



**The Biochemical and Cellular Analysis
of Caspase-2 Activation
During Apoptosis.**

By

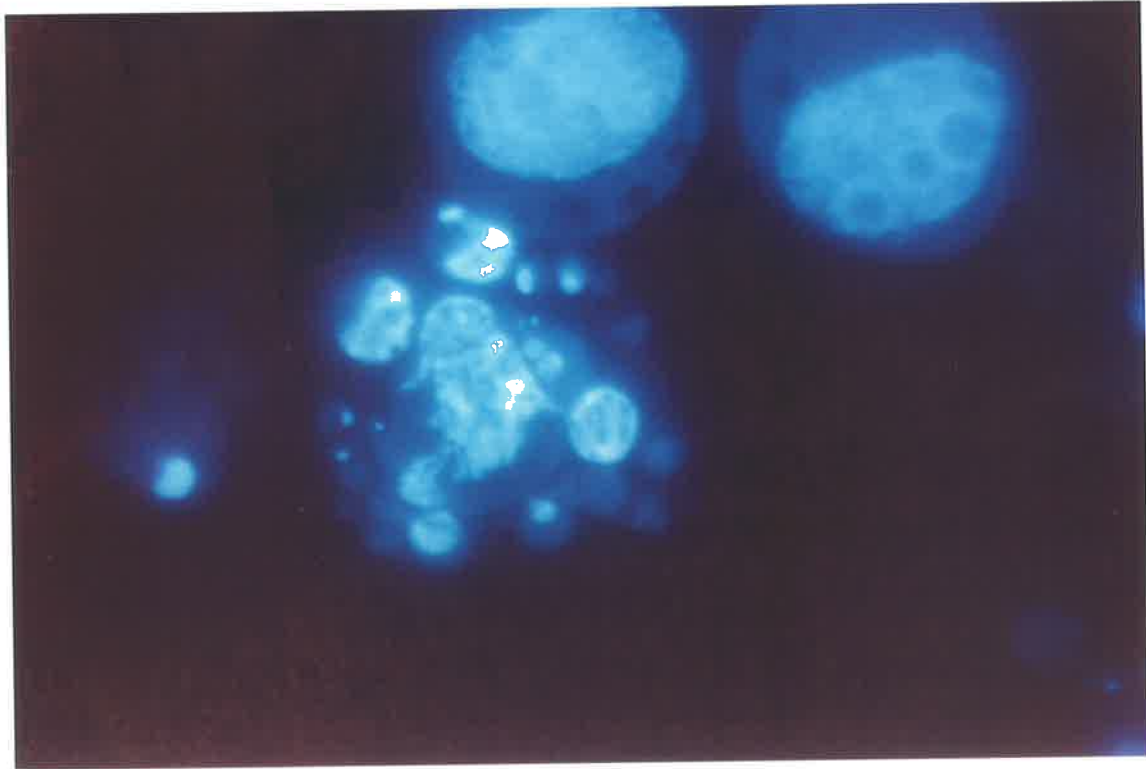
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An FDC-P1 cell displaying the morphological features of apoptosis

FDC-P1 cells were treated with etoposide for 6 h, stained with DAPI
and analysed by fluorescence microscopy.

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Abstract

Apoptosis, or programmed cell death, is a fundamental process during both embryonic development and adult homeostasis. Aberrant apoptosis has been associated with a number of human diseases, which may arise either from a failure of cells to undergo apoptosis, or from excessive apoptosis. Understanding of the cellular components of the apoptotic machinery should contribute to the design of therapeutic agents which may be beneficial in the treatment of diseases which have disruptions in the apoptotic pathway.

A unique family of cysteine proteases, termed caspases, plays a central role in the execution of apoptosis by mediating the regulated cleavage of a group of cellular proteins responsible for apoptotic cell death. Thirteen members of the caspase family have been identified so far and the role that each contributes to the execution of apoptosis in response to a variety of stimuli is being intensively researched. The focus of the studies presented in this thesis is the second caspase family member to be identified; caspase-2. The aim of this project was to characterise the biochemical and cellular aspects of caspase-2 activation during apoptosis, in order to delineate the role that caspase-2 plays in the apoptotic pathway.

To initially investigate the mechanism of caspase-2 activation, caspase-2 protein was produced *in vitro* and incubated with cytoplasmic extracts from apoptotic cells, isolated caspases and the serine protease granzyme B. Caspase-2 was shown to be proteolytically processed from its 51 kDa proenzyme form to subunits 19 kDa and 12 kDa in size by apoptotic cell extracts, caspase family members and granzyme B. In addition, caspase-2 was shown to undergo self-proteolysis. These results suggested that caspase-2 is likely to

be activated during apoptosis and that the mechanism of caspase-2 activation could be one of self-proteolysis, or of cleavage by other caspases. The ability of granzyme B to cleave caspase-2 suggested that caspase-2 may be important in apoptosis mediated by cytotoxic lymphocytes.

To determine whether caspase-2 was activated *in vivo* in response to a variety of apoptotic signals, immunoblot analysis using a caspase-2 antibody was performed. Caspase-2 activation was shown to occur rapidly in response to apoptotic signals mediated by death receptors such as Fas and TNFR1, and also in response to apoptotic stimuli such as growth factor deprivation, γ -irradiation and treatment with the chemotherapeutic agent, etoposide. Caspase-2 activation preceded that of caspase-3, suggesting that caspase-2 activation occurs upstream of caspase-3 in the sequence of caspase proteolysis induced by apoptotic stimuli.

To determine the aspartate residues in procaspase-2 which are critical for caspase-2 self-proteolysis and resultant enzymatic activity, a site-directed mutagenesis approach was used. The processing of caspase-2 aspartate mutants was assessed both *in vitro* and *in vivo*. These studies demonstrated that cleavage at particular residues is essential for the generation of active caspase-2. Both the *in vitro* and *in vivo* processing of procaspase-2 directly correlated with its ability to induce cell death in transfection experiments.

The role of the caspase-2 prodomain in mediating the characteristics typical of an apical, or upstream, caspase, was analysed in a series of experiments in which the caspase-2 prodomain was fused to caspase-3. Fusion of the caspase-2 prodomain to caspase-3

conferred to caspase-3 the ability to dimerise, self-process and efficiently induce apoptosis when expressed in transfected cells. Caspase-3 alone does not exhibit these properties. Chimeric molecules such as these have potential therapeutic application for inducing apoptosis in cells which have a block in the apoptotic pathway that lies upstream of caspase activation.

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by any other person, except where due reference has been made in the text. I give consent for this thesis to be made available for loan and photocopying.

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Publications

Colussi PA, **Harvey NL**, Shearwin-Whyatt LM and Kumar S. (1998) Conversion of Procaspase-3 to an Autoactivating Caspase by Fusion to the Caspase-2 Prodomain. *J. Biol. Chem.* In press.

