart et al., 1996).
In addition, stimulation of lymphocytes leads to clustering of LFA-1 providing stronger adhesion at the sites of cell-cell contact. Clustering also provides the means of localizing activated LFA-1, thereby increasing the collective signal necessary to interact with the underlying cytoskeleton. The ins and out of LFA-1 cross talk shows that the activation of LFA-1 on a lymphocyte membrane has impact on the activity of other types of integrin and possibly other classes of receptors.

1.4.6 Affinity and Avidity of LFA-1

LFA-1 requires activation by high concentrations of divalent cations, engagement of the TCR and chemokine receptors to bind to its major counter-receptor ICAM (Dustin and Springer 1989, Campbell et al., 1998). Divalent cations such as Mg\(^{2+}\) regulate ligand interactions through selective binding to several sites on integrins; Mg\(^{2+}\) directly associates with the ligand-binding site and control access to a cryptic binding site through altering the conformation of the integrin (Dransfield and Hogg 1989, Tominaga et al., 1998). Although Mg\(^{2+}\) is directly involved in the affinity of LFA-1 for its ligand, Ca\(^{2+}\) correlates with avidity regulation of LFA-1 by clustering LFA-1 molecules at the cell surface of T cells, thus facilitating LFA-1-ligand interaction (Van Kooyk et al., 1994). Ca\(^{2+}\) mobilizing agents induce an increase in adhesion to the LFA-1 ligand ICAM-1 with the activation and redistribution of PYK2 (the proline-rich tyrosine kinase-2) to the MTOC in T cells (Rodriguez-Fernandez et al., 2002). Ca\(^{2+}\) flux is directly responsible for LFA-1 clustering with ligand binding a secondary event. Other reports showed that Ca\(^{2+}\) inhibits Mg\(^{2+}\)-induced T cell adhesion by inhibiting the expression of the Mg\(^{2+}\)-induced 24 epitope on LFA-1 (Stewart et al., 1996). Increase in cytoplasmic Ca\(^{2+}\) accompany triggering via the TCR, and acts as an essential component of other adhesion-inducing mechanisms. T cell stimulation by Ca\(^{2+}\) mobilizers via the TCR receptor or phorbol ester increase integrin avidity by clustering LFA-1 over the entire cell membrane. In addition, Ca\(^{2+}\) activates kinases and mediators such as PKC, Calmodulin, calreticulin, myosin light chain and calpain (Stewart et al., 1998). Moreover, several lines of evidence point to a direct link between cytoskeletal structure and intracellular Ca\(^{2+}\) levels. Treatment of cells with Cytochalasins that disrupt the actin cytoskeleton can lead to increase in intracellular Ca\(^{2+}\) (Janmey 1998). On the other hand, some aspects of IP\(_3\)-dependent Ca\(^{2+}\) release require an intact actin and microtubule system (Hajnoczky et al., 1994). Phorbol-ester induced lymphocyte adhesion to its ligand is Ca\(^{2+}\) dependent (Rothlein and Springer 1986, Stewart et al.,
1996) and involves activation of calpain (Stewart et al., 1998). Calpain is highly expressed in T cells and increased during LFA-1 adhesion by phorbol esters, Ca^{2+} ionophore and anti-CD3 treatment (Deshpande et al., 1995).

1.5 LFA-1 Mediated T Cell Locomotion

LFA-1 plays an important role in lymphocyte trafficking and locomotion (Hauzenberger et al., 1997). LFA-1 induced T cell activation results in a dramatic cell polarization resulting in cell migration.

1.5.1 LFA-1 Mediated Adhesion and The Cytoskeleton

The interaction of LFA-1 with the cytoskeleton is dynamic, depending on both LFA-1 signaling and the state of the cytoskeletal organization within the cell. LFA-1 association with cytoskeletal structures is brought about by both integrin cross-linking and ligand binding (Porter et al., 2002). Cytoskeletal rearrangements that are typical of actively migrating cells are mainly based on coordinated networks of intracellular signaling. The microtubular cytoskeleton plays an important role in the LFA-1 mediated adhesion (Rodriguez-Fernandez et al., 2002). In a polarized activated T cell, the MTOC localizes at a specific cytoplasmic location, in the region between the cell body and the long cellular extension, which is characterized by strong anchorage to the substrate ICAM-1 (Rodriguez-Fernandez et al., 1999). Activation of T cells induces tyrosine phosphorylation of α-tubulin (Marie-Cardine et al., 1995, Ley et al., 1994).

1.5.2 LFA-1 cytoplasmic domain

The cytoplasmic domain of LFA-1 is essential for the control of its function, it is required for the transduction of the bidirectional information across the plasma membrane, interacting directly with different cytoskeletal and signaling molecules. The β subunit of LFA-1 has short cytoplasmic tail with no catalytic domain. Therefore, ligand induced interactions at the cell surface must organize cytoskeletal complexes that serve as a frameworks for association of proteins in the complex. Candidate proteins include adaptor or scaffolding proteins.

1.5.3 LFA-1/Cytoskeletal Interactions
The cytoskeleton is intimately involved in the adhesion process. The cytoskeletal filaments comprise linear elements that span the cell and its network of signaling pathways are closely linked and directly respond to immediate conditions of the cell (Hollenbeck 2001). After activation, LFA-1 associates with multiple cytoskeletal elements (Pardi et al., 1992). LFA-1 is linked indirectly to actin filaments via adaptor proteins and several actin-binding proteins, including α-actinin, talin, and filamin (Pavalko and LaRoche 1993, Sharma et al., 1995). In resting T cells, LFA-1 is constitutively linked to the actin cytoskeleton via the protein talin (Fig. 1.12). When T cells become activated talin becomes dissociated from the β2 tail. This phase is transient followed by reattachment of actin filaments via another protein α-actinin. Association of α-actinin with LFA-1 helps to stabilize the cytoskeleton and promote firm adhesion and migration across the endothelium (Sampath et al., 1998). Mutagenesis experiments revealed that the affinity of α-actinin binding to the β2 tail is regulated by a change in the conformation of the tail that unmasks a cryptic α-actinin binding domain. Deletion of the β subunit tail prevents association of integrins with the cytoskeleton and disrupts normal integrin-ligand interactions (Williams et al., 1994).

The actin cytoskeleton influences several aspects of leukocyte adhesion. For example, it is involved in the shape changes of the adhering and migrating cell strengthening the contact zone between the T lymphocyte and its target. Inhibiting actin assembly prevents LFA-1 clustering and T cell adhesion (Bubb et al., 1994), although low levels of cytochalasin D promote integrin-mediated adhesion and allows movement of LFA-1 in the membrane (Lub et al., 1997, Yauch et al 1997, Kucik et al., 1996). Recent results showed that when T cells are activated, LFA-1 can direct the remodeling of the F-actin cytoskeleton, and this remodeling is essential for the LFA-1-mediated adhesion of T lymphocytes to ligand ICAM-1 (Porter et al., 2002). The activity of LFA-1 is directly influenced by the state of the actin cytoskeleton and, in turn, LFA-1 can signal into the T lymphocyte and reorganize the cytoskeleton. An initial instant interaction of clustered high affinity LFA-1 on T cell with multivalent ligand on the target cell, triggers actin reorganization and enhanced cell adhesion. Thus, LFA-1 acts not only as an adhesion receptor for T lymphocytes, but also as a signaling receptor by initiating actin reorganization (Leitinger and Hogg 2000, Porter et al., 2002).
1.5.4 LFA-1/JAB-1 Association: Role in Signal Transduction and Nucleocytoplasmic Trafficking

Trafficking of signaling molecules between the cytoplasmic and nuclear compartments has chief implications for the degree and specificity of gene expression. Cytoplasmic domains of LFA-1 are essential in the establishment of organized association with the cytoskeleton, hence, important for signal transduction. Since cytoplasmic domains of integrins have no catalytic activity, molecules attached to them are crucial for signal transduction. Bianchi et al. (2000) using the β2 cytoplasmic domain of LFA-1 as bait in yeast two hybrid screen, were able to characterize an important component, JAB1 (Jun activation domain-binding protein 1). JAB1 is also a known coactivator of the c-Jun transcription factor (c-Jun), and localizes both in the nucleus and at the cell membrane of T cells. A fraction of JAB1 colocalized with LFA-1 at the cell membrane. LFA-1 engagement is accompanied by an increase in the nuclear pool of JAB1, paralleled by enhanced binding of c-Jun-containing AP-1 complexes and enhanced activation of an AP-1-driven promoter. Crosslinking of LFA-1 induced a rapid increase in endogenous JAB1 in the nuclear fraction, as compared with unstimulated T lymphocytes. These studies demonstrate that signaling through the LFA-1 could control c-Jun-driven transcription by regulating JAB1 nuclear localization; JAB1 seems to directly convey information from ligated LFA-1 altering gene expression (Fig. 1.13), which is a novel pathway for integrin-dependent modulation of gene expression (Bianchi et al., 2000).

1.6 LFA-1/ICAM-1 Interactions

1.6.1 ICAM-1

LFA-1 mediates important adhesive phenomena via interaction with its ligands (Fig. 1.14) from the intercellular adhesion molecule (ICAM) family, ICAM-1 (CD54), ICAM-2 (CD102), and ICAM-3 (CD50) (Springer 1990, Springer 1994, Gahmberg 1997). ICAM-1 (CD54) is a ligand for at least two members of the β2 family of leukocyte integrins, LFA-1 (αLβ2) and Mac-1 (αMβ2). ICAM-1 is a cell-surface membrane glycoprotein protein, member of the immunoglobulin superfamily (the immunoglobulin supergene family which consists of at least 15 molecules responsible for mediating adhesion). Members of this family have a common structural feature represented by the presence of the immunoglobulin domain, composed of 90-100 amino acids arranged in a sandwich of two sheets of antiparallel β strands, stabilized by
Figure 1.12: A Model showing the role of talin and α-actinin interactions with LFA-1 in resting and activated lymphocytes (From Sampath et al 1998).

Figure 1.13: Regulation of nucleocytoplasmic trafficking of signaling molecules linked to β2 integrin cytoplasmic domains. β2 acts as a cytoplasmic anchor for JAB-1; JAB-1 interacts with c-Jun containing AP-1 complexes, and enhances Transactivation from AP-1 dependent promoters.
disulphide bonds. ICAM-1 has five Ig extracellular domains, a hydrophobic transmembrane domain, and a short cytoplasmic domain (Simmons et al., 1988, Staunton et al., 1988). The LFA-1 binding site is located in domain 1 of ICAM-1 (Staunton et al., 1990). ICAM-1 is expressed at low levels on many cell types including leukocytes, epithelial and endothelial cells. On exposure to cytokines, endotoxins/lipopolysaccharide, or phorbol esters, ICAM-1 levels rise (Springer 1990, Gahmberg et al., 1997).

ICAM-1 is important for cell-cell interactions (Dougherty et al., 1988, Altmann et al., 1989, Xu et al., 1994), leukocyte extravasation (Smith et al., 1989, Diamond et al 1991), and lymphocyte-mediated cytotoxicity (Makgoba et al., 1988, Mentzer et al., 1988). In addition, ICAM-1 promotes a wide range of cellular interactions important in inflammation (Springer 1995, Iigo et al., 1991).

1.6.2 Importance of LFA-1/ICAM Interactions

LFA-1/ICAM interactions are crucial for lymphocyte homing (Springer 1994), extravasation and strengthening the complex between T cell antigen receptor and major histocompatibility molecules (Dustin and Cooper 2000, Dustin and Springer 1989, Van Kooyk et al., 1989). ICAM-1/LFA-1 interaction includes adhesion of leukocytes to the endothelium, followed by their extravasation at sites of inflammation, costimulatory signaling for T cell activation, and adherence of killer T cells to target cells (Springer 1990). Interaction between LFA-1 and ICAM-1 has a pleiotropic effect, as it not only plays an important role in T cell recirculation but also in T cell activation (Springer 1994, Sligh et al., 1993). In addition, LFA-1/ICAM mediated adhesion is crucially important for T cell in inflammatory and immune responses (Staunton et al., 1990, Oppenheimer-Marks and Lipsky 1996, McMurray 1996). LFA-1/ICAM-1 association is essential for cell spreading (Pyszniak et al., 1997). Adhesion necessary for T cell function is abrogated by the use of antibodies to LFA-1 and ICAM-1. Also, T cell stimulation is inhibited by antibodies to LFA-1 and ICAM-1 (Rothlein and Springer 1986). LFA-1 up regulates ICAM-1 on monocytes and serves as signaling partner for other leukocyte receptors (Yamada et al., 1997, Todd and Petty 1997). LFA-1 and ICAM-1 can interact at 40 nm and can thereby initiate adhesive interactions when appropriately activated. Cytoskeletal interactions with activated LFA-1 triggers expansion of close contact areas. LFA-1 clusters are also transported in a directed fashion resulting in movement of the T cell over the substrate. Whereas, areas adjacent
to LFA-1 clusters are forced into close contact with the apposing membrane. Blocking ICAM-1/LFA-1 interaction can suppress T-cell activation in autoimmune diseases and organ transplantation (Yusuf-Makagiansar et al., 2002, Dedrick et al., 2003).

1.6.3 The I Domain in LFA-1/ICAM Interactions

Multiple studies indicate that under physiological conditions, the ligand binding activity of LFA-1 correlates mostly with the organization of this integrin into clusters on the membrane of T cells and not with conformational changes of individual integrin molecules, which are characterized by increased affinity for ligands (Van Kooyk et al., 1999, Van Kooyk and Figdor 2000). Mutagenesis, epitope mapping and structural studies suggest that the ICAM-1 binding site is located on the upper face of the I domain (Fig. 1.15) (Emsley et al., 1998, Huth et al., 2000) approximately 200 residues located in the amino-terminal region α₄ subunit of LFA-1 (Gahmberg et al., 1997, Diamond and Springer 1993). The I domain of about 200 amino acids is inserted between β-sheets 2 and 3 of the β-propeller domain. This is sufficient for maximal ligand binding affinity and adhesiveness (Lu et al., 2001, Shimaoka et al., 2001). Using three-dimensional crystal structures of the I domain, a high similarity to that of small G proteins was detected (Lee et al., 1995, Emsley et al., 2000). The I domain is essential in controlling integrin activation (Leitinger and Hogg 2000). Removal of the ligand binding I domain from LFA-1 creates an integrin with the hallmarks of a constitutively active receptor. The I domain has been expressed on the cell surface of T cells in isolation from other integrin domains, it also supports rolling on immobilized ICAM-1 under shear flow (Knorr and Dustin 1997, Champe et al., 1995, Huth et al., 2000) and cooperate with other ligand-binding domains for firm adhesion. The I domain has been shown to directly mediate conformation- and cation-dependent ICAM-1-binding through its metal ion-dependent adhesion site.

1.7 Protein Kinases and LFA-1 Signaling in T Cells

The adhesive properties of T cell integrins are regulated by intracellular proteins (Fernandez et al., 1998). LFA-1 cross-linking induces the formation and the recruitment of a stable multi-component protein complex involving LFA-1, cytoskeletal and signaling proteins (Volkov et al., 2001). One of the major kinases studied that play a role in the regulation of the LFA-1-mediated T cell migration is the PKC family of
Figure 1.14: LFA-1 on the T cell interacting with its ligand ICAM on the APC or endothelial cell (Taubes 1999).

Figure 1.15: Structure of β2 integrin I domain. The αβ heterodimeric structure is common to all integrins. The α chain includes seven extracellular N-terminal homologous repeats organized into a β propeller structure. The α chain I domain is shown in red with the embedded MIDAS motif in orange, and the β chain I-like domain with MIDAS motif is shown in corresponding fashion. The GFFKR sequence (dark green) in the cytoplasmic tail of the α subunit is involved in heterodimer assembly and regulation of ligand recognition. The heterodimer is illustrated in the "closed" or inactive state that undergoes tertiary and quaternary changes in response to inside-out signals. From Harris et al 2000.
isoenzymes. LFA-1-induced signaling involves multiple phosphorylation events mediated by protein kinase C family (Kelleher et al., 1995).

1.7.1 Structure and Function of Protein Kinase C
PKC isoenzymes are grouped as follows: classical (Ca\(^{2+}\)-dependent and activated by diacylglycerol and PMA (\(\alpha, \beta (I), \beta (II), \) and \(\gamma\)), novel Ca\(^{2+}\)-independent (\(\delta, \epsilon, \eta, \theta, \mu\)) and atypical phospholipid- Ca\(^{2+}\)-independent \(\xi, \lambda, \iota\))(Toker 1998). The primary structure of the three sub-families of PKC is illustrated in Fig 1.16. Each PKC family member plays an individual role in various signaling pathways and specifically affect diverse cellular processes, such as growth, differentiation, apoptosis, and tumorigenesis (Bhr et al., 2000). Their specific action involves a sophisticated network of regulation of PKC activity, including subcellular localization, and substrate phosphorylation. Beside the enzyme regulation by signal-induced second messengers, such as diacylglycerol, PKC is regulated by up- and down-modulation of its expression by phosphorylation and by interaction with other proteins involved in signal transduction, such as other protein kinases and anchoring or adaptor proteins. These anchoring proteins are important for specific subcellular localization of the enzyme and a selective phosphorylation of substrate proteins (Mochly- Rosen and Gordon 1998).

The PKC family of isoenzymes play critical roles in transducing signals from a plethora of extracellular receptors, including those for hormones, neurotransmitters, growth factors, and antigens (Stabel and Parker 1991, Newton 1995).

1.7.2 Role of PKC in T Cells
PKC plays a vital role in T cell activation and IL-2 secretion. The T lymphocyte expresses the PKC isoforms \(\alpha, \beta(I), \delta, \epsilon, \xi, \eta, \) and \(\theta\) (Nishizuka 1992). PMA activates members of the PKC family by binding a cysteine-rich region that is physiologically recognized by DAG (diacylglycerol) (Newton 1997, Nishizuka 1992). Increase in intracellular calcium using calcium ionophores or elevation of PKC (via phorbol esters) can induce T cell activation (Berry et al., 1989).

PKC \(\epsilon\) and to a lesser extent PKC \(\alpha\) were found to regulate the transcription factors AP-1 and NF-AT-1 (Genot et al., 1995). Also, PKC \(\theta\) (PKC \(\theta\) is a component of the supramolecular complex at the interface of the T cell and APC) has been shown to be an
Figure 1.16: Schematic diagram of the primary structure of the extended PKC family. A comparison of the primary structure of cPKCs (alpha, betaI, betaII and gamma), nPKCs (delta, epsilon, eta and theta), and aPKCs (PKCzeta, iota/lambda). Shown are the pseudosubstrate domain (PS), cysteine-rich region (C1), calcium-binding domain (C2), catalytic domain (C3 + C4). Reproduced from Toker 1998.
upstream regulator of c-Jun kinase/stress activated protein kinase and IL-2 promoter activation in Jurkat cells (Ghaffari-Tabrizi et al., 1999). Redistribution of novel PKC isoforms δ and ε between cytosolic and cytoskeletal fractions can be modulated by PKC agonists and specific inhibitors (Kiley et al., 1992, Keenan et al., 1995). In addition, PKC β plays an important role in the cytoskeletal organization of T cell cytoskeleton, and motile behavior (Entschladen et al., 1997, Thorp et al., 1996).

1.7.3 PKC-B and motile T cell
PKC-B has been demonstrated to undergo translocation to the plasma membrane from the cytosolic pool and cytoplasmic vesicles containing β2 integrins in response to phorbol ester treatment (Kiley et al., 1995). Moreover, PKC-B co-localizes with microtubule-associated proteins, actin-binding protein, spectrin, and membrane anchorage protein, ankyrin, following T cell activation (Gregorio et al., 1994) and its has been shown to be physically linked with the actin cytoskeleton (Blobe et al., 1996). Previous studies have shown that PKC β-deficient T cell line (K-4) failed to develop a motile phenotype when stimulated via LFA-1 (Volkov et al., 1998), and of equal importance, this PKC β-deficient T cell line had no IL-2 secretion when activated with PMA (Long et al., 2001). The role of PKC-β is underscored by the findings that it plays a key role in the proliferation and stimulated interleukin-2-secretion in lymphoma cells (Kelleher and Long 1992, Long and Kelleher 1993).

1.8 Role of Chemokines in Directing Cell Migration
Leukocyte chemoattractants, including chemokines, offer directional cues for leukocyte motility. Chemokines are responsible for the polarization of leukocytes with the establishment of two different regions: the leading edge and the uropod (del Pozo et al., 1995). Chemokines trigger G-proteins on tethered leukocytes where integrin-ligand interactions take place leading to the arrest of the rolling leukocytes. The arrested leukocyte utilizes these interactions to cross the endothelial barrier and migrate to tissues where it follows a chemotactic gradient (Sigal et al., 2000). Chemokines are released by leukocytes and other cells in response to different stimuli, they induce chemotaxis of different leukocyte types and regulate the adhesive properties of leukocyte integrins (Oppenheim et al., 1991, Lukacs et al., 1995, Tan et al., 1995, Rollins 1997, Baggioni 1998). Activation of cells by chemokines enhances the
functional activity of integrins, resulting in increased adhesion. Such "inside-out" signaling to integrins involves both cytoskeletal-dependent reorganization of integrins on the cell surface and activation-dependent increases in integrin affinity caused by conformational changes (Stewart et al., 1996, Shattil et al., 1998, Van Kooyk and Figdor 2000, Vinogradova et al., 2002, Takagi et al., 2002).

Chemokines are a group of small (8-14 kDa), mostly basic, structurally related molecules. The chemokines regulate cell trafficking of various types of leukocytes through interactions with a subset of seven-transmembrane G protein-coupled receptors. The human chemokine system currently includes more than 50 chemokines and 18 chemokine receptors. According to NH$_2$-terminal cysteine-motifs, the chemokines are divided into the C, CC, CXC and CX$_3$C subfamilies (Loetscher et al., 2000, Zlotnik and Yoshie 2000, Murphy and Baggiolini 2000). The large superfamily of chemokines consists of four subfamilies which display 2-4 highly conserved N-terminal cysteines. Most of them fall into the $\alpha$ or C-X-C (including SDF-1, IL-8/NAP-1, IP-10) groups and the $\beta$ or C-C (RANTES, MCP-1, -2 and -3, MIP-1 $\alpha$ and $\beta$) groups, whereas there is only one C (lymphotactin) and one CX$_3$C (fraktalkine) chemokine (Baggiolini et al., 1997).

T cells response to chemokines are different from phagocytes and monocytes. Interaction of resting cells with a chemokine MIP-1 $\beta$ induces functional activation of the T cell integrins either when the chemokine is in solution or is immobilized by proteoglycans (Tanaka et al., 1993). The versatile migratory pathways of lymphocytes are tightly regulated by changes in chemokine receptor expression and exposure to chemokines as well as the state of cell activation.

Chemokines can be divided into two categories: homeostatic and inflammatory. The former are those constitutively expressed in a certain tissue or organ, suggesting a specific function involving cell migration. The inflammatory chemokines in contrast are strongly upregulated by inflammatory or immune stimuli in various cell types including T cells. The latter are important in the development of immune and inflammatory reactions (Zlotnik and Yoshie 2000). Alternative classification uses functional features, which include conditions and locations of chemokine production as well as cellular
distribution of chemokine receptors, to distinguish between inflammatory chemokines and homeostatic (housekeeping or lymphoid) chemokines.

In terms of chemokine receptor expression and modulation of responses to chemokines, lymphocytes represent the most complex group of leukocytes (Von Andrian and Mackay 2000, Moser and Loetscher 2001). The most striking consequences of chemokines effects on leukocytes are the rearrangement of the cytoskeleton and the formation of integrin-mediated focal adhesions. Subsequently, cell binds and detaches from the substrate in a coordinated manner with extension and retraction of the uropod to accomplish the directional migration (Bokoch 1995, Ward et al., 1998). Binding of chemokines to their receptors is followed by the involvement of heterotrimeric G proteins, the Rho family of small GTPases (Reif and Cantrell 1998, Ward et al., 1998), phospholipases C, A, D, protein tyrosine, serine/threonine kinases, lipid kinases such as PI3K and the triggering of intracellular second messengers such as cAMP, phosphoinositides and calcium. Distinct sets of chemokines and their receptors are responsible for directing lymphocyte migration in the course of T cell development, antigen-driven differentiation into memory and effector T cells and immune responses at inflammatory sites. T cell migration along a chemokine gradient involves the sensing of subtle differences in chemokine concentrations and the establishment of cell polarity. This is followed by directional cell locomotion via cytoskeletal rearrangements and adhesive interactions with the extracellular matrix (Sanchez-Madrid and del Pozo 1999). In addition to navigation of cells within tissues, chemokines represent another aspect in the control of tissue-specific homing of circulating lymphocytes. Chemokines presented on endothelial cells trigger integrin activation and arrest of those leukocytes that carry the corresponding receptors (Cyster 1999, Campbell and Butcher 2000).

Chemokines have been found in tissues in different pathological conditions where there’s leukocyte infiltration such as arthritis, sepsis, atherosclerosis, asthma, pulmonary disorders and ischaemia-reperfusion (Taub 1996). Strategies undertaken to inhibit chemokine activity in order to limit leukocyte migration in chronic inflammatory disease include either the use of antireceptor or antiligand (Baggiolini 1997), or blocking leukocyte adhesion (Cornejo et al., 1997), or blocking downstream effectors such as NFκB activity (Bondeson et al., 1999).
1.9 Immunological Synapse

The interaction of MHC-presented antigenic peptides and their cognate TCRs results in the formation of a nanometer-scale intercellular junctional gap between T cells and APC, referred to as the immunological synapse (Fig. 1.17). The organization of this junction, both in the intercellular and lateral dimensions, is vital for integration of antigen and innate cues (Dustin 2002). The immunological synapse is one class of informational synapses that relay information across quasistable cell-cell junctions, others being the neuromuscular junction and many classes of CNS synapses. It consists of a central cluster of T cell receptors surrounded by a ring of adhesion molecules. The immunological synapse coordinates naive T cell activation and migration by stopping T cell migration with antigen-presenting cells bearing appropriate MHC peptide complexes (Fig. 1.18, Fig. 1.19). Furthermore, the immunological synapse allows full T cell activation through sustained signaling. The synapse is able to maintain persistent TCR engagement, while its formation requires substantial changes in T cell cytoskeletal architecture brought about by costimulatory molecules.

The well-organized interplay between TCR and costimulatory signals decides whether successful immune response or immune failure pursue (Kiefer et al., 2002). This is the role of the immunological synapse which allows activation in the absence of continued T cell migration; this is required for T cell activation through serial encounters, as it allows T cells to distinguish potential antigenic ligands. Initially, T cell receptor ligands are engaged in an outermost ring of the nascent synapse. Transport of these complexes into the central cluster is dependent on T cell receptor-ligand interaction kinetics.

The immunological synapse develops over a period of minutes following initial interactions of the T cell and APC surface. At the early stages of synapse formation, the TCR is not necessarily engaged in the center, but as the cell-cell interaction ensues, it translocates from the periphery into the center of the synapse. Formation of the synapse is concurrent with early TCR signals and depends upon an intact actin cytoskeleton.

1.9.1 Role of The Immunological Synapse in LFA-1 Mediated Adhesion

The immunological synapse organizes and segregates adhesion molecules and TCR-associated components into two major compartments (Bromley et al., 2001). These
Figure 1.17: Diagram showing the process of T cell adhesion and formation of the immunological synapse (Das et al 2002).

Figure 1.18: Model for immunological synapse formation. Side view of T cell forming an immunological synapse with an APC. Stage 1: Junction formation. LFA-1 anchors the central region of the nascent immunological synapse, providing a fulcrum for cytoskeletal protrusive mechanisms that force an outermost ring of T cell membrane into close opposition with the substrate. Stage 2: MHC-peptide transport this could be mediated by actin-based transport mechanisms. Stage 3: Stabilization. The intermediate ring is enriched in LFA-1-ICAM-1 complexes, whereas the outermost ring of close membrane opposition lacks LFA-1/ICAM-1 complexes, but promotes efficient TCR-MHC-peptide interaction in the nascent synapse. From Grakoui et al 1999.
Figure 1.19: The organization of the immunological synapse formed at the interface between APC and a responding T cell. The central zone of this structure, from which large adhesion molecules such as the integrin LFA-1 (blue) are excluded, contains T cell receptors (TCRs; pink), CD28 costimulatory receptors (yellow), and other smaller molecules. (A) Engagement of the TCR with peptide-MHC on the APC surface results in peptide-MHC signaling within the APC. Interaction of the costimulatory receptor CD28 with its ligands, which results in secondary signaling, is enhanced in the central region of the immunological synapse. (B) Key effector molecules, such as the cytokine IL-4 and the inhibitory protein CTLA-4, delivered to the central area by exocytosis, limit effects on bystander cells. (C) The immunological synapse may be required for internalization of TCRs, which may be one way that TCR signaling is down regulated. From van der Merwe and Davis 2002.
areas, referred to as supramolecular activation clusters (SMACs) include the central (c) SMAC, which is enriched in TCRs, and the peripheral (p) SMAC, which contains LFA-1 and talin (Monks et al., 1998, Delon and Germain 2000). APC surface components are also integral to these clusters, such that MHC-peptide complexes are found in the cSMAC, whereas ICAM-1 is concentrated in the pSMAC. The cSMAC also contains engaged CD28, while the pSMAC contains CD2 and LFA-1 in segregated domains (Grakoui et al., 1999, Bromley et al., 2001). These are not homogenous structures, but they seem to be composed of smaller clusters that can sometimes be interspersed when LFA-1 and CD2 penetrate the cSMAC region.

Segregation of surface receptors is a central feature of the immunological synapse and represents a vital step in the mechanisms of T cell regulation as it places constraints on direct collaboration between LFA-1 and the TCR (Monks et al., 1998). Recently, LFA-1 was shown to directly participate in the immunological synapse during TCR ligation by antigen, elucidating the pivotal role of LFA-1 in immunomodulation (Grakoui et al., 1999). In immunological synapse formation, not all of the molecules that are engaged at the interface move to the same point in the synapse. This is important, as for example, the adapter protein SLAP-130 (SLP-76-associated phosphoprotein) associates physically both with the TCR and functionally with LFA-1 (Hunter et al., 2000). It links TCR signaling to LFA-1 activation, but molecules of SLAP-130 that interact with the TCR will be far from the site of LFA-1 activation (Griffiths et al., 2001, Peterson et al., 2001). Thus, active SLAP-130 may have to diffuse across micrometers of cytoplasm to transduce a signal from TCR to LFA-1. Members of the Rho/Rac family of GTPases such as Vav play a role in mediating LFA-1 function at the immunological synapse. Vav1 is activated following TCR stimulation, Vav functions to transduce TCR signals to the activation of some actin-controlled processes, which is required for TCR-induced activation of the integrin LFA-1 (Ardouin et al., 2003). Thus, Vav1 transfers signals to a subset of cytoskeleton-dependent events at the immunological synapse.

1.9.2 Organization of MTOC at The Immunological Synapse
The microtubule cytoskeleton is organized into a single array with their minus ends associated with the MTOC near the nucleus. At the immunological synapse, the MTOC is drawn strongly to the contact site by a microtubule sliding mechanism. When the MTOC arrives at the contact site, it oscillates laterally. Then, microtubules loop through
and anchor to the pSMAC defined by the dense clustering of LFA-1 at the target contact site. Microtubules that run straight between the MTOC and pSMAC and then turn sharply can indicate the action of microtubule motors (Kuhn and Poenie 2002).

1.10 The Cytoskeleton

The multicomponent network of cytoskeletal elements is a central integrator in migratory and signal transduction pathways. Two major cytoskeletal related processes that regulate migration include the broad and important role of actin based filopodia and lamellipodia, the reciprocity of the small GTPases, Rho, Rac and Cdc 42 activities, the role of microtubules as a delivery system for local messages, and the regulation of adhesion complex turnover. Understanding how these elements are integrated will be the cornerstone in our understanding of cell migration and the associated signaling pathways.

1.10.1 Microtubules

Microtubules (MTs) play an essential role in multiple cellular functions. They organize and control signaling networks both spatially and temporarily and have a vital role in chromosome segregation in mitosis, in determining organelle positioning, cell polarity and cell movement. A number of studies have further emphasized the importance of the tubulin cytoskeleton in determining cellular polarity and motile behavior (Waterman-Storer and Salmon 1999, Entschladen et al., 2000). In most cells, MTs are organized in an orderly array, with their minus ends associated with the MTOC near the nucleus in the center of the cell and their plus ends towards the periphery of the cell. This gives the cell a defined polarity inherent in every microtubule. The two motor proteins kinesin and dynein move along the microtubule plus and minus ends respectively. The central localization of the Golgi apparatus (GA) around the nucleus is maintained by the microtubules. MTs provide sufficient surface area for protein-protein interactions as they occupy a huge distribution in the cell with a total protein surface area of 1000 \( \mu \text{m}^2 \) in an average fibroblast (which is the same surface area as the plasma membrane and ten times the nuclear envelope). The microtubules play a role in mostly all cells; in epithelial cells the MTOC changes location when cell-cell contact is made (Bacallao 1995). Neuronal cells utilize the microtubules for axonal transport and endocrine cells
require microtubules to move the secretory granules from cell surface to the cell interior through long processes produced by the cells (Kreis et al., 1989).

1.10.2 Microtubules and Signal Transduction
MTs are unique in that they fill the whole cytoplasm spanning the plasma membrane up to the nucleus, providing the way for directional flow of information, which make them ideal for transmitting signals. The discovery of signaling molecules that interact with microtubules, indicate that microtubules are critical to the spatial organization of signal transduction (Rios and Bornens 2003, Gundersen and Cook 1999). Many of the signal transduction components that interact with microtubules are represented by large multimeric complexes and the assembly of these complexes may be promoted by microtubules. MTs are also involved in the late step in endocytosis to the cell center (Gruenberg and Howell 1989) through microtubule based vesicle movement; this movement involves salvage of intermediates molecules between the ER and the Golgi complex (Pelham 1989, Lippincott Schwartz and Cole 1995).

The microtubule cytoskeleton plays a key role in the modulation of gene expression. MTs have been shown to regulate the function of the major transcription factor NFκB, although NFκB has not been demonstrated to be directly localized on the microtubules. IκB on the other hand has been found to interact with motor protein dynein (Crepieux et al., 1997); these interactions may sequester NFκB or lead to its delivery. Microtubule depolymerization by drugs leads to IκB destruction through a kinase-dependent mechanism allowing NFκB to bind DNA and stimulate transcription (Rosette and Karin 1995). Stability of IκB apparently depends on its anchorage to microtubules, possibly by its ankyrin repeat domain. The JNK (C-Jun N-terminal kinase), a component of the MAPK (mitogen activated protein kinase) was also found to physically associate with the microtubules (Nagata et al., 1998). The ERK (extracellular signal regulated kinase) members, ERK1 and ERK2 MAPKs also interact with MTs (Reszka et al., 1995, Morishima-Kawashima and Kosik 1996). Translocation of MAPK to the nucleus does not change the level of MT-associated MAPK, suggesting that cytoplasmic to nuclear translocation of MAPK is not regulated by MT association, rather MT association might be important for retaining activated MAPK in the cytoplasm thus regulating cytoplasmic events.
1.10.3 Microtubule Targeted Drugs (Nocodazole, Taxol) and Signal Transduction

Microtubules can be disrupted by drugs that lead to their breakdown such as Nocodazole or Colchicines or drugs that hyperstabilize them such as Taxol. These drugs have specific effects on processes involved in signal transduction, they generally bind specifically to tubulin (Gundersen and Cook 1999) and have different effects on the cell including gene expression, proliferation, polarization and apoptosis. Microtubule disrupting agents can induce interleukin 1 gene expression (Maine et al., 1993).

