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Cloning and Functional Characterisation of Genes involved in Cell Death and Cell Survival

By

Helen Conroy

A thesis presented to the University of Dublin in fulfilment of the requirements for the degree of Doctor of Philosophy

Molecular Cell Biology Lab
Department of Genetics
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October 2003

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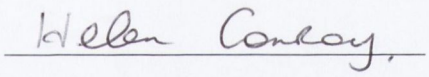

Helen Conroy

October 2003

For My Parents

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SUMMARY

During development and as a result of infection or injury, individual cells of multicellular organisms are required to mount appropriate survival or death responses. Two main signalling pathways are responsible for these outcomes. Apoptosis is a physiological cell death pathway responsible for a tightly controlled dismantling of cells and is regulated by a family of proteases called caspases. In opposition to this, the induction of the transcription factor NFκB results in the activation of a vast number of gene products which prevent cell death, promote immune responses and encourage cell growth and survival. The pathways of NFκB activation and caspase activation rely on interactions between effector proteins with distinctive domain structures. One such domain is the Caspase Recruitment Domain (CARD) domain, which facilitates protein-protein interactions between proteins in both caspase and NFκB induction pathways. As such, the CARD motif occupies a pivotal position in cell death and cell survival. This thesis explores the role of two CARD proteins, NAC and ASC, in these pathways.

CHAPTER III

Caspase-1 is a member of a family of enzymes implicated in both cell death and immunity. The role of caspase-1 in cytokine production has been confirmed, however, the details of caspase-1 activation and regulation remain obscure. The yeast two-hybrid approach was used to search for binding partners for this caspase. However, no confirmed caspase-1 interacting proteins were identified in the screen.

CHAPTER IV

This chapter describes the identification and characterisation of a novel, CARD-containing protein, NAC. This protein also contains a CARD-like domain known as the PYRIN domain, whose function has not yet been defined. Given the tendency for many CARD-containing proteins to induce NFκB activation, this possibility was explored but ruled out as a function for NAC. NAC was also unable to induce

apoptosis upon transient transfection in human cell lines. However, NAC was found to cooperate with the only other PYRIN /CARD protein identified to date, ASC, in the induction of apoptosis. The synergy between these proteins relied on the CARD domain of NAC, suggesting a direct interaction between these proteins though their CARD domains. Co-immunoprecipitation experiments confirmed this interaction. Thus ASC and NAC may represent a novel apoptosis-inducing complex.

CHAPTER V

To further characterise apoptosis induced by ASC and NAC, immunolocalisation studies were carried out. These experiments revealed a NAC-dependent relocalisation of ASC into large complexes within cells. In addition, co-expression of these proteins led to processing and activation of endogenous caspases and appearance of the distinctive apoptotic morphology in cells. RNA interference experiments aimed at exploring ASC function revealed a siRNA-dependent, non-specific sensitization of cells to apoptotic stimuli.

ABBREVIATIONS

Ac-DEVD-AFC	Acetyl-DEVD-Aminofluormethylcoumarin
AD	Activation Domain (GAL4 transcriptional activation domain)
APAF-1	Apoptotic Protease Activating Factor 1
ASC	Apoptosis-inducing Speck-like protein with a CARD
ATP	Adenosine triphosphate
Bcl-1	B-cell Lymphoma
BD	Binding Domain (GAL4 DNA binding domain)
BLAST	Basic Local Alignment search tool
BSA	Bovine Serum Albumin
CAD	Caspase-dependent deoxyribonuclease
CARD	Caspase Recruitment Domain
CARDINAL	CARD Inhibitor of NFκB activating ligands
CASPASE	Cysteinyl Aspartate Specific Protease
CED	Cell Death Defective
CFU	Colony Forming Unit
Crm A	Cytokine Response Modifier
CTL	Cytotoxic T-Lymphocytes
DEFCAP	Death Effector Filament Forming Ced-4 apoptosis protein
DD	Death Domain
DEAE	Diethylaminoethanol
DED	Death Effector Domain
DISC	Death Inducing Signalling Complex
DMSO	Dimethylsulphoxide
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
DNA-PK	DNA-dependent Protein Kinase
DR	Death Receptor

DTT	Dithiothreitol
EDTA	EthyleneDiamineTetraAcetic Acid
ER	Endoplasmic Reticulum
FADD	Fas Associated Death Domain
FCS	Fetal Calf Serum
FITC	Fluorescein IsoThioCyanate
FL	Full length
FLIP	FLICE-like inhibitory protein
FMF	Familial Mediterranean Fever
GST	Glutathione S-Transferase
HEK	Human Embyonic Kidney cells
HEPES	N-(2-hydroxyethyl) peperazine-N′-(2-ethanesulphonic acid)
HRP	Horse Radish Peroxidase
HSP	Heat Shock Protein
IAP	Inhibitor of Apoptosis Protein
ICAD	Inhibitor of Caspase-dependent deoxyribonuclease
ICE	Interleukin-1β converting enzyme
IκB	Inhibitor of κB
IKK	IκB kinase
IL-1	Interleukin-1
IL-1R	IL-1 Receptor
IP	Immunoprecipitation
IPAF	ICE protase activating factor
IPTG	Isopropylthio-β-D-galactoside
IRAK	IL-1 receptor Associated Kinase
KDa	Kilo Daltons
LB	Luria Bertrani
LiAC	Lithium Actate
LPS	LipoPolySaccharide

LRR	Leucine Rich Repeats
MALT	Mucosa Associated Lymphoid Tissue
MCS	Multiple Cloning Site
MHC	Major Histocompatibility Complex
MMP	Mitochondrial Membrane Permeability
MOPS	4-Morpholinepropanesulfonic acid
MyD88	Myeloid Differentiation factor 88
NAC	NB domain and CARD protein
NBD	Nucleotide Binding Domain
NFκB	Nuclear factor κB
NK	Natural Killer
OD	Optical Density
ONPG	o-nitrophenyl β-D-galactopyranoside
PAGE	Poly Acrylamide Electrophoresis
PAMP	Pathogen Associated Molecular Pattern
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffered saline
PCD	Programmed Cell Death
PCR	Polymerase Chain Reaction
PEG	Poly Ethylene Glycol
PIPES	Piperazine-N-N′-bis(2-ethanesulphonic-acid)
PMSF	Phenylmethylsulphonylfluoride
PS	Phosphatidyl Serine
PT	Permeability transition
RAIDD	RIP-associated Ich-1/CED homologous protein with a DD
RICK	RIP-like interacting CLARP domain
RIP	Receptor interacting protein
RNA	RiboNucleic Acid
RPMI	Roswell Park Memorial Insitute

SD	Synthetic Dropout Medium
SDS	Sodium Dodecyl Sulphate
TAE	Tris Acetate EDTA
TBST	Tris buffered saline with Tween
TE	Tris EDTA
TIR	Toll/IL-1R homology
TLR	Toll-like receptor
TNF	Tumour Necrosis Factor
TNFR	Tumour Necrosis Factor Receptor
TRADD	Tumour Necrosis Factor Associated Protein with a Death Domain
TRAIL	Tumour Necrosis Factor Related Apoptosis Inducing Ligand
UV	Ultra-violet
VDAC	Voltage Dependent Anion Channel
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
XIAP	X-linked inhibitor of apoptosis protein
YPD	Yeast Extract/Peptone/Dextrose
zVAD.fmk	Benzyloxycarbonyl-Val-Ala-Asp-(OME) fluoromethylketone

PUBLICATIONS

Bouchier-Hayes, L., Conroy, H., Egan, H., Adrain, C., Creagh, E.M., MacFarlane, M. and Martin, S.J. (2001) CARDINAL, a novel caspase recruitment domain protein, is an inhibitor of multiple NF-κB activation pathways. *J. Biol Chem.* 47, 44069-44077.

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CHAPTER I

Introduction

1.1 INTRODUCTION

During the lifespan of a multicellular organism, the requirement for certain cells can change. Whether part of the natural development of the organism or as a response to variations in the environment, the body's overall composition of cells is constantly changing. The most significant of these changes occur through cellular proliferation and through the death of cells. Permanent changes to the body's complement of cells occur during the sculpting of tissue, where excess cells are removed (Zuzarte-Luis and Hurle, 2002). However, in other situations such as an immune response, the body maintains cellular homeostasis by co-ordinating the rapid increase in cell number with a balanced degree of cell death (de Alboran *et al.*, 2003).

1.1.1 *Determining Cell Fate*

The processes of cell death and cell survival are controlled by complex signalling networks involving the co-operation of multiple protein effectors. The complex pathways involved in determining the fate of cells are beginning to be unravelled, revealing highly organised and regulated processes. While the alternatives of cell death, cell survival and proliferation would seem to occupy opposite ends of a spectrum, they are not completely separate. Common proteins, signalling pathways and structural motifs are involved in bringing about both outcomes. These overlapping themes will be discussed in the following sections.

A concerted effort can be made by a cell to promote its own survival when it is faced with the threat of infection or injury. Furthermore, in the case of infection, speedy proliferation of necessary effector cells specifically equipped to cope with this threat is also required (Freitas and Rocha, 2000). However, the survival route is not always initiated. Sometimes, limited cell death is necessary to prevent a threat to the organism as a whole. Surprisingly, the elimination of healthy cells (as in the removal of inter-digital tissue during development) or their unhealthy counterparts

(damaged or pathogen infected cells) is achieved by similar means. The mechanism responsible for the controlled removal of all types of unwanted cells is known as Programmed Cell Death.

1.1.2 *Programmed Cell Death*

Programmed cell death (PCD) or apoptosis is a form of death that involves the cell being dismantled in a coordinated fashion from within (Kerr *et al.*, 1972). The original studies on apoptosis used morphological characteristics to define this distinct type of death (Robertson *et al.*, 1978). These features include cell shrinkage, chromatin condensation, membrane-blebbing and cellular fragmentation. The dying cell detaches from its neighbours and collapses inwards. The structure of the cell rearranges until all that remains are membrane-bound cell fragments called 'apoptotic bodies'. In this way the cell does not burst and the contents are not released, avoiding an inflammatory response and causing minimal destruction to the surrounding tissue (Weedon *et al.*, 1979). This controlled form of cell death differs greatly from necrosis which is a result of a harsh insult which overcomes the cells ability to survive or activate a more organised cell death (Rafrey and Cohen, 1997).

The membranes around the apoptotic cell and apoptotic bodies undergo alterations to facilitate their recognition and removal by phagocytes. During apoptosis, phosphatidylserine (PS), a phospholipid normally found in the inner leaflet of the plasma membrane, moves to the outer leaflet (Fadok *et al.*, 1992; Martin *et al.*, 1995). This 'PS flip' is critical for recognition and phagocytosis by either neighbouring cells or circulating phagocytes (Henson *et al.*, 2001). Recently, another signalling molecule, lysophosphatidylcholine, was identified as being released after activation of calcium independent phospholipase A2 during apoptosis. Lysophophatidylcholine, a phospholipid, is a chemotactic factor secreted by cells to attract phagocytes to the dying cell and ensure its removal (Lauber *et al.*, 2003).

1.2 THE CASPASE FAMILY

The morphological and biochemical changes associated with apoptosis are achieved though the activation of a family of proteases known as the caspases (Cysteinyl Aspartate-specific Proteases). Although not originally discovered in a cell death context, the role of these enzymes in cell death was suspected when their homology to the CED-3 gene of the nematode *Caenorhabditis elegans (C. elegans*) was discovered (Yuan *et al.*, 1993). The programmed cell death pathway is responsible for the removal of 131 of 1090 cells during development of the hermaphrodite form of *C. elegans*. Mutations in any of the CED (cell death abnormal) family of genes interferes with the normal cell death phenotype (Liu and Hengartner, 1999). Although PCD in the nematode is greatly simplified compared to mammals, other members of the CED family have since been found to be homologues of other key proteins involved in mammalian apoptosis (Hengartner and Horvitz, 1994; Zou *et al.*, 1997).

1.2.1 *Caspase Function*

Thus far 11 human caspases have been identified (Figure 1.1). However, not all caspases have a central role in apoptosis, for example, caspases -1, -4, and -5 are believed to function predominantly in cytokine processing (Thornberry *et al.*, 1992; Faucheu *et al.*, 1995; Kamens *et al.*, 1995; Munday *et al.*, 1995; Faucheu *et al.*, 1996; Wang *et al.*, 1996). This role has been confirmed for caspase-1 and the extensive similarities in sequence, or in some cases substrate preference, between this subgroup of caspases led to the prediction that they have similar roles (Fassy *et al.*, 1998). Caspase-11 is likely to be the mouse equivalent of human caspase-5 as they share an additional characteristic of being Lipopolysaccharide (LPS)-inducible (Lin *et al.*, 2000). The remaining caspases are thought to have roles (of varying importance) in cell death. Through cleaving key cellular components, eliminating survival responses and demolishing internal scaffolding, the caspases are capable of completely disrupting the viability of the cell (Martin and Green, 1995; Fischer *et al.*,

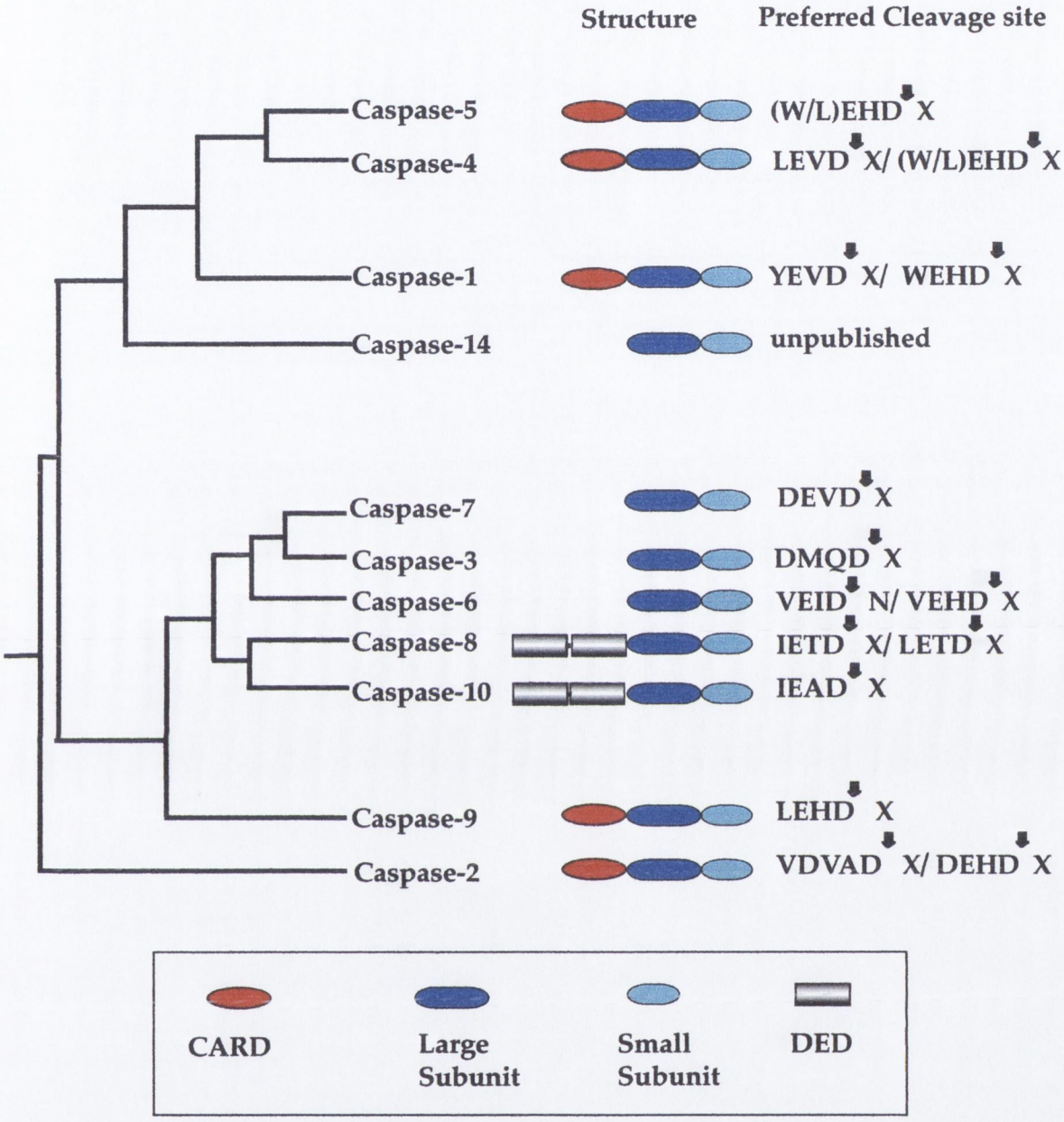


Figure 1.1:
Phylogenetic Relationships and Substrate Preferences of the Human Caspases
The caspases have evolved different structures and substrate preferences to form sub-families of similar enzymes. Caspases -1, -4, -5 and -14 have extensive homology and are predicted to be involved in cytokine processing. The other caspases which comprise both executioner and initiator caspases function in apoptosis.

2003). It is estimated that approximately 500 substrates exist in the cell (Martin, 2002) however, it is unclear at present which of these are crucial in orchestrating the apoptotic death phenotype. Consideration of some of the individual substrates provides a guide as to how specific features of apoptosis may be produced. For example, proteolysis and inactivation of substrates such as the DNA repair enzymes PARP (Poly-ADP-ribose-Polymerase) and DNA-PK (DNA-dependent Protein Kinase), prevents repair of damaged DNA (Lazebnik *et al.*, 1994; Casciola-Rosen *et al.*, 1995). Conversely, cleavage of ICAD (Inhibitor of Caspase Activated Deoxyribonuclease) allows the activation of the CAD endonuclease, which produces a particular pattern of nuclear DNA fragmentation, characteristic of apoptosis (Sakahira *et al.*, 1998). However, the precise effect that the cleavage of the majority of caspase substrates has during apoptosis is not yet known. These details will no doubt become clearer when the role of each substrate is investigated through the generation of non-cleavable mutants.

1.2.2 *Caspase Structure*

Caspases are synthesised as a single chain with an N-terminal pro-domain followed by a large subunit and a C-terminal small subunit (Nicholson, 1999). Cleaving of the immature molecule between the large and small subunit occurs at aspartate residues. The active caspase enzyme comprises a heterotetramer of two small and two large subunits (Stennicke and Salvesen, 1999). The active site is formed through interactions between the large and small units with a conserved QACXG motif at its core. The typical caspase cleavage site in substrates is a DXXD motif, with cleavage occurring after the second aspartate residue. The preferred substrate cleavage site differs between caspases (Figure 1.1). As the caspases are themselves processed at aspartate residues between the large and small subunits, the possibility that self or autoprocessing and caspase cascades occur was suggested and has since been confirmed (Kumar, 1999; Slee *et al.*, 1999).

The caspases can be structurally divided into two groups; the initiator and the executioner caspases (Cohen, 1997). The initiator or apical caspases possess long pro-domains as well as the small and large subunit, whereas the executioner caspases have short prodomains. The division made in terms of structure may also hold for function. The initiator caspases are first to be processed and activated in response to a death signal and are responsible for cleaving downstream effector caspases in a defined order (Slee *et al.*, 1999) (Figure 1.2). The activated effector/executioner caspases then cleave distinct cellular components in order to dismantle the cell. Interestingly, feedback loops that involve so called effector caspases cleaving initiator caspases downstream in the activation cascade also exist (Slee *et al.*, 1999; Fujita *et al.*, 2001).

1.2.3 *Caspase Activation through autoprocessing*

There are three known methods by which caspases become activated. The first of these is the means by which the initiator group of caspases are believed to be activated. Initiator caspases are activated through auto-processing of their internal aspartate residues. Initiator caspases contain a long pro-domain region which enables these caspases to interact with adaptor molecules during activation. The 'induced proximity model' was put forward to describe how activation of the proximal initiator caspase occurs (Salvesen and Dixit, 1999). This model proposes that clustering of the proenzymes occur, when they are recruited to adaptors (via their pro-domains). The adjacent enzymes can then autoprocess to produce the mature enzyme. This clustering of proenzymes can occur at membrane receptor complexes or through interactions with cytoplasmic, scaffold proteins, which will be discussed later. This mode of activation is not possible for executioner caspases which lack a significant prodomain, and so do not have the typical surface for interaction with adaptor molecules.

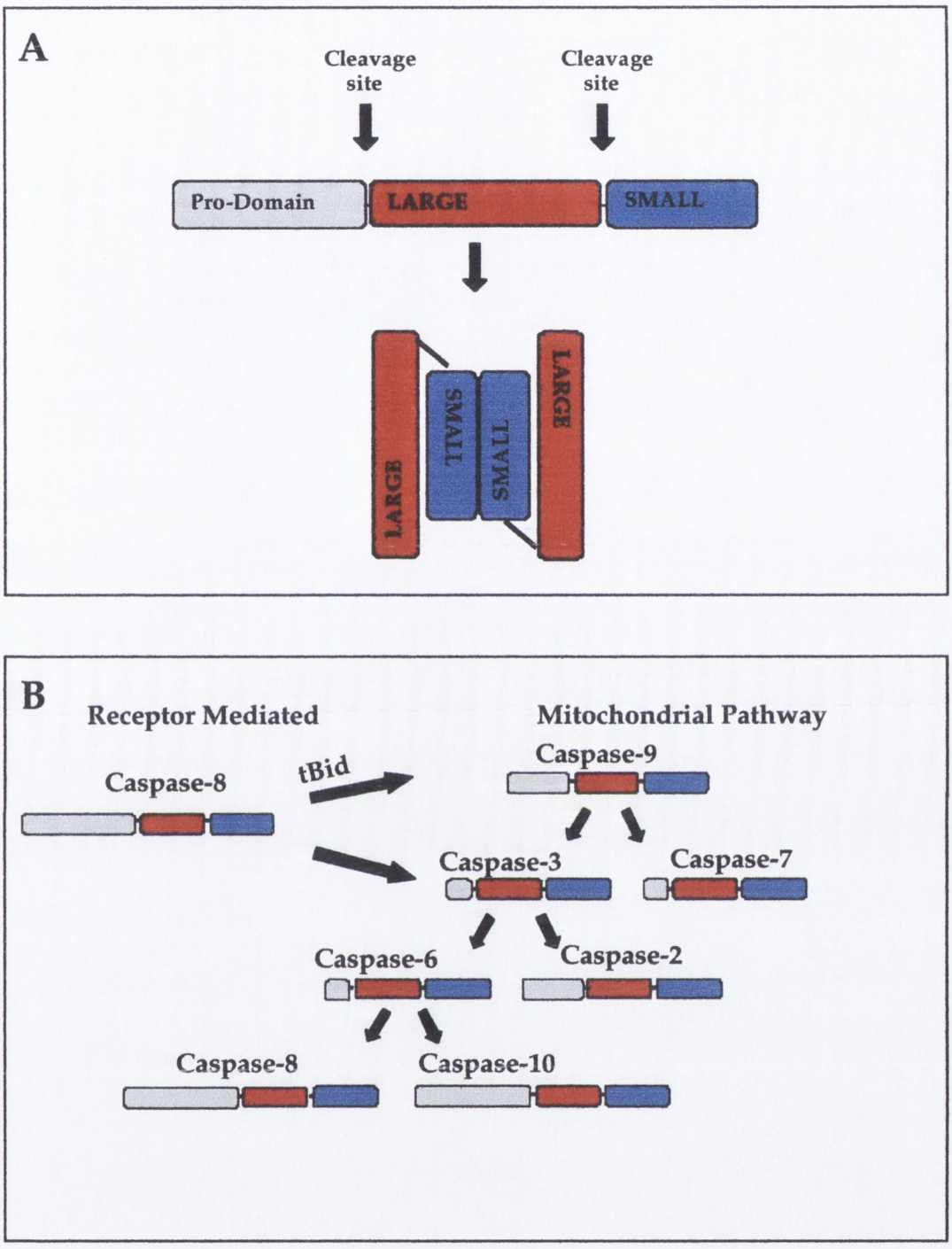


Figure 1.2:

A. Schematic representation of caspase activation through autoproteolysis
Cleavage of full length caspases at aspartate residues after the pro-domain and between the large and small sub-units frees these regions to form the mature enzyme as shown.

B. The caspase activation cascades.
Activation of the receptor mediated and mitochondrial apoptotic pathways result in a cascade of caspase activation.

1.2.4 *Caspase processing by Initiator caspases*

The executioner subset of caspases rely on the second type of activation mechanism. This involves processing by the activated upstream caspases between the large and small subunits, followed by auto-processing to remove the prodomain. In the case of caspases -3 and -7, the initial cleavage is carried out by caspase-9 (Srinivasula *et al.*, 1998). For caspase-6 the initial processing is actually done by another executioner, caspase-3. When the various executioner caspases were originally discovered, it was not known whether the members of this group of caspases had distinct roles or were redundant or interchangeable. This has been explored through systematic immunodepletion of each of the enzymes and examining the effect of this on substrate cleavage events (Slee *et al.*, 2001). In this study it was demonstrated that caspase-3 was responsible for the majority of substrate cleavage events examined. Thus, although caspases -6 and -7 are thought to be important executioner caspases, their role in apoptosis remains to be clarified.

1.2.5 *Caspase processing by Granzyme B*

In addition to auto-processing and processing by upstream caspases, a third activation mechanism, involving activation by non-caspase enzymes is also known to occur. The most common example of this mode of processing is exhibited by the apoptosis-inducing enzyme, Granzyme-B (Darmon *et al.*, 1995; Martin *et al.*, 1996). This enzyme is contained within the granules of cytotoxic T lymphocytes (CTLs) and Natural Killer (NK) cells along with a pore forming molecule, perforin (Barry and Bleakley, 2002; Waterhouse and Trapani, 2002). CTLs are responsible for recognising virally infected cells, displaying a viral epitope complexed to a Class I MHC (Major Histocompatibility Complex) molecule, on the plasma membrane of target cells. Recognition of such an epitope by CTLs results in granule mediated attack of the infected cell and release of Granzyme B into the cytoplasm. Granzyme B can directly process caspases -3 and -8 and also some known caspase substrates (Martin *et al.*, 1996; Medema *et al.*, 1997; Atkinson *et al.*, 1998). This enzyme can also

feed into the caspase activation cascade by cleaving a molecule called Bid (producing gtBid) to trigger the mitochondrial pathway which will be discussed below (Barry *et al.*, 2000; Heibein *et al.*, 2000).

1.3 CELL DEATH PATHWAYS

Although there are numerous reasons why a cell might undergo programmed cell death, at a molecular level caspases are activated through two main pathways; the receptor mediated pathway, and the mitochondrial pathway (Figure 1.3).

1.3.1 *The Fas pathway*

The receptor-mediated pathway begins at transmembrane receptors which belong to the death receptor subfamily of the nerve growth factor (NGF)/tumor necrosis factor (TNF) receptor superfamily (Ashkenazi and Dixit, 1998). This sub-family of membrane bound receptors includes; Fas, TNF-R1, DR3, DR4 (TRAIL-R1) and DR5 (TRAIL-R2). Two members of this family of receptors, the TNF and Fas receptors, are the best characterised of the group, with the majority of their downstream signalling events identified (Loetscher *et al.*, 1990; Schall *et al.*, 1990; Itoh, *et al.*, 1991).

Three main circumstances in which the Fas pathway is used to kill cells are known. First, the Fas receptor is known to play a role in the elimination of large numbers of superfluous immune cells which are produced during an infection when they have served their purpose. Second, the Fas-Fas-Ligand system is required for the removal of immune cells which have entered an immune-privileged area (such as the eye, central nervous system or the reproductive organs). Finally, the Fas system is involved in elimination of cells which have been infected with a virus (Peter and Krammer, 1998).

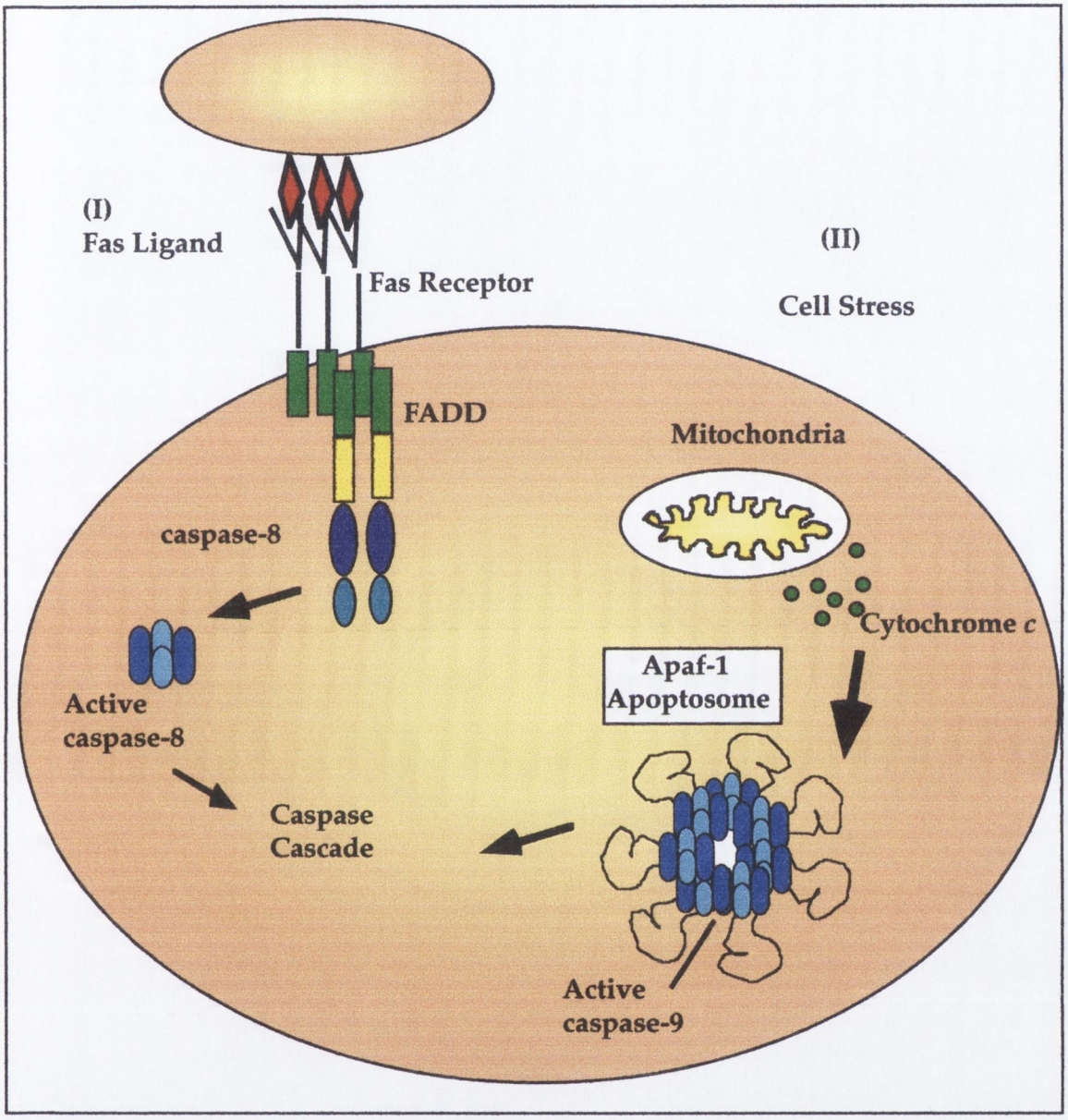


Figure 1.3:
Schematic represention of the two main pathways to apoptosis.
(I) In the death receptor pathway FADD associates with the Fas receptor via its DD (green), which recruits Caspase-8 via its DED (yellow) association. **(II)** General cellular stresses (such as chemotherapeutic drugs, DNA damage and heat shock) promote release of mitochondrial cytochrome *c*. In the cytosol, cytochrome *c* binds to the WD40 region of APAF-1, inducing its oligomerisation and association with caspase-9 via CARD-CARD (red) interactions. Both caspase-8 and -9 become activated via induced proximity (see text).

1.3.2 *Caspase activation at the Fas Receptor*

The Fas receptor is characterised by having an intracellular cytoplasmic death domain (DD) motif. Binding of the trimeric Fas ligand to the cognate receptor causes aggregation of the death domains and induces important conformational changes. These changes to the intracellular domain allows the recruitment of an adaptor molecule FADD (Fas Associated Death Domain) via its death domain (Boldin *et al.*, 1995; Chinnaiyan *et al.*, 1995). The FADD adaptor is a bipartite protein and aswell as a death domain, also contains a DD-like region called the DED (Death Effector Domain) capable of interaction with other proteins through DED-DED binding. The most significant DED-containing protein, which is recruited to FADD during Fas signalling is the initiator caspase, caspase-8 (Boldin *et al.*, 1996; Muzio *et al.*, 1996). Auto-processing of caspase-8 at the Fas signalling complex is one means of initiating the caspase cascade.

While apoptosis initiated at DR3, DR4, DR5, Fas and TNFRI are all believed to involve interactions between homologous death domain regions, it is worth noting that other members of the NGFR superfamily can promote apoptosis by other means. TNFRII and the neurotrophin receptor p75 are membrane receptors of the NGFR family which do not possess the typical cytoplasmic death domain found in the death receptors mentioned above. TNFRII like TNFRI also binds the cytokine TNFα, but with a greater affinity than TNFR1. While TNFRII lacks a death domain, it can promote apoptosis in two ways. The first of these relies on its superior ability to bind cytokine and increase its local concentration in the vicinity of TNFRI. TNFRII-bound cytokine is then 'passed' to TNFRI which then transduces a signal which promotes processing of caspase-8 and apoptosis (Tartaglia *et al.*, 1993). Another means by which TNFRII promotes death, which does involve signaling through the receptor itself, also exists. Although unable to recruit factors which promote apoptosis directly such as FADD and caspase-8, TNFRII like TNFRI, can promote signaling which results in activation of the transcription factor NFκB. This

transcription factor when activated promotes the expression and secretion of TNFα by the cell, which provides a feed back loop where the cytokine can bind to membrane TNFRI to promote apoptosis (Lazdins *et al.*, 1997; Vercammen *et al.*, 1995; Weiss *et al.*, 1998).

The details of neuronal apoptosis induced by the neurotrophin receptor p75 are not yet fully elucidated, however it is clear that the pathway involves an alternative mode of signal transduction to those mentioned above. The p75 receptor has a cytoplasmic type II death domain, similar but not identical to the type I receptor found in Fas or TNFRI (Liepinsh et al., 1997). This type of death domain is not believed to require self-association for its role (Rabizadeh *et al.*, 2000). Growth factor withdrawal provides the stimulus for p75-dependent apoptosis (Hofer and Barde, 1988) and two alternatives have been put forward to explain how the death signal is transduced. The first of these suggests that p75 is a 'dependence receptor' which will activate a death signal in the absence of ligand binding but binding of growth factors inhibits this death signal (Bredesen *et al.*, 1998). An alternative argument is that p75 does act in a manner similar to other death receptors where binding of a ligand is the stimulus for promoting death (Aloyz *et al.*, 1998; Frade *et al.*, 1996). A number of downstream cytoplasmic binding factors for p75 have been identified which may mediate apoptosis (Mukai *et al.*, 2000; Salehi *et al.*, 2000;Ye *et al.*, 1999) but the exact details of this mode of cell death are still unclear.

1.3.3 *The Mitochondrial Pathway*

Activation of the caspase cascade and completion of the apoptosis program without the engagement of membrane receptors also occurs. A variety of stimuli can initiate an alternative pathway mediated through mitochondria. Because mitochondria rupture during necrosis, maintainance of mitochondrial organelle integrity was one feature which was originally used to differentiate apoptosis from necrosis (Robertson and Orrenius, 2000). However, it later became clear that mitochondria

play a very important role during certain instances of apoptosis. Triggers for the mitochondrial route to cell death, also known as the intrinsic pathway, are numerous. These include cytotoxic drugs which target mitochondria directly, such as Antimycin A. Other drugs can antagonise the Endoplasmic Reticulum (ER), which functions in calcium homeostasis and the modification of proteins. ER antagonists can cause an accumulation of unfolded proteins. In addition, DNA damaging agents such as cisplatin or etoposide can induce expression of a number of pro-apoptotic proteins through activation of the transcription factor p53. All of the above stress signals converge, though mechanisms not yet fully delineated, on mitochondria (Ravagnan *et al.*, 2002).

1.3.4 *Mitochondrial Permeability*

The major activation event in the intrinsic pathway is the release of proteins from the mitochondrial inter membrane space. Two mechanisms have been suggested to explain the selective release of proteins from mitochondria. The first involves the formation of a permeability transition (PT) pore, through interactions between the two mitochondrial complexes, ANT in the inner mitochondrial membrane and voltage dependent anion channel (VDAC), in the outer mitochondrial membrane. The second and most widely accepted mechanism involves the activation of pro-apoptotic members of the bcl-2 family of proteins, BAX and BAK (Griffiths *et al.*, 1999, Wolter *et al.*, 1997). BAK resides in the mitochondrial outer membrane and can form oligomers with BAX when the latter translocates to mitochondria as a result of stress signals. These BAX/BAK oligomers form pores in the mitochondrial outer membrane. The resulting increase in mitochondrial membrane permeability (MMP) allows the release of a number of proteins capable of acting in the apoptosis pathway. When one of the most significant of these (cytochrome *c*) escapes, it plays a pivotal role in the formation of caspase activation complex by binding to Apaf-1 (Apoptosis protease activating factor-1) in the cytoplasm.