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Chapter 1

Introduction



1.1 *The Apoptosis Concept*

Apoptosis is the term first introduced by Kerr and colleagues (1972) for an intrinsically programmed process of cell death that is distinct from necrosis and is defined by a procession of morphological changes in the dying cell. The morphological features characteristic of apoptosis include cellular shrinkage, nuclear condensation, plasma membrane blebbing and fragmentation of DNA at internucleosomal intervals (Kerr *et al.*, 1972, Wyllie, 1980). In contrast to necrosis, apoptosis occurs in the absence of inflammation and is not confined to cells receiving cytotoxic stimuli; apoptosis also occurs in normal tissues during both development and homeostasis (Kerr *et al.*, 1972, Wyllie *et al.*, 1980). Although Kerr and colleagues were the first to propose and define the concept of apoptosis in 1972, the occurrence of apoptosis has been recognised and documented as early as last century (reviewed in Majno and Joris, 1995).

1.2 *Aberrant Apoptosis and Disease*

It is now recognised that the execution of apoptosis is fundamental during embryonic development and adult homeostasis and dysregulated apoptosis has been implicated in a number of human diseases (reviewed in Thomson, 1995, Hetts, 1998). Diseases associated with the failure of cells to undergo apoptosis include autoimmune disorders and some cancers. Conversely, excessive apoptosis has been implicated in neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease and spinal muscular atrophy. In addition, inappropriate apoptosis leading to premature cell death is associated with AIDS, ischaemic heart damage and aplastic anaemia. Elucidation of the mechanisms involved in the execution of apoptosis is thus an area of intense research. The development of agents that selectively interact with the apoptotic machinery to either inhibit inappropriate apoptosis, or selectively induce apoptosis in cells that are

abnormally resistant will be crucial to combat diseases in which aberrant apoptosis occurs.

1.3 Programmed Cell Death in *C. elegans*

The analysis of developmental cell death in the nematode *Caenorhabditis elegans* (*C. elegans*) has been integral in the identification of the molecular components of the cellular apoptotic machinery. Of 1090 cells generated during *C. elegans* development, 131 are removed by apoptosis (Sulston and Horvitz, 1977, Sulston *et al.*, 1983). To date, 14 genes have been identified that are involved at various stages of the cell death pathway (reviewed in Horvitz *et al.*, 1994). The products of these genes function in the initiation of apoptosis, the execution of apoptosis and the engulfment of apoptotic cells. Of the genes involved in the execution of cell death, three, *egl-1*, *ced-3* and *ced-4*, are absolutely required for cell death to occur. Loss of function mutations in any of these genes results in extra cells in *C. elegans* embryos (Ellis and Horvitz, 1986, Conradt and Horvitz, 1998). In contrast, the *ced-9* gene product is involved in the protection of cells against apoptosis; loss of function mutations in this gene result in the death of cells that normally survive (Hengartner *et al.*, 1992). Genetic studies show that *ced-9* functions upstream of *ced-3* and *ced-4* to negatively regulate their apoptosis inducing activity and thus maintain cell survival (Hengartner *et al.*, 1992). Of these two death inducing genes, *ced-4* has been shown to act upstream of *ced-3* (Shaham and Horvitz, 1996); cell death induced by *ced-4* overexpression is significantly reduced in the absence of *ced-3* expression. Recent evidence suggests that the negative regulation of *ced-3* and *ced-4* by *ced-9* is mediated by direct physical interaction between these three proteins. The apoptosis inhibiting activity of CED-9 is dependent on its ability to bind CED-4 (Chinnaiyan *et al.*, 1997, James *et al.*, 1997, Otilie *et al.*, 1997a, Spector *et al.*, 1997,

Wu *et al.*, 1997a). CED-4 can, in turn, bind to and activate CED-3 (Chinnaiyan *et al.*, 1997, Seshagiri and Miller, 1997a, Wu *et al.*, 1997b) and appears to be reliant on the activation of CED-3 for its apoptosis inducing activity (Chinnaiyan *et al.*, 1997). *egl-1* has recently been shown to be the most apical of the *C. elegans* cell death execution genes and promotes cell death by negatively regulating the *ced-9* gene, a process thought to be mediated via physical association between the EGL-1 and CED-9 proteins (Conradt and Horvitz, 1998). Binding of EGL-1 to CED-9 has been postulated to sequester CED-9 from the CED-9/CED-4 complex. Free CED-4 is then able to mediate the activation of CED-3 which results in apoptotic death (Conradt and Horvitz, 1998).

The cellular death apparatus has been conserved during evolution. Mammalian homologues of each *ced-9*, *egl-1*, *ced-4* and *ced-3* exist, all of which function in the mammalian cell death pathway. *ced-9* encodes a protein homologous to the product of the mammalian *bcl-2* proto-oncogene (Hengartner and Horvitz, 1994) which protects cells from apoptosis (Vaux *et al.*, 1988). *bcl-2* expression can prevent apoptosis in *C. elegans* (Vaux *et al.*, 1992) and partially compensate for *ced-9* mutations (Hengartner and Horvitz, 1994), indicating that these molecules are functionally similar. The *egl-1* gene shares homology with a family of cell death activators including Bik, Bid, Harakiri and Bad (Conradt and Horvitz, 1998). These proteins all share a short region of homology similar to the BH3 domain (Bcl-2 homology region 3) which mediates both their interaction with Bcl-2-like molecules and their ability to induce cell death (Wang *et al.*, 1996, Inohara *et al.*, 1997a, Kelekar *et al.*, 1997, Otilie *et al.*, 1997b, Zha *et al.*, 1997). Apaf-1 has recently been identified as a mammalian homologue of CED-4 (Zhou *et al.*, 1997) and has been shown to be required for the activation of a mammalian CED-3 homologue (Li *et al.*, 1997, Zhou *et al.*, 1997). There now exists a large family of

mammalian CED-3 homologues termed caspases, for aspartate specific cysteine proteases (Alnemri *et al.*, 1996). Caspases have been shown to play a central role in the execution of apoptosis, serving to cleave the cellular proteins which mediate apoptotic demise (reviewed in Kumar and Lavin, 1996, Nicholson and Thornberry, 1997). The presence of multiple mammalian counterparts of *C. elegans* apoptosis genes suggests that the execution of apoptosis is much more complex in mammals than it is in *C. elegans*.

1.4 ICE and Nedd2, the First Mammalian Homologues of CED-3

The cloning and characterisation of *ced-3* (Yuan *et al.*, 1993) and its protein product revealed homology with the human interleukin-1 β -converting enzyme (ICE) and murine *Nedd2*. ICE is a cysteine protease required for the cleavage of the inactive 31 kDa precursor of human interleukin-1 β to its mature 17.5 kDa active form (Cerretti *et al.*, 1992, Thornberry *et al.*, 1992), while *Nedd2* was identified as a neural precursor cell expressed gene that is downregulated during development of the murine brain (Kumar *et al.*, 1992). *Nedd2* was subsequently shown to encode a cysteine protease homologous to ICE (Kumar *et al.*, 1994, Wang *et al.*, 1994). Comparison of the *C. elegans* CED-3 protein sequence with the related nematodes *C. briggsae* and *C. vulgaris* demonstrated most conservation in the carboxy terminal half of the protein, the region most similar to ICE and Nedd2 (Yuan *et al.*, 1993). The three nematode CED-3 proteins share 29% amino acid identity with ICE (Yuan *et al.*, 1993); absolutely conserved residues including those that surround the catalytic cysteine of ICE, QACRG (Thornberry *et al.*, 1992). The homology shared by ICE and CED-3 suggested that CED-3 may exert its apoptosis inducing function in *C. elegans* by acting as a cysteine protease, and also that ICE and Nedd2 may play an equivalent role to that of CED-3 in the execution of

mammalian apoptosis. Indeed, ICE and Nedd2 were subsequently shown to possess apoptosis inducing activity (Miura *et al.*, 1993, Kumar *et al.*, 1994, Wang *et al.*, 1994), indicating that in mammals, at least two functional homologues of CED-3 exist.