1.10.4 Microtubule and Actin Interactions

In most cell types, microtubules and actin filament networks cooperate functionally to produce a wide variety of functions including directed cell migration (Goode et al., 2000, Gundersen and Cook 1999). Depolymerization of microtubules leads to the 'collapse' of intermediate-filament organization in a microfilament-dependent process (Klymkowsky 1999). Therefore, pharmacological disruptors of one filament will have profound effects on the organization of the other filament. The formation of lamellipodia together with its position in the crawling cell is affected by microtubules. Decreasing microtubule dynamics by nanomolar concentration of Taxol or Nocodazole, which doesn’t disrupt microtubules or their organization, lead to a proportional reduction in lamellipodial area (Mikhailov and Gundersen 1998). Furthermore, MTs can modulate actin stress fibre formation. Tension produced by MTs on actin has been shown to counteract Rho-dependent stress fibre formation, though depolymerization of MTs (Bershadsky et al., 1996, Enomoto 1996). Rho has been shown to be necessary for the generation of stabilized microtubules in serum-starved fibroblasts implying the coordination between actin and the MT cytoskeleton. In T cells, Rho and Vav has been shown to associate with tubulin cytoskeleton (Huby et al., 1995). MAPs convey signaling molecules to the actin cytoskeleton where they have been shown to have a functional role (Cunningham et al., 1997).

1.10.5 The Uropod in T Cell Migration

The uropod is the main feature of a migrating T lymphocyte; it forms at the site of the MTOC implying the importance of tubulin cytoskeleton in determining cellular polarity and motile behavior (Ratner et al., 1997). The uropod contains cytoskeletal and signaling elements including microtubules, microfilaments, centrioles, Golgi complex,
mitochondria, and rough ER (Hauzenberger et al., 1995). In motile T cell, the uropod varies in length from short cytoplasmic protrusions as shown (Fig. 1.20 A) to a very elongated cytoplasmic extensions observed in migrating T cell activated with phorbol esters (Fig. 1.20 B). During T cell migration, actin becomes concentrated at the leading edge of the cell (lamellipodia), while microtubules retract towards the rear of the cell in the uropod.

1.10.6 Microtubules Dynamics and Focal Adhesions

Microtubules have recently been shown to control focal adhesion and focal complex dynamics (Kaverina et al., 1999, Waterman-Storer and Salmon 1999). Microtubules play a well-established role in the polarization and motility of different cell types (Schliwa and Höner 1993, Small et al., 2002). The degree to which cells require microtubules for maintaining polarity generally parallels the extent of anchorage of cells to the extracellular matrix and the degree of focal adhesion formation (Small and Kaverina 2003). It has been hypothesized that microtubules exert their influence on cell polarity by affecting anchorage and protrusion through a modulation in the activities of Rho-family of GTPases (Wittmann and Waterman-Storer 2001, Kaverina et al., 2002). Depolymerization of microtubules in fibroblasts leads to a decrease in the turnover of focal complexes, which results in reduced cell spreading, large peripheral focal adhesions, increased tension, and reduced protrusive activity (Horwitz and Parsons 1999). Upon restoration of the microtubular network, there is improved turnover of focal adhesions along with increased Rac activation and protrusive activity. Direct contact with microtubules leads to the dissociation of focal adhesions, and either the cell edge retracts or a new protrusion forms. The effect of microtubules on cell polarity is dependent on the turnover of specific sets of substrate adhesions in a cell, to allow protrusion at the front, and promote retraction at the rear. The turnover of focal adhesions at the front is needed to remodel the actin cytoskeleton and to recycle components required for protrusion, consistent with the dramatic ruffling response induced by the dissolution of focal adhesions by Rho kinase inhibition (Rottner et al., 1999). Retraction at the rear also helps promote protrusion at the front (Small et al., 2002), though different data had shown that microtubules generally inhibit actin cytoskeleton contractility (Geiger and Bershadsky 2001, Kaverina et al., 2002) and it was thus suggested that the destabilization of adhesions could be attributed to local, microtubule-linked relaxation at adhesion foci (Kaverina et al., 1999).
Figure 1.20: The uropod in crawling T cells. (A) Confocal image of a uropod in a crawling T cell showing microtubule retraction into the uropod. Actin becomes concentrated in the anterior lamellipodium (green) and microtubules retract to the posterior uropod (red), from Ratner et al., 1997. (B) T cell activated with phorbol esters with long extending uropod, from Volkov et al., 2001.
1.10.7 Microtubule Nucleation

γ-Tubulin concentrates at the MTOC. The microtubule nucleating capacity resides in the PCM (pericentriolar matrix), rings of 25 nm in diameter called γ-TuRC (γ-tubulin ring complexes) responsible for the MTs nucleation at the centrosome (Moritz et al., 1995, Vogel et al., 1997, Zheng et al., 1995, Weise and Zheng 1999). The major MTOC in eukaryotes is the centrosome. The centrosome has a unique localization in both MTs nucleation and organization, as newly formed MTs are proposed to remain tightly bound to the pericentriolar material. The centrosome activity is more complex and dynamic; cells constantly release MTs from centrosomes that are then anchored to centrosomal and non-centrosomal sites. The mechanisms for MTs nucleation and anchoring at non-centrosomal sites are still poorly understood. MTs release from the centrosome depends on the cell type and the concentration of MTs-anchoring activities at the centrosome (Bornens 2002).

1.10.8 Role of Protein Phosphorylation in Microtubule Dynamics

Several studies have shown that phosphorylation of tubulin and microtubule associated proteins have functionally significant effects on microtubule stability. An example is MARK (microtubule associated protein-microtubule affinity associated kinase), which phosphorylates microtubule associated proteins and triggers MT disruption (Drewes et al., 1997). In addition, the modulation of PKC activity affects antigen-induced reorientation of the MTOC in cytotoxic T cells (Nesic et al., 1998). Also, PKC-β association with the MT is crucial as diminished association with the MT could result in defective MT re-organization in some cells (Kiley and Parker 1997). The effects of some microtubule disrupters such as Nocodazole depend on protein phosphorylation (Walker et al., 1997), and the microtubule-destabilizing drug Taxol exerts some of its effects through PKCδ stimulation (Das et al., 1998).

1.11 Cytoskeletal Reorganization in T Cell Motility

T cell motility requires multiple rapid cycles of cytoskeletal assembly and reassembly, co-ordinated by actin and microtubules. Actin-based filopodia and lamellipodia is responsible for a direct role in T cell adhesion and locomotion (Mitchison and Cramer 1996, Stewart et al., 1996). Actin functions are largely dependent on the small G proteins (GTPases), Rho, Rac and Cdc42 (Nobes and Hall 1995). The first physical step
in directional motility is the extension of cellular processes termed pseudopods (Cassimeris and Zigmond 1990). Pseudopodia are flattened, fan shape cellular protrusions enriched in signaling molecules and cytoskeletal elements including filamentous actin, which is preferentially nucleated at the leading edge of a migrating cell (Weiner et al., 1999). The MTs also play a key role in T cell motility (Ratner et al., 1997). During locomotion, the MTs retract into the cellular uropod, increasing the deformability thus facilitating migration through narrow spaces, such as the gaps between the endothelial cells. The radial array of MTs anchored to the centrosome and projecting to the cell periphery could supply the cell with a mechanism able to regulate MTs cytoskeleton as a whole by controlling centrosomal activity.

1.11.1 Rho and The Actin Cytoskeleton in T Cell Motility
Cell movement is driven by the regulated and polarized turnover of the actin cytoskeleton and of the adhesion complexes that link it to the extracellular matrix. Regulation of actin polymerization is crucial for multiple functions of T cells, especially cell polarization and migration. Polarization requires the engagement of actin and microtubules, which exert their effect by mediating changes in the activity of the Rho GTPases. Rho GTPases undergo reversible association with the cytoskeleton; their association is partly related to the regulation of the actin cytoskeleton (Tapon and Hall 1997). In some cells, binding of small GTPases to the cytoskeleton is mediated by adaptor proteins (Symons et al., 1996). The multiplicity of GTPases and associated proteins that undergo regulated binding to the cytoskeleton seem to be not entirely related to signals directly leading to cytoskeletal reorganization but may serve other purposes, i.e. to sequester and organize elements of signaling pathways.

Rho activity is required for T lymphocyte spreading and motility (Woodside et al., 2003), in addition to their role in polarization (Fig. 1.21) and cytoskeletal dynamics (Stowers et al., 1995, Allen et al., 1998, Servant et al., 2000). Furthermore, Rho GTPases are involved in regulating integrin dependent events such as leukocyte homotypic aggregation and in chemokine upregulation of integrin-mediated adhesion (Laudanna et al 1996, Tominaga et al., 1993). In migrating cells, the production of protrusions, formation of new adhesions, and stabilization of existing adhesions are regulated by members of the Rho family of small GTPases (Hall 1998). Rho induces the assembly of contractile actinomyosin filaments involved in the formation of
Figure 1.21: Role of Rho in cell motility. The diagram shows the different classes of adhesion assemblies observed in migrating cells. The earliest recognizable adhesions are focal complexes (FX), which arise in association with lamellipodia and filopodia, although signaling via Rac and Cdc42. They support protrusion and can either turnover on the minute scale or differentiate into longer-lived focal adhesions via the upregulation of Rho. The existence of an intermediate form of focal complex (FX*) is suggested by the finding that Rac can be activated via mDia, which also stimulates actin polymerization. The differentiation to focal adhesions requires the recruitment of myosin (grey bars) and the activation of contractility via Rho/Rho kinase, by exchange factors such as GEF-H1. Focal adhesions (FA) behind an advancing front remain stationary, and turn over by disassembly. Focal adhesions in retracting edges slide and subsequently disassemble (slFA). Microtubules (green bars) target focal adhesions and also some focal complexes (presumably FX*) formed when Rho kinase is inhibited. Microtubule-linked signals could retard the growth or promote the disassembly of adhesion complexes. Reproduced from Small and Kaverina 2003.
lamellipodial and filopodial membrane protrusions respectively (Ridley et al., 1992). Inhibition of Rho blocked the effect of microtubule disruption by Nocodazole on β2-integrin mobility (Zhou and Kucik 2001). The Rho-related GTP binding protein Rho is primarily responsible for mediating the reorganization of the actin cytoskeleton (Hall 1998). Moreover, the Rho family of G proteins has been described as a link between the actin cytoskeleton functioning and gene expression cell growth regulation and induction of malignant transformation (Craig and Johnson 1996, Symons et al., 1996).

Studies by Vicente-Manzanares et al demonstrated that RhoA is involved in the control of actin balance through the RhoA effector mDia, which is critical for T cell responses and can be induced in vitro in activated PBL. mDia is highly expressed in vivo in diseased tissue-infiltrating activated lymphocytes and localizes at the leading edge of polarized T lymphoblasts. Also, overexpression of an activated mutant of mDia results in an inhibition of both spontaneous and chemokine-directed T cell motility (Vicente-Manzanares et al., 2003).

1.12 Microtubule Motor Protein: Dynein

Dynein is a large multisubunit protein divided into inner and outer arms, comprising two heavy chains (DHC1a, approx. 500-530 kDa), two to three intermediate chains, DIC, approx. 74 kDa, a set of light intermediate chains, approx. 51-61 kDa; (Holzbaur and Vallee 1994) and three different light chains, approx. 8, 14 and 22 kDa (King et al., 1996). Conventional cytoplasmic dynein is a minus-end directed microtubule-based motor involved in the movement of various intracellular cargoes (Thaler and Haimo 1996). It is responsible for retrograde transport, centripetal transport of endosomes, lysosomes and elements of Golgi apparatus. Dynein associates with actin cytoskeleton thought the dynactin complex (Glotzer and Hyman 1995).

1.13 PKN and Cytoskeleton Organization

PKN belongs to the AGC (Cyclic nucleotide regulated protein kinase) family of protein kinases (Taylor and Radzio-Andzelm 1994), which also include PKA (Cheng et al., 1998, Dutil et al., 1998), PKB (Alessi et al., 1997, Stokoe et al 1997) and PKC (Chou et al., 1998). PKN was the first identified serine/threonine protein kinase that could bind to and be activated by the small GTPases Rho in a GTP-dependent manner. PKNα purified
from a bovine brain membrane fraction is stimulated by the addition of the GTPγS-bound form of RhoA (Mukai and Ono 1994, Amano et al., 1996, Flynn et al., 1998). PKN is widely distributed in various organisms and mammals. There are three different isoforms of PKN (PKNα/PAK-1/PRK-1, PKNβ, and PRK2/PAK-2/PKNγ) each of which shows varied functional properties and tissue distribution (Mukai et al., 1997). Activated Rho binds to PKN and induces a conformational change that is permissive for binding to PDK1.

The exact role of PKN in regulating the reorganization of the actin cytoskeleton is still being evaluated. Previous data showed that ectopic expression of PKNα stimulates actin stress fiber depolymerization and membrane ruffling (Dong et al., 2000). Several studies showed that the expression of a kinase-negative form of human PRK2/PKNγ in microinjected NIH 3T3 fibroblasts results in the disruption of actin stress fibers, suggesting an important role for PKNγ in regulating actin reorganization (Vincent and Settleman 1997, Mukai 2003). PKN could regulate the actin cytoskeleton with associated cell shape changes downstream of Rho family GTPases in Drosophila (Lu and Settleman 1999). Recent studies demonstrated that PKN is directly linked to the cytoskeletal network through associations with α-actinin (Mukai et al., 1997). PKNα binds to the third spectrin-like repeats of actin cross-linking protein α-actinin.

PKN has a catalytic domain similar to that of PKC. PKN efficiently phosphorylates substrates based on the pseudosubstrate sequence of PKC. PKNα phosphorylates basically the same sites as PKC in vitro which includes MARCKS and vimentin (Palmer et al., 1996). In some cases, however, PKNα phosphorylates sites of the same protein different from those phosphorylated by PKC (Taniguchi et al., 2001).

1.14 Centrosome and The MTOC

The centrosome represents the major (and usually the only) microtubule organizing centre in mammalian cells including T cells. MTOC or centrosome is the center of important processes in the cell. The centrosome nucleates microtubules, which is important for cell shape, motility and division (Zimmerman et al., 1999). The centrosome organizes microtubules in interphase cells and is essential for the formation of the mitotic spindle during cell division. Mammalian centrosome is composed of a pair of centrioles surrounded by electron-dense cloud of amorphous PCM.
(pericentriolar matrix) (Kellog et al., 1994). PCM comprises a number of large coiled-coil proteins that are connected to the centrosome and serve to recruit other proteins to the centrosome.

The MTOC is a critical organizing point within the cell; it becomes reoriented during cell polarization, and participates in the redistribution and concentration of surface molecules in membrane caps, including different adhesion receptors (Dustin et al., 1997). The binding of the T cell to another or to an APC induces the rapid reorganization of the T-cell cytoskeleton with the formation of a tight collar of polymerized actin at the T cell-APC interface and the reorientation of MTOC towards the bound APC (Kupfer and Singer 1989, Kupfer et al., 1994, Dustin et al., 1997). The reorientation of MTOC allows the repositioning of the T-cell secretory apparatus into close proximity with the APC (Kupfer et al., 1994).

1.14.1 γ-Tubulin Ring Complexes

The PCM contains components of the γ-TuRC that nucleate and organize microtubules and provides template for the addition of tubulin subunits to make a microtubule (Stearns 2001, Takahashi et al., 2002). γTuRC belongs to the group of centrosome proteins whose centrosomal localization is independent of microtubules. γTuRC is a ring shaped multiprotein complex containing γ-tubulin found in the cytoplasm and the centrosome (Zheng et al., 1995, Schiebel 2000). Only γTuRC is essential for centrosome-mediated microtubule nucleation (Moritz et al., 1998). The cytoplasmic pool of γ-TuRC may be a source of nucleating complexes that are recruited to the centrosome when increased microtubule nucleation is required (Khodjakov and Rieder 1999). In humans, 6 proteins have been already discovered in γ-TuRC, including GCP2, GCP3 (also named HsSpc98), and γ-tubulin, which are the orthologues of Spc97p, Spc98p, and Tub4p, respectively (Tassin et al., 1998, Murphy and Preble 2001). The Tub4p complex is equivalent to the γ-tubulin small complex (γTuSC), a major building block of the γTuRC (Gunawardane et al., 2000).

1.14.2 The Pericentrosomal Region

The pericentrosomal region is the site where essential activities for the functioning of the cytoskeletal and secretory systems concentrate. A major component of the
pericentriolar material is pericentrin (Doxsey et al., 1994, Dictenberg et al., 1998). Pericentrin interacts with PKA and dynein, scaffolding a range of centrosomal components (Purohit et al., 1999, Diviani et al., 2000). The PCM encompasses a large array of interconnected meshwork of coiled-coil proteins, these proteins are important to anchor and cluster components of the signaling pathways, serving to connect centrosome activity with signaling pathways (Doxsey 2001, Bornens 2002). Since microtubules nucleate from centrosomes, the centrosomal proteins associated with γ tubulin might have a direct role for microtubule dynamics. During the past few years, an increasing number of kinases and phosphatases has been shown to be targeted to the centrosome and to regulate its function (Bischoff et al., 1998, Hinchcliffe et al., 1999, Nigg 1998; Zhou et al., 1998). Targeting serves to bring molecular complexes bound at the tips and along microtubules in close proximity with adhesion complexes.

The type II PKA was initially detected at the centrosome over 15 years ago (De Camilli et al., 1986, Nigg et al., 1985). Phosphorylation of centrosomal proteins is suggested to be involved in the regulation of centrosomal function (Vandre et al., 1984). Various protein kinases and phosphatases are localized to centrosome (Faux and Scott 1996), and some of the kinases are implicated in the regulation of centrosome separation (Glover et al., 1995, Fry et al., 1998) and microtubule nucleation (Verde et al., 1992).

1.15 Centrosome at Mitosis

Centrosome ensures the fidelity of chromosome segregation during mitosis by guiding microtubule nucleation towards the formation of a bipolar spindle. This organelle is involved in cell cycle checkpoint control and cell cycle progression (Bornens 2002, Lange 2002, Khodjakov and Rieder 2001, Hinchcliffe et al., 2001). Both centrioles and their surrounding pericentriolar material, PCM are capable of self-assembly. Centrosomes enlarge dramatically after mitotic entry. During S phase of the cell cycle, the single centrosome that is present in a G1-phase cell is duplicated. The two centrosomes then set up the poles of the mitotic spindle and each daughter cell receives one centrosome. Whereas centrioles are normally duplicated during G1-S phase, PCM components may be loaded onto centrosomes in both a microtubule-dependent and independent manner at all stages of the cell cycle. The duplication and segregation
cycles of centrosomes and chromosomes need to be coordinated to avoid chromosome missegregation or ploidy changes (Nigg 2002).

1.15.1 Centrosome Aberration and Tumor Formation
Many tumors display numerical and structural centrosome aberrations. Extra copies of centrosomes could, in principle, arise through overduplication within a single cell cycle, through aborted cell division, cell fusion or de novo genesis. Centrosome aberrations and abnormalities in tumors, can give rise to chromosomal instability (Fig. 1.22) and altered tissue architecture, they can lead to the generation of invasive, genetically unbalanced cells during cancer progression. Importantly, centrosome aberrations and chromosomal instability are expected to enhance each other. Most multipolar divisions cause severe chromosome missegregation and lead to lethal outcome. On the other hand, they might give rise to cells with chromosomal compositions that favor survival in the microenvironment of the tumor (Nigg 2002).

1.16 Golgi Localization and Microtubule Dynamics
The Golgi apparatus (GA) of mammalian cells has a unique localization at the cell center, and its existence as an integrated organelle is dependent on microtubules. MTs are required both for determining the localization and organization of the Golgi apparatus. Other factors, however, also appear necessary for regulating both the static steady-state distribution of this organelle and its relationship with microtubule minus-end-anchoring activities of the centrosome. The Golgi localizes at the MTOC during cell migration (Thyberg and Moskalewski 1999). A fraction of cytosolic γ-tubulin has been found recently to associate with purified Golgi membranes (Chabin-Brion et al., 2001). The Golgi membrane fraction was found to nucleate MTs, this activity required peripherally associated Golgi proteins, specifically γ-tubulin, molecular motors and MT-anchoring proteins which also have a key role in maintaining the organization and positioning of the Golgi (Lippincott-Schwartz et al., 2001, Allan et al., 2002). Proteins associated with the Golgi might have a vital role in microtubules dynamics in the context of T cell motility. Association of the Golgi complex with cytoskeletal elements, in particular microtubules, is required for maintenance of the Golgi's characteristic spatial location (Fig. 1.23) within cells and for efficient delivery of proteins and lipids to diverse cellular sites. In T cells treated with Taxol, stable MTs remain anchored to the centrosome and Golgi localization does not change (Bornens 2002, Knox et al.,
Figure 1.22: Co-ordination between the centrosome and the chromosome cycles. Aberration in one cycle can lead to tumour formation (Nigg 2002).
Figure 1.23: Signaling at the Golgi. From Donaldson and Lippincott-Schwartz 2000.
Recent work has suggested the mechanisms underlying this association involve components, such as ankyrin and spectrin that facilitate Golgi membrane association with motor proteins, including cytoplasmic dynein, kinesin and myosin (Xu et al., 2002, Allan et al., 2002).

The Golgi apparatus of mammalian cells is organized into stacks of cisternae. Each stack has 4-8 cisternae, 40-80 stacks are attached to each other and anchored to the pericentriolar area. The GA is localized centrally in the secretory pathways and it is in dynamic equilibrium with the ER. The Golgi receives de novo synthesized proteins from the ER and sort cargo to their final destinations (Mellman and Simons 1992). The cargo exiting the ER is packaged into pre-Golgi transport intermediates that are subsequently translocated along the MTs toward the centrosome. These pre-Golgi elements fuse to generate the first Golgi cisternae, which then mature from a cis (entry) to a trans (exit) form. This emphasizes the Golgi central location around the centrosome. Concurrently, other membrane tubules and vesicles form the Golgi cisternae and are transported back to the ER (Lippincott-Schwartz et al., 2001, Pelham 2001).

The Golgi/centrosome region plays important roles in several physiological processes, such as intracellular signaling, mitosis or apoptosis. The Golgi highly dynamic nature possibly relies on a wide array of MT-anchoring proteins. In addition, The GA plays important roles in cell motility, polarization and differentiation; the interplay between Golgi apparatus and centrosome could participate in other physiological processes such as intracellular signaling, mitosis and apoptosis (Donaldson and Lippincott-Schwartz 2000).

1.16.1 The Golgi During Mitosis
When cells enter mitosis, the pericentriolar stacks of the Golgi cisternae undergo massive fragmentation into thousands of vesicles and tubules where they are dispersed throughout the cytoplasm (Zaal et al., 1999), and some membranes are reabsorbed into the ER. The GA is assembled early during mitosis and reassembled at the end of mitosis. This is important for the equal division of the GA into two daughter cells. Its inheritance utilizes several strategies: de novo formation, fission and disassembly and reassembly (Bevis et al., 2002, Warren 1993, Uchiyama et al., 2003). The
fragmentation and dispersal of the GA has been found to be a pre-requisite for entry into mitosis in mammalian cells, and this pericentriolar GA organization is a sensor for controlling entry into mitosis (Sutterlin et al., 2002). More surprisingly, the fragmentation and dispersal of the GA has been found to be a pre-requisite for entry into mitosis in mammalian cells (Sutterlin et al., 2002). It has been suggested that the pericentriolar GA organization is a sensor for controlling entry into mitosis. Both the centrosome and GA can initiate apoptosis by specific stress sensors and relay apoptosis-modulating signals to the rest of the cell (Ferri and Kroemer 2001, Piekorz et al., 2002). Golgi fragmentation occurs in at least two steps, first, the pericentriolar Golgi stacks are converted into small fragments, and then these fragments either undergo vesiculations or fuse with the ER (Nelson 2000, Rossanese and Glick 2001). Prevention of Golgi fragmentation inhibits entry into mitosis (Sutterlin et al., 2002). Thus, fragmentation of the Golgi apparatus is not an effect of mitosis-specific events but a key cause regulating entry of cells into division cycle. PKA RIIα associates with the Golgi-centrosomal region during interphase, and dissociates from its centrosomal localization at metaphase-anaphase transition (Keryer et al., 1998).

1.16.2 Golgi/ Microtubule Association

Marsh et al., 2001 utilized for the first time high-pressure freezing, freeze-substitution and electron tomography to study the three-dimensional structure of the Golgi and Golgi related structures. Their results showed that there is an association between the Golgi stacks and MTs at the cis face (Figure 1.24) where individual MTs appeared to closely follow the membranes of the first cisternae (separated by ≤30 nm) and frequently make contact with it. A fraction of cytosolic γ-tubulin has been found recently to associate with purified Golgi membranes (Chabin-Brion et al., 2001). The Golgi membrane fraction was reported to nucleate MTs both in a permeabilized cell system and in vitro assays. Furthermore, MTs traversing the Golgi stacks via cisternal openings at multiple points were seen. These data indicate an important role of MTs in the cis face of the Golgi where new cisternae are generated. Maturation of cis to trans cisternae would therefore implicate also the relocalization of MT-binding activities associated to the cis-Golgi membranes, either by membrane recycling or by association/dissociation from a cytoplasmic pool (Marsh et al., 2001, Rios and Bornens 2002). In lymphocytes all of MTs are anchored to the centrosome with the Golgi surrounding the centrosome. Such an intimate relationship between Golgi and MTs-
Figure 1.24: Three-dimensional reconstruction of a part of the Golgi ribbon, revealing in situ physical relationships between the cis-most cisternae of the Golgi and MTs. MTs (green) closely follow and occasionally form contacts with the membranes of the cis-most cisternae of the Golgi (blue). From Rios and Bornens 2003.
anchoring activities is also revealed by treatment of cells with Taxol or during cell differentiation. In lymphoblasts treated with Taxol, stable MTs remain anchored to the centrosome and Golgi localization does not change (Bornens 2002). In addition, a fraction of cytosolic γ-tubulin has been found recently to associate with purified Golgi membranes (Chabin-Biron et al., 2001). The Golgi membrane fraction was reported to nucleate MTs both in a permeabilized cell system and in vitro assays. The Golgi/centrosomal area has been shown to be involved in several physiological processes including intracellular signalling and mitosis. The importance of this area has been further elucidated by the identification of multiple signal transduction molecules associated with these organelles (Donaldson and Lippincott-Schwartz 2000, Lange 2002). Microtubule motors and non-motor MT-binding proteins have been shown to associate at the cis face of the Golgi (Allan et al., 2002, Grissom et al., 2002, Xu et al., 2002).

GMAP-210 is a peripheral membrane protein that behaves like the Golgi matrix proteins in response to brefeldin A (BFA), a Golgi disrupting agent (Infante et al., 1999). It mediates interactions between Golgi membranes and stable MTs, and binds to MTs through its carboxy-terminal domain at the MT minus ends. This protein plays a role in the biogenesis of the Golgi around the centrosome. Overexpression of GMAP-210 induces the loss of the MT aster, and the formation of a dense network of short MTs that co-localize with the Golgi, suggesting a MT-anchoring and stabilizing activity for this protein. Another protein in this group is Hook protein. These are MT-binding proteins linking Golgi membrane organelles to MTs (Walenta et al., 2001). Hook3 in particular localizes the Golgi near the centrosome. This localization is MT-dependent and mostly insensitive to BFA. During prophase, Hook3 accumulates at mitotic poles with a fraction of Hook3 was shown to be associated with cis-Golgi membranes and to redistribute to peripheral sites after BFA treatment. On top, Hook3 overexpression induces fragmentation and dispersion of the Golgi.

Stable MTs might be important in the maintenance of Golgi structure and localization. Stabilization can be achieved through the CLIP-associated proteins CLASPs, CLASPs (Akhmanova et al., 2001) have been recently shown to stabilize the subset of MTs implicated in the organization of the Golgi, when overexpressed they exhibit MT-stabilizing effects and increase the number of detyrosinated MTs. There is a substantial
Golgi-associated pool of CLASPs, and CLASP2 is targeted to Golgi membranes. Another protein from the CLIP-170 family (CLIP-170 is implicated in membrane trafficking and plays a role in dynein mediated transport), CLIPR-59, has been shown recently to be associated with membranes of the trans-Golgi network (Perez et al., 2002).

1.17 NFκB and IκB

Cytoskeletal proteins regulating the function of NFκB in T cell functions are not completely elucidated. The activity of the transcriptional factors that reside in the cytoplasm and undergo translocation from the cytoplasm upon stimulation can be modulated by cytoskeletal plasticity. This is the case with the NFκB/Rel family of transcription factors, which exist in a latent form in the cytoplasm of unstimulated cells (Baeuerle and Baltimore 1988). NFκB is retained in the cytoplasm by the IκB inhibitory molecules. The most studied member of the IκB is IκBα protein. All IκB are characterized by 5-7 ankyrin motifs responsible for the p65 association and DNA binding inhibition (Jaffray et al., 1995). These motifs are also responsible for mediating cytoskeletal binding (Lux et al., 1990). When the cell is stimulated, IκBα is inducibly degraded, this unmasking the nuclear localization of the NFκB complex, this allows then for nuclear translocation and gene stimulation (Verma et al., 1995). Stability of IκB could depend on its anchorage to microtubules, possibly by its ankyrin repeat domain (Rosette and Karin 1995). When microtubules depolymerize, IκB is released, becomes degraded, and releases NFκB to the nucleus.

1.17.1 IκBα

IκBα is the key molecular target controlling NFκB activity, thus characterization of effector proteins that mediate inducible IκBα degradation is crucial. A 700 KDa multisubunit complex has been isolated as a potential IκBα kinase activator (Chen et al., 1996). Interestingly, although IκBα is linked to NFκB Rel (p65), studies shows that it also associates physically with the motor protein dynein through DIC-1 (dynein light chain) using yeast two-hybrid screen with N terminal region of IκBα as bait in B lymphocytes (Creipieux et al., 1997). Dynein light chain was found in both nuclear and cytoplasmic areas and co localized with DIC-1 in the cytoplasm. Not only IκBα associates with DIC-1, but also with the microtubules particularly at the MTOC region.
1.18 Adaptors in T Cell Function

Adaptors play a role in the recruitment of proteins to the plasma membrane; facilitate phosphorylation, enzyme activation, and interactions with other proteins and substrates. Adaptor proteins that functionally play a role in T cells include LAT (linker for activation of T cells) (Zhang et al., 2000), SLP-76 (SH2-domain containing leukocyte protein of 76 kDa), (Kennedy et al., 1999, Wardenburg et al., 1996), BLNK (B cell linker protein)/SLP-65 (SH2 domain-containing leukocyte protein of 65 kDa), FYB (Fyn T-binding protein)/SLAP-130/Fyb/ADAP (SLP-76-associated protein), SKAP55 (Src kinase-associated protein of 55 kDa), the 3BP2 protein and a 'pure adaptor' (GADS)(Burack et al., 2002). These adaptors lack enzymatic and transcriptional domains, but they have multiple domains that allow binding to other proteins. These multiple binding sites create various combinations of multiprotein complexes that help to integrate signals from surface receptors (Rudd 1999). LAT plays a major role in TCR signaling acting as a central adaptor; it is critical in the coupling of proximal events to downstream signaling. LAT contains a transmembrane domain followed by a long cytoplasmic tail containing several tyrosine phosphorylation sites (Zhang et al., 1998). LAT acts in response to TCR ligation by recruiting multiple proteins required for downstream signaling. Phosphorylated LAT binds directly to key proteins such as phospholipase Cγ-1 (PLCγ-1) and Grb-2/Grap (Grb2-like accessory protein)(Van Leeuwen and Samelson 1999). LAT recruitment of PLCγ-1 regulates the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP2) to inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG) (Fig. 1.25). IP3-induced increase in intracellular Ca^{2+} is required to activate the serine/threonine phosphatase calcineurin to dephosphorylate the transcription factor NF-AT. Dephosphorylated NF-AT translocates to the nucleus and participates in IL-2 transcription (Rao et al., 1977).

Adaptors have a critical role in the immune system. Toll-like receptors (TLRs) are a family of receptors involved in microbial recognition by the immune system. TLR3 and TLR4 specifically regulate interferon (IFN)-dependent gene expression during anti-viral responses. Adaptors that link TLRs with downstream signaling events such as TRIF (a key component of the MyD88-independent signaling cascade activated by TLR3 and TLR4) are central to the anti-viral host response involving TLR3 and TLR4 activation.
Figure 1.25: Schematic diagram showing adaptor proteins in T cell signaling. Derived from Leo and Schraven 2001.
by controlling the specificity of gene expression in response to individual TLR stimulation, macrophages from mice lacking the *Trif* gene, exhibit impaired expression of IFN-beta and IFN-inducible genes (Yamamoto *et al.*, 2003).

### 1.19 Scaffolding Proteins/AKAPs

Research by Michel and Scott over the past years demonstrated that in order to insure tight control of hormonally initiated phosphorylation events, certain mechanisms must be involved. Firstly, the activity of multifunctional kinases and phosphatases should be precisely regulated and responds to fluctuations in diffusible second messengers such as Ca\(^{2+}\), phospholipid, and cAMP. A second mechanism that contributes to their regulation is the restriction of the location of these enzymes to certain subcellular compartments. Subcellular targeting enhances the selectivity of serine/threonine phosphatases and kinases by favoring their accessibility to certain substrate proteins. Compartmentalization is achieved by a "targeting moiety", which is defined as that part of a phosphatase or kinase that directs the catalytic subunit to a certain subcellular environment (Michel and Scott 2002).