1.3.5 *Formation of the Apoptosome*

Apaf-1 is a multi-domain scaffold protein, which is ordinarily present in the cytoplasm in an inactive, closed conformation (Adrian *et al.*, 1999). During mitochondrial apoptosis cytochrome *c* binds to the C-terminal WD-40 repeat region of Apaf-1, followed by binding of dATP (Li *et al.*, 1997). Oligomerisation of multiple Apaf-1 molecules can then occur through a region of Apaf-1 homologous to the C. *elegans* protein, CED-4. The oligomerised Apaf-1 structure is a heptamer and resembles a wheel shape (Acehan *et al.*, 2002; Figure 1.3). Oligomerised Apaf-1 recruits pro-caspase-9 through its N-terminus. Caspase-9 then becomes activated through auto-processing. It is thought that the active enzyme may also rely on its association with Apaf-1 for full activity. Active caspase-9 can then feed into the caspase cascade by processing caspase-3 and caspase-7 (Figure 1.2B).

In some cell types the receptor mediated pathway feeds into the mitochondrial pathway. This is possible because one substrate of active caspase-8 is the pro-apoptotic Bcl-2 family member Bid (mentioned earlier as a substrate of Granzyme B; Li *et al.*, 1998). The cleavage product known as tBid, is capable of translocating to mitochondrial membranes and promoting release of cytochrome-c through activation of BAX and BAK (Korsmeyer *et al.*, 2000).

1.4 CELL SURVIVAL

Cells require a number of factors to be present at relatively constant levels in order to survive and proliferate. Changes in some of these, such as the removal of growth factors or introduction of heat shock, can trigger apoptosis. However, below a certain threshold level, not all damage is life-threatening. One set of threats which the body has developed a survival strategy against is infection and injury. When such a threat is detected the goal is to minimise the spread and eradicate the source of the infection.

A reaction termed 'the innate immune response' is initiated by damaged cells at the site of physical injury or infection due to release biochemicals such as histamine or prostaglandins. These chemicals induce localised inflammation, characterised by swelling and redness of the affected area. This is due to expansion of the blood vessels and increased blood supply in the proximity of the injury. The walls of the blood vessels also increase in permeability to allow the entry of specialised immune cells. Migration of these cells into the area of the infection is aided by the production of adhesion proteins.

The lymphocytes which arrive as part of the inflammatory response include short-lived neutrophils which phagocytose pathogens, and longer lived monocytes or macrophage which can clear pathogens and debris from dead neutrophils. These cells, as well as functioning in clearing up debris are also capable of participating in the signalling which amplifies the general innate immune response. This leads to the initiation of a more specialised adaptive immune response.

1.4.1 *Signalling in Cell Survival*

As with the induction of programmed cell death, a number of triggers are capable of promoting cell survival and proliferation. The most obvious of these are pathogens which result in the initiation of an immune response. As with apoptosis, various molecular signals converge at a small number of effector elements to signal a survival pathway (Figure 1.4).

1.4.2 *The Toll-Like Receptors (TLRs)*

The Toll-like receptors are a family of ten receptors, that derive their name similarity to the *Drosophila melonogaster* Toll protein. In mammals, these membrane bound receptors are responsible for the recognition of pathogen associated molecular patterns (PAMPs), which are conserved elements of a pathogen's structure. The Toll-like receptors have a leucine rich repeat (LRR) motif in their

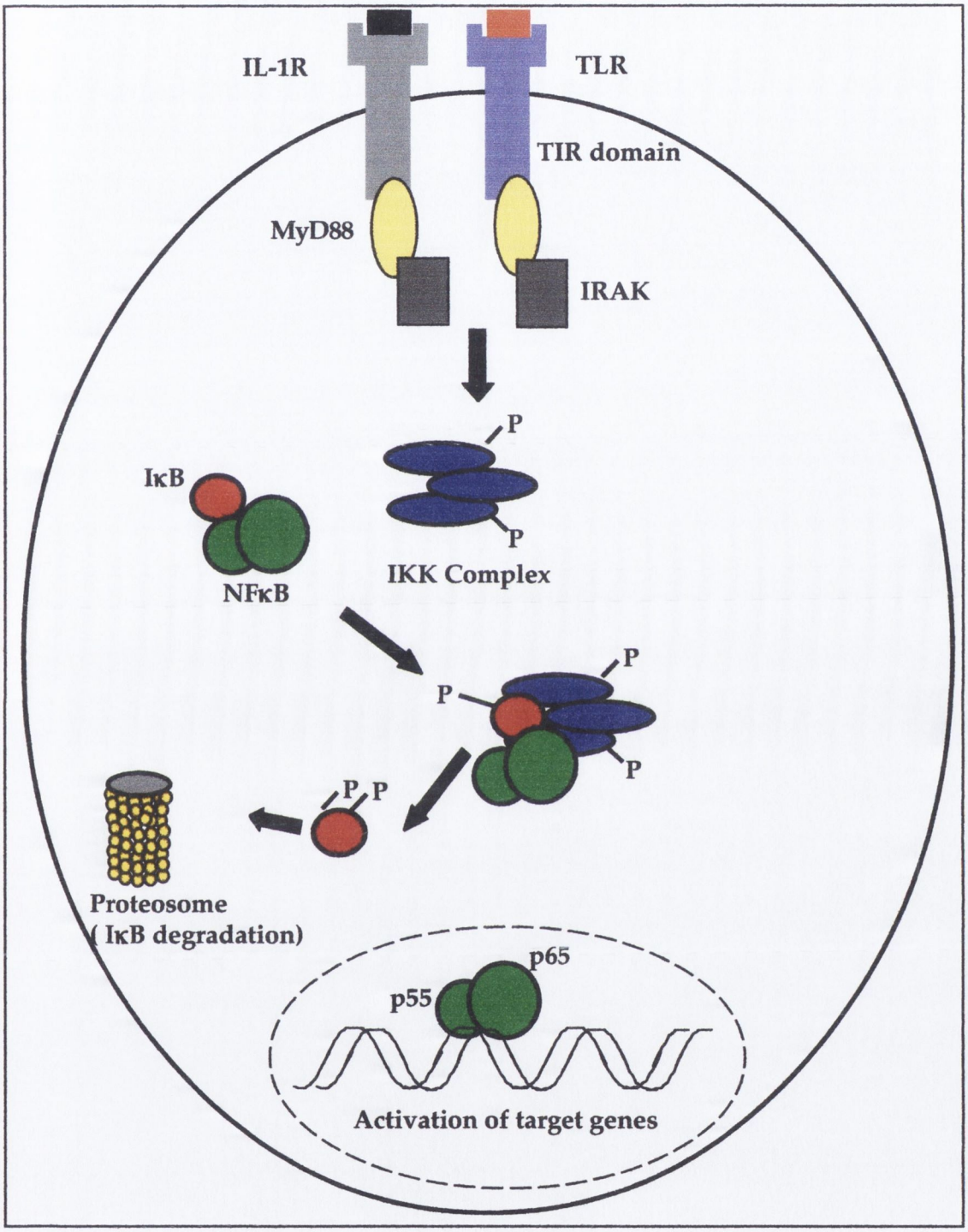


Figure 1.4:
Activation of NFκB through degradation of its inhibitor IκB
Inactive NFκB is held in the cytoplasm through binding of IkB to its nuclear targeting signal. Phosphorylation of IκB by the IKK complex promotes its degradation by the proteosome. Release of NFκB from its inhibitor allows its translocation into the nucleus.

extracellular region, which is associated with PAMP binding. Examples of PAMPs include LPS in the outer membrane of gram-negative bacteria or mannan from the cell wall of yeast. It has emerged that different Toll receptors recognise specific PAMPs e.g. TLR2 is specific for peptidoglycan and TLR4 recognises LPS. Interaction between a PAMP from an infectious agent and the LRR region of its respective TLR results in the transduction of downstream signals which eventually leads to the activation of a chief pro-survival transcription factor, NFκB (Kopp and Medzhitov, 1999). The cytoplasmic domains of Toll receptors contain TIR (Toll/IL-1R) motifs and share homology with the intracellular region of the IL-1 receptor. These two receptors also share a number of signalling intermediates that are involved in the induction of NFκB (O'Neill, 2002).

1.4.3 *NFκB Induction*

NFκB activation is primarily associated with combating infection and promotion of cell survival (Caamano and Hunter, 2002). It does this by switching on the expression of sets of genes, which can illicit a variety of responses. NFκB is a dimeric transcription factor made up of p65 and p50 subunits (Baeuerle and Baltimore, 1989; Ghosh *et al.*, 1990). It is constitutively present in the cell but is normally sequestered in the cytoplasm away from its gene targets, by its inhibitor IκB. Removal of inhibition by IκB is initiated when IκB is phosphorylated by the IκB kinase (IKK) complex, and targeted to the proteosome for degradation (DiDonato *et al.*, 1997). Free, active NFκB can now move to the nucleus and finds its transcriptional targets (Figure 1.4). The IKK complex consists of three proteins, IKKα, IKKβ and IKKγ. The α and β subunits are responsible for the kinase activity, with the γ subunit having a regulatory role, through interaction with upstream activators of the complex (Karin, 1999). Activation of distinct subsets of genes by NFκB under different circumstances is achieved though regulation by phosphorylation of the NFκB dimer as well as interactions with other transcriptional elements.

While the activation of NFκB is of greatest importance in a number of facets of the immune response, the induction of NFκB is also known to have a direct role in inhibiting apoptosis. This became evident when RelA (p65) null mice were found to be embryonic lethal due to excessive apoptosis in the liver (Bet *et al.*, 1995). This inhibition is thought to be a result of direct competition for receptors capable of inducing signals for both apoptosis and NFκB, and also through its ability to transcriptionally up-regulate many anti-apoptotic genes.

1.4.4 *NFκB and the Immune Response*

Targets of NFκB include genes involved in inflammation such as the interleukins IL-2, IL-6 and IL-8, as well as cell adhesion molecules (Hoyos *et al.*, 1989; Libermann and Baltimore, 1990; Stein and Baldwin, 1993). The transcription of cytokines which are important for initiating the adaptive immune response, such as TNF and IL-1β, are also turned on by NFκB. Interleukin-1 (IL-1) and TNF are key cytokines secreted by lymphocytes during pathogen infection. Interestingly, many of the signalling molecules which are upregulated by NFκB, including IL-1 and TNF, are also capable of inducing NFκB, resulting in a positive feedback effect to efficiently combat infection.

1.4.5 *Interleukin-1 Signalling*

Interleukin-1β is the prototypic member of the interleukin family of cytokines. It is synthesised as a 31kDa molecule which is processed to an active 17 kDa form by caspase-1 (Black *et al.*, 1988; Thornberry *et al.*, 1992). When IL-1β is secreted, predominantly by lymphocytes, it can have effects on most other cell types through interaction with the membrane-bound IL-1 receptor. The IL-1 receptor is structurally very similar to the Toll-like receptor (Heguy *et al.*, 1992). Stimulation of the IL-1 receptor promotes increased expression of cytokines such as IL-1 itself and TNFα, cytokine receptors, growth factors, and clotting factors, predominantly through NFκB. In addition to the above survival signals, IL-1 also promotes clonal

proliferation of Helper T-cells as part of the adaptive immune response. As mentioned previously, the IL-1 receptor shares a number of intracellular signalling elements induced by Toll-like receptor stimulation, from the adaptor proteins MyD88 and TRAF6, to the kinases IRAK and NIK.

1.4.6 *The Tumor Necrosis Factor Receptor*

The prototypic member of the death receptor family, TNFR1, contrary to its name, actually plays a greater role in promoting cell survival. The intracellular death domain of TNFR1 binds an adaptor protein TRADD (TNF Receptor Associated Death Domain), which can promote caspase-8 processing by interacting with FADD and, as in the Fas pathway, activate the apoptotic caspase cascade. However, this usually only occurs when protein synthesis has been inhibited. The most common downstream effect of TNFR signalling is the activation of NFκB (Hsu *et al.*, 1995). A protein called RIP binds to TRADD and recruits and activates the IKK complex. TNF signalling results in the phosphorylation of IKK through RIP and activation of NFκB (Hsu *et al.*, 1996).

1.4.7 *Inhibition of Apoptosis by NFκB*

As well as promoting proliferation and survival in cells by upregulating molecules such as cytokines and growth factors, NFκB is capable of directly inhibiting apoptosis. Most significantly for TNF signalling, NFκB activation results in increased expression of proteins which are also recruited to the TNFR, such as TRAF2, c-IAP-1 and -2 (Chu *et al.*, 1997; Wang *et al.*, 1998). TRAF2 is an adaptor molecule which associates with the TNFR intracellular domain and recruits the inhibitor of apoptosis proteins cIAP1 and cIAP2 (Rothe *et al.*, 1995). The latter proteins are known to bind active sites of caspases -3, -7 and -9 thus inhibiting their activity (Deveraux *et al.*, 1998). The potential importance of recruitment of c-IAP-1 and c-IAP-2 to TNFR in inhibiting TNFR induced apoptosis has not yet been determined.

Other anti-apoptotic proteins upregulated by NFκB include; c-Flip, Bfl-1, XIAP and Bcl-2 (Kreuz *et al.*, 2001; Zong *et al.*, 1999; Stehlik *et al.*, 1998; Heckman *et al.*, 2002). These proteins act at different points of the apoptosis pathway. Bcl-2 and Bfl-1 both block the release of cytochrome *c* from mitochondria to inhibit apoptosis, whereas c-Flip is homologous to caspase-8 and acts to inhibit the processing of this caspase. XIAP functions in a similar manner to c-IAP -1 and -2 by binding to the active site of caspases (Deveraux *et al.*, 1998).

1.5 FORMATION OF SIGNALLING COMPLEXES

1.5.1 *Protein motifs*

The first steps in the pathways to apoptosis and NFκB induction involve the formation of protein complexes, involved in processing caspases (apoptosis) or activating IKK (NFκB induction). Generation of these complexes relies heavily on homotypic interactions between like motifs in different molecules, many of which will be discussed in detail below (Bouchier-Hayes and Martin, 2002).

In apoptosis, a series of protein motifs have been repeatedly found to be involved in facilitating protein-protein interactions between the caspases and upstream caspase-activating molecules (Weber and Vincenz, 2001). This family of motifs consists of the DD (Death Domain), DED (Death Effector Domain) and CARD (Caspase Recruitment Domain). All three motifs are composed a bundle of six or seven α- helical regions.

The Death Domain is predominantly associated with membrane receptors of the NGFR family, such as the Fas or TNFR described earlier. This domain forms the intracellular region responsible for transducing death signals by recruitment of other DD containing molecules. In some cases, such as with the TNFR, the DD binds to a DD containing protein which is not involved in death. Interestingly, the Death Domain motif is also found at an interaction interface between the adaptor

protein MyD88 and the kinase IRAK which are involved in NFκB induction through the Toll-like receptors (Muzio *et al.*, 1997; Wesche *et al*, 1997)).

The DED is found in adaptor molecules such as FADD, and also in the prodomain of caspases -8 and -10. Homologues of human caspase-8 with DED-containing prodomains are found in a number of other organisms including the mouse, *Xenopus*, Zebrafish, and *Drosophila* (Lamkanfi, *et al.*, 2002). Interactions between the DEDs of these molecules are responsible for recruiting caspase-8 to the Fas receptor or to provide a link between the TNF receptor and caspase-8.

The most common member of the group of related motifs, the CARD domain, is found within Apaf-1, the adaptor for Caspase-9. A number of the caspases themselves (caspases -1, -2, -4, -5 and -9) also contain a CARD in their prodomain region. Binding of the CARD of Apaf-1 to that of caspase-9 as well as Apaf-1-Apaf-1 interactions through the CED-4 homologous region of Apaf-1, create the apoptosome complex responsible for the processing of caspase-9 (Adrain *et al.*, 1999; Cain *et al.*, 2002; Hill *et al.*, 2004).

1.5.2 *CARD Proteins*

Previously, the presence of a CARD motif in a protein was thought to be a direct indication that this protein was involved in recruiting caspases and participating in pathways leading to apoptosis. This, however, is not always the case. Recently, a substantial number of CARD proteins have been identified which neither have a role in cell death or recruitment of caspases (Bouchier-Hayes and Martin, 2002). The CARD domain is now known to be an interaction surface implicated in numerous pathways. In some cases the broad function of CARD containing proteins can be evident from looking at other domains within the protein. CARD-containing proteins can be subdivided into four main categories; the bipartite

CARDS, the CARD only proteins the (Nuceotide-Binding-Domain) NBD-CARDs and the coiled-coil CARDs (Bouchier-Hayes and Martin, 2002).

The CARD-containing caspases fall into the bipartite CARD category, as each of these molecules consists of a protease region in addition to a CARD. The CARD caspases (caspases -1, -2, -4, -5, -9 and -11) are in the initiator caspase group, and the function of the CARD is thought to allow clustering of multiple pro-enzymes to facilitate auto-processing and activation. Other bipartite CARDs include adaptor proteins which can form a bridge between molecules in a pathway, such as RAIDD (CRADD) which can interact with the intracellular region of the TNF receptor and can also bind caspase-2 (Ahmad *et al.*, 1997; Duan and Dixit, 1997).

The CARD-only proteins, as their name suggests, are single domain proteins. These proteins can engage in binding to other CARD proteins to co-operate in the formation of larger complexes. These single-motif proteins are also known to compete with other proteins for binding to the same partner and in thais way act as competitive inhibitors. These proteins have been found to regulate a number of pathways including inhibition of NFκB-induction (CARDINAL; Bouchier-Hayes *et al.*, 2001) and prevention of caspase-1 activation (ICEBERG; Humke *et al.*, 2000).

The coiled-coil CARD group are multi-domain proteins. The coiled-coil region is an oligomerisation domain usually at the centre of the formation of a complex. The majority of proteins belonging to this group are involved in NFκB activation through recruitment and modification of other CARD containing proteins. Examples include CARD9 and CARD11 (Bertin *et al.*, 2000; Bertin *et al.*, 2001).

The NBD-CARDs (Nucleotide Binding Domain) are perhaps the most interesting members of the CARD group. This collection of multi-domain proteins have now emerged to have a function in mammalian cells as sensors of stress. Detection of

stress by these molecules is probably achieved by direct interaction with intracellular indicators of stress. Depending on the particular NBD-CARD, binding of the signal molecule to the NBD-CARD promotes either caspase activation or NFκB induction. One of the best characterised NBD-CARD proteins is Apaf-1, the caspase-9 activator. The NBD region of Apaf-1 is associated with the ability to oligomerise (Adrian *et al.*, 1999) and so the members of this group probably also form multimeric signalling complexes upon stimulatation. In the case of Apaf-1, the stimulus for oligomerisation is the binding of the mitochondrial protein cytochrome *c* to the WD-40 repeat region of Apaf-1 (Liu *et al.*, 1996; Zou *et al.*, 1997). Cytochrome *c*, a key component of the mitochondrial respiratory chain, is usually found exclusively within mitochondria, but cellular stress can induce mitochondrial permeability and cytochrome *c* release into the cytoplasm.

1.5.3 *Sensors within the Cell*

Apaf-1 is not the only NBD-CARD protein which can physically detect a stress signal and mount an appropriate response. Echoing this functional strategy, two of the most interesting CARD proteins to be characterised in the past four years are Nod1/CARD4 (Inohara *et al.*, 1999; Bertin *et al.*, 1999) and Nod2/CARD15 (Ogura *et al.*, 2001). The Nod1 molecule was initially described by two groups as inducing NFκB through Rip2 (Cardiak/Rick)-mediated recruitment of IKKγ. The structures of Nod1 and Nod2 show similarities to Apaf-1, having N-terminal CARD regions and central NBD oligomerisation domains. The WD-40 repeat region of Apaf-1 is replaced in the Nod proteins by Leucine Rich Repeats (LRRs).

The Leucine Rich Repeat motif is a feature that was first associated with plant receptor proteins responsible for the recognition of pathogens. The R genes in plants encode a variety of receptor molecule, which interact directly with pathogen produced proteins (encoded by Avr genes) through the LRR (Dangl and Holub, 1997). Variations in the LRR determine specificity for different Avr products. LRRs

are also found in the extracellular region of the Toll like receptors, as discussed earlier.

The link between LRRs and recognition of pathogen infection led to investigations on the role of the LRRs of Nod-1 and Nod-2 (Inohara *et al.*, 2001). The expression of these molecules, not on the cell surface but inside the cell meant they were unlikely to recognise extracellular PAMPs. Studies using epithelial cells showed that although these cells are unresponsive to extracellular LPS, they can respond to microinjected LPS, suggesting the existence of an intracellular recognition mechanism (Philpott *et al.*, 2000). This lead to experiments which showed a Nod-dependent response to LPS in cells (Inohara *et al.*, 2001). Thus, it appears that Nod-1 and Nod-2 could be the first intracellular LPS receptors. Bolstering this idea was the discovery of an association between a frameshift mutation in Nod-2 and the inflammatory bowel disorder, Crohns disease (Hugot *et al.*, 2001; Ogura *et al.*, 2001).

1.5.4 *PYRIN proteins*

Since the discovery of Nod-1 and Nod-2, an array of LRR containing proteins have been identified with as yet uncharacterised functions. More recently a new category of molecules has been defined, that consists of LRRs associated with a novel motif called the PYRIN domain (Staub *et al.*, 2001; Pawlowski *et al.*, 2001). The PYRIN domain is now placed in the death domain family because of its similar six α-helical structure (Martinon *et al.*, 2001; Bertin and DiStefano, 2000). This motif derives its name from the protein, Pyrin (also known as Marenostrin), which is mutated in the disorder Familial Mediterranean Fever (FMF Consortium, 1997). The exaggerated inflammatory response in this disorder is consistent with Pyrin having a role in modifying the inflammatory response. Furthermore, a Zebrafish caspase containing a PYRIN motif has also been identified (Inohara and Nunez, 2000), and this raises the possibility of an association between PYRIN domains and either apoptosis or cytokine production. For many of the PYRIN-containing

proteins, it is predicted that this motif acts in a similar manner to a CARD, as a protein-protein binding region.

1.6 CROSSTALK BETWEEN NFκB AND APOPTOSIS PATHWAYS

The pathways of cell death and cell survival constitute opposing alternatives for the fate of a cell. In keeping with this, a key feature of both pathways is the disabling of the alternative route. A number of caspase substrates are key players in cell survival signalling including the IAP proteins, Bcl-2, IκB and the p50 and p65 subunits of NFκB (Levkau *et al.*, 2001; Kirsch *et al.*, 1999; Reuther and Baldwin, 1999; Ravi *et al.*, 1998; Levkau *et al.*, 1999). Cleavage of these proteins disrupts their pro-survival activity, removing obstacles in the apoptosis program (Fishcer *et al.*, 2003). As mentioned earlier, the NFκB survival pathway includes the upregulation of proteins which directly inhibit the activation of caspases and interfere with apoptosis.

1.7 PROJECT AIMS

The primary goal of this project was to identify and characterise novel proteins involved in cell death and cell survival. As detailed in the introduction, these seemingly opposing pathways share a number of common features, most notably the prevalence of particular protein motifs. For this reason it was predicted that searches for novel proteins specifically containing such motifs would be an effective way of identifying new players in these pathways.

Large gaps in our knowledge of the activation and regulation of members of the caspase family as well as the regulation of NFκB survival signal pathways exist. The first approach adopted was a yeast two-hybrid screen to search for binding partners for caspase-1, a molecule with connections to both cell death and cell survival. The second approach involved using public sequence databases to identify novel CARD containing proteins by searching with the CARD domain of caspase-1. This resulted in the identification of a multi-domain CARD protein called NAC. The role of this protein in regulating cell death and cell survival was investigated during this study, and is described in the following chapters.

CHAPTER II

Materials and Methods

2.1 MATERIALS

2.1.1 Antibodies

Antibodies used for this work were obtained from the following sources;

Anti-Actin	ICN, UK
Anti-ASC	MBL (Stratech, UK)
Anti-Caspase-1	Santa Cruz, UK
Anti-Caspase-2	Transduction Laboratories, UK
Anti-Caspase-3	R and D Systems, UK
Anti-Caspase-3 (Active)	NEB (Cell Signalling), Uk
Anti-Caspase-7	Transduction Laboratories, UK
Anti-Caspase-7 (Active)	NEB (Cell Signalling), UK
Anti-Caspase-8	Alexis (Apotech), UK
Anti-Caspase-9	Oncogene Research Products, UK
Anti-FLAG	Sigma, UK
Anti GAL4 BD	Santa Cruz, UK
Anti-NAC	Generated by Harlan Sera Labs, UK

(HRP-conjugated, Anti-mouse and anti-rabbit secondary antibodies were obtained from Jackson Labs)

2.1.2 Reagents

Restriction enzymes and T4 DNA ligase were obtained from NEB (New England Biolabs). Chemical and general reagents unless otherwise stated were obtained form Sigma-Aldrich or BDH.

2.2 METHODS

DNA-RELATED PROTOCOLS

2.2.1 *Generation of competent Bacteria*

Escherichia coli DH5α were made ultra-competent by the Inuoe Method (Inuoe *et al.*, 1990). Briefly, a freshly streaked bacterial colony was grown overnight in 3 ml SOB (2 % Bacto-tryptone, 0.5 % (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ pH 6.7-7.0). This culture was then used to inoculate a larger (250 ml) SOB culture, which was grown at 18°C until it reached an OD_{600} of ~0.6. The culture was then incubated on ice for 10 min before pelleting the cells by centrifugation (2,500 g for 10 min at 4°C). The SOB supernatant was then removed and the bacterial pellet was resuspended in ice cold TB (10 mM Pipes, 55 mM $MnCl_2$, 15 mM $CaCl_2$, 200 mM KCl, pH 6.7), using 80 ml TB/250 ml original culture). The resuspended cells were then incubated on ice for 10 min. Again the cells were pelleted for 10 min at 2,500 g (4°C). The TB was removed and cells were resuspended in fresh ice-cold TB (20 ml/250 ml original culture). DMSO (Dimethylsulphoxide) was added to a final concentration of 7 %. Cells were aliquoted and stored at -70°C until required.

2.2.2 *Transformation of Competent Bacteria*

50 µl of competent cells were typically used per transformation. Approximately 1 µg of plasmid was added to the cells and allowed to coat the cell surface at 4°C for 30 min. Cells were then heat-shocked at 42°C for 40 s before being left to recover on ice for 2 min. 450 µl of LB (Luria Bertrani) broth (Bacto-Tryptone 10 g/L, Yeast Extract 5 g/L, NaCl 10 g/L, pH 7.0) was then added and cells were allowed to grow and express antibiotic resistance genes for 1 h at 37°C. 100 µl of the cells were plated on LB Agar plates containing antibiotic (100 µg/ml for Ampicillin and 50 µg/ml for Kanamycin).

2.2.3 *Plasmid DNA Preparation*

Plasmid preparation was carried out as in Sambrook *et al.* (1989) on mini-scale or midi-scale, using culture volumes of 1 ml or 50-100 ml respectively. Overnight LB cultures were pelleted at 21,000 g (mini) or 3,500 g (midi) and LB was removed. Pellets were resuspended in 100 µl /4 ml P1 buffer (50 mM Tris-HCl pH 8, 10 mM EDTA). 200 µl /4 ml of an alkaline lysis buffer P2 (200 mM NaOH, 1 % SDS) was then added and cells were inverted ~5 times before the addition of 200 µl/4 ml of P3 buffer (3M KAc pH 5.5) and further inversion ~5 times. Addition of the P3 buffer precipitates the bacterial genomic DNA. This precipitate is pelleted through centrifugation at 21,000 g (miniprep) or 11,000 g (midiprep) for 15 min. The supernatant which contains plasmid DNA was retained and 0.7 volumes of isopropanol was added to precipitate the plasmid DNA for 20 min. The precipitate was pelleted by centrifugation at 21,000 g (mini) 15,000 g (midi) for 15 min. Supernatant was removed and the plasmid DNA pellet was washed twice with 1 ml 70% ethanol. The DNA pellet was allowed to air-dry before resuspending in 10 µl ddH_20 +10 µl TE (10 mM Tris-HCl pH 8, 1 mM EDTA) for the miniprep and 50 µl ddH_20 +50 µl TE for midipreps.

2.2.4 *Qiagen Preps*

Plasmids which were for use in transient transfections were prepared using endotoxin free P1, P2 and P3 buffers (Qiagen). Plasmid DNA was cleaned, after the P3 step, by passing through DEAE (Diethylaminoethanol) columns which was previously equilibrated with QBT (750 mM NaCl, 50 mM MOPS pH, 15 % (v/v) isopropanol, 0.15 % (v/v) Triton X-100). The column bound plasmid was then washed with QC (1 M NaCl, 50 mM MOPS, pH 7, 15 % (v/v) isopropanol). DNA was eluted into the Qiagen QF buffer (1.25 M NaCl, 50 mM Tris-HCl pH 8.5). DNA was then washed as before with 70% ethanol and resuspended in the appropriate amount of 0.5X TE.

2.2.5 *Cloning of Yeast Two hybrid bait and prey genes*

Constructs generated for yeast two hybrid studies were all made in a similar manner. The fragment of human caspase-1 gene encompassing the CARD (base pairs 1-357/amino acids 1-119) was amplified with appropriate forward and reverse primers;

SMAI start
Forward **5'**ATA GGA TCC CCC GGG **ATG** GCC GAC AAG GTC **3'**

BAMHI
Reverse **5'** TGT GGA TCC CTC GAG TTA GTC CTG CAC TGC CTG **3'**

using the following PCR (Polymerase Chain Reaction) conditions;

94°C for 3 min,
94°C for 2 min,
68°C for 1 min, } 35 cycles
72°C for 2 min,
72°C for 10 min

Approximately 50-100ng of pCDNA3-Caspase-1 was used as a template along with Taq polmerase and buffers (Qiagen) used according to manufacturers instructions. The fragment was then digested using SmaI/BamHI restriction enzymes. The fragment and the pAS2.1, pACT2, pGBKT7 and pGADT7 vectors which had been digested with the same enzymes were then cleaned using 100 mg/ml silica in 3 M NaI and eluted into ddH_20. The insert and cut vectors were then ligated with T4 ligase at 16°C overnight. After ligation the DNA was transformed into competent DH5α. A selection of colonies were grown to miniculture stage and the DNA was prepared as described earlier. Plasmid DNA was electrophoresed and the 'upshifted' plasmids were selected. The presence of the correct insert was checked by restriction digest and sequencing.

2.2.6 *Subcloning of KIAA0926 insert*

pBluescript KIAA0926 obtained from the Kasuza institute, Japan. This construct encodes a protein corresponding to DEFCAP short. This plasmid was digested with Kpn1 and Not1 using NEB buffers and enzymes overnight (~14 h) to excise the insert from the vector. The total digest was electrophoresed on an agarose gel and stained with Ethidium Bromide. A fragment corresponding to the insert was excised from the gel and cleaned using 100 mg/ml silica in 3 M NaI and eluted into ddH_20. pCDNA3 digested with Kpn-1 and Not-1 was also cleaned from solution using silica and eluted into ddH_20. T4 DNA ligase was used to ligate the digested KIAA0926 insert to the digested pCDNA3 at 16°C for ~14 h. The ligation mixture was used to transform ultra-competent DH5α. A selection of colonies were grown in 3 ml cultures and the plasmids prepared as before. Plasmids were electrophoresed and those which ran at a size consistent with the expected size were digested with Kpn1/Not1.

PGEX4T2-NAC, the plasmid used to express a recombinant NAC CARD-GST fusion protein was produced by amplification of the CARD region of NAC (base pairs 3963-4287 / amino acids 1321-1429) using the following primers:

EcoRI
Forward **5′** CCC GAA TTC GAA TGC CTG CAA CTA CTC TG **3′**

XhoI
Reverse **3′** ATA CTC GAG TCA GCT GCT GAG TGG 3′

The amplified insert was then digested with EcoRI and Xho I and ligated into the PGEX4T2 plasmid which had digested with these enzymes.

2.3 PROTEIN PURIFICATION AND DETECTION

2.3.1 *Induction of GST-NAC* $^{1312-1429}$

Freshly transformed DH5α (containing pGEX-NAC-CARD) were grown in a 3 ml LB overnight culture. The following day this culture was used to inoculate a 1 L culture and bacteria were grown at 37°C to an OD_{600} of 0.1-0.2. At this point 100 μM

IPTG (isopropylthio-β-D-galactoside) was added to induce expression of GST-NAC. Bacteria were grown for 2 h before pelleting at 3,500 g. Culture supernatant was removed and the cells were resuspended in 20 ml NEMN (20 mM MOPS-HCl, 100 mM NaCl, 1 mM EDTA, 0.5 % NP40). The cells were sonicated to break the outer membrane using five 30 s pulses. Insoluble material was then pelleted at 15,000 g. The supernatant containing the induced GST-NAC protein was then incubated with a 50 % slurry of Glutathione-Sepharose beads and rotated at 4°C for 4 h to allow capture of the protein.

2.3.2 *Elution of GST-NAC*

The GST-NAC protein was eluted from the Glutathione Beads using the following two methods. The first method used a Glutathione-elution buffer (50 mM MOPS-HCl pH 8, 150 mM NaCl, 10 mM Glutathione). Equal volumes of elution buffer and captured protein were incubated at room temperature, under rotation for 30 min. The beads were pelleted and supernatant containing eluted protein was removed. Fresh elution buffer was added to elute additional protein, and this was repeated for a third elution.

The second method of elution involved incubating equal volumes of beads and 100 mM glycine pH 3 at 37°C for 15 min. Again, the beads were pelleted and the eluate removed in the supernatant.

2.3.3 *Concentration of GST-NAC eluate*

Total eluate was pooled and concentrated by passing through a Viva-spin 500 concentrator, to a volume of 300 µl. The concentrated protein was then applied to the inner chamber of a dialysis cassette (10,000 MW cut off). This cassette was then floated in 2 L of 100 mM MOPS pH 7.5, at 4° C overnight with constant stirring.

2.3.4 *Production of Polyclonal anti-NAC antibody*

Purified recombinant $NAC^{1312-1429}$ was used in an immunization program by Harlan SeraLabs to produce rabbit polyclonal serum. This involved 6 immunizations of two rabbits over a 77-day period. 100 µg of purified protein was used per immunization.

2.3.5 *Preparation of Affinity Column for Antibody Purification*

A 1:1 ratio (500 µl : 500 µl) of Affigel-10 and Affigel-15 (Biorad Protein binding agarose) was washed to remove solvent supernatant and re-suspended in 0.1 M MOPS pH 7.5. The gel was then coupled to the concentrated GST-NAC through incubation for 4 h under rotation. 100 mM ethanolamine pH 8 was added and the beads were rotated for a further hour to block any unreacted esters on column. The Affigel-coupled protein was then added to an Econo-column (Biorad). The column was pre-washed with 20 ml PBS, 10 ml 100 mM MOPS pH 7.5, 10 ml PBS, 10 ml 100 mM glycine pH 3, and finally 10 ml PBS. The column was stored in PBS (containing 0.2 % azide) at 4°C until ready to use.

2.3.6 *Purification of Polyclonal Antibody*

Before application of terminal bleed of α-NAC antibody, the Affigel column was washed with 10 ml PBS, 10 ml 100 mM Glycine pH 2.4/ 150 mM NaCl and a final 10 ml wash with PBS. Serum was then added to column, collected as it flowed through and reapplied 4 times. The column was then washed 5 times with 10 ml PBS. The antibody was eluted using 10 ml 100 mM Glycine HCl pH 2.4/150 mM NaCl, in successive 1 ml fractions. Each fraction was neutralised with 200 µl of 1 M Tris-HCl pH 8.

2.3.7 *SDS Poly-Acrylamide Gel Electrophoresis*

Proteins were separated using standard SDS PAGE conditions (Sambrook *et al.* (1989).

2.3.8 *Coomassie Staining of Gels*

To determine successful induction of GST-NAC, lysates and purified proteins were electrophoresed and gels were stained for total protein using Coomassie blue Staining (0.25 g Coomassie Brilliant Blue in 45 ml H_2O, 45 ml methanol and 10 ml glacial acetic acid).

2.3.9 *Western Blotting*

Proteins which had been separated using standard electrophoresis protocols were transferred onto 0.45 μm nitrocellulose membranes (Schleicher and Schull) at 30 mA for ~14 h. Nitrocellulose blots were blocked with TBST (10 mM Tris HCl, 150 mM NaCl, 0.05 % Tween-20) containing 5 % (w/v) non-fat dried milk for 30 min and then probed with primary antibody (typically at 250 ng/ml) for 2 h. Blots were then washed for 30 min TBST (substituting fresh TBST every 10 min). The blots were then incubated with the appropriate horse-radish peroxidase coupled secondary antibody (Jackson Labs) in TBST/NFDM for a further hour. This was followed by washing in TBST for 30-40 min. Enhanced chemiluminescense was used to detect bound secondary antibody.