1.5 The Caspase Family

Subsequent to the description of the pro-apoptotic function of CED-3, ICE and Nedd2, a family of CED-3 homologues have been identified. For uniformity, these proteases were recently termed caspases (Alnemri *et al.*, 1996) and have been numbered according to the chronological order of their discovery (Figure 1.1). To date 13 caspases have been identified and the role that each plays in various apoptotic pathways is being actively investigated. The caspases can be divided into three subfamilies on the basis of sequence similarity and substrate specificity as determined by using a positional scanning synthetic combinatorial library (Table 1.1). The caspase-1 subfamily comprises caspases-1, 4 and 5, and displays a substrate specificity for the (W/L)EHD sequence (Thornberry *et al.*, 1997). Caspases-11, 12 and 13 probably belong to this family on the basis of sequence homology. The caspase-3 subfamily comprises caspases-3, 6, 7, 8, 9 and 10. This family can be divided into two groups on the basis of substrate specificity; caspases-3 and 7, together with CED-3 have a preference for DEXD sequences. Caspases-6, 8 and 9 optimally recognise (I/L/V)EXD sequences (Thornberry *et al.*, 1997). It can be postulated that by virtue of its close homology with caspase-8, caspase-10 is probably a member of this subfamily. On the basis of sequence homology and substrate specificity caspase-2 appears to stand alone in the caspase family; although Thornberry and colleagues (1997) demonstrated that like caspases-3 and -7, caspase-2 prefers DEXD sequences, studies by Talanian and colleagues (1997a) have determined that caspase-2 actually prefers a pentapeptide sequence; VDVAAD.

Figure 1.1 Phylogenetic analysis of caspase family members. Alias for the caspases are shown in parentheses. (Reproduced from Humke *et al.*, 1998)

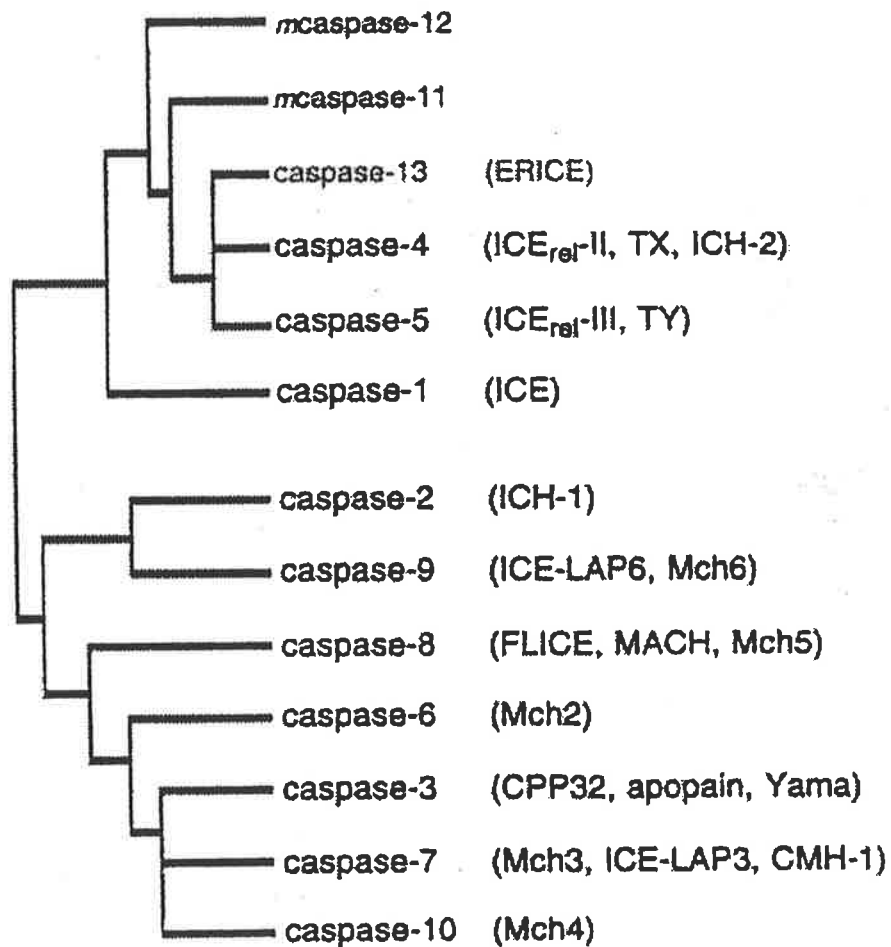


Table 1.1 Caspase subfamilies and substrate specificities. Caspase substrate specificities were derived as described by Thornberry *et al.*, 1997 and Talanian *et al.*, 1997a. Class I / Class II classification is based on the presence of a long (Class I), or short (Class II) amino terminal prodomain.

<u>Caspase subfamily</u>	<u>P4-P1 substrate specificity</u>	<u>Class I / Class II</u>
<u>Caspase-1</u> Caspase-1 Caspase-4 Caspase-5 Caspase-11 Caspase-12 Caspase-13	WEHD (W/L)EHD (W/L)EHD	Class I Class I Class I Class I Class I Class I
<u>Caspase-3</u> Caspase-3 Caspase-7 Caspase-6 Caspase-8 Caspase-9 Caspase-10	DEVD DEVD VEHD LETD LEHD	Class II Class II Class II Class I Class I Class I
<u>Caspase-2</u> Caspase-2	VDVAD	Class I

In addition to classification on the basis of sequence homology and substrate specificity, caspases can be further divided into two classes based on the presence or absence of an amino-terminal prodomain. Emerging evidence suggests that caspases containing a long amino-terminal prodomain (Class I caspases) are the first to be activated in response to apoptotic signals, by virtue of direct recruitment via specific adaptor molecules to death signalling complexes. Recruitment of Class I caspases to these complexes is a prodomain dependent process which results in caspase autoactivation. Caspases which have a short or absent prodomain appear to rely on the activity of Class I caspases for their activation and as such, lie downstream of Class I caspases in the apoptotic pathway. Class II or “effector” caspases are responsible for the cleavage of all known cellular apoptotic substrates so far identified and are also able to process various Class I caspases which may result in amplification of the apoptotic cascade (reviewed in Kumar and Lavin, 1996, Nicholson and Thornberry, 1997).