Scaffolding proteins have been intensively studied recently and were shown to play essential roles in the coordination of signal transduction complexes by recruiting multiple signaling enzymes near potential substrates. Acting as a common strategy for preventing the wrong kinases and phosphatases from acting on a target, scaffolding proteins facilitate the proper reactions by holding selected kinases and phosphatases near the precise proteins they are supposed to regulate (Scott and Pawson 2000). Spatial and temporal organization of signal transduction is critical in determining the speed and precision by which signaling events occur. Targeting subunits for the type II cAMP-dependent protein kinase PKA, is achieved by the AKAPs (stands for A-Kinase anchor proteins). The first PKA II binding protein was discovered 15 years ago and named microtubule-associated protein 2 (MAP-2) (Theurkauf and Vallee 1982). Since that time, several AKAPs have been identified, localizing PKA II to thyroid cytoskeleton (Carr *et al.*, 1992), mitochondria (Lin *et al.*, 1995), the Golgi apparatus (DeCamilli *et al.*, 1996), centrosomes (Nigg *et al.*, 1985, Keryer *et al.*, 1993), and microtubules (Nigg *et al.*, 1985, Vallee 1986). More than fifty AKAP genes have been isolated so far by interaction cloning techniques some of which have complex gene families with numerous splice variants. AKAPs are classified on the basis of their ability to bind the R
subunits of PKA (Michel and Scott 2002, Edwards and Scott 2000). There are a number of cAMP-dependent signaling pathways operating simultaneously in the cell; AKAPs thus facilitate the stimulation of a single unique target at the appropriate time and place (Edwards and Scott 2000).

AKAPs were named according to the apparent molecular weight of the proteins on SDS-PAGE gels. Structure-function analysis suggest that each anchoring protein binds to the RII dimer of PKA through a conserved amphipathic helix region (Fig. 1.26, Fig. 1.27) of twenty residues that is responsible for high affinity interaction with the regulatory subunit of PKA and is tethered to specific subcellular sites via association of a targeting domain with structural proteins or cellular organelles (Schillace and Scott 1999, Carr et al., 1992). This targeting domain confers specificity on each AKAP as they direct the localization of the kinase to the cytoskeleton, nuclear matrix, endoplasmic reticulum, peroxisomes, and cell membranes. Peptides corresponding to this region are antagonists of PKA/AKAP interaction and disrupt the localization of the kinase when presented into cells; hence they were named “anchoring inhibitor peptides”. These peptides have been used by many researchers to uncouple certain cAMP-responsive events, such as the activation of glutamate receptors, and to suppress hormone-mediated insulin secretion from beta islet cells.

All AKAPs possess not only a subcellular targeting domain but also a regulatory domain that binds type II regulatory subunit of the PKA thus enabling compartmentalization of PKA (Michel and Scott 2002). This is crucial for the signaling pathway due to the following. Firstly, by anchoring PKA close to the site of c-AMP generation, AKAPs can increase the sensitivity to incoming c-AMP signals. Secondly, by targeting PKA to multiple subcellular organelles, AKAPs can influence the enzyme substrate specificity of PKA (Dodge and Scott 2000). AKAP with binding capacity for the regulatory subunits of PKA RI has also been reported (Huang et al., 1997, Burton et al 1997. D-AKAP-1 contains dual targeting domains that are utilized based upon an amino-terminal splice that selects for the expression of either mitochondrial or endoplasmic reticulum targeting domains (Huang et al., 1999). AKAP79 acts as multivalent adaptor for PKC, PKA and PP2B at the postsynaptic densities of mammalian synapses (Klauck et al., 1996).
Figure 1.26: Targeting proteins for kinases and phosphatases. Targeting proteins are depicted for (A) the cAMP-dependent protein kinase, which is bound to its AKAPs by an amphipathic helix on the AKAP; (B) PKC, which is attached to its binding protein through protein-phospholipid interactions; (C) PP-1, which binds its targeting subunit through a consensus-binding motif (indicated in the single-letter code); (D) the B subunit of PP-2A, which binds and targets the A and C subunit complex. From Pawson and Scott 1997.
Figure 1.27: Functional motifs of AKAPs. (1) A conserved binding domain interacts with the AKAP binding surface on the regulatory subunit dimer of PKA. (2) Unique targeting domains direct AKAP signaling complexes to distinct intracellular locations. (3) Additional binding sites for other signaling components such as kinases, phosphatases, or potential substrates. From Michel and Scott 2002.
Although AKAP have been named on the basis of interaction with PKA, AKAPs can also target other signaling proteins to specific subcellular compartments, thus forming a scaffold for a multi-enzyme complex. AKAPs can also bind other signaling molecules, such as phosphatases and other kinases that regulate AKAP targeting and activate other signaling pathways acting as a multivalent scaffold, assembling and integrating signals from different pathways (Faux and Scott 1996). Accordingly, AKAPs may serve as points of integration for numerous signaling pathways acting as a “transduceosome”. This transduceosome optimizes the signal/noise ratio of cAMP/PKA stimuli traveling from the membrane to the nucleus and other subcellular components (Feliciello et al., 2001).

1.19.1 Role of Phosphorylation/Dephosphorylation in Cell Signaling

A few decades ago it was discovered that phosphorylation could reversibly alter the activity of an enzyme through the combined action of a protein kinase and a protein phosphatase (Krebs and Beavo 1979). Variations in protein phosphorylation provide the major means of enzymatic regulation now known in the body, especially in the regulation of signal transduction from cell surface receptors. Protein phosphorylation is a reversible process involving two classes of signaling enzymes: protein kinases, which catalyze the transfer of phosphate from ATP onto substrate proteins, and phosphoprotein phosphatases, which are responsible for the dephosphorylation step. To insure tight control of signaling initiated phosphorylation events, the activity of multifunctional kinases and phosphatases is carefully regulated and respond to fluctuations in diffusible second messengers such as Ca\(^{2+}\), phospholipid, and cyclic AMP (Lester et al., 1997).

Signaling networks of kinases and phosphatases frequently orchestrate the synchronization of phosphorylation/dephosphorylation events. Many proteins implicated in cell migration are phosphorylated, which regulates their folding, enzymatic activities and protein-protein interactions. Co-ordinated control of kinases and phosphatases is best illustrated during the eukaryotic cell division cycle where decisions to proceed through different stages are made by the timely phosphorylation and dephosphorylation of specific cell cycle regulators (Lechward et al., 2001). Thus, phosphorylation-dephosphorylation events act as switches or a checkpoint ensuring that cell has fulfilled all the requirements needed to proceed to the next cell cycle stage.
Errors in checkpoint control form the most prevalent basis for aberrant cell growth (Oliver and Shenolikar 1998).

1.19.2 Compartmentalization of PKA Signaling by AKAPs and Role of AKAPs in T Cells

It has always been a challenge understanding how multiple PKA isozymes with different biochemical properties are targeted in the cell, until the discovery of AKAPs. PKA association with various AKAPs may convey specificity in the cAMP-signaling pathway. AKAPs orchestrate regulation of several proteins, which serve as substrates for PKA and enzymes essential for signal transduction in different cells. This mode of regulation ensures that PKA is exposed to isolated cAMP gradients, which allows for efficient catalytic activation and accurate substrate selection. Cyclic AMP (cAMP)/PKA-signaling pathway is activated by a vast number of receptors that upon binding of their respective ligands transduce signals over the cell membrane by coupling to G-proteins. Then, G-proteins interact with adenyl cyclase on the inner membrane surface either to activate or to inhibit the production of cAMP. Receptors that activate PKA through generation of cAMP regulate major cellular processes, including cell growth and division (Boynton and Whitfield 1983), sperm motility (Tash et al., 1984), cell differentiation (Liu 1982, Schwartz and Rubin 1983, Francis and Corbin 1994), metabolism (Krebs and Beavo 1979), and gene regulation (Roesler et al., 1988). Understanding the functional complexities of how the kinase is activated in the right place and time inside the cells is crucial in many levels. This specificity is achieved in part through the compartmentalization of PKA at different subcellular locations through interaction with the AKAPs (Fig. 1.28). Examples include gravin (AKAP 250), which targets PKA RII, PKC and PKN to the intracellular cytoskeleton and filopodia of cells (Nauert et al., 1996) and AKAP79, which is targeted to the nerve terminals and anchors signal transduction enzymes important for the transmission of neuronal impulses. These signal transduction enzymes include the phosphatase calcineurin, calcium-calmodulin dependent protein phosphatase 2B, PKA RII and PKC (Klauck et al., 1996). Experiments have shown that disruption of PKA/AKAP79 impairs the transfer of excitatory neuronal impulses from one nerve to the next.
Figure 1.28: PKA/AKAP interactions in the cell. A schematic representation of the subcellular localization of AKAPs. The localization of PKA to different cellular compartments is mediated through interactions with AKAPs. A selection of AKAPs, the signaling molecules that they bind, and their subcellular location are depicted here. From Schillace and Scott 1999.
Most of the PKA-AKAP interactions have been described for non T cell lineages. The importance of these or similar associations in T cells remains largely unknown. Recently a crucial role for AKAP signaling in T cells was revealed by Williams (2002), who showed that elevated intracellular levels of cAMP in lymphocytes have a negative regulatory effect on proliferation and cytokine expression and that the cAMP signaling pathway represents a potentially important inhibitory influence on T cell activity. AKAPs contribute to the maintenance of T cell homeostasis. Disruption of the AKAP-PKA interaction stimulate T cell cytokine production and desensitizes T cells to cAMP-elevating agent. Though disruption of the AKAP-PKA interaction causes the release of cytokines, it is still under investigation whether this involves an increase in the rate of T cell proliferation. Therefore, AKAPs regulate the activity of T cells by determining the sensitivity to incoming c-AMP signals by targeting PKA to different subcellular compartments which is important in maintaining T cell homeostasis. The findings of Williams (2000) led to the speculation whether the interaction between AKAP and PKA inhibits T cell activity by rendering the cAMP/PKA pathway constitutively active, or it sensitizes the cells to very low levels of endogenous cAMP-elevating agents. Since disruption of the AKAP-PKA interaction renders T cells resistant to the inhibitory effects of cAMP this might be crucial for the treatment of retroviral infection, as it could be possible to use this approach to desensitize T cells to virally induced activation of the cAMP-PKA pathway.

1.19.3 Cyclic AMP/PKA in Cell Signaling

Cyclic AMP was discovered by Sutherland and his colleagues in the 1957 as a second messenger for the hormone adrenaline (Wosilait and Sutherland 1957, Butcher and Sutherland 1967). A large number of hormones, neurotransmitters and other signaling molecules utilize cAMP as an intracellular second messenger. Cyclic AMP regulates a number of different cellular processes such as cell growth and differentiation, ion channel conductivity, synaptic release of neurotransmitters and gene transcription. The term second messenger in relation to the cAMP means that it that carries signals from the cell surface to proteins within the cell, including protein kinases, which are subsequently broken down in cells by enzymes called phosphodiesterases. In mammals, the cAMP-dependent protein kinase PKA mediates most of the effects of cAMP. Cyclic AMP activates PKA; PKA phosphorylation of proteins produces various functional
effects. However, some phosphorylations events are functionally silent. The following should be noted for cAMP actions in mammalian cells.

- Not all cAMP gains access to all PKA
- Not all PKA has access to its substrate
- Not all cAMP has access to all cellular PDEs.

The physiological substrates for PKA include phosphorylase kinase, glycogen synthase, hormone-sensitive lipase, pyruvate kinase, tyrosine hydroxylase, PDEs, Ca\(^{2+}\) Channels, c-AMP response element binding protein (CREB), phosphatases inhibitor 1, cholesterol ester hydrolase, acetyl CoA carboxylase, myriad hormone receptors and ion channels (Shacter et al., 1988, Gettys and Corbin 1989). PKA phosphorylation is reversed by protein phosphatases; these have complex regulatory schemes, which exhibit diverse and selective phosphoprotein substrate specificities. The balance between protein kinases and phosphatases plays a key role in regulating many signaling functions in the cell (Shacter et al., 1988). Disturbing this balance can lead to disease or tumor formation.

1.19.4 Structure of PKA

PKA is composed of a dimer of regulatory subunits (R), and two monomeric catalytic subunits(C). The PKA isozymes differ mainly in their R subunit composition and are divided into type I (RI\(\alpha\)C\(_2\), RII\(\beta\)C\(_2\)) and type II (RII\(\alpha\)C\(_2\), RII\(\beta\)C\(_2\)). PKA I is predominantly cytosolic, whereas up to 75% of PKA II is compartmentalized to specific cellular compartments via binding AKAP (Rubin 1994). R and C interact with each other, the interaction involves a consensus phosphorylation sequence in the R that acts as a competitive inhibitory substrate for C and is known as the auto inhibitory domain. This auto inhibition involving a substrate-like sequence is utilized by many kinases. The biochemical properties of PKA isoenzymes account for differential cellular responses to discrete extracellular signals that activate adenylate cyclase. Upon binding of cAMP to the tetrameric PKA holoenzyme (Fig. 1.29), the active catalytic subunits are released from the regulatory subunit dimer and are free to phosphorylate substrates in their vicinity. One of the regulatory mechanisms in place to restrict the movement of C subunits and prevent nonspecific phosphorylation events is the subcellular localization of the PKA holoenzyme through association with AKAPs (Schillace and Scott 1999).
Figure 1.29: How PKA works. PKA is a holoenzyme consisting of regulatory (R) and catalytic subunits (C). When a ligand binds to a G-protein coupled receptor (Receptor 1 and receptor 2) that activates adenylyl cyclase (AC), free cAMP may stimulate and alter the activity of different cAMP receptor molecules, which includes various PKA holoenzymes. PKA is the major target for cAMP action. RI, RII and C denote subunits of PKA. Adapted from Skalhegg and Tasken 2000.
The immense diversity of cellular processes regulated by PKA implicates the presence of mechanisms generating specificity in the PKA signaling pathway in order for different signals generated by cAMP to achieve discrete cellular responses. The mechanisms include heterogeneity in R and C subunit resulting in a number of PKA isozymes with different biochemical properties and differential expression. The R subunit is differentially distributed in mammalian tissues. The distinctiveness of the PKA holoenzymes is largely dependent by the structure and properties of their R subunits. The regulatory subunits RIIα and RIIα are ubiquitous, present in almost all cell types, whereas expression of RIIβ and RIIβ are less distributed, cell and tissue-specific mainly in the brain, endocrine, reproductive and fat tissues (Scott 1991, Skalhegg and Tasken 1997, Edelman 1987, Taylor 1992). PKA regulatory subunit is linked to microtubules by its interaction with MAP2 (Obar 1989). In addition, the binding affinity to cAMP of RIIβ is lower than RIIα and much lower when compared to RIIα.

1.19.5 Regulation of Lymphocyte Function by Protein Phosphorylation: Role of Cyclic AMP and PKA

PKA-dependent phosphorylation of different cellular substrates controls multiple cell functions (Fig. 1.30), including metabolism, differentiation, gene transcription, growth, motility and more (Taylor et al., 1992, Edelman et al., 1987, Meinkoth et al., 1993, Montminy 1997). Eukaryotic cells express multiple forms of PKA regulatory and catalytic subunits which assemble together at different holoenzyme isoforms, each having distinctive expression and distribution. In addition, PKA switches off the effects of stathmin, a centrosome and microtubule associated phosphoprotein involved in the regulation of microtubule dynamics (Gradin et al., 1998). Receptors for T lymphocytes associate functionally with nonreceptor protein tyrosine kinases. Protein tyrosine kinases are implicated in signaling from the CD4 and CD8 co receptors and the beta chain of the IL-2 receptor. Protein serine/threonine kinases and several different phosphatases also participate in the intracellular propagation of antigen receptor-derived signals (Perlmutter et al., 1993). T lymphocytes express both PKA I and II, consisting of RII2C2 and RIIα2C2, respectively (Skalhegg et al., 1992). Upon T cell receptor triggering, an initial peak of cAMP and PKA activity is observed that may serve as an acute negative modulator and a negative feedback of signaling through the TCR/CD3 complex (Laxminarayana and Kammer 1996). This is followed by a decrease (40-45%) in PKA specific phosphotransferase activity (Skalhegg and Tasken 2000). cAMP has
been known to be a potent inhibitor of T cell activation and elevated intracellular levels of cyclic AMP have negative regulatory effect on proliferation and cytokine expression (Kammer 1988).

1.19.6 Protein Phosphatases, PP2A, in Cell Migration and Mitosis

Phosphatases are essential at each stage of the migration process. Phosphatases can control the formation and maintenance of the actin cytoskeleton, regulate GTPase molecular switches, and modulate the dynamics of cell matrix interaction, actin contraction, rear release and migratory directionality (Larsen et al., 2003). Protein phosphatase 2A (PP2A) controls the activities of several major protein kinase families, in particular PKB, PKC, p70 S6 kinase, calmodulin dependent kinases, MAP kinases and cyclin-dependent kinase 1 (CDK). At the submolecular level PP2A localizes to the microtubules, where it has been shown to directly bind MTs and tau in neuronal cells (Sontag et al., 1999). PP2A plays a role in the G2/M transition, particularly in negatively regulating Cdc2 protein kinase, Cdc2 activity is dependent on Thr 161 phosphorylation, and PP2A seems to be the phosphatase involved in its dephosphorylation and inactivation. PP2A has been implicated in many different functions of a living cell, including cell cycle progression and malignant transformation.

1.19.7 PKA and Mitosis

During mitosis, dramatic changes occur in the cell. The nuclear envelope breaks down, the chromosomes condense, the centrosomes move apart, membrane-bound compartments like the endoplasmic reticulum and the Golgi apparatus fragment into vesicles, and a highly dynamic bipolar spindle of microtubules replaces the relatively static interphase microtubule network (Fry 2002). During interphase, PKA localizes around the centrosome and Golgi apparatus and is tethered to these organelles through anchoring by scaffolding proteins. At the start of mitosis, PKA moves to the chromatin in the nucleus where it becomes tethered by another anchoring protein AKAP95. Although, PKA (RIIα, RIIβ) are localized at the Golgi-centrosomal region during interphase, RIIβ remains associated at this region whereas RIIα relocates to the nucleus and binds in part to the condensing chromosomes during mitosis. Thus, PKA dissociates from its centrosomal localization at metaphase-anaphase transition (Keryer et al., 1998). This magnificent biological process is mediated in part by the CDK1 (cyclin dependent kinase 1) together with other protein kinases specifically PKA which is important in
different stages of mitosis. During metaphase-anaphase transition, drop of CDK1 upregulates PKA to stimulate exit from mitosis. At the onset of mitosis, PKA moves towards its mitotic substrates where CDK1 phosphorylates PKA. This causes PKA to switch from one binding anchoring protein to another. Such change of binding affinity probably explains why PKA RIIα relocates from the centrosome/Golgi region to the nucleus at the transition between interphase and mitosis (Tasken et al., 2001).

1.19.8 PKA and Immune regulation
A full immune response requires proper presentation of the antigen, interaction with cognate ligands and fine-tuning of the proximal signaling. Fine-tuning of signaling occurs at different levels in the cell, and the levels of activation and inhibition reflect the activation status of the T cell. Activation of PKA by c-AMP elevation represents a way for regulating antigen receptor signaling (Torgersen et al., 2002). PKA regulation of NFκB activity is another way by which PKA can modulate the transcription of genes involved in immune activation.

1.19.9 PKA and The Regulation of NFκB Activity
The only source of PKA is cytosolic. Cyclic AMP elevation causes it's binding to the regulatory subunits of PKA and the consequent release of PKA catalytic subunits (Scott 1991, Walsh and Van Patten 1994). Activated PKA plays a role in the regulation of essential metabolic enzymes and the expression of target genes. NFκB Rel family members have a PKA consensus phosphorylation site (RRXS) close to the NLS (nuclear localization sequence), and some groups have reported NFκB activation following PKA phosphorylation (Blank et al., 1992, Verma et al., 1995). The transcriptional activity of NFκB is regulated through phosphorylation of NFκB p65 by PKA (Zhong et al., 1997, Skalhegg and Tasken 2000).

1.19.10 PKAc Association with IκB in The NFκB–IκB Complex
PKAc is maintained in an inactive state through association with IκBα or IκBβ in an NFκB–IκB–PKAc complex. Degradation of IκB results in activation of PKAc in a cAMP-independent manner with the subsequent phosphorylation of p65. Stimulation of cells with inducers of NFκB activity lead to degradation of IκB proteins and the consequent activation of IκB-bound PKAc. The active PKAc then phosphorylates NFκB p65 at the PKA consensus site in the Rel domain and leads to an increase in the
transcriptional activity of NFκB. Inhibition of PKA activity in cells stimulated with inducers of NFκB potently inhibits transcriptional activation by NFκB (Zhong et al., 1997).

1.19.11 PKA and The Cytoskeleton
PKA is implicated in the regulation of the MAP kinase pathway (Graves et al., 1997, Houslay and Kolch 2000). In addition, cAMP regulation of B-Raf coupled to the small G-protein Rap-1, is activated by PKA phosphorylation (Hafner et al., 1994, Vossler et al., 1997). PKA is also involved in the regulation of the phospholipase pathway; members of the PLC family are targets for PKA phosphorylation thus, providing cross talk between G-proteins and PLC (Barton et al., 1988, Liu and Simon 1996). In addition, there’s a PKA phosphorylation site on several members of PLC that map to regions important for interaction with the G proteins (Yue et al., 1998). Furthermore, actin reorganization is achieved via the Rho family of G-proteins and several data have shown that PKA-mediated phosphorylation can regulate the activity of RhoA (Lang et al., 1996, Laudanna et al., 1997, Mackay et al., 1997).

1.19.12 Protein Kinase C Adaptor Proteins
Compartmentalization of PKC isoforms requires the association with a variety of interacting proteins, including RACKs (receptors for activated C kinase), PICKs (proteins that interact with C kinase) or STICKs (substrates that interact with C kinase) and DIK (PKC-delta-interacting protein kinase) (Schechtman and Mochly-Rosen 2001, Bhr et al., 2000). Although each class of interacting protein connects PKC through a slightly different mechanism, common functions include influencing the substrate specificity of PKC and recruiting the enzyme into signalling networks (Bauman and Scott 2002). RACKs not only function in anchoring activated PKC isozymes but also other signaling enzymes (Fig. 1.31). RACK1, the anchoring protein for activated βIIIPKC, binds Src tyrosine kinase, integrin, and a phosphodiesterase. RACK2, the εPKC-specific RACK, is involved in vesicular release and cell to cell communication. Thus, RACKs are not only adaptors for PKC, but also serve as adaptor proteins for several signaling enzymes. This is important since proteins that bind to RACKs, including PKC itself can regulate cell growth and differentiation modulating their interactions with RACKs could reveal signaling pathways involved in carcinogenesis
**Figure 1.30: PKA signaling pathways.** Left: Binding of a ligand to a G protein-coupled receptor (GPCR) leads to activation of heterotrimeric G proteins (Ga, Gbg) and the stimulation of adenylyl cyclase (AC), which increases production of the second messenger molecule cAMP. An increase in cAMP promotes activation of PKA, which activates various downstream effector molecules (active effectors). One such effector is the L-type calcium channel (effector 4) whose activity is modulated by PKA-mediated phosphorylation of its C subunit. This linear signaling pathway is nonselective because a single class of receptor can engage many pathways, and different classes of receptors can activate the same downstream effector. Right: a more integrated model of GPCR signaling in which, for example, b2AR forms a multiprotein complex with specific signaling molecules such as the Ga and Gbg subunits, AC, PKA and its anchoring protein AKAP, the phosphatase PP2A, and the final effector, the L-type calcium channel. Such multiprotein signaling complexes ensure rapid and specific activation of the correct signaling pathway. Adapted from Laporte et al 2001.

**Figure 1.31: Schematic diagram of PKCβ interacting proteins.** From Bauman and Scott 2002.
and help in the identification of novel therapeutic targets (Schechtman and Mochly-Rosen 2001).

1.20 Importance of Scaffolds in Cell Functions

High fidelity signaling within the cells depends on the interlocking of proteins via highly dedicated linker adaptor proteins molecules or scaffolding proteins (Scott and Pawson 2000). Scaffolding proteins form complexes (Fig. 1.32), which ensure that enzymes and their targets are brought together promptly as soon as receptor at the cell surface is activated. This is how cells guarantee that only the right proteins combine to form a specific signaling pathway. The name scaffolding proteins was given as these proteins permanently hold groups of signaling proteins together in one place. This means that certain signaling networks are hardwired into cells, this hardwiring can increase the speed and accuracy of information transfer. It should be noted that a large number of human disorders are due to aberrant signaling in cells; examples include cancer, hereditary diseases and immune disorders. Cancer is characterized by uncontrolled cell proliferation and migration and it results from genetic mutations, where over activity of proteins in signal relaying pathways within the cells causes the cells to divide more rapidly in response to extracellular signals. Some drugs that stop this excessive signaling are currently in use to treat some cancerous conditions (Pawson and Scott 2000).

1.20.1 Scaffolding Complexes and The Cytoskeleton

In neuronal and other cells, association of protein kinases with the cytoskeleton is dependent on binding to scaffold proteins. Targeting of PKA to the cytoskeleton is achieved through AKAP (Fig.1.33) (Diviani and Scott 1999). Proteins associated with the microtubules are important for stabilization of microtubules, regulation of organ transport and anchoring of signaling enzymes (Diviani and Scott 2001). MAP2 is one of the AKAPs predominately expressed in neurons and it was shown to interact with PKA (Theurkauf and Vallee 1982). MAP2 attaches to the microtubule through its tubulin-binding domain, which binds to the C-terminus of tubulin (Hirokawa 1994). Another example is MARK, which phosphorylates microtubule associated proteins and triggers MT disruption (Drewes et al., 1997).
Figure 1.32: A model developed by Pawson and Scott 2000, demonstrating how scaffolds speed signaling in cells. The InaD Scaffolding protein operates in the cell of the fruit fly and participates the sending visual messages to the brain. Three of the scaffold’s five “PDZ” linker domains separately grasp an ion channel, an enzyme that opens the channel when light hits nearby light receptor (rhodopsin) and an enzyme that closes the channel promptly thereafter.
Figure 1.33: AKAPs and the cytoskeleton. Schematic diagram showing targeting of signaling enzymes to (A) the centrosome and microtubules, and (B) the actin cytoskeleton (gravin signaling complex) through anchoring proteins. Reproduced from Diviani and Scott 2001.
Scaffolding complexes associated with the centrosome include pericentrin, AKAP350/AKAP450/CG-NAP (refer to section 1.21 described below). Pericentrin associate with γ-tubulin and the motor protein dynein (Dictenberg et al., 1998, Purohit et al., 1999). Binding to γ-tubulin is required for the regulation of microtubule nucleation, where the association with dynein is necessary for the transport of pericentrin-γ-tubulin complexes along microtubules to the centrosome (Purohit et al., 1999, Young et al., 2000). Targeting to the actin cytoskeleton is accomplished by gravin (an antigen for the autoimmune disease myasthenia gravis) and WAVE (a member of the Wiskott-Aldrich syndrome protein (WASP) family of adapter proteins) are AKAPs (Nauert et al., 1997, Westphal et al., 2000). As mentioned before, gravin interacts with PKA, PKC and actin, this interaction is important in the regulation of cytoskeletal interactions.

1.20.2 Other Important Scaffolding Proteins

Another important scaffolding proteins is yotiao (Fig. 1.34). This molecule grasps a dual-purpose membrane spanning protein that is both a glutamate receptor and an ion channel. It also clasps a kinase that adds phosphate to, and thereby opens the ion channel when the receptor is activated by glutamate. In addition, it anchors a phosphatase; the bound phosphatase closes the ion channels whenever glutamate is absent from the receptor, thus ensuring the ions flow through the channel only when glutamate is docked with the receptor (Bauman and Scott 2002).

Fig. 1.35 and Fig. 1.36 illustrate some other scaffolding proteins, which have been shown to play an important role in various cell systems.

1.21 CG-NAP (Centrosome and Golgi PKN Associated Protein)

The scaffolding protein CG-NAP (centrosome and Golgi localized PKN-associated protein) also reported as AKAP450/AKAP350 was found to anchor several protein kinases (PKN and PKA) and phosphatases (PP1 and PP2A) on centrosome and Golgi apparatus in COS7 and Hela cells acting as a multivalent adaptor protein at these organelles (Fig. 1.37). CG-NAP interacts with PKA through RIβx subunit, PP2A through its regulatory B subunit PR130, and the catalytic subunit of PP1 (et al., 1999). Recent data suggest that CG-NAP plays a role in MT nucleation (Takahashi et al., 2002, Steadman et al., 2002). Due to the association of CG-NAP with the regulatory subunit
Figure 1.34: A schematic diagram of the Yotiao signaling complex. Yotiao directs a phosphatase/kinase-signaling complex towards its substrate, the NMDA receptor. Associated enzymes and signaling proteins are indicated. The background shows the cellular distribution of Yotiao (green), PKA (blue) and PP1 (red) in cultured neuronal cells. From Bauman and Scott 2002.

Figure 1.35: Scaffolding protein in a skin cell. Scaffolding protein is stained green and molecules attached are stained in blue and red. The cell in the left is quiet, as soon as the signaling pathway is activated by external signals; the scaffolding protein moved its target enzymes to their targets deeper in the cell this is revealed by the yellow hue (right)(Scott and Pawson 2000).
Figure 1.36: Scaffolding proteins in the nerve cell. Yotiao protein (green) and PSD-95 (red). The blue in both neurons marks the location of specific signaling enzyme (Scott and Pawson 2000).
Rlα of PKA, CG-NAP was classified as an AKAP. CG-NAP showed a high homology to yotiao, AKAP120, and relative homology to pericentrin (Takahashi et al., 1999). In addition, CG-NAP and pericentrin share a conserved domain (the pact domain) at their C-termini, which is responsible for anchoring CG-NAP and pericentrin to the centrosome (Gillingham and Munro 2000). Furthermore, CG-NAP associates with PKCe, serving as a protein scaffold for phosphorylation of this PKC isoform (Takahashi et al 2000).

### 1.21.1 CG-NAP Biography

In 1993, Keryer et al were first to identify an AKAP in purified centrosomal fractions with a molecular mass around 350 kDa (Keryer et al., 1993). Later studies by three different independent groups: Takahashi et al (Takahashi et al., 1999), Schmidt et al (Schmidt et al., 1999), and Witczak et al (Witczak et al., 1999) subsequently led to the characterization of this giant AKAP which was called CG-NAP, AKAP350, and AKAP450 respectively. Using cloning and biochemical studies of CG-NAP revealed a cDNA encoding a coiled coil protein with molecular mass of 450 kDa, identified as a PKN-interacting protein by a yeast two-hybrid screen using the N-terminal regulatory region of PKN as bait (Takahashi et al., 1999). This protein was localized to centrosome throughout the cell cycle and to the Golgi apparatus at interphase, hence named centrosome and Golgi localized PKN-associated protein. Although several groups identified its centrosomal localization, Takahashi et al., were the first to identify Golgi localization of CG-NAP, which was later confirmed by other groups (Shanks et al., 2002).

### 1.21.2 Structure and Expression of CG-NAP

The genomic region containing CG-NAP is found on chromosome 7q21 and is multiply spliced, producing at least three distinct CG-NAP isoforms as well as yotiao, a protein associated with the N-Methyl-D-Aspartate receptor (NMDA), which shares the same genomic DNA region as CG-NAP. CG-NAP contains four leucine zipper-like motifs and many stretches of coiled-coil structure (Fig. 1.38, Fig. 1.39). These structural features are thought to be involved in association with other proteins and/or homodimerization/homo-oligomerization. The original clone 2-43, the EE and BH fragments (Fig. 1.40) were bacterially expressed as GST-fused proteins to generate rabbit polyclonal antisera αEE and αBH (Takahashi et al., 1999). In addition, BLAST
search yielded two proteins that are highly homologous to partial regions of CG-NAP: human yotiao (Lin et al., 1998) and rabbit AKAP120 (Dransfield et al., 1997), corresponding to aa 1-1626 and 2049-3060 respectively. Yotiao is 99% identical to amino acids 1-1249 of CG-NAP, approximately 50% of the coding sequence for yotiao is found in exons for CG-NAP. CG-NAP also shows limited and relatively weak homology with pericentrin (Doxsey et al., 1994).

1.21.3 Detailed Molecular Structure of CG-NAP
Exons composing the DNA sequence for both yotiao and CG-NAP are labeled according to the BAC in which they are found (Fig. 1.41). S1 and S2 denote the sites of alternative splicing to generate isoforms (Schmidt et al., 1999). CG-NAP sequence contains 58 phosphorylation sites for protein kinase C, 56 for casein kinase II, and three for tyrosine kinase. PKA phosphorylation sites located at residues 1860, 2285, 2727, and 3076. PKA II binding region is found from amino acid 2174 to 2187 in the human CG-NAP. These sites are identical except for a single isoleucine to valine substitution, which should not affect the integrity of the amphipathic helix. Four leucine zipper motifs are present between amino acids 313 and 334, 391 and 412, 2651 and 2672, and 3211 and 3239 (Schmidt et al., 1999). The CG-NAP protein is 15.3% glutamate, 9.3% glutamine, and 12.4% leucine by total amino acid composition. In 62 individual locations, two consecutive glutamate residues are found, and in 21 locations, two consecutive glutamine residues are found.

1.21.4 Centrosomal Proteins with Homology to CG-NAP
Other centrosomal associated proteins sharing similarity with CG-NAP include CENP-F (20.7%), and CEP250 (20.2%). Other cytoskeletal proteins with 20-24% identity include two intermediate filament-binding proteins, plectin and trichohyalin, as well as a number of other proteins with extensive coiled-coil domains including myosin heavy chain, giantin, and the Golgi antigen GCP372 (Schmidt et al., 1999).

1.22 PKN and CG-NAP
PKN is a multifunctional protein kinase involved in both cytoskeletal reorganization and nuclear events. PKN is a serine/threonine protein kinase with a catalytic domain highly homologous to PKC in the carboxyl-terminal region and a unique regulatory domain in
**Figure 1.37:** Schematic diagram of the CG-NAP/AKAP350/AKAP40 signaling complex. Adapted from Bauman and Scott 2002.

**Figure 1.38:** Full-length CG-NAP: amino acid sequence is shown with dark boxes indicating leucine residues in leucine zipper-like motifs. The coding region of the original clone 2-43 is shaded (Takahashi *et al.*, 1999).
**Figure 1.39:** Coiled-coil of CG-NAP analyzed by COILS program. CG-NAP has coiled-coil regions that could form filamentous complex (Takahashi et al., 1999).

**Figure 1.40:** Schematic representation of various constructs of CG-NAP and the corresponding positions of cDNA sequences yielded by BLAST search (Takahashi et al 1999). Schematic representation of the structure of CG-NAP is shown on the top. LZ, leucine zipper-like motif; PP1, putative PP1 binding motif; RII, putative RII binding motif. Aligned below are locations of polypeptide P#2-43 encoded by the original clone 2-43, the EE and BH fragments bacterially expressed as GST-fused
proteins to generate rabbit polyclonal antisera αEE and αBH, respectively, and various
deletion constructs of CG-NAP. cDNAs representing high sequence homology obtained
by BLAST search, yotiao, AKAP120, and pericentrin, are also shown at corresponding
regions of CG-NAP with percentage of amino acid sequence homology in parentheses.

Figure 1.41: Multiple splicing in CG-NAP: At least three major sites of alternate
splicing exist in the CG-NAP sequence (Schmidt et al., 1999).
the amino-terminal region (Mukai et al., 1994, Mukai and Ono 1994). PKN family comprises at least three gene products, including PKNα/PRK1, PKNβ, and PRK2 (Oishi et al., 1999). The amino-terminal region of PKNα functions as a binding interface with various proteins, including small GTP-binding protein Rho (Amano et al., 1996, Shibata et al., 1996), intermediate filament proteins (Matsuzawa et al., 1997), actin cross-linking protein α-actinin (Mukai et al., 1997), phospholipase D1 (Oishi et al., 2001), the transcription factor PCD-17 (Takanaga et al., 2001), and the basic Helix-Loop-Helix transcription factor NDRF/NeuroD2 (Shibata et al., 1999). PKN also delays mitotic timing by inhibiting the mitotic regulatory phosphatase, Cdc25C (Misaki et al., 2001). PKN also links Rho to the nucleus (Marinissen et al., 2001). Recently PKN was found to regulate the p38γ MAPK signaling pathway (Takahashi et al., 2002). Using yeast two-hybrid screen and immunoprecipitation studies, Takahashi et al., (1999) showed that PKN directly interacted with CG-NAP through the N-terminal region. Certain populations of PKN and RIIα are localized to centrosome and centrosome/Golgi apparatus where CG-NAP is located. Furthermore, immunocytochemical study of human brain tissues showed that PKN is also enriched in the Golgi bodies (Kawamata et al 1998).

1.23 CG-NAP and PKCe Association

CG-NAP could coordinate the location and activity of specific enzymes and regulate the phosphorylation states of specific substrates at the centrosome and the Golgi apparatus. CG-NAP was found to interact with PKCe, serving as a protein scaffold for phosphorylation of this PKC isoform (Takahashi et al., 2000). Hypophosphorylated or immature PKCe associates with CG-NAP at Golgi/centrosome area, and adequately phosphorylated PKCe dissociates from CG-NAP as a "mature" PKCe responsive to the incoming second messenger signals.

1.24 CG-NAP and Calmodulin

Calmodulin (CaM) is the most ubiquitous and abundant Ca^{2+} binding protein in cells. Calcium binds to CaM by means of a structural motif called an EF-hand, and a pair of these structures is located in both globular ends of the protein. Several proteins that anchor γ-TURC associate with calmodulin (Sundberg et al., 1996). Data suggest that CaM and CaM-binding proteins might be required for centrosome-mediated

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microtubule nucleation by tethering γTuRC to centrosomes in animal cells (Sundberg et al., 1996, Stirling et al., 1996). CaM binds to and activates target enzymes. Ca\(^{2+}\)/CaM-dependent protein kinases include CaM-kinase kinase, CaMKI and CaMKIV, which are phosphorylated and activated by CaM-kinase kinase, and CaMKII. Subcellular localization of Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) and interaction with specific anchoring proteins may be an important mechanism contributing to the regulation of CaMKII. CaMKII is somewhat unique in that it exhibits broad substrate specificity and is found in most tissues. CaMKIV is a monomeric multifunctional enzyme expressed in a number of cell types in the body, including T lymphocytes. CaMKIV is present in the nucleus of the cells in which it is expressed and has been implicated in regulation of transcription of a number of genes including those encoding interleukin 2, which is responsible for T cell activation. CG-NAP associates with the centrosome through their carboxy terminal region, which was found to associate with calmodulin in a calcium independent fashion. However, deletions studies also revealed a calcium-dependent calmodulin-binding site (Gillingham and Munro 2000). Centrosomal targeting of CG-NAP could be mediated by calmodulin in mitosis; calmodulin could also serve as a chaperone for the carboxy-terminal region of CG-NAP and which becomes released after centrosomal targeting (Takahashi et al., 2002).

1.25 Calmodulin/NFκB Association
Jang et al in 2001 have shown that CaMKIV is a key mediator of Ca\(^{2+}\) induced gene expression. CaMKIV was found to directly associate with and phosphorylate NFκB component p65. The phosphorylation of p65 by CaMKIV resulted in recruitment of transcription coactivator cAMP-response element-binding protein. Thus, NFκB component p65 could serve as a novel downstream phosphorylation target of CaMKIV acting as one of its effector molecules (Jang et al., 2001).

1.26 CG-NAP and Pericentrin
Pericentrin (also called kendrin; Flory et al., 2000) is a large 220-kDa coiled-coil protein found in centrosomes and forms a component of the pericentriolar material (PCM). Pericentrin forms a complex with γ-tubulin ring complex, and both proteins form a unique lattice within the PCM (Dictenberg et al., 1998). Anti-pericentrin antibodies disrupt meiotic and mitotic divisions when injected into frog embryos
Pericentrin associates with PKA, and with cytoplasmic dynein, (Purohit et al., 1999; Diviani et al., 2000). This interaction with dynein is thought to play a role in the recruitment of pericentrin and γ-tubulin to centrosomes (Purohit et al., 1999, Young et al., 2000). Pericentrin has a significant homology to CG-NAP; it shares a 21% identity and 47% similarity with CG-NAP. These two large coiled coil proteins situated at the centrosome and share a stretch of highly related sequence near their C-termini outside of their coiled coil regions and PKA binding sites, this is responsible for anchoring CG-NAP and pericentrin to the centrosome (Gillingham and Munro 2000). Overexpression of the C-terminal domain of CG-NAP displaces endogenous pericentrin indicating competition for a shared binding site at the centrosome (Gillingham and Munro 2000). A large isoform of pericentrin, human kendrin also known as pericentrin B (Li et al., 2001), was shown to share high homology with calmodulin binding proteins. Furthermore, pericentrin binds to calmodulin and mutation of some residues inhibits calmodulin binding but not its centrosomal localization (Flory et al., 2000).

1.27 The PACT Domain of CG-NAP

Centrosomal localization of CG-NAP is achieved through the C-terminal domain which is also called the PACT domain (pericentrin-AKAP450 centrosomal targeting). The PACT domain is found in two carboxy-terminal splice variants of CG-NAP. Antibodies specific for one splice variant localize to the Golgi apparatus. The localization of CG-NAP to the Golgi is achieved through a distinct carboxy-terminal Golgi targeting domain located adjacent to the PACT domain. Differential subcellular targeting of CG-NAP is thus determined by the presence of centrosomal PACT domain and a distinct Golgi targeting domain (Schmidt et al., 1999). The PACT domain binds to calmodulin in a calcium independent manner, although, deletion of this domain revealed the presence of calcium dependent calmodulin site (Gillingham and Munro 2000). A similar calmodulin binding protein acts in this manner, namely phosphorylase kinase that binds calmodulin in a calcium independent fashion through multiple sites some of which however, are calcium dependent (Jurado et al., 1999). One theory imply that calmodulin could serve to chaperone for the PACT domain in the cytoplasm. Thus it could either remain bound upon centrosomal association, or be released to allow assembly with other proteins (Gillingham and Munro 2000).
Moreover, overexpression of the CG-NAP pact domain leads to defects in cytokinesis, cell cycle progression and centriole replication (Keryer et al., 2003). This displacement of CG-NAP from the centrosomes did not disrupt the centrosomal localization of pericentrin or γ-tubulin. It is hypothesized that a functional redundancy occurs between CG-NAP and pericentrin, as pericentrin can interact with PKA (Diviani et al., 2000), and CG-NAP can interact with components of the γ-tubulin ring complexes (Takahashi et al., 2002).

1.28 CG-NAP, PKA and PDE4D

The broad specificity PKA and phosphodiesterases (PDEs) have complementary roles in cAMP signaling. Whereas PKA phosphorylation is activated by cAMP, PDEs degrade cAMP and inactivate the cAMP signal (Fig. 1.42). PKA-mediated phosphorylation of PDE4D3 increases the rate of cAMP metabolism (Conti and Jin 1999, Oki et al., 2000). In addition, PDE4D3 has also been reported to associate with CG-NAP (Tasken et al., 2001). Stimulation of T cells by CD3 and CD28 induces expression of PDE resulting in IL-2 production (Li and Yee 1999).

1.29 CG-NAP in Cell Cycle Progression and Mitosis

1.29.1 Association of CG-NAP with the γ-tubulin ring complexes

Microtubule nucleation and assembly are accomplished by the γ-TuRC, which localizes to the PCM and provides template for the addition of tubulin subunits to produce a microtubule. Proteins of the γ-TuRC include GCP2, GCP3 (Tassin et al., 1998, Murphy et al., 1998, Murphy and Preble 2001). Studies to elucidate the anchoring proteins for γ-TuRCs showed that CG-NAP and kendrin anchor γ-TuRC through binding with GCP2 and GCP3 at their amino terminal regions providing sites for MT nucleation or anchoring (Takahashi et al., 2002). In addition, both CG-NAP and kendrin were found to form complexes with GCP2 and γ-tubulin in vivo. In addition, pretreatment with antibodies to CG-NAP suppressed microtubule aster formation, microtubule nucleation and decreased γ-tubulin in the centrosomes. The antibody suppression of microtubule nucleation could be through displacement of γ-TuRCs from CG-NAP in the centrosome. CG-NAP could form the matrix for integral components of PCM providing anchoring for γ-TuRC on top of serving as a targeting for important signaling
components to the centrosome. Moreover, perturbation of CG-NAP blocks mitosis at an early stage.

1.29.2 Interaction of CG-NAP and CK1: Role in Mitosis

The protein kinase CK1 (formerly termed casein kinase1) is involved in a wide range of cellular activities, primarily cell division (Behrend, Milne 2000). CK1 comprises a family of serine/threonine protein kinases, which are ubiquitous in eukaryotic cells. Members of CK1 (up to 14 isoforms) including the alternatively spliced isoforms α isoform, γ1, γ2, δ and ε isoforms. Substrates for CK1 include the cytoskeletal proteins spectrin, myosin, troponin, ankyrin and tau as well as other nuclear proteins (Gross and Anderson 1998). CK1δ and CK1ε are enriched in the centrosomes in interphase cells and at the spindle during mitosis. CK1δ and CK1ε are constitutively active signaling enzymes; they play an important role in many cellular processes including cell cycle progression. Previous reports showed that CK1δ interacted with the trans Golgi network and cytoplasmic, granular particles that associate with MTs (Behrend, Stoter 2000). Recent studies by Silliboume et al 2002 have revealed further association between CG-NAP and CK1 isoforms, CK1δ and CK1ε (Silliboume et al., 2002). This interaction might have crucial implications in cell cycle progression and centrosomal regulation. Treatment of cells with a CK1 inhibitor leads to mitotic arrest resulting in centrosomal dispersion and changes in spindle architecture (Behrend, Milne 2000) consistent with the idea that CK1δ and CK1ε might likely phosphorylate proteins involved in controlling pericentriolar structure and centrosome segregation. CG-NAP acts as a scaffold for multiple protein kinases and phosphatases at discrete subcellular locations with a high level of specificity. Anchoring CK1 to the centrosome provides yet another role for CG-NAP in cell cycle regulation and mitosis. CG-NAP may serve as a substrate for CK1 anchoring this kinase in immediate proximity to a number of physiological substrates.

1.29.3 Interaction of CG-NAP and TACC

Further studies led to the identification of another CG-NAP interacting proteins using the C-terminal domain of CG-NAP (AKAP350A) as bait in a yeast two-hybrid screen. The resulting protein is a member of the transforming acidic coiled-coil-containing (TACC) protein family, TACC4 (Steadman et al., 2002). TACC4 colocalizes with CG-NAP at the centrosome only in interphase in T cells. Early in mitosis, TACC4
translocates to the spindle apparatus accumulating at the spindle poles, whereas CG-NAP remains at the centrosome. The amino acids 247-404 of TACC4 act as the region responsible for CG-NAP interaction and corresponding centrosome localization. In addition, a separate amino-terminal TACC4 region from amino acid 1 to 380 is responsible for spindle localization. TACC4 could play an important role in spindle dynamics, as it is sequestered to the centrosome during interphase by CG-NAP interaction. However, this interaction is lost in mitosis where TACC4 translocates to the spindle apparatus (Steadman et al., 2002). There are two distinct targeting regions in TACC4, one region for CG-NAP binding and sequestration to the centrosome during interphase, the other for TACC4 localization to the spindle during mitosis. The TACC4 region responsible for interaction with CG-NAP is localized at the 247 to 404 aa of TACC4. Truncation in the amino terminal of TACC4 revealed a second subregion at the beginning of the coiled-coil TACC domain between aa 247 and 284 necessary for CG-NAP interaction. Localization of TACC4 to the centrosome, but not the spindle apparatus, requires interaction with CG-NAP. CG-NAP anchoring of TACC4 provides a unique spatial localization for TACC4 enabling its functional role in regulating the interaction with the spindle apparatus during mitosis (Steadman et al., 2002).

1.29.4 CG-NAP and The Centrosome

The centrosome encompasses multiple signaling components, and provides docking sites for regulatory molecules involved in the control of cell division. The centrosome concentrates several kinases and phosphatases including PKA type II, cdc2, NEK2 (NIMA-related kinase, the polo-like kinase and the PP1 and PP4 (Keryer et al., 2003). In addition, the coiled coil protein C-Nap1 was found to associate with the proximal ends of the centrioles and forms a complex with Nek2 and PP1 (Helps et al., 2000). CG-NAP directly interacts with the centrosomes via its C-terminal domain in a microtubule-independent manner and binds directly to centrioles. It is hypothesized that the centriole walls could be the candidates for docking CG-NAP, where CG-NAP could bind directly to centriole triplet microtubules (Keryer et al., 2002).

1.29.5 Interaction of CG-NAP with CLIC

CG-NAP not only scaffolds enzymatic signaling molecules but also conveys organelle-specific interactions with non-kinase/phosphatase effector molecules namely the chloride intracellular channel (CLIC) family of proteins (Shanks et al., 2002). The
region of CG-NAP known as pericentrin homology region (PHR), which has 41% similarity with pericentrin interacts with the CLIC family of proteins (Schmidt et al., 1999). The CLIC family of proteins consists of parchorin, bovine p64 and CLIC1-CLIC5; each protein has a high amino acid homology at the C-terminal end of the protein. All the members these CLIC proteins associate with membranous organelles. The CLIC proteins are considered integral membrane proteins that can exist as cytosolic and membrane proteins based on conformational change in the amino terminus (Harrop et al., 2001). Each CLIC family member contains multiple spliced variants. CLIC 5B associates with CG-NAP at the Golgi apparatus, although CG-NAP can potentially bind to all CLICs. BFA treatment caused dispersion of CG-NAP and CLICB in the cytoplasm. CLIC4, p64, CLIC5A, and CLIC5B all share a conserved PKA phosphorylation site in their extreme carboxyl termini (Molloy et al., 1998, Martin et al., 1999). Cyclic AMP-dependent phosphorylation of CLIC proteins by PKA could regulate their distribution and function.

1.30 Ran GTPase in Nucleocytoplasmic Trafficking and Mitosis

Ran is a highly conserved protein that belongs to the small GTPase family. Ran plays a role in the control of microtubule functions and centrosome-associated activities (Carazo-Salas et al., 2001, Fleig et al., 2000), together with a major role in nucleocytoplasmic transport (Fig. 1.43), reorganization of import and export complexes (Mattaj and Englmeier 1998, Gorlich and Kutay 1999). Nucleotide exchange and hydrolysis of Ran are catalyzed by regulator of chromosome condensation (RCC1) and Ran GTPase-activating protein (RanGAP), while the rate of nucleotide turnover is further modulated by the Ran binding protein 1 (RanBP1) and a Ran-interacting protein that increases hydrolysis and inhibits nucleotide exchange (Bishoff et al., 2002).

During interphase, Ran regulators are localized in distinct subcellular compartments, i.e. RanGAP and RanBP1 in the cytoplasm and RCC1 in the nuclei. Consequently, RanGTP is confined to the nuclei whilst RanGDP to the cytoplasm (Kunzler and Hurt 2001). Ran plays a role in nuclear envelope reconstitution at the mitosis-to-interphase transition (Zhang and Clarke 2000). Ran acts as an important factor controlling chromatin-dependent reactions in the cell, mitotic processes, such as spindle assembly during metaphase and the reformation of the nuclear envelope during telophase (Greber and Carafoli 2002). Ran-GTP is generated on M-phase chromatin and activates the
Figure 1.42: Model of the PKA-PDE signaling complex. The signaling complex can be divided into three steps, Step 1: The functions of c-AMP are mediated by PKA phosphorylation of substrate proteins. Step 2: PKA phosphorylates and activates PDE4D3. Step 3: The activated PDE4D3 degrades c-AMP and terminates the signal. From Tasken et al., 2001.

Figure 1.43: Signals and trafficking in nucleo-cytoplasmic communications. An integrated view of signals and trafficking in nucleo-cytoplasmic communications. CG-NAP is referred to as AKAP450 (red arrow) in this diagram. From Greber and Carafoli 2002.
microtubule assembly factor TPX2, thus stabilizing the plus ends of microtubules and contributing to chromosome segregation in M-phase. Ran’s role in nucleocytoplasmic transport and microtubule array formation can be summarized as follows: during interphase, Ran-GTP destabilizes importin-containing complexes that regulate protein translocation through nuclear pores, while during mitosis, at the stage of nuclear envelope breakdown, Ran-GTP produced in the vicinity of the chromosomes, similarly dissociates complexes that contain aster-promoting factors, such as NuMA (Nachury et al., 2001, Wiese et al., 2001) and TPX2 (Gruss et al., 2001). Ran-GTP also acts on microtubule dynamics by stabilizing microtubule plus-ends (Wilde et al., 2001). In eukaryotic cells, overexpressing RanBP1 yields multipolar spindles (Guarguaglini et al., 2000) by disrupting centrosome cohesion (Di Fiore et al., 2003).

1.30.1 CG-NAP Interaction with Ran GTPase at The Centrosome
Keryer et al., (2003a) studying the subcellular targeting of Ran at the centrosome, found that Ran staining colocalized with CG-NAP, and that Ran dissociated in situ from the centrosome when centrosomal CG-NAP was displaced by overexpression of C-terminal domain of CG-NAP-GFP, leading to the belief that Ran might be docked within the pericentriolar matrix through CG-NAP. This was verified by immunoprecipitation experiments, which demonstrated that Ran and centrosomal CG-NAP participate in the same protein complex. In addition, cells in which the Ran-CG-NAP-containing complex was displaced from the centrosome had defects in microtubule regrowth despite the persisting association of tubulin with the centrosome (Keryer et al., 2003a). Therefore, Ran/CG-NAP association could control the microtubule anchorage at the centrosome.

Accomplished research studies by our group on the role of LFA-1 in T cell motility include:

- Characterization of LFA-1 and its role as a signaling molecule for cytoskeletal changes in HUT-78 (Kelleher et al., 1990).
- The use of K-4 cell line as a tool in the dissection and involvement of PKC isoenzymes in T cell function (Kelleher and Long 1992).
- The expression and organization of neurofilament regulated by LFA-1 (Murphy et al., 1993).
The role of VLA-4 and D2.1 in the adherence of intraepithelial lymphocytes to human enterocytes (Kelleher et al., 1994).

Early research in our lab on demonstrated the role of PMA treatment in the activation of the TCR-CD3 complex and the development of motile phenotype in normal PBTLs (Kelleher et al., 1995).

LFA-1 crosslinking in T cells targets PKC β(1) and δ to the cytoskeleton localizing at the MTOC and microtubules(Volkov et al., 1998).

LFA-1 crosslinking in T cells induces the chemokine secretion of macrophage inflammatory protein (MIP-1)-alpha and MIP-1 beta important for directional cell motility (Murphy et al., 2000).

The critical importance of the microtubule cytoskeleton in T cell locomotion and the role of the microtubule-directed intracellular signaling pathway mediated by PKC β. PKC β activation represents an essential event in the induction of active T cell motility. PKC β deficient T cell line (K-4) was not able to rearrange its microtubule cytoskeleton or display a motile phenotype when stimulated via LFA-1 (Volkov et al., 2001).

Objectives:

- Study of the locomotory phenotype of T cells induced by LFA-1 crosslinking
- Characterize the signaling/cytoskeletal components that associate with the LFA-1 signaling complex in motile T cells
- Determine if CG-NAP is expressed in T cells
- Define if CG-NAP is a component of the LFA-1-mediated signaling complex and its functional role
- Establish the role of CG-NAP in T cell signaling and other signaling/cytoskeletal components that could interact with CG-NAP
- Examine the role of PKA in T cell locomotion
CHAPTER II
MATERIALS AND METHODS

2.1 Antibodies and Reagents

Polyclonal antisera against CG-NAP designated αEE and αBH (described in detail in chapter I, 1.22.2) were a gift from Dr. Takahashi. The abs were prepared by immunizing rabbits with bacterially synthesized GST-fused fragments of aa 423-542 and 2875-2979, respectively (Takahashi et al., 1999). Antibodies used for induction of T cell motility were of the clone SPV-L7 (mAb to the α-chain of LFA-1 purchased from Monosan Monoclonal antibodies, Sanbio, Uden, the Netherlands). Goat anti-mouse immunoglobulins (GAM) and goat anti-rabbit immunoglobulins (GAR) were from Sigma Chemical Co, St. Louis, MO. For immunoprecipitation of LFA-1, mAb to human α-LFA-1 clone YTH-81.5 was used at 6 μg/ml (Serotec, Oxford, UK). Anti-C-Nap1 antibody was a kind gift from Prof. Erich A. Nigg (Max-Planck Institute of Biochemistry in Martinsried/Munich, Germany). Anti PKC δ and anti PKC θ were from Transduction Labs, BD (New Jersey, US). PKC β used for immunostaining, was from R&D (Minneapolis, USA). Other antibodies and reagents used for immunofluorescent staining (all purchased from Sigma Chemical Co, St. Louis, MO) were α- tubulin (clone B-5-1-2), γ- tubulin (clone GTU-88), FITC conjugated Monoclonal anti-LFA-1 and anti- 58 Golgi (clone 58K-9). For blocking, we used normal goat serum (R&D). Normal mouse serum and normal rabbit serum were used as a negative control (Sigma). Nuclei were visualized with Hoechst 33342 (Sigma). Secondary antibodies were FITC conjugated goat anti-rabbit, TRITC conjugated goat anti-mouse from Sigma. Alexa Fluor® 488 and Alexa Fluor® 568 antibodies were from Molecular Probes (Eugene, OR). Human purified fibronectin was purchased from Sigma. For control studies (resting cells) cells were directly placed on Super Frost Plus positively charged slides (BDH chemicals, Poole, UK).

C-terminal GFP linked AKAP450 (CG-NAP) construct was a gift from Dr. Guy Keryer, Unité Mixte Recherche, Paris, France (Keryer 2003). Polyclonal antibodies to IκB-α were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-NFkB
P65 was from Chemicon international, Temecula, CA. Mouse monoclonal anti-dynein (D5167, intermediate chain, clone 70.1) purchased from Sigma. Mouse anti-PKA RII was obtained from Transduction Labs. In transmigration studies, cell motility was assessed using Costar transwells (Costar, Cambridge, MA). CD3 mAb OKT3 was also obtained from DAKO (Glostrup, Denmark).

2.2 Peptides
InCELLect™AKAP St-Ht31 inhibitor peptide (Promega, Madison, WI) and InCELLect™ St-Ht31 control peptide were used to inhibit the interaction of the RII subunit of PKA with CG-NAP. The presence of the stearated moiety renders Ht31 cell permeant (Vijayaraghavan et al., 1997). AKAP St-Ht31 inhibitor peptide binds to RII of PKA and disrupts interaction with AKAP. St-Ht31 control peptide varies in 2 amino acids (two isoleucine residues have been replaced by proline residues) thereby blocking its ability to disrupt the AKAP-PKA interaction, and doesn’t disrupt RII-AKAP binding.

2.3 Chemicals
Acrylamide: bisacrylamide (29:1), Nonidet P40 (NP40), phorbol-12-myristate-13 acetate (PMA), leupeptin, 2-β-mercaptoethanol, ethylenediamine tetra acetic acid (EDTA) and phenyl methyl sulphonyl fluoride (PMSF) were obtained from BDH chemicals and Sigma. PMA was dissolved in dimethysulphoxide (DMSO) (Sigma) and stock solutions were kept at -20 °C. Sodium chloride (NaCl), magnesium chloride (MgCl2), calcium chloride (CaCl2), ethanol, methanol, Tween-20, acrylamide, bisacrylamide, chloroform, glycerol, acetone, acetic acid, dimethylsulphoxide, N,N,N',N'-tetra-methylenediamine (TEMED), hydroxymethyl aminomethane (Tris) and Triton X100 were obtained from BDH chemicals.

2.4 Cell Tissue Culture
2.4.1 Source Of Cell Lines
HUT-78 (human T lymphoma line) was obtained from American Type Culture Collection (ATCC, Rockville, MD). Cells of the PKC-β-deficient K-4 clone derived from parental HUT-78 line in this research center. K-4 cells did not differ from the parental line in respect to other PKC isoenzymes (Kelleher and Long 1992).
2.4.2 Cell Culture Reagents

CO₂ independent medium, foetal calf serum, penicillin, streptomycin, L-glutamine, Hank’s balanced salt solution (HBSS) and trypsin were obtained from GIBCOBRL (Life Technologies Renfrewshire, Paisley, Scotland). ‘Lymphoprep’ density gradient was obtained from Nycomed Pharma AS (Oslo, Norway). RosetteSep was obtained from Stem Cell Technologies (Vancouver, BA, Canada).

2.4.3 Maintenance of Cell Cultures

Cell lines were grown in CO₂ independent medium supplemented with 10% foetal calf serum (FCS), 100 Units/ml penicillin, 100 μg/ml streptomycin and 2mM L-glutamine. The medium was stored at 4 °C and used within two weeks after opening, as glutamine can become enzymatically converted by serum enzymes (Griffiths 1992). A stock solution of penicillin/streptomycin and L-glutamine was prepared and stored in aliquots at -20 °C until required. Aliquots of FCS were prepared by incubation at 56 °C in a water bath for 60 min, to heat inactivate complement, and stored at -20 °C. Cells were maintained in culture flasks (Nunclon, Kamstro, Roskilde, Denmark) at 37 °C in a CO₂ incubator and examined regularly by phase contrast microscopy.

2.4.4 Cell Preparation

A suspension of HUT-78 cells (20 ml) was overlaid on ‘lymphoprep’ density gradient and centrifuged at 1200 rpm for 25 min. The interphase ring containing the cells was collected, washed twice with HBSS solution at 2000 rpm for 10 min, and then resuspended in complete medium.

2.4.5 Freezing and Recovery of Cells from Liquid Nitrogen

Stocks of viable cells were maintained by cryopreservation. Cell lines were frozen in aliquots (1x10⁶ cells/ml) in filtered FCS containing 10% DMSO (Hay 1992) in sterile cryostat tubes (Sarstedt, Numbrecht, Germany) at -70 °C, and then transferred to liquid nitrogen (Harlow and Lane 1988). Frozen cells were resuscitated by rapid defrosting and resuspended in 10 ml CO₂ independent medium. Cells were then centrifuged at 1500 rpm for 5 min, washed twice with HBSS and then resuspended in CO₂ independent medium in a tissue culture flask in a CO₂ incubator at 37 °C.
2.4.6 Cell Counting and Viability

In order to assess cell number and viability, acridine orange (AO) and ethidium bromide (EB) fluorescence staining (Lee et al., 1975) was used. Cell counting was performed by diluting cells with EB/AO working solution (1/10) (Appendix B) and counted on a Neubauer haemocytometer on an inverted microscope with immunofluorescence attachment as described (Hudson and Hay 1976). Viable cells stained green and dead cells stained orange.

2.5 Preparation of Blood Samples

2.5.1 Isolation of Peripheral Blood T Lymphocytes (PBTLs)

Peripheral blood samples were obtained by venupuncture from healthy volunteers into 10 ml heparinized vacutainers (Becton Dickinson UK Ltd., Cowley, Oxford, UK). RosetteSep™ (Stem Cell Technologies, Va, CA) technique was used for the isolation. RosetteSep™ antibody cocktail was added to the whole blood and incubated for 20 minutes at room temperature. The blood collected was then diluted 1:1 with PBS layered over ficoll, and centrifuged for 20 minutes at 1200g. Post centrifugation, the enriched cells were collected from the ficoll interface and washed once. The purity of isolated peripheral blood T cells obtained by the RosetteSep™ technique is over 97%, which is better than the results obtained using the conventional Lymphoprep density separation gradient. For cell motility studies, PBTLs were preactivated with 25 ng/ml PMA for 72 hours at 37 °C, or used in the resting state in control experiments.

2.6 Induction of LFA-1 Mediated Cell Motility

8-well Permanox plastic chamber slides (Nunc, Naperville, IL) were coated with 50-μg/ml recombinant ICAM Fc protein (r-ICAM)(R&D, Minneapolis, USA) overnight at 4 °C. In several experiments, slides were alternatively coated with goat anti-mouse immunoglobulins (1:100 v/v) overnight at 4 °C and washed gently twice with sterile phosphate-buffered saline (PBS), followed by motility inducing anti-LFA-1 antibodies clone SPVL-7 at 1:50 v/v for 60 min at room temperature. The wells were further washed twice in PBS. PBTLs or HUT-78 were loaded into the chambers at 5x10^5 cells per well. The chambers were incubated in 5% CO₂ incubator at 37 °C. For the dynamic functional studies involving high-resolution microscopy, cell were directly observed in
the chambered coverslips without fixation. In separate experiments, chamber slides were covered with human purified fibronectin (to study cell motility induced via different integrin ligands, for example, the β1 integrin ligand fibronectin) at 1mg/ml for 12 hours at 4 °C. For control studies (resting cells), cells were directly placed on Super Frost Plus positively charged slides.

2.7 Cytoskeletal Inhibitors
The cytoskeletal modulator cytochalasin D purchased from Calbiochem (Novabiochem Corp., La Jolla, CA) prepared according to manufactures instructions; the stock solution was kept at −20 °C. Cytochalasin D was used at a final concentration of 1μM dissolved in medium. Taxol and Nocodazole (Sigma) were used at final concentration of 5μM. Colchicine (Sigma) was used at 5μM. All the above cytoskeletal inhibitors were added to the medium for 30-45 minutes prior to induction of cell motility.

2.8 PKC Inhibitors
The PKC inhibitors G66976 was purchased from Calbiochem. G66976 was dissolved in DMSO and the stock solution was kept at −20 °C. Appropriate concentrations were made in cell culture medium prior to use. Crossponding final concentration of DMSO was used in vehicle controlled studies.

2.9 Immunofluorescent Staining and Microscopy
For immunostaining, the slides were fixed in 100% methanol at −20 °C for 10 min, or in some experiments with 4% v/v paraformaldehyde in PBS, and then permeabilized for 10 min in 0.2% v/v Triton X100 in PBS. After permeabilization, the slides were blocked with normal goat serum for 20 min. The slides were incubated with primary antibodies (most of the antibodies were used at 5μg/ml) for two hours at room temperature, then washed three times in 0.1% v/v Tween-20 in PBS, followed by incubation with 1:1000 v/v FITC GAR and 1:1000 v/v TRITC GAM secondary antibodies for 30 min. Immunofluorescent studies of LFA-1 and CG-NAP colocalization were performed as follows: Activated PBTLs (5x10^5 cells) were allowed to adhere to the bottom of glass chamber slides coated with recombinant ICAM Fc protein. After 2 hours, FITC conjugated monoclonal anti-LFA-1 antibodies (1:100) were added for 30 min at room
temperature. Wells were then washed gently twice with prewarmed culture medium. In several studies, Alexa Fluor®488 labeled goat anti-mouse IgG (1:100 v/v) was added for further 15 minutes to improve green signal performance for confocal microscopy. Cells were then washed gently and fixed with 3% formaldehyde in culture medium. Cells were next permeabilized in 0.1% Triton x-100 in PBS, followed by anti-CG-NAP antibodies for 1 hour and Alexa Fluor® 568 labeled goat anti-rabbit IgG (1:100 v/v) for 15 min.

Conventional microscopy and microphotography was performed on Nikon TE 300 inverted microscope equipped with Leica DC-100 color digital camera. Confocal microscopy was performed on the Perkin Elmer LCI imaging workstation (Cambridge, UK). Images were acquired using UltraView acquisition software (Perkin Elmer) and processed with Volocity 2.0.1 program (Improvision). For the 3-D acquisition, 32-50 image slices where acquired in Z-plane with 0.2 μm step intervals.

### 2.10 In Situ Immunoprecipitation

The *in situ* immunoprecipitation assay (Fig. 2.1) utilized in this study was based on microtubule resistance to non-ionic detergents at room temperature in the presence of GTP and Mg$^{2+}$ ions (Volkov et al., 2001). HUT-78 or activated PBTLs $1\times10^6$ cells /ml were exposed to LFA-1 antibodies immobilized on a 75 cm² tissue culture flasks as described above (in 2.6 Induction of cell motility). Cells were incubated either at 37 °C or 4 °C (low temperature preserves cell attachment but blocks active locomotion). After 3 hours, flasks were washed 3 times in warmed PBS to remove non-attached cells prior to lysis. Cells remaining adherent to the flasks were lysed in 2 ml microtubule friendly NP40 buffer (described in Appendix B) at 25 °C. After double washing with the lysis buffer for 1 min and finally with PBS for another minute, proteins associated strongly with LFA-1 (LFA-cytoskeletal complexes) remained attached to the flask bottom via LFA-1/ligands interactions. These complexes were extracted with SDS-containing buffer (Appendix B) and concentrated by acetone precipitation. Proteins obtained from equal number of cells kept at 4 °C or 37 °C were loaded on the gels to enable quantitative analysis. Protein loading was standardized by analysing the intensity of the immunoglobulin chains of the "in situ" precipitated anti-LFA-1. Proteins were resolved by 4.5% SDS-PAGE, electrotransferred onto PVDF membrane and probed with anti-CG-NAP antibodies and developed by ECL method (Appendix B).
Figure 2.1: Schematic representation of the in situ immunoprecipitation technique. The diagram is modified from Volkov et al., 2001. T cells were activated with anti-LFA-1 immobilized to the bottom of tissue culture flasks. Cells incubated at 37°C developed a polarized locomotory phenotype with extending uropod; those incubated at 4°C remained attached to the flasks but did not acquire active locomotion. After 3 hours, cells were lysed in optimised microtubule friendly buffer. The remaining LFA-1 cytoskeleton complexes were extracted from the flasks by scrapping into SDS-containing buffer and concentrated by acetone precipitation prior to SDS-polyacrylamide gel electrophoresis (SDS-PAGE).
2.11 Preparation of Cell Protein Samples

2.11.1 Preparation of Whole Cell Extracts
HUT-78 or PBTL cells were lysed in ice cold 1% NP40 buffer (20mM Tris-HCl pH 7.5, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin) vortexed by inverting the tubes gently upside-down every 5 min for 30 min at 1200rpm. The supernatants were removed as cell lysates concentrated by acetone precipitation and stored at -20 °C until required.

2.11.2 Protein Estimation
The protein concentration of cell extracts was determined by the dye-binding method of Bradford (Bradford 1976) in order to standardize the amount of the protein of each lysate for Western blotting (Appendix B). Bovine serum albumin (BSA) obtained from Sigma was used as the protein standard.

2.12 Immunoprecipitation
Cells were lysed with buffer containing 20mM Tris-HCl at pH 7.5, 1% NP-40, 0.15 M NaCl, 1mM EDTA, 1mM EGTA, 1 mM dithiothreitol, 1.5 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, 20 µg/ml aprotinin and 10 µg/ml leupeptin on ice, inverted briefly every 5 min, sonicated for 3 min and centrifuged at 100,000g for 30 min at 4 °C. Cleared lysates were incubated with the appropriate antibody (CG-NAP or γ-tubulin) for 2 hours at 4 °C with gentle shaking. 10 µl of protein G-Sepharose beads or protein A Sepharose beads (Amersham Pharmacia Biotech) were added and the reaction was continued for another 1 hour using the rotator at 4 °C. The resin was washed extensively 3 times in the same buffer. The bound proteins were dissociated from the beads by boiling for 5 minutes in 50 µl of SDS sample buffer and immunoprecipitation of endogenous CG-NAP was performed by a combination of polyclonal αEE and αBH (at 2 µg/ml).

2.13 Polyacrylamide Gel Electrophoresis (PAGE)

2.13.1 Preparation of Protein Samples and Molecular Weights Standards
Following protein estimation, test samples were aliquoted to yield 50 µg of sample protein. This volume was diluted 1:5 with ice-cold acetone to concentrate the protein
sample, vortexed and allowed to incubate at -20 °C for at least 30 min. The tubes were then centrifuged at 15,000g for 2 min and the supernatant was discarded. Excess acetone was allowed to evaporate at room temperature for 15 min. Samples containing 50 μg of proteins, were suspended in 20 μl 1x sample buffer (Appendix B). Samples and molecular weight markers (SDS-Sigma) standards were boiled for 5 min and centrifuged to precipitate any insoluble solids.

2.13.2 SDS-PAGE
Equal amounts of proteins in cell extracts were separated by polyacrylamide gel electrophoresis under reducing conditions according to the method of Laemmli (Laemmli 1970). For proteins with different molecular mass, the gel concentration was selected. In case of CG-NAP, 4.5% gels were used. Both resolving and stacking gels and were prepared in the order indicated in Table 2.1 A and B. 10% gels were used for detection and study of most proteins in this study, while 4.5% gels were specifically utilized for CG-NAP studies due to its large molecular weight. The APS and TEMED were added last with gentle swirling of the mixture. Gels were run at 30 mA for 1-1.5 hours until dye level was approximately 5mm from the base of the gel.

2.13.3 Western Blotting
Following electrophoresis, the proteins on the resolving gel were electrophoretically transferred to PVDF membrane (Gelman Sciences Inc., Ann Arbor, MI, USA) by semi-dry transfer as described by Towbin et al., (1979) using the Multiphor II semi-dry transfer apparatus (Pharmacia). PVDF membranes cut 9 cm x 6.5 cm in size were saturated in methanol for 10-15 s. Whatman 3mm thick filter paper cut to 9 cm x 6.5 cm was saturated in transfer buffer prior to assembly of semi dry blot sandwich construction. The transfer sandwich was assembled in the order of cathode, filter paper, PVDF membrane, electrophoresed gel, filter paper and the anode. Electrophoretic transfer was conducted at 100mA per gel for 2 h. Following transfer, the PVDF membrane was removed and processed for immunoblotting. The lane containing the molecular weight markers was stained with Commassie Blue (Appendix B) and then destained with 50% methanol. On some occasions, the gel was fixed in 40% v/v methanol, 7% acetic acid and stained in 0.01% w/v Comassie Blue in
Table 2.1: Composition of gels for PAGE electrophoresis

A: For 10% gel

<table>
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<th>Component</th>
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<th>Stacking gel (10%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>8.23 ml</td>
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<td>5.0 ml</td>
<td>--</td>
</tr>
<tr>
<td>(1.5 M Tris, pH 8.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stacking buffer</td>
<td>--</td>
<td>3.05 ml</td>
</tr>
<tr>
<td>(1 M Tris, pH 6.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acrylamide/Bisacrylamide</td>
<td>6.66 ml</td>
<td>1.33 ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>100 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>10 µl</td>
<td>10 µl</td>
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</tbody>
</table>

B: For 4.5% gel

<table>
<thead>
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<th>Component</th>
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<th>Stacking gel (4.5%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>11.9 ml</td>
<td>5.55 ml</td>
</tr>
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<td>Resolving buffer</td>
<td>5.0 ml</td>
<td>--</td>
</tr>
<tr>
<td>(1.5 M Tris, pH 8.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stacking buffer</td>
<td>--</td>
<td>3.05 ml</td>
</tr>
<tr>
<td>(1 M Tris, pH 6.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acrylamide/Bisacrylamide</td>
<td>3.11 ml</td>
<td>1.33 ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>100 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>10 µl</td>
<td>10 µl</td>
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</table>
fixative to check for efficient transfer or to perform visual comparative analysis of corresponding protein bands intensity under various experimental conditions.

2.13.4 Immunoblot Detection
Membranes were blocked to prevent non-specific binding by incubation with freshly prepared PBS containing 5% non-fat dried skimmed milk and 0.05% v/v Tween-20, Blotto Tween solution (Appendix B) for 1h with gentle shaking (Orbital Shaker S03, Stuart Scientific, UK) at room temperature. The membranes were then washed twice with PBS and incubated with specific primary antibodies in a sealed plastic bag with shaking. Following incubation with primary antibody for 1 h at room temperature or overnight at 4 °C, the membranes were then washed for 10 min in PBS-0.05% Tween-20 (x3). The membranes were then incubated with the relevant secondary antibody (horseraddish peroxidase conjugate) in Blotto PBS-Tween-20 for 1h at room temperature with gentle shaking, washed three times for 10 min with PBS-Tween and placed in PBS prior to development.

2.13.5 Enhanced Chemiluminescence (ECL)
Development of the immunoblots was performed by enhanced chemiluminescence (ECL) method. The membranes were incubated for 1 min in a solution of iodophenol (400 µM), luminol (1.25 mM) and hydrogen peroxide (0.01% v/v) in 0.1M Tris-HCl (pH 8.8) (Appendix B). The membrane was put between two acetate sheets and exposed to Kodak X-OMAT S film for 10-30 s. Exposed films were then developed using automatic developer (CURIX 60, AGFA, Type 9462/100/140, Agfa-Gevaert AG München, Germany).

2.14 Microinjection
2.14.1 Microinjection of CG-NAP Construct
CG-NAP plasmid and "empty" vector were obtained from Prof. Keryer G (Keryer et al., 2003). CG-NAP construct covering the C terminus of CG-NAP (aa 3707-3908). Direct intranuclear injection of plasmid DNA (0.2 µg/ml) was performed (Fig. 2.2) within the first 30 minutes after the initial adhesion of HUT-78 cells to the immobilized anti-LFA-1 antibodies in borosilicate glass chambered coverslips (Nunc). On the average, 50-100 cells were injected using glass capillary microneedles (inner diameter 0.1 µm) with Narishige microinjection equipment (Tokyo, Japan). Cells were analyzed 6 h post
Figure 2.2: Microinjection Technique

Note the dark shadow of the tip of the needle prior to injecting the cell (arrow).
microinjection when GFP fluorescent signal was clearly detectable. At least 25-30 GFP expressing cells were observed per each experimental condition.

2.15 Time-Lapse Video Recording and Image Analysis
Time-lapse video recording and image analysis was performed on Nikon TE 300 inverted microscope equipped with digital CCD TKC1380 color video camera (JVC, Tokyo, Japan). Analysis of acquired video and still image frames was performed on Pentium III PC using the Scion image and Adobe Photoshop 6 software.

2.16 Studies of Lymphocytes Migration Using Antibody-Coated Beads
Polystyrene beads of 0.8-μm diameter (Sigma) were washed three times in PBS, and covered with rabbit anti mouse immunoglobulins at 4 °C overnight. Unbound antibodies were removed by triple washing in PBS. The beads were subsequently covered with antibodies to LFA-1 clone SPVL-7 (1:100 v/v) for 1 h at room temperature, followed by further washing in PBS. Beads were then added to the cell-containing chambers (1000:1, bead: cell ratio) at the stage when the cells had established a locomotory phenotype after contact with the immobilized LFA-1. Unbound particles were removed by gentle washing and refilling of the chambers with warmed culture medium. Images were recorded as described above.

2.17 Transmigration Assay
PBTLs isolated from peripheral blood were preactivated with PMA as described. Anti-LFA-1 mAb or control antibodies (rabbit anti-mouse immunoglobulins) were immobilized on the upper side of the 3-μm-pore size, 6.5-mm diameter Costar transwells (Fig. 2.3). The wells were washed twice in PBS, PBTLs were added to the upper chamber of the transwells at 5 x10^6 per well and maintained in the incubator at 37 °C in 5% CO₂. PBTLs were allowed to transmigrate for 2 h and then the upper chamber was removed to stop the assay. Transmigrated cells in the bottom chamber were counted using the microscope with the micrometric grid.

2.18 Transfection of HUT-78 Cells
Figure 2.3: Transmigration assay. Phase contrast image of the Costar transwell insert (upper panel). The lower panel shows PBTLs migrating through the inserts coated with immobilized anti-LFA-1 antibodies. Migrating cells are represented by the yellow arrows.
Cell transfection was performed by either lipofection using Gene Porter™ transfection reagent (Gene Therapy Systems, San Diego, CA) or using electroporation (Bio-Rad). EGFP plasmid was from Clontech (Palo Alto, CA).

2.18.1 Transfection using Gene Porter lipofection:
DNA was diluted in CO₂ serum free media (prewarmed to 37 °C) in half the transfection volume (Table 2.2), gene porter reagent vortexed and left at room temperature for no longer than 15 min. The gene porter reagent was diluted in CO₂ serum free media (prewarmed to 37 °C) in half the transfection volume. Diluted gene porter reagent was added to the diluted DNA and mixed rapidly by pipetting up and down 4-5 times and then incubated at room temperature for 45 min to allow complex formation. The culture medium was aspirated and gene porter/DNA mix added to the cells and incubated for 4 h at 37 °C. The CO₂ independent medium with 20% FCS was added to the cells and incubated for 24 h at 37 °C. Expression of the GFP construct was evaluated 24 h after transfection.

Table 2.2: Schematic of Transfection Protocol

<table>
<thead>
<tr>
<th>DNA (μg)</th>
<th>Gene porter (μl)</th>
<th>Transfection Volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8well chamber slide</td>
<td>1</td>
<td>4</td>
</tr>
</tbody>
</table>

2.18.2 Transfection using Electroporation:
HUT-78 cells were transfected by electroporation, using a BioRad Gene Pulser (Hercules, CA) at 240 V, 960 μF. Approximately 1 x 10⁶ cells in a final volume of 400 μl were transfected with 10 μg of EGFP (enhanced GFP) construct. Cells were cultured for 32 h after transfection in 5 ml complete media before preparation of cellular extracts. Cells were harvested, washed twice in PBS, and then studied for presence of fluorescence using microscopy.
CHAPTER III

CYTOSKELETAL AND SIGNALING COMPONENTS ASSOCIATED WITH LFA-1 IN LOCOMOTORY T CELLS

3.1 Introduction

T cell migration requires a series of ligand-receptor interactions between LFA-1 on the T cell surface and ICAM-1 on the endothelium. T cells circulating in the blood are non-adherent; only when they are activated do they acquire the ability to roll and subsequently adhere to endothelial cells. This is regulated largely by interaction of the LFA-1 with the underlying cytoskeleton. Complex signaling pathways are delivered across the cell membrane to the cytoskeletal elements when LFA-1 is activated. Once activated, LFA-1 undergoes a rapid change in its ligand avidity with conformational modification in its extracellular domain and a clustering of the receptors at the membrane (Van Kooyk et al., 1999, van Kooyk and Figdor 2000, Lu et al., 2001, Salas et al., 2002, Kim et al., 2003, Ma et al., 2002, Lupher et al., 2001). The association of cytoskeletal proteins with LFA-1 plays a crucial role in regulating intracellular signaling events that affect the conformation of LFA-1 extracellular domains and promote LFA-1 clustering (Williams et al., 1994, Pardi et al., 1995, Kolanus et al 1996, Sampath et al., 1998, Stewart et al., 1998, Porter et al., 2002, Lub et al., 1997). T cell adhesion is initiated first by LFA-1 ligand binding, which is followed by changes in cell morphology leading to cell attachment, polarization, spreading, formation of additional integrin-matrix contacts (Small et al., 1999) and eventually cell motility or locomotion. LFA-1 engagement activates a number of intracellular signaling proteins including tyrosine kinases, serine/threonine kinases, and small GTP-binding proteins (Miranti and Brugge 2002).

Motile lymphocytes develop trailing extensions, which contain cytoskeletal, signaling elements and organelles including microtubules, microfilaments, centrioles, Golgi complex, mitochondria, and rough ER (Hauzenberger et al., 1995). Cross-linking of cell surface adhesion receptors by mAbs mimicking to a certain extent multivalent...
interactions with natural ligands (Miyamoto et al., 1995) has been effectively used as a model to study intracellular signaling processes mediated by integrins (Hauzenberger et al., Volkov et al., 1998). mAb-induced effects have been assessed by cell motile characteristics, homotypic aggregation or clustering of cytoskeletal or signaling proteins (Hauzenberger et al., 1997, Vermot-Desroches et al., 1995, Miyamoto et al., 1995).

LFA-1 plays a crucial role in connecting the extracellular matrix with the cell interior. To investigate LFA-1-mediated signaling in T cells, a reporter system based on the induction of cell locomotion was used. This model is more closely related to physiological phenomena taking place, for instance, at the stage of cell migration during the process of extravasation (Springer 1994, Siegelman 2001), where LFA-1 induced signaling ensues resulting in firm adhesion, cell spreading, polarization and finally transendothelial migration. The model used here in this study is adapted from the method developed by Kelleher et al., (1995) in which cells of the human T lymphoma line HUT-78 or activated human peripheral blood T lymphocytes (PBTLs) were exposed to a triggering signal via LFA-1 by immobilized mAb (mAb (i)) specific for its α-chain. In this system, T cells acquired a locomotion-associated phenotype on anti-LFA-1 mAb. Preactivation via PMA treatment was required for the development of motile phenotype in normal PBTL but not HUT-78 cells. PMA is a potent activator of the intracellular phosphorylation enzymes of the PKC family, which was found to be critical for the development of motile phenotype in PBTLs and represent an essential step in LFA-1 mediated T cell migration. The PKC’s role is essential in adhesion and motility (Downey et al., 1992, Kucik et al., 1996, Thorp et al., 1996, Volkov et al., 1998, Volkov et al., 2001) as well as activation, differentiation and survival of T cells (Baier 2003).

The cytoplasmic domains of integrins, including LFA-1 are crucial in the establishment of organized complexes with the cytoskeleton (Burridge and Chrzanowska-Wodnik 1996, Bianchi et al., 2000). These complexes are required for the transduction of the bi-directional information across the plasma membrane, interacting directly with a number of cytoskeletal and signaling molecules and participating in formation of adhesion complexes (Pardi et al., 1992, Fernandez et al., 1998, Coppolino and Dedhar 2000, Hemler 1998, Kolanus et al., 1996). LFA-1 complexes act as both recipients and generators in the signaling pathways. Cross talk between LFA-1 and the cytoskeleton
plays a central role in T cell migration (Williams et al., 1994, Coppolino et al., 1995, Lauffenburger and Horwitz 1996, Stewart et al., 1996, Penninger and Crabtree 1999). To identify molecules associated with LFA-1 cytoskeletal/signaling complex, the in situ-immunoprecipitation technique was deployed. This technique was utilized earlier by Volkov et al., (2001), based on single-ligand-triggering of T cell locomotion via immobilized recombinant ICAM-1 or motility inducing antibodies to LFA-1 that mimic the effects of natural ligands.

3.2 T Cells Locomotory Phenotype Activated Via LFA-1 Crosslinking

Resting PBTLs and HUT-78 cells are spherical in shape and non-adherent. Crosslinking of LFA-1 in HUT-78 via immobilized antibodies resulted in cell adherence followed by spreading and polarization with the development of a locomotory phenotype (Fig. 3.1) characterized by long trailing cytoplasmic projections. Similar experiments were performed using a control anti-CD3 mAb OKT3, which is highly expressed on the surface of T cells. Cells crosslinked with anti-CD3 antibodies were adherent but failed to produce a motile phenotype seen in cells crosslinked via LFA-1 (Fig. 3.1). PBTLs were studied similarly to examine the phenotypic changes associated with LFA-1 crosslinking. PBTLs (Fig. 3.2) were used either non-activated (upper panel), or preactivated with PMA (lower panel) and exposed to a triggering signal from anti-LFA-1 mAb (i). Cells shown in the lower panel display a locomotory phenotype characterized by a strong adhesion and long trailing cytoplasmic tails due to dramatic cytoskeletal changes associated with LFA-1 crosslinking. The acquisition of this locomotory phenotype was directly accompanied by active cell body translocation with repeated cytoskeletal rearrangements (also refer to Fig. 3.8 A, B). This migratory phenotype was induced in PBTLs by anti-LFA-1 mAb (i) clone SPVL-7. Clone SPVL-7 was utilized in the induction of motility in all experiments for immobilizing LFA-1.

Similar active locomotory behaviour was observed in HUT-78 cells exposed to immobilized recombinant ICAM-1 (r-ICAM-1)(Fig. 3.3).

Time-lapse video microscopy was deployed to study step by step the development of the motile phenotype. HUT-78 cells were exposed to immobilized anti-LFA-1 antibody and video recording of images was taken with 10-second intervals between the frames (Fig. 3.4). Cells started spreading with consequent cytoskeletal changes resulting in a
Figure 3.1: Phenotypic changes in HUT-78 crosslinked with anti-LFA-1 and anti-CD3 antibodies. HUT-78 exposed to immobilized anti-LFA-1 antibodies showed active locomotory behaviour characterized by long cytoplasmic extensions, whereas, cells exposed to anti-CD3 antibodies (OKT3), which is also expressed on the cell surface, did not show a similar locomotory phenotype.
Figure 3.2: Phenotypic changes in PBTLs induced by LFA-1 cross-linking in the presence and absence of PMA. **Upper panel**, Non-activated PBTLs crosslinked via LFA-1 cross-linking. **Lower panel**, PBTLs preactivated with PMA and subsequently on mAb to LFA-1. The images were taken 30 minutes after LFA-1 cross-linking. Consistent cell phenotypes were reproduced in >8 independent experiments.
Figure 3.3: Locomotory phenotype of HUT-78 exposed to human chimeric ICAM-1-Fc fusion protein (recombinant ICAM, r-ICAM) and anti-LFA-1 antibodies. Similar locomotory phenotype of PBTLs exposed to r-ICAM and anti-LFA-1 antibodies.
Figure 3.4: T cell polarization in response to anti-LFA-1 crosslinking. Time course video recording of HUT-78 dependent polarization after exposure to immobilized anti-LFA-1 antibodies.
polarized phenotype with the development of cytoplasmic projections within 3 minutes of LFA-1 crosslinking. Cell polarization involves the formation of two distinct poles: the leading edge characterized by lamellipodia, filopodia (black arrow) and cytoplasmic tails (grey arrow).

The microtubules play a major role in T cell locomotion. To study the effects of LFA-1 crosslinking on the microtubules cytoskeleton in T cells. HUT-78 cells were attached using Poly-L-lysine (Fig. 3.5, control, upper image), or cells were activated by LFA-1 (lower image of the same figure). In the cells migrating on anti-LFA-1, polarization is reflected by long cytoplasmic tails rich in microtubules, and the positioning of the MTOC between the nucleus and the cytoplasmic tails. The acquired T cell polarization is a crucial step for motility.

To study the distribution of LFA-1 in resting and motile T cells, an FITC conjugated anti-LFA-1 monoclonal antibody was utilized. In resting cells, anti-LFA-1 stained the circumferential surface of the cell (Fig. 3.6A) and the areas of contact between the cells was clearly visualized when high resolution was employed (Fig. 3.6B). Whereas, in motile cells, LFA-1 staining was distributed around the area of the MTOC (Fig. 3.7) and the base of the uropod (also refer to Fig. 4.13) in addition to membrane localization. Clustering of LFA-1 was further examined using beads coated with immobilized anti-LFA-1 Abs (Fig. 3.8 A, B). Beads were added to the chamber slides after the cells acquired a motile phenotype induced by LFA-1 as described before. The distribution of the beads and hence LFA-1 was attributed to the base of the uropod in the motile cells at the area of the MTOC. This process is dynamic as shown by the forward and backward clustering of the beads (arrow) along the uropod.

3.3 Characterization of The Multi-Component Cytoskeleton/Signaling Complex Assembled Upon LFA-1 Crosslinking

To elucidate cytoskeletal components associated with LFA-1 complex, the insitu immunoprecipitation technique was deployed. This technique detects components associated with the LFA-1 intracellular signaling complex, which forms upon LFA-1 cross-linking in T cells. Using the in situ immunoprecipitation assay described in the experimental procedure (Chapter II, 2.10), T cells were activated with anti-LFA-1
Figure 3.5: T cell polarization and the microtubule cytoskeleton in response to LFA-1 crosslinking. Immunofluorescent microscopy of microtubules, α-tubulin as a marker for the microtubules stained in green. Microtubule rearrangement in resting cells, control (upper image). HUT-78 directly plated on Poly-L-Lysine. Cells migrating on mAb (i) to LFA-1 (lower image). White arrows indicate the localization of the MTOC in control and LFA-1 crosslinked cells. Note, microtubule elongation in motile cells crosslinked via anti-LFA-1 with the formation of the uropod (yellow arrows).
Figure 3.6: LFA-1 distribution in resting T cells.

A- Live staining of FITC conjugated anti-LFA-1 in resting PBTLs.
B- Live staining of FITC conjugated anti-LFA-1 in resting HUT-78 cells
(at higher resolution)
Figure 3.7: LFA-1 distribution in motile T cells. Live staining of FITC conjugated anti-LFA-1 in motile PBTLs exposed to human chimeric ICAM-1-Fc fusion protein.
Fig 3.8 A: Dynamic clustering of LFA-1 in locomotory HUT-78. Phase contrast images taken consecutively after 30 and 35 minutes from establishing a locomotory phenotype induced by LFA-1 in HUT-78. The images show the redistribution of the polystyrene beads coated with anti-LFA-1 mAb. Arrows are indicative of the distribution of the beads and change in migration.
Fig 3.8 B: Dynamic clustering of LFA-1 in locomotory HUT-78. Phase contrast images taken consecutively after 60 minutes from establishing a locomotory phenotype induced by LFA-1 in HUT-78
immobilized on the bottom of tissue culture flasks. Cells incubated at 37 °C developed a polarized locomotory phenotype with extended trailing tails, while those incubated at 4 °C remained attached to the flasks without acquiring a locomotory phenotype. After 3 hours, cells were lysed in optimized microtubule friendly buffer. The remaining LFA-1-cytoskeleton complexes were extracted from the flasks by scraping into the SDS-containing buffer, concentrated by acetone precipitation and separated by SDS-polyacrylamide gel electrophoresis.

3.4 The Cytoskeletal Protein γ-Tubulin is a Component of The LFA-1 Complex

The MTOC is a major nucleating point in mammalian cells. γ-Tubulin is mainly concentrated at the MTOC. As mentioned before, the MTOC becomes reoriented during cell polarization, and participates in the redistribution and concentration of surface molecules in membrane caps, including different adhesion receptors (Dustin et al., 1997). In addition, the MTOC helps position the T cell secretory apparatus (Kupfer et al., 1991). The fact that LFA-1 clusters over the MTOC in motile T cells led to the speculation that γ-tubulin might be an important cytoskeletal component in the LFA-1 complex. The level of γ-tubulin association with the LFA-1 signaling complex was examined (Fig. 3.9) using the in situ-immunoprecipitation technique described previously. The amount of precipitated γ-tubulin was higher when cells were incubated at 37 °C compared to cells incubated at 4 °C. Experiments were performed at 4 °C due to the fact that low temperature preserves LFA-1 mediated adhesion but inhibits the process of cytoskeletal reassembly and signaling, which normally proceeds at 37 °C. Similar differences between T cells kept at 4 °C and 37 °C were observed for α-tubulin (Volkov et al., 2001).

3.5 PKCδ is a Component of The LFA-1 Complex

Previous studies clearly demonstrated the localization PKCβ and PKCδ at the MTOC (Volkov et al., 1998). PKCδ was studied to elucidate if it represented a component of the complex. Further analysis of the in situ immunoprecipitate revealed that PKCδ associates with the complex. The association of PKCδ with the LFA-1 associated
complex was increased at 37 °C in the locomotory cells compared to the complex from the cells maintained at 4 °C (Fig. 3.10).

3.6 PKCε is a Component of The LFA-1 Signaling Complex
PKCε is a member of the so-called atypical PKCs, which are Ca\(^{2+}\) independent. In resting lymphocytes, PKCε is located near the centrosome at the Golgi complex. The association of PKCε with α-actinin in some cells and the direct association between LFA-1 and α-actinin led to the hypothesis that PKCε might be a component of the anti-LFA-1 induced signaling complex. To prove this, in-situ immunoprecipitates from HUT-78 were probed for PKCε. PKCε was detected in the complex, the amount of precipitated proteins were higher in the cells at 37 °C compared to the cells at 4 °C (Fig. 3.11). By contrast PKCθ, which associates with the TCR-associated complex at the immunological synapse, was not detectable in the in situ-immunoprecipitates (Fig. 3.12). The absence of PKCθ from the complex could prove that the proteins previously detected in the complex are not merely due to passive or non-specific interactions.

3.7 Detection of The Motor Protein Dynein in The LFA-1 Cytoskeletal/Signaling Complex
The microtubule motor protein dynein forms inner and outer arms, each composed of heavy, intermediate and light chains (Vallee 1993, Holzbaur and Vallee 1994). Dyneins are motors that drive the molecular force for microtubule-based transport in cells (Hunter and Wordeman 2000). Dynein is responsible for retrograde, centripetal transport of endosomes, lysosomes and elements of Golgi apparatus (Theulaz and Rfeffer 1992, Holzbaur and Vallee 1994). An impressive body of evidence proved that microtubule-based motors such as dynein can influence microtubule dynamics (Hunter and Wordeman 2000); furthermore, additional studies suggested that the presence of dynein increased the dynamic behavior of microtubules.

Using the in-situ immunoprecipitation technique, the intermediate chain of dynein was examined to determine if it participates in the complex. A band was detected around the 70kDa range; the amount of precipitated proteins was higher at 37 °C compared to the 4 °C band (Fig. 3.13).
Figure 3.9: Western blot detection of γ-tubulin in the LFA-1 cytoskeletal /signaling complex in HUT-78. γ-Tubulin represents a component of the LFA-1 complex. γ-tubulin association with the complex is significantly increased at 37 °C compared to 4 °C.

Figure 3.10: Detection of PKCδ in the LFA-1 complex. PKCδ association with the complex is higher at 37 °C compared to that at 4 °C.
Figure 3.11: Detection of PKCε in the anti-LFA-1 complex. Western blot analysis of the in situ-immunoprecipitate of HUT-78 crosslinked with LFA-1 at 37 °C, 4 °C. PKCε association with the complex is higher at 37 °C compared to the non-motile cells at 4 °C.

Figure 3.12: Kinase association specificity of in situ immunoprecipitated LFA-1-assembled complex. PKC isoform with distinctive cellular functions (PKC0) was not detectable in the in situ-LFA-1 immunoprecipitates. PKC0 was detected in the total cell lysates in HUT-78 around 80kDa. Equal amounts of the “in situ” precipitating anti-LFA-1 heavy (H) and light (L) chains were also recognized as “non-specific” bands by secondary HRP-anti mouse conjugates.
Figure 3.13: Dynein in the anti-LFA-1 induced complex in T cells.
Detection of dynein intermediate chain using western blot analysis of the in situ-immunoprecipitates from locomotory HUT-78 cells at 37 °C and non-motile cells at 4 °C. The blot was probed with monoclonal anti-dynein (intermediate chain).
3.8 PKA RII: Another Protein Kinase as A Component of The LFA-1 Induced Signaling Complex

Protein phosphorylation is a pivotal mechanism in signal transduction, and the activities of protein kinases and phosphatases are highly regulated. PKA is activated as a result of interaction with c-AMP. The role of PKA RII in cell motility is not completely identified, and the available data is largely controversial. Studies in Dictyostelium discoideum showed that PKA activity must be suppressed in order for cells to elongate along a substratum and crawl (Zhang et al., 2003), elevation of cAMP diminished neutrophil adhesion to endothelial cells (Derian et al., 1995). On the other hand, other data demonstrated that PKA inhibition produced a dramatic decrease in lymphocyte random motility and chemokine-directed chemotaxis (del Pozo et al., 1999). Moreover, PKA has been reported to be involved in cell polarization and uropod formation induced by chemokines (del Pozo et al., 1995). In addition, Whittard and Akiyama (2001) demonstrated that PKA is essential for β1 integrin induced cell substrate adhesion (Whittard and Akiyama 2001). Thus, we decided to investigate whether this kinase is involved in the process of T cell motility.

To analyse the presence of PKA RII in cells crosslinked with LFA-1, the in situ immunoprecipitation technique was performed. PKA RII was detected in the LFA-1 signaling complex (Fig. 3.14). However, in locomotory T cells the amount of PKA RII precipitated in the LFA-1 induced complex was less in contrast to the non-motile cells incubated at 4 °C. This suggests that PKA RII may be inhibited during the process of T cell locomotion.

3.9 NFkB Rel A and IkBα association with LFA-1

Nuclear translocation of the NFkB family of transcription factors is a proximal step in the signal transduction of a pleiotropic group of genes (Ghosh and Karin 2002, Dixit and Mak 2002). Rel/NFkB family is composed of heterodimers and homodimers involving Rel A (p65) and p50. Under unstimulated conditions, p65 is restricted to the cytoplasm by inhibitory proteins of the IkB family members. Immunologic stimuli induce posttranslational modifications with subsequent phosphorylation and ubiquitination of IkB, particularly IkBα, thereby freeing the p50/p65 complex to
translocate to the nucleus. Direct interaction between NFκB and microtubules has not been established in any cell system. IkB on the other hand has been found to interact with motor protein dynein (Crepieux et al., 1997) and to colocalize with α-tubulin in Hela cells. In addition, microtubule depolymerization by drugs leads to IkB destruction through a kinase-dependent mechanism allowing NFκB to bind DNA and stimulate transcription (Rosette and Karin 1995).

In view of the major involvement of tubulins in LFA-1 mediated signaling, we aimed to determine whether IkBα was related to microtubules, if IkBα is participating in LFA-1 mediated signaling cascades in T cells. Similar experiments were performed on the NFκB component: Rel A (p65).

3.9.1 IkBα, NFκB Rel A: Transcriptional Participants in The LFA-1 Induced Signaling Complex

IkBα was detected in the LFA-1 associated complex in HUT-78 cells (Fig. 3.15 A) using in situ immunoprecipitation. To verify that IkBα association in the complex is specific to LFA-1, the antibody to a different adhesion molecule, ICAM-1 (anti-CD54) was utilized in the study. Anti-ICAM-1 activates intracellular signaling pathways distinctive from anti-LFA-1. Anti-ICAM-1 was used as a control (Fig. 3.15 B). Cells activated with anti-CD54 immobilized on the bottom of the tissue culture flasks at 37 °C only adhered to the flasks but did not polarize or acquire active locomotion. Similar experiments were performed to deduce if any of the NFκB components associate with the LFA-1 associated signaling complex. The NFκB Rel A (p65) was identified in the complex. However the amount of precipitated proteins did not show significant difference at 4 °C and 37 °C (Fig. 3.16).

3.10 Discussion

T cell adhesion and migration is a finely regulated and organized but complex process entailing coordinated assembly and disassembly of adhesion complexes, controlled in part by reversible, activation-dependent up-regulation of β2-integrin function (Mitchison and Cramer 1996, Stewart et al 1996, Hauzenberger et al., 1997). Considerable progress has been made in identifying adhesion components and the molecules they regulate. The breakthrough in imaging technology has allowed better characterization of components
PKA RII was detected in the complex, the amount of precipitated proteins are higher at 4 °C in the non-motile HUT-78 compared to the motile cells at 37 °C.

Figure 3.15 A: Identification of IκB α in the multicomponent signaling complex assembled upon LFA-1 cross-linking. Analysis of the in situ immunoprecipitates in HUT-78 revealed relatively equal amounts of IκBα. T represents the total cell lysates.
Figure 3.15B: IκB α in the LFA-1 associated complex but not in the ICAM-1 linked complex at 37 ºC. Similar experiment to that in figure 3.15A was performed using in situ anti-ICAM-1 together with the in-situ form the anti-LFA-1 in motile cells at 37 ºC.

Figure 3.16: NFκB-p65: a potential component in the LFA-1 complex. In situ immunoprecipitation detected a weak band corresponding to the molecular weight of NFκB -p65 in non-motile and motile HUT-78 cells.
associated with adhesion molecules, and monitoring these components during migration. Circulating lymphocytes are spherical yet acquire a polarized morphology when activated (Anderson and Anderson 1976). The change from a non-adherent circulating to a firmly adherent phenotype is essential for efficient immune surveillance. This is achieved largely by interaction of LFA-1 integrin receptor on the T cell and its ligands (Springer 1994, Stewart et al., 1996, Hauzenberger et al., 1997). LFA-1/ligand interactions participate in the guided movement of leukocytes from the bloodstream across the vascular wall toward the site of inflammation or injury (Porter et al., 2002).

At the onset of T cell migration, a cascade of intracellular signaling events is initiated, resulting in an organized redistribution of cell surface receptors and cytoskeletal elements. Two distinct cell compartments characterize motile T cells. First, the leading edge at the front of the cell where β-actin is enriched. The second compartment, the uropod or the trailing edge, which protrudes from site of the contact with the substratum, represents the area where important motor proteins, the Golgi apparatus and microtubules directed from the MTOC are packed (Serrador et al., 1997, Ratner et al., 1997). Leukocyte polarization requires protein cycling either to the leading edge, to a central polarizing compartment or into the uropod. The net forward movement of the cell over stable adhesions leads to an accumulation of adhesion components towards the cell away from the leading edge (Friedl and Brocker 2000). Motility is characterized by multiple repeated cycles of cytoskeletal rearrangements that are largely based on coordinated networks of intracellular signaling. The morphological asymmetry characteristic of a polarized T cell reflects the spatial rearrangement of cytoskeletal proteins (Drubin and Nelson 1996). Studies have shown that activated T lymphocytes are highly motile with speeds reaching as high as 7-10 μm/min, which is ten times faster than fibroblasts (Gudima et al., 1988).

T cell adhesion and locomotion requires intact functioning of the two major cytoskeletal systems, actin and microtubules. It is generally documented that the integrity of actin and microtubule cytoskeleton provides an essential structural support for T cell activation (Cerottini and Brunner 1972, Monks et al., 1998, Serrador et al., 1999). Actin based lamellipodia and filopodia are important for adhesion and cell spreading (Mitchison and Cramer 1996, Stewart et al., 1996). This is dependent on the activation of the small GTPases such as Rho. The Rho family of small GTPases is a key regulator
of adhesion dynamics at the front of the cell (Nobes and Hall 1995, Hall 1998). Rho is also involved in disassembly of adhesions in the rear of the cells (Smith et al., 2003).

LFA-1 can mediate strong binding of T cells to ICAM-expressing cells, or to purified immobilized ICAM ligands (Springer 1990). In addition, the adhesion and cytoskeletal rearrangements in T cells can be triggered by recombinant soluble ICAM-1-Fc fusion protein (Stewart et al., 1996). This study deployed a reporter system utilizing the induction of cell locomotion via natural LFA-1 ligands based on previous models (Hauzenberger et al., 1997, Volkov et al., 1998). In these models, mAbs mimicking to a certain extent multivalent interactions with natural ligands were used to study intracellular signaling processes mediated by integrins. This model could be considered as physiologically relevant triggers for LFA-1 activation. Active conformation of LFA-1 is induced via receptor-mediated signaling.

Clustering of LFA-1 initiates the activation of other intracellular signaling pathways (Miyamoto et al., 1995). Clustering would provide the means of localizing active integrins, thereby increasing the collective signal necessary to generate and remodel the cytoskeleton (Porter et al., 2002). Unique signaling via LFA-1 is highlighted by experiments demonstrating activation of phosphorylation events upon LFA-1 engagement (Wang et al., 1995).

This study shows that clustering of LFA-1 associated with its signaling function also occurs at the membrane sites adjacent to the MTOC. LFA-1 interaction with the cytoskeleton has not been completely elucidated (Sanchez-Madrid and del Pozo 1999). Cytoskeletal and signaling proteins associated with LFA-1 are not well recognized. Cytoplasmic proteins identified so far that directly bind to LFA-1 and regulate its function include cytohesin-1, talin (Liddington and Ginsberg 2002) and α-actinin (Sampath et al., 1998). Cytohesin-1 associate with LFA-1 and regulate LFA-1 mediated adhesion in response to activation of phosphatidylinositol 3-kinase (Nagel et al., 1998).

The microtubule cytoskeleton plays a key role in different stages of T cell locomotion, including polarization and the direction of pseudopodial route (Bershadsky et al., 1991, Soll and Wessels 1998). MTs are likely to be critical to the spatial organization of signal transduction. MTs themselves are also affected by signaling pathways and thus may
contribute to the transmission of signals to downstream targets. This study shows that LFA-1 mediated signaling in T cells involves the microtubule cytoskeleton. The MTOC is one of the key orchestrating compartments required for cell functioning within the cell. It becomes reoriented during cell polarization (Dustin et al., 1997) and, in addition, the MTOC helps position the T cell secretory apparatus. The reorientation of the MTOC is followed by the polarized concentration of cytokines and cytotoxic mediators at the T cell-APC surface (Kupfer et al., 1991). In a polarized activated T cell, the MTOC localizes in the region between the cell body and the long cellular extension, which is characterized by strong anchorage to the extracellular ICAM-1 ligands (Rodriguez-Fernandez et al., 1999). Data presented here show that LFA-1 induced motility results in dramatic rearrangement of the microtubule-based cytoskeleton and involves signaling pathways dependent on protein kinases.

Cells use motor proteins to modulate microtubule polymerization dynamics (Hunter and Wordeman, 2000). Dynein is recruited to a variety of locations within the cell, at different times in the cell cycle. The presence of dynein increases the dynamic behavior of microtubules. In addition to tubulin, the microtubule motor protein dynein could constitute an integral component of the LFA-1 mediated complex. In the present study, dynein has been shown to participate in the complex; however, more studies are required to clarify the precise role of dynein in the process of T cell motility.

Protein kinases such as PKCβ were shown to play a key role in T cell locomotion. PKC activation represents an essential event in induction of active T cell motility. In crawling T cells triggered via LFA-1 cross-linking, PKC isoenzymes β(I) and δ, are targeted to the cytoskeleton localizing at the area MTOC and microtubules (Volkov et al., 1998). The data presented here defined PKC δ as a component of the complex in addition to the previously identified PKC β(I) (Volkov et al., 2001). PKC δ could interact with PKC β(I) to control T cell motile functions since both proteins are localized at the area of the MTOC. Although, PKC β(I) redistributes along the microtubules during motility, PKC δ, however, lacked this character.

Another PKC isozyme, whose role in T cell function is still not determined, is the PKCε. PKCε is localized at the Golgi complex close to the area of the MTOC. Due to this specific location, we considered it appropriate to study if this particular PKC

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isoform in LFA-1 associated complex in addition to previously described isoforms β and δ. Results presented here showed that this PKC isoform integrates with the complex, this is pronounced more in motile cells. PKCe/LFA-1 interaction and PKCe localization at the Golgi complex could provide a novel role for LFA-1 induced cytokine secretion and the relevant secretory pathways, however, these interaction needs further investigation.

Integrins are known not only as adhesion receptors but also as signaling molecules that can signal across the plasma membrane in both directions (Hynes 2002). Leukocyte integrins transduce signals, which affect genetic programs, consequently defining cell phenotype and function. Many integrin signals converge on cell cycle regulation, directing cells to live or die, to proliferate, or to exit the cell cycle and differentiate (Giancotti and Ruoslahti 1999). Binding of extracellular ligands induces, in turn, structural changes that convey distinct signals to the cell interior (Xiong et al., 2003). LFA-1 not only plays a key role in adhesion and locomotion in T cells but it also controls signal transduction events leading to gene expression. LFA-1 engagement enhances transcriptional activation of numerous genes by regulating its association with transcription modulators such as JAB-1 (Rossetti et al., 2002).

Nuclear translocation of the NFκB family of transcription factors is a proximal step in the signal transduction of a pleiotropic group of proinflammatory genes. However, NFκB/ microtubule interaction has not been previously examined. Many protein kinases have been found to bind to microtubules in vivo (Gundersen and Cook 1998, Lim et al., 2003, Kawauchi et al., 2003). In the resting cell, NFκB is held inactive in the cytoplasm by its association with the inhibitory factor IκB. Stability of IκB apparently depends on its anchorage to microtubules, possibly by its ankyrin repeat domain (Rosette and Karin 1995). When microtubules depolymerize, IκB is released, becomes degraded, and releases NFκB to allow its nuclear import (Gundersen and Cook 1998). The detection of RelA (p65) in the LFA-1 complex provides evidence that NFkB could potentially modulate LFA-1 functions and NFκB localization could be regulated by microtubules. Thus IκBα was studied to see whether it is a component of LFA-1 signaling in T cells, being a component of the cytoplasm at the same time regulated by microtubules (Rosette and Karin 1995). IκBα detection in the LFA-1 signaling complex highlights a
new interaction between LFA-1 and IκB, however, it was not clear if the levels of IκBα varied during motility. Thus, further studies are needed to explain their functional significance.

The PKA regulatory subunit is linked to microtubules by its interaction with the microtubule-associated protein MAP2 (Rubino et al., 1989, Obar et al., 1989). PKA is a negative regulator in T cell activation. The elucidation of PKA RII as a participant in the LFA-1 mediated complex implies that it could potentially play a role in LFA-1 mediated signaling. It could be speculated that PKA role could be to limit LFA-1 signaling thus, pointing out a modulatory role for PKA. This is verified by its increased association in the complex in the non-motile T cells compared to the locomotory cells (i.e. 4 °C and 37 °C respectively). More studies have demonstrated that signals that cause the degradation of IκB result in activation of PKAc in a cAMP-independent manner with the subsequent phosphorylation of p65 (Zhong et al., 1997). Therefore, this pathway represents a novel mechanism for the cAMP-independent activation of PKA and the regulation of NFκB activity.

Taken together, these data demonstrate that LFA-1 mediated signaling in T cells involves the formation of a multicomponent protein complex rich in cytoskeletal and signalling molecules. The question remaining is how this multicomponent LFA-1 protein complex is targeted in the cell, and how is such precise signaling accomplished? It could be concluded that perhaps specific adaptor and or scaffolding proteins participate in this process.

Scaffolding proteins represent a relatively recently discovered novel class of molecules involved in signal transduction (Scott and Pawson 2000). Scaffolding proteins can hold multiple kinases, phosphatases and ensure that various signaling molecules act almost simultaneously (Scott and Pawson 2000). Understanding if scaffolding proteins play a role in LFA-1 mediated signaling in T cell will be discussed in the following chapter.
CHAPTER IV

THE SCAFFOLDING PROTEIN CG-NAP: An INTEGRATING COMPONENT OF LFA-1 INDUCED CYTOSKELETON/SIGNALING COMPLEX IN LOCOMOTORY T CELLS

4.1 Introduction

The elucidation of multiple protein kinases and cytoskeletal elements associated with the LFA-1 cytoskeleton/signaling complex not only adds to the complexity of LFA-1 mediated signaling in T cells, but also highlights the importance of each component. Stimulation of signaling pathways is dependent on protein phosphorylation, which requires activation of kinases and phosphatases. The compartmentalization of these dynamic signaling molecules is crucial for effective signaling. The study of how cells achieve this task revealed the existence of scaffolding proteins; these scaffolds allow spatial control of signaling processes inside cells (Faux and Scott 1996, Burack and Shaw 2000). With the discovery that signaling molecules are scaffolded in specific locations within the cell was a major breakthrough in the understanding of how signal transduction is controlled. Furthermore, emerging evidence proves that signaling molecules are transported into complexes to and from functional regions of the cell by large perinuclear cytoplasmic components (Webb et al., 2002).

Scaffolding proteins localize signaling molecules near their precise targets ensuring proper signal transduction. AKAPs (A-Kinase anchoring proteins) are a class of scaffolding proteins required to anchor the regulatory subunit of PKA with other cellular components to specific cytoskeletal locations within the cells (Lester and Scott 1997). Moreover, AKAPs can assemble protein kinases and phosphatases close to their specific substrates. Most AKAPs have additional binding sites for other signalling molecules, making them points for integration of signals from diverse sources. The
function of each AKAP is defined by its localization and proteins bound to it (Keryer et al., 1993). A number of adaptor proteins have been shown to play a role in T cells, but the role of AKAPs or scaffolding proteins was not examined. The giant scaffolding protein CG-NAP, which is a product of multiply spliced gene family localizing at the centrosome and Golgi apparatus, scaffolding an array of kinases and phosphatases was characterized in a number of cells but not T cells (Takahashi et al., 1999). CG-NAP was a likely candidate for this study; as a scaffold that could regulate the phosphorylation, dephosphorylation and/or motile characteristics in T cells. The results of the extensive studies of CG-NAP role in T cells, its expression, localization and association with LFA-1 are presented in this chapter.

Aims of this study:

1. To establish whether CG-NAP is expressed in T cells
2. To determine if CG-NAP is part of the LFA-1 cytoskeletal/signaling complex in locomotory T cells
3. To identify the functional importance of CG-NAP in the LFA-1-associated complex in motile T cells

4.2 CG-NAP Expressions and Localization in T Cells

To detect the presence and localization of the scaffolding protein CG-NAP in T cells, immunofluorescence studies were initially employed. Double immunofluorescence staining of normal and lymphoma T cells for CG-NAP and α-tubulin was performed to determine the subcellular localization of CG-NAP (Fig. 4.1 and Fig. 4.2). In resting, non-adherent cells, CG-NAP staining was detected as compact perinuclear spots around the centrosome. In addition, there were some weaker stained diffuse scattered patterns present throughout the cell in the cytoplasm. Cells activated on immobilized anti-LFA-1 mAb (Fig. 4.1, lower panel) acquired a polarized phenotype with trailing extensions. In these locomotory cells, CG-NAP was localized to the area of the centrosome, and distal to the centrosome in a distribution related to the microtubules. Similar pattern of CG-NAP staining was also detected in resting and locomotory PBTLs (Fig. 4.3). Western blotting in HUT-78 cells (Fig. 4.4) and PBTLs (Fig. 4.5) confirmed the presence of CG-NAP as a high molecular weight species (approximately 450kDa). Colocalization of CG-NAP and α-tubulin was also verified using confocal microscopy.
Figure 4.1: Subcellular localization of CG-NAP in resting and motile HUT-78 lymphoma cells. Upper panel, (left to right): staining of resting cells for CG-NAP, α-tubulin and merged images, respectively. CG-NAP staining localizes at the centrosome and in a scattered network around it. Lower panel, motile HUT-78 cells activated by immobilized LFA-1 antibodies. CG-NAP is localized to the area of the centrosome and to some extent along the microtubules in the trailing uropod.
Figure 4.2: Distribution of α-tubulin (top image) and CG-NAP (lower image) in the identical microscopic fields. Distribution of α-tubulin (top image) and CG-NAP (lower image) in the identical microscopic fields. Locomotory phenotype was induced by LFA-1 cross-linking and cells were subsequently exposed to mild detergent extraction (0.2% Triton X100).
Figure 4.3: Localization of CG-NAP in resting and locomotory PBTLs.

In resting PBTLs, CG-NAP staining (green) is attributed to the centrosome and some scattered patterns throughout the cytoplasm. The scattered component of CG-NAP can be visualized using higher magnification of the cell (inset on the right extracted from the figure in the upper panel). The lower panel, in motile cells CG-NAP staining is also present along microtubules in the trailing uropod (arrows), similar to Fig.4.1.
Figure 4.4: Detection of CG-NAP in HUT-78 total cell lysates. Total cell lysates from HUT-78 cells were probed with anti-CG-NAP antibodies or isotype matched IgG normal rabbit serum, NRS. Western blotting confirmed the presence of CG-NAP as a high molecular weight species (approximately 450kDa), which corresponds to the predicted molecular weight of CG-NAP.

Figure 4.5: Detection of CG-NAP in PBTLs total cell lysates. CG-NAP was identified in normal human peripheral blood lymphocyte cell lysates. The band detected was exactly at the same level as in HUT-78 cells.
to establish spatial distribution of both proteins in locomotory T cells in detail (Fig. 4.6).

Taken together, these findings demonstrate the expression of CG-NAP in normal T lymphocytes and the lymphoma T cell line HUT-78. In addition, CG-NAP undergoes dramatic redistribution along microtubules in proximity to the centrosome upon LFA-1 crosslinking.

4.3 CG-NAP Co-Localizes with γ- Tubulin at The Centrosome
The relation of the compact and scattered CG-NAP staining components to the respective intracellular structures was examined. CG-NAP colocalized with γ- tubulin to the centrosome using co-staining with γ-tubulin antibodies in resting and LFA-1 activated HUT-78 cells (Fig. 4.7A, B). Colocalization with γ-tubulin confirmed that CG-NAP is localized to the centrosome.

4.4 Interaction of CG-NAP with γ- Tubulin
To seek further evidence for CG-NAP and γ-tubulin colocalization, immunoprecipitation of CG-NAP and γ- tubulin (Fig. 4.8) was deployed. HUT-78 extracts were immunoprecipitated with anti-CG-NAP or control rabbit serum followed by immunoblot with anti-γ- tubulin. Immunoprecipitates from anti-CG-NAP lysates detected a band coinciding with the molecular weight of γ-tubulin 48kDa, a band appearing at the same position as the one detected from the total cell lysates of the cells. Later studies by Takahashi et al. 2002, gave further evidence on γ-TuRC and CG-NAP colocalization, their results showed that the amino-terminal region of CG-NAP associates with γ-TuRC (γ- tubulin ring complexes) through interaction with GCP (γ-tubulin complex protein), GCP2 and/or GCP3 (Takahashi et al., 2002).

4.5 CG-NAP Co-Localizes with The Golgi Apparatus
HUT-78 cells were also double stained for CG-NAP and the Golgi apparatus using anti-Golgi p58 marker antibody. The non-centrosomal scattered pattern of CG-NAP colocalized but not exclusively with the Golgi staining (Fig. 4.9). Takahashi et al. (1999) detected similar colocalization patterns in Hela cells. To study if other centrosomal
Figure 4.6: Confocal microscopy of a motile PBTLs crosslinked via LFA-1. CG-NAP stained green and microtubules (a-tubulin) stained red, the area of colocalization is seen in yellow.
Figure 4.7A: Localization of CG-NAP at the centrosome in resting and motile HUT-78. Intracellular staining for γ-tubulin (red) (centrosomal marker) and CG-NAP (green) demonstrates co-localization of the centrosomal component of CG-NAP and γ-tubulin at the centrosome/MTOC area.
Figure 4.7B: Double immunofluorescent staining of CG-NAP (green) and γ-tubulin (red) in locomotory HUT-78 triggered via LFA-1 crosslinking (upper photo). Lower photo, high magnification images of the HUT-78 cells activated and stained as above. Note, CG-NAP colocalized with γ-tubulin at the area of the centrosome (MTOC).
Figure 4.8: Co-precipitation of CG-NAP and γ-tubulin in T cells. (i) Detection of γ-tubulin in total HUT-78 cell lysates (Total) and in immunoprecipitates obtained with anti-CG-NAP (α-BH) and control rabbit serum (NRS). MW molecular weight marker band corresponds to 50kDa. Specific bands of γ-tubulin appear in the total lysates and α-EH immunoprecipitate at approx. 48kDa. ii) Cell lysates from HUT-78 cells immunoprecipitated with anti-γ-tubulin (α-γ-tubulin), control antibody anti-IE (α-IE) followed by immunoblotting with anti-γ-tubulin antibody. Heavy chains (H) of the precipitating antibody indicated by the arrowhead. The molecular weight of the heavy chains is around 52kDa. Total, γ-tubulin detected in total cell lysates.
Figure 4.9: Co-localization of CG-NAP with the Golgi apparatus. In both resting cells (upper panel) and motile cells triggered via anti-LFA-1 (lower panel), the scattered component of CG-NAP staining (arrows) localizes but not exclusively to the Golgi apparatus visualized with anti-p58 k antibody (Golgi marker), as seen on the merged overlay panel.
proteins undergo similar redistribution upon LFA-1 crosslinking, a centrosome-associated protein C-Nap1 (Fry et al., 1998) was examined. C-Nap1 associates with the centrioles but lacks similar Golgi and microtubule association (Fig. 4.10 and Fig. 4.11). Cytoskeletal modulators were used to determine if C-Nap1 centrosomal localization is microtubule dependent (Fig. 4.12). In cells treated with nocodazole, which depolymerizes the microtubules, C-Nap1 was still attached to the centrosome with little or no change in the intensity of the characterized centrosomal staining. In cells treated with a microtubule stabilizer taxol, C-Nap1 was still associated with the centrosome with no change in its localization. Thus, C-Nap1 association with centrosomes is independent of microtubule assembly. Similarly, cytochalasin, which inhibits actin polymerization, had no effect on C-Nap1 centrosomal localization. These results show that C-Nap1 failed to redistribute on LFA-1 activation demonstrating that LFA-1 activation involves the participation of a pericentriolar component not associated with C-Nap1.

4.6 CG-NAP Is a Component of LFA-1 Induced Signaling Complex in T Cells

In situ immunoprecipitation using motility-inducing monoclonal anti-LFA-1 antibody SPV-L7 was utilized to determine whether CG-NAP associated with the LFA-1 signaling complex. CG-NAP was detected in the complex in HUT-78 cells and PBTLs (Fig. 4.13A and B). There was little CG-NAP association with the LFA-1 complex when the experiment was performed in metabolically inert conditions at 4 °C wherein cells were adherent but non-motile. Association of CG-NAP was significantly enhanced at 37 °C in LFA-1 in actively motile cells triggered by LFA-1 cross-linking. This was confirmed by the immunofluorescent staining of LFA-1 associated components remaining attached to plastic after cell lysis with in-situ immunoprecipitation buffer (Fig. 4.14). Significantly lower amount of CG-NAP and α-tubulin were recruited in the complex in low temperature conditions blocking active intracellular signaling events. To prove that the cells were completely lysed, Hoechst 33342 was used to visualize any remaining nuclei. No nuclear staining was observed as seen in Fig. 4.14.

In the studies described in chapter 3, it was demonstrated that LFA-1 in locomotory T cells is localized over the area of the MTOC. Therefore, we decided to establish if such
Figure 4.10: C-Nap1 distribution in resting and motile HUT-78 cells. In contrast to CG-NAP, another centrosomal marker protein C-Nap1, C-Nap1 localizes at the centrioles, but lacks the scattered Golgi-attributed component and fails to redistribute along microtubules on LFA-1 activation (note the arrows).
Figure 4.11: C-Nap1 in locomotory HUT-78 cells migrating on immobilized anti-LFA-1 antibodies. Triple staining for C-Nap1 (green), α-tubulin (red), and nucleus (blue) illustrate the localization of C-Nap1 in motile HUT-78 cells.
Figure 4.12: Localization of C-Nap1 in HUT-78 cells exposed to cytoskeletal modulators. A) Control, cells migrating on LFA-1 antibody and stained for C-Nap1 in green. B and C, Cells pretreated with Nocodazole and Taxol show no change on the centrosomal localization of C-Nap-1. D) Actin disruption by Cytochalasin similarly did not alter C-Nap-1 localization pattern.
Figure 4.13: *In situ* precipitation analysis of LFA-1 associated complex. Western blot detection of proteins associated with LFA-1 cytoskeletal/signaling complex in HUT-78 (A) and PBTLs (B) revealed that CG-NAP represents a component of the complex. CG-NAP association with the complex was significantly increased at 37 °C compared to 4 °C.
Figure 4.14: Detection of the components of the \textit{in situ} immunoprecipitated complex by immunofluorescence. Exposure to motility inducing antibodies via LFA-1 as described in the methods, cells were lysed in membrane detergent containing buffer (the same buffer used in the \textit{in situ} immunoprecipitation technique for detection of CG-NAP in the complex) and the LFA-1 associated intracellular components were stained for tubulin (red), CG-NAP (green) and nucleus (blue). Cells were kept either at 4 °C or 37 °C as indicated on the figures. Note that tubulin at 4 °C is detected \textit{in situ} as faint red spots after cell lysis compared to partially preserved filamentous structures at 37 °C.
distribution was associated with CG-NAP and LFA-1 colocalization. With this aim, 3-D confocal microscopy of cells immunostained for CG-NAP and LFA-1 was utilized (Fig. 4.15A and B). Co-localization was observed at the distal portions of the elongated CG-NAP scaffold and a subset of LFA-1 adjacent to the plasma membrane.

To compare CG-NAP distribution in a cell locomotion system different from LFA-1 triggered motility, a different model of cell migration on immobilized purified fibronectin was deployed (Fig. 4.16). Although the polarized morphology of T cells on fibronectin is somewhat similar to the cells migrating on ICAM-1, CG-NAP in these cells appears to be located in a compact spot around the centrosome and does not show the typical pattern of intracellular distribution observed in the cells triggered by LFA-1.

4.7 Overexpression of C-Ter Domain of CG-NAP Blocks LFA-1 Mediated T cell Motility

To elucidate the functional significance of CG-NAP in LFA-1 mediated T cell locomotion, transfection studies were carried out. We originally attempted to transfet HUT-78 cells using two established techniques: Lipofection (Gene-Porter), and electroporation. Both techniques proved to be unsuccessful; the percentage of transfected cells was less than 10%. However, microinjection of CG-NAP construct was deployed instead and proved to be very successful. Injection of HUT-78 cells with a plasmid encoding C-terminal domain of CG-NAP resulted in the loss of cell polarity in response to LFA-1 ligation and blocked the development of typical locomotion associated phenotype (Fig. 4.17A and B). Cells overexpressing C-terminal domain of CG-NAP had a rounded morphology with clearly defined isolated spots corresponding to the centrioles and limited free cytosolic pool of GFP. However, the attachment of the cells and formation of associated filopodial structures remained intact in these cells (Fig. 4.17B). Cells injected with the empty vector GFP (EV-GFP) retained their ability to polarize and migrate when triggered via LFA-1 (Fig. 4.17C).

4.8 CG-NAP Localization in Different Stages of Mitosis

To characterize the cell cycle dependent redistribution of CG-NAP, we used immunofluorescent staining for CG-NAP, α-tubulin and nucleus of HUT-78 T lymphoma cell at different stages of cell cycle (Fig. 4.18A and B). CG-NAP staining is
Figure 4.15: Co-localization of CG-NAP and LFA-1 in locomotory T cells. A, B- 3D reconstruction of the confocal images illustrating localization of LFA-1 (green) predominantly as a compact sheet around the base of the trailing cell projections and CG-NAP (red). Co-localization is reflected as orange/yellow merge colour (arrows) in two different spatial orientation planes, predominantly at the distal portions of the CG-NAP scaffolding in proximity to the LFA-1 pool at the plasma membrane. Inset in fig. B shows transmitted light and fluorescent overlay of the same cell.
Figure 4.16: Spatial distribution of LFA-1 and CG-NAP scaffolding in HUT-78 cells migrating on fibronectin. Note the compact spot of CG-NAP (red) around the MTOC, a different intracellular distribution pattern compared to the one registered in cells triggered via LFA-1. LFA-1 integrin (green) is no longer seen in the characteristic location around the base of the trailing tail projections (compare to Fig. 4.15).
Figure 4.17: Over-expression of the C-terminal domain of CG-NAP in HUT-78 cells triggered via LFA-1. A, B. Note clearly defined spots indicated by arrows corresponding to the location of the C-ter GFP/CG-NAP protein at the centrioles in adherent, but non-polarized cells (arrows). B, X-Y-Z planes of the representative transfected cell illustrating spatial intracellular location of the C-ter CG-NAP GFP.
Figure 4.17C: Over-expression of the C-terminal domain of CG-NAP/AKAP450 in HUT-78 cells triggered via LFA-1. X-Y-Z confocal images of migrating cells cross-linked with LFA-1 and transected with EGFP vector alone. Cells retained their elongated morphology and locomotory characteristics.
Figure 4.18: CG-NAP, microtubules and nucleus during different stages of mitosis.

Subcellular localization of CG-NAP (green), α-tubulin (red), and nucleus (blue) in interphase (A) and at different stages of mitosis (B). HUT-78 cells were attached to the coverslips via Poly-L-Lysine. Coverslips were mounted on a heated stage kept at 37°C. Right column shows merged images.
attributed to the area of the MTOC (centrosome) during interphase (Fig. 4.18A). At the start of mitosis, early in prophase, CG-NAP staining disappears. Prometaphase and late metaphase show reorganization of CG-NAP, which eventually reappears again in late anaphase (Fig. 4.18B).

4.9 Effect of Cytoskeletal Modulators on CG-NAP Localization

The effect of cytoskeletal disrupters on CG-NAP localization was examined. Following pre-treatment with the microtubule disrupter Nocodazole, microtubule stabilizer Taxol and the actin disrupter Cytochalasin, cells rounded up and failed to acquire a motile phenotype or to undergo cytoskeletal reorganization when crosslinked with LFA-1 (Fig. 4.19A, B and C). Complete inhibition of cell polarization was more pronounced with Taxol and Cytochalasin D. Pretreatment with Nocodazole (Fig. 4.19B) caused dispersion of the Golgi associated component of CG-NAP throughout the cytoplasm and reduction in intensity of the MTOC-associated component, though, targeting of CG-NAP to the centrosome was not affected by this treatment. Some reports also address this issue; they show that CG-NAP localization to the centrosome is independent of microtubules, whereas that to the Golgi apparatus is disrupted by microtubule depolymerization (Takahashi et al., 2000). Pretreatment with Cytochalasin D (Fig. 4.19A) caused partial dispersion of the Golgi component of CG-NAP near the Golgi area and a stronger intensity of the centrosomal localized component of CG-NAP. The organized microtubule architecture is completely lost with Cytochalasin treatment potentially due to disruption of actin/microtubule association. In contrast to the findings observed with Nocodazole, CG-NAP staining was observed at the centrosome as an intense spot when cells were pretreated with Taxol (Fig. 4.19C).

4.10 Co-localization of CG-NAP and PKC-δ at the MTOC in T cells

Results described in the previous chapter showed that PKC-δ, another isoenzyme from the PKC family is a further cytoskeletal component in the LFA-1-induced signaling complex. In addition, previous results from Volkov et al. (2001) demonstrated the association of this isoform at the area of the MTOC on LFA-1 cross-linking (Volkov et al., 2001). Other studies verified its involvement in mitotic division and MTOC-orchestrated cell functions (Watanabe et al., 1992, Volkov et al., 1998). Since CG-NAP has also been shown to be a component of the LFA-1 complex in T cells, subcellular
Figure 4.19A: Effect of the cytoskeletal modulator Cytochalasin D on the intracellular localization of CG-NAP in HUT-78. Cells were pretreated with Cytochalasin D at 1µM for 30 minutes and then exposed to motility inducing immobilized anti-LFA-1 antibodies. CG-NAP staining in green, microtubules in red and the nucleus in blue. The lower picture is a higher resolution immunofluorescent image of the cell stained for CG-NAP only (arrow).
Figure 4.19B: Effect of the cytoskeletal modulator Nocodazole on the intracellular localization of CG-NAP in HUT-78. Cells were pretreated with Nocodazole at 5μM for 30 minutes and then exposed to motility inducing immobilized anti-LFA-1 antibodies. CG-NAP staining in green, microtubules in red and the nucleus in blue. The merged image in the middle obtained from different experiment visualized using higher resolution. The lower image is a separate immunofluorescent staining for CG-NAP only, the long arrow points to the MTOC-associated component, the short arrows point to the dispersed Golgi-component of CG-NAP.
Figure 4.19C: The effect of the microtubule stabilizer Taxol on CG-NAP localization in HUT-78. Cells were similarly pretreated with Taxol at 5μM for 30 minutes and then exposed to motility inducing immobilized anti-LFA-1 antibodies. CG-NAP staining is illustrated by the arrow.
relationship between both CG-NAP and PKC-δ was examined using double immunofluorescence in resting T cells. As shown on the overlay image (Fig. 4.20), significant co-localization was visualized between CG-NAP and PKC-δ. Previous studies by Volkov et al. (2001) demonstrated that PKC-δ remained localized at the MTOC as a localized compact spot when activated via LFA-1 in T cells; therefore, we did not proceed to study its distribution in motile cells.

4.11 Interaction of CG-NAP, PKC-β (I) and Microtubules at The MTOC in Motile T Cells

The results described early in this study established the localization of CG-NAP at the area of the MTOC and its involvement in the LFA-1 cytoskeletal complex. Furthermore, the PKC-β(I) isoenzyme has been shown to be a crucially important for orchestrating LFA-1 mediated locomotion in T cells, also localizing at the area of the MTOC (Volkov et al., 2001). Moreover, α-tubulin was shown to be a component of the complex associated with PKC-β(I) at the MTOC in motile T cells (Volkov et al., 2001). Volkov et al. (2001) hypothesized that PKC-β(I) interaction with the microtubules in the LFA-1 induced complex could be mediated via intermediate scaffolding proteins. We deduced, therefore, that CG-NAP might interact with PKC-β(1) at the MTOC in motile cells. Subcellular redistribution of PKC-β(I) and CG-NAP was determined by immunofluorescence together with their relation to the microtubule cytoskeleton in motile cells (Fig. 4.21). A dramatic degree of colocalization of these three intracellular components was observed. The arrow in Fig. 4.21 indicates the area of the MTOC, where colocalization of the three proteins is very pronounced.

Taken together with the data on the PKC δ described above, these findings could indicate that CG-NAP could potentially scaffold more than one member of the PKC family at the area of the centrosome or Golgi apparatus, thus adding to the importance of CG-NAP in T cell signaling.

4.12 Detection of CG-NAP in Total Cell Lysates of K4 Cells

We next examined whether CG-NAP localization is dependent on PKC-β expression. A PKC-β deficient HUT-78 clone (K-4) was utilized to determine if CG-NAP expression is altered when PKC-β is absent. Total cell lysates obtained from HUT-78 and K-4 cells
Figure 4.20: Co-localization between CG-NAP and PKC-δ at area of the MTOC in resting T cells. Immunofluorescence analysis of CG-NAP (green), and PKC-δ (red). The merged image at the bottom demonstrates the area of co-localization of these proteins (yellow). The nuclei are highlighted in blue.
Figure 4.21: Intracellular localization of CG-NAP, PKC-β in relation to the microtubules cytoskeleton in motile T cells. Triple immunofluorescent staining of CG-NAP (red), PKCβ (green), and α-tubulin (blue) in migratory HUT-78 cells activated via LFA-1 crosslinking. The left column shows the co-localization of CG-NAP and PKC-β at the MTOC (arrows), the right column shows the identical microscopic field stained for α-tubulin. The arrows point to the centrosomes.
were probed with anti-CG-NAP antibody; CG-NAP expression was not changed in K-4 cells (Fig. 4.22A) implying that the co-localization observed in Fig. 4.21 between PKC-β and CG-NAP is not necessarily a direct association but could rather be mediated via other interlinking molecules. Another explanation is that both proteins interact with the microtubule cytoskeleton at different loci. We further verified these findings using the selective PKC-β inhibitor, Gö6976 (Fig. 4.22B). Gö6976 inhibits conventional PKC isoenzymes (Volkov et al., 1998). PKC-β inhibition suppressed cell locomotion triggered via LFA-1; however, the distribution of CG-NAP was unaffected.

4.13 CG-NAP/NFκB(Rel A) Interaction in Resting and Motile T Cells

Nuclear translocation of the NFκB family of transcription factors is a proximal step in the signal transduction in many cellular processes including motility. Under unstimulated conditions, Rel A, p65 is restricted to the cytoplasm by inhibitory proteins, designated IκB. IκB are family of proteins degrading in response to immune and oxidant stimuli. Exposure to immune stimuli induces the posttranslational modifications of this factor by phosphorylation and subsequent proteolysis of IκB, thereby freeing the p50/p65 complex to translocate to the nucleus (Verma et al., 1995). Cytoskeletal proteins controlling NFκB activity in T cell functions are not completely elucidated. The subcellular localization of Rel A p65 in resting and locomotory T cells activated via LFA-1 crosslinking was examined by double immunofluorescence using anti-p65 and anti-CG-NAP antibodies. In resting T cells, Rel A, p65 was observed in diffuse granular distribution at the peri-centrosomal area (Fig. 4.23). Negative control staining is shown in Fig. 4.24. At high power magnification, CG-NAP and p65 showed no co-localization in resting cells (Fig. 4.25), instead, Rel A, p65 showed scattered spots surrounding the MTOC area encircling CG-NAP. In the cells with motile phenotype, however, p65 redistributed around CG-NAP at the base of the trailing cytoplasmic tails (Fig. 4.26), and was more pronounced in cells with very long uropods. We next determined the effect of the microtubule stabilizer Taxol on p65 distribution. Taxol treatment rendered the cells non-motile but did not alter the subcellular distribution of p65 (Fig. 4.27).

4.14 IκBα/microtubules interactions

The IκBα is an important molecular target controlling the NFκB transcriptional activity.
Figure 4.22A: Western blot analysis of the total cell lysates of HUT-78 and K-4 cells. The band detected in both HUT-78 and K-4 cells corresponds to the molecular weight of CG-NAP (450 kDa).

Figure 4.22B: Immunofluorescent staining of CG-NAP in HUT-78 cells pretreated with the PKCβ inhibitor Gö6976. HUT-78 cells pretreated with the PKCβ inhibitor Gö6976 at 5μM for 30 minutes and then exposed to motility inducing immobilized anti-LFA-1 antibodies. CG-NAP localization is depicted by the arrow.
Figure 4.23: Dual color immunostaining for CG-NAP and p65 in resting PBTLs. P65 staining (red), CG-NAP (green). Nuclei are counterstained blue. Yellow/orange color in the merged panel illustrates co-localization of the two proteins of interest.
Figure 4.24: Control Immunostaining for CG-NAP and p65. An isotype matched IgG (anti-IE) is used as a control for p65, while normal rabbit serum (NRS) is used as a control for CG-NAP. Nuclei are stained blue to indicate the position of the cells.
Figure 4.25: Double immunofluorescent staining of CG-NAP and p65 in resting HUT-78 cells visualized at high magnification. The merged photo showing p65 (red), CG-NAP (green), and nuclear staining (blue). HUT-78 cells were attached to the coverslips via Poly-L-Lysine.
Figure 4.26: Double immunofluorescent detection of CG-NAP and p65 in motile HUT-78. The merged image of p65 (red) and CG-NAP (green). Arrows indicate the distribution of p65 corresponding to the centrosomal CG-NAP component, which is more pronounced in the cells with long uropods.
Figure 4.27: Double immunofluorescent staining of CG-NAP and p65 in motile HUT-78 cells treated with Taxol. HUT-78 cells pretreated with 2μM of Taxol for 30 minutes prior to LFA-1 crosslinking. The distribution of p65 (red) around the perimeter of CG-NAP (green) is depicted by the arrows.
Since 1kBα is the key molecular target controlling NFκB activity, it is important to characterize effector proteins that interact with 1kBα. Characterization of 1kBα in the LFA-1 complex (chapter III, 3.9.1) led to the belief that it could potentially bind to the microtubules. Immunofluorescence studies were performed to study the subcellular localization of 1kBα and its relation to the microtubule cytoskeleton. 1kBα showed a diffuse cytoplasmic localization in resting cells with no co-localization with tubulin (Fig. 4.28). In motile cells 1kBα shows minimal co-localization with tubulin at the base of the trailing projections (Fig. 4.29). A control for the immunostaining is depicted in Fig. 4.30. These data demonstrate that 1kBα and microtubules do not necessarily interact with each other in resting cells or motile T cell activated by cross-linking of LFA-1.

4.15 Co-localization of CG-NAP and Dynein in T Cell

The microtubule-based molecular motor dynein plays an important role in the assembly of the mitotic and microtubule sliding movement during the progression of mitosis (Wittmann and Waterman-Storer 2001, Sharp et al., 2000). Dynein also plays a role in the regulation of the MTOC reorientation in migrating cells and centrosome assembly (Palazzo et al., 2001, Young et al., 2000). The association of the dynein intermediate chain with the LFA-1 complex (chapter III, 3.7) led to the assumption that dynein might potentially interact with CG-NAP.

We determined the localization of dynein in relation to CG-NAP. Double immunofluorescence analysis using anti-CG-NAP and anti-dynein antibodies showed co-localization of both target proteins in resting and motile PBTLs (Fig. 4.31 and Fig. 4.32). The dynein intermediate chain showed a compact spot at the area of the centrosome together with a scattered pattern around it in resting cells, co-localizing with CG-NAP. In motile cells, dynein followed the distribution of CG-NAP at the area of the MTOC.

4.16 Disruption of AKAP/PKA Interaction Suppresses LFA-1 Mediated T Cell Motility and Leads to CG-NAP Dispersion Around The Centrosome
Figure 4.28: IKBα subcellular distribution in resting T cells. Triple staining for IKBα (green), α-tubulin (red) and the nucleus (blue). Resting PBTLs placed on Poly-L-Lysine.
Figure 4.29: IκBα subcellular distribution in motile T cells. Triple staining for IκBα (green), α-tubulin (red) and nucleus (blue) in motile PBTLs crosslinked via anti-LFA-1 Abs.
Figure 4.30: Control Immunostaining for IκBα in resting T cells. PBTLs stained with normal rabbit serum (NRS) as a control for IκBα, anti-IE as a control for α-tubulin. The nuclear stain in blue.
Figure 4.31: Subcellular localization of CG-NAP and dynein in resting PBTLs.
Double immunofluorescent staining for CG-NAP (green), and dynein intermediate chain (red), the lower panel is the merged picture of the two images.
Figure 4.32: Subcellular localization of CG-NAP and dynein in locomotory T cell activated via LFA-1 cross-linking. Double immunofluorescence staining showing CG-NAP (green), and dynein intermediate chain (red), the bottom panel is the merged image of the two images.
Tethering of PKA to subcellular loci is achieved through the A-kinase Anchoring Proteins (AKAPs). Several studies reported the association of PKA RII at the centrosome and the Golgi complex (De Camilli et al., 1986, Griffiths et al., 1990). Because CG-NAP directly interacts with PKA RII at the centrosome and Golgi complex (Takahashi et al., 1999), we questioned whether disrupting the PKA/AKAP interaction could affect the localization of CG-NAP (as one of the AKAPs) and whether this disruption induces a change in the motile behavior induced by LFA-1 in T cells.

Peptides encompassing the amphipathic region of St-Ht-31 (residues 493-515) have been utilized to study the functional significance of PKA anchoring inside cells by disrupting PKA RII/AKAP interaction by competitive inhibition (Jones 2002). HUT-78 cells treatment with St-Ht31 for 30 minutes caused noticeable reduction in the cells acquiring the motile phenotype upon exposure to immobilized anti-LFA-1 (Fig. 4.33). Cells were observed with rounded phenotype compared to the cells with no treatment or the cells treated with the control peptides. However, cells incubated with St-Ht31 were still able to retain their adhesive properties. A control peptide differs in two amino acids in which isoleucine have been replaced by proline residues, thereby blocking its ability to disrupt AKAP/PKA interaction (Vijayaraghavan et al., 1997).

The effect of AKAP/PKA disruption on CG-NAP distribution was also documented. CG-NAP distribution remained intact in cells treated with the control peptide. However, exposure of cells to St-Ht31 led to the dispersion of the Golgi component of CG-NAP in the cytoplasm, partially dissociating from the centrosomal component and forming multiple spots around the centrosome (Fig. 4.33 arrows). This is mainly observed in the cells that did not display locomotory phenotype after St-Ht31 treatment.

4.17 Disruption of AKAP/PKA Interaction Inhibits LFA-1 Mediated T Cell Transmigration

To determine if AKAP/PKA interaction influences the migratory ability of T cells induced by LFA-1, equal number of PBTLs were allowed to transmigrate through inserts coated with immobilized anti-LFA-1 antibodies (Fig. 4.34). In separate inserts, cells were also pretreated with St-Ht31 peptide or control peptide for 30 minutes prior to their plating on filter inserts. Coating with RAM only was used as a double negative
Figure 4.33: Effect of St-Ht31 peptide treatment on HUT78 motile phenotype mediated by LFA-1 and CG-NAP distribution. Motile HUT-78 cells exposed to immobilized anti-LFA-1 Abs (upper image) were pretreated with either St-Ht31 control peptide or St-Ht31 inhibitor peptide for 30 minutes prior to LFA-1 crosslinking. Most of the cells treated with St-Ht31 inhibitor did not develop a locomotory phenotype. CG-NAP subcellular localization was studied by immunofluorescence. In contrast to CG-NAP, localized spot is seen in motile cells and cells pretreated with the control peptide (arrow heads). Most of the cells pretreated with St-Ht31 inhibitor peptide showed diffused pattern of localization (arrows). The background intensity was artificially increased to demonstrate the overall cell shapes (rounded or elongated).
control. The numbers of transmigrated PBTLs pretreated with St-Ht31 peptide were 4-fold lower compared to those with no treatments and approached the negative control values (Fig. 4.35). It could be hypothesized that disruption of AKAP/PKA interactions might destabilize CG-NAP functions or affect other AKAPs potentially involved in T cell motility.

4.18 Role of C-AMP And PKA in T Cell Motility Mediated by LFA-1

The role of c-AMP and PKA in T cell motility is not been characterized before. Several data have demonstrated PKA as a negative regulator of the $\beta_2$ integrin avidity and PKA activation led to diminishing in neutrophil adhesion to endothelial cells (Jones 2002, Derian 1995). Results presented in the previous chapter (Chapter III, 3.8) demonstrated that PKA RII is a component of the LFA-1 complex, however PKA RII participation was more evident in non-motile cells. We further verified these findings using a c-AMP analogue (8-Bromo), which activates PKA and a PKA inhibitor H-89. HUT-78 cells were pretreated with both reagents for 30 minutes prior to LFA-1 crosslinking (Fig. 4.36). 8-Bromo treatments resulted in the appearance of cells with less motile phenotype (multiple shorter uropods) compared to the non-treated cells crosslinked with LFA-1. However, much longer trailing projections (uropods) were observed in motile cells crosslinked with anti-LFA-1 after exposure to the PKA inhibitor, H-89.

4.19 Discussion

This study presents the novel findings characterizing the expression of the scaffolding protein CG-NAP in peripheral blood lymphocytes and in the T cell lymphoma. Expression of this molecular scaffold is localized to the region of the centrosome and the Golgi apparatus. Furthermore, CG-NAP was found to be a component of the LFA-1 signaling complex. CG-NAP could potentially contribute to the orchestration of LFA-1-induced intracellular signaling cascades. CG-NAP could play a functional role in LFA-1 mediated events, the finding that the association is greatly reduced when cells are maintained at low temperature conditions hence indicating an active metabolic requirement for this association. CG-NAP association with LFA-1 could target this adhesion molecule to a location close to its specific intracellular substrates at the centrosome or Golgi apparatus. The interaction between LFA-1 and CG-NAP is not
Figure 4.34: Effect of AKAP/PKA disruption on T cell transmigration mediated by LFA-1. Anti-LFA-1 mAb or control (RAM) were immobilized on the upper side of the inserts. The inserts were coated with motility inducing anti-LFA-1 antibodies. PBTLs were added to the upper chamber and examined for transmigration using the microscope.

Figure 4.35: The number of PBTLs transmigrated with and without AKAP/PKA disruption. Cells were counted in three representative high power (100X) fields per insert. The graph shows the mean values and standard errors of 3 independent experiments.
Figure 4.36: Effect of c-AMP analogue and PKA inhibitor on motile T cells activated by LFA-1 crosslinking. HUT-78 cells were either untreated (upper panel), pretreated with PKA inhibitor H89, or the c-AMP analogue 8-Bromo prior to migration on motility inducing anti-LFA-1 antibodies.
direct, possibly involving the participation of other molecules assembled on CG-NAP scaffold.

Previous data on CG-NAP showed that it interacted with a number of critical kinases including the regulatory subunit of PKA, and the Rho-activated protein kinase PKN, in addition, CG-NAP interacted with the protein phosphatases PP1, and PP2A (Takahashi et al., 1999). Furthermore, a novel interaction between CG-NAP and a PKC family member, PKCε was established (Takahashi et al., 2000), demonstrating the involvement of CG-NAP in the regulation of PKC phosphorylation. CG-NAP was found to associate with the immature non-phosphorylated form of PKCε (Takahashi et al., 2000). This targeting interaction was required for the phosphorylation of PKCε, which occurs upon phosphorylation at several sites on the enzyme. In concert with the previous studies, the results presented here reveal the association of PKCε as a component of the LFA-1 induced signaling complex supported by CG-NAP scaffolding.

Another potential interaction could exist between CG-NAP and PKC β. Both CG-NAP and PKC β are found partially associated with the MTOC. A critical role of PKCβ in LFA-1 mediated T cell migration has been established; PKCβ is an essential component of the LFA-1-mediated signaling complex (Volkov et al., 2001). In addition, PKCβ plays a crucial role in the transport and secretion of the cytokine interleukin-2 in T cells potentially interfering with the intracellular microtubule mediated (Long et al., 2001).

PKC θ on the other hand, is primarily required around the plasma membrane at the contact zones between T cells and APCs (Monks et al., 1997). It is clearly important to distinguish between the effects of LFA-1 as an adhesion co-factor in lymphocyte-APC interactions and the effects of LFA-1 as a signaling molecule for locomotion. The absence of PKC θ to from the LFA-1-associated signaling complex implies that it might not directly influence these LFA-1 related functions in T cells.

The Golgi apparatus is a critical station for transport and secretion of newly synthesized proteins to the extracellular milieu (Ponnambalam and Baldwin 2003). LFA-1 activation has been shown to be important for the secretion of chemokines involved in regulation of directional T cell migration (Murphy et al., 2000). Such secretion would be predicted
to be dependent on Golgi apparatus function and the maintenance of cell polarity. Hence, it is possible that the generation of this signaling complex brings together not only molecules involved in migration but also molecules involved in intracellular transport and secretion. In addition to the critical role of PKCβ in T cell migration, PKCβ expression plays a crucial in the transport and secretion of the cytokine interleukin-2 (Long et al., 2001), further emphasizing the connectivities between the signaling events involved in these cellular functions.

Maintaining the integrity of the Golgi apparatus in non-polarized cells requires the motor protein dynein (Roghi and Allan 1999). Microinjection of function blocking antibodies directed against dynein resulted in the dispersal of the Golgi apparatus (Burkhardt et al., 1997, Roghi and Allan 1999). Furthermore, microinjection of dynein heavy chain antibodies causes dispersal of the Golgi complex, even in cells lacking cytoplasmic dynein (Roghi and Allan 1999). It is also speculated that cytoplasmic dynein which also identified on Golgi membranes (Roghi and Allan 1999) might be required to some extent for assembling and/or docking γ-tubulin on the Golgi (Chabin-Brion et al., 2001). In addition, impairment of dynein function results in mitotic spindle defects, including spindle fragmentation and disorganization (Purohit et al., 1999). The microtubule-dependent motor protein dynein has been implicated in vesicular transport to and from the Golgi complex (Burkhardt et al., 1997, Burkhardt 1998). Other evidence showed that dynein associates with Golgi membranes through proteins other than the spectrin/ankyrin membrane lattice; however, the identity of the proteins involved was not known (Fath et al., 1997). It is possible to speculate that CG-NAP could be responsible for this association; thus, CG-NAP might be involved in the regulation of dynein dynamics. CG-NAP anchors PKA, and dynein is a substrate for PKA (Inaba et al., 1998). CG-NAP/dynein co-localization at the Golgi/centrosomal area, in addition to dynein participation in the LFA-1 cytoskeletal complex could thus be crucial for the process of T cell migration.

Microtubules play a central role in cell locomotion and migration. Furthermore, microtubules have a critical function in maintaining the integrity and intracellular location of the Golgi apparatus in cells. Disruption of microtubules using depolymerizing drugs results in the fragmentation of the Golgi complex and its redistribution into multiple functional Golgi mini stacks dispersed throughout the
cytoplasm (Thyberg and Moskalewski 1999). Thus, the microtubules have a role in subcellular localization of organelles, in addition to their role in locomotory events. Tubulin represents an essential component of the LFA-1 cytoskeletal complex. CG-NAP showed a significant colocalization with both α and γ-tubulin, in addition to its direct interaction with γ-tubulin ring complex (Takahashi et al., 2002). The functional significance of CG-NAP for LFA-1 induced motility is further underscored by the results of the studies in cell transfectants. The finding that T cells overexpressing C-terminal domain of CG-NAP failed to polarize and acquire locomotory phenotype indicates that CG-NAP is crucially involved in one of the MTOC dependent cell functions such as active locomotory behavior. These data are in concert with the recent findings by Keryer et al. (Keryer et al., 2003), who demonstrated that overexpression of CG-NAP in Hela cells impairs cytokinesis and increased ploidy in these cells implying a significant functional role of CG-NAP in the integrity of the centrosome and related signaling pathways.

The LFA-1/cytoskeletal interactions are co-ordinated during lymphocyte migration. Association of CG-NAP with the LFA-1 signaling complex may be dependent on intact microtubule functioning. Disruption of microtubules caused dispersion of CG-NAP localization implying that this association may be important in at least some of the signaling process involved in migration.

Another role for CG-NAP has been established during the process of cell division and mitosis (Keryer et al., 2003). Data presented here show that CG-NAP is localized to the centrosome and Golgi complex in all stages of mitosis except early in mitosis at the stage of early prophase, this could be explained in many ways. First, since CG-NAP has interaction domains for PKA RIIα subunits, and that PKA RIIα relocates from the centrosome/Golgi region to the nucleus at the transition between interphase and mitosis (Carlson et al., 2001), presumably relocating to sites either near its mitotic substrates or away from interphase substrates. In addition, phosphorylation of PKA by CDK1 causes it to switch from binding one anchoring protein to binding another, and since these anchoring proteins are located in different compartments of the cell. Another explanation is related to CG-NAP association with the Golgi complex. During mitosis, disassembly of the Golgi complex is followed by reassembly when mitosis is completed (Rabouille and Jokitalo 2003, Colanzi 2003). In addition, it has been suggested that the
pericentriolar Golgi organization acts as a sensor for controlling entry into mitosis (Rios and Bornens 2003). These findings prove the importance of CG-NAP in the process of mitosis.

The importance of CG-NAP in regulation of mitosis and cell cycle progression is further elucidated by Keryer et al., 2003. Keryer et al. proposed that the ability of CG-NAP to act as a scaffold for multiple regulatory proteins could be vital not only for the activity of the centrosome as a microtubule organizing center but also for its stability and biogenesis. Their results suggest that CG-NAP scaffolding function could be to organize local pools of signaling molecules or cell cycle regulators into close proximity of centrioles in order to regulate both centrosome activity and centriole biogenesis. Keryer et al. addressed the role of CG-NAP in cell cycle control and centrosomal activity, by selectively modifying the activity of the centrosome through expressing a C-terminal region of CG-NAP (the PACT domain) reported to contain the centrosomal targeting domain of this protein (Gillingham and Munro 2000). This domain is shared by other large coiled-coil centrosomal proteins kendrin/pericentrin as well as CG-NAP. This approach led to the displacing PKA type II and delocalization of CG-NAP but not centriolar or centrosomal matrix components such as γ-tubulin, centrin or pericentrin, and did not affect localization of other pools of PKA bound to AKAPs elsewhere in the cell, because the C terminus of CG-NAP does not contain the RII binding motifs and should not disrupt anchored PKA activities at other loci in the cell. Keryer et al. (2003) showed that cells expressing C-terminal of CG-NAP, in which the centrosome is principally targeted, entered mitosis but did not complete cytokinesis. Independent studies by Takahashi et al. 2002 also showed that perturbation of CG-NAP blocks mitosis at an early stage.

CG-NAP directly interacts with PKA RIIα at the centrosome and Golgi complex (Takahashi et al., 1999). Although, here we show that PKA RIIα represents a component of the LFA-1 signaling complex in T cells, increased levels of PKA RIIα are attracted to this complex in the cells that have stopped moving rather than in the actively migrating cells, suggesting a potential modulatory role for PKA in active T cell motility. PKA has been implicated in the inhibition of the MAP kinase pathway. In addition, JNK activation is delayed by c-AMP (Stork and Schmitt 2002). Rovere et al. 1996, showed that intracellular PKA activation, promotes T cell de-adhesion by
disassembling the actin-based cytoskeleton, thus dissociating LFA-1 from cytoskeletal anchoring proteins. Same author also showed that cells stimulated via LFA-1 receptors by specific antibodies or by binding to intercellular adhesion molecule-1 display elevated intracellular cAMP due to the synergistic induction of a PKC-dependent adenylyl cyclase isoform, acting in feedback mode for short term regulation of LFA-1. This involves sequential, LFA-1 dependent activation of the PKC and adenylyl cyclase/cAMP-dependent kinase enzymatic pathways and leads to disengagement of the adhesion receptor from its specific ligand (Rovere et al., 1996). Hence, it is possible to speculate that PKA and PKC-β may have opposite roles in the promotion of the LFA-1 mediated migratory phenotype in T cells. It is hypothesized that decrease in c-AMP and increase in Rho GTPases at the front of the cell increases integrin affinity and cytoskeletal adhesions, whereas, high c-AMP and low Rho GTPases at the cell rear promotes release of adhesions and uropod formation (Sanchez-Madrid and del Pozo 1999).

The NFκB/Rel family of transcription factors exist in a latent form in the cytoplasm of unstimulated cells (Baeuerle and Baltimore 1988), where cytoplasmic retention is kept by IκB inhibitory molecules. This interaction could allow the activity state of NFκB to be modulated by the cytoarchitecture, and provide machinery for gene regulation in response to cytoskeletal changes. Agents that depolymerize the microtubules but not actin cytoskeleton rapidly activate NFκB and induce NFκB-dependent gene expression (Crepieux et al., 1997, Rosette and Karin 1995). On the other hand, IκBα associates physically with dynein light chain (Dlc-1) while it is still linked to NFκB Rel (p65), in addition to IκBα association with the microtubules particularly at the MTOC (Crepieux et al., 1997). It is still unclear which cytoskeletal components associate with NFκB. The microtubule cytoskeleton plays a key role in the modulation of gene expression; in addition, microtubule-disrupting agents can induce gene expression (Maine et al., 1993). NFκB has not been previously shown to be localized on microtubules, however, microtubule depolymerization leads to IκB destruction through a kinase-dependent mechanism allowing NFκB to bind DNA and stimulate transcription (Zhong et al., 1997). Many protein kinases have been found to bind to microtubules in vivo. Scaffolding proteins such as CG-NAP could modulate the activity of the transcriptional factors that reside in the cytoplasm and undergo translocation from the cytoplasm upon
stimulation. The data presented here reveals that LFA-1 mediated signaling in motile T cells is associated with the translocation of the scaffolding protein CG-NAP to the microtubule cytoskeleton along the uropod to a location close to the NFkB Rel A.

Ca\(^{2+}\) represents an important signaling molecule in cell division; calmodulin acts in a Ca\(^{2+}\)-dependent fashion in initiating changes involved with mitotic progression. (Berridge et al., 1998). CaMKIV is a key mediator of Ca\(^{2+}\)-induced gene expression, CaMKIV was found to directly associate with and phosphorylate the NFkB component p65; the phosphorylation of p65 by CaMKIV resulted in recruitment of transcription coactivator cAMP-response element-binding protein-binding protein (Jang et al., 2001). NFkB p65 might serve as a novel downstream phosphorylation target of CaMKIV and act as one of its effector molecules in vivo. In addition, there’s a recent speculation that calmodulin could be involved in the attachment of kinetochore microtubules to the centrosome (Moisoi et al., 2002). Though, calmodulin localization at the centrosome is independent of \(\gamma\)-tubulin (Moisoi et al., 2002), which could be via other scaffolding protein that functions in the microtubule-nucleating machinery or as binding site for other proteins. CG-NAP could be the obvious candidate as it contains the Ca\(^{2+}\)-independent calmodulin-binding motif (Gillingham and Munro 2000).

\(\gamma\)-TuRC is a multicomponent complex containing \(\gamma\)-tubulin (Zheng et al., 1995), present in both the centrosome and the cytoplasm (Schiebel 2000). From interphase to metaphase, the amount of \(\gamma\)-tubulin at the centrosome increases threefold and decreases rapidly in late metaphase (Khodjakov and Rieder 1999, Takahashi et al., 2002). \(\gamma\)-TuRC is necessary for microtubule nucleation from the centrosome (Martin et al., 1998, Moritz 1998, Zhang et al., 2000). \(\gamma\)-TuRC belongs to the group of centrosome proteins whose centrosomal localization is independent of microtubules. CaM and CaM-binding proteins might be required for centrosome-mediated microtubule nucleation by tethering \(\gamma\)-TuRC to centrosomes in animal cells (Kawaguchi and Zheng 2003). Mutations of the five amino acids necessary for calmodulin-binding completely abolished the centriolar targeting of the C-terminal CG-NAP fragment (Kawaguchi and Zheng 2003). However, calmodulin might not be essentially required for CG-NAP binding to centrioles, as calmodulin does not concentrate on the interphase centrosome (Moisoi et al., 2002), but it does on the spindle pole body of budding and fission yeasts (Moser et al., 1997). CG-
NAP provides a structural scaffold necessary for proper centrosomal architecture and microtubule nucleation during mitosis, binding to γ-tubulin is required for the regulation of microtubule nucleation. This function is very similar to that of pericentrin, which shares a high homology to CG-NAP (Dictenberg et al., 1998, Doxsey et al., 1994). Thus, CG-NAP could act as a centrosomal scaffold for the microtubule nucleation events during mitosis.

In addition, it is speculated that γ-tubulin could be recruited from the cytosolic pool to promote microtubule nucleation at the Golgi apparatus, similar to γ-tubulin at the centrosomes which is organized in a nucleating complex known as γ-tubulin ring complex (Zheng et al., 1995). Interaction of CG-NAP with γ-tubulin and the Golgi complex could be vital for these processes. CG-NAP could well serve as a link that recruits γ-tubulin on Golgi membranes. The Golgi stacks are usually arranged as an interconnected network in the region around the centrosome where microtubule nucleation occurs. After drug-induced disruption of microtubules, the Golgi stacks are disconnected from each other, dispersed in the cytoplasm, and redistributed to endoplasmic reticulum exit sites (Thyberg and Moskalewski 1999). Following removal of the drugs, scattered Golgi elements move along reassembling microtubules back to the centrosomal region and reunite into a continuous system.

These findings suggest that CG-NAP provides a point for the integration of diverse signaling pathways, including those involving PKC, PKA, motor protein dynein and NFκB in LFA-1-mediated T motility. Future studies utilizing the use of yeast two-hybrid screen would be necessary to characterize important intracellular components in the cytoskeleton that interacts with the cytoplasmic domain of LFA-1 and control its function.
CHAPTER V

General Discussion

In many cells, the process of signal transduction depends on AKAPs that directly coordinate signaling (Michel and Scott 2002). Some AKAPs are scaffolding proteins that coordinate multiple signaling pathways within cells by targeting effector kinases, phosphatases and substrates at the same subcellular location (Scott and Pawson 2000, Tomlinson 2000, Myung 2000, Michel and Scott 2002, Bauman and Scott 2002, Schillace et al., 2002). AKAPs are present in regions of high microtubule nucleating activity. CG-NAP is a scaffolding protein from the AKAP family of anchoring proteins (Takahashi et al., 1999).

CG-NAP as a Multikinase Scaffolding Protein

CG-NAP is the product of a multiply spliced gene that generates numerous isoforms of a large protein, scaffolding many enzymes typically protein kinases and phosphatases. CG-NAP structurally represents a giant molecule with four leucine zipper-like motifs, a binding domain that interacts with PKA through its regulatory subunit PKA II, and a docking site for Rho-activated protein kinase PKN (Takahashi et al., 1999). Moreover, CG-NAP also has binding sites for protein phosphatases PP1, and PP2A. CG-NAP is localized at both the centrosome and the Golgi complex; its localization to the centrosome is independent of MTs, whereas that to the Golgi complex is disrupted by MT depolymerization or BFA treatment (Witzczak et al., 1999). Only recently, the amino-terminal domain of CG-NAP has been found to interact with GCP2/GCP3 components of γ-TuRC providing sites for MT nucleation or anchoring (Takahashi et al., 2002). Disclosed interaction of CG-NAP with members of the centrosomal MT-interacting TACC protein family further expands these findings (Steadman et al., 2002). CG-NAP was also found to interact with the immature non-phosphorylated form of
PKCe (Takahashi et al., 2000). The complex coiled-coil architecture of the molecule adds to the characteristic features of CG-NAP likely to be involved in multiple associations with several proteins.

**CG-NAP as an integrating component in the LFA-1 mediated cytoskeletal and signaling complex in locomotory T cells**

This study illustrated for the first time expression of CG-NAP in peripheral blood lymphocytes and in the T cell line HUT-78 localizing at the region of the MTOC and Golgi complex. Furthermore, a novel role of CG-NAP in the LFA-1 mediated signaling has been established in this study. Crosslinking of LFA-1 resulted in the re-distribution of CG-NAP in the trailing cell extensions. Using the *in situ* immunoprecipitation approach, CG-NAP has been found to physically associate with the LFA-1 in the multi-molecular signaling complex. These results led to the speculation of a functional involvement of this scaffolding protein in the process of LFA-1 induced T cell motility.

The thesis highlights the role of CG-NAP as an integral component of the LFA-1-induced cytoskeletal and signaling complex. Specifically by linking together the MTOC, microtubules and possibly protein kinases involved in cytoskeletal modifications in locomotory T cells, CG-NAP could potentially contribute to the orchestration of LFA-1-induced intracellular signaling cascades. The LFA-1 cytoskeletal interactions are coordinated during lymphocyte migration. Association of CG-NAP with the LFA-1-associated signaling complex is dependent on intact microtubule functioning. Disruption of microtubules caused partial dispersion of CG-NAP localization implying that this association may be important in at least some of the signaling process involved in migration. Furthermore, LFA-1 activation induces the secretion of chemokines involved in regulation of directional cell migration (Murphy et al., 2000). This phenomenon would be predicted to be dependent on Golgi apparatus function and also on the maintenance of cell polarity. Hence, it is possible that the generation of this signaling complex brings together not only molecules involved in migration but also proteins involved in intracellular transport and secretion.
CG-NAP Is Associated With the Major Components of the Microtubule Cytoskeleton

CG-NAP showed strong association with the tubulin cytoskeleton. Microtubules play an active role in both migration and secretion (Rodriguez-Fernandez et al., 1999). Furthermore, γ-Tubulin is a major component of the pericentriolar matrix at the MTOC and is involved in centrosomal microtubule nucleation (Oakley and Oakley 1989). The association of CG-NAP with the γ-tubulin ring complex at the centrosome (Takahashi et al., 2002), and the potential association between CG-NAP and γ-tubulin itself as shown in this thesis, using co-immunoprecipitation, suggests the possibility that CG-NAP may play a crucial role in microtubule-dependent functions in both processes.

CG-NAP Plays A Major Functional Role in the LFA-1 Mediated T cell Motility

The functional significance of CG-NAP in LFA-1 induced motility is underscored in the cell transfectants data. Cells overexpressing the C-terminal domain of CG-NAP could neither polarize nor achieve motile phenotype demonstrates a role for CG-NAP in controlling specific MTOC related functions or motile behavior in T cells. Furthermore, the findings illustrated in this thesis provide evidence that intracellular distribution of CG-NAP was typical of the cells triggered by recombinant ICAM-1 but not by fibronectin which indicates that the involvement of this scaffolding protein might play a key role in β2 integrin induced T cell motility but may not necessary play a similar role in the motility induced via different integrin ligands, for example, the β1 integrin ligand fibronectin.

Model for Control of Mitosis by CG-NAP

CG-NAP localization at the centrosome also plays a crucial role during mitosis, CG-NAP could anchor several signaling molecules and cell cycle effectors, hence, another vital role of the centrosome and CG-NAP in the regulation of cell cycle progression at the level of the whole cell population (Keryer et al., 2003). CG-NAP could play a similar role in cell cycle progression in T cells. Cell cycle regulation documented for CG-NAP could be due to its ability to cluster several regulatory molecules at the
centrosome via an orchestrated signaling complex capable of modulating their activities, or it might be due to a concerted action of several cross-talking signaling events that could come into play.

**A Potential Interaction between CG-NAP and PKCβ at the Centrosome**

A major protein kinase implicated in the LFA-1 mediated T cell motility is PKCβ. In addition to the critical role of PKCβ in T cell migration, PKCβ also plays an important role in T cell activation. PKCβ expression is crucial for transport and secretion of the cytokine interleukin-2 (Volkov *et al.*, 2001, Long *et al.*, 2001). Immunofluorescence studies elucidated a striking colocalization between CG-NAP and PKCβ at the area of the MTOC and the base of the uropod, which could provide a new role for interaction between CG-NAP and PKCβ, however, it is not yet clear if CG-NAP has direct interactions with PKCβ. There may be other interconnecting molecules associated with CG-NAP that also associate with PKCβ at the MTOC in motile T cells. Alternatively, CG-NAP association with the MTOC is independent of PKCβ, as CG-NAP was expressed in the T cell line deficient in PKCβ. Yet, a novel interaction between pericentrin (which shares a significant homology with CG-NAP) and PKCβII (Chen *et al.*, 2004) has just emerged. PKC/Pericentrin interactions occur at the C1A domain in PKC and residues 494-593 in pericentrin. Disruption of PKC/pericentrin interactions by overexpressing the binding domain on pericentrin (494-593) results in the mislocalization of PKCβII from the centrosome, microtubule disorganization, cytokinesis failure, spindle dysfunction, chromosome missegregation and aneuploidy. These findings raise the interesting possibility that both pericentrin and CG-NAP act as scaffolding proteins for PKC β at the region of the MTOC and function simultaneously at different domains to modulate its function.

**A Model for Negative Regulation of T Cell Motility by PKA**

The cAMP signaling pathway represents a potentially important inhibitory influence on T cell activity. Elevated cAMP has a negative regulatory effect on proliferation and
cytokine expression in lymphocytes. PKA has been suggested to hamper T cell activation by inhibiting IL-2 receptor alpha-chain (CD25) expression and IL-2 production in activated T-lymphocytes (Ramstad et al., 2000). The suppressive effect of PKA could be due to inhibition of signaling pathways operating downstream of the TCR such as NFκB. PKA inhibits NFκB by modifying the C-terminal transactivation domain of p65 or the kappa B site in the IL-2 promoter (Takahashi et al., 2002a, Neumann et al., 1995). Thus, PKA-mediated suppression of NFκB activity plays an important role in the control of activation of T lymphocytes. In the context of T cell migration, PKA inhibition led to the development of longer uropods on LFA-1 crosslinking in the absence of chemokines. Other data on the role of cAMP and PKA in cell polarization and uropod formation induced by chemokines show that cAMP analogs induce uropod development whereas PKA inhibitors prevents its formation (del Pozo et al., 1995). However, The system used by del Pozo et al. was different from ours as they utilized different cell activation model (T lymphoblasts) and these blasts were additionally exposed to chemokines. This might explain the differences between the phenomena observed in our studies and their findings. Other data from the same group indicate that PKA inhibition led to a dramatic decrease in lymphocyte random motility and chemokine-directed chemotaxis (del Pozo et al., 1999). In addition, PKA can phosphorylate RhoA leading to inactivation of this GTPase (Lang et al., 1996), thus suggesting a connection between chemokine receptors and Rho GTPases.

**PKA and PKC-β Association with CG-NAP is Potentially Critical in T cell Locomotion**

PKA and PKC-β could have contrasting roles in T cell motility (Fig. 5.1). It is possible that the balance between these enzymes may be essential for the migratory process. It is also possible that both are required for the adhesion/de-adhesion events involved in the locomotory process in lymphocytes. PKCβ is an important component of the LFA-1 signaling complex located at the area of the MTOC where CG-NAP is situated. PKCβ is enriched in the complex in motile cells at 37 °C, whereas, PKA becomes more associated in the complex when the cells are non motile, i.e. at 4 °C. Thus, it could be concluded that both PKCβ and PKA act in different directions, and have opposing roles in T cell migration. CG-NAP interaction with PKA and PKC, in addition to the
Figure 5.1: Schematic representation of the reversible relationship between PKA and PKCβ in T cell migration. A) Motile T cell activated by LFA-1 crosslinking is associated by upregulation of PKCβ and downregulation of PKA, whereas, the reverse occurs when the cell becomes non motile (B).
potential opposing role of both PKA and PKC in T cell locomotion mediated by LFA-1 lead to the speculation that CG-NAP could play a critical role in maintaining a balance between these kinases. Thus, it could be possible to design new-targeted therapies based on CG-NAP itself or CG-NAP interaction with PKA or PKC.

**PKA Tethering by CG-NAP Regulates T Cell Homeostasis**

AKAPs contribute to the maintenance of T cell homeostasis. It has been speculated (Williams 2002) that the interaction between AKAP and PKA may either inhibit T cell activity by rendering the cAMP/PKA pathway constitutively active, or may sensitize the cells to very low levels of endogenous cAMP elevating agents. However, disruption of the AKAP-PKA interaction stimulates T cell cytokine production. It is still under investigations whether this disruption involves an increase in the rate of T cell proliferation. Several lines of evidence indicate that PKA II could play a role in stabilizing the minus end of MTs that originate from the centrosome (Carlson et al., 2001). PKA is recruited to the AKAPs where it can influence microtubule dynamics by regulating microtubule-destabilizing factors. PKA II is targeted to the Golgi/centrosome area by interaction of its regulatory subunit with pericentrin (Diviani et al., 2000) and CG-NAP. However, the direct involvement of this process in lymphocyte migration remains unclear.

**LFA-1 Plays a Crucial Role in Physiological and Pathological Disease Processes**

Cell motility is crucial for normal functioning of the immune system in lymphocyte infiltration and in mediating inflammation. The recruitment of the scaffolding protein CG-NAP into the multicomponent LFA-1-associated protein complex in locomotory T cells underlines a novel function for scaffolding proteins in T cells. These findings may have further physiological significance at advanced stages of lymphocyte extravasation. Leukocyte integrins are pivotal for the emigration of leukocytes from the circulation toward the site of inflammation. These molecules play a critical role in mediating leukocyte endothelial interactions. Leukocyte-endothelial adhesion plays a central role in leukocyte extravasation, a key feature of inflammation. Inflammatory signals induce
the expression of proteins on the endothelial cell surface that promote the adhesion and extravasation of activated immune cells from the circulation into the underlying tissues (Dedrick et al., 2003). Cell-cell and cell-extracellular matrix interactions are required for lymphocyte activation and migration of cells during the inflammatory response (Petruzzelli et al., 1998).

LFA-1 is required for leukocyte extravasation and subsequent immune and inflammatory responses. LFA-1 mediated leukocyte locomotion and extravasation at the sites of inflammatory response has been implicated in the pathogenesis of conditions such as Rheumatoid arthritis, inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn’s disease (CD), and also multiple sclerosis (MS)(Vanier et al., 2000, Dedrick et al., 2003, Correale et al., 2003). These conditions are characterized by leukocyte chemotaxis and adhesion, lymphocyte activation with the expression of LFA-1 on activated blood T lymphocytes (Szekanecz and Koch 2000), thus, emphasizing the critical role of cell migration in the pathogenesis of both acute and chronic inflammatory diseases.

Rheumatoid arthritis is a chronic polyarthritis leading to joint destruction as a consequence of inflammatory processes. The disease is progressive, resulting ultimately in significant disability. Long-term outcome studies documented that nearly 3 out of 4 RA patients become disabled despite treatment. LFA-1 is reported to play a role in the pathogenesis of Rheumatoid arthritis. Activated functional LFA-1 present at sites of RA acts as a central molecule in the onset and maintenance of RA (Yokota et al., 1995).

Leukocyte infiltration is a hallmark histopathological feature of IBD; these cells may also contribute to the increased vascular permeability. There is increasing evidence to suggest that adhesion and emigration of leukocytes from inflamed postcapillary venules results in an increased plasma protein extravasation at the sites of leukocyte transendothelial migration (Granger and Kubes 1994).

LFA-1 proved to be required for transepithelial migration in UC, and leukocyte locomotion in CD (Vainer et al., 2000). Antibodies to LFA-1 inhibit effector immune responses, and leukocyte migration to the site of infection (Furie et al., 1991, Calhoun et al., 1999). Monoclonal antibody therapy directed against LFA-1 has been utilized in
various animal models and human trials. MAbs interfering with LFA-1 blocking selective aspects of immune reaction could be promising therapeutic tools in autoimmune and inflammatory diseases such as RA, IBD and MS, and proved to have significant efficacy (Elovaara et al., 2000, Yusuf-Makagiansar et al., 2002). Interestingly, anti-LFA-1 therapy has been successfully used in preventing or reducing RA activity and other forms of arthritis (McMurray 1996, Yokota et al., 1995), also it has been effectively utilized to reduce graft failure in children with immunodeficiencies, prolong allograft survival and the induction of donor-specific tolerance (Fischer et al., 1991, Nicolls et al., 2000). In vivo and in vitro studies using antibodies to LFA-1 have demonstrated reduced migration of lymphocytes to lymph nodes and Peyer’s patches emphasizing the critical role of LFA-1 in lymphocyte trafficking to peripheral lymph nodes (Andrew et al., 1998). Furthermore, activated lymphocytes from LFA-1 deficient mice were unable to cross endothelial cell monolayers in response to chemotactic gradient and were unable to reject immunogenic tumors or demonstrate priming responses against tumor-specific antigens (Schmits et al., 1996, Andrew et al., 1998).

The importance of LFA-1 in immune and inflammatory processes was recognized in a rare genetic human disease, the lymphocyte adhesion deficiency (LAD)-1 syndrome, in which lymphocyte migration to inflamed tissues is blocked resulting in severe recurrent life threatening bacterial infections, impaired pus formation, wound healing, recurrent skin infections and increased leukocytosis (Kobayashi 1994). The profound pathogenesis of LAD has been attributed to impairment of adhesion-dependent T cell functions due to lack of cell surface expression of LFA-1 and other β2 integrins (Krensky et al., 1985).

Greater understanding of the process of cell migration can give rise to potential molecular targets for the development of improved therapeutic candidates. Targeting leukocyte-mediated extravasation may be useful for the future management of all related inflammatory arthritis diseases. Strong data showed that the inhibition of LFA-1 and ICAM-1 could be an effective therapy. Suggested therapeutic approaches designed to block the binding of LFA-1 to ICAM-1 for the treatment of immunological disorders have been mostly mAb based (Fischer et al., 1991, Hourmant et al., 1996, Kavanaugh et al 1994, Cornejo et al., 1997). Such antibody treatment blocks normal as well as abnormal functions of the immune system. Clinical studies with humanized monoclonal
antibodies that interfere with LFA-1/ICAM-1 interactions have shown significant efficacy and good safety profiles in autoimmune disease, including multiple sclerosis and inflammatory bowel disease (Dedrick et al., 2003). Another cutting edge data showed that a small molecule antagonist to LFA-1 had the ability to inhibit leukocyte function both in vitro and in vivo (Kelly et al., 1999).

On the other hand, physiological T cell activation requires the interaction of T cell antigen receptors with the proteins of the MHC, which takes place at the immunological synapse (Monks et al., 1998, Grakoui et al., 1999, Dustin et al., 2002). This process is critical for adaptive immune responses. LFA-1/ICAM-1 interactions are required for the proper functioning of the immunological synapse (Monks et al., 1998). Anti-LFA-1 therapy block cell motility, migration and pathologic responses. However, in addition to its influence on cellular pathology and trafficking is also required for T cell activation, hence, LFA-1 therapy could affect physiological T cell responses such as those at the immunological synapse. Therefore, we need to design new therapies that selectively distinguish between LFA-1 at the immunological synapse and LFA-1-mediated migration at the inflammatory sites. Blocking of LFA-1 functions can affect its association with interacting molecule involved in cell migration and thus block inflammation, but can lead to suppression of T cell functions at the immunological synapse. Identification of mechanisms in which CG-NAP associates with the LFA-1 complex is of crucial importance particularly in the design of molecular therapeutics with defined targets and effector functions.

Functional Role of CG-NAP and Other Components Associated With LFA-1: Future Perspectives

This study elucidated the recent advances in understanding the nature and function of proteins associated with the LFA-1 signaling complex with particular emphasis on the scaffolding protein CG-NAP as an integrating component of the complex in locomotory T cells. Future work on the diverse structural nature of CG-NAP will provide significant information on its functional role as a major coordinator in the cells. Proteomic characterization using mass-spectrometric instrumentation and RNA technology as well as biochemical structural analysis will be the cornerstone to the understanding of the exact molecular features of CG-NAP.
CHAPTER VI

Conclusions and Perspectives

This study describes the scaffolding protein CG-NAP that may play a crucial role in cell motility in addition to its role in microtubule organization and cytokinesis. Another centrosomal coiled-coil protein "pericentrin" which shares homology with CG-NAP has been shown recently to function in a similar manner to CG-NAP interacting with complexes and signaling molecules at the MTOC. Therefore, it is important to explore the relationship between pericentrin and CG-NAP and to delineate the putative functional roles shared by both proteins and the important domains responsible for binding selective kinases at the centrosome.

CG-NAP and Pericentrin share Structural and Functional Similarities at the MTOC

The two major AKAPs present at the centrosome are CG-NAP and pericentrin serving as structural and regulatory scaffolds. Both are divergent centrosomal proteins sharing few common domains. CG-NAP and pericentrin are large proteins forming a coiled-coil over most of their length except for N- and C-terminal regions of about 200 amino acids (Gillingham and Munro 2000). In addition, both proteins have been shown to be involved in the control of microtubule organization at the MTOC (Doxsey et al., 1994), anchoring \( \gamma \)-tubulin ring complexes that nucleates microtubules in the pericentriolar material (Flory et al., 2000, Takahashi et al., 2002), and both have a binding site for PKA RII subunits (Diviani et al., 2000). This visible redundancy in PKA anchoring may ensure that the kinase is always anchored to this organelle for an as yet unidentified essential role in some aspect of centrosomal function.

Like CG-NAP, pericentrin remains with the centrosomes through mitosis. Although pericentrin and CG-NAP both contain the same targeting domain, they share little homology otherwise, except for the C-terminal region, are therefore likely to drive
PKA-mediated phosphorylation of different substrates at the centrosome, thus, they might interact with the same structural elements at the centrosome.

**The C-terminal Domain Shared by CG-NAP and Pericentrin Is Responsible For Centrosomal Targeting**

The C-terminal domain of CG-NAP and pericentrin is directly responsible for recruiting CG-NAP and pericentrin to the centrosome. This domain is also present at the C-terminus of coiled-coil proteins from *Drosophila* and *S. pombe*, and that from the *Drosophila* protein is sufficient for targeting to the centrosome in mammalian cells (Gillingham and Munro 2000). Searching the database with the C-terminal region of CG-NAP revealed a strong homology to the equivalent region of pericentrin (Fig. 6.1). In addition, fusion of the C-terminal region from pericentrin or CG-NAP to a reporter protein confers a centrosomal localization, and overexpression of the domain from CG-NAP displaces endogenous pericentrin, suggesting recruitment to a shared site (Gillingham and Munro 2000), which led to the speculation by the authors that the PACT domain is well conserved in evolution as it could provide a critical link between the core of the centrosome and the components of the pericenteriolar material such CG-NAP and pericentrin, which themselves serve to recruit a number of proteins to the centrosome. This PACT domain is outside of both their coiled-coil regions and their PKA binding site.

**Both CG-NAP and Pericentrin Control Microtubule Nucleation and Centrosomal Assembly**

The microtubule nucleating capacity of the centrosome is localized to the PCM. The pericenteriolar material contains a high proportion of pericentrin and CG-NAP. The pericenteriolar material also contain a large protein complexes of γ-tubulin and associated proteins that have a ring-like structure and mediate the nucleation of microtubules or γ-TuRCs (Zheng et al., 1995). If these large coiled-coil proteins serve to recruit other factors to the centrosome this raises the question of how they themselves are localized. The amino-terminal region of CG-NAP indirectly associated with γ-tubulin through binding with γ-tubulin complex protein 2 (GCP2) and/or GCP3. GCP2 was efficiently
Figure 6.1: Structural similarities between CG-NAP and Pericentrin.
A) The related region between CG-NAP and pericentrin is shown in black, and regions predicted to be predominantly coiled-coil are shown in grey. B) Alignment of the C-terminal sequences of CG-NAP and pericentrin. Residues identical (black) or related (grey) in two or more of the sequences are shown, with the two particularly well conserved sections are underlined (Adapted from Gillingham and Munro 2000).
coimmunoprecipitated with the amino-terminal region of CG-NAP, CG-NAP\textsubscript{16-1229}, and weakly with CG-NAP\textsubscript{1229-1917}. Though, GCP2/3 binding site of pericentrin to the C terminus of the protein, a region that shows no apparent homology to CG-NAP (Takahashi et al., 2002). In somatic cells, a fraction of γ tubulin remains at centrosomes/spindle poles under conditions that disrupt the GCP2/3-pericentrin interaction. This fraction could be anchored by other proteins that have been shown to bind γTuRC components such as CG-NAP (Takahashi et al., 2002).

However, recent data demonstrated that CG-NAP and a larger isoform of pericentrin, human kendrin (or pericentrin B) (Li et al., 2001) anchor γ-TuRC through binding with GCP2 and/or GCP3 at their amino-terminal regions. CG-NAP and kendrin are localized to the centrosome via their carboxyl-terminal regions, which interact with calmodulin. Furthermore, endogenous CG-NAP and kendrin form complexes together with GCP2 and γ-tubulin in vivo. It was concluded that CG-NAP and kendrin provide microtubule nucleation sites by anchoring γ-TuRC at the centrosome (Takahashi et al., 2002). The carboxyl-terminal region containing the amino acid residues 3510–3810 is responsible for the centrosomal localization of CG-NAP. BLAST search using full-length CG-NAP yielded kendrin at two regions with relatively high homology (Fig. 6.2A, shaded areas), and the carboxyl-terminal part contained the sequence shown in Fig. 6.2B. BLAST search of CG-NAP\textsubscript{3510-3828} revealed that this region shares high homology with the carboxyl-terminal region of kendrin (Fig. 6.2B). CG-NAP and kendrin have three coiled-coil regions flanked by noncoiled regions (Fig. 6.2A).

Thus, CG-NAP and pericentrin may serve as a structural scaffolds necessary for microtubule nucleation in cooperation with γ-TuRCs. Both may also act as a link between structural elements like the microtubule network and regulatory elements such as PKA and downstream targets. Determining the function of these scaffolding proteins, in addition to the proteins and signaling molecules they bring together, may have important implications for understanding cell cycle signaling events. Moreover, overexpression of pericentrin causes microtubule defects, chromosome missegregation, and aneuploidy (Pihan et al., 2001).
Figure 6.2: Schematic representation of CG-NAP, pericentrin/kendrin. A) Schematic structure of CG-NAP and kendrin are shown with predicted coiled-coil regions in shaded boxes. Shaded areas between CG-NAP and kendrin represent the regions sharing homology found by BLAST search. B) Sequence homology of the centrosomal-localization region of CG-NAP with kendrin. Aligned sequences are the result of BLAST search with CG-NAP_{3510-3828} (Adapted from Takahashi et al., 2002).
Pericentrin: Novel PKC β Binding Protein. The Potential Missing Link

Anchoring PKC β to CG-NAP: Critical Implications For T Cell Motility

PKCβ plays a critical role in T cell motility (Volkov et al., 1998, Volkov et al., 2001) and is emerging as a major regulator of microtubule organization and dynamics. Volkov et al., (2001), have shown that PKCβI-deficient T cells failed to develop a polarized microtubule network, a defect that can be rescued by expressing PKCβI.

The PKC family requires phosphorylation of itself to become competent for responding to second messengers. Their functions are extensively regulated by subcellular location mediated by protein interactions (Mochly-Rosen, 1995). It is likely that the PKC family is also recruited to the particular place through binding with anchoring proteins to receive phosphorylation.

Only recently have CG-NAP and pericentrin been found to play critical roles in anchoring PKC members at the MTOC and surrounding areas (Takahashi et al., 2000, Chen et al., 2004). CG-NAP anchors hypophosphorylated PKCe at the Golgi/centrosome area during maturation and serves as a scaffold for the phosphorylation reaction. Binding regions of PKCe and CG-NAP were located within the catalytic domain of PKCe and at the C terminus of CG-NAP (Takahashi et al., 2000). The role of CG-NAP for the phosphorylation of PKCe may provide insight into the regulation of other members of PKC kinase superfamily in particular PKCβ.

Recently, pericentrin was also established to have a functional role anchoring PKCβII to the centrosome (Chen et al., 2004). Using yeast two-hybrid screen, pericentrin was identified as a binding partner for PKCβII. Co-immunoprecipitation and biochemical studies revealed that PKCβII interacted directly with pericentrin via the C1A domain in pericentrin and residues 494-593 in pericentrin. Moreover, Chen and his colleagues also tested the effect of disrupting pericentrin-PKC interaction by overexpression of the PKC-binding domain of pericentrin, the authors found that disruption of this interaction led to the release of PKCβII from the centrosome and resulted in microtubule defects, cytokinesis failure, spindle defects and chromosome missegregation. Thus, the authors
suggested that uncoupling of PKC from pericentrin is sufficient to induce cytokinesis failure. Taken together, these finding show that pericentrin akin to CG-NAP functions as a multikinase scaffold directing PKC together with PKA and other signaling kinases to the centrosome/MTOC to modulate centrosomal and other cellular functions. This raises intriguing questions on whether CG-NAP and pericentrin work cooperatively to mediate such functions or these scaffolding proteins work separately.

Although results presented in this thesis using immunofluorescence show a strong colocalization between CG-NAP and PKCβ, yet, immunoprecipitation studies did not show a direct binding between both proteins, suggesting an interlinking molecule such as pericentrin could be responsible for the association or different domains such as the N-terminal domain of CG-NAP might be responsible for the binding as it is the case with pericentrin. Therefore, different antibodies directed to the N-terminal region of CG-NAP would potentially give some idea about this interaction.

Thus, CG-NAP has the potential to organize numerous enzyme combinations in a context-specific manner. This increases the repertoire of signals that can be processed through the CG-NAP associated complex.

**Direct Correlation Between CG-NAP and Pericentrin In Mitosis**

Pericentrin alone can anchor PKA by binding RII and tethering it at the centrosome, therefore, giving pericentrin a role similar to CG-NAP. Pericentrin, PKA RII, and the catalytic subunit of PKA were all detected in pericentrin immunoprecipitates (Diviani et al., 2000). These results indicate that, similar to CG-NAP, pericentrin acts to concentrate PKA at specific subcellular compartments.

Akin to CG-NAP, pericentrin remains with the centrosomes throughout mitosis. On the face of it, if PKA RIIa is no longer detected at spindle poles, it must lose its association not only with CG-NAP but also with pericentrin. In reality, the size of the centrosomal PKA pool associated with pericentrin may be much smaller than the pool associated with CG-NAP, in which case, any PKA RIIa remaining associated with pericentrin during mitosis may not be detected (Diviani et al., 2000).
Future Perspectives

Taken together the data presented in this thesis illustrate a new role for CG-NAP in the integration of various intracellular signaling networks. The challenge ahead is to discriminate the system that controls how each kinase is assembled and anchored to CG-NAP and how this assembly could control the migratory process in T cells. Some of the emerging techniques that might enable us to achieve these goals include protein-purification techniques and proteomic analyses; these refined analytical techniques can be used to dissect each component associated with CG-NAP. Other techniques would be to selectively displace individual CG-NAP binding partners and monitor signal flow through this modified complex. This approach requires the removal of the endogenous CG-NAP and rescue with a mutant form, which can be carried out by RNA interference or the generation of 'knock-in' animals. Using this technology the components of the CG-NAP complex could be examined.

Other lines of investigation could include real-time experiments that explore the impact of CG-NAP/LFA-1 signalling in living cells, using fluorescence resonance energy transfer (FRET) to examine different kinases associated with CG-NAP. However, there is a need for additional fluorescent probes that allow the simultaneous detection of multiple binding partners. Also, the use of GFP-like fluorescent proteins of different colors. Although, there are limitations to both of these potentially practical approaches, future developments and emerging techniques will help us to clarify all the speculations and shed a new light on our understanding of the functional roles of anchoring proteins particularly CG-NAP and its role in cell signaling.
References


phosphatidylinositol-3,4,5-trisphosphate in the activation of protein kinase B. *Science* **277**: 567-570.


membranes are absorbed into and reemerge from the ER during mitosis. *Cell* 99: 589-601.


### Appendix A

#### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKAP</td>
<td>PKA-kinase anchoring protein</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>ATCC</td>
<td>American type cell culture</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BFA</td>
<td>Brefeldin A</td>
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<tr>
<td>BLNK</td>
<td>B cell linker protein</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CaM</td>
<td>Calmodulin</td>
</tr>
<tr>
<td>CaMK</td>
<td>Calmodulin kinase kinase</td>
</tr>
<tr>
<td>CLIC</td>
<td>Chloride intracellular channel</td>
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<td>C-AMP</td>
<td>Cyclic AMP</td>
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<tr>
<td>CDK-1</td>
<td>Cyclin dependent kinase-1</td>
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<td>CG-NAP</td>
<td>Centrosome and Golgi PKN associated protein</td>
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<td>c-AMP response element protein</td>
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<tr>
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<td>Diaeylglycerol</td>
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<td>DMSO</td>
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<td>Epithelial cell</td>
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<td>ECM</td>
<td>Extracellular matrix</td>
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<td>EDTA</td>
<td>Ethylenediamine tetra acetic acid</td>
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<td>EGTA</td>
<td>Ethylglycolis (aminoethlether) tetra acetic acid</td>
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<td>Endoplasmic reticulum</td>
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<td>ERK</td>
<td>Extracellular signal regulated kinase</td>
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<td>F-actin</td>
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<td>Fyn T binding protein</td>
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<td>Abbreviation</td>
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<tr>
<td>γTuRC</td>
<td>γ-Tubulin Ring Complex</td>
</tr>
<tr>
<td>γTuSC</td>
<td>γ-Tubulin Small Complex</td>
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<tr>
<td>GAD</td>
<td>Glutamic acid decarboxylase</td>
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<td>GADS</td>
<td>Grb2-like adaptor downstream of Shc</td>
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<td>GCP</td>
<td>Gamma-tubulin complex protein</td>
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<td>GFP</td>
<td>Green fluorescent protein</td>
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<td>GRKs</td>
<td>G-protein coupled receptor kinases</td>
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<td>Guanosine triphosphate</td>
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<td>Guanosine triphosphatases</td>
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<td>Human leukocyte antigen</td>
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<td>Intercellular adhesion molecule</td>
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<td>Interferon</td>
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<td>IκB</td>
<td>I kappa B</td>
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<td>Interleukin</td>
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<td>IP3</td>
<td>Inositol 1,4,5-triphosphate</td>
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<td>JAB</td>
<td>Jun activation domain</td>
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<tr>
<td>JNK</td>
<td>C-Jun N-terminal kinase</td>
</tr>
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<td>LAD</td>
<td>Leukocyte adhesion deficiency type-1</td>
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<td>LAT</td>
<td>Linker for activation of T cells</td>
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<tr>
<td>LFA-1</td>
<td>Leukocyte function associated antigen –1</td>
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<td>MAP</td>
<td>Microtubule associated protein</td>
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<td>MARCK</td>
<td>Microtubule associated protein-microtubule affinity regulating kinase</td>
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<td>MCP</td>
<td>Monocyte chemoattractant protein</td>
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<td>MEK1</td>
<td>Mitogen-activated protein kinase kinase 1</td>
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<td>MHC</td>
<td>Major Histocompatibility complex</td>
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<td>MIDAS</td>
<td>Metal ion-dependent adhesion site</td>
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<td>MIP</td>
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<td>MT</td>
<td>Microtubule</td>
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<td>MTOC</td>
<td>Microtubule organizing center</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
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<td>-------------</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
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<tr>
<td>NEK</td>
<td>NIMA-related kinase</td>
</tr>
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<td>NFAT</td>
<td>Nuclear factor of activated T cells</td>
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<td>NFκB</td>
<td>Nuclear factor kappa B</td>
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<tr>
<td>NLS</td>
<td>Nuclear localization sequence</td>
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<td>NMDA</td>
<td>N-methyl-D-Aspartate</td>
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<td>Normal mouse serum</td>
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<td>Normal rabbit serum</td>
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<td>Orthoclone 3</td>
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<td>PACT</td>
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<td>Phosphate buffered saline</td>
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<td>PBTLs</td>
<td>Peripheral blood T lymphocytes</td>
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<td>PCM</td>
<td>Pericenteriolar matrix</td>
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<td>PDE</td>
<td>Phosphodiesterases</td>
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<td>Phosphoinositide-dependent protein kinase</td>
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<td>Pleckstrin homology</td>
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<tr>
<td>PHR</td>
<td>Pericentrin homology region</td>
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<tr>
<td>PICKs</td>
<td>Protein that interact with C kinase</td>
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<td>PIP2</td>
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<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinase B</td>
</tr>
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<td>Protein kinase C</td>
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<td>Protein kinase N</td>
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<tr>
<td>PLC</td>
<td>Phospholipase C</td>
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<td>PMSF</td>
<td>Phenyl methyl sulphonyl fluoride</td>
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<td>Protein Phosphatase</td>
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<td>Protein tyrosine kinase</td>
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<td>Proline-rich tyrosine kinase</td>
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<td>Receptor for activated C kinase</td>
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<td>Rabbit anti-mouse immunoglobulin</td>
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<td>Description</td>
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<td>RCC</td>
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<td>Tris buffered saline</td>
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<td>T cell receptor</td>
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<td>N,N,N',N'-tetra-methylenediamine</td>
</tr>
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<td>T cell growth factor</td>
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<td>Toll like receptors</td>
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<td>TNF</td>
<td>Tumor necrosis factor</td>
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<td>TRITC</td>
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<td>XLP</td>
<td>X-linked lymphoproliferative disease</td>
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<tr>
<td>ZAP-70</td>
<td>μ-chain associated 70 kDa protein</td>
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Appendix B

Reagents and Buffers

1-Cell Culture Reagents

Culture cocktail
Penicillin (100U/ml) / Streptomycin (10μg/ml) 100 ml
L-glutamine (20 mM) 100 ml
2-mercaptoethanol 37 μl
This mixture was stored at 20°C in aliquots of 10 ml.
One aliquot was used for each medium bottle.

Complete CO₂ independent medium
CO₂ independent medium 500 ml
FCS 50 ml

Hanks balanced salts solution (HBSS)
HBSS 500 ml
HEPES buffer (1 M) 10 ml

Cryopreservative solution
Foetal calf serum 9 ml
Dimethylsulphoxide (DMSO) 1.0 ml

Ethidium bromide (EB) stock
EB 100 mg
PBS 20 ml

Acridine orange (AO) stock
AO 20 mg
20 ml

**EB/AO working solution**
- EB solution: 4 ml
- AO solution: 4 ml
- PBS: 100 ml

**2-Cell Preparation**

10X Phosphate Buffered Saline (PBS)

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<tr>
<td>Na$_2$HPO$_4$.2H$_2$O</td>
<td>8 mM</td>
<td>14.24 g</td>
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<tr>
<td>KH$_2$PO$_4$</td>
<td>1.5 mM</td>
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<td>NaCl</td>
<td>137 mM</td>
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<tr>
<td>KCl</td>
<td>2.7 mM</td>
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Adjust to pH 7.4 and made up to 1 litre

1% Nonidet P40

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<tr>
<td>PBS</td>
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<tr>
<td>EGTA</td>
<td>0.038 g</td>
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<tr>
<td>Nonidet P40</td>
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**Protease inhibitors**

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<td>Leupeptin</td>
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<td>PMSF</td>
<td>1 M</td>
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**3- Protein Determination**

Bradford reagent

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<td>Coomassie Blue G</td>
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<tr>
<td>95% ethanol</td>
<td>50 ml</td>
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<tr>
<td>0.85% orthophosphoric acid</td>
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</table>

This solution was made up to 1 litre with distilled water

**4-Polyacrylamide Gel Electrophoresis**

5x Sample buffer for sample preparation
Glycerol 5 ml
2-mercaptethanol 6.25 ml
20% SDS 5 ml
Stacking gel buffer 1.25 ml
0.2% Bromophenol blue 0.3 ml
Make up to 25 ml with distilled water

**Resolving gel buffer**
Tris base 36.3 g
Distilled water 100 ml
Adjust to pH 8.8 with conc. HCl

**Stacking gel buffer**
Tris base 6.0 g
Distilled water 100 ml
Adjust to pH 6.8 with conc. HCl

**1% Ammonium persulphate (APS)**
APS 0.1 g
Distilled water 10 ml
Make up fresh before use.

**10% Sodium dodecyl sulphate (SDS)**
SDS 10 g
Distilled water 100 ml

**Acrylamide/Bisacrylamide Mix**
Acrylamide 30 g
Bisacrylamide 0.8 g
Distilled water 100 ml

**Water-saturated butanol**
Butanol 10 ml
Distilled water 10 ml
This was mixed well, allowed to settle and pipetted from top layer

10X Electrode running buffer
Tris base 30 g
Glycine 114 g
SDS 5.0 g
Distilled water 1000 ml
Dilute 1/10 in distilled water before use

5- Semi-Dry Transfer
Transfer buffer for 10-12% gels
Tris base 2.9 g
Glycine 1.45 g
SDS 0.185 g
Methanol 100 ml
Make up to 500 ml with distilled water

10X 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS)
CAPS 22.13 g
Adjust pH with NaOH to pH 11.0 and Make up to a 1 liter

Transfer buffer for 4.5 % gels (CAPS buffer)
1X CAPS buffer
CAPS 100 ml
Methanol 100 ml
Make up to 800 ml with distilled water

Coomassie blue gel stain
Coomassie Brilliant Blue R 0.5 g
Methanol 200 ml
Glacial acetic acid 35 ml
Distilled water 265 ml

**Gel destain solution**
- Methanol 400 ml
- Glacial acetic acid 70 ml
- Distilled water 530 ml

**6-Immunoblotting**

**Blotto-Tween blocking solution**
- Skimmed dried milk (Marvel) 5 g
- PBS 100 ml
- Tween 20 46 μl

Make up fresh before use

**0.05% PBS-Tween washing solution**
- PBS 1000 ml
- Tween 20 500 μl

**Primary antibody solution**
- Primary antibody 10 μl
- Blotto-Tween 10 ml

**Secondary antibody solution**
- Swine anti-rabbit peroxidase-conjugated Ig 10 μl
- Blotto-Tween 20 ml

**7- Enhanced Chemiluminescence**

**Developing solution**
- Luminol 14 mg
- Iodophenol 4 mg
- DMSO 500 μl
- H2O2 18 μl
- 0.1 M Tris-HCl (pH 8.8) 50 ml
8- 4% Para-formaldehyde solution

Para-formaldehyde 4 g
PBS 100 ml
The solution was heated up to 65°C to be dissolved, cooled, filtered and stored at 4°C

9- Immunoprecipitation Buffers

NP40 lysis buffer (membrane lytic, microtubule friendly buffer)

PBS (PH 7.4) 48.6 ml
0.5% Nonidet P-40 (NP-40) 250 μl
MgCl$_2$ (1M) 250 μl
EGTA (100mM) 500 μl
PMSF (1M) 50 μl
Leupeptin (2 mg/ml) 250 μl
Okadaic acid (100 μM) 100 μl

SDS lysis Buffer

Tris. HCL (20 mM, PH7.5) 41.625 ml
1% SDS 0.5g
NaCl (1 M) 7.5 ml
EGTA (100mM) 0.5 ml
EDTA (0.5 M) 0.1 ml
PMSF (1M) 25 μl
Leupeptin (2mg/ml) 250 μl
Appendix C

Presentations and Publications

Presentations

The Novel Scaffolding Protein CG-NAP Is A Component of LFA-1 Induced Cytoskeleton/Signalling Complex in Locomotory T Cells.

5th Annual Meeting, Institute of Molecular Medicine. November 2000

ISI Meeting 2000, Maynooth, Dublin Ireland. 1ST PRIZE for ORAL PRESENTATION

Trinity Center for Health Sciences Meeting. September 2000

11th International Congress for Immunology, Stockholm, Sweden. 22nd - 27th July 2001

Cell Adhesion and Migration in Inflammation and Cancer, 3rd Amsterdam "Zoo" Meeting, Amsterdam, The Netherlands, October 17-20, 2001
CG-NAP Is A Component of LFA-1 Cytoskeleton/Signaling Complex In Locomotory T Cells.

ELSO (European Life Scientist Organization) NICE, France, June 29th -July 3rd, 2002


42 Annual Meeting, American Society for Cell Biology, San Francisco, California
14-18 December 2002

Basma Salah El Din El Homasan, Yuri Volkov, Mikiko Takahashi, Yoshitaka Ono, Guy Keryer, Annie Delouvee, Eileen Looby, Aideen Long, and Dermot Kelleher. CG-NAP: An Essential Component of LFA-1 Induced Cytoskeleton/Signaling Complex.

BSI Annual Congress, Harrogate. 2-5 December 2003

Basma Salah El Din El Homasan, Yuri Volkov, Mikiko Takahashi, Yoshitaka Ono, Guy Keryer, Annie Delouvee, Eileen Looby, Aideen Long, and Dermot Kelleher. CG-NAP: An Essential Component of LFA-1 Induced Cytoskeleton/Signaling Complex.

43 Annual Meeting, American Society for Cell Biology, San Francisco, California.
14-18 December 2003

Basma Salah El Din El Homasan, Yuri Volkov, Mikiko Takahashi, Yoshitaka Ono, Guy Keryer, Annie Delouvee, Eileen Looby, Aideen Long, and Dermot Kelleher. The Scaffolding Protein CG-NAP/AKAP450 Is An Integrating Component Of LFA-1 Induced Cytoskeleton/Signaling Complex In Locomotory T-Cells.

12th International Congress of Immunology, Montreal, Canada. July 18-23 2004

Publications