2.4 MAMMALIAN CELL CULTURE AND TECHNIQUES

2.4.1 *Transient Transfection using Calcium Phosphate Precipitation*

293T (Human Embryonic Kidney) cells were used for immunoprecipitation experiments and NFκB assays and were cultured in DMEM (Dulbecco's Modified Eagle's Medium) containing 10 % FCS (Fetal Calf Serum). The cells were transiently transfected using calcium phosphate precipitation in small-scale experiments (6 well dishes, 10^5 cells/well) and on large scale (10 cm dishes, $2X10^6$ cells per plate). Small-scale transfection consisted of a DNA mixture made up to 100 μl along with 25 μl 2.5 M $CaCl_2$. This was added dropwise to an equal volume (125 μl) of 2X HBS (280 mM NaCl, 10 mM KCl, 1.5 mM Na_2HPO_4, 12 mM Dextrose, 50 mM Hepes, pH

7.1). Complexes were allowed to form for 20 min before they were added drop-wise to the cells.

2.4.2 *Transient Transfection using Lipid reagents*

MCF7 cells used for apoptosis assays were cultured in RPMI containing 5 % FCS. These cells were transfected on a small scale (6 well dishes -10^5 cells per well) using Fugene-6 (Roche) transfection reagent. 50 ng of pCMVβ-gal plasmid was typically added per treatment to determine which cells had been successfully transfected. Fugene at 2 µl per well was added to RPMI without serum (100 µl per treatment) and left to form complexes for 15 min, before being added to the plasmid mixtures. The treatments were then left for a further 20 min before being added drop-wise to the cells.

2.4.3 *Transfection of siRNA oligonucleotides*

siRNA oligonucleotides obtained from Dharmacon Research were resuspended in RNAse free ddH_2O to a final concentration of 20 µM and strored in aliquots at -70 °C. For transfections on a 10 cm dish scale 20 µl of oligofectamine transfection reagent (Invitrogen) was diluted into 80µl of serum free medium and allowed to stand for 5 min. This mixture was then added dropwise to a mixture containing 50 µl of siRNA in 450 µl of serum free medium. The siRNA was allowed to form complexes for 15-20 min. Cells to be transfected were washed in serum free medium and incubated with 4.4 ml of serum free medium. The complexes were then added dropwise and the plate was swirled gently to distribute the siRNA complexes. 4-6 h later media containing 2XFCS was added to the cells to restore the normal FCS conditions.

2.4.4 *NFKB-Induction Assays*

Luciferase Assay

The induction of NFκB was determined by transfecting a plasmid encoding the protein of interest along with the pGL3.5xκB-luc reporter plasmid. This plasmid contains 5 NFκB binding sites in the promoter of the luciferase gene. Induction of NFκB results in expression of the luciferase gene in cells. 24 h after transfection cells were washed with PBS and lysed using 200 µl of reporter lysis buffer (100 mM Hepes pH8, 2 mM $MgCl_2$, 2 % (v/v) Triton X-100, 5 µM DTT). Lysates were then frozen at -70°C and thawed to improve lysis. 50 µl of lysate was added to 100 µl of luciferase assay reagent (20 mM Glycylglycine, 1 mM $MgCl_2$, 100 µM EDTA, 278 µg/ml ATP, 213 µg/ml Co-Enzyme A, 2.5 µM DTT, and 50 µl/ml beetle luciferin (Promega). Light emission from 3 X 50 µl samples for each treatment were read in a white 96 well plate using a luminometer (Tecan SpectraFluor).

Liquid β-Gal Assay

In order to determine the transfection efficiency of cells used in the NFκB assay, pCMVβ-gal was transfected along with each of the plasmid treatments. 50 µl of each lysate was added to 950 µl of Z buffer (16.1 g/L Na_2HPO_4, 5.5 g/L NaH_2PO_4,, 0.75 g/L KCl, 0.246 g/L $MgSO_4$) which contained 0.66 mg/ml ONPG (2-nitrophenyl-b-D-galactopyranoside) a substrate for β-galactosidase. After incubation at 37°C for ~30 min (or until a yellow colour had developed) the absorbance readings at OD_{420} were read on a spectrophotometer. These readings were used to normalise luminometer readings.

2.4.5 *Immunoprecipitation*

Cells which had been transfected for IP experiments were typically lysed at 10^7 cells/ml in a single detergent IP lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 8, 1 % NP40, 1 mM PMSF, 10 µg/ml leupeptin, 1 µg/ml aprotinin) at 4°C for 30 min. Insoluble material was pelleted by centrifugation at top speed in a microfuge.

Lysates were precleared using 30 µl Protein A/G Agarose beads (Santa-Cruz), for 2 h under rotation at 4°C. 1 µg of IP antibody was incubated with fresh 30 µl of protein A/G beads in order to couple the antibody to the beads. Antibody-coupled beads were added to lysates, and IPs were incubated overnight under rotation at 4°C. Following rotation, beads were pelleted and washed with IP lysis buffer (3X5min washes). Washed beads were then pelleted and resuspended in 1X SDS PAGE loading buffer.

2.4.6 *Apoptosis Assays*

MCF7 cells which had been transfected with plasmids for 24 h were washed in PBS, and fixed using Paraformaldehyde fixative (2 % paraformaldehyde, 0.2 % gluteraldehyde in PBS pH 7.2) for 5 min. Cells were then washed with PBS (5 min room temp). Cells were stained using β-Gal staining buffer (5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6.3H_2O$, 2 mM $MgCl_2$, 0.02 % (v/v) NP-40, 0.01 % (w/v) SDS, 1 mg/ml X-gal in PBS pH7.2) using approximately 0.8 ml per well. Plates were incubated at 37°C to allow a blue colour to develop. Cell death was then scored according to morphology, using a light microscope to visualise the transfected cells. Three hundred transfected (blue) cells were counted for each treatment.

2.4.7 *Peptide Hydrolysis Assay*

Cells were lysed in Assay lysis buffer (150 mM NaCl, 20 mM Tris pH7.5, 1 % Triton X-100, 10 µg/ml leupeptin, 5µg/ml aprotinin, 100 µM PMSF) or standard IP lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 8, 1 % NP40, 1 mM PMSF, 10 µg/ml leupeptin, 1 µg/ml aprotinin). Each well in the assay typically comprised the lysate from 10^5 cells. Lysate was added to well containing a fluorometric substrate at a final concentration (when lysate is added) of 50 µM. Ac-DEVD-AFC (Acetyl-Asp-Glu-Val-Asp-aminofluormethylcoumarin), Ac-LEHD-AFC (Ac-Leu-Glu-His-Asp-

AFC) and Ac-IETD-AFC (Ac-Ile-Glu-Thr-Asp-AFC) were obtained from Bachem. The assay was carried out at 37°C in a fluorimeter (Tecan Spectrofluor) measuring relative fluorescence units over a two hour period.

2.4.8 *Immunofluorescence Analysis*

For analysis of expression patterns of endogenous or overexpressed protein, cells were grown on glass coverslips before transfection or before analysis. Cells were then washed in PBS (3 x 2 ml) to remove serum proteins. Cells were then fixed using 3% parafomaldehyde in PBS pH 7.2 for 10 min at room temperature. The fixative was removed by washing with 3 x 2 ml PBS pH 7.2 at room temperature on rocking platform (15 min per wash). The cells were permeabilised by incubation with 0.15 % v/v TX-100 in PBS for 15 min. To reduce background binding, the cells were then incubated with a blocking buffer (2 % BSA w/v in PBS) for 30 min at room temp. The coverslips were then incubated with the primary antibody diluted in 2 % w/v BSA (PBS) for 1 h. Non-specifically bound antibody was removed by washing with 2 % w/v BSA (PBS) (3 x 10 min on rocking platform). The slides were then incubated with a flourochrome-coupled secondary antibody appropriated to the primary antibody (anti-mouse, rabbit etc.). Again, non specifically bound antibody was removed by washing with PBS pH 7.2, 3 x 10 min on rocking platform. Coverslips were then mounted in antifade containing 20μM Hoechst stain to visualise nuclei.

2.5 YEAST TWO HYBRID ASSAY METHODS

2.5.1 *Expansion of pACT2 Jurkat cDNA library*

E. *coli* BNN 132 containing a library of Jurkat (Human Leukemia) cDNAs cloned into the yeast two hybrid vector pACT2 were obtained from Clontech. This commercial library contained inserts ranging in size from 0.5 to 4 Kb cloned into XhoI/EcoRI sites of the pACT2 vector. Serial dilutions of the bacteria were grown for 18 h on LB agar plates to calculate the titre of the library. The titre was found to

be 4.4×10^9 cfu/ml (colony forming units/ml). The library contains 3.0×10^6 independent clones, which need to be represented at least twice in the expansion. Therefore ($4.4\times10^9/6\times10^6$) 1.36 µl of library were used to inoculate 15 ml of LB. 100 µl aliquots of the inoculate were plated individually on 150 ×15 cm LB Agar plates and incubated at 37°C. After 18 h growth 5 ml of LB : Glycerol (3:1) was added to each plate to aid scraping of the colonies. The colonies were pooled into GSA tubes and centrifuged at 7,000 rpm for 10 min. The plasmid DNA from the bacterial pellet was prepped as previously described using 140 ml P1/P2/P3 buffers. The supernatant was purified through at total of 6 Qiagen Tip 500 columns using the Qiagen plasmid purification protocol and with each column eluted using 15 ml QF buffer. The plasmid DNA was precipitated using 0.7 volumes (63 ml) isopropanol. The pellets were washed in 70% Ethanol and resuspended in 3 ml 1:1 dH_2O : TE pH 8.

2.5.2 *Yeast Transformation*

Yeast media

YPD 1L (10 g Yeast Extract, 20 g Peptone, 2 % Dextrose pH 5.8)

Synthetic Dropout medium (SD) (6.7 g/L Yeast Nitrogen base, 100 ml/L 10× Amino Acid stock solution, 2 % Dextrose pH 5.8).

I L of 10× Amino Acid Stocks were made up using 300 mg/L L-Isoleucine, 1500 mg/L L-Valine, 200 mg/L L-Adenine Hemisulphate, 200 mg/L L-Arginine HCl, 200 mg/L L-Histidine HCl monohydrate, 1000 mg/L L-Leucine, 300 mg/L L-Lysine HCl, 200 mg/L L-Methionine, 500 mg/L L-Phenylalanine, 2000 mg/L L-Threonine, 200 mg/L L-Tryptophan, 300 mg/L L-Tyrosine, 200 mg/L Uracil. $Leucine^-$, $Tryptophan^-$, $Leucine^-Trypyophan^-$ and $Histidine^-Leucine^-Tryptophan^-$ synthetic dropout medium was made using a 10× stock which did not include the respective amino acid/s. HLT- media also contains 25 mM 3-Amino Triazole to prevent leaky expression of the HIS3 gene.

Yeast Strains Y190 was obtained from Clontech. Transformations were carried out on untransformed parental Y190 and Y190 carrying one or other of the yeast two hybrid vectors. These two situations require different growth conditions to prepare for the transformation. In the case of untransformed yeast 50 ml YPD was inoculated with a single colony of Y190 and grown overnight at 30°C with shaking at 250 rpm in a shaker incubator for 18 h. When the culture reached an OD_{600} ~2.0 a 1:11 dilution was made using 30 ml culture into 300 ml fresh YPD medium. The yeast were then grown to an OD_{600} ~0.5±0.1.

Where the yeast already carried either bait or prey plasmids, the colony was used to inoculate 5 ml Trp^- (bait) or Leu^- (prey) initially and grown for 16 h 30°C. This 5 ml culture was then used to inoculate a larger 50 ml culture and again grown for 16 h to an OD_{600} ~2.0. The yeast were then diluted into total volume of 300 ml YPD and then grown to an OD_{600} ~0.5±0.1.

On reaching the appropriate OD_{600}, the cells were harvested by centrifugation for 5 min at 1000 g. The yeast pellet was then washed with 25 ml of TE pH 7.5 and centrifuged again 1000 g/5 min. LiAc/TE was added to the cell pellet (227 µl per 50 ml original culture) and cells were resuspended by vortexing vigorously every 2 min over a 10 min period. The plasmid/s to be transformed were added in a total volume of 5 µl (5 µg maximum) to a universal along with 10 µg herring sperm DNA. 100 µl (10^8) cells were then added to the DNA and vortexed to mix. 600 µl of 40% PEG 4000/LiAc/TE was added to the tube, vortexed for 10 s and incubated at 30°C for 30 min with shaking at 200 rpm in shaker incubator. 70 µl DMSO was then added, mixed by swirling before the cells were heat-shocked at 42 °C for 15 min. Cells were incubated on ice to recover for 2 min before being washed with 20 ml sterile TE. The transformed yeast were then pelleted by centrifugation at 1,000 g for 5 min and resuspended in 1 ml TE. 100 µl of these cells were then plated on the appropriate SD Agar plates and grown at 30°C.

2.5.3 *Protein Expression in Yeast*

In order to verify expression of bait and prey proteins in the yeast host, protein lysates were made. Single colonies of transformed yeast containing bait plasmids were used to inoculate 5 ml of Trp^- SD medium which were grown for 18 h at 30°C. The overnight cultures were then used to inoculate 50 ml of YPD and the yeast were then grown at 30°C for 4-8 h until they reached an OD_{600} of 0.4-0.6. On reaching the required OD_{600} the cultures were added to a pre-chilled GSA tube half filled with ice and centrifuged at 1000 g for 5 min and 4°C. The yeast were then resuspended in 50 ml ice-cold dH_2O and re-centrifuged under the same conditions. The pellet was then frozen using liquid nitrogen and stored and -70°C until ready for use.

2.5.4 *Plasmid Rescue*

Colonies that had grown on HLT^- medium, were selected for further processing and prey plasmid isolation. First the colonies were restreaked to generate yeast stocks. A colony was then used to inoculate 5 ml of Leu^- medium and grown overnight at 30°C. Cultures were centrifuged at 14,000 rpm for 5 min and resuspended, by pipetting in 200 µl plasmid rescue solution (10 mM Tris pH 8, 100 mM NaCl, 1 mM EDTA, 2 % Triton X-100, 1 % SDS). 100 µl of glass beads were added along with 200 µl 25:24:1 Phenol: Chloroform:Isoamylalchohol. The tubes were then vortexed vigorously for 2 min. The disrupted cells were then centrifuges at 14,000 rpm for 5 min and the top aqueous phase was removed to a fresh tube. 0.1 volumes 3 M NaOAc pH and 2 volumes 100% Ethanol were added and the tube was placed after mixing at 70°C for 20 min. Plasmid DNA was then pelleted at 14,000 g for 15 min, and washed with 1 ml 70% Ethanol. Pellets were resuspended in 20 µl of 10 mM Tris HCl pH 8.5.

2.5.5 *Amplification of Plasmid Inserts*

Inserts were typically amplified using 5 µl of a 1:20 dilution of the yeast plasmid preparation and standard buffer conditions, using the parameters below.

94°C for 3 min,

94°C for 1 min,
54-56°C for 1 min, } 30 cycles
72°C for 2-3 min,

72°C for 10 min

The following primers were used in all cases to amplify library clones;

Forward **5′** TTC GAT GAT GAA GAT ACC CC **3′**

Reverse **5′** GAA CTT GCG GGG TTT TTC AG **3′**

Optimum annealing temperatures were determined for individual inserts by lowering of this temperature in successive rounds of PCR until sufficient amounts of product were obtained. Amplification times were increased from 2-3 minutes in for longer inserts.

The inserts were sequenced commercially by MWG Biotech (Germany).

2.6 BIOINFORMATICS

Identification of novel CARD-containing proteins was carried out by using the BLAST (Basic Local Alignment Search Tool) program on the NCBI (Entrez) website. The BLASTP tool was used to align the protein sequence of interest (Caspase-1 CARD) against all entries in the protein database and provide a list of proteins which contained similar regions of sequence. The entries in the database are collected from the Genbank CDS translations, RefSeq Proteins, PDB and Swissprot public databases. The default matrix Blosum62 was used for the alignments which assigns gap costs of 11 for existence and 1 for extension. Previously uncharacterized proteins with recognizable CARD domains were then identified from the list.

CHAPTER III

Searching for novel Caspase-1-Interacting Proteins by Yeast Two-Hybrid Analysis

3.1 INTRODUCTION

As described in the introduction, the caspases are a family of proteases involved in key cellular processes from cytokine production to programmed cell death. Thus far, the activation mechanisms of only a few members of this family have been elucidated, leaving a large void in our understanding of these important enzymes.

3.1.1 Caspase-1

Caspase-1, the first member of the caspase family to be identified, was originally isolated in a search for the enzyme responsible for processing of the cytokine, Interleukin-1β (IL-1β; Thornberry *et al.*, 1992). For this reason, when the IL-1β converting enzyme (ICE) was originally identified, it was already known that this protease was activated upon stimulation of cells with LPS and was required for cleavage of pro-IL-1β to promote its secretion. Upon isolation of ICE/caspase-1, work began on determining exactly how this novel protease is activated. This challenge, has proven to be a difficult one, however, with the exact details of caspase-1 activation still unsolved.

Structurally, caspase-1 belongs to the initiator group of caspases and so is believed to be activated through oligomerisation followed by autoprocessing (Gu *et al.*, 1995). The caspase-1 prodomain contains a CARD region, predicted to be capable of binding to other CARD proteins which may act as adaptors and regulators of caspase-1 activation. Indeed, a number of CARD proteins have been identified that are capable of binding to caspase-1. The first protein put forward as a putative caspase-1 activating molecule was CARDIAK (RIP2/RICK) (Inohara *et al.*, 1998; McCarthy *et al.*, 1998; Thome *et al.*, 1998). Co-expression of CARDIAK and caspase-1 led to weak caspase-1 processing. However, when the RIP2-null mouse was characterised, no defect in caspase-1 activation was found (Kobayashi *et al.*, 2002). An Apaf-1-like molecule called IPAF has also been shown to be capable of promoting caspase-1 processing. However, this affect was achieved through

overexpression analysis and has not yet been confirmed at physiological levels of either protein (Poyet *et al.*, 2001). ICEBERG and Pseudo-ICE are two CARD-containing proteins that have a high degree of similarity to the caspase-1 prodomain (Druihle *et al.*, 2001). These proteins can negatively regulate caspase-1 activity by acting as dominant interfering proteins, preventing association between caspase-1 proenzymes. Active IL-1β can induce a wide variety of effects on cells, and so production of this cytokine is likely to be highly regulated. For this reason there may be a number of as yet unidentified modulators of caspase-1 activity, which ensure that activation of this caspase remains under tight control.

Caspases are constitutively expressed in most cells and are therefore believed to require proteolytic processing for activation. However, one puzzling feature of caspase-1 activation is that processed caspase-1 is very difficult to detect in cells under circumstances where the enzyme is known to be active (Ayala *et al.*, 1994; Miossec *et al*, 1996). This presents the possibility that either the unprocessed form of caspase-1 has activity, or is rapidly secreted extracellularly. As IL-1β does not have a classic signal peptide for secretion, the suggestion that secretion of this cytokine may be facilitated through interactions with caspase-1 has also been proposed (Singer *et al.*, 1995).

Identification of caspase-1-interacting proteins is important for many- reasons. First, understanding the normal activation mechanism would provide a basis for dissection of IL-1β production. Aberrant production of IL-1β has been implicated in common diseases such as rheumatoid arthritis (Dinarello, 1996). Target areas for design of drugs for intervention are likely to become evident once the exact details of how the process is clarified.

Generation of the caspase-1 knockout mouse revealed the importance of caspase-1 in the production of mature IL-1β, and in the mounting of an immune response to

pathogen products such as LPS (Kiuda *et al.*, 1994; Li *et al.*, 1995). Macrophages from these animals did not secrete IL-1β when stimulated *ex vivo* with LPS. Moreover, when LPS was injected into knockout mice at doses that were lethal for wild type mice, the caspase-1-null mice survived. The failure to undergo systemic inflammatory response syndrome (SIRS) or septic shock, under these conditions, was explained by the failure to produce mature IL-1β and also reduced levels of another cytokine, TNFα. No defect in apoptosis was found in these animals. However, a number of groups have suggested that caspase-1 may have a role in inducing apoptosis under certain conditions. Experiments in a mouse model of Huntington's disease, a neurodegenerative disorder characterised by excessive neuronal cell death, showed that inhibition of caspase-1 through introduction of a dominant negative caspase-1 mutant, slowed progression of the disease in these animals (Ona *et al.*, 1999). Overexpression of caspase-1 in rat fibroblasts has been shown to induce apoptosis (Miura *et al.*, 1993). These studies did not however rule out the possibility of non-specific interference (activation or inhibition) with other members of the caspase family. In addition, in the case of the Huntington's disease mouse model, the observed effects may be associated with the role of caspase-1 in inflammation. The role of caspase-1 in apoptosis versus inflammation will undoubtedly become clearer once the activation mechanism of this caspase is solved.

To search for caspase-1 interacting partners from a large pool of proteins, we decided to use the yeast two-hybrid assay. This assay is a protein screening method which has proven successful for identifying novel protein-protein interactions and characterising the interaction domains of known interaction partners.

3.1.2 The Yeast Two Hybrid Interaction Trap Assay

The yeast two-hybrid interaction trap assay was designed as a means of identifying, confirming or characterising protein-protein interactions (Fields and Song, 1989).

The method of co-expressing a protein of interest along with a library of putative interacting proteins, allows for screening of large numbers of molecules at once. The principle of the assay involves manipulation of a yeast transcription factor which can control metabolic genes in the yeast. In this case the transcription factor is the dimeric protein GAL4 (Figure 3.1). GAL4 promotes transcription of genes by binding to activating sequences upstream of the promoter region of a gene. GAL4 contains two domains, a DNA binding domain (GAL4-BD) and an activation domain (GAL4-AD). For the purposes of a screen, these two domains are separated and a protein of interest is fused to the DNA binding domain. A library of cDNAs containing potential interactors are fused to the second domain, GAL4-AD. Interaction between the protein of interest (the bait) and a library protein brings the two GAL4 domains together. Reconstitution of the GAL4 transcription factor restores its activity as an activator of the HIS3 gene. Expression of the HIS3 gene is required for synthesis of the amino acid histidine, required by yeast for survival. The transformed yeast is plated on media deficient in histidine, to select for colonies which are producing this amino acid. In this way, transformants carrying an interacting clone from the library are isolated.

3.1.3 Finding Caspase-1-Binding Partners using the Yeast-Two Hybrid Approach

Caspases have a well-defined domain structure which makes them good candidates for the yeast two hybrid assay as truncation of proteins used as bait is often required. This is necessary to decrease toxicity and increase the uptake of the bait plasmid by the yeast. As described in the general introduction, the CARD domain has been established as a critical protein-protein interaction domain in the formation of caspase activating complexes. For this reason the CARD of caspase-1 is very likely to be the region responsible for associating with regulatory molecules. Therefore this domain was chosen as the region to be used as the bait for this screen to identify novel binding partners for caspase-1.

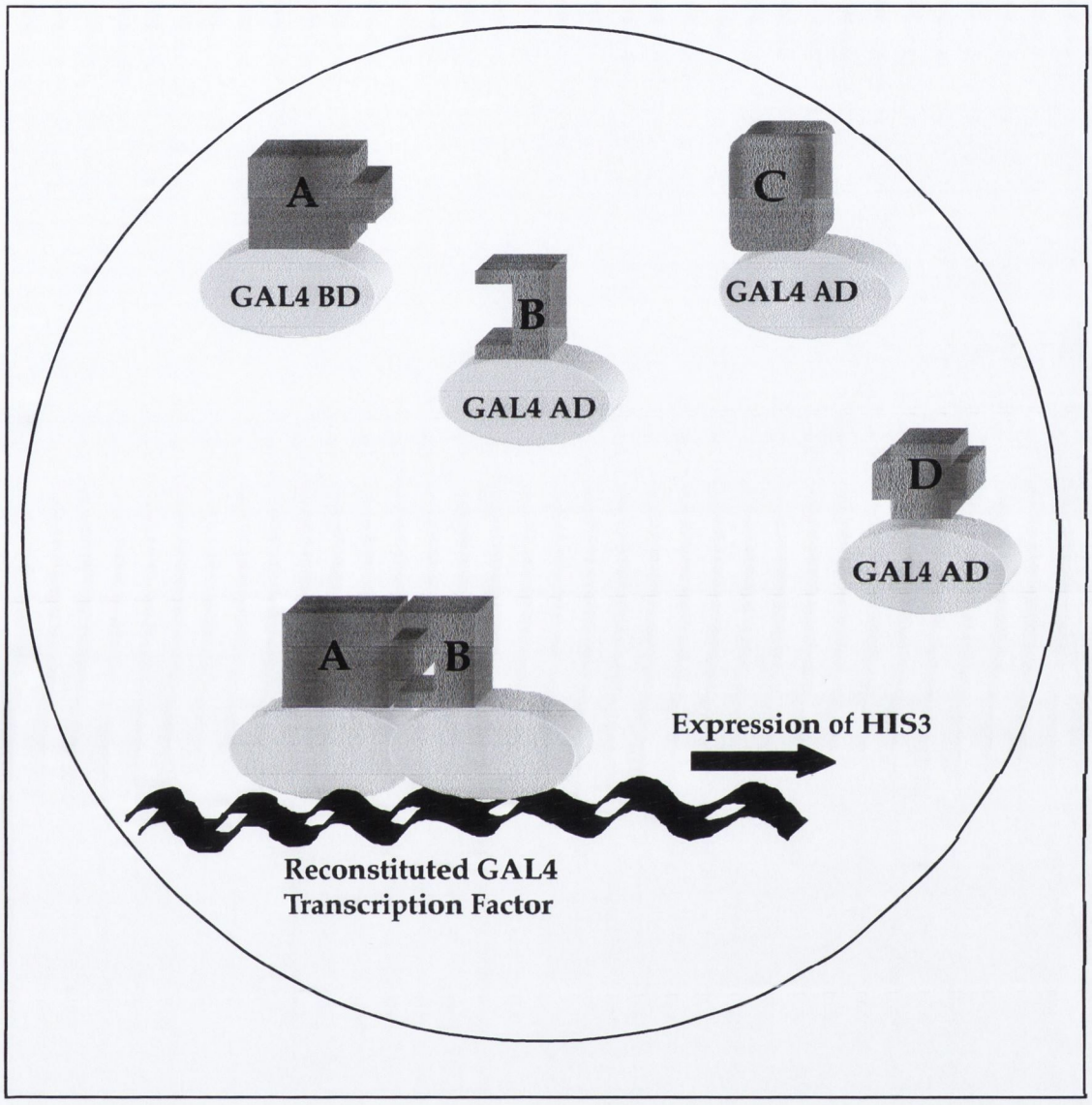


Figure 3.1

Principle of the Yeast Two -Hybrid Interaction Trap Assay:

The protein of interest (A above), known as the bait protein, is fused to the DNA binding domain of the GAL4 transcription factor (GAL4-BD). A library of proteins fused to the GAL4 Activation domain (GAL4-AD) are co-expressed with the bait protein in yeast. Interaction between a clone in the library and the bait protein brings the two GAL4 domains together and allows for trancription of the HIS3 gene and growth of yest plated on Histidine deficient medium.

3.2 RESULTS

3.2.1 *Generation of a GAL4BD-Caspase-1 CARD fusion protein*

The N-terminal prodomain of caspase-1, comprising amino acids 1-119, encodes a consensus CARD domain. This domain is predicted to function as an interface in associations with other CARD containing proteins. For this reason, the CARD fragment of caspase-1 was chosen as the bait for the two-hybrid screen. Amino acids 1-119 of caspase-1 were cloned into the yeast expression vector pAS2.1, downstream of and in frame with the GAL4 DNA binding domain, generating a GAL4BD-Caspase-1 CARD fusion protein (Figure 3.2).

Initial test transformations with this construct yielded quite low tranformation efficiencies ~$5x10^3$ cfu per μg (colony forming units /μg of DNA transformed) compared with the expected efficiency (up to 10^5 cfu/μg). The pAS2.1 vector is relatively large (8.4Kbp) before the addition of an insert. The size of this construct is likely to affect the success of transformation as plasmid uptake decreases with larger vectors. In order to improve the number of transformants, the caspase-1 prodomain was also cloned into a smaller vector pGBKT7 (6.3 Kbp), which also encodes the GAL4BD (Figure 3.2). Transfomations with this smaller construct yielded improved transformation efficiency ~$1x10^4$ cfu/μg.

3.2.2 *Expression of the Bait Proteins in Saccharomyces Cerevisiae*

In order to ensure both constructs, pAS2.1-Caspase-1^{1-119} and pGBKT7-Caspase-1^{1-119} efficiently expressed the fusion protein, these plasmids were transformed independently into the host strain, *Saccharomyces Cerevisiae* (Y190). Cell lysates of these Y190 transformants were made along with control lysates expressing the empty pAS2.1 and pGBKT7 vectors, which express the GAL4-BD region only. Overexpressed proteins were detected using an antibody specific for the GAL4-BD and an antibody directed towards the prodomain region of caspase-1 (Figure 3.3),

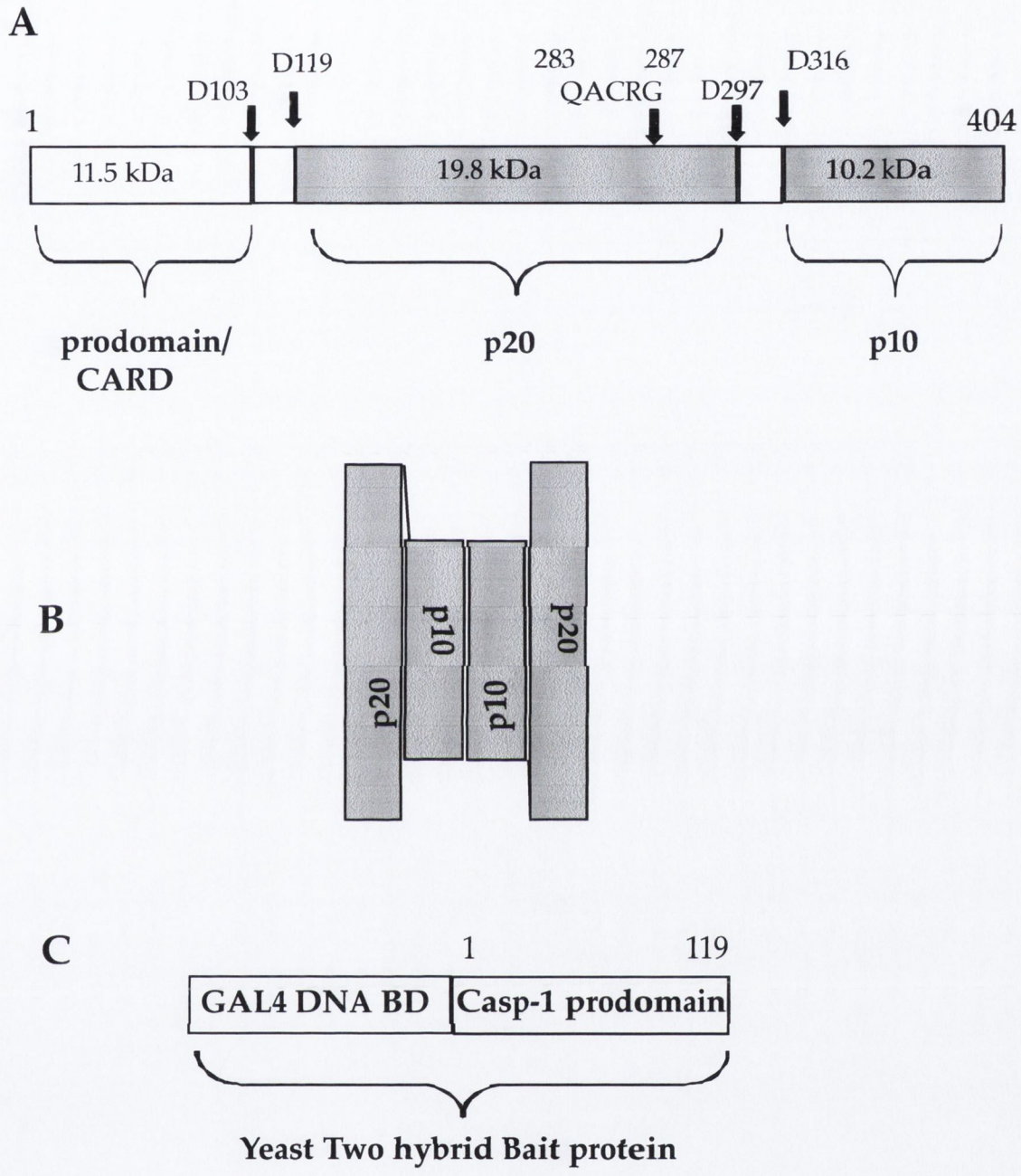


Figure 3.2

Generation of a Yeast Two Hybrid caspase-1 bait protein:

(**A**) The N-terminal prodomain of caspase-1 encompassing amino acids 1-119 encodes a CARD domain. This region is suspected to interact with adaptors which facilitate its oligomerisation and processing. Arrows indicate processing sites and the active site (QACRG). (**B**) The active caspase-1 enzyme consists of a heterotetramer of two large and two small subunits. (**C**) The yeast two-hybrid bait plasmids encode a fusion protein which consists of the GAL4-DNA binding domain fused to the N-terminus of the caspase-1 prodomain.

demonstrating that both constructs had successfully incorporated the caspase -1^{1-119} insert.

3.2.3 *Confirmation of Suitability of Bait Construct*

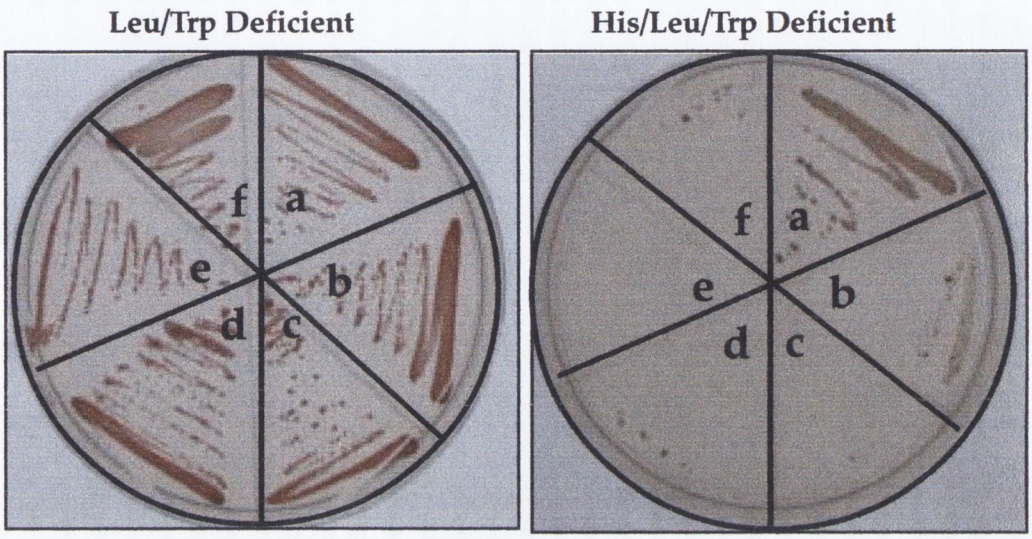
Occasionally, bait constructs are capable of activating transcription of the HIS 3 reporter gene in the absence of an interaction with a GAL4-AD fusion protein. This 'auto-activation' of the HIS 3 reporter renders a bait protein unsuitable for use in a screen. To ensure that the caspase-1 bait protein did not auto-activate, the bait plasmid was transformed alone alongside positive controls. GAL4-BD-casp-1^{1-119} expressed by pAS2.1 or pGBKT7 was incapable of autoactivating the HIS 3 reporter and yeast harbouring these constructs failed to grow on His- plates. This confirmed that both constructs were suitable bait proteins for a yeast two hybrid screen (Figure 3.4 A and B)

3.2.4 *The caspase-1 prodomain does not self-associate*

Self-association of the caspases through the CARD domain is known to occur at high concentrations and can facilitate autoprocessing. To assess the ability of the caspase-1 CARD to self-associate, bait and prey plasmids expressing this region were co-expressed. However, no interaction was detected between these regions of the protein under these conditions (Figure 3.4 A and B)

3.2.5 *Strategies to optimise the yeast two hybrid screen*

The Jurkat cDNA library used for the screen contains $3x10^6$ independent clones, known as the complexity of the library. In order to maximise the chances of a successful screen, at least twice the complexity of the library is co-transformed with the bait. Each 15 cm plate used for the screen supports the transformants resulting from 1.25 μg of library. To keep the number of plates to a minimum it is necessary to optimise the number of transformants resulting from each 1.25 μg of library. Some of the parameters that were altered from standard protocols to optimise the efficiency of transformation included using the highest quality grade of components

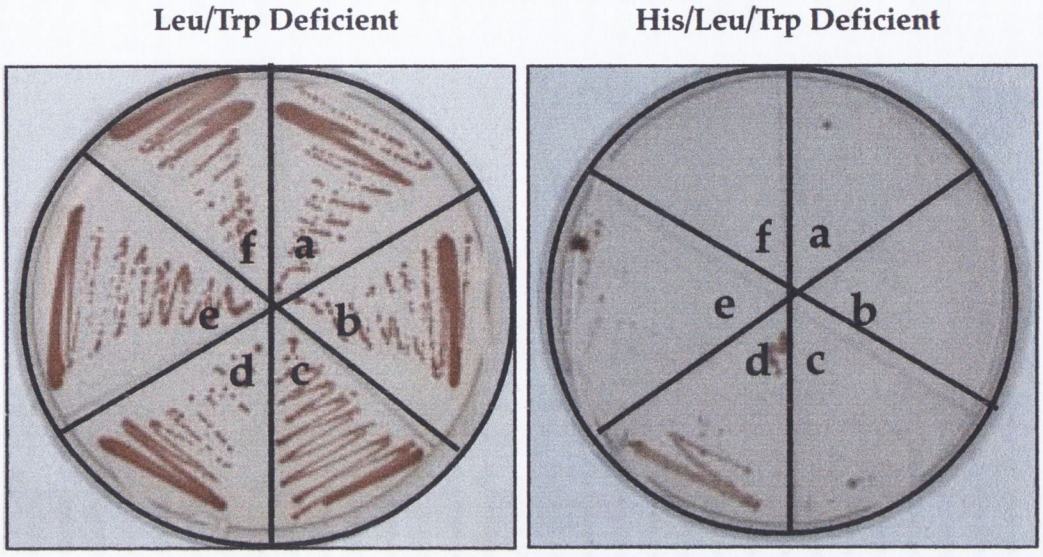


Bait Plasmid		Prey plasmid
a.	pAS2.1-Casp-9^{1-130}	pACT2-Apaf-1^{1-601}
b.	pAS2.1-Casp-8^{1-216}	pACT2-$FADD^{FL}$
c.	pAS2.1-Casp-1^{1-119}	pACT2 empty
d.	pAS2.1-Casp-1^{1-119}	pACT2-Casp-1^{1-119}
e.	pAS2.1-Casp-1^{1-119}	pGADT7 empty
f.	pAS2.1-Casp-1^{1-119}	pGADT7-Casp-1^{1-119}

Figure 3.4 (A)

The caspase-1 prodomain does not autoactivate or self-interact in yeast:

To ensure that the GAL4BD-Caspase-1 fusion protein did not spontaneously activate the HIS3 gene, the pAS2.1-caspase-1 plasmid was co-transformed with empty prey vectors into yeast (**c.** and **e.**) and grown on selective histidine-deficient medium (abovc RHS). Growth on Leu/Trp -deficient media confirms both plasmids are present (above LHS). pAS2.1 expressing the caspase-1 fusion, when expressed alone did not grow on the selective medium. Yeast which had been co-transformed with bait and prey plasmids expressing the caspase-1 CARD (**d.** and **f.**) did not grow on the selective media suggesting no capase-1 self-association was occuring. Positive contols showing caspase-8/FADD and caspase-9/Apaf-1 interactions are shown (**a.** and **b.**).



	Bait Plasmid	Prey Plasmid
a.	pGBKT7-Casp-1^{1-119}	pACT2 empty
b.	pGBKT7-Casp-1^{1-119}	pACT2-Casp-1^{1-119}
c.	pGBKT7-Casp-1^{1-119}	pGADT7 empty
d.	pAS2.1-Casp-9^{1-130}	pACT2-Apaf-1^{1-601}
e.	pAS2.1-Casp-8^{1-216}	pACT2-$FADD^{FL}$
f.	pGBKT7-Casp-1^{1-119}	pGADT7-Casp-1^{1-119}

Figure 3.4 (B)

The caspase-1 prodomain does not autoactivate or self-interact in yeast:

As in figure 3.4 (A), it was necessary to confirm that the GAL4BD-Caspase-1 expressed from the pGBKT7 vector did not spontaneously activate the HIS3 gene. As before, pGBKT7-caspase-1 plasmid was co-transformed with empty prey vectors into yeast (**c.** and **e.**) and grown on selective histidine-deficient medium (abovc RHS). Like pAS2.1-caspase-1, pGBKT7- caspase-1 , when expressed alone did not grow on the selective medium. Yeast which had been co-transformed with pGBKT7-caspase-1 bait and prey plasmids expressing the caspase-1 CARD (**d.** and **f.**) did not grow on the selective media suggesting no capase-1 self-association was occuring with protein expressed from the pGBKT7 vector. Again, positive contols showing caspase-8/FADD and caspase-9/Apaf-1 interactions are shown (**a.** and **b.**).

for all solutions and introducing a recovery step after the transformation procedure. The recovery step involved inoculating the transformed yeast into YPD medium to allow the cell walls time to recover from the harsh transforming conditions, and so increase the chances of survival for the transformants. pAS2.1-caspase-1^{1-119} and pGBKT7-caspase-1^{1-119} displayed contrasting characteristics including differences in efficiency of bait protein expression and transformation efficiency. Therefore it was decided to screen using both bait plasmids. The transformation efficiency when conditions were optimised was calculated to be approximately $2x10^4$ cfu/μg. In order to screen twice the complexity of the library, 300 μg of library, and 240 15 cm plates were required.

Growth of colonies on the selective histidine deficient media was monitored closely on the days immediately following the transformations. While there was a detectable amount of background growth on both sets of plates (containing either the pAS2.1-caspase-1 bait or the pGBKT7-caspase-1 bait), positive colonies whose growth significantly exceeded the background were readily apparent. The background growth found on the pGBKT7 set of plates was observed to be greater than that on the pAS2.1. The growth phase for the screen was terminated on day 10 after plating of yeast transformants.

Control transformations carried out at the same time and under the same conditions as the screen were plated to determine the transformation efficiency of the screen. This revealed that the actual efficiency of the screen was lower than expected with approximately $6.3x10^3$ cfu/ μg obtained. This meant that $1.9x\ 10^6$ library clones were screened, 0.63 times the total library complexity. Therefore not all cDNAs contained in this library have been tested for an interaction with caspase-1.

3.2.6 *Selection of Positive Clones for Identification*

All colonies that had grown significantly more than the background, ~500 in total, were restreaked on duplicate Leu/Trp dropout and His/Leu/Trp dropout, 15 cm plates, which had been divided into sectors (Figure 3.5). These plates were incubated for 4 days (pGBKT7) or 6 days (pAS2.1). The regrowth on selective HLT-media was assessed as weak (+), average (++), strong (+++), or very strong (++++) after 4-6 days. Equal numbers of colonies from each category were chosen to undergo the plasmid recovery procedure. Plasmid DNA from the prey vector was isolated and the cDNA inserts were amplified through PCR. These PCR products were then sequenced and from this approximately 100 sequences were obtained.

3.2.7 *Identification of Clones*

Sequences obtained were identified using the BLASTX tool, which aligns the sequence against all nucleotide sequences in the database and provides a list of the nearest matches. Table 3.1 shows the sequence identities obtained, some sequences did not match any database entries. In most cases this was because the sequence obtained was only a short fragment of the yeast clone or the sequence was not of a high enough quality.

HIS/Leu/Trp Deficient

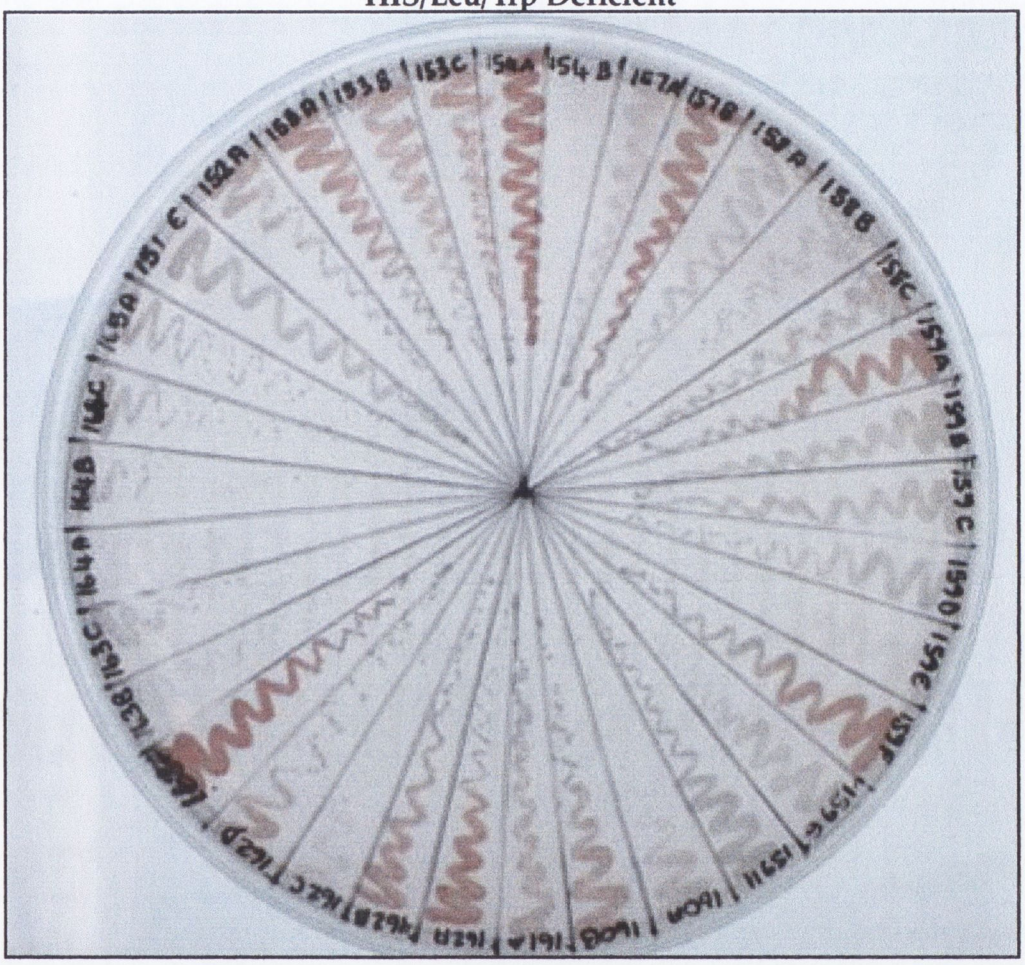


Figure 3.5

Selection of positive clones for sequencing:

Positive clones from the yeast two hybrid screen which had grown significantly above background were restreaked on sectored histidine-deficient master plates, and allowed to grow to reconfirm the interaction. The photograph above gives an example of the regrowth of colonies after restreaking. The strength of the HIS3 expression is reflected in the growth towards the centre of the plate and by the appearance of a pink colour in the yeast.

TABLE 3.1

Clone ID	Accession Number	BLASTX (Human)	BLASTX (Non-Human)	*Score	**E value
51C	338929	T-cell receptor active B-chain		484	e-136
58C	16226067	Putative methyltransferase WBMT		412	e-114
33F	3183544	Polyadenylate binding protein 1		400	e-110
89B	4507873	Von Hippel-Lindau binding protein		394	e-109
51B	5174447	Guanine Nucleotide Binding protein		382	e-121
59A	12654695	Similar to COP9 subunit 5		368	e-101
174A	1709217	Ran binding protein 2		349	e-118
233A	13129150	HSP90		347	6e-95
35A	7706254	Nucleolar protein NOP5/NOP58		339	1e-92
135B	4826870	Nucleobindin 2		322	2e-87
159B2	6912634	Ribosomal protein L13a		312	7e-85
52A	7435520	adenosylhomocysteinase		300	e-117
58D	4506691	Ribosomal protein S16 (40S)		291	3e-78
199B	20149594	HSP90		288	e-101
44A	4506605	Ribosomal protein L23		276	4e-74
160A	6912634	Ribosomal protein L13a		269	4e-72
48B	4506605	Ribosomal protein L23		261	1e-69
33B	4506605	Ribosomal protein L23 (60S)		256	5e-68
223C	4506713	Ubiquitin and ribosomal protein S27a precursor		246	1e-65
160A	6912634	Ribosomal protein L13a		269	4e-72
234A	13376410	NM_024936		229	4e-60
214A	14251209	Chloride Intracellular channel		228	3e-59
230A	14251209	Chloride Intracellular channel NM_001288		220	5e-57
194A	14251209	Chloride Intracellular channel 1		210	6e-54
88B	5031601	Actin related protein 2/3 complex subunit 1B		193	4e-49
57A	18031722	Keratin protein K6irs		165	9e-41
236A	13643342	High mobility group protein		162	2e-39
162A	728836	Alu subfamily SP seq		162	4e-39
153A	181967	Elongation Factor 1 alpha		150	4e-39
2B	31092	Elongation Factor 1 Alpha X16869		150	2e-65
210A	14740717	Similar to FLJ0075		143	1e-33
195B	21263420	ADP-ribosylation factor		133	4e-31
212A	30645	GAPDH		102	1e-21
159F	181967	Elongation factor 1 alpha		97	8e-25
151A	14743802	Protein Tyrosine Kinase		96	1e-19

Clone ID	Accession Number	BLASTX (Human)	BLASTX (Non-Human)	*Score	**E value
135C	14743802	Protein Tyrosine Kinase β		96	1e-19
135C	14743802	Protein Tyrosine Kinase β		96	1e-19
211A	12230015	Cytochrome b5 outer mitochondrial membrane precursor		94	7e-19
197C	13650330	Similar to heterogeneous nuclear ribonucleoprotein		92	3e-18
150A	20470607	Hypothetical protein XP_166629		90	2e-17
163A	4759244	Thymosin beta 4		83	1e-15
129A	20470607	Hypothetical protein XP_166629		78	7e-14
140D	340062	Pro-Ubiquitin		70	1e-11
144E	340062	Pro-Ubiquitin		68	1e-14
23B	7706266	CGI-20 protein		57	6e-08
81B2	279650	Ribosomal protein L23		52	1e-06
116A	20538705	Similar to TCRBV6S8		51	3e-06
95B	14017399	Humanin		49	5e-05
97B	17447945	Similar to ribosomal protein L5		47	8e-05
124D	13652116	Plakophilin 2		47	2e-04
169A	14720869	Similar to Alu subfamily SB		47	1e-04
58B	1550084		Putative protein XG976	45	5e-04
89A	347527	Human ribosomal protein S3		42	3e-04
239A	18603988	Hypothetical protein XP_097121		37	0.041
229E	12644407	Diacylglycerol kinase zeta		36	0.077
146A	17486564	Similar to melanoma antigen		36	0.37
90A2	20544010	Hypothetical protein XM_099866		35	0.21
81D	15610114		ThiL (*Mycobacterium tuberculosis*)	35	0.99
142A	21292778		AgCP4253 (*Anopheles Gambiae*)	35	0.48
118B	16164234	KIAA1538		35	0.13
183A	7243087	KIAA1353		34	0.90
190C	9845342		PR50 (Rat cytomegalovirus)	34	0.44
130A	17539448		Putative ER protein (*C.elegans*)	34	1.9
172A	1335985	VP16 transactivating domain (cloning vector)		33	1.1
27A	3789970		Fininogen A-aloha (*Macropus rufus*)	33	0.89
29C	21295272		AgCP14645	33	0.86
138C	11493552	Pro 1933		33	0.87
140B	11493552	Pro 1933		33	0.85

Clone ID	Accession Number	BLASTX (Human)	BLASTX (Non-Human)	*Score	**E value
151B	15220448		Putative ovule specific homeotic protein (*Arabidopsis Thaliana*)	32	3.8
229B	18553140	Hypthetical protein XM_104110		32	3.4
122C	8150454		Disease resistance protein (*Arabidopsis Thaliana*)	32	2.8
110B	18266907	Unknown protein		32	1.4
219A1	4507399	Transcription factor 4 isoform beta		32	1.3
157B	16121811		Putative siderophore biosynthetic enzyme (*Yersinia pestis*)	32	0.98
135A	18860864	MAP kinase activating DD containing protein		31	5.3
226C	18606370	Hypothetical protein XP_108825		31	4.8
51A	15610931		Emb B (Mycobacterium tuberculosis)	30	27
56A	19773541	Fat3		30	25
237A	9653270		NADH dehydrogenase subunit 5 (*Physeter Catdon*)	30	8.2
142B	15807139		ABC Transporter (*Deinococcus radiodurans*)	30	7.5
186A	19921984		CG11887 gene product (*Drosophila melanogaster*)	30	7.5
25A	17987083		Propionyl-Co A Carboxylase alpha chain (*Brucella Melitensis*)	30	7.3
81B1	12329141		ORF 212 (*Shigella Flexneri*)	30	3e-78
143E1	21289510		EbiP9880	25	248
198A1	17539448		C55c3.2p (*C. elegans*)	24	2.2

Clones shaded in this way are known DNA-binding proteins which occur frequently as false positives.

Clones shaded in lighter grey are known to occur as false positives, as a result of their tendency to non-specifically bind other proteins.

*Score: This value refers to the bit score, which is determined from the number of aligned identical or similar residues, unaligned gaps and also takes into account the length of the sequence. The higher the score the better the alignment.

**E Value: This value is the number of hits expected for this alignment. The lower the value the more significant the match.

3.3 DISCUSSION

3.3.1 *Introduction*

Analysis of the identities of clones isolated in the screen revealed a high number of known false positives. These proteins are commonly encountered in yeast two hybrid screens irrespective of the bait used. False positives fall into a number of categories. The majority of these molecules are DNA binding proteins which, when fused to the GAL4-AD as part of the screen, non-specifically activate the HIS3 gene. Another category are proteins whose role in the cell involves interacting with, or becoming conjugated to wide ranges of other proteins. This might be as a chaperone to aid folding of proteins or as a degradation signal as in the case of ubiquitin ligases. False positives may also occur where expression of a particular protein allows the yeast to overcome the nutritional selection which the clones are subjected to (Hengen, 1997). Unfortunately, growth characteristics of the false positives did not show any common pattern, with the growth ranging from just above what was considered background growth to significantly strong growth compared to the background colonies.

In addition to this category of proteins, a large number of proteins were believed not to be genuine caspase-1 adaptors, as they did not contain any recognisable domain such as a CARD or oligomerisation domain, which would facilitate their interaction with and the activation of caspase-1. For this reason they were not pursued any further. It is possible that the above criteria are not required for interaction or activation of caspase-1 but looking at the modes of activation of other apical caspases (eg caspases -8 and -9) it would seem most likely that formation of a complex possibly through the oligomerisation of a scaffold protein should occur. It is also possible however, that an as yet unidentified domain is capable of interaction with caspases. New domains predicted to have roles in apoptosis and inflammation (Pyrin and NACHT) have been identified recently and it is likely that more will be added as analysis of seqence databases continues. In undertaking a

large-scale screen such as the yeast two hybrid approach it is inevitable that large amounts of data will be produced which is not always possible to analyse in full. For this reason it was decided that that absence of any proteins containing a recognible CARD region and lack of multiple copies of proteins was a weak basis for the continued analysis of the proteins identified in the screen.

3.3.2 *Putative Caspase-1 Interacting molecules*

A large number of the clones obtained are suspected to be false-positives, however two clones did stand out as having as association with caspase-dependent pathways and so the possibility of a genuine association with caspase-1 was considered.

Von Hippel-Lindau Syndrome Binding protein

Clone 89B was identified as the Von Hippel-Lindau binding protein. Von Hippel-Lindau syndrome is a hereditary disorder characterised by the development of multiple tumours of the blood, kidneys and nervous system (Kaelin, 2002). The Von Hippel-Lindau tumour suppressor gene is mutated in the germline of sufferers of this disease (Latif *et al.*, 1993). Tumour suppressor genes encode various proteins that are responsible for inhibiting excessive cell proliferation by inducing apoptosis. Inactivation of these proteins allows cells to multiply unchecked and go on to form tumours. The protein encoded by the Von Hippel-Lindau gene, Von Hippel-Lindau protein (pVHL), is responsible for the down regulation of a transcription factor known as the hypoxia inducible factor (HIF) (Kim and Kaelin, 2003). The role of HIF is to transcriptionally activate the hypoxia inducible genes, which include a number of growth factors that stimulate cell proliferation. HIF is downregulated by pVHL, which is part of a multi-protein complex. This pVHL ubiquitin ligase complex targets proteins such as HIF to the proteasome for degradation by attaching ubiquitin moieties to the protein in question (Kamura *et al.*, 2000). The VHL-binding protein is a component of this complex (Tsuchiya *et al.*, 1996). It is possible that a positive interaction between caspase-1 and VHL-BP in the screen,

reflects a functional association that occurs under physiological conditions, where caspase-1 is a target for pVHL–ubiquitin ligase complex. However, it is also possible that this interaction is non-specific as the pVHL protein complex consists of chaperone molecules which function in protein–binding and might therefore be 'sticky' proteins. As only one copy of this protein was obtained in the screen, the role of VHL-binding protein in caspase-1 regulation has not been pursued to date.

Humanin

Familial Alzheimer's Disease (FAD) is a neurodegenerative disorder characterised by apoptosis of neurons as a result of excessive production of a known number of gene products. Humanin is a polypeptide which was identified in screens for genes whose products could suppress death induced by genes associated with Alzheimer's disease (Hashimoto *et al.*, 2001). Overexpression of this polypeptide could prevent death induced by three FAD-associated gene products, amyloid precursor protein, presenilin-1 and presenilin-2. This inhibition of apoptosis has been suggested to be as a result of interactions between humanin and the pro-apoptotic protein Bax (Guo et al., 2003). As described in the general introduction, Bax is a pro-apoptotic member of the Bcl-2 family which is thought to be involved in facilitating cytochrome *c* release. The interaction with the humanin peptide results in the inactivation of Bax preventing its role in promoting cytochrome-*c* release from the mitochondria. As with the Von-Hippel Lindau binding protein clone, only one copy of humanin was obtained in this screen for caspase-1 binding partners. For this reason the importance of this interaction is not certain. However, the fact that only a proportion of the total clones in the library were screened, the number of copies of genuine interactors would also be reduced.

3.2.3 *Evaluation of the Yeast Two-Hybrid strategy*

Although the yeast two-hybrid approach has proven to be effective in the identification of a number of novel interaction partners, it may be that these successes are the exception rather than the rule. A number of disadvantages are associated with this tool. One of the most significant of these disadvantages is the degree to which the bait protein influences every aspect of the screen. From deciding on the size of the screen to the probability that a given protein will make a suitable bait for the screen, huge variations exist. The results of a screen are also affected by the possibility that true interactions can be missed, i.e. false negatives. A number of factors are responsible for this, such as the possibility that the GAL4 fusion hinders interactions with genuine binding partners by altering the secondary structure or merely obstructing the binding interface. It is also significant to point out that the expression of human proteins in a yeast host may not allow typical interactions to occur. Post-translational modifications required for binding which are not provided by the yeast or intracellular localisation differences- as GAL4 interactions must occur in the nucleus to activate the HIS3 gene, can both result *in* a false negative.

In the case of the caspase-1 screen a number of factors are likely to have influenced the outcome. Firstly, it is probable that the GAL4 BD-caspase-1 construct was slightly toxic to the host yeast, as the efficiency of transformation was quite low compared with other constructs which have been used previously in our laboratory. Initial experiments found no interaction between GAL4 BD-caspase-1 and caspase-1-GAL4 AD fusion proteins, contrary to reports which suggest that CARD domains of caspase-1 self-associate. This may mean that truncation of the caspase CARD may have disrupted its secondary structure and the new folding pattern may not be capable of interacting in the correct manner. This may have impacted on the ability to associate with binding partners in the library. One alteration to the existing yeast two-hybrid tool, which would improve its efficiency would be the elimination

of false positives, one way which this has been done is to introduce additional reporter genes to apply a more stringent selection process. Ideally, the most efficient method would be to remove clones which encode known false positives from libraries.

If, in the future, further information emerged regarding proteins identified as possible caspase-1 interacting molecules in this screen, it would be possible to re-investigate these molecules more thoroughly. Cloning of the full-length molecules into mammalian expression vectors to assess their function in another system would provide additional information and allow confirmation of their ability to interact with caspase-1. The list of results from this screen will also provide a useful source of information for those continuing yeast two hybrid analysis and those investigating caspase-1 activation.

Because the yeast two-hybrid approach failed to identify proteins with motifs (e.g. CARDs or DDs) that suggested they may be involved in activation of this caspase, we decided to pursue an alternative approach to this problem. BLAST-based searches of the human DNA and protein databases were performed using the CARD motif of caspase-1 as a query sequence. This approach succeeded in identifying novel CARD-containing proteins, one of which will be discussed in the following chapter.

CHAPTER IV

Identification of a Novel Death-inducing complex between the PYRIN proteins DEFCAP and ASC

4.1 INTRODUCTION

4.1.1 *Identifying novel CARD proteins*

The advantage of the yeast two-hybrid approach, in principle, is that very large numbers of molecules are included in the screen. This of course maximises the chances of finding at least one binding partner for the bait protein. An alternative strategy, which involves *in silico* screening a large number of proteins, was adopted to increase the likelihood of identifying novel CARD-containing proteins. Using the public databases as a source of sequences, proteins were identified which showed sequence similarity to the CARD of caspase-1. One advantage of this type of screen, in addition to the scale, is that it is not affected by expression patterns of proteins as the database contains all ESTs from a wide variety of sources and it requires little experimental effort. The CARD domain has been shown to be a motif involved in pathways of signalling for cell death and cell survival, as discussed in the introduction. For this reason, novel proteins identified in this screen could potentially have roles in a number of different pathways.

The database interrogation method of identifying novel proteins has previously proven to be successful by our own group, and was used to identify CARDINAL, a novel inhibitor of multiple NFκB activation pathways (Bouchier Hayes et al., 2001). This approach has also been successfully used by several other groups for example in the identification of the CARD proteins NOD1 and NOD2 (Inohara *et al.*,1999; Bertin *et al.*,1999; Ogura *et al.*, 2000).

While it has been established that CARD domain containing proteins are involved in a number of signalling pathways, a number of questions remain about the features of this large family of proteins. As mentioned earlier, the CARD belongs to the death domain family of protein motifs, which share a similar structure. It is known that the death domain-containing protein FADD is capable of binding more than one upstream binding partner, the Fas receptor or TRADD. It is not however,

known if CARD proteins have a similar capacity to bind multiple partners or whether these motifs are more specific than the death domain.

By using database searches, a screen for novel CARD proteins was carried out with the aim of broadening the current knowledge of the pivotal role these important proteins play in numerous pathways. This search resulted in the identification of a novel multi-domain CARD protein, NAC. This protein belongs to the NBD-CARD category and as such shares common features with Nod1/2 and Apaf-1. In this chapter the role of this protein is explored. We found that NAC, through synergising with a bipartite-CARD, ASC, promotes apoptosis. These data suggest the existence of a novel multimeric apoptosis-inducing complex.

4.2 RESULTS

4.2.1 Identification of NAC

To identify novel CARD-containing protein, the tBLASTn and BLASTP homology search tools were used to scrutinize the public sequence databases. Using the pro-domain of Caspase-1 (amino acids 1-119, which contains the CARD) as a search sequence, a CARD-containing hypothetical protein, KIAA0926 (Genbank™ accession number NP_055727), was identified. The full-length protein encoded by this clone is predicted to be 1429 amino acids in length and contains a CARD domain at its C-terminus. This protein was subsequently identified by two other groups (Hlaing *et al.*, 2001; Chu *et al.*, 2001). The protein is now referred to as NAC or DEFCAP. A longer isoform which contains 1473 amino acids (an additional 44 amino acids) is also known to exist. Figure 4.1 illustrates the amino acid sequence of the full length NAC isoform. Detailed sequence analysis revealed several other protein motifs within the NAC sequence. The position of each of the domains is indicated in Figure 4.1. The C-terminal CARD represents the region homologous to the Caspase-1 pro-domain. In addition to the CARD domain the protein also contains an NBD region, several LRR, and a PYRIN domain (Figure 4.1A and B). The longer isoform contains 12 LRRs while the shorter isoform is missing one of these. At its N-terminus, NAC contains a PYRIN domain, which is a recently characterised domain similar to the CARD.

To gain some information on the protein family to which NAC belongs, alignments were made to illustrate the similarities between the PYRIN and CARD regions of the protein, and those in other characterised proteins. In Figure 4.2, a selection of CARD and PYRIN domains are compared. The CARDs of ASC and NAC are most similar, with Apaf-1 sharing stretches of identical residues with these two molecules. Alignment of the PYRIN domain, which is approximately the same length as a CARD domain, also reveals a high degree of similarity between ASC and NAC. Overall, NAC bears a high degree of similarity to the Nod and Apaf-1

A

MAGGAWGRLACYLEFLKKEELKEFQLLLLANKAHSRSSSGETPAQPEKTSG
MEVASYLVAQYGEQRAWDLALHTWEQMGLRSLCAQAQEGAGHSPSFPYSP
SEPHLGSPSQPTSTAVLMPWIHELPAGCTQGSERRVLRQLPDTSGRRWRE
ISASLLYQALPSSPDHESPSQESPNAPTSTAVLGSWGSPPQPSLAPREQE
APGTQWPLDETSGIYYTEIRERERERKSEKGRPPWAAVVGTPPQAHTSLQP
HHHPWEPSVRESLCSTWPWKNEDFNQKFTQLLLLQRPHPRSQDPLVKRSW
PDYVEENRGHLIEIRDLFGPGLDTQEPRIVILQGAAGIGKSTLARQVKEA
WGRGQLYGDRFQHVFYFSCRELAQSKVVSLAELIGKDGTATPAPIRQILS
RPERLLFILDGVDEPGWVLQEPSSELCLHWSQPQPADALLGSLLGKTILP
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IRAFRLVKSNKELWALCLVPWVSWLACTCLMQQMKRKEKLTLTSKTTTTL
CLHYLAQALQAQPLGPQLRDLCSLAAEGIWQKKTLFSPDDLRKHGLDGAI
ISTFLKMGILQEHPIPLSYSFIHLCFQEFFAAMSYVLEDEKGRGKHSNCI
IDLEKTLEAYGIHGLFGASTTRFLLGLLSDEGEREMENIFHCRLSQGRNL
MQWVPSLQLLLQPHSLESLHCLYETRNKTFLTQVMAHFEEMGMCVETDME
LLVCTFCIKFSRHVKKLQLIEGRQHRSTWSPTMVVLFRWVPVTDAYWQIL
FSVLKVTRNLKELDLSGNSLSHSAVKSLCKTLRRPRCLLETLRLAGCGLT
AEDCKDLAFGLRANQTLTELDLSFNVLTDAGAKHLCQRLRQPSCKLQRLQ
LVSCGLTSDCCQDLASVLSASPSLKELDLQQNNLDDVGVRLLCEGLRHPA
CKLIRLGLDQTTLSDEMRQELRALEQEKPQLLIFSRRKPSVMTPTEGLDT
GEMSNSTSSLKRQRLGSERAASHVAQANLKLLDVSKIFPIAEIAEESSPE
VVPVELLCVPSPASQGDLHTKPLGTDDDFWGPTGPVATEVVDKEKNLYRV
HFPVAGSYRWPNTGLCFVMREAVTVEIEFCVWDQFLGEINPQHSWMVAGP
LLDIKAEPGAVEAVHLPHFVALQGGHVDTSLFQMAHFKEEGMLLEKPARV
ELHHIVLENPSFSPLGVLLKMIHNALRFIPVTSVVLLYHRVHPEEVTFHL
YLIPSDCSIRKAIDDLEMKFQFVRIHKPPPLTPLYMGCRYTVSGSGSGML
EILPKELELCYRSPGEDQLFSEFYVGHLGSGIRLQVKDKKDETLVWEALV
KPGDLMPATTLIPPARIAVPSPLDAPQLLHFVDQYREQLIARVTSVEVVL
DKLHGQVLSQEQYERVLAENTRPSQMRKLFSLSQSWDRKCKDGLYQALKE
THPHLIMELWEKGSKKGLLPLSS

B

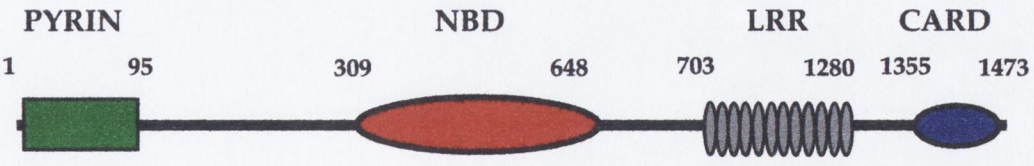


Figure 4.1:
Sequence Analysis of NAC and domain structure of the full length NAC protein
(**A**)The full length sequence of the long isoform of NAC/DEFCAP is 1473 amino acids. The sequence is colour-coded to indicate the domain structure. Green for PYRIN domain, Red for Nucleotide binding domain (NBD), Grey for each of the 12 Leucine Rich Repeats (LRRs) and Blue for CARD domain. The short isoform of NAC lacks one of the LRRs. (**B**) Domain structure of NAC/DEFCAP amino acid positions are indicated at the beginning and end of each domain.

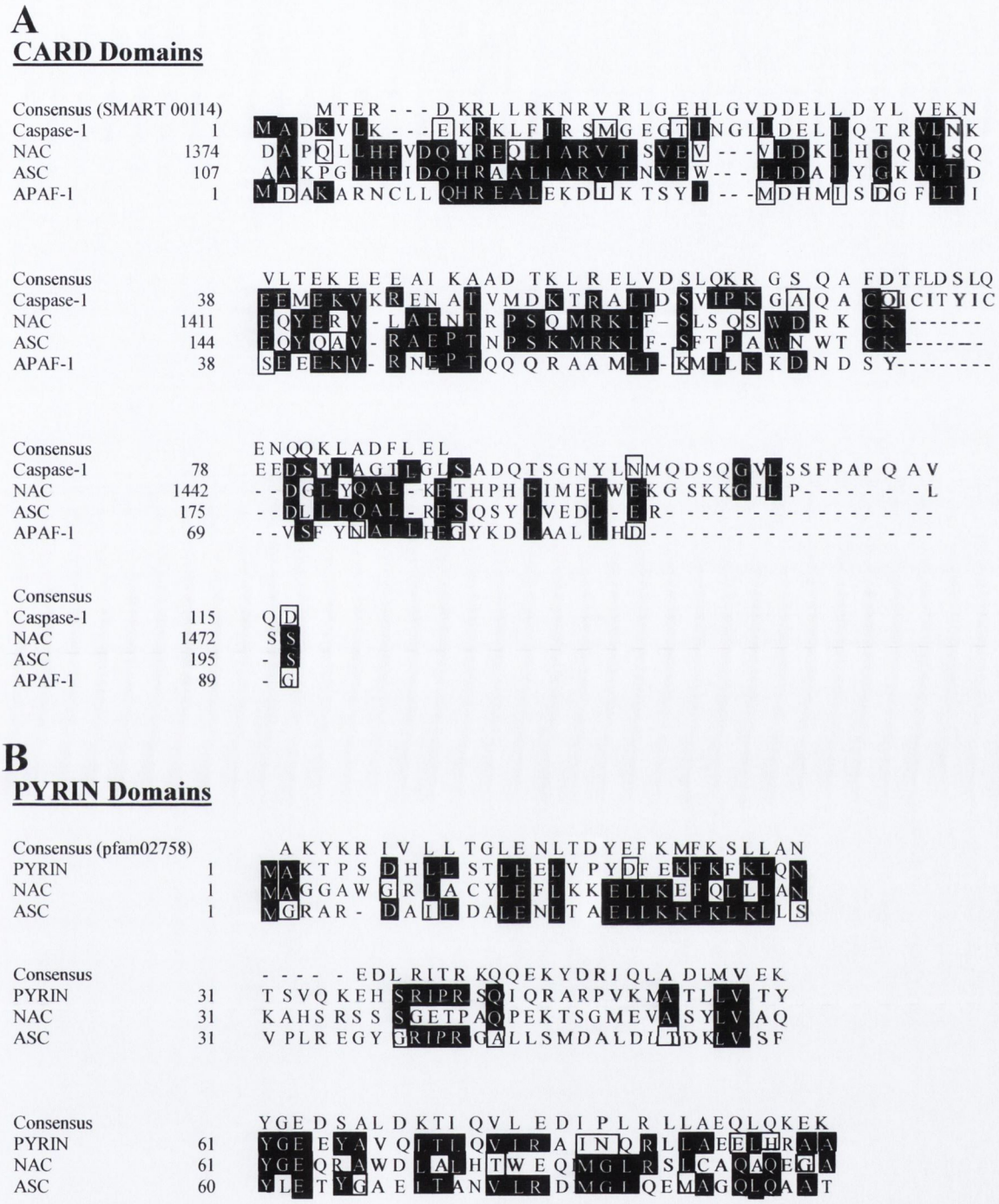


Figure 4.2:

Alignment of NAC protein motifs with those of other proteins

Sequence alignments were carried out using the Clustal program with the Lasergene application.

(A) Alignments of the CARD domain of NAC (amino acids 1374-1473) with other known CARDS.

(B) Alignment of the PYRIN domain of NAC (amino acids 1-90) with other PYRIN motifs. Identical residues are shaded in black while conserved residues are boxed.

multi-domain CARD protein family that have been implicated in caspase and NFκB activation pathways in response to cell stress or infection.

4.2.2 Cloning of NAC

To carry out NAC functional studies and generate deletion, the KIAA0926 cDNA (in the pBluescript vector) was obtained from the Kazusa Institute (Japan). The cDNA insert was then cloned into pcDNA3 to facilitate efficient expression in mammalian cells. A panel of DEFCAP (NAC) truncation constructs (of both the long and short isoforms) were also obtained from Dr. Tom Hlaing (University of Michigan; Figure 4.3). Each of these constructs contained an N-terminal Myc epitope tag, to facilitate detection of expressed constructs by western blot analysis.

4.2.3 Generation and Affinity Purification of anti-NAC antibody

To facilitate western blot analysis of NAC protein expression, a polyclonal anti-NAC antibody was generated. This required the production of large quantities of recombinant NAC protein (approximately 100 μg of protein per immunisation, for 3 separate immunisations). A GST-NAC$^{1312-1429}$ fusion protein was constructed by cloning the appropriate coding sequence into the pGEX4TK2 bacterial expression vector. GST-NAC$^{1312-1429}$ was expressed in DH5 *E. coli* and purified as described in materials and methods. Rabbits were immunised with purified GST-NAC $^{1312-1429}$ spanning the CARD domain (Figure 4.4). Large-scale induction resulted in the protein becoming quite insoluble. This was most likely due to the production of inclusion bodies by the bacteria to store this foreign protein. Formation of these membrane bound structures was reduced by slowing down the growth of the bacterial cultures (through lowering the growth temperature) and decreasing the duration of protein expression.

To ensure that the antibodies generated were capable of specifically recognising NAC, lysates of cells transiently overexpressing NAC were prepared. As expected

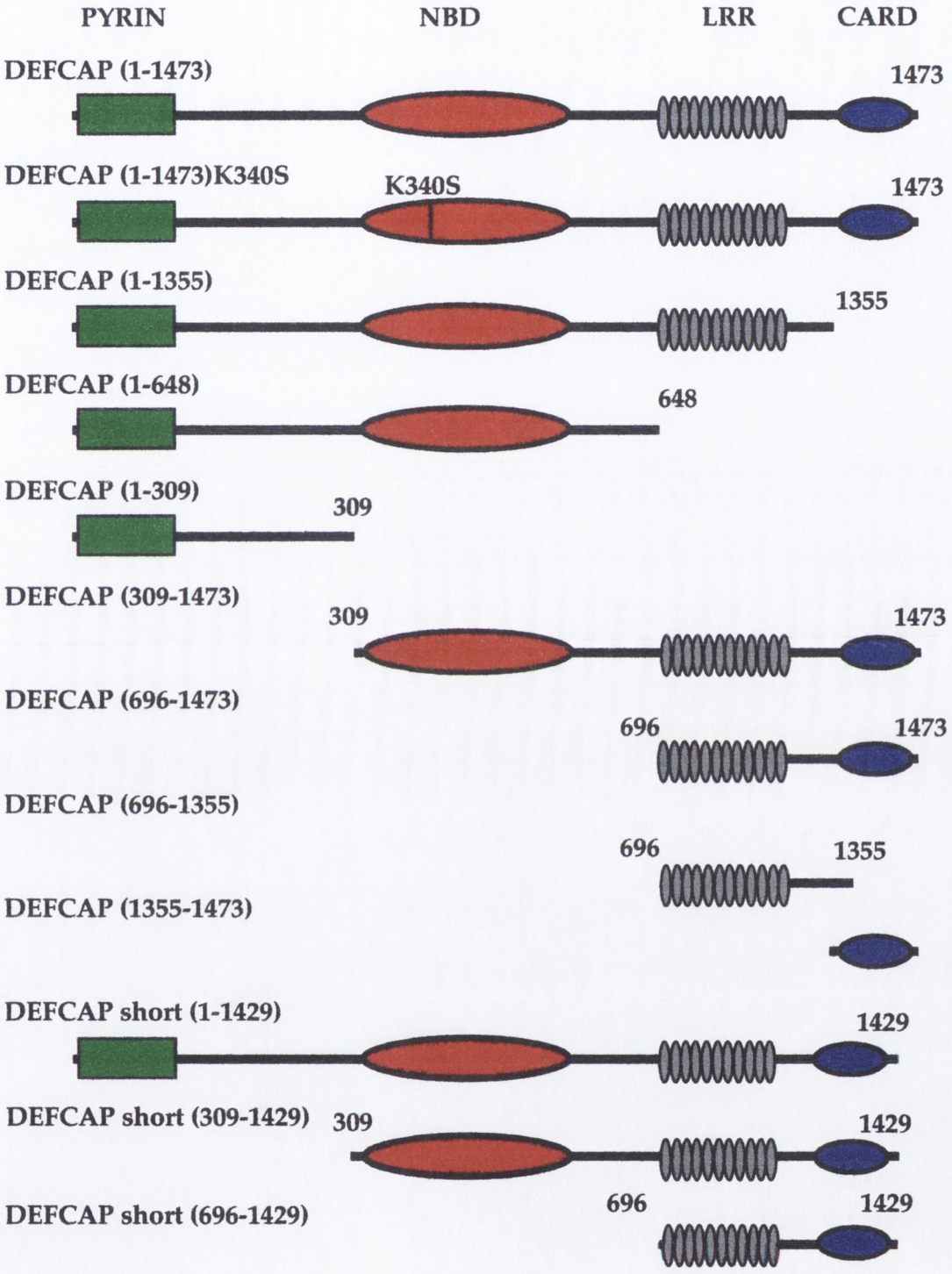
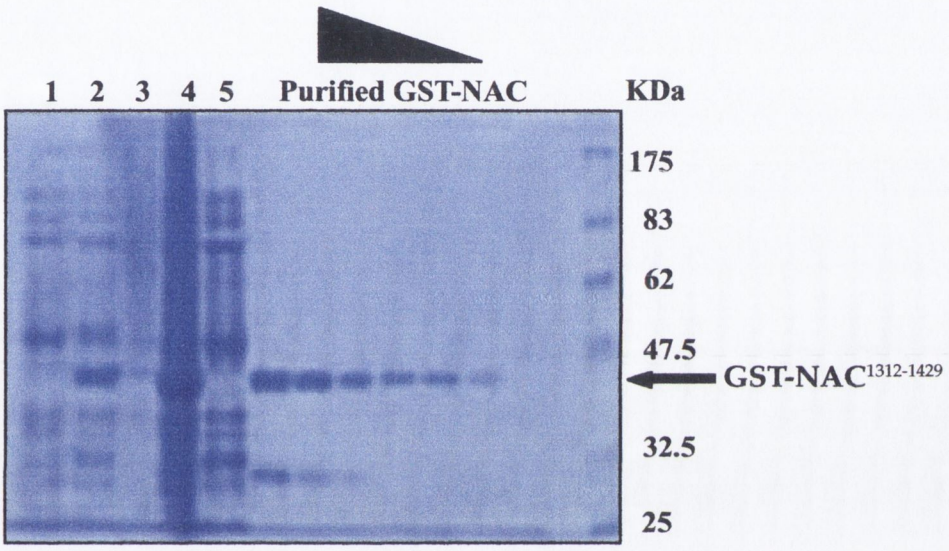


Figure 4.3:
Panel of DEFCAP/NAC full length and truncated proteins.
Schematic representation of N-terminal myc-tagged full length and truncated versions of NAC/DEFCAP (long and short isoforms) that were used for overexpression studies. Numbers indicate amino acid positions.



1. Pre-Induction bacterial lysate.
2. Post-Induction bacterial lysate.
3. Protein released by sonication.
4. Insoluble protein fraction.
5. Protein remaining after incubation with Glutathione beads.

Figure 4.4:
Bacterial expression and Purification of GST-NAC recombinant protein.
GST-NAC (amino acids 1312-1429) was induced in *E.coli* by addition of 100 μM IPTG. The protein was released by disruption of the bacteria through sonication. The solublised protein was then purified by binding to Glutathione-sepharose beads. A substantial portion of GST-NAC when overexpressed became insoluble (lane 4). Some degradation of purified GST-NAC is visible.

the antiserum recognised NAC deletion mutants which contained the CARD motif and not those which had this region deleted (Figure 4.5).

To improve the specificity of the anti-NAC polyclonal Antibody, the serum was affinity-purified using GST-NAC$^{1312-1429}$ immobilised on Affigel beads, and packed into an Econo-column with a bed depth of 1 ml. This resulted in significant improvement of the specificity of the anti-serum for overexpressed NAC (Figure 4.6). Purified antibody was then concentrated using a centrifugation-based protein concentrator. The purified anti-NAC antibody was not capable of efficiently detecting endogenous NAC expression in a panel of cell lines tested, suggesting that this protein is expressed at low levels in these cell lines (Figure 4.6).

4.2.4 *NAC fails to induce NFκB or augment LPS dependent NFκB activation*

Data from a number of groups support the idea that the Nod proteins function as intracellular pathogen receptors. Because of the structural similarities between NAC/DEFCAP and Nod1, we wondered whether NAC/DEFCAP might act as a sensor for pathogen components. Using Nod1 as a positive control we established that the panel of NAC truncations do not have any intrinsic NFκB activating abilities (Figure 4.7). During the transfection procedure, LPS was added to the medium which still contained the transfection complexes used to introduce the plasmids. This method has been used previously by Inohara *et al.*, to carry LPS across the plasma membrane and into the cytoplasm. The addition of LPS resulted in a Nod-1-dependent increase in NFκB activation as expected. However, no significant increase in NFκB activation was found in any of the NAC transfected cells upon LPS addition (Figure 4.7).

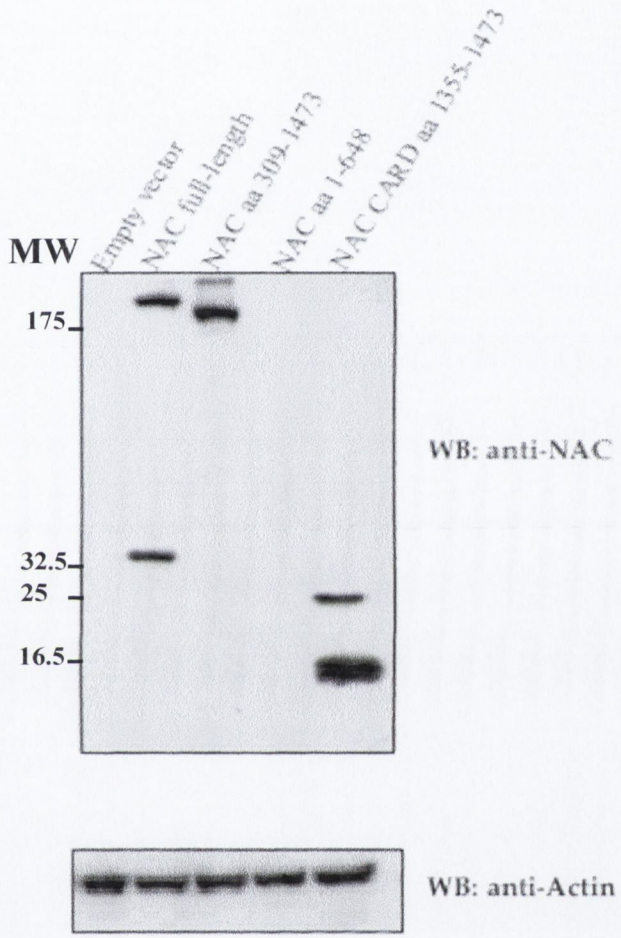


Figure 4.5:
Characterisation of anti-NAC polyclonal antibody by Western blot analysis
Cell lysates were prepared from 293T cells which had been transfected with the indicated NAC deletion constructs or empty vector. The blot was reprobed with anti-Actin to act as a loading control.

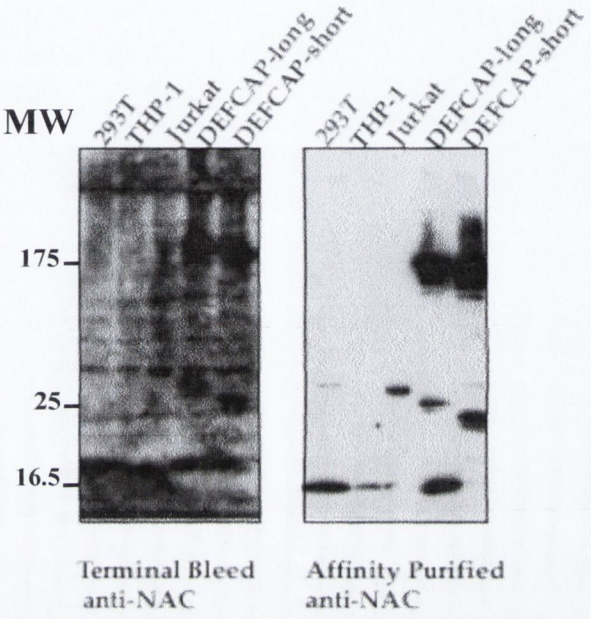


Figure 4.6:

Affinity Purification of anti-NAC antibody

Lysates were prepared from the following cell lines; Human Embryonic Kidney (HEK) 293T cells, THP-1 monocytic cells, and Jurkat T-lymphocytes. Lysates from 293T cells transfected with the long and short isoforms of DEFCAP were also assessed. Replicate blots were probed with the crude terminal bleed or the affinity purified anti-NAC antibody.

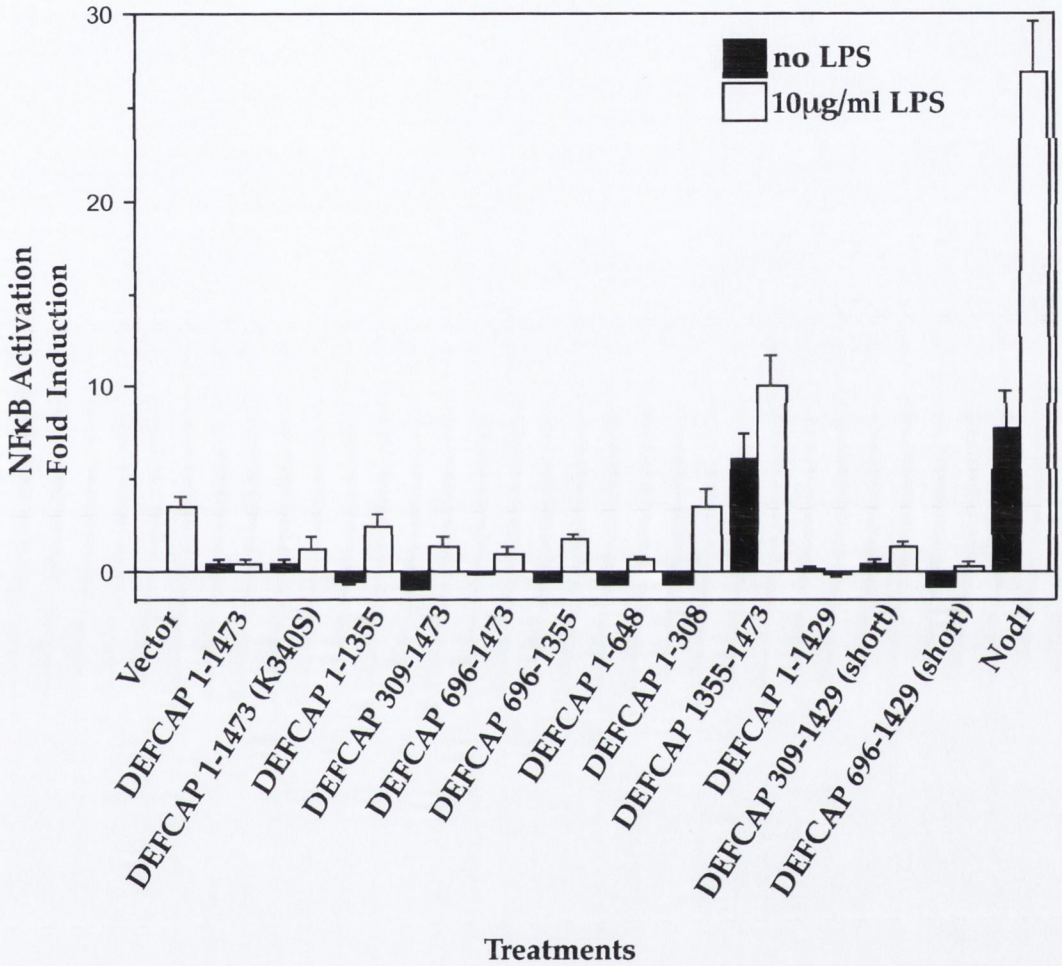


Figure 4.7:
NAC/DEFCAP fails to induce NFκB alone or in an LPS-dependent manner.
1 μg of each of the indicated plasmids was transfected along with 100 ng Luciferase reporter gene to assess NFκB activation. Relative transfection efficiency was monitored by co-transfection of 50 ng β-Gal reporter gene. LPS was added at 10 μg/ml to provide a stimulus for NFκB activation.

4.2.5 *Tissue and Cell Line Expression of ASC*

As NAC/DEFCAP did not appear to induce NFκB, to any significant extent upon overexpression in HEK 293T cells, we next decided to investigate the possibility that NAC/DEFCAP might require an adaptor molecule to recruit an effector molecule. The most likely candidate for an adaptor for NAC/DEFCAP seemed to be ASC, because of the fact that both molecules are the only CARD proteins that also contain PYRIN domains. To explore the tissue distribution of ASC a panel of human tissue lysates and transformed cell line lysates were probed with an anti-ASC mouse monoclonal antibody (Figure 4.8). The highest expression of ASC was seen in the spleen, with little or no expression of the protein in heart or skeletal muscle. This pattern mirrors the RT-PCR data on NAC RNA expression available from the Kazusa institute (www.kazusa.or.jp/huge/gfpage/KIAA0926). The high level of ASC expression in the spleen may suggest a role for this protein in the immune system as this is a site which stores large numbers of lymphocytes and antigen processing cells. Transformed cell lines showed variable expression of ASC. The human embryonic kidney cell line HEK293T had no detectable ASC expression, which may suggest developmentally regulated expression. The THP-1 and U937 monocytic cell lines showed very high levels of the protein, again indicating an association between ASC and immune related cells.

4.2.6 *ASC and NAC/DEFCAP do not synergise to promote NFκB activation*

As the domain structures of ASC and NAC/DEFCAP indicated that these molecules may have a role in immune processes, we investigated whether they could synergise to promote NFκB activation. The ability of ASC alone and DEFCAP alone to activate NFκB was first assessed. Transient expression of ASC alone did not induce significant NFκB activation (Figure 4.9). Moreover, when transfected along with each of the DEFCAP truncations there was no significant

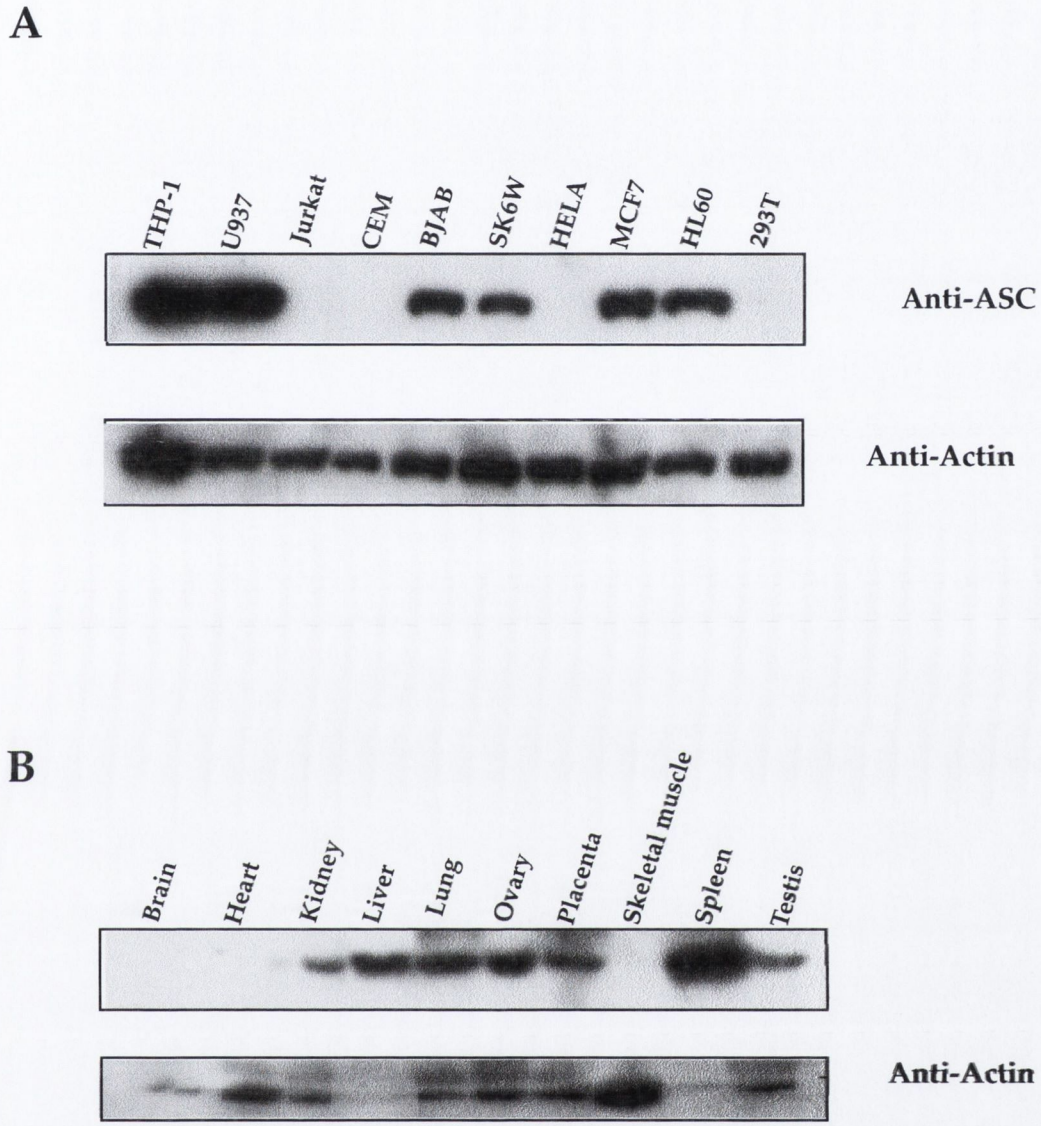


Figure 4.8:
Protein expression of ASC in transformed cell lines and normal human tissues.
(**A**) Cell lysates from panel of transformed cell lines (100 μg/lane) were analysed for ASC expression by Western blot analysis (actin was used as a loading control).
(**B**) Expression of ASC was determined by loading 50 μg of protein from normal human tissues followed by Western blot analysis (again actin was used as a loading control).

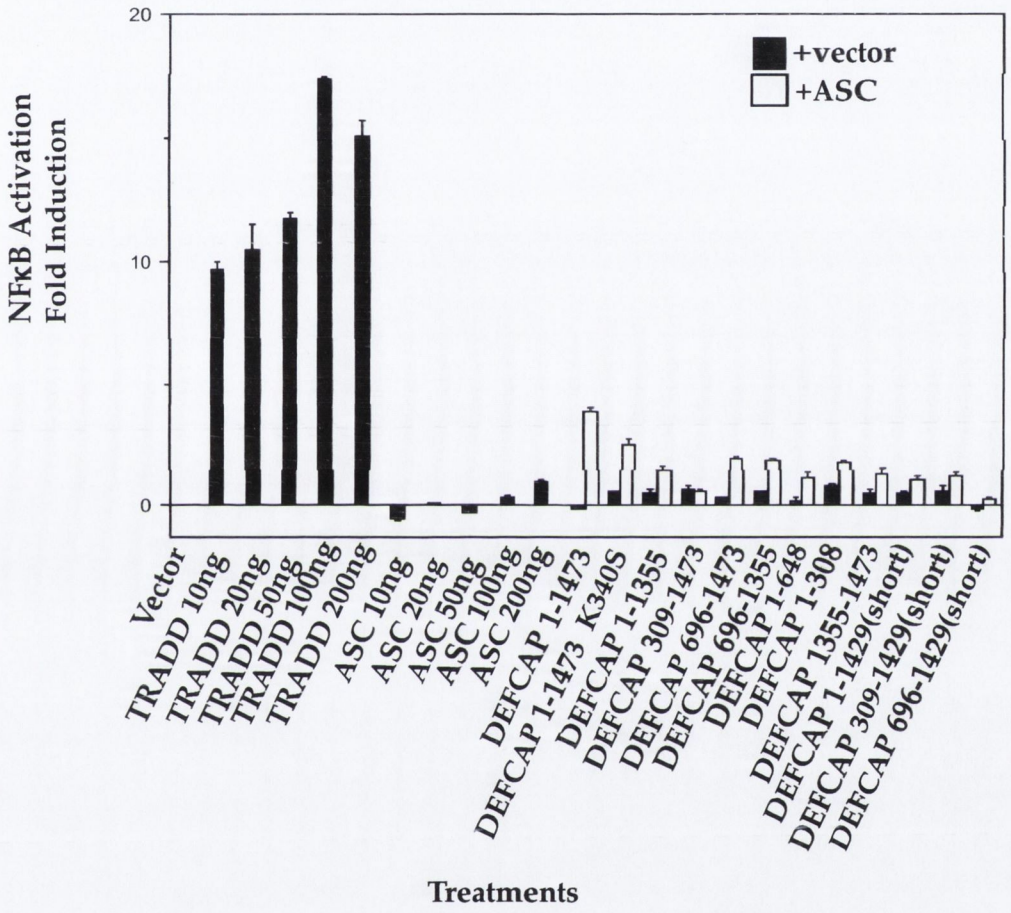


Figure 4.9:

ASC alone or in combination with DEFCAP *fails* to *induce* NFκB activity

Titrations of ASC and TRADD (a known NFκB inducer) were compared to assess the ability of ASC to induce NFκB. ASC and the panel of DEFCAP truncations were combined in a 1:1 ratio (100 ng:100 ng), to explore the possibility of synergy between the two molecules to induce NFκB activity.

increase in NFκB activation (Figure 4.9). Thus NAC and ASC are unlikely to collaborate in a pathway leading to NF□B activation.

4.2.7 *ASC and NAC/DEFCAP synergise to promote apoptosis*

While transfecting 293T cells with combinations of ASC and NAC/DEFCAP for NFκB assays, a substantial degree of apoptosis was observed in certain treatments. To explore this further we switched to the MCF7 cell line which is more amenable to analysis of cell death. This is an adherent breast carcinoma cell line which does not express caspase-3 and therefore does not undergo several of the normal apoptotic morphological changes. For this reason MCF7 cells do not detach from the plate surface when apoptosis is initiated, which allows these cells to be stained and counted more effectively than other cell lines. Using MCF7 cells it was established that the full length, long isoform of DEFCAP and ASC synergise to promote apoptosis to levels comparable to positive controls (Figure 4.10). Deletion of the CARD domain of NAC completely abolished its ability to synergise with ASC in this context. Removal of the Pyrin domain of NAC did not decrease the levels of apoptosis, in fact an increase over background death was observed in cells transfected with this region. The full- length shorter isoform of NAC did not show any synergy with ASC in assays. This may suggest that the LRR region is involved in the ability of NAC to synergise with ASC. Surprisingly the LRR-CARD region of NAC (amino acids 696-1473) constitutively promoted apoptosis. The information gained from this panel of deletion mutants suggests that the CARD region of NAC is critical for the activation of the apoptotic pathway (Figure 4.10).

4.2.8 *NAC-associated Apoptosis is ASC dependent*

At low levels of overexpression neither ASC or NAC promoted apoptosis. However, to confirm that the pro-apoptotic effect of ASC and NAC/DEFCAP was due to synergy, and not an additive effect of two moderately pro-apoptotic proteins, we titrated both to observe whether they could induce apoptosis

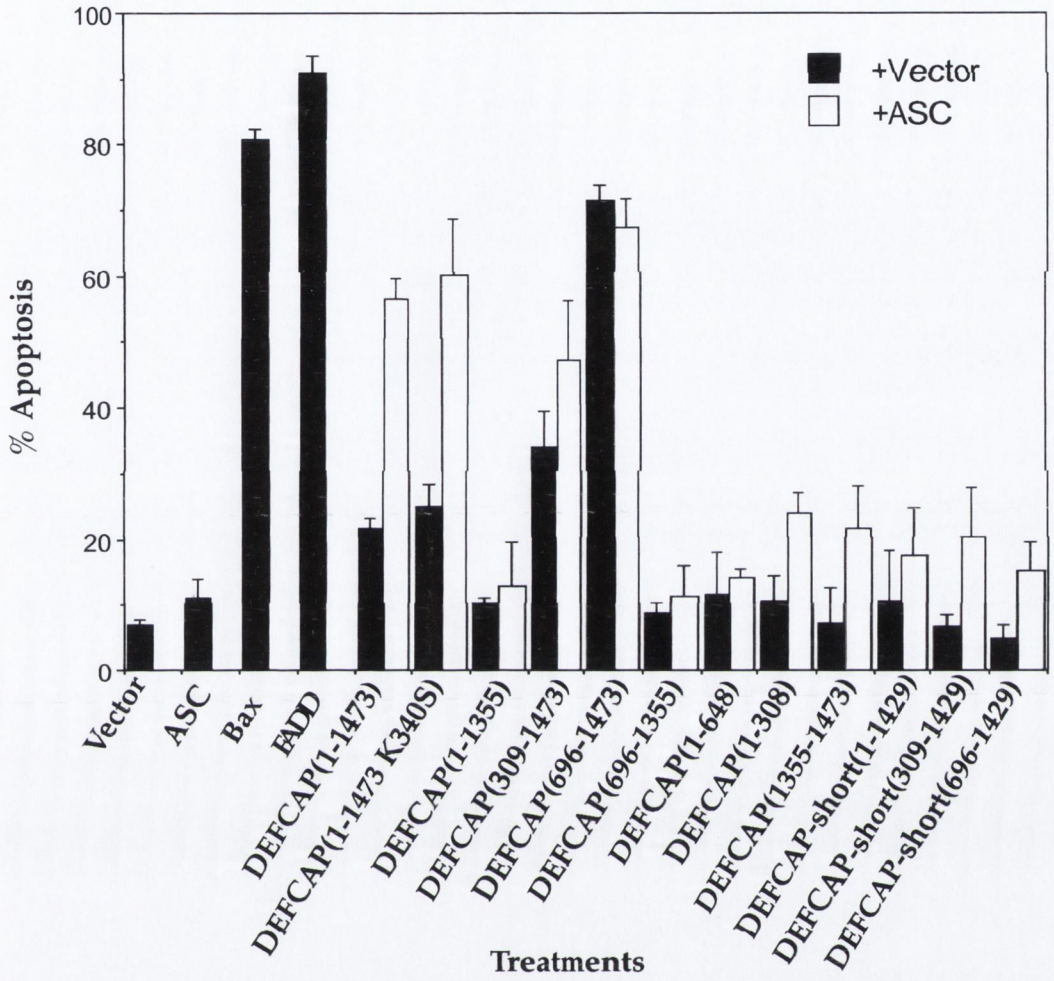


Figure 4.10:

Full length NAC/DEFCAP synergises with ASC to promote Apoptosis

100 ng of ASC , FADD and Bax and 400 ng of the DEFCAP plasmid constructs were transfected to determine their ability to promote apoptosis. ASC and the DEFCAP truncations were then combined in a ratio of 4:1 (400 ng DEFCAP:100 ng ASC) to assess any co-operation between these proteins. 50 ng of β-Gal reporter plasmid was included in each treament to determine transfection efficiency. Proteins were allowed to express for 24 h before staining with β-Gal staining buffer. Apoptosis was judged by morphological assessment of transfected cells.

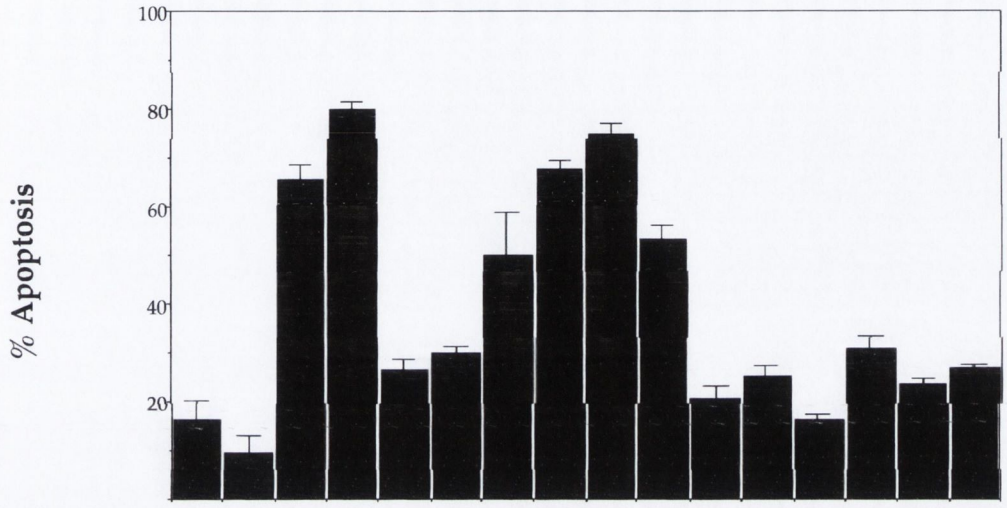
independently. We first titrated NAC alone and in combination with a fixed concentration of ASC. When transfected alone NAC was unable to kill cells even when high concentrations of plasmid were used. However when ASC was added at a fixed concentration, titration of NAC produced a corresponding increase in death (Figure 4.11).

4.2.9 ***High level overexpression of ASC is sufficient to promote apoptosis***

DEFCAP alone was unable to promote apoptosis, probably due to its requirement for an adaptor such as ASC. It has been shown, however, that the overexpression of caspase adaptors such as FADD is sufficient to promote apoptosis. Although caspase adaptor proteins may require oligomerisation for maximum activity, high concentrations can promote spontaneous aggregation of the adaptor and can exhibit activity independent of oligomerisation platforms. Thus, even though NAC may act as an oligomerisation platform for ASC, high concentrations of ASC alone may be able to oligomerise in the absence of NAC. To test this, ASC expression plasmid was titrated from 100 ng-800 ng in MCF7 cells. As shown in Figure 4.12, cells overexpressing high concentrations of ASC did indeed undergo apoptosis suggesting that ASC may indeed be the caspase-activating component of the NAC-ASC pro-apoptotic complex.

4.2.10 ***Expression of NAC with ASC, and high doses of ASC alone produce the classical apoptotic phenotype***

MCF7 cells were transfected with different plasmid combinations of NAC and ASC along with the β-Gal plasmid to confirm transfection. After staining the cells with β-Gal staining buffer containing X-Gal, transfected blue cells were assessed for viability. Cells which had received both ASC and NAC showed the classsical shrunken, rounded features typical of apoptosis (Figure 4.13). Figure 4.14 shows the dose dependent increase in apoptotic morphology with increased concentrations of ASC.



Vector (ng)	900	–	–	–	750	700	600	400	200	–	750	700	600	400	200	–
ASC	–	+	–	–	+	+	+	+	+	+	–	–	–	–	–	–
FADD	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
Bax	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
DEFCAP (ng)	–	–	–	–	50	100	200	400	600	800	50	100	200	400	600	800

Treatments

Figure 4.11:
The synergy between NAC and ASC to promote apoptosis is dose dependent
Increasing concentrations of DEFCAP/NAC alone fails to result in increase in apoptosis, however when ASC (100 ng) is present DEFCAP/NAC can promote apoptosis. 100 ng of the positive controls, FADD and Bax, were transfected where indicated. Plasmids were transfected into MCF7 cells at the indicated concentrations and allowed to express for 24 h. 50 ng of β-Gal reporter plasmid was added to each treatment to assess transfection efficiency. Cells were stained with β-Gal staining buffer and apoptosis was assessed by morphology.

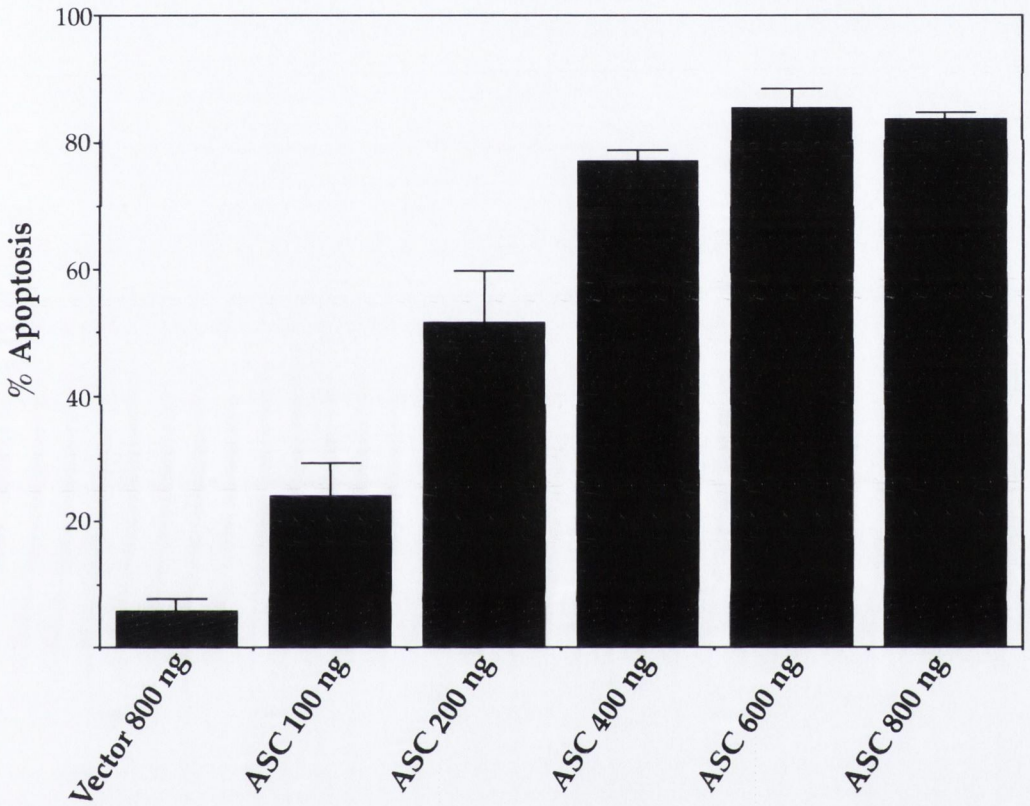


Figure 4.12:
ASC promotes apoptosis when titrated to high concentrations
MCF7 cells were transfected with the indicated amounts of ASC expression plasmid and allowed to express for 24 h. Cells were then stained and death was assessed as before.

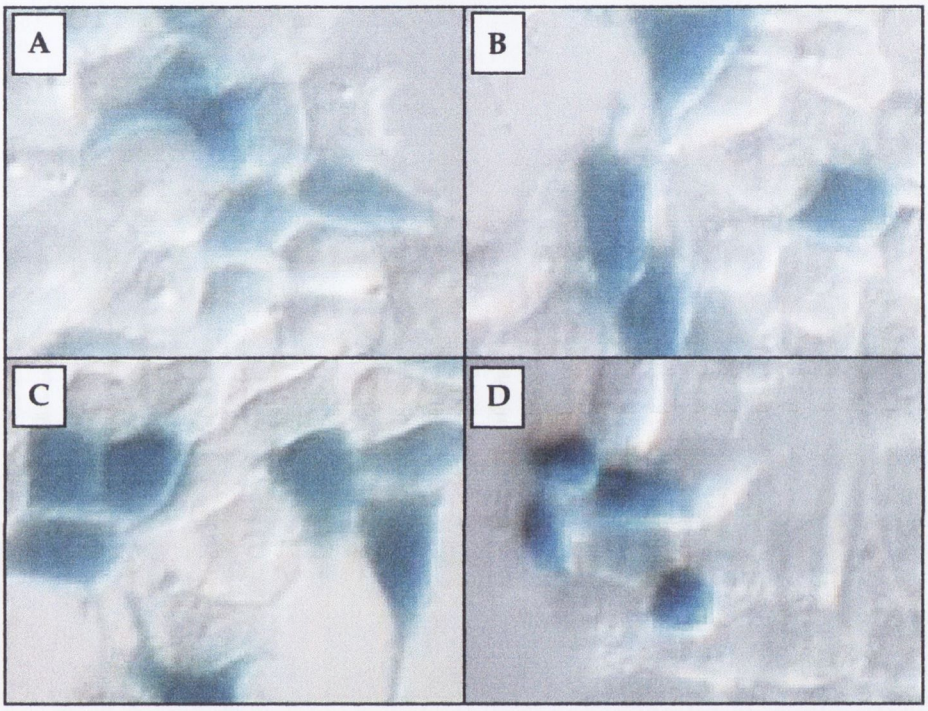


Figure 4.13:
DEFCAP/NAC and ASC synergise to promote apoptosis.
MCF7 cells transfected with (**A**) Vector (500 ng) (**B**) ASC (100 ng) (**C**) NAC/DEFCAP (400 ng) (**D**) NAC/DEFCAP (400 ng) + ASC (100 ng). Blue cells indicate the presence of β-Gal plasmid and plasmids of interest.

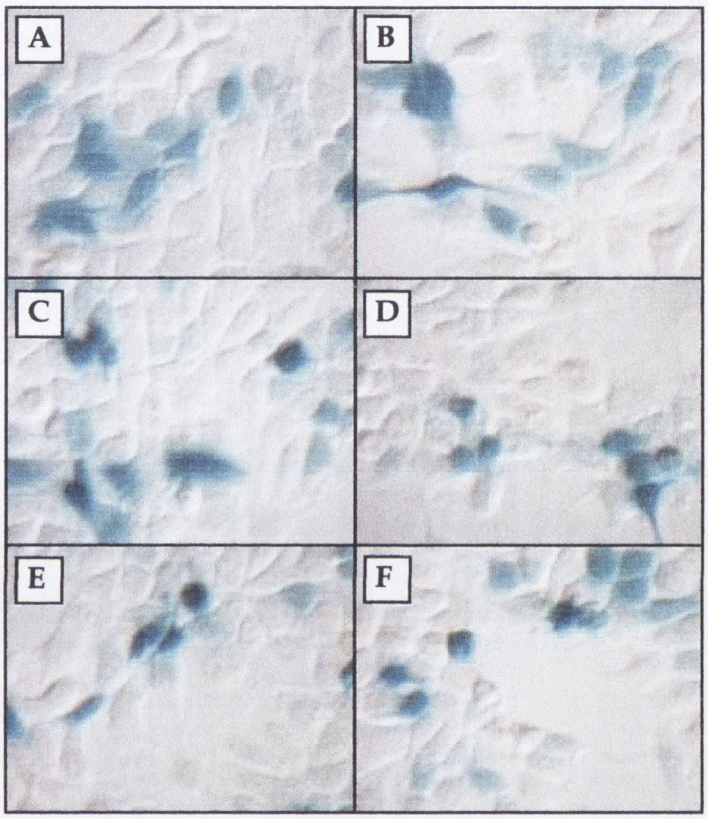


Figure 4.14:
Titration of ASC to promote Apoptosis
MCF7 cells transfected with (**A**) Vector 800 ng (**B**) ASC 100 ng (**C**) ASC 200 ng (**D**) ASC 400 ng (**E**) ASC 600 ng (**F**) ASC 800 ng
Plasmids were allowed to express for 24 h and cells were then stained with β-Gal staining buffer containing 1 mg/ml X-Gal.

4.2.11 *Full length NAC interacts with ASC*

To determine whether the synergy between NAC and ASC involves a direct interaction between these two proteins, immunoprecipitation studies were carried out. The full length NAC protein and a selection of truncations which lacked motifs predicted to participate in protein-protein interactions were chosen. Overexpression of this range of NAC deletion mutants with ASC revealed a strong interaction between full length NAC and ASC. Interestingly, NAC deletion mutants lacking the CARD or PYRIN domains were also capable of interacting with ASC suggesting that the latter may interact with NAC through CARD-CARD or PYRIN-PYRIN interactions (Figure 4.15). These results, while not identifying an exact binding interface do suggest that these molecules are capable of interacting. It is also possible that in addition to PYRIN-PYRIN and CARD-CARD interactions, PYRIN-CARD heterotypic interactions may also occur between the two molecules.

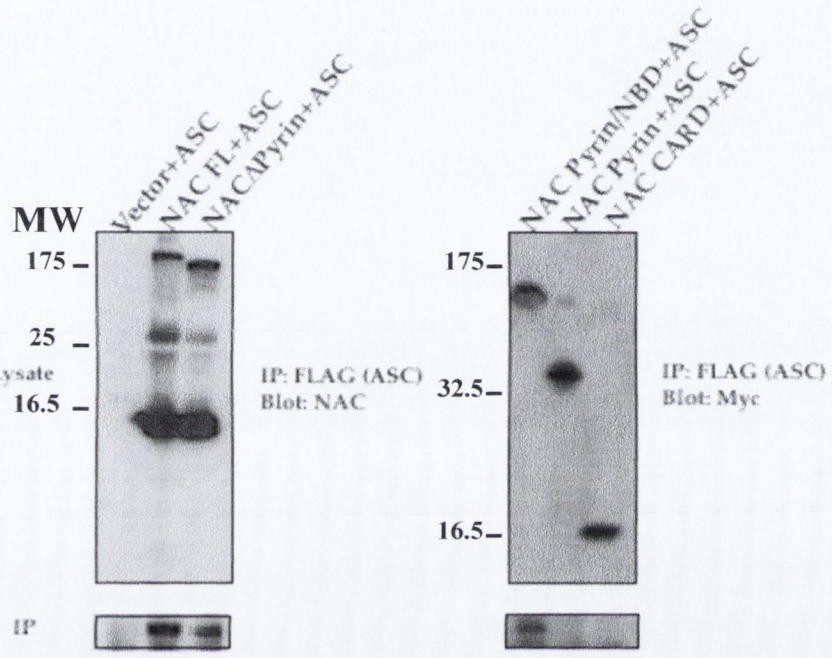


Figure 4.15:

Interaction of ASC and NAC.

293T cells (2 x10^6 per treatment) were transfected with 2.5 µg of Flag-ASC along with 5 µg of DEFCAP/NAC truncations, and left to express for 24 h. Overexpressed Flag-ASC was then immunoprecipitated using 1 µg of anti-FLAG antibody over-night.

4.3 DISCUSSION

4.3.1 *Isolation of Novel CARD proteins*

The discovery of numerous CARD-containing proteins in recent years has revealed their importance in a variety of pathways (Martin, 2001; Bouchier-Hayes, 2002). Many of the studies in this area describe the role of the CARD as a protein-protein interaction motif, which is consistent with models implicating CARDs in the formation of multimeric complexes. The large family of CARDs have been sub-divided according to their domain structures, as discussed in the introduction. This grouping together of similar structures often correlates with similarities in mode of function, thus providing a direction for investigation into the role of newly identified molecules.

Using homology searches of public databases we were able to identify novel proteins with CARD domains. This allowed us to find new potential members of apoptosis or NFκB signalling networks. We decided to use the caspase-1 pro-domain in these searches as caspase-1 contains a consensus CARD domain and adaptors for this caspase have not been described prior to this study. Searching with this domain resulted in the identification of established CARD domains in known proteins in addition to other novel proteins. Among the latter was the putative CARD protein encoded by the KIAA0926 clone deposited in the Kasuza Institute cDNA repository. This protein (NAC/DEFCAP) has a complex multi-domain structure with an N-terminal PYRIN domain and C-terminal CARD domain. The presence of both of these domains in the one molecule makes for an unusual configuration, as most proteins contain one or the other motif but rarely both.

The overall structure of NAC/DEFCAP bears striking similarities to both Apaf-1 and the Nod proteins, which are all members of the NBD-CARD family. The presence of the NBD region suggests the possibility that NAC may be capable of

forming homo-oligomers. This is a feature of the Apaf-1 apoptosome assembly, where Apaf-1 self-associates in this manner to generate the large apoptosome complex. By extension, other NBD-CARD molecules may also self-associate to generate large signalling complexes.

Typically, the occurence of stretches of leucine rich repeats (LRRs) in molecules that also contain nucleotide-binding domains is taken to implicate the molecule in recognition of pathogen components. Variability in this region is thought to correlate with the diversity seen in PAMPS. It is likely that the LRRs in NAC have a specific ligand or associated PAMP. The involvement of the Nod proteins in sensing intracellular LPS suggests that an entire range of intracellular PAMP receptors comparable with the Toll receptor family of extracellular pathogen sensors may exist.

4.3.2 *NAC does not have a role in NFκB Pathways*

The Toll and Nod protein signalling pathways converge at the point of NFκB activation. Accordingly, it seemed likely that if NAC had a similar immune-related signalling function, a downstream consequence would be the activation of NFκB. It is typical for proteins involved in the various NFκB pathways to induce activation of the latter when overexpressed (eg RIP, TRADD). This is usually as a direct result of spontaneous oligomerisation when present at high levels of expression within the cell. However, overexpressed DEFCAP/NAC was unable to induce NFκB either alone, or when cells were treated with PAMPS such as LPS. Although we did not find that LPS or a number of the most commonly available PAMPs produced any NAC or ASC-dependent NFκB activation, an exhaustive screen of all available PAMPs would be necessary to rule out a role for this molecule in immune-related signalling.

During the course of this work two papers were published concerning the identification of NAC/DEFCAP, both of which suggested a role for this molecule in promoting apoptosis. Hlaing and co-workers identified the protein as DEFCAP (Hlaing *et al.*, 2001) and found that expression of the CARD region of the molecule in cells resulted in the formation of "death effector filaments". These filamentous structures formed during apoptosis have been suggested to have a role in the recruitment and activation of caspases. Hlaing *et al.* did not however find that the full-length DEFCAP/NAC protein induced apoptosis when overexpressed in cells. Using co-immunoprecipitation methods these authors found a weak interaction between DEFCAP and caspase-9 and a strong interaction between DEFCAP and caspase-2. However the latter study did not investigate these interactions in the context of DEFCAP function. As both caspase -9 and -2 are CARD-containing caspases, it is significant to note that CARD-CARD interactions can occur as a result of overexpression of proteins that would not occur at physiological concentrations.

Reed and colleagues (Chu *et al.*, 2001) similarly did not find that full-length NAC induced apoptosis. They suggested that the role of this protein was to enhance the pro-apoptotic activity of Apaf-1. Using gel filtration methods they found that Apaf-1 and NAC co-eluted in the same fractions. They showed that stimulation of the mitochondrial apoptosis pathway could be attenuated using the CARD of NAC as a dominant negative mutant.

In agreement with both of these studies, we did not find that the full-length NAC protein promoted cell death. Positive controls such as FADD and BAX are effective in promoting apoptosis even at low concentrations. Having established that NAC/DEFCAP overexpression was not sufficient to either induce NFκB or promote apoptosis we suspected that additional elements may be required. The existence of both CARD and PYRIN motifs, presents the possibility that NAC may interact with one or more either CARD or PYRIN containing proteins. We considered likely

candidate molecules which contained suitable interaction domains. To date, only one other molecule has been identified in humans which contain both a PYRIN and a CARD domain. This protein ASC (TMS1; Masumoto *et al.*, 1999; Conway *et al.*, 2000) has a bipartite structure comparable to that found in adaptor proteins such as FADD, TRADD and Bcl-10. We postulated that association between NAC and ASC may result in initiation of one or other of the CARD-related pathways.

Initially, we investigated a possible role for NAC/DEFCAP and ASC in NFκB induction. The bipartite CARD-protein, RICK, has been shown to be capable of recruiting the IKK complex directly (Inohara *et al.*, 2000). Oligomerisation of this molecule through interaction with upstream CARD proteins is important for facilitating its recruitment role. We wondered whether ASC worked in a similar manner, possibly becoming oligomerised through binding to NAC and recruiting the IKK complex. We found that coexpression of ASC and NAC in 293T cells, failed to induce NFκB.

Recent papers have described the proteins PYPAF-1 and PYPAF-7 (Manji *et al.*, 2002; Wang *et al.*, 2002) which are homologous to NAC in their PYRIN/NBD/LRR regions but lack the CARD motif. It has been suggested that these proteins are capable of interacting with ASC to promote NFκB induction. However, the NFκB assay data in these papers lacked a number of positive controls necessary to make meaningful comparisons. However, if true, this may suggest that ASC is capable of acting as an adaptor in both NFκB and apoptotic signalling pathways. This would be analogous to FADD, which is recruited to the TNF, TRAIL and Fas receptors. Also the bipartite adaptor TRADD has been shown to be able to recruit FADD and RIP to allow the dual activity of the TNF receptor. These two examples are representative of situations where adaptors can have more than one upstream or downstream partner.

4.3.3 *NAC and ASC co-operate to induce Apoptosis*

Co-transfection studies using ASC and NAC, which were carried out as part of the NFκB assessment assays revealed that certain combinations of ASC and NAC were pro-apoptotic. Using the MCF7 cell line, it was clear that at low levels neither ASC nor NAC killed when transfected on their own. However, when combined at these low levels a substantial degree of apoptosis was seen. Using the panel of deletion mutants the regions of NAC required for this effect were established. Deletion of

the CARD region of NAC completely abolished the synergy between the two molecules, suggesting the interaction is through this region. Interestingly, the construct consisting of the LRR/CARD region was capable of promoting death constitutively and did not show any increase when ASC was added. This construct did not however have the same effect in 293T cells which do not express endogenous ASC (data not shown). The short form of NAC which lacks one LRR did not display any ability to promote apoptosis. This may reveal a crucial role for the LRR in the ability of NAC to co-operate with ASC.

To date it appears that the most common protein-protein interactions in apoptosis and NFκB induction occur between like motifs (i.e. CARD-CARD, DED-DED etc.). This paradigm would predict that interactions between NAC and ASC are likely to be either CARD-CARD or PYRIN-PYRIN. The results of the cell death assays were consistent with an interaction between the CARDs of NAC and ASC

Interestingly, the full-length NAC molecule was seen to co-immunoprecipitate with ASC suggesting a direct interaction. Furthermore, a construct lacking the PYRIN domain and one lacking the CARD also bound to ASC. This suggests either that the PYRIN and CARD domains are dispensible for interactions between NAC and ASC or that these proteins can interact through either domain. Direct interactions betweeen PYRIN domains and CARD domains have also been observed

(Masumoto *et al.*, 2001) so it is possible that this occurs in the case of NAC and ASC. Perhaps at high concentrations permutations of CARD-CARD, CARD-PYRIN or PYRIN-PYRIN interactions can occur.

More recently Tschopp and colleagues (Martinon *et al.*, 2002) have claimed that the short isoform of NAC (also referred to as NALP-1), functions as part of a caspase-1 activating complex they term the 'inflammasome'. Gel filtration analysis of THP-1 lysates were used to determine the elution patterns of NAC and ASC along with the pro-inflammatory caspases -1 and -5. Although some co-elution was seen, significant association was not evident. Martinon *et al* suggest that this is due to the transient nature of the complex formation. Moreover, only truncated versions of NALP-1 were used to produce much of the data on the "inflammasome" complex in the latter study. However, this group also showed that a dominant negative ASC molecule could inhibit processing of IL-1β through inhibition of caspase-1 processing. Another group (Srinivasula *et al.*, 2002) has also claimed that ASC functions as an adaptor for caspase-1 but contradict the claim that NALP plays a role. Both of these papers implicate ASC in caspase recruitment, and show its ability to act as an adaptor molecule

Our data suggest that the NAC and ASC can co-operate in a novel apoptosis pathway, through direct interaction. Apoptosis assays in MCF7 cells revealed that the CARD domain of NAC is critical for synergy between the proteins. In other to further characterise this complex, experiments to determine how it maybe regulated or initiated were undertaken and are described in the following chapter.

CHAPTER V

Examination of the Features of Cell Death induced by the NAC/ASC Complex

5.1 INTRODUCTION

5.1.1 *Characterisation of NAC/ ASC-induced Apoptosis*

In Chapter IV the identification of a novel apoptosis inducing complex between the PYRIN-CARD proteins ASC and NAC was described. Having established conditions where the synergy between these two molecules to promote cell death could be observed *in vivo*, the features of ASC/NAC induced apoptosis could be explored in further detail. Two main lines of investigation were followed. The first of these involved confirming that the cell death phenotype induced by the ASC/NAC complex was apoptosis, and was associated with the activation of caspases. In addition, attempts were made to determine the proximal caspase in the apoptotic pathway induced by the ASC/NAC complex. Secondly, the trigger for ASC/NAC complex formation and apoptosis was explored.

Because the bipartite protein ASC was capable of inducing apoptosis alone when expressed at high concentrations, it seemed likely that this protein was responsible for directly recruiting a caspase. Although the activation pattern of downstream caspases is conserved between the different apoptosis pathways, the proximal caspase differs, depending on the stimulus. At this time, only two members of the initiator group of caspases are known to initiate a caspase cascade *in vivo*, caspase-8 and caspase-9. The role of these, as well as other initiator caspases in ASC/NAC induced apoptosis, are examined in this chapter.

As discussed in the introduction, there are numerous stimuli which can promote apoptosis, from the activation of membrane receptors to the disruption of organelle function. In an attempt to place activation of the ASC/NAC complex downstream of an apoptotic stimulus, the effect that removal of ASC had on a wide range of pro-apoptotic stimuli was examined. This was done by using a gene knockdown strategy known as RNA interference (RNAi) (Dykxhoorn, 2003).

5.1.2 *RNA Interference*

RNA interference is a phenomenon that was originally identified in *C. elegans*, where introduction of double stranded RNA (dsRNA) initiates the silencing of genes containing a region homologous to the RNA (Fire *et al.*, 1998). For nematodes, techniques to introduce the dsRNA include; injection, soaking in an RNA solution and feeding the worm on bacteria expressing the RNA. Shortly after its discovery in *C.elegans*, similar phenomena in other organisms such as *Drosophila* were confirmed (Boutla, 2001). A strategy has now been developed to induce RNAi in mammalian cells (Elbashir *et al.*, 2001).

Two models have been proposed to explain how RNAi may work by examining the process in *Drosophila* and *C. elegans* (Ramaswamy and Slack, 2002). These are the endonucleolytic cleavage model and the random degradative PCR model. In the first of these models, it is proposed that siRNAs complex with other proteins in the cell to form the RNA-induced silencing complex or RISC (NyKanen *et al.*, 2001). The dsRNA is then unwound in an ATP-dependent manner by the RISC. The single stranded RNA then acts as a guide, binding homologous endogenous mRNA. After binding, the target is cleaved by an endonuclease within the cell thus destroying the specific mRNA species. In the second model, it is suggested that the siRNA acts in the same manner as a PCR primer (Tijsterman *et al.*, 2002). Binding of the siRNA to its homologous target is followed by extension of the siRNA by an RNA-directed RNA polymerase. This results in production of a long dsRNA molecule. This is then cleaved by an RNAse enzyme, Dicer, into smaller ineffective fragments.

In the mammalian system there is an added complexity. Doubled stranded RNA in the cytosol can trigger an anti-viral, 'interferon response' if the oligos are longer than 30bp. In mammalian cells, dsRNA of 21bp (short interfering RNAs, siRNA) have been demonstrated to mediate specific silencing of endogenous genes

(Elbashir *et al.*, 2001). This chapter describes experiments using a number of 21bp siRNA oligos targeted toward ASC, alongside a number of control oligos. These experiments reveal a previously unreported effect of siRNA which has important implications for the use of such oligos in cell death studies.

5.2 RESULTS

5.2.1 NAC/ASC coexpression induces the formation of ASC Specks associated with Apoptosis

ASC was originally identified as an insoluble protein within HL60 cells which had undergone apoptosis as a result of treatment with a range of cytotoxic drugs (Masumoto *et al.*, 1999). ASC was observed to form large aggregates or 'specks' in these cells following exposure to cytotoxic agents. To explore the effect of NAC on cellular distribution of ASC, exogenous ASC was initially expressed in Hela cells. In all cells expressing ASC, a common diffuse cytoplamic distribution pattern was observed and the cells appeared healthy. However, at the same concentrations of ASC, co-expression of NAC induced the reorganisation of ASC into 'Specks' (Figure 5.1). The presence of these ASC aggregates coincided with the cells taking on an apoptotic morphology. It is also possible that the relocalisation of ASC into 'Specks' is downstream of ASC/NAC induced apoptosis and that the formation of aggregates by ASC is associated with apoptosis generally and not required as part of apoptosis induction.

5.2.2 ASC/NAC induced cell death is Caspase-dependent

To explore the events occurring in cells during death induced by ASC/NAC co-expression, the effects of a variety of caspase-inhibitors were investigated (Figure 5.2). MCF7 cells were co-transfected with ASC and NAC along with plasmids encoding various inhibitors of apoptosis were. Cell death induced by ASC/NAC or Bax was inhibited by the synthetic broad-spectrum caspase inhibitor *z-VAD* at a concentration of 50 μM. This confirmed that NAC/ASC-induced cell death was caspase-dependent. More specifically NAC/ASC- induced death was also inhibited by p35 which targets a narrower range of caspases, being particularly effective against caspase-8 but not effective for inhibition of caspase-9 *in vitro* (Zhou *et al.*, 1998). The inhibitor of apoptosis protein XIAP which is specific for caspases –3, -7 and –9 was also capable of inhibiting apoptosis induced by NAC/ASC (Figure 5.2).

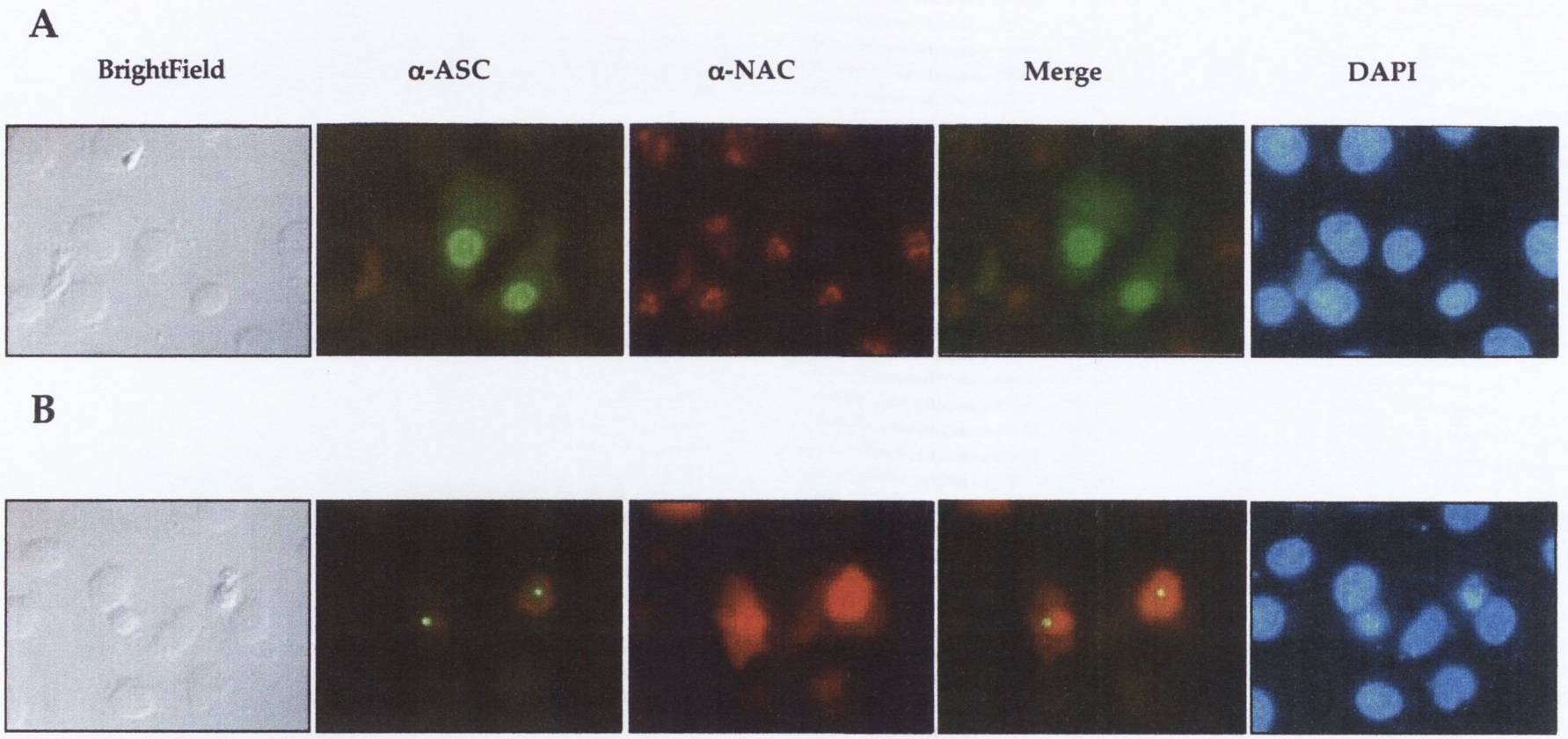


Figure 5.1
NAC induces relocalisation of ASC into Specks associated with cell death:
Hela cells which do not express endogenous ASC were transiently transfected for 24h with 100 ng of ASC expression plasmid alone (**A**) or in combination with 400 ng of NAC -endoding plasmid (**B**). When expressed alone ASC shows a diffuse expression pattern, localising throughout the cytoplasm and nucleus. In cells where NAC is co-expressed (**B**) ASC relocalises to a discrete 'speck' and the cells become apoptotic. DAPI (Hoechst) staining shows condensed nuclei typical of cells undergoing apoptosis. ASC expression was detected using a mouse anti-ASC primary antibody and an anti-mouse FITC coupled secondary antibody. NAC expression was detected using a rabbit anti-NAC polyclonal primary antibody and a Rhodamine coupled secondary antibody.

5.2.3 ASC/NAC induced death is not inhibited by Dominant Negative Caspase-9

An active site mutant of caspase-9, which acts as a dominant negative, was capable of inhibiting BAX induced apoptosis. However, this mutant failed to prevent ASC/NAC induced death. This may indicate that caspase-9 is not proximally activated in this pathway (Figure 5.2).

5.2.4 ASC/NAC-induced Apoptosis promotes Caspase-processing

Cell death induced by ASC/NAC was accompanied by the processing of endogenous caspases similar to that seen with apoptosis induced by the Bcl-2 family protein Bax. Cleavage products of caspases -2, -3, -7, and -8 were found to have similar patterns and intensity in lysates of 293T cells transfected with plasmids encoding Bax or ASC/NAC. Interestingly, no caspase-9 processing could be detected under these conditions so no comparisons could be made (Figure 5.3).

5.2.5 Active Caspases are present in lysates from ASC/NAC transfected cells

To ensure that the caspase processing observed in lysates of 293T cells overexpressing ASC and NAC produced functional enzymes, these lysates were assayed for activity. Using the hydrolysis of synthetic caspase substrates as a measure of catalytic activity, caspase -3, -8 and -9 activity was detected. Cells were transfected with the indicated plasmids and allowed to express the proteins for 24 h. Cells were then lysed and assayed using the relevant substrates (see materials and methods for details). Lysates from Bax and ASC/NAC transfected cells showed levels of substrate cleavage substantially greater than the control lysates (Figure 5.4 A-C).

Attempts were made to identify a proximal caspase activated by the ASC/NAC complex during apoptosis by immunoprecipitating (IP) ASC and looking for co-immunoprecipitaion of endogenous caspases. This was done initially with caspase-1 as it had been reported that ASC was an adaptor for this caspase. After extensive

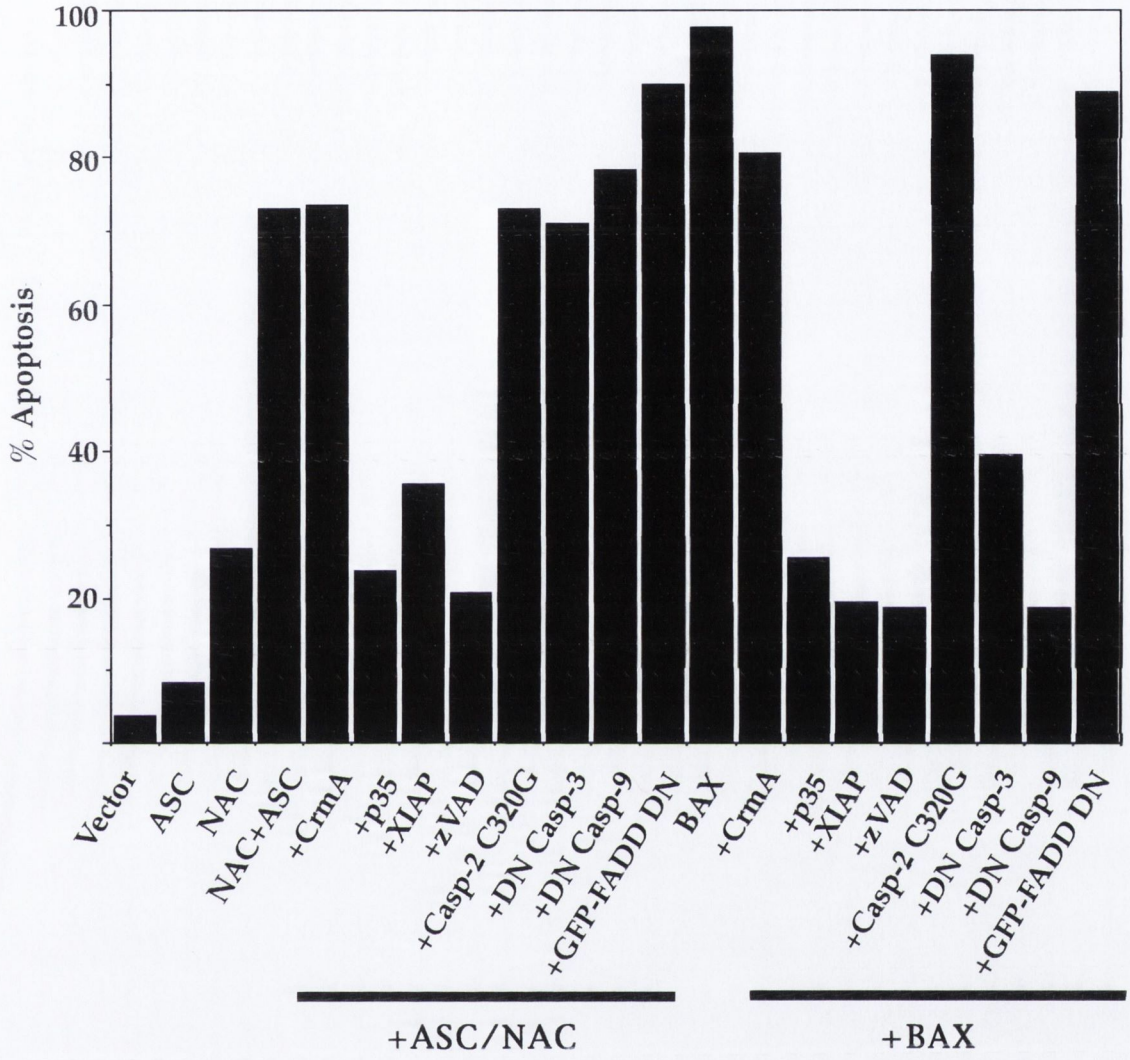


Figure 5.2

Investigation of the effect of caspase inhibitors on ASC/NAC -induced apoptosis: MCF7 cells were co-transfected with 100 ng ASC + 400 ng NAC along with 1 μg of each of the inhibitors indicated. 50 ng of a B-Gal reporter plasmid was included to allow assessment of transfection efficiency. Cells were allowed to express the p roteins for 24 hrs, and then were scored for apoptosis, by morphology. 200 ng of the pro-apoptotic protein Bax was used as a positive control.

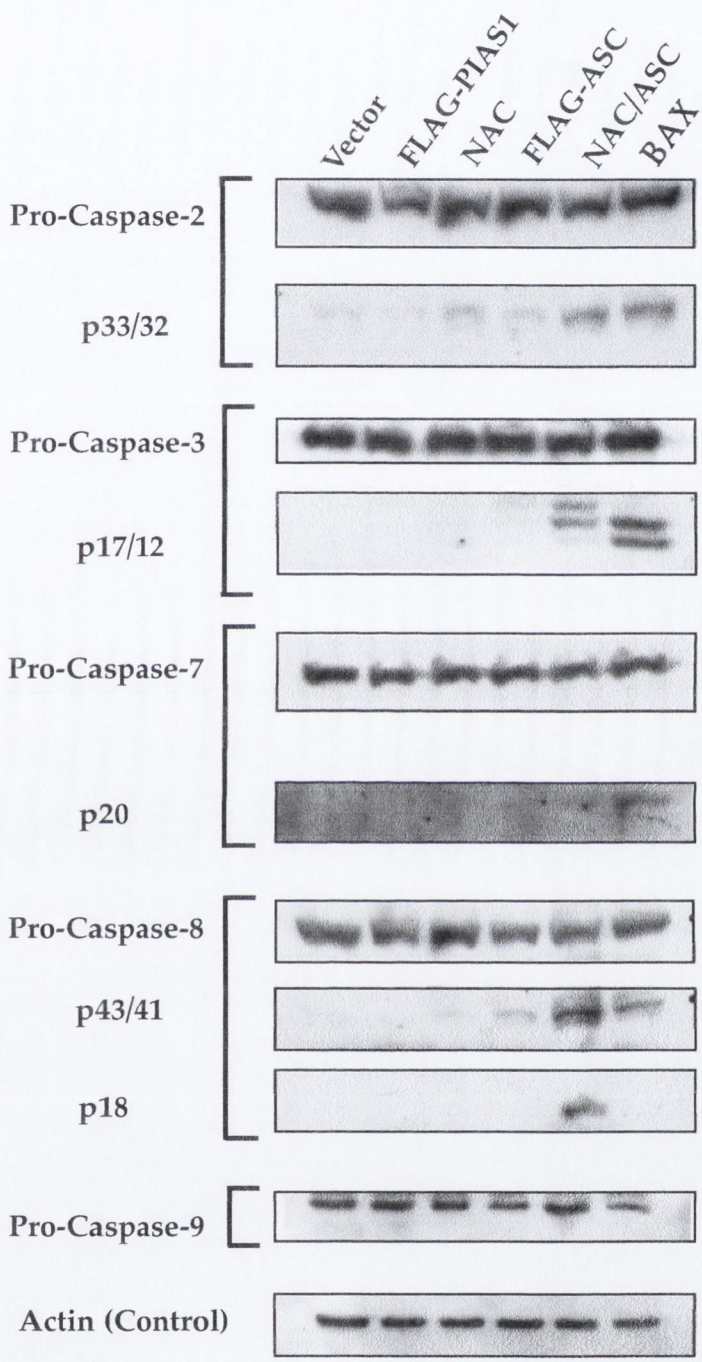


Figure 5.3

Processing of Endogenous Caspases by ASC/NAC.

293T cells were transfected with the indicated plasmids at the following concentrations; 11.25 µg Vector, 2.25 µg PIAS1 (control) 2.25 µg ASC, 9 µg NAC, or 4.5 µg Bax. Cells were allowed to express the proteins for 24 h and were lysed in standard lysis buffer. Samples were electrophoresed and examined for caspase processing by Western blot.

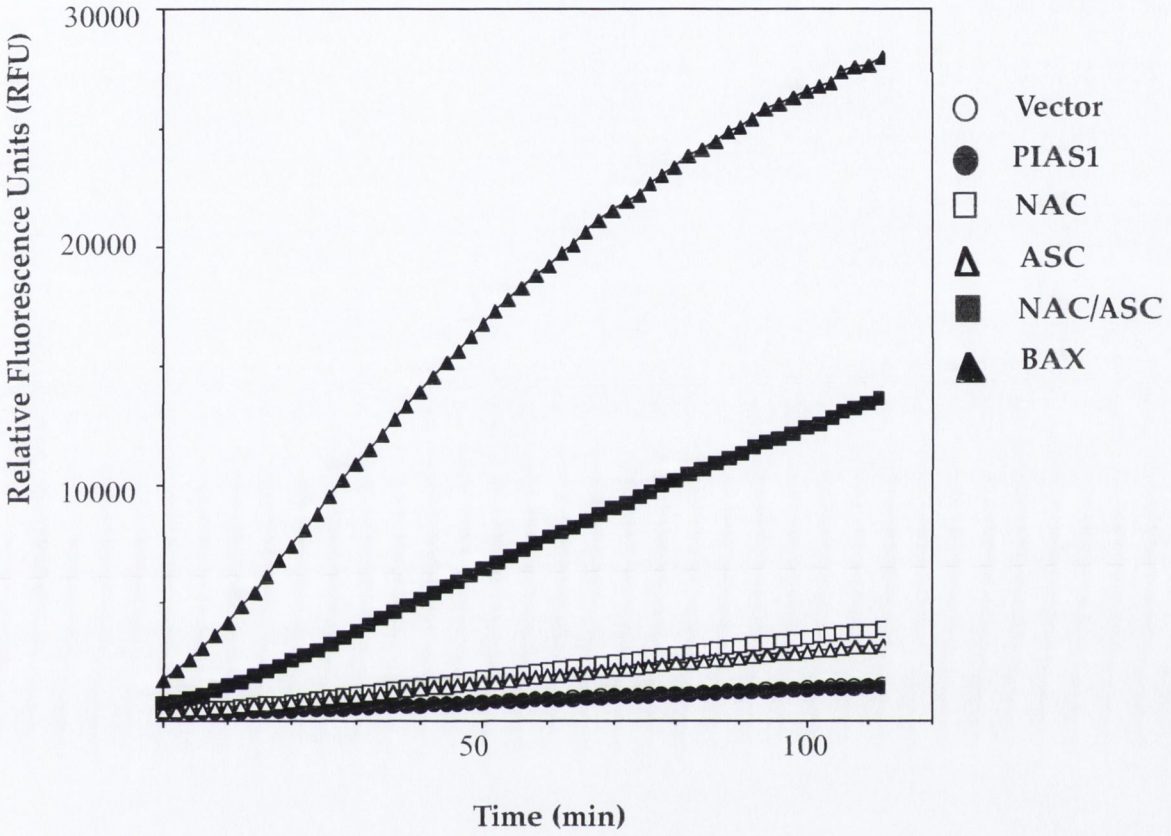


Figure 5.4 A

Hydrolysis of the Fluorometric Substrate DEVD-AFC:

Cleavage of the AC-DEVD-AFC peptide motif is used as an indication of caspase-3 or -7 activity in the lysate. Human embryonic kidney cell line (293T) were transfected with the indicated plasmids and left to express the proteins for 24 h. Samples of each lysate were added to buffer containing the substrate. Hydrolysis of the substrate results in the relese of fluorescence. Bax and NAC/ASC show significant amounts of activity above control lysates.

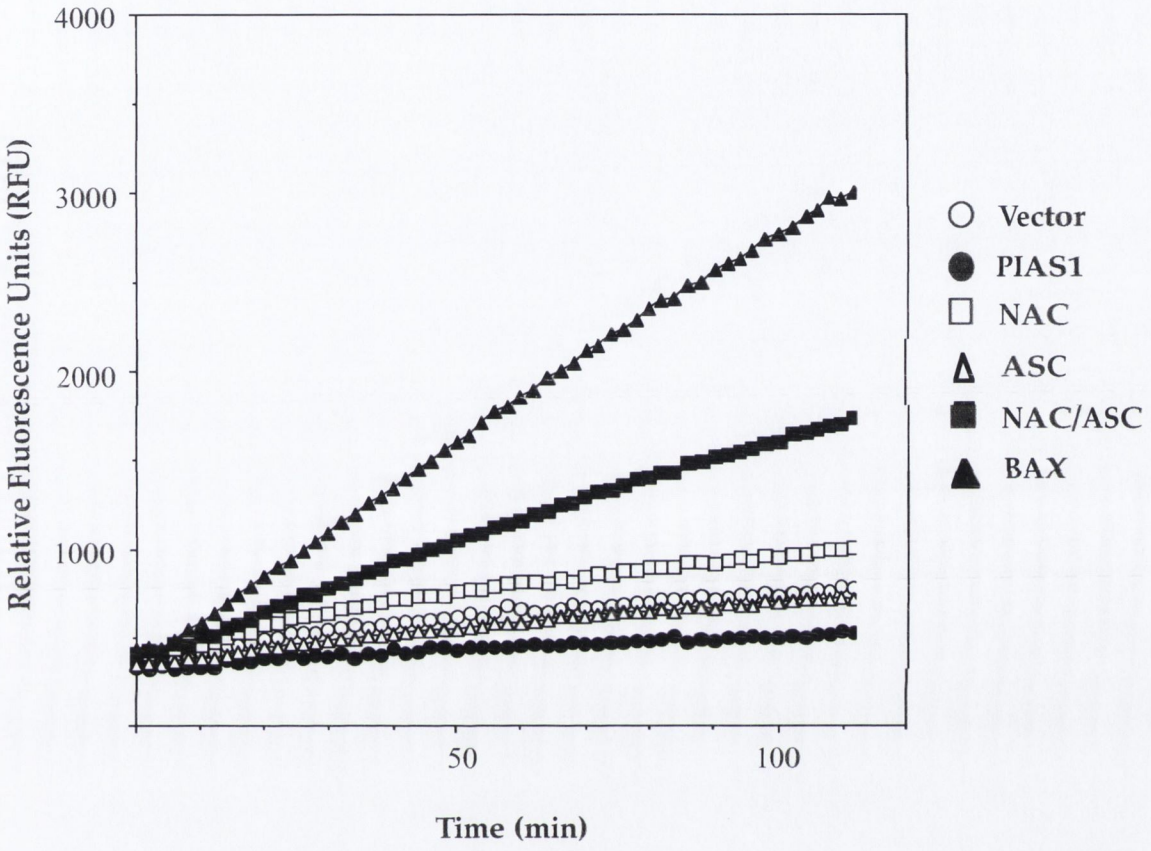


Figure 5.4 B

Hydrolysis of the Fluorometric Substrate LEHD-AFC:

LEHD hydrolysis is achieved most efficiently by active caspase-9. Again lysates from both Bax and ASC/NAC transfected cells show significantly higher levels of activity than the control lysates.

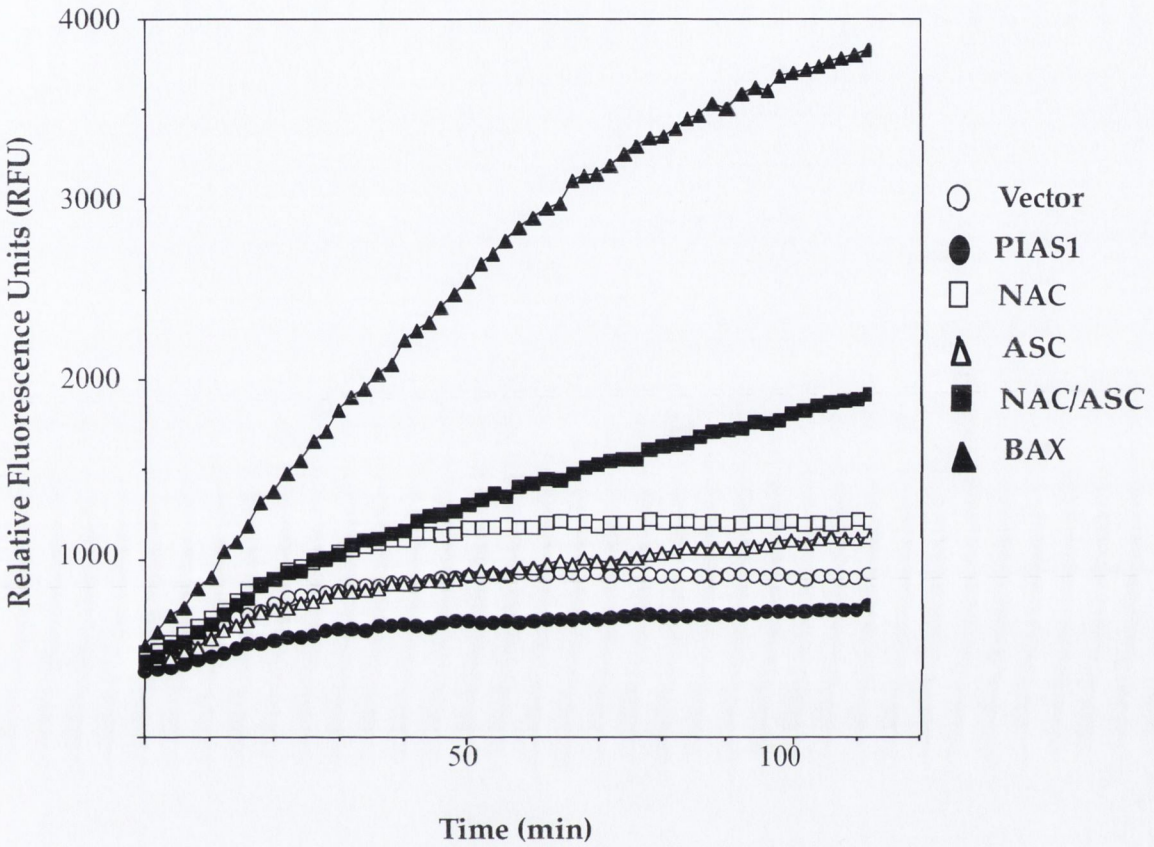


Figure 5.4 C

Hydrolysis of the Fluorometric Substrate IETD-AFC:

IETD-AFC hydrolysis is used to measure caspase-8 activity. Bax shows a significant increase on contol lysates for this enzymatic activity whereas ASC/NAC is higher than the controls but significantly lower than the Bax lysate. The activity in the NAC/ASC lysates however, looks to be still increasing so may have shown a more significant increase at later timepoint.

attempts to assess binding between ASC and caspase-1 both through overexpression and immunoprecipitation, no evidence could be found for this interaction (data not shown). The immunoprecipitation of ASC was optimised by performing large scale transfections with increased amounts of IP antibody. While this greatly improved the IP of ASC, in relative terms it was still low. No co-immunoprecipitation of caspases -2, -8 or -9 could be detected (data not shown), but this was inconclusive because of the suboptimal IP conditions. Therefore, it remains unclear whether ASC is capable of directly recruiting a caspase.

5.2.6 *Design of ASC siRNA oligos*

Short double stranded RNA oligonucleotides, 21bp in length have been shown to be able to specifically downregulate expression of genes to which they are homologous. This approach was used to ablate expression of ASC, with a view to assessing the effect of ASC knockdown on apoptosis induced by a range of stimuli. Two siRNAs, homologous to different regions of ASC were designed using the two different strategies commonly used for creating efficient siRNA oligonucleotides. These strategies are known as the AA based and the AA/G(N17) C based methods and were devised by the Tuschl lab (Elbashir *et al.*, 2001). Briefly, for the AA method, a region of the gene approximately 75 bp downstream of the start codon is chosen and an AA dimer in close proximity is selected. The 19 bases directly downstream of this doublet are recorded and a 3′ overhang (UU or dTdT is added). The second method is principally the same with the added feature that the 19 bases should begin with a G and end with a C. Oligonucleotides targeted to ASC at base pair positions 213 and 522 were designed using the above methods. Additional siRNA oliognucleotides were designed against other target mRNAs to serve as controls.

5.2.7 *Knockdown of ASC using siRNA shows variable efficiency*

Ideally the effect of knockdown of ASC on apoptotic stimuli would be assessed using suspension cells which can be analysed by flow cytometry. This would provide a means of counting large numbers of cells giving a more accurate reflection of events. However although a number of methods (lipid-based transfection reagents and multiple electroporation protocols) were tried to introduce siRNA oligos into the THP-1 suspension cell line, no knockdown was achieved. As yet no effective means of attaining knockdown in suspension cells has been reported.

MCF7 cells, which express high levels of ASC and are used frequently for cell death assays, were chosen for examining the effect of ASC knockdown on apoptosis induced by a range of stimuli. To first assess the ability of the oligos to knock down expression of ASC, cells were transfected with 200 nM of each siRNA and incubated for 48 h to permit knockdown to occur. Lysates were then made and assessed for expression of ASC, Figure 5.5. The efficiency of knockdown between experiments differed greatly.

5.2.8 *siRNA oligos induce non-specific sensitisation to apoptotic stimuli*

The effect of ASC knockdown on apoptosis induced by diverse pro-apoptotic stimuli was then explored. MCF7 cells were treated with 200 nM of each oligonucleotide and incubated for 48 h. The cells were then reseeded in 6-well plates and incubated overnight. The next day cells were treated with TNF, Daunorubicin, Actinomycin D or were left untreated. Cells which were not treated with a cytotoxic drug appeared healthy with no discernable difference between any of the siRNA treatments.

After exposure to siRNAs, cells were incubated for an appropriate amount of time, in the presence of the various stimuli, to allow approximately 50% death in each of

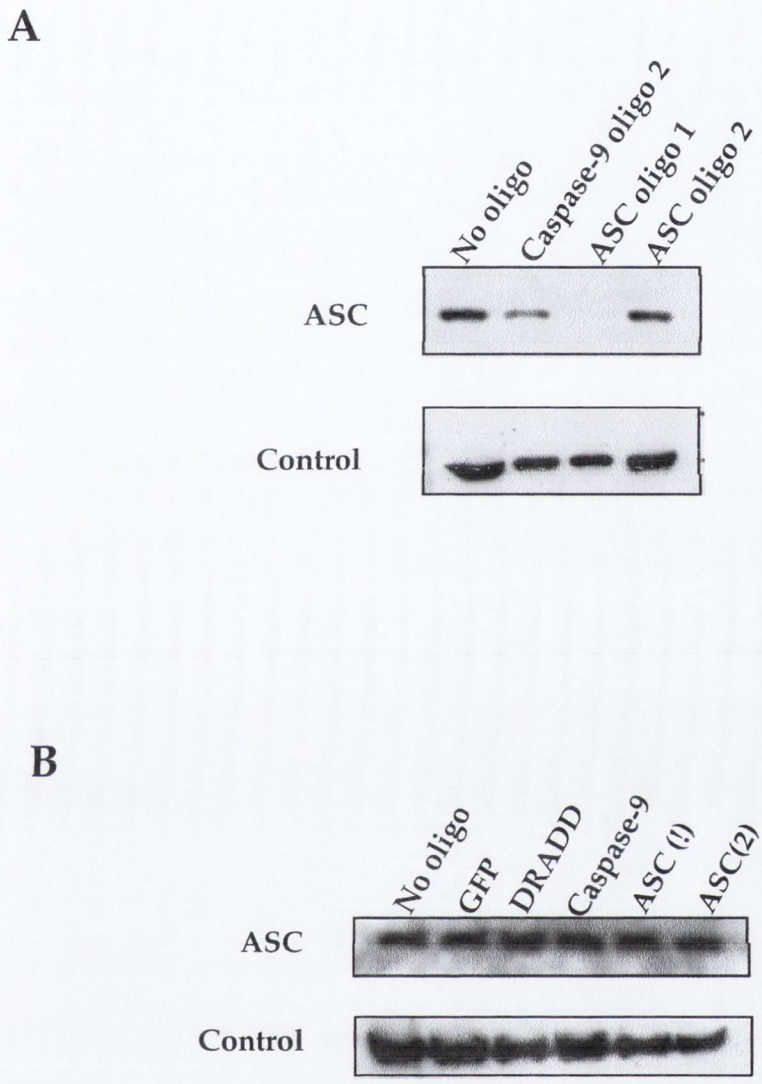


Figure 5.5

Knockdown of ASC expression by siRNA oligos shows high degree of variability

MCF7 breast carcinoma cells which have a high level of endogenous ASC and are also suitable for death assays were chosen for ASC knockdown studies. An example of the most successful results is shown in (**A**). Cells treated with both oligos were lysed after 48 h and assessed for ASC expression. In this case ASC oligo 1 had successfully decreased the expression of ASC.. (**B**)This panel shows the most frequent result obtained with the ASC oligos, where expression has not been decreased.

the treatments. At these timepoints a dramatic increase in cytotoxicity was observed in the cultures which had been treated with ASC oligo 1 and the GFP oligo. Cells within the latter cultures had undergone huge levels of apoptosis with the majority of cells having detached from the plate (Figure 5.6 A-D)). Figure 5.7 A-D show the graphical representation of the extent of death in these experiments. Due to the large scale of detachment of cells in the ASC1 and GFP oligo-treated wells, the numerical representation is an underestimate of the death seen in these treatments. This effect was repeated a number of times, and was not dependent on successful knockdown of ASC as determined by Western blot analysis.

Alignments of the ASC and GFP oligos did not reveal any significant similarity which could account for their shared ability to sensitize cells to cytotoxic stimuli. Furthermore, siRNA oligos targeted against should clearly have no effect as this gene is not expressed in mammalian cells. Clearly the sensitization to apoptosis observed with certain siRNAs was non-specific and is unrelated to specific knockdown of the target gene. These observations have important implications for studies employing the siRNA strategy to study apoptosis.

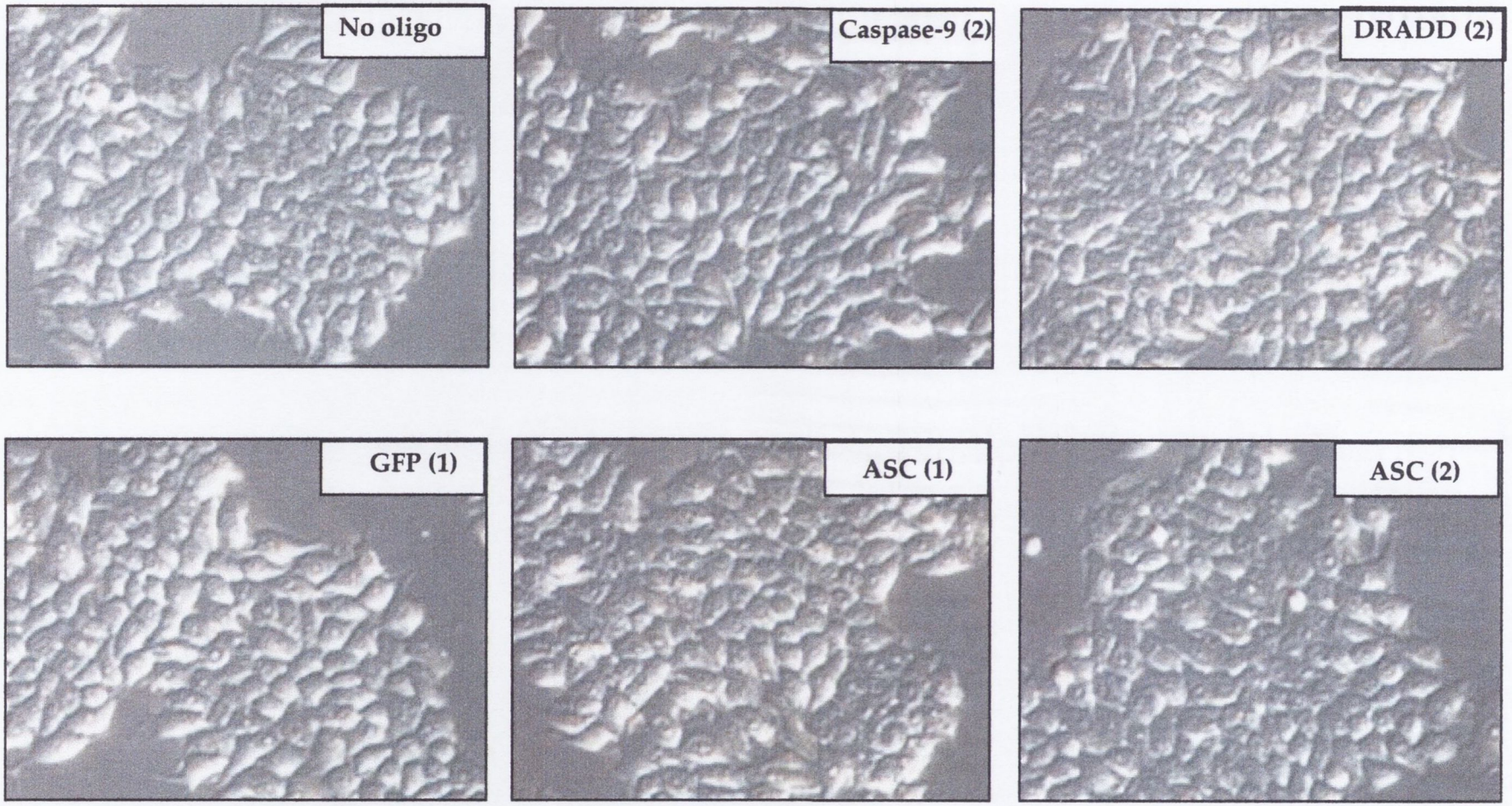


Figure 5.6 A

Cells treated with a range of oligos appear healthy:

MCF7 cells were transfected with 200 nm of each siRNA oligo and cultured for 48 h before reseeding. This montage shows the appearance of the cells after reseeding with no cytotoxic drug treatment.

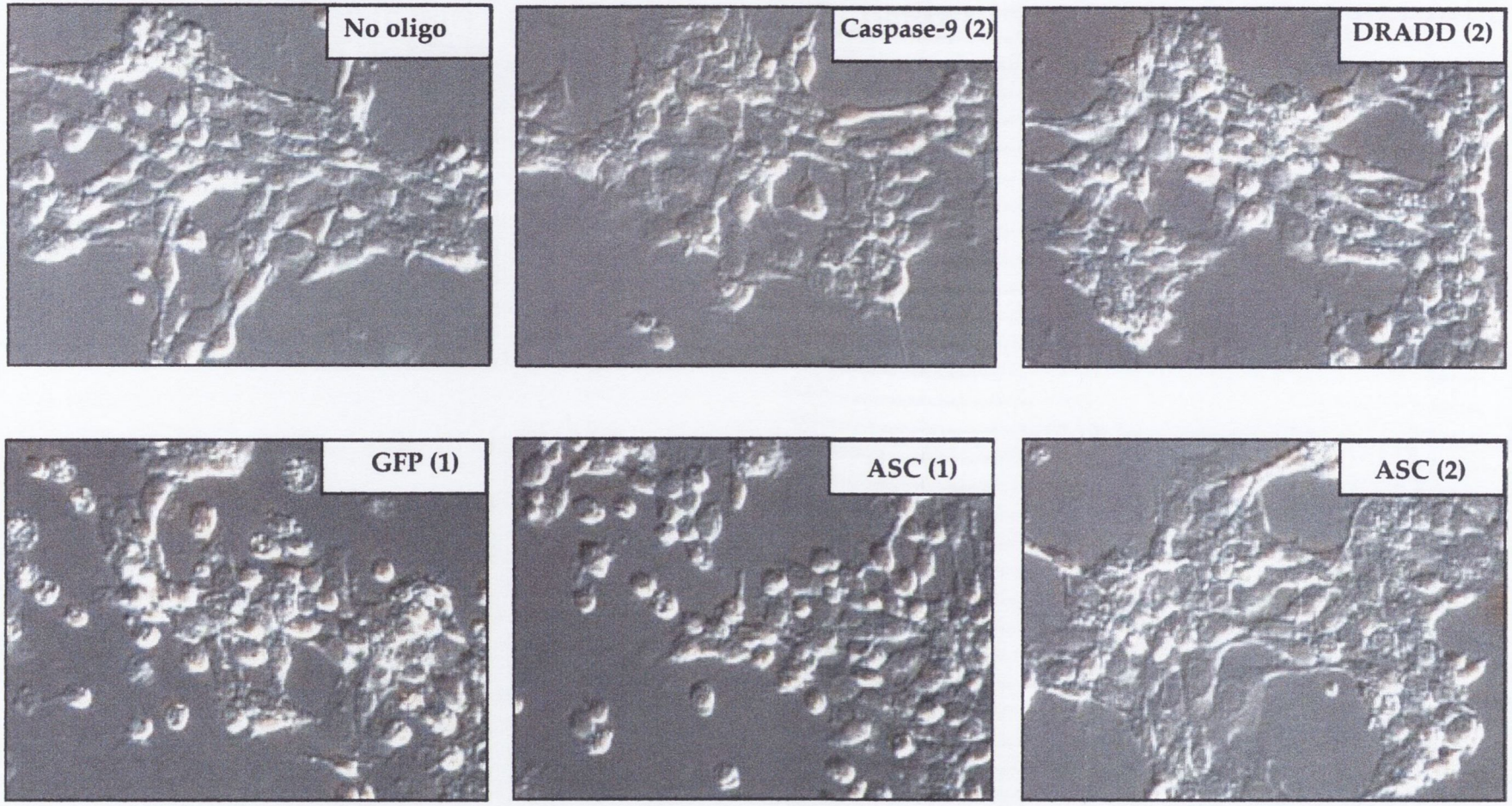


Figure 5.6 B

Cells treated with siRNA oligos targeted against ASC(1) and GFP oligo(1) show increased sensitivity to Actinomycin D. MCF7 cells were transfected with 200 nM of each siRNA oligo and were incubated for 48 h before reseeding. This montage shows the cells after treatment with 5 μM Actinomycin D for 20 h. Cells which received the oligos GFP(1) and ASC(1) show significantly higher levels of death than cells which were transfected with the other oligos.

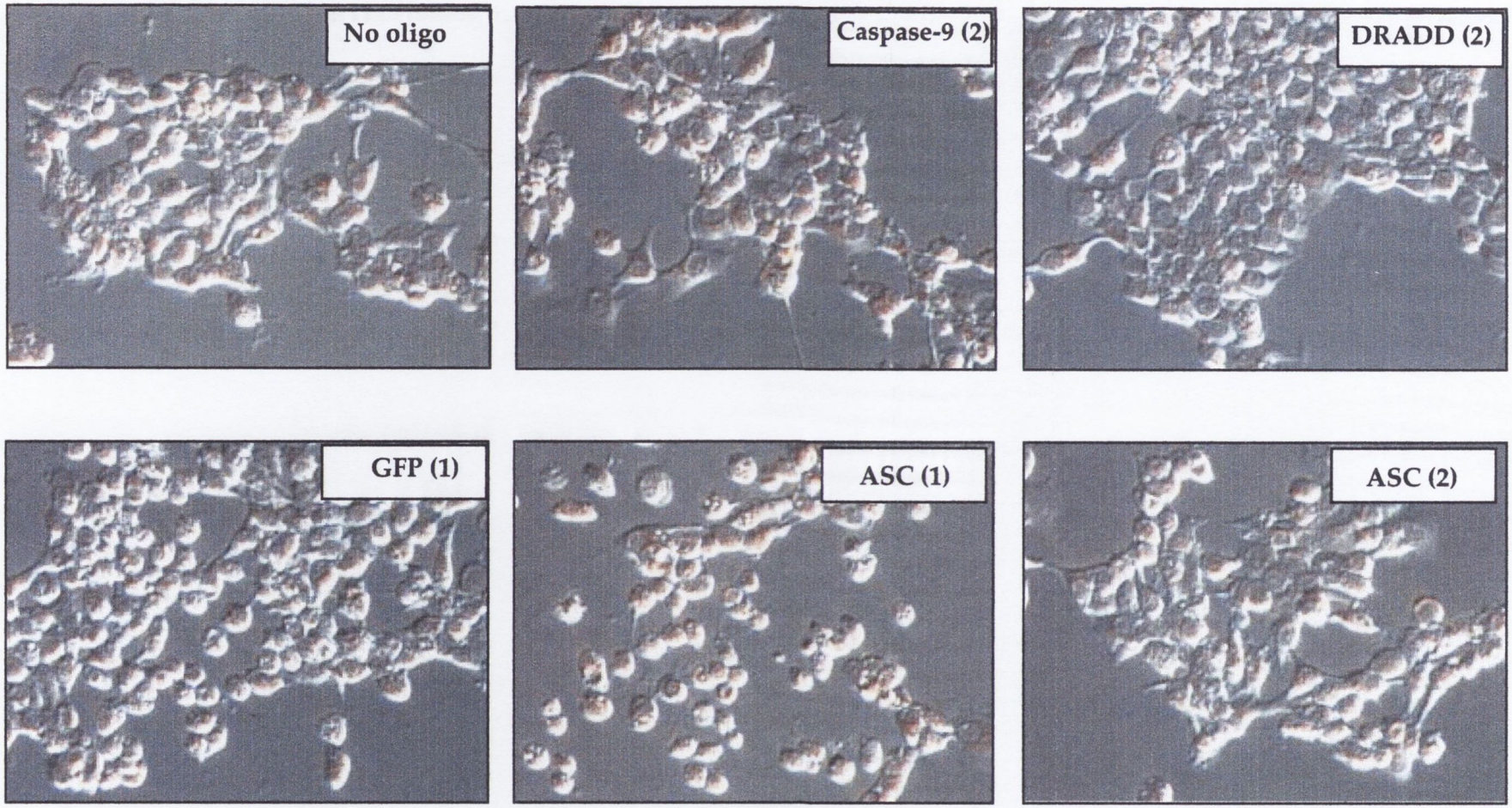


Figure 5.6 C

Cells treated with siRNA oligos ASC(1) and GFP oligo(1) show increased sensitivity to Daunorubicin:

Cells were transfected with oligos and reseeded. 24 h after reseeding the cells were treated with 10 μM Daunorubicin. After incubation with this cytotoxic agent for 20 h the apoptosis induced in the GFP(1) and ASC(1) wells was observed to be much greater than that seen in the other oligo treatments.

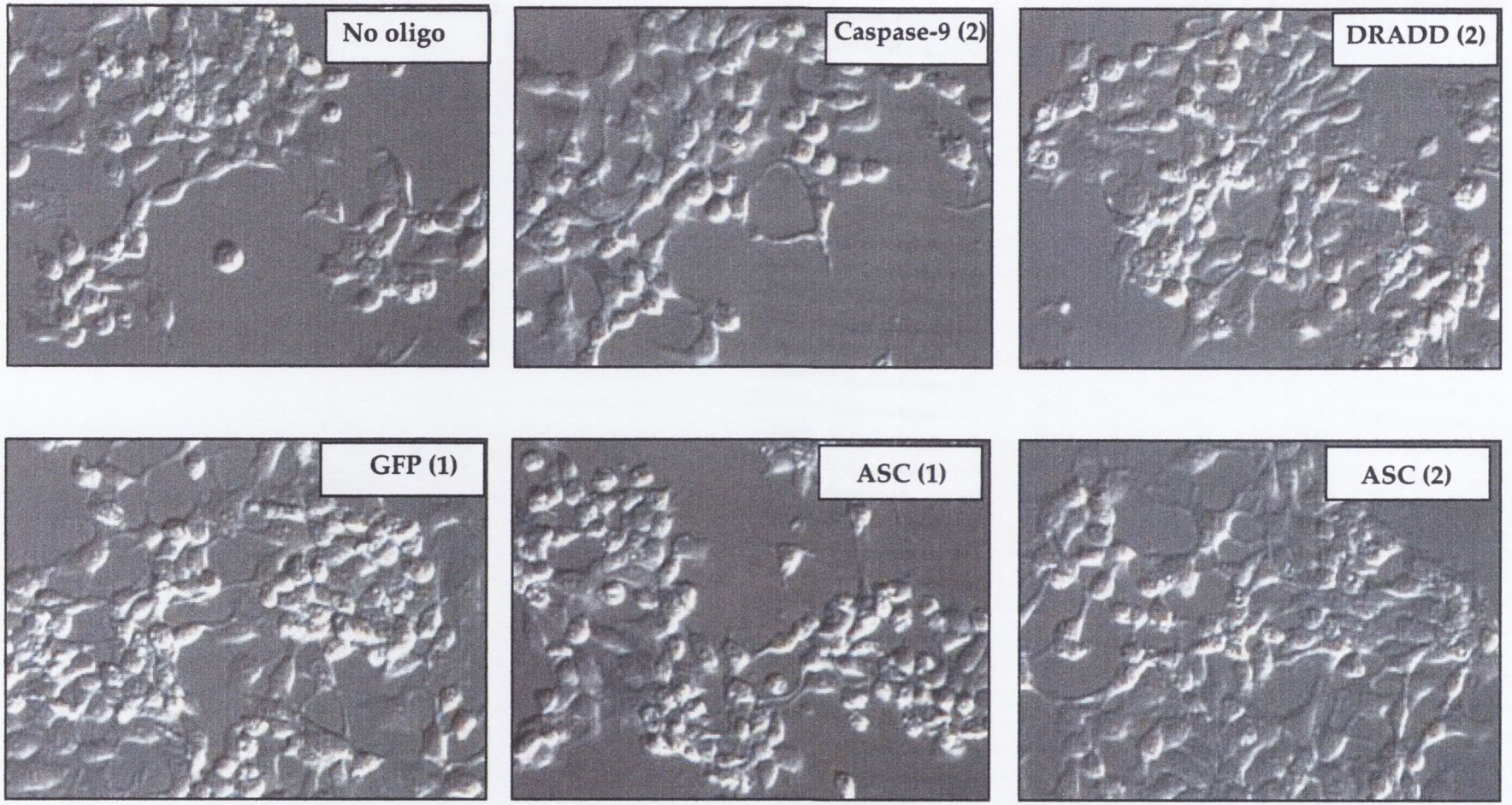


Figure 5.6 D

Cells treated with siRNA oligos ASC(1) and GFP oligo(1) show increased sensitivity receptor-mediated apoptosis:
Cells were transfected with oligos and reseeded. 24 h after reseeding the cells were treated with 25 ng/μl TNF. This stimulus differs from the others in that it targets a membrane receptor and not the intrinsic mitochondrial pathway. As with the other stimuli, cells which had been treated with the GFP or ASC(1) oligos showed increased sensitivity to the stimulus.

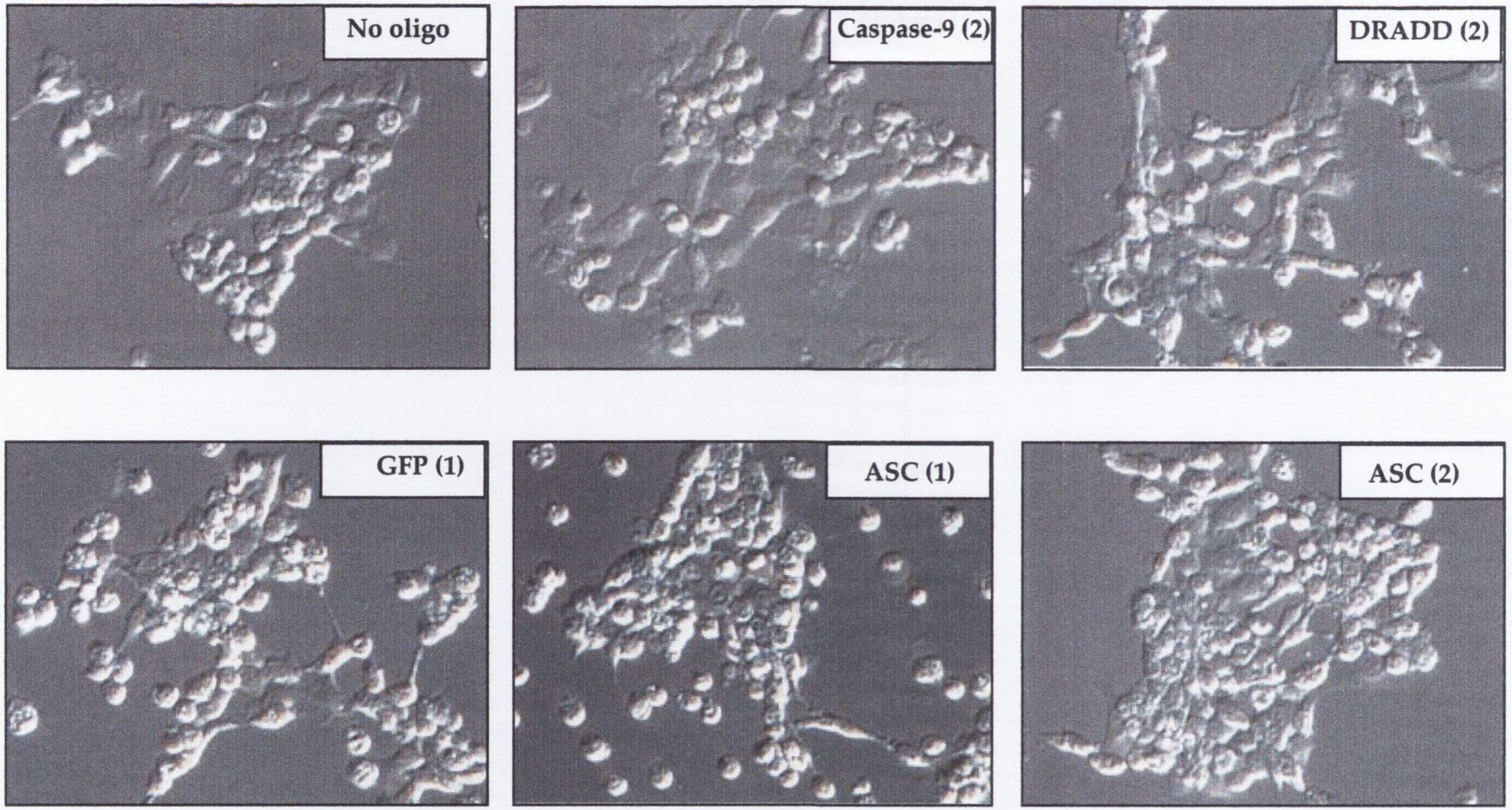


Figure 5.6 E

Cells treated with siRNA oligos ASC(1) and GFP oligo(1) show increased sensitivity to UV induced apoptosis:
Cells were transfected with oligos and reseeded. 24 h after reseeding the cells were treated with 20 s pulse of UV exposure. 17 h later, apoptosis was significantly higher in the wells where ASC or GFP had been transfected.

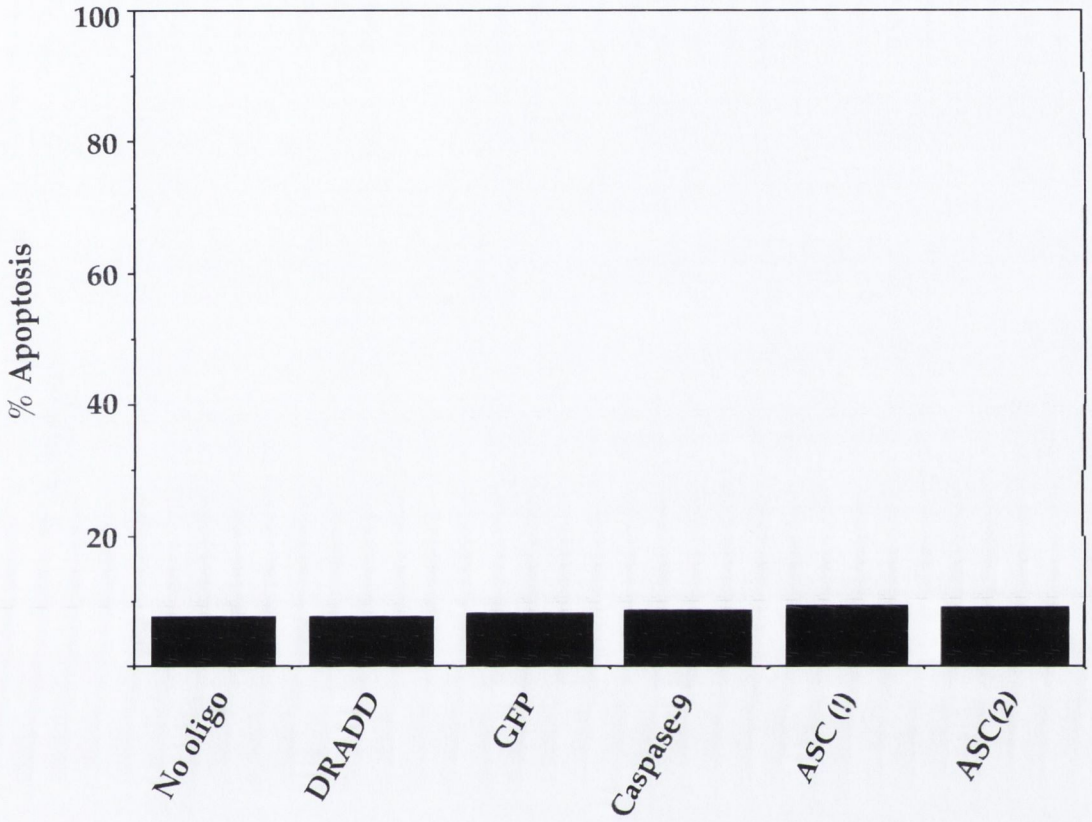


Figure 5.7 A

Cells treated with a range of si RNA oligos do not have an increased rate of spontaneous apoptosis

MCF7 cells were transfected with 200 nm of each siRNA oligo and left for 48 h before reseeding. Cells were scored for apoptosis by morphology.

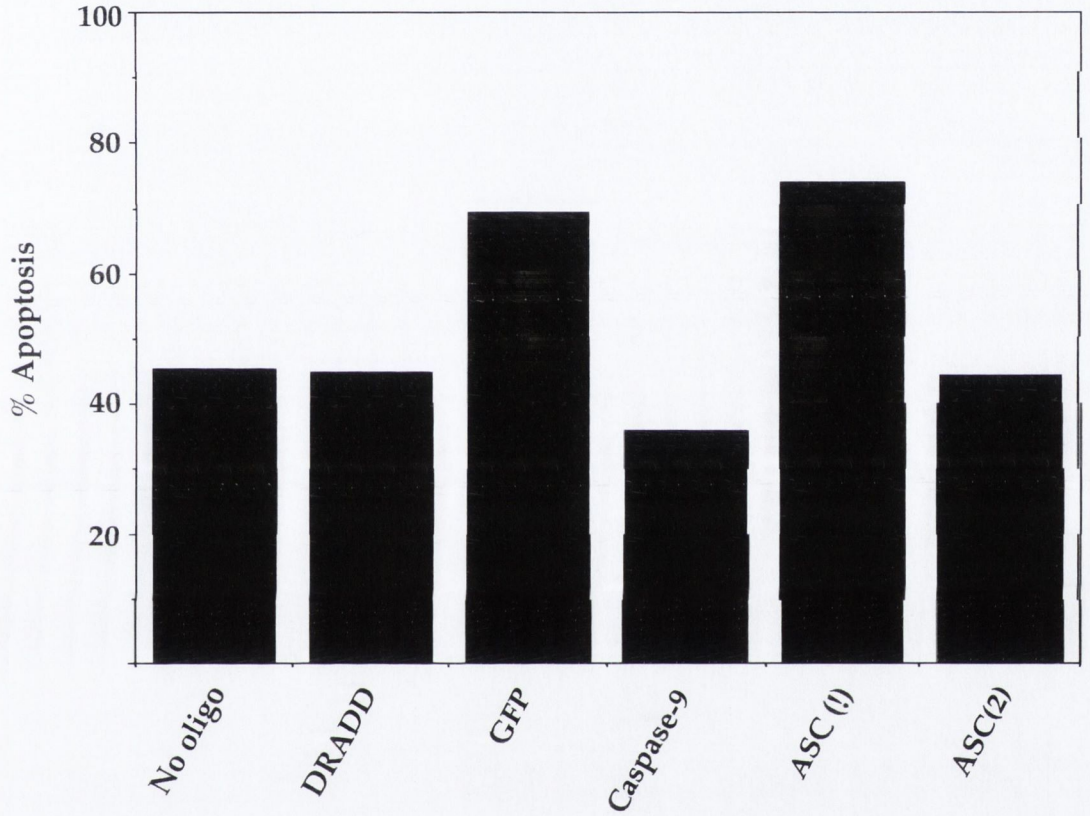


Figure 5.7 B

Cells treated with oligos ASC(1) and GFP oligo(1) show increased sensitivity to Actinomycin D:

MCF7 cells were transfected with 200 nm of each siRNA oligo and 48 h before reseeding. This montage shows the cells after treatment with 5 μM Actinomycin D for 20 h. Cells which received the oligos GFP(1) and ASC(1) have significantly higher levels of death than cells which were transfected with the other oligos.

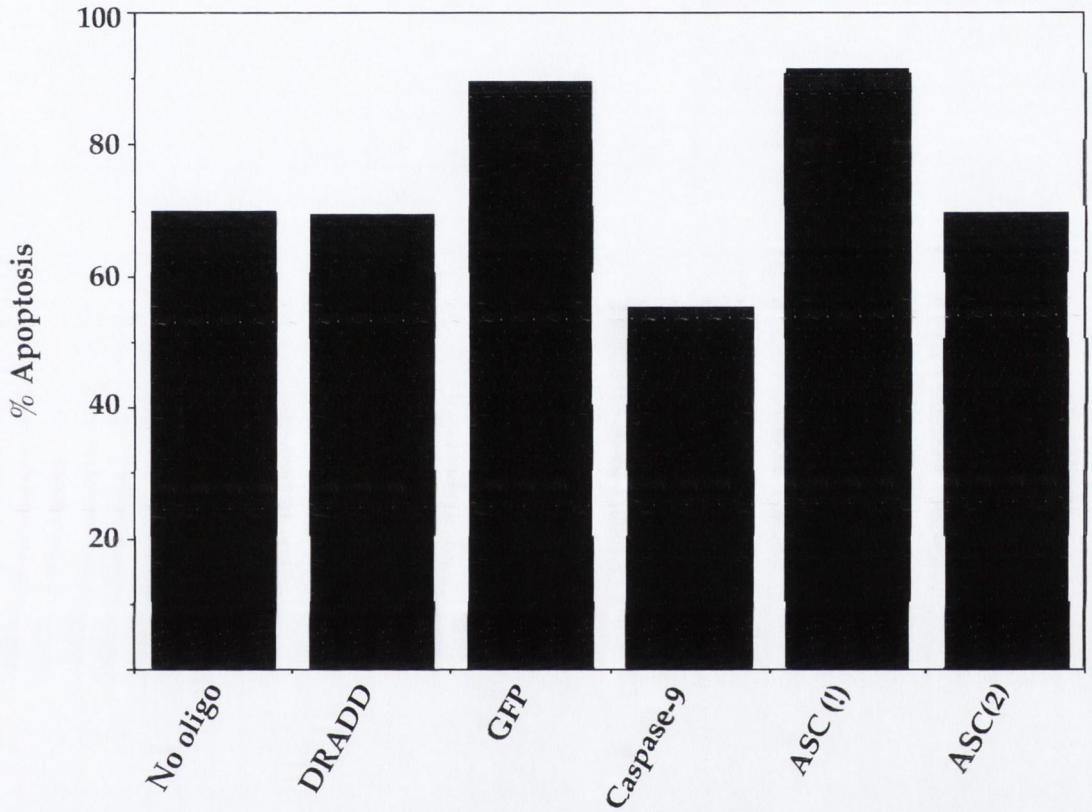


Figure 5.7 C

Cells treated with oligos ASC(1) and GFP oligo(1) show increased sensitivity to Daunorubicin:

Cells were transfected with oligos and reseeded. 24 h after reseeding the cells were treated with 10 μM Daunorubicin. After 20hrs the apoptosis induced in the GFP(1) and ASC(1) wells was observed to be much greater than that seen in the other oligo treatments.

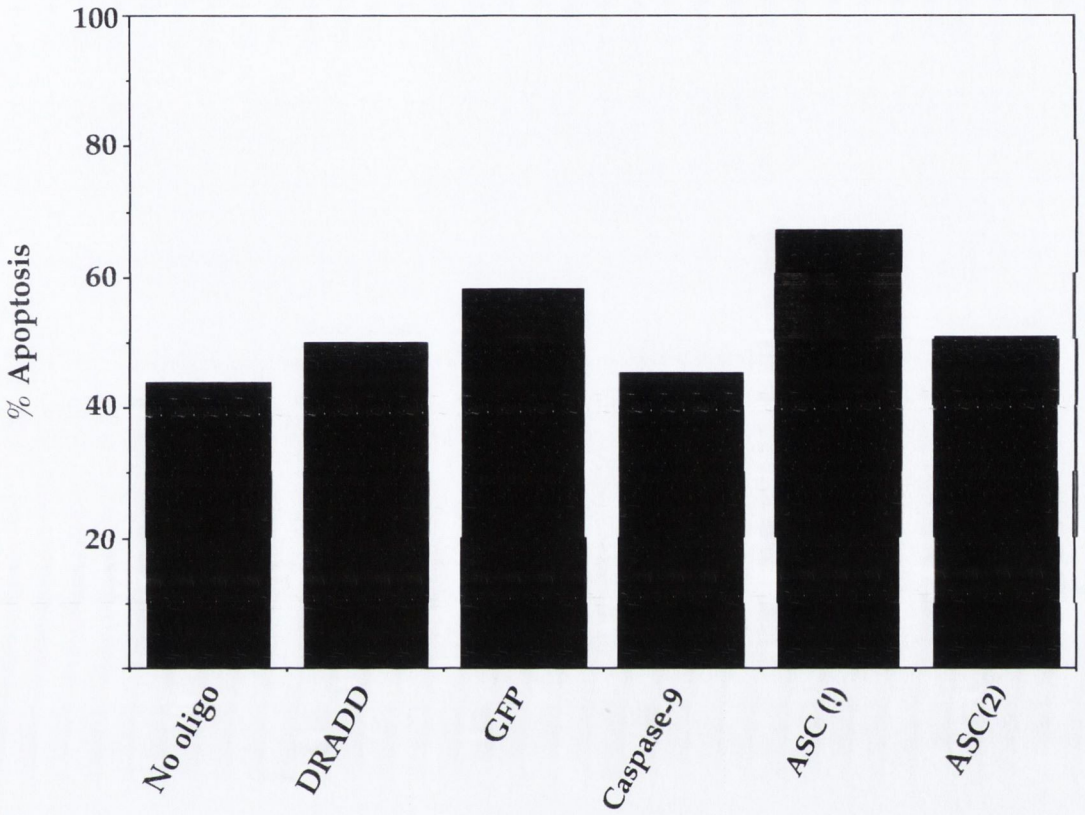


Figure 5.7 D

Cells treated with oligos ASC(1) and GFP oligo(1) show increased sensitivity to receptor-mediated apoptosis:

Cells were transfected with oligos and reseeded. 24 h after reseeding the cells were treated with 25 ng/µl TNF. This stimulus differs from the others in that it targets a membrane receptor and not the intrinsic mitochondrial pathway. As with the other stimuli, cells which had been treated with the GFP or ASC(1) oligos showed increased sensitivity to the stimulus.

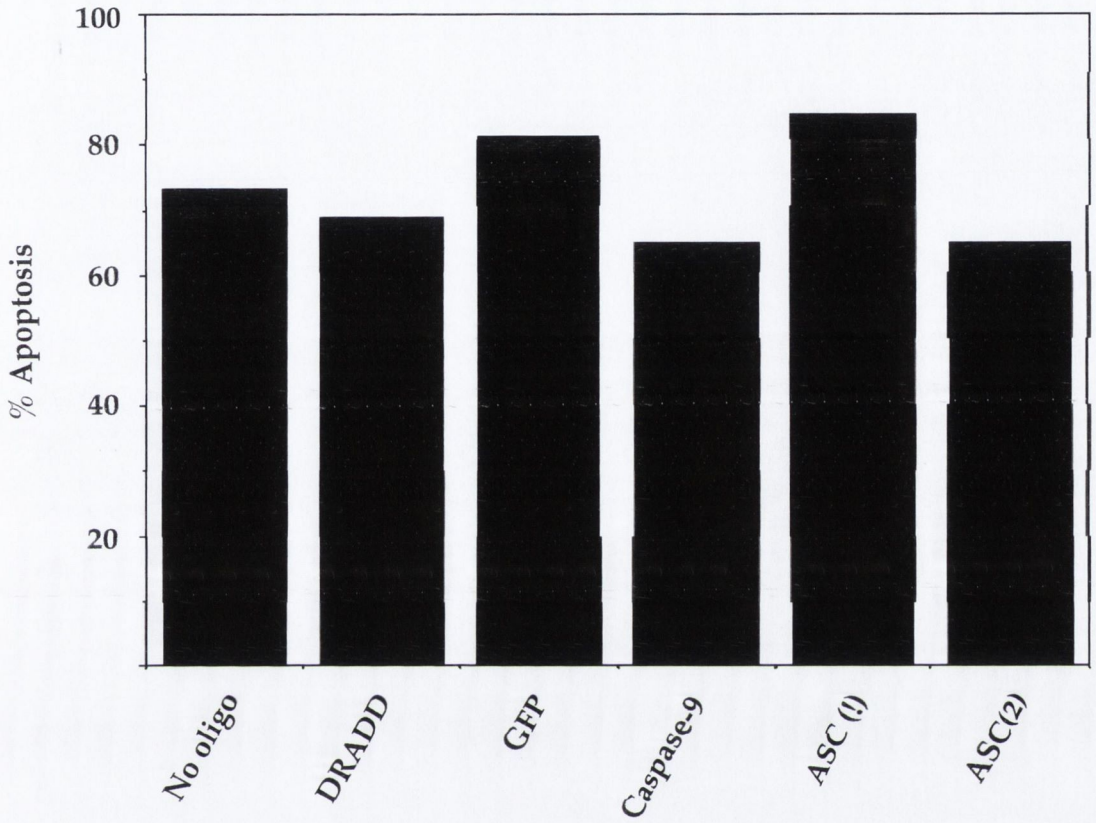


Figure 5.7 E

Cells treated with oligos ASC(1) and GFP oligo(1) show increased sensitivity to UV irradiation:

Cells were transfected with oligos and reseeded. 24 h after reseeding the cells were treated with 30 sec UV irradiation. As with the other stimuli, cells which had been treated with the GFP or ASC(1) oligos showed increased sensitivity to the stimulus.

5.3 DISCUSSION

5.3.1 *NAC induced re-localisation of ASC*

Chapter IV descrbed the ability of NAC to promote the apoptosis-inducing effects of ASC. As this effect became NAC-independent at high concentrations of ASC, apoptosis in this context most likely occurs as a result of oligomerisation of NAC, which then recruits ASC and allows multiple ASC molecules to come into close proximity with each other. Caspase molecules bound to ASC would then be in a position to undergo autoprocessing. In chapter IV an interaction between ASC and NAC was confirmed through co-immunoprecipitation. In this chapter a direct effect of NAC on the expression pattern of ASC was visible using immunofluorescence on cells overexpressing the two proteins. ASC expressed alone at a concentration which is not cytotoxic had a diffuse cellular pattern, but dramatically relocalised to a single speck per cell when NAC was co-expressed. As ASC is a bipartite protein composed of two interaction domains, namely CARD and PYRIN domains, it is possible that multiple ASC–ASC interactions as well as ASC-NAC interactions between CARD-CARD, PYRIN-PYRIN or PYRIN-CARD produce these specks.

Unfortunately the propensity of ASC to form aggregates under the conditions which induce apoptosis introduced great difficulty in experiments involving immunoprecipitation. This is probably due to the insolubility of ASC aggregates formed. Using the standard transfection conditions, very little ASC was immunoprecipitated. While this was greatly improved by scaling up the experiments, the amount of ASC which was successfully immunoprecipitated was relatively little compared with the total overexpressed protein. Furthermore, although detectable amounts of ASC protein could be precipitated, this fraction may in fact represent soluble protein, which is not involved in binding to caspases.

5.3.2 *NAC/ASC and Caspase Activation*

Lysates made from cells undergoing apoptosis induced by ASC/NAC overexpression had detectable processing of caspases -2, -3, -7, and -8. Interestingly a DN-caspase-9 plasmid, which had inhibited BAX-induced apoptosis, did not affect apoptosis induced by NAC/ASC. The caspase processing pattern of ASC/NAC was assessed through western blotting of cell lysates. These lysates had been made from 293T cells which were transiently transfected with ASC/NAC at ratios which induce significant cell death (approximately 60%). The analysis revealed processing of a range of caspases which are known to be activated during apoptosis, including caspases -2, –3, -7 and -8. Although ASC or NAC expression alone were not capable of inducing caspase processing, their coexpression induced caspase processing directly comparable to expression of the pro-apoptotic Bcl-2 family protein Bax. High levels of caspase-8 processing were evident with significant production of the p18 cleavage fragment. Caspase-8 can be activated either proximally or downstream in the caspase cascade and so while the strong caspase-8 processing seen in ASC/NAC induced apoptosis may be an indication of a proximal role, it is also possible that this is downstream activation occuring at the end of the caspase cascade.

5.3.2 *Non-Specific Effects of siRNA*

The use of siRNA as a tool to obtain data about gene function has become increasingly popular in the past year. The efficiency of siRNA in the knockdown of targets seems to far exceed previous techniques such as antisense DNA. More recently, concerns have been raised over the need for such experiments to be carefully controlled to ensure meaningful results. Experiments carried out to ablate ASC expression and examine the resulting effect on various stimuli revealed some novel surprising with regard to the use of siRNA.

The first major problem associated with the use of siRNAs was the variable efficiency of the technique. Using the same conditions the success of the knockdown varied from no decrease in expression to almost full ablation of the target gene expression. This introduces difficulty in setting up experiments which involve treatment of cells after knockdown as knockdown of the target gene must be confirmed in each experiment.

To ensure suitable controls for all siRNA experiments, a panel of siRNA oligos were used. Some of these had previously been confirmed as achieving RNAi of target genes (DRADD oligo), others were used as negative controls (caspase-9 oligo), while others targeted proteins known not to be expressed by mammalian cells (GFP oligo). In all cases, cells appeared healthy after treatment with siRNA oligos. However, a significant sensitization to apoptotic stimuli was caused with the GFP siRNA and one of the ASC oligos.

Although RNAi phenomena have been observed in a range of organisms, it is likely that further differences between the organisms, in the mechanisms and regulation of the process will emerge. These differences are important to consider while analysing the results of experiments using this technique. One of the most important considerations which must be kept in mind for mammalian siRNA is the potential immune response. When the RNAi system was being optimised for mammalian cells it was initially carried out using large sequences of RNA up to over 1kb in length. These studies were carried out succesfully in oocytes and mouse embryos. However, when the same approach was used in mammalian cell lines RNAi was not achieved (Ui-Tei *et al.*, 2000). This is predicted to be a result of the cellular response to dsRNA greater than 30bp in length, known as the interferon response. The interferon response is an anti-viral immune response which is based on the fact that many viral genomes are comprised of dsRNA. An RNA binding protein called PKR binds to dsRNA greater than 30bp in length resulting in

activation of its kinase activity. Activated PKR then phosphorylates a number of target proteins responsible for a dramatic cellular response. The first of these is the translation initiation factor, eIF2α. Once phosphorylated eIF2α becomes inactive and translation shuts down. PKR is also capable of activating the transcription factors NFκB and p53. In addition to the activation of PKR, dsRNA also activates 2'-5'-oligodenylate synthetase and enzymes which are required for activation of RNaseL, a ribonuclease which non-specifically degrades RNA in the cell.

During the course of our experiments using a range of oligos, it was found that certain oligos could non-specifically sensitize cells to a range of apoptotic stimuli. This sensitization has not been reported as an effect of siRNA oligos to date. Considering the effects that dsRNA can have on cells, we predict that this sensitzation is a result of activation of the interferon response. Two outcomes of this response which are likely to have a direct effect on the cell's apoptosis threshold are already known. The first of these is the activation of the transcription factor p53, this protein has been found to be required for apoptosis induced by stimuli such as UV irradiation. The second factor is the finding that certain genes contain alternative translation initiation sites which are used when eIF2α becomes inactivated. One of these proteins is the pro-apoptotic Bcl-2 family protein Bax.

The exact explanation for this increased sensitization to apoptosis induced by siRNA oligos is being pursued and is likely to impact greatly on the use of siRNA in the study of apoptosis.

CHAPTER VI

GENERAL DISCUSSION

6.1 INTRODUCTION

The purpose of the work described in this thesis was to identify and characterise novel proteins which function in the signalling pathways of cell death and cell survival. Understanding what makes up the pathways which result in cell death or survival has important implications above and beyond merely adding to our knowledge of all cellular processes. Many common diseases are a direct result of either excessive cell death or defects that interfere with the normal cell death program. By defining the normal steps in promotion or inhibition of cell death, a better understanding of the manner in which these defects bring about disease can be established. In turn, treatments targeted towards correcting specific defects can then be designed.

The accessibility of public protein and DNA sequence databases in recent years has helped to reveal the extensive homology between different organisms (Aravind *et al.*, 2001). The conservation of many protein motifs can be seen from organisms as unrelated as nematode worms and man. Moreover, the patterns of protein interactions which make up different signalling networks are often similar, regardless of the signal being transduced. Signalling pathways involved in cell death and cell survival have been shown to rely on families of proteins which contain a small number of protein motifs. Examples of these recurring domains are; the caspase recruitment domain (CARD), the death effector domain (DED) and the death domain (DD). However, assigning function to novel proteins on the basis of a recognisable domain, such as those mentioned above, is not as clear-cut as it first seems. Not all CARD domains are involved in caspase recruitment for example and not all death domains always transduce a death signal. What has been established is the important role that these domains play in many different protein-protein interactions, whether to promote cell death or cell survival.

Database searches for proteins containing specific domains has proven to be an effective means of identifying key molecular players in the formulation of signalling pathways. Rather than isolating proteins on the basis of their ability to bind other proteins by using methods such as the two-hybrid approach, the database method is a rapid way to identify novel sequencesthat might have a role in the pathway of interest. While approaches such as the yeast two-hybrid screen can be successful in identifying novel interaction partners, it could be argued that the results of this approach don't often merit the amount of effort involved in setting up a screen. As shown in Chapter III the success of such a screen is heavily dependent on the bait protein being used. Because various parameters, such as the size of the screen, have to be decided on a screen-by-screen basis this is a time consuming and laborious process. The results of such a screen need to be scrutinised very carefully as the incidence of false positives is very high.

The use of the yeast two-hybrid assay to identify novel caspase-1 interacting proteins was unsuccessful. However, solving the caspase-1 activating mechanism remains a priority in the fields of cell death and cytokine research. Although a number of proteins have been put forward as putative adaptor molecules, large gaps remain in the signal pathway from pathogen recognition by Toll-like receptors, to caspase-1 activation and finally IL-1β processing and secretion. Unfortunately attempts to identifying caspase-1 interacting molecules, as described in chapter III did not shed any further light on the activation mechanism.

Using the activation of other initiator caspases such as caspase-9 as a model, we predicted that novel caspase adaptor molecules would minimally contain a CARD domain capable of interaction with the prodomain of a caspase. BLAST search analysis with the caspase-1 prodomain against sequences within the human public databases revealed proteins with CARDs of varying similarity. A novel protein, NAC with a multidomain scaffold-type structure was identified.

6.2 The PYRIN proteins NAC and ASC co-operate in a novel Apoptosis-pathway.

The presence of a number of defined domains in NAC presented a range of possibilities in terms of function. As many of these putative roles as possible were investigated, in order that some could be ruled out and the options narrowed down. A number of structural features indicated that NAC might have a role in an inflammatory pathway. The N-terminal PYRIN domain is so-called because of its homology to the PYRIN protein. Mutations in this protein are associated with Familial Mediterranean Fever, a disorder characterised by disruption of the inflammatory response. In addition, the overall structure of NAC, which contains a CARD, NBD and Leucine Rich Repeats bears striking resemblance to the Nod proteins. Both Nod1 and Nod2 have been implicated as intracellular LPS receptors (Inohara *et al.*, 2001). The possibility that NAC may represent a member of a family of intracellular receptors specific for different PAMPs in a manner similar to Nod1/2 was also considered. No data in support of this hypothesis could be found.

The bipartite adaptor-like protein ASC was found to induce apoptosis when expressed at high concentrations, presumably through concentration-dependent aggregation. We found that NAC was able to induce ASC-dependent apoptosis at low concentrations of ASC, which alone were not cytotoxic to cells. Apoptosis induced by ASC/NAC was dependent on caspases, and was inhibited by the pan-caspase inhibitor zVAD-fmk. Caspase processing was detected during cell death induced by these molecules, confirming that apoptosis was taking place.

The synergy between ASC and NAC was found to be dependent on the CARD domain of NAC, indicating that this region was involved in the interaction between the two proteins. Removal of the PYRIN domain did not affect the co-operation between the two molecules, suggesting that recruitment of proteins via this domain is not required for the induction of apoptosis. An N-terminal deleted construct consisting of LRR-CARD of NAC constitutively induced apoptosis suggesting that

the N-terminal region may negatively regulate the full-length molecule. Such negative regulation is found in Apaf-1, where the WD-40 region inhibits binding of caspase-9 to Apaf-1 in the absence of cytochrome *c*. The leucine rich repeats of NAC are likely to be responsible for interacting with a ligand, which induces ASC/NAC complex formation. It is also possible that a family of NAC like molecules exist which respond through the LRR to different stimuli as is seen with TLRs. In fact recently a group of proteins have been identified with a similar structure to NAC varying in length, numbers of LRR and presence of PYRIN domains (Tschopp *et al.*, 2003; Chamaillard *et al.*, 2003). The exact role of these PYPAF/NALP proteins has yet to established.

The possibility that intracellular caspase activating complexes other than the apoptosome, exist is beginning to be investigated by a number of groups. Until recently, it was presumed that all intrinsic death pathways converged at the Apaf-1 apoptosome. This idea is now beginning to be challenged by a number of groups. These challenges have their basis in the finding that Apaf-1 null cells are not resistant to all stress stimuli and therefore must contain another caspase-activation mechanisms (Marsden *et al.*, 2002; Rao *et al.*, 2003). More specifically the idea that caspase-2 acts upstream of mitochondria in response to certain stress stimuli has been proposed (Lassus *et al.*, 2002). This study showed that in the absence of caspase-2, Bax failed to translocate to mitochondria in response to certain stress stimuli. In keeping with this idea another group has implicated caspase-2 in the formation of a high molecular weight complex similar to the apoptosome (Read *et al.*, 2002). If this proves to be true there may be alternative modes of producing caspase-9 activation or indeed other proximal caspases activated in response to cellular stress, possibly caspase-2.

The details of how caspase-8 is engaged by activation complexes, at the beginning of the caspase cascade, are also being redefined (Harper *et al.*, 2003). It had been

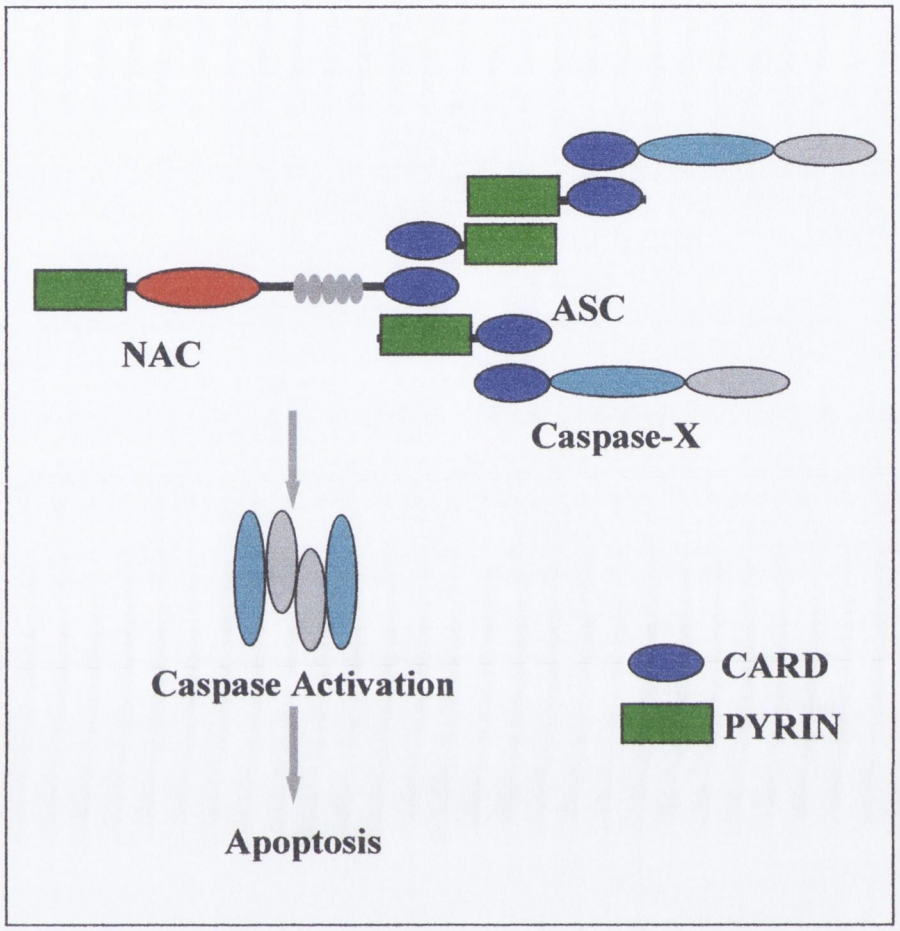


Figure 6.1:
Predictive model for ASC/NAC complex formation and Caspase activation
Binding of ASC to the CARD domain of NAC occurs through either the CARD or PYRIN domain of ASC. This interaction facilitates the recruitment of a caspase and induction of apoptotic cell death.

assumed that all death receptors have a similar method of recruiting and activating caspase-8 when stimulated, although not all membrane complexes have been purified. However, very recently it has emerged that processing of caspase-8 at the TNFR and other death receptors such as the Fas receptor proceeds in more than one way. It seems that rather than recruiting caspase-8 and FADD to the TNF Receptor via TRADD to form one complex, the activation process is divided into the initial recruitment of TRADD upon receptor stimulation, followed by its release into the cytoplasm to interact with FADD and caspase-8, forming a second complex (Micheau and Tschopp, 2003). This new model suggests the possibility that caspase-8 can be activated in cytoplasmic complexes in addition to membrane receptor complexes.

The above are examples of the rapid changes which are coming about in the knowledge of different pathways to apoptosis. It is becoming more evident that assigning caspase activation to either the Fas-like membrane pathway or the intrinsic Apaf-1-dependent pathways may not be always possible. There may be a range of caspase activating complexes as well as differential regulation of Apaf-1. However, thus far evidence for such complexes is scant.

6.3 Non-Specific effects induced by siRNA oligos

Ablating the expression of proteins is an invaluable tool for generating data on gene-function. RNAi technology and in particular the use of siRNA oligos to knock down proteins is becoming increasingly common. This relatively new method can be very effective in mirroring gene-knockout phenotypes and so provides a less expensive alternative.

Two siRNA oligos targeted towards ASC were generated for use in exploring ASC/NAC-induced death. It was hoped that ablation of ASC expression would directly interfere with apoptosis induced by particular stimuli and therefore

identify the context in which ASC/NAC complex formation occurs. Variable results were obtained with these oligos, with knockdown rarely achieved. In addition, a significant, non-specific sensitisation to a variety of apoptosis stimuli was observed in cells which had been treated with certain oligos. This effect highlights the importance of the inclusion of control oligos in experiments as well as no oligo controls. Original papers which outlined the development of siRNA oligos, emphasised that the length of these short dsRNA molecules meant they did not induce an interferon response in cells. However, very recently it has now been reported that RNAi achieved through vector expression of siRNA oligos, or transfection of siRNA can induce genes associated with the interferon response (Bridge *et al.*, 2003; Sledz *et al.*, 2003). This increased sensitization of cells to apoptosis is an important consideration for further studies using siRNA oligos which involve the assessment of levels of apoptosis. To further complicate this issue, it is also known that different cell lines react differently to dsRNA and not all mount a strong interferon response (Stojdl *et al.*, 2000). This fact will necessitate the control of all experiments for each cell type being used

The further characterisation of the role of the ASC/NAC complex in apoptosis will require the use of approaches which involve examining the endogenous complex. Unfortunately the use of siRNAs to do this may not be possible. However, proteomic analysis of the complex would identify its exact components and would constitute a novel approach to this problem.

6.4 CONCLUSIONS

The specific conclusions which this thesis supports are;

(i) The PYRIN proteins ASC and NAC interact to form a novel apoptosis inducing complex.

(ii) NAC/ASC coexpression induces the relocalisation of ASC into distinct Speck structures which are associated with apoptosis.

(iii) ASC and NAC co-operate to induce processing of endogenous caspases to their active forms.

(iv) siRNA oligos used for the knockdown of endogenous genes can induce non-specific sensitization to a range of apoptosis stimuli.

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