1.6 The Caspase-1 Subfamily

1.6.1 Caspase-1

The interleukin-1 β converting enzyme (ICE, caspase-1) was originally identified in monocytes as a unique cysteine protease which is responsible for the cleavage of the 31 kDa interleukin-1 β precursor molecule between Asp-116/Ala-117 and Asp-27/Gly-28 to liberate the active 17.5 kDa cytokine (Black *et al.*, 1989, Kostura *et al.*, 1989, Cerretti *et al.*, 1992, Thornberry *et al.*, 1992). The *CASP-1* gene encodes a 45 kDa protein which is proteolytically processed at aspartate residues to generate two subunits of 20 kDa and 10 kDa in size which heterodimerise to form the active enzyme (Cerretti *et al.*, 1992, Thornberry *et al.*, 1992). The catalytic cysteine residue of caspase-1 is located in the p20 subunit although both subunits are required for enzyme activity (Thornberry *et al.*,

1992). The fact that procaspase-1 is cleaved at aspartate residues to liberate the p20 and p10 subunits suggested that autocatalysis may be the mechanism of caspase-1 activation. This has been shown to be the case *in vitro* (Thornberry *et al.*, 1992, Gu *et al.*, 1995, Ramage *et al.*, 1995).

Based on the site at which caspase-1 cleaves IL-1 β , initial studies investigating the minimal recognition sequence of caspase-1 showed that there is a stringent requirement for an aspartate residue in the P1 cleavage position as well as a minimum requirement of four residues amino terminal to the cleavage site (Sleath *et al.*, 1990, Howard *et al.*, 1991). Substitution studies performed by Thornberry and colleagues (1992) demonstrated an increased affinity of caspase-1 for the tetrapeptide sequence Ac-YVAD-NH-CH₃, which differs by one amino acid from the YVHD recognition sequence in proIL-1 β . YVAD is still the most commonly used peptide substrate for the detection and inhibition of caspase-1-like activity. Recent studies employing a positional scanning library have identified the optimal tetrapeptide sequence for caspase-1 as WEHD (Rano *et al.*, 1997), which may facilitate the identification of additional caspase-1 substrates and more specific caspase-1 inhibitors. In addition to playing a crucial role in interleukin-1 β production, caspase-1 has recently been shown to regulate the production of IFN- γ by cleaving and activating the precursor of the interferon- γ inducing factor (Gu *et al.*, 1997).

1.6.2 Crystal Structure of Caspase-1

Derivation of the crystal structure of caspase-1 complexed with the YVAD peptide demonstrated that the active caspase-1 enzyme is a tetramer comprised of two (p20 + p10) heterodimers (Walker *et al.*, 1994, Wilson *et al.*, 1994). The spatial arrangement of

the p20 and p10 subunits in the caspase-1 tetramer illustrates the contribution that each subunit makes to the active site and overall conformation of the enzyme. Residues in both the p20 and p10 subunits contribute to formation of the active site and to dimer-dimer interaction (Walker *et al.*, 1994, Wilson *et al.*, 1994). The prodomain of caspase-1 appears to play an important role in caspase-1 processing and activity (Van Crielinge *et al.*, 1996). Expression studies in yeast showed that procaspase-1 was able to undergo autocatalytic processing while p30 caspase-1 lacking the prodomain was not. Importantly, this study demonstrated that procaspase-1 molecules containing mutations of the active site cysteine are able to dimerise, suggesting a model for self-proteolysis where prior dimerisation of procaspase-1 molecules is essential for proteolytic processing.

1.6.3 The Role of Caspase-1 in Apoptosis

Caspase-1 was the first homologue of CED-3 to be implicated in the apoptotic pathway when its overexpression in Rat-1 cells was shown to induce apoptosis (Miura *et al.*, 1993). Apoptosis induced by caspase-1 was inhibited by overexpression of *Bcl-2* or of the cowpox virus encoded gene *crmA* (Miura *et al.*, 1993), first identified as a specific inhibitor of caspase-1 (Ray *et al.*, 1992). Further evidence of a role for caspase-1 or caspase-1-like proteases came from Gagliardini and colleagues (Gagliardini *et al.*, 1994) who observed that CrmA could suppress apoptosis initiated by the withdrawal of NGF from neurons, suggesting that caspase-1 may participate in the execution of apoptosis in neural cells. CrmA has subsequently been shown to inhibit apoptosis in response to a variety of stimuli including serum deprivation (Wang *et al.*, 1994), Fas/APO-1 (Enari *et al.*, 1995, Los *et al.*, 1995, Strasser *et al.*, 1995, Tewari and Dixit, 1995), tumour necrosis factor (TNF) (Miura *et al.*, 1995, Tewari and Dixit, 1995), γ -irradiation

(Dorstyn and Kumar, 1997) and cytotoxic T lymphocytes (Tewari *et al.*, 1995c). In addition, tetrapeptide inhibitors of caspase-1 such as YVAD-CMK, YVAD-CHO and z-VAD-FMK (a broad caspase inhibitor) have been shown to inhibit apoptosis induced by a variety of stimuli in a variety of cell types (Lazebnik *et al.*, 1994, Chow *et al.*, 1995, Enari *et al.*, 1995, Fearnhead *et al.*, 1995, Los *et al.*, 1995, Zhivotovsky *et al.*, 1995). In retrospect, the inhibition of apoptosis observed in these experiments is probably not due solely to the inhibition of caspase-1, but due to the ability of CrmA and YVAD peptide inhibitors to inhibit additional members of the caspase family when present in high concentrations. CrmA has been shown to inhibit caspase-8 with high affinity (Zhou *et al.*, 1997), while it inhibits CED-3 and caspases-2, 3 and 10 relatively poorly (Miura *et al.*, 1993, Wang *et al.*, 1994, Xue and Horvitz, 1995, Srinivasula *et al.*, 1996a, Dorstyn and Kumar, 1997).

The clearest picture of the contribution that caspase-1 makes to the apoptotic pathway comes from studies in caspase-1 knockout mice (Kuida *et al.*, 1995, Li *et al.*, 1995). Caspase-1 null mice display an apparently normal phenotype, but are deficient in the production of IL-1 α and IL-1 β . Their apparently normal passage through development suggests that caspase-1 is not the mammalian equivalent of *ced-3*. The only apoptotic aberration in these mice was observed in thymocytes induced to undergo apoptosis by Fas/APO-1. While caspase-1 (-/-) thymocytes retain the ability to undergo apoptosis in response to dexamethasone and ionising radiation, they were resistant to apoptosis induced by Fas/APO-1 (Kuida *et al.*, 1995). Abrogation of the Fas/APO-1 pathway in these cells was not complete as is the case in *lpr* and *gld* mice which have mutated Fas/APO-1 or Fas/APO-1 ligand respectively (Watanabe-Fukunaga *et al.*, 1992, Takahashi *et al.*, 1994). These mice display lymphadenopathy and splenomegaly due to

a failure of T lymphocytes to undergo apoptosis, and some mice develop autoimmune diseases. These observations suggest that other caspases in addition to caspase-1 are involved in Fas/APO-1 mediated apoptosis. With the benefit of hindsight, it now appears that caspase-1 plays a more significant role in cytokine processing and inflammation than in the execution of apoptosis.

1.6.4 Caspase-4, Caspase-5, Caspase-11, Caspase-12 and Caspase-13

Caspases-4, 5 and 11 were identified based on sequence similarity with caspase-1 and have all been shown to induce apoptosis when overexpressed (Faucheu *et al.*, 1995, Kamens *et al.*, 1995, Munday *et al.*, 1995, Faucheu *et al.*, 1996, Wang *et al.*, 1996). None of the caspase-1 family members except caspase-1 itself are capable of cleaving proIL-1 β , indicating divergent substrate specificity. Substrate binding studies by Kamens and colleagues (1995) demonstrated that the binding affinity of caspase-1 for the YVAD substrate (K_m 83 $\pm$ 10 μ M) is approximately 8 fold higher than that of caspase-4 (K_m 681 $\pm$ 84 μ M) and correspondingly, the IC_{50} of caspase-4 for YVAD-CHO is approximately 20 fold greater (748nM) than for caspase-1 (38 nM). These observations suggest that caspase-1 family members process different substrates and fulfil divergent cellular functions. Several studies illustrate a functional hierarchy between members of the caspase-1 subfamily. Caspase-4 is able to process caspase-1 *in vitro* (Faucheu *et al.*, 1995), while murine caspase-11 promotes proIL-1 β processing by caspase-1 (Wang *et al.*, 1996), suggesting that both caspase-4 and caspase-11 may function upstream of caspase-1 to induce caspase-1 activation. The generation of caspase-11 mutant mice confirms this suggestion, demonstrating that murine caspase-11 is absolutely essential for murine caspase-1 activation and the subsequent generation of mature IL-1 α and IL-1 β in response to endotoxic shock (Wang *et al.*, 1998). Caspase-12

was identified in a PCR screen based on regions of homology shared between CED-3, caspase-1 and caspase-2 and was shown to share an expression pattern similar to other members of the caspase-1 subfamily (Van de Craen *et al.*, 1997). A human counterpart of caspase-12 has not yet been identified. Caspase-13 was recently identified on the basis of sequence similarity with caspase-4, sharing 75% sequence identity to caspase-4, 61% identity to caspase-5 and 47% identity to caspase-1 (Humke *et al.*, 1998). As with all caspases, overexpression of caspase-13 induces apoptosis in various cell types (Humke *et al.*, 1998). Caspase-13 has been shown to be activated by caspase-8 and is postulated to play a role in the pathways of cell death in which caspase-8 is involved (Humke *et al.*, 1998). The relative contribution that caspase-13 makes to these pathways remains to be seen, however, a major role seems unlikely given that other members of the caspase-1 family play a primary role in cytokine maturation and inflammation.

1.7 The CED-3/Caspase-3 Subfamily

1.7.1 Caspase-3

The caspase-3 subfamily is composed of caspase-3 and its closest homologues caspases-6, 7, 8, 9 and 10. Caspase-3 was first cloned by Fernandes-Alnemri and colleagues (1994) and shown to encode a 32 kDa cysteine protease homologous to CED-3, caspase-1 and caspase-2 that was capable of inducing apoptosis when overexpressed in insect cells. By virtue of the homology shared with caspase-1, the active caspase-3 enzyme was thought to consist of two subunits 20 kDa and 11 kDa in size. Nicholson and colleagues (1995) subsequently demonstrated by electrospray mass spectroscopy analysis that the subunits comprising active caspase-3 are 17 kDa and 12 kDa in size. Unlike caspase-1, caspase-3 lacks an amino terminal prodomain. Caspase-3 was subsequently identified as the protease responsible for cleaving poly(ADP-ribose)

polymerase (PARP) during apoptosis (Nicholson *et al.*, 1995, Tewari *et al.*, 1995b). PARP is a nuclear enzyme involved in DNA repair and was one of the first cellular substrates shown to be cleaved in apoptotic cells (Kaufmann, 1989). The cleavage of PARP from a 115 kDa protein to an 85 kDa fragment first described by Kaufmann (1989) is one of the most commonly used hallmarks of apoptotic cells. The substrate specificity of caspase-3 was determined from the DEVD sequence at which PARP is cleaved, and tetrapeptides of this sequence are still routinely used for the detection and inhibition of caspase-3 activity. Comparison of the effect of caspase-1 specific inhibitors Ac-YVAD-CHO and CrmA on caspase-3 activity showed that these were much less effective at inhibiting caspase-3 than was DEVD-CHO (Nicholson *et al.*, 1995), confirming a significant divergence in substrate specificity between these two caspases.

1.7.2 Crystal Structure of Caspase-3

Determination of the crystal structure of caspase-3 complexed with a specific tetrapeptide inhibitor revealed homology to caspase-1 structure and also highlighted structural differences responsible for the divergence in substrate specificity of these two proteases (Rotonda *et al.*, 1996, Mittl *et al.*, 1997). As with caspase-1, active caspase-3 is a tetramer of two large and two small subunits. The most marked difference in the substrate binding region of the enzymes is at the P₄ position, consistent with the caspase-3 preference for aspartate in this position as opposed to the caspase-1 preference for tyrosine or a hydrophobic residue. The enzyme subsite that binds the P₄ substrate residue in caspase-3 differs in both spatial and chemical composition from that of caspase-1, being a compact pocket that closely surrounds the P₄ aspartate side chain as distinct from the large, shallow region in caspase-1 that binds the more extensive

tyrosine side chain (Rotonda *et al.*, 1996). The regions of caspase-3 and caspase-1 that bind the P₁-P₃ residues of the substrate share a higher level of similarity, with most conservation displayed at the P₁ binding position. Conserved homology at this region probably accounts for the absolute requirement of all caspases for aspartate at P₁.

1.7.3 The Role of Caspase-3 in the Apoptotic Pathway

Several recent studies have attempted to analyse the specific role that caspase-3 plays in the apoptotic pathway. Caspase-3 null mice die at 1-3 weeks of age and their brain development is profoundly disturbed due to decreased apoptosis (Kuida *et al.*, 1996). This suggests that caspase-3 plays a critical role in the execution of apoptosis during development, at least in cells of the central nervous system. Thymocytes from these mice retain the ability to undergo apoptosis in response to a variety of stimuli, suggesting that other caspases may compensate for caspase-3 function in tissues other than the CNS. Woo and colleagues (1998) used embryonic stem (ES) cells and mouse embryonic fibroblasts (MEF) from mice lacking exon 3 of caspase-3 to investigate the consequences of caspase-3 deficiency on a variety of apoptotic processes in response to a variety of stimuli. They demonstrated that caspase-3 inactivation results in a dramatic decrease in both activation-induced cell death of peripheral T lymphocytes and drug-induced apoptosis of oncogenically transformed MEFs. In caspase-3 null ES cells, UV-induced apoptosis was impaired, but γ -irradiation induced apoptosis was unaffected. These observations demonstrate a death stimulus-specific requirement for caspase-3 activity. Additionally, it was shown that a particular apoptotic stimulus could display a cell type dependence on caspase-3. TNF α was found to induce normal levels of apoptosis in caspase-3 null thymocytes, but decreased apoptosis in oncogenically transformed MEFs. This study also determined that caspase-3 was required for certain

apoptotic events, but not others; selected caspase-3 null cell types undergoing apoptosis did not display chromatin condensation or DNA degradation, but did display PARP cleavage and phosphatidylserine (PS) relocalisation to the outer membrane. These observations suggest that caspase-3 plays an important role in the apoptotic pathway in a tissue and stimulus-specific manner. Jänicke and colleagues (1998) also reported that caspase-3-deficient cells treated with apoptosis inducing agents do not display the characteristic apoptotic hallmarks of DNA fragmentation and cellular blebbing. The fact that these cells still undergo apoptotic death reveals that caspase-3 activity is required for only selected apoptotic events.

1.7.4 Discovery of Caspase-3 Homologues

Caspases-6 and 7 were first identified by Fernandes-Alnemri and colleagues (1995a, 1995b) using a PCR-based approach and were shown to be more homologous to caspase-3 than to caspase-1. Caspase-7 was also identified independently by two other groups (Duan *et al.*, 1996a, Lippke *et al.*, 1996). Caspase-6 is a 293 amino acid caspase that shares 38% sequence identity with caspase-3 and, like caspase-3, lacks a long amino-terminal prodomain. Caspase-6 cleaves PARP to the identical sized fragments produced by caspase-3 *in vitro* and observed in apoptotic cells, indicating that it may fulfill some of the same functions as caspase-3 in apoptosis (Fernandes-Alnemri *et al.*, 1995a). A novel property of caspase-6 is its ability to cleave nuclear lamins (Fernandes-Alnemri *et al.*, 1995a), an activity implied earlier by Lazebnik and colleagues (1995) who showed that cleavage of nuclear lamins in a cell free system required the activity of a caspase distinct from the one that cleaves PARP. Orth and colleagues (1996) also demonstrated that caspase-6 was responsible for lamin cleavage during apoptosis and showed that lamin cleavage activity was restricted to caspase-6 and could not be

mediated by caspase-3 or caspase-7. These results indicate that although caspase-6 is closely related to caspase-3 and caspase-7, it possesses unique substrate specificity and apoptotic function.

Caspase-7 has proven to be a much closer relative of caspase-3 than caspase-6 is, sharing 52% amino acid identity with caspase-3 compared to 37% identity with caspase-6. Caspase-7 is a 303 amino acid protein with a very short amino-terminal prodomain (Fernandes-Alnemri *et al.*, 1995b, Duan *et al.*, 1996a, Lippke *et al.*, 1996). Interestingly, two forms of caspase-7 were identified by Fernandes-Alnemri *et al.*, (1995b) and shown to be the result of alternative splicing; caspase-7 α is the apoptotic form while the alternatively spliced β form encodes truncated protein that does not contain the QACRG active site and its function remains unknown (Fernandes-Alnemri *et al.*, 1995b). Like caspases-3 and 6, caspase-7 can cleave PARP (Fernandes-Alnemri *et al.*, 1995b, Lippke *et al.*, 1996), indicating possible functional similarity between these caspases. Caspase-7 is distributed diffusely throughout the cytoplasm and, like caspases-3 and -6, is proteolytically activated *in vivo* in response a variety of apoptotic stimuli (Chinnaiyan *et al.*, 1996a, Duan *et al.*, 1996a, Orth *et al.*, 1996, Schlegel *et al.*, 1996, Faleiro *et al.*, 1997, Harvey *et al.*, 1997, Keane *et al.*, 1997, MacFarlane *et al.*, 1997, Martins *et al.*, 1997, Mukasa *et al.*, 1997).

1.7.5 Caspases-8, -9 and -10

Although caspases-8,-9 and -10 belong to the caspase-3 subfamily on the basis of sequence similarity and substrate specificity, they differ to other members of this subfamily by virtue of the presence of a long, amino-terminal prodomain (Boldin *et al.*, 1996, Duan *et al.*, 1996b, Fernandes-Alnemri *et al.*, 1996, Muzio *et al.*, 1996,

Srinivasula *et al.*, 1996a, , Srinivasula *et al.*, 1996b, Vincenz and Dixit, 1997). Caspase-8 was the first of these caspases to be identified and was isolated on the basis of its physical interaction with the Fas/APO-1 associated adaptor molecule FADD/MORT1 (Boldin *et al.*, 1996, Muzio *et al.*, 1996). Physical coupling of these two proteins was shown to be mediated by the death effector domain (DED) of FADD/MORT1, binding to analogous domains contained in the amino-terminal prodomain of caspase-8. This interaction serves to recruit caspase-8 to the Fas/APO-1 signalling complex in response to receptor ligation (Boldin *et al.*, 1996, Muzio *et al.*, 1996), resulting in caspase-8 activation (Medema *et al.*, 1997). The proteolytic activation of caspase-8 has recently been shown to occur by self-proteolysis, a process which is dependent on the prior death effector domain-mediated oligomerisation of procaspase-8 molecules (Muzio *et al.*, 1998, Yang *et al.*, 1998). The association of FADD/MORT1 with TRADD, an analogous adaptor molecule that binds to TNF-R1 (Hsu *et al.*, 1995), implicates caspase-8 recruitment and activation in TNF-mediated apoptosis in addition to Fas/APO1-mediated apoptosis. The seminal discovery that caspase-8 could be directly recruited via specific adaptor molecules to cellular death signalling machinery was the first evidence illustrating a mechanism for caspase activation in response to apoptotic signals.

Caspase-10 has a high level of sequence similarity with caspase-8, displaying 52% identity in amino acid composition, excluding the prodomain (Fernandes-Alnemri *et al.*, 1996). In a similar manner to caspase-8, the prodomain of caspase-10 contains two DEDs (Fernandes-Alnemri *et al.*, 1996, Vincenz and Dixit, 1997) that bind the corresponding domain in FADD (Vincenz and Dixit, 1997). This serves to recruit caspase-10 to both Fas/APO1 and TNF receptors in a FADD-dependent manner. An

important role for caspase-8 and caspase-10 in death signalling through these receptors is suggested by the fact that active site mutants of these caspases inhibit both Fas/APO1 and TNF-mediated apoptosis (Vincenz and Dixit, 1997). Both caspase-8 and caspase-10 have been shown to activate several other caspase members *in vitro* (Srinivasula *et al.*, 1996a, Muzio *et al.*, 1997), suggesting that their primary function once activated *in vivo*, may be to mediate the activation of downstream Class II caspases which possess very short prodomains. It is interesting to note that caspases-8 and 10 are the first observed caspases to deviate from the otherwise conserved QACRG sequence surrounding the catalytic cysteine; they contain a QACQG pentapeptide sequence (Fernandes-Alnemri *et al.*, 1996).

Caspase-9 also possesses a deviation of the active site pentapeptide from QACRG to QACQG (Duan *et al.*, 1996b, Srinivasula *et al.*, 1996b). Like caspases-8 and 10, caspase-9 possesses a long amino-terminal prodomain which mediates the recruitment and activation of caspase-9 via association with the adaptor molecule Apaf-1 (Li *et al.*, 1997). As is the case with caspase-8, caspase-9 activation is mediated by intrinsic autocatalytic activity, a process facilitated by Apaf-1 dependent oligomerisation of procaspase-9 molecules (Srinivasula *et al.*, 1998). Caspase-9 is unique in comparison to caspases-8 and 10 in that it appears to be part of a subcellular death complex rather than a cell surface receptor death complex. Caspase-9 was originally identified on the basis of homology with other caspases (Duan *et al.*, 1996b, Srinivasula *et al.*, 1996b) and was subsequently shown to be identical to Apaf-3, (apoptotic protease activating factor-3) a protein factor required for the activation of caspase-3 (Zou *et al.*, 1997). Caspase-9 appears to be the apical caspase in the cytochrome *c*/dATP triggered apoptotic pathway (Li *et al.*, 1997); depletion of caspase-9 from cellular cytoplasmic extracts results in a

significant decrease in the level of caspase-3 activation observed and mutation of the caspase-9 active site results in the abrogation of both caspase-3 activation and cellular apoptotic events. In an analogous association to that of CED-9, CED-4 and CED-3, mammalian homologues Bcl-X_L, Apaf-1 and caspase-9 have been shown to form a ternary complex in mammalian cells (Pan *et al.*, 1998). The interaction of Bcl-X_L with Apaf-1 is interrupted by pro-apoptotic Bcl-2 family members BAX and BAK, suggesting a model of regulation in which the competitive association of pro-apoptotic molecules such as BAX and BAK with Bcl-X_L releases Apaf-1 from the Bcl-X_L complex so that it is free to recruit and activate caspase-9 (Pan *et al.*, 1998). Hu and colleagues (1998) draw similar conclusions from their experiments demonstrating that Bcl-X_L associates with Apaf-1 and caspase-9 and that binding of Bcl-X_L to Apaf-1 inhibits Apaf-1-mediated activation of caspase-9.

1.8 Caspase-2

Caspase-2 was originally identified as a neural precursor cell expressed, developmentally downregulated gene in the murine central nervous system (Kumar *et al.*, 1992) and was subsequently shown to encode a protein with homology to CED-3 and caspase-1 (Kumar *et al.*, 1994, Wang *et al.*, 1994). To date, caspase-2 stands alone in the caspase family based on sequence homology and substrate specificity. Caspase-2 shares 29% sequence identity with caspase-1 and 31% identity with CED-3. Several lines of evidence suggest a role for caspase-2 in apoptosis. Overexpression of caspase-2 induces apoptosis in a variety of cell types (Kumar *et al.*, 1994, Wang *et al.*, 1994, Allet *et al.*, 1996), while expression of antisense caspase-2 in factor-dependent cells delays the initiation of apoptosis induced by factor withdrawal (Kumar, 1995, Troy *et al.*, 1997). The expression of antisense caspase-2 did not, however, protect against apoptosis

induced by superoxide dismutase (SOD1) downregulation (Troy *et al.*, 1997), suggesting that caspase-2 activity may be confined to selected apoptotic pathways. In an analogous manner to antisense caspase-2, an alternatively spliced form of caspase-2 that encodes a truncated protein has been shown to protect against cell death induced by serum withdrawal in Rat-1 and NIH-3T3 cells (Wang *et al.*, 1994, Kumar *et al.*, 1997). Upregulation of caspase-2 mRNA has been observed in response to ischaemia-induced cell death in the Mongolian gerbil and in rat brain (Kinoshita *et al.*, 1997, Asahi *et al.*, 1997), while downregulation of caspase-2 has been observed during gonadotropin-promoted follicular survival (Flaws *et al.*, 1995). Like other Class I caspases (eg. caspases-8, -9 and -10), the amino-terminal prodomain of caspase-2 has recently been shown to mediate association with a death adaptor molecule, RAIDD/CRADD (Duan and Dixit, 1997, Ahmad *et al.*, 1997). RAIDD interacts with RIP, a protein kinase that associates with the TNF receptor through TRADD. The recently identified death receptor DR3/Apo3/WSL-1/TRAMP is closely related to TNFR1 and has been shown to bind TRADD and subsequently recruit RIP and FADD molecules (Bodmer *et al.*, 1996, Chinnaiyan *et al.*, 1996b, Kitson *et al.*, 1996, Marsters *et al.*, 1996). It is therefore feasible that caspase-2 may be recruited to the DR3 death receptor as well as to the TNF-R1 and Fas/APO-1 receptors in response to apoptotic signals, and thereby initiate the cascade of proteolysis that mediates receptor-induced cell death. Caspase-2 has recently been shown to undergo rapid activation in response to a variety of apoptotic stimuli in various cell lines that are sensitive to apoptosis (Harvey *et al.*, 1997, MacFarlane *et al.*, 1997, Harvey *et al.*, 1998), but not in cells that are resistant to apoptosis (Harvey *et al.*, 1997). The activation of caspase-2 in response to apoptotic signals other than those mediated through death receptors indicates that caspase-2 may be recruited to other, as yet unidentified death signalling complexes. The most recent

and compelling evidence of caspase-2 function in apoptosis comes from studies in caspase-2 null mice (Bergeron *et al.*, 1998). These mice have excess numbers of female germ cells and oocytes from these animals are resistant to apoptosis induced by doxorubicin, suggesting that caspase-2 plays a role in both developmental apoptosis and in apoptosis induced by cytotoxic drugs in this cell type. In addition, caspase-2 null B lymphoblasts are resistant to apoptosis mediated by granzyme B and perforin, demonstrating that caspase-2 has an important role in the response of these cells to cytotoxic lymphocyte-mediated killing.

Although no cellular substrates of caspase-2 have yet been identified, studies with peptide substrates indicate that caspase-2 is unique in the caspase family in that it has preference for a residue in the P₃ position and is only weakly active on a tetrapeptide substrate (Talanian *et al.*, 1997). Caspase-2 prefers the sequence VDVAD↓, with a strong preference for aspartate in the P₄ position similar to caspases-3 and -7. The unique substrate specificity of caspase-2 was confirmed in a recent study which demonstrated that caspase-2 does not cleave other caspase family members *in vitro*, even though they contain DXXD sequences (Ahmad *et al.*, 1997). Correspondingly, the peptide inhibitor Ac-DEVD-CHO is a relatively poor inhibitor of caspase-2 (K_i 1.7μM) compared to Ac-VDVAD-CHO which inhibits caspase-2 with a K_i of 3.5 nM (Talanian *et al.*, 1997). All but the initial observations that caspase-2 induces apoptosis in mammalian cells when overexpressed were unpublished at the time this PhD study was initiated.

1.9 Caspase Substrates in Apoptosis

Apoptosis is accompanied by the specific degradation of a number of cellular proteins which serve to mediate the apoptotic process. Although the caspase-mediated cleavage of PARP is one of the hallmarks of apoptotic cell death, it is not sufficient to mediate apoptosis, as mice deficient in PARP develop normally (Wang *et al.*, 1995). Many other cellular substrates that are cleaved during apoptosis have since been described. These include nuclear proteins, cytoskeletal proteins and cytoplasmic proteins. One of the first nuclear proteins other than PARP found to be cleaved during apoptosis was the U1 small ribonucleoprotein particle 70 kdal protein (U1-70 kdal) which is involved in mRNA splicing (Casciola-Rosen *et al.*, 1994). Cleavage of U1-70 kdal occurs in response to UV irradiation, nutrient deprivation, Fas/APO1 and TNF-induced apoptosis (Casciola-Rosen *et al.*, 1994, Tewari *et al.*, 1995a) and appears to be mediated by caspase-3 (Casciola-Rosen *et al.*, 1996). A second nuclear protein shown to be cleaved during apoptosis is the catalytic subunit of the DNA repair enzyme, DNA-dependent protein kinase (DNA-PK_{CS}) (Casciola-Rosen *et al.*, 1995, Song *et al.*, 1996a, Song *et al.*, 1996b, McConnell *et al.*, 1997). DNA-PK_{CS} is cleaved in a variety of cell types in response to a variety of stimuli. Song and colleagues identified that caspase-3, but not caspases-1, 4 or 6, executes DNA-PK proteolysis to liberate the protein fragments observed in apoptotic cells (1996a). Other nuclear proteins cleaved by caspases during apoptosis include the nuclear mitotic apparatus protein (NuMA) (Hsu and Yeh, 1996, Weaver *et al.*, 1996) and the sterol regulatory element binding proteins 1 and 2 which are involved in regulating cholesterol biosynthesis (Wang *et al.*, 1995, Pai *et al.*, 1996, Wang *et al.*, 1996). The nuclear lamins A, C (Ucker *et al.*, 1992, Lazebnik *et al.*, 1995) and B1 (Kaufmann, 1989, Oberhammer *et al.*, 1994, Lazebnik *et al.*, 1995) are all cleaved during apoptosis induced by a range of stimuli, a process which contributes to

dismantling of the nuclear architecture (Rao *et al.*, 1996). As previously mentioned, the cleavage of lamin A is mediated specifically by caspase-6, but not by caspase-3 or caspase-7 (Orth *et al.*, 1996). Cleavage of the proteins mentioned above may reflect the shutdown of processes which require cellular energy and in some cases, may contribute to the morphological changes that take place in apoptotic cells.

Cytoskeletal proteins cleaved by caspases during apoptosis include actin (Kayalar *et al.*, 1996, Mashima *et al.*, 1996), fodrin (Martin *et al.*, 1995b, Cryns *et al.*, 1996, Vanags *et al.*, 1996) and Gas2, a microfilament protein (Brancolini *et al.*, 1995). Cleavage of gelsolin, an actin associated protein, liberates a protein fragment that promotes the rounding up of cells that is characteristic of apoptosis (Kothakota *et al.*, 1997). The caspase-mediated cleavage of PAK2, a serine/threonine kinase involved in regulation of the actin cytoskeleton, results in its activation, contributing to the formation of apoptotic bodies (Rudel and Bokoch, 1997). The cleavage of cytoskeletal proteins therefore appears to facilitate the morphological changes in cell shape that are characteristic of apoptotic death. Cytoplasmic substrates that are cleaved during apoptosis include Nedd4, a ubiquitin protein ligase that is involved in ubiquitin-mediated protein degradation (Harvey *et al.*, 1998), protein kinase C δ (Emoto *et al.*, 1995), D4-GDI; a GDP dissociation inhibitor for the Rho family GTPases (Na *et al.*, 1996) and PITSLRE kinases which are a family of Cdc-2-like kinases thought to be involved in apoptotic signalling and tumourigenesis (Beyaert *et al.*, 1997). Additional caspase substrates cleaved during apoptosis include the tumour suppressor gene product Rb (Janicke *et al.*, 1996, Chen *et al.*, 1997, Tan *et al.*, 1997), hn RNP C1 and C2 (Waterhouse *et al.*, 1996), the large subunit of replication factor C (Song *et al.*, 1997) and the cytosolic phospholipase A2 (Wissing *et al.*, 1997). While the apoptosis induced cleavage of most

proteins inactivates their normal cellular function, in some cases, caspase-mediated cleavage can contribute to increasing the magnitude of the proapoptotic signal. In the case of the two cyclin A-associated cdk inhibitors p21^{Cip1/Waf1} and p27^{Kip1}, caspase-3 mediated cleavage results in a reduction of their association with nuclear cyclin-cdk2 complexes, leading to a dramatic increase in cdk2 activity. Cdk2 activation appears to be necessary for apoptosis to proceed (Levkau *et al.*, 1998). The serine/threonine kinase Mst1 is also cleaved by caspase-3-like activity during Fas/APO1 and staurosporine induced apoptosis, resulting in removal of the C-terminal regulatory domain (Graves *et al.*, 1998). Removal of this domain leads to increased Mst1 activity, which has been proposed to induce apoptosis by inducing caspase activity and activating the SAPK and p38 MAPK pathways (Graves *et al.*, 1998). All known apoptotic substrates appear to be cleaved during apoptosis by the action of Class II “effector” caspases-3, -6 and -7.

1.10 The DNA Fragmentation Factor/Caspase-Activated DNase

The caspase substrate that mediates the characteristic DNA laddering in apoptotic cells is the DNA Fragmentation Factor (DFF) 45 kDa subunit (Liu *et al.*, 1997) or Inhibitor of Caspase-Activated DNase (ICAD) (Enari *et al.*, 1998, Sakahira *et al.*, 1998). DFF was purified from the cytoplasm of HeLa cells as the protein that induces DNA fragmentation in isolated nuclei following activation by caspase-3. DFF is a novel, heterodimeric protein that is composed of two subunits 40 kDa and 45 kDa in size. Caspase-3 is responsible for cleavage of the 45 kDa subunit at two sites, thus generating the active factor capable of inducing the DNA fragmentation which is a hallmark of apoptosis. ICAD appears to be a murine homologue of DFF 45 (Enari *et al.*, 1998, Sakahira *et al.*, 1998). ICAD and CAD (Caspase-Activated DNase) were identified in the cytoplasm of mouse lymphoma cells. CAD is a 343 amino acid protein which carries

a nuclear localisation signal and was shown by Enari and colleagues to possess DNase activity, while ICAD exists in a long form of 331 amino acids (ICAD-L) and a short form of 265 amino acids (ICAD-S). Both ICAD-L and ICAD-S are able to inhibit CAD DNase activity (Enari *et al.*, 1998). ICAD therefore acts as a chaperone for CAD, inhibiting CAD activity during the absence of apoptotic signals and maintaining localisation of the CAD/ICAD complex in the cytoplasm. Once ICAD is cleaved by caspase-3 or caspase-3 relatives in response to apoptotic stimuli, CAD is liberated to enter the nucleus and degrade chromosomal DNA.

1.11 Granzyme B Functions by Activating Caspases

Cytotoxic lymphocytes mediate the death of their target cells by inducing apoptosis, a process which requires the action of serine proteases termed granzymes which are contained in their cytotoxic granules (reviewed in Smyth and Trapani, 1995). Of the granzymes contained in cytotoxic lymphocytes, granzyme B is the most abundant and demonstrates the rare substrate specificity of cleavage C-terminal to aspartate residues, a property shared only by caspases. This common substrate specificity led to the suggestion that granzyme B may induce apoptosis in target cells by directly activating members of the caspase family (Vaux *et al.*, 1994), and this has been shown to be the case. *In vitro*, granzyme B activates caspases-2, 3, 6, 7, 8, 9, 10, 11, (Darmon *et al.*, 1995, Duan *et al.*, 1996b, Fernandes-Alnemri *et al.*, 1996, Harvey *et al.*, 1996, Orth *et al.*, 1996, Wang *et al.*, 1996, Talanian *et al.*, 1997b) but not caspase-1 (Darmon *et al.*, 1994). Granzyme B cleaves caspases-3, 7 and 10 at an IXXