The Scaffolding Protein CG-NAP/AKAP450 Is a Critical Integrating Component of the LFA-1-Induced Signaling Complex in Migratory T Cells[1]

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T cell migration represents a complex highly coordinated process involving participation of surface receptor/ligand interactions, cytoskeletal rearrangements, and phosphorylation-dependent signaling cascades. Members of the A-kinase anchoring protein (AKAP) family of giant scaffolding proteins can assemble and compartmentalize multiple signaling and structural molecules thereby providing a platform for their targeted positioning and efficient interactions. We characterize here the expression, intracellular distribution, and functional role of the scaffolding protein CG-NAP (centrosome and Golgi localized protein kinase N-associated protein)/AKAP450 in the process of active T cell motility induced via LFA-1 integrins. This protein is predominantly localized at the centrosome and Golgi complex. T cell locomotion triggered by LFA-1 ligation induces redistribution of CG-NAP/AKAP450 along microtubules in trailing cell extensions. Using an original in situ immunoprecipitation approach, we show that CG-NAP/AKAP450 is physically associated with LFA-1 in the multimolecular signaling complex also including tubulin and the protein kinase C β and δ isoenzymes. CG-NAP/AKAP450 recruitment to this complex was specific for the T cells migrating on LFA-1 ligands, but not on the β_1 integrin ligand fibronectin. Using the GFP-tagged C-terminal CG-NAP/AKAP450 construct, we demonstrate that expression of the intact CG-NAP/AKAP450 and its recruitment to the LFA-1-associated multimolecular complex is critically important for polarization and migration of T cells induced by this integrin. *The Journal of Immunology,* 2005, 175: 7811–7818.

I ntegrins deliver "outside-in" and "inside-out" signals that control important physiological and pathological cellular functions including survival, gene induction, cell adhesion, migration, cell proliferation, differentiation, inflammatory disorders, atherosclerosis, and metastasis (1–3). The cytoplasmic domains of integrins are required for the transduction of the bidirectional information across the plasma membrane, interacting directly with a number of cytoskeletal and signaling molecules and participating in formation of adhesion complexes (4–7). These complexes act as both recipients and generators in the signaling pathways. Cross-talk between integrins and the cytoskeleton plays a central role in lymphocyte migration.

The β_2 leukocyte integrin, LFA-1, is essential for normal immune responses, T cell activation, and effector functions (8, 9). Abs to LFA-1 inhibit effector immune responses and leukocyte migration to the site of infection (10, 11). In the context of T cell motility, LFA-1 not only acts as a vital molecule in lymphocyte adhesion but also mediates transmembrane signaling, leading to T cell migration (12). LFA-1 activation has been shown to mediate a tyrosine kinase-dependent activation of phospholipase Cγ1 (13) and is further associated with consequent activation of protein kinase C (PKC)[4] (14).

In motile T cells activated through LFA-1, reorganization of the cytoskeleton is accompanied by translocation of PKC isoenzymes to sites adjacent to the centrosome, the major microtubule-organizing center (MTOC) in mammalian cells (15, 16). A number of studies have further emphasized the importance of the microtubule cytoskeleton in determining cellular polarity and motile behavior (17, 18). The motile lymphocytes develop trailing extensions which contain cytoskeletal and signaling elements (19).

Stimulation of signaling pathways is dependent on protein phosphorylation which requires activation of kinases and phosphatases. Linker or adapter (also called scaffolding) proteins facilitate signaling by recruiting kinases and phosphatases to their respective targets (20–22). A number of scaffolding/adaptor proteins have been identified in lymphocytes, including T cells (23–25). The proteins of this group known to functionally play a role in T lymphocytes include linker for activation of T cells (LAT; Ref. 26) and Src homology 2-domain-containing leukocyte protein of 76 kDa (SLP-76; Refs. 27 and 28). Both adaptor proteins are interconnected; LAT plays a major role in TCR signaling (29), acting as a central adaptor, while SLP-76 binds multiple cytosolic effector proteins.
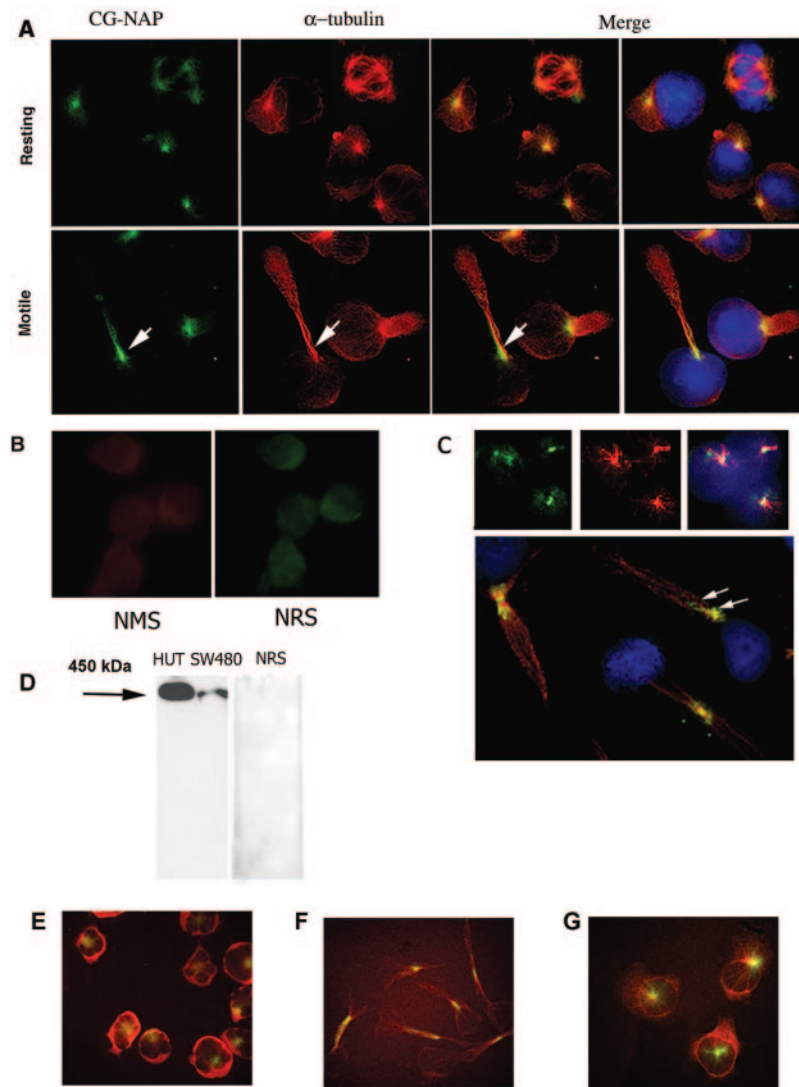
FIGURE 1. *A*, Subcellular localization of CG-NAP/AKAP450 in resting and motile HUT-78 lymphoma cells on immobilized anti-LFA-1. *Upper panel* (*left* to *right*), Staining of resting cells for CG-NAP/AKAP450, α-tubulin and merged images, respectively. Nuclei are highlighted blue. CG-NAP/AKAP450 staining localizes at the centrosome and a scattered network around it. *Lower panel*, Motile HUT-78 cells activated by immobilized LFA-1 Abs. CG-NAP/AKAP450 is localized to the area of the centrosome and to some extent distal to the MTOC in the trailing uropod (arrows). *B*, Negative control staining using NMS(red) and NRS (green). *C*, Localization of CG-NAP/AKAP450 in resting and locomotory PBTLs. CG-NAP/AKAP450 staining is attributed to the centrosome and is also present along microtubules in the trailing uropod (arrows) similar to *A*. See also supplementary Movie 1. *D*, Detection of CG-NAP/AKAP450 in lymphoma cell HUT-78 and human colonic epithelial line SW480 total cell lysates. Expected molecular mass of CG-NAP/AKAP450 is around 450 kDa. Normal rabbit serum (NRS) was used as a negative control and total cell lysate from epithelial cells SW480 was used as a positive control. *E*, Abs supporting T cell adhesion and spreading, but not motilly, do not induce the formation of the CG-NAP/AKAP450-associated complex. Hut 78 lymphoma cells were exposed to immobilized anti-CD54 and subsequently fixed and stained for CG-NAP/AKAP450 (green) and tubulin (red). Cells failed to display an orderly CG-NAP/AKAP450 and microtubule association typical of the stimulation via immobilized β_2 integrins. *F* and *G*, Formation of the LFA-1-associated complex depends on Ab specificity. Abs of the clone YTN-81.5 (motility-inducing) support the formation of the CG-NAP/AKAP450-associated complex (*F*) whereas clone MEM-83 (inducing the adhesion but not motility) do not initiate the complex assembly (*G*). PBTL were exposed to immobilized Abs and subsequently fixed and stained for CG-NAP/AKAP450 (green) and tubulin (red).

Recently, a novel scaffolding protein CG-NAP (centrosome and Golgi localized protein kinase N (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 (30, 31). Through the association of CG-NAP/AKAP450 with the regulatory subunit RIIα of PKA, it is considered as an A-kinase anchoring protein (AKAP). CG-NAP/AKAP450 showed a high homology to yotiao, AKAP120, and relative homology to kendrin/pericentrin. In addition, it was found that CG-NAP/AKAP450 and kendrin/pericentrin share a conserved domain at their C termini which targets CG-NAP/AKAP450 and kendrin/pericentrin to the

centrosome (32). Furthermore, CG-NAP/AKAP450 was found to be associated with PKC-ϵ, serving as a scaffolding protein for the hypophosphorylated form of this PKC isoform (33). However, CG-NAP/AKAP450 has not previously been described in T lymphocytes.

Most recent functional studies on CG-NAP/AKAP450 had shown that overexpression of the C-terminal domain of CG-NAP/AKAP450 in HeLa cells (34) significantly perturbed the centrosomal function, elucidating the importance of this scaffolding protein for orchestrating centrosome-related cell functions. Previous results from our group (15) have demonstrated that in crawling T cells triggered through LFA-1 cross-linking, PKC-β(I) and PKC-δ
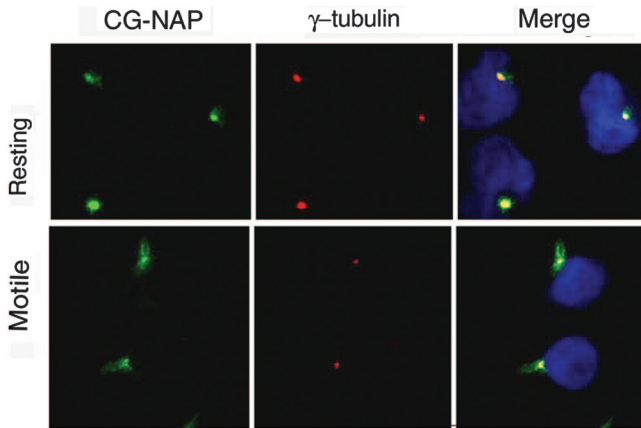
FIGURE 2. Localization of CG-NAP/AKAP450 at the centrosome in resting and motile HUT-78. Intracellular staining for γ-tubulin (red) and CG-NAP/AKAP450 (green) demonstrates colocalization of the centrosomal component of CG-NAP/AKAP450 and γ-tubulin at the centrosome.
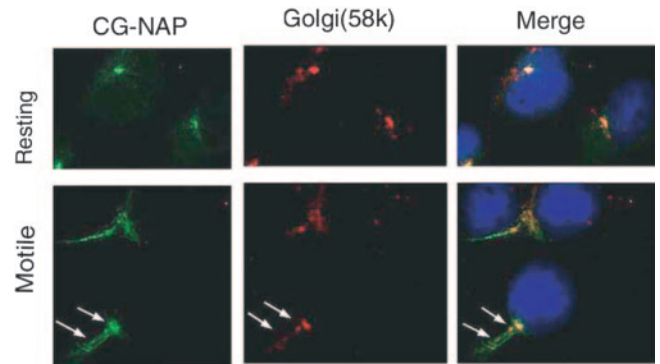

FIGURE 3. Colocalization of CG-NAP/AKAP450 with the Golgi apparatus. In both resting cells (*upper panel*) and motile cells triggered via LFA-1 (*lower panel*), the scattered component of CG-NAP/AKAP450 staining localizes to the Golgi apparatus visualized with p58 k Abs (Golgi marker), as seen on the merged overlay panel (arrows).

are targeted to the cytoskeleton, localizing at the centrosome and microtubules. PKC subcellular localization has also been shown to be mediated by anchoring proteins (35). In this study, we demonstrate that CG-NAP/AKAP450 represents a component of the LFA-1-dependent multimolecular protein complex assembled upon LFA-1 cross-linking in motile T cells. This complex also includes the key signaling molecules PKC-β (16) and PKC-δ. Using the GFP-tagged C-terminal CG-NAP/AKAP450 construct, we provide direct evidence that intact CG-NAP/AKAP450 function and its recruitment to the LFA-1-associated multimolecular complex is critically important for polarization and migration of T cells induced by this integrin.

Materials and Methods

Cells

HUT-78 (human T lymphoma cell line) was obtained from the American Type Culture Collection (ATCC). Human peripheral blood T lymphocytes (PBTLs) were obtained from healthy volunteers, using the RosetteSep technique (from Stem Cell Technologies), preactivated with 25 ng/ml PMA (Sigma-Aldrich) for 72 h at 37°C as previously described (36). Cells were grown in CO_2-independent medium (Invitrogen Life Technologies) supplemented with 10% FCS and antibiotics (penicillin, streptomycin, and L-glutamine).

Abs and reagents

Polyclonal antisera against CG-NAP/AKAP450 designated αEE and αBH were described previously (30). Abs used for induction of T cell motility as previously described (36) were of the clone SPV-L7 (mAb to the α-chain of LFA-1 from Sanbio). Other LFA-1 clones used were YTN-81.5 from Serotec and MEM83 (Sanbio). Recombinant ICAM-Fc protein was from R&D Systems. Monoclonal AKAP-450 (clone 7) was purchased from BD Biosciences/BD Pharmingen. Anti-PKC-δ and anti-PKC-θ were from BD Transduction Laboratories. Fibronectin was purchased from Sigma-Aldrich. Other Abs and reagents used for immunofluorescent staining (all purchased from Sigma-Aldrich) were: anti-α-tubulin (clone B-5-1-2), anti-γ-tubulin (clone GTU-88), FITC-conjugated monoclonal anti-LFA-1, and anti-58 K-Golgi (clone 58K-9). Normal goat serum (R&D Systems) was used for blocking. For negative control, we used normal mouse and normal rabbit sera (NMS and NRS, respectively). Nuclei were visualized with Hoechst 33342 (Sigma-Aldrich). Secondary Abs were FITC-conjugated goat anti-rabbit, TRITC-conjugated goat anti-mouse (Sigma-Aldrich); Alexa Fluor 488 and Alexa Fluor 568 Abs were from Molecular Probes. The C-terminal GFP-linked CG-NAP/AKAP450 construct was prepared as described (34).

Induction of cell motility

Eight-well Permanox plastic chamber slides (Nunc) were coated with 50 μg/ml rICAM/Fc overnight at 4°C. In several experiments, slides were alternatively coated with goat anti-mouse Igs overnight at 4°C and washed gently twice with sterile PBS, followed by motility inducing anti-LFA-1Abs clone (SPVL-7) for 1 h at room temperature. The wells were further washed twice in PBS. PBTLs or HUT-78 were loaded into the chambers at 5×10^5 cells/well. In separate experiments, chamber slides were either covered as above with anti-human β_1 integrin Abs (clone BV7; Abcam), anti-CD54 (MEM-111; Monosan) or with human purified fibronectin at 1 mg/ml for 12 h at 4°C. For control studies (resting cells), cells were directly placed on Super Frost Plus positively charged slides (BDH).

Immunofluorescent staining and microscopy

For immunostaining, the slides with attached cells were fixed by immersion in 100% cold methanol, or in some experiments fixed in 4% paraformaldehyde in PBS, and permeabilized in 0.2% Tween 20 in PBS. After permeabilization, the slides were blocked with normal goat serum. The slides were incubated with primary Abs for 2 h at room temperature, then washed three times in 0.1% Tween 20 in PBS, followed by incubation with FITC-conjugated goat anti-rabbit and TRITC-conjugated goat anti-mouse secondary Abs for 30 min.

Immunofluorescent studies of LFA-1 and CG-NAP/AKAP450 colocalization were performed as follows: activated PBTLs were allowed to adhere to the bottom of glass chamber slides, coated with rICAM-Fc protein. After 2 h of incubation, FITC-conjugated monoclonal anti-LFA-1 Abs were added for 30 min. Wells were then washed gently twice with prewarmed culture medium. In several studies, Alexa Fluor 488-labeled goat anti-mouse IgG were added for further 15 min to improve green signal performance for confocal microscopy. Cells were washed gently and fixed with 4% formaldehyde in culture medium. Cells were then permeabilized in 0.1% Triton X-100 in PBS, followed by anti-CG-NAP/AKAP450, or monoclonal anti-AKAP450 Abs for 1 h and Alexa Fluor 568-labeled goat anti-rabbit IgG for 15 min.

Conventional microscopy and microphotography was performed on a Nikon TE 300 inverted microscope equipped with a Leica DC-100 color digital camera. Confocal microscopy was performed on the Live Cell Imager workstation (PerkinElmer). Images were acquired using UltraView acquisition software and processed with the Volocity 2.0.1 program (Improvision). For the three-dimensional reconstruction, 32–50 image slices where acquired in the Z-plane with 0.2-μm step intervals.

Microinjection

Direct intranuclear injection of plasmid DNA (0.2 μg/ml) was performed within the first 30 min after the initial adhesion of HUT-78 cells on anti-LFA-1 Abs immobilized in borosilicate glass-chambered coverslips. On average, 50–100 cells were injected using glass capillary microneedles (inner diameter 0.1 μm) with Narishige microinjection equipment. Cells were analyzed 6 h postmicroinjection when the GFP fluorescent signal was clearly detectable. At least 25–30 individual cells were observed per each experimental condition.

Analysis of the subcellular localization of the GFP-tagged expressed C-terminal domain of CG-NAP/AKAP450 in relation to the endogenous CG-NAP/AKAP450 and LFA-1 was performed as described earlier, in which HUT-78 were allowed to adhere to the bottom of glass chamber
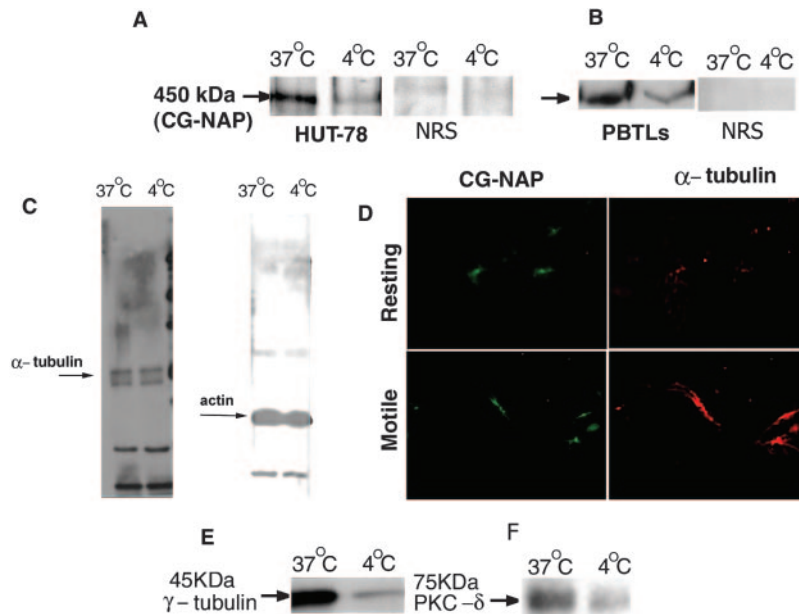
FIGURE 4. In situ precipitation analysis of the LFA-1-associated complex. Western blot detection of proteins associated with the LFA-1 cytoskeletal/ signaling complex in HUT-78 (*A*) and PBTLs (*B*) revealed that CG-NAP/AKAP450 represents a component of the complex. CG-NAP/AKAP450 association with the complex was significantly increased at 37°C compared with 4°C. NRS, probing with control nonimmune rabbit serum. *C*, Cross-linking of CD54 does not induce the cytoskeleton/signaling complex in T cells. Western blot analysis of the α-tubulin component present in in situ immunoprecipitates performed using immobilized anti-CD54. Equally low amounts of this cytoskeletal protein are detected in the immunoprecipitates performed at 37°C and 4°C. Actin levels demonstrate equal loading of proteins as its expression previously has been shown to be independent of the cell migratory potential (16). *D*, Detection of the components of the in situ immunoprecipitated complex by immunofluorescence. The cells were exposed to motility induced Abs via LFA-1 as described in *Materials and Methods*, were lysed in membrane detergent-containing buffer and the LFA-1-associated intracellular components were stained for tubulin (red) and CG-NAP/AKAP450 (green). Cells were kept either at 4°C or 37°C as indicated on the figures. Note that tubulin at 4°C is detected in situ as faint red spots after cell lysis compared with partially preserved filamentous structures at 37°C. *E*, The amount of γ-tubulin associated with the LFA-1 signaling complex was examined. At 37°C, the amount of γ-tubulin is higher when compared with the nonmotile cells at 4°C, due to the fact that low temperature preserves LFA-1-mediated adhesion but inhibits the process of cytoskeletal reassembly and signaling. *F*, Further analysis of the in situ immunoprecipitate revealed that PKC-δ associates with the complex; the association is increased at 37°C in the locomotory phenotype.

slides coated with rICAM-1/Fc, followed by microinjection with the C-terminal GFP-tagged CG-NAP/AKAP450 construct. Four hours postmicroinjection, cells were fixed with 4% formaldehyde in culture medium and permeabilized in 0.1% Triton X-100 in PBS. Cells were subsequently immunostained with rat monoclonal anti-LFA-1 (Serotec) followed by goat anti-rat and 7-amino-4-methylcoumarin-3-acetic acid-conjugated anti-goat reagents (DAKO) or anti-PKC-β-1 (Sigma-Aldrich) and goat anti-rabbit TRITC conjugate (DAKO). AKAP450 was detected with mouse mAbs (BD Transduction Laboratories) followed by Alexa Fluor 568 anti-mouse conjugates (Molecular Probes).

Immunoprecipitation and Western blotting

The in situ immunoprecipitation assay used in this study was largely based on the method developed earlier (16) with modifications. HUT-78 or activated PBTLs (1×10^6/ml) were exposed to purified fibronectin or monoclonal anti-LFA-1 immobilized on 50-ml tissue culture flasks as described above. Cells were then incubated either at 37°C or 4°C (low temperature preserves cell attachment but blocks active locomotion). Following a 3-h incubation, flasks were washed three times in warmed PBS to remove nonattached cells prior to lysis. Cells remaining adherent to the flasks were lysed with buffer containing (PBS (pH 7.4), 0.5% Nonidet P-40, 5 mM MgCl$_2$, 1 mM EGTA, 1 mM PMSF, 10 μg/ml leupeptin, 0.2 μM okadaic acid) at 25°C. After double washing with the lysis buffer and finally with PBS, proteins associated strongly with LFA-1 (LFA-cytoskeletal complexes) remained attached to the flask bottom via LFA-1/ligands interactions. These complexes were next extracted with SDS-containing buffer (20 mM Tris-HCL (pH 7.5), 1% SDS, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 0.5 mM PMSF, 10 μg/ml leupeptin) 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 comparative analysis. Proteins were resolved by 4.5% SDS-PAGE, electrotransferred onto a polyvinylidene difluoride membrane and probed with anti-CG-NAP/AKAP450, PKC-δ and γ-tubulin Abs and developed by the ECL method.

For the immunofluorescent detection of the components of the LFA-1 signaling complex, cells were lysed in the same buffer (above), followed by incubation with Abs as described above.

To detect the presence of CG-NAP/AKAP450 in total cell lysates, cells were lysed in boiling buffer containing 20 mM Tris-HCl (pH 7.5), 1% SDS, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 0.5 mM PMSF, and 10 μg/ml leupeptin and spun down at $16,000 \times g$ for 5 min. The resulting supernatant was collected and concentrated by acetone precipitation and Western blotting was performed as described above.

Results

CG-NAP/AKAP450 is expressed in T lymphocytes and localizes at the centrosome

Double immunofluorescence staining of T cells for CG-NAP/ AKAP450 and α-tubulin was performed to determine the localization of CG-NAP/AKAP450 in HUT-78 cells (Fig. 1*A*). In resting cells, CG-NAP/AKAP450 staining was detected as a compact area around the centrosome and a weaker stained scattered network in the cytoplasm. Cells activated on immobilized anti-LFA-1 mAb (Fig. 1*A*, *lower panel*) acquired a polarized locomotory phenotype (see also supplementary movie 4)[5] with a trailing extension (on average, >90% of cell population). Cells exposed to immobilized rICAM-1 displayed the same morphological changes (see Fig. 8). In these cells that acquired a motile phenotype, CG-NAP/ AKAP450 was localized to the area of the centrosome, and distal to the centrosome along the microtubules. Similar distribution of

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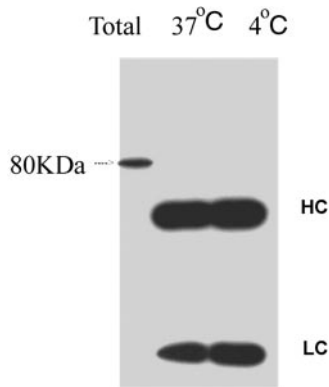[5] The online version of this article contains supplemental material.

FIGURE 5. Specificity of kinase association with the in situ immuno-precipitated LFA-1-assembled complex. A PKC isoform with distinctive cellular functions (PKC-θ) was not detectable in the in situ-LFA-1 immuno-precipitates. PKC-θ was detected in the total cell lysates in HUT-78 at 80 kDa. In situ immunoprecipitates performed either at 4°C or 37°C displayed the presence of equal amounts of H and L chains (HC and LC) of the precipitating Abs (serving here also as a loading control), but not the PKC-θ-specific band. Please note that due to a significant difference in gel concentrations and ECL exposure times used throughout the study for identification of particular proteins, relevant chains of the precipitating Abs did not always get detected at the same positions or appear clearly visible on the probed membranes.

CG-NAP/AKAP450 was also detected in resting and locomotory PBTLs (Fig. 1C). Western blotting in HUT-78 cells (see Figs. 1D and 4A) and PBTLs (see Fig. 4B) confirmed the presence of CG-NAP/AKAP450 as a high molecular mass species (~450 kDa). Therefore, our findings demonstrate that CG-NAP/AKAP450 is expressed in normal T lymphocytes and lymphoma T cell line, and that CG-NAP/AKAP450 undergoes distribution along microtubules upon LFA-1 cross-linking. The Abs which did not induce a locomotory phenotype in T cells, but supported adhesion and spreading, e.g., anti-CD54 (Fig. 1E) and anti-LFA-1 clone MEM83 (Fig. 1G) also failed to induce cell polarity and the redistribution of either CG-NAP/AKAP450 or microtubules.

CG-NAP/AKAP450 colocalizes with γ-tubulin at the centrosome and with the Golgi apparatus

We next examined the relationship of the compact and scattered CG-NAP/AKAP450 staining components to the respective intracellular structures. We found that CG-NAP/AKAP450 was localized to the centrosome using costaining with γ-tubulin Abs in resting and LFA-1-activated HUT-78 cells (Fig. 2). HUT-78 cells were also double stained with anti-Golgi p58 Ab. The noncentrosomal associated scattered pattern of CG-NAP/AKAP450 colocalized with the Golgi staining (Fig. 3).

CG-NAP/AKAP450 is a component of the LFA-1-induced signaling complex

In situ immunoprecipitation using mAb SPV-L7 as the motility-inducing LFA-1 mAb (Fig. 4, A and B) was used to determine whether CG-NAP/AKAP450 associated with the LFA-1 signaling complex. There was little CG-NAP/AKAP450 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. However, association of CG-NAP/AKAP450 was significantly enhanced at 37°C in LFA-1-activated cells triggered to undergo motility. The Abs to a distinctive cell receptor, CD54, previously shown not to induce cell polarity and locomotion under

similar experimental conditions (Fig. 1E), did not stimulate the assembly of the cytoskeleton/signaling complex as confirmed by Western blotting for anti-tubulin (Fig. 4C), previously reported to be a marker component of the complex (16). This was confirmed by the immunofluorescent staining of the in in situ-immunoprecipitate complex (Fig. 4D), where significantly lower amounts of CG-NAP/AKAP450 and α-tubulin were recruited in the complex in low temperature conditions blocking active intracellular signaling events. Similar association with γ-tubulin in the LFA-1 signaling complex was observed (Fig. 4E). We have now shown that PKC-δ (Fig. 4F) in addition to the previously reported PKC-β isoform (16) associates with the LFA-1 signaling complex during lymphocyte migration. By contrast, PKC-θ, which associates with the TCR-associated complex at the immunological synapse, is not detectable in the in situ immunoprecipitates (Fig. 5).

These findings have been confirmed by confocal microscopy of cells immunostained for CG-NAP/AKAP450 and LFA-1 (Fig. 6 and supplementary movie 2). Colocalization was observed at the distal portions of the elongated CG-NAP/AKAP450 scaffolding and a subset of LFA-1 adjacent to the plasma membrane.

We then compared CG-NAP/AKAP450 distribution in a cell locomotion system different from LFA-1 triggered motility, i.e., on immobilized purified fibronectin (Fig. 7A). Although the polarized morphology of T cells on fibronectin is somewhat similar to the cells migrating on ICAM-1, CG-NAP/AKAP450 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. In addition, the amount of CG-NAP/AKAP450 associated with fibronectin ligands represented by β_1 integrins (Fig. 7B) was noticeably less in comparison to that observed after LFA-1 cross-linking at 37°C. A weak basal amount of CG-NAP/AKAP450 brought down by the in situ immunoprecipitates performed on the immobilized fibronectin or Abs specific for β_1 integrin was equivalent to the level of CG-NAP/AKAP450 recruitment under metabolically inert conditions.

Overexpression of the C-terminal domain of CG-NAP/AKAP450 blocks LFA-1-dependent T cell polarization and migration

Transfection of HUT-78 cells with a plasmid encoding C-terminal domain of CG-NAP/AKAP450 resulted in the loss of cell polarity in response to LFA-1 ligation and blocked the development of the typical locomotion-associated phenotype (Fig. 8, A, B, and D). Cells injected with the plasmid 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 filopodia remained intact in these cells (Fig. 8B and supplementary movie 4). Cells injected with the empty vector GFP retained their ability to polarize and migrate when triggered via LFA-1 (Fig. 8C and supplementary movie 5). In the cells overexpressing the C-terminal domain of CG-NAP/AKAP450, both endogenous CG-NAP/AKAP450 and the C-terminal mutant were present at the compact centrosomal location displaying a partial colocalization (Fig. 8D). Wild-type CG-NAP/AKAP450 no longer colocalized with LFA-1, which retained a circumferential cellular distribution, strikingly different to motile untransfected cells where LFA-1 moved to a location adjacent to the base or "neck" of the trailing uropod. In the cells expressing the C-terminal mutant, there was a notable disruption of the ability of PKC-β to translocate to the centrosome and the associated complex was supported by the microtubules (Fig. 8E). Instead, it retained a largely diffuse cytosolic pattern of staining. The loss of cell polarity and PKC-β redistribution was displayed in a 100% of those cells which remained viable following the microinjection and which expressed the GFP-tagged mutant protein.

FIGURE 6. Colocalization of CG-NAP/AKAP450 and LFA-1 in locomotory T cells. *A* and *B*, Three-dimensional 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/AKAP450 (red). Colocalization is reflected as an orange/yellow merge color (arrows) in two different spatial orientation planes, predominantly at the distal portions of the CG-NAP/AKAP450 scaffolding in proximity to the LFA-1 pool at the plasma membrane. *Inset* in *B*, Transmitted light and fluorescent overlay of the same cell. See also corresponding supplementary movie 2.
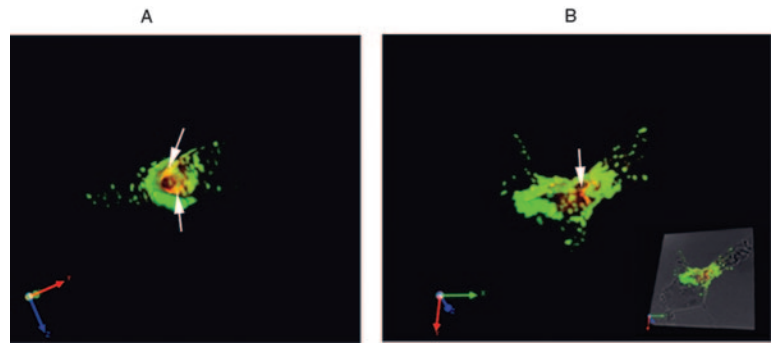
Discussion

It has become clear that in many cells, the process of signal transudation depends on cytoskeletal scaffolding proteins that directly coordinate signaling. By targeting effector kinases, phosphatases, and substrates at the same subcellular location, these scaffolding proteins organize multiple signaling pathways within cells (20–25). Moreover, it has been shown that AKAPs play a role in the maintenance of T cell homeostasis (37).

CG-NAP/AKAP450 is a scaffolding protein from the family of related AKAPs which was shown to associate with the centrosome and the Golgi apparatus in a number of mammalian cell lines (30). CG-NAP/AKAP450 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. CG-NAP/AKAP450 has binding sites for protein phosphatases PP1 and PP2A, and was also found to interact with an immature nonphosphorylated form of PKC-ϵ (33). The complex coiled-coil architecture of the molecule adds to the characteristic features of CG-NAP/AKAP450 likely to be involved in multiple associations with several proteins.

In this study, we show for the first time that the scaffolding protein CG-NAP/AKAP450 is expressed in PBL PBTLs and in the T lymphoma cell line HUT-78. Expression of this molecular scaffold is localized to the region of the centrosome and the Golgi apparatus. LFA-1 cross-linking results in the development of the signaling complex that contains cytoskeletal components along with the PKC isoenzymes β and δ. We demonstrate here that CG-NAP/AKAP450 represents an integral component of this complex. Specifically by linking together the centrosome, microtubules, and

two enzymes involved in cytoskeletal modification in locomotory T cells, CG-NAP/AKAP450 could potentially contribute to the orchestration of LFA-1-induced intracellular signaling cascades. The functional importance of this is further underscored by 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.

The LFA-1 cytoskeletal interactions are coordinated during lymphocyte migration. Association of CG-NAP/AKAP450 with the LFA-1 signaling complex may be dependent on intact microtubule functioning. Disruption of microtubules caused partial dispersion of CG-NAP/AKAP450 localization (data not shown), implying that this association may be important in at least some of the signaling process involved in migration (38). We have previously demonstrated that LFA-1 activation also induces the secretion of chemokines involved in regulation of directional cell migration (39). Such secretion would be predicted to be dependent on the 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 molecules involved in intracellular transport and secretion. In addition to the critical role of PKC-β in T cell migration, we have also demonstrated that PKC-β expression is crucial for transport and secretion of the cytokine IL-2, further emphasizing the connectivities between the signaling events involved in these cellular functions. Microtubules play an active role in both migration and secretion. The association of CG-NAP/AKAP450 with the γ-tubulin ring complex at the centrosome (40) suggests the
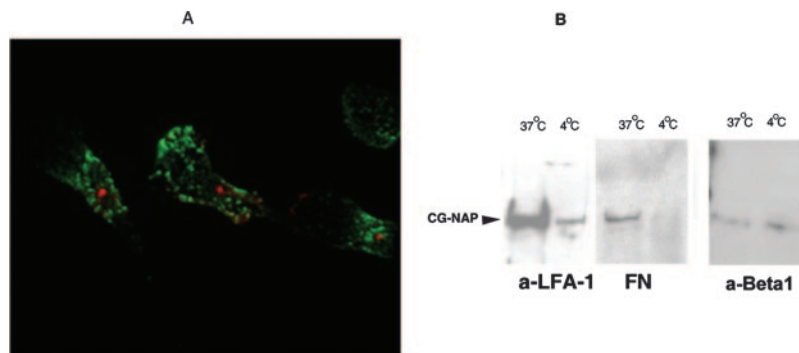

FIGURE 7. *A*, Spatial distribution of LFA-1 and CG-NAP/AKAP450 scaffolding in T cells migrating on fibronectin. Note the compact spot of CG-NAP/AKAP450 around the MTOC (red), a different intracellular distribution pattern compared with the one registered in cells triggered via LFA-1. LFA-1 is no longer seen in the characteristic location around the base of the trailing tail projections (compare to Fig. 6). See also supplementary movie 3. *B*, Comparative analysis of CG-NAP/AKAP450 detected in in situ immunoprecipitates from the cells migrating on fibronectin and after LFA-1 and β_1 integrin cross-linking. The amount of CG-NAP/AKAP450 associated with the complex in T cells exposed to fibronectin or anti-β_1 Abs was markedly lower than that assembled on LFA-1 cross-linking under identical experimental conditions. Labels above the lanes indicate immunoprecipitates obtained in metabolically active (37°C) and inert (4°C) conditions. a-LFA, Immunoprecipitates from the motile cells triggered via LFA-1 receptor. FN, Immunoprecipitates from the cells migrating on fibronectin. A-Beta-1, anti-β_1 integrin Ab used for in situ immunoprecipitation.
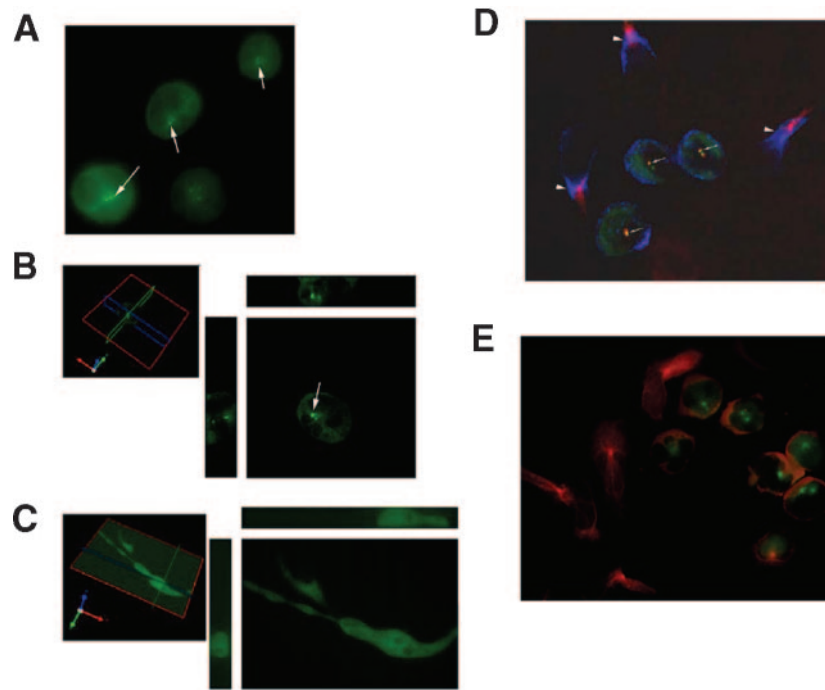
FIGURE 8. Overexpression of the C-terminal domain of CG-NAP/AKAP450 in HUT-78 cells triggered by ICAM-Fc via LFA-1. *A* and *B*, Note clearly defined spots indicated by arrows corresponding to the location of the C-terminal GFP/CG-NAP/AKAP450 protein at the centrioles in adherent, but nonpolarized cells (arrows). *B*, X-Y-Z planes of the representative transfected cell illustrating spatial intracellular location of the C-terminal CG-NAP/AKAP450/GFP (see also relevant supplementary movie 4). In cells microinjected with the C-terminal GFP/CG-NAP/AKAP450 construct, the absolute majority (95%) of the population gave an identical response. Cells which did not survive the microinjection procedure were excluded from the analysis. *C*, X-Y-Z confocal projections of cells transected with EGFP vector alone. Cells reserved their elongated morphology and locomotory characteristics (see also relevant supplementary movie 5). *D*, Overexpression of the C-terminal domain of CG-NAP/AKAP450 blocks the association of wild-type CG-NAP/AKAP450 with the LFA-1 signaling complex. T cells of the HUT-78 line exposed to a motility-triggering stimulus via immobilized ICAM-1/Fc were analysed for intracellular distribution of GFP-tagged C-terminal CG-NAP/AKAP450 domain in combination with endogenous CG-NAP/AKAP450 (red) and LFA-1 (blue). Cells injected with plasmid-encoding GFP-tagged C-terminal CG-NAP/AKAP450 (arrows) lost their motile phenotype and no longer display a characteristic concentrated staining for LFA-1 at the base of the trailing uropods typical of locomotory cells (arrowheads). Partial colocalization of wild-type CG-NAP/AKAP450 and GFP-tagged C-terminal CG-NAP/AKAP450 is retained at the centrosome area (yellow/orange overlay color, arrows). *E*, Overexpression of the C-terminal domain of CG-NAP/AKAP450 disrupts the recruitment of the PKC-β into the LFA-1-associated signaling complex. Experimental conditions and microinjection were performed as described in *D*. Green, GFP-tagged C-terminal domain of CG-NAP/AKAP450; red, PKC-β.

possibility that CG-NAP/AKAP450 may play a crucial role in microtubule-dependent functions in both processes.

The functional significance of CG-NAP/AKAP450 for LFA-1-induced motility is further underscored by the results of the studies in cell transfectants. The fact that cells overexpressing the C-terminal domain of CG-NAP/AKAP450 failed to polarize and acquire a locomotory phenotype indicates that CG-NAP/AKAP450 is crucially involved in one of the MTOC-dependent cell functions such as active motile behavior. These data are in concert with the recent findings by Keryer et al. (34) who demonstrated that overexpression of CG-NAP/AKAP450 in Hela cells impairs cytokinesis and increases ploidy implying a significant functional role of CG-NAP/AKAP450 in the integrity of the centrosome and related signaling pathways.

In addition, the results of studies in the fruit fly demonstrate that when the *Drosophila* homolog of CG-NAP/AKAP-450 (*Drosophila* pericentrin-like protein (D-PLP)) is mutated, mitosis is not severely perturbed. However, D-PLP is essential for microtubule-supported cilia/flagella function and D-PLP-mutant flies are uncoordinated and have nonfunctional neurons (41). Furthermore, pericentrin, which shares homology to CG-NAP/AKAP450, has recently been shown to scaffold PKC to the centrosome and to control major centrosomal functions including microtubule organization, spindle function, and cytokinesis (42).

The findings that intracellular distribution of CG-NAP/AKAP450 was typical of the cells triggered via ICAM-1, but not fibronectin, indicate that the involvement of this scaffolding protein might play a key role for β_2 integrin T cell motility but may not necessarily play a similar role in adhesion and motility induced via different integrin ligands, for example, the β_1 integrin ligand fibronectin or corresponding Abs. This is further supported by the data showing that the amount CG-NAP/AKAP450 associated with fibronectin ligands in migrating cells was significantly lower in comparison to that associated with LFA-1 in similar experimental conditions.

The fact that LFA-1 and wild-type CG-NAP/AKAP450 no longer colocalized in the cells overexpressing C-terminal GFP-tagged CG-NAP/AKAP450 strongly indicates that the formation and function of the motility-associated protein complex containing both LFA-1 and CG-NAP/AKAP450 was inhibited under these conditions. Taken together, our findings provide the first definitive evidence that the protein CG-NAP/AKAP450 is a key scaffolding component of the multimolecular complex assembled in T cells upon LFA cross-linking and is functionally indispensable for cell polarity and migration induced by this integrin.

Disclosures

The authors have no financial conflict of interest.

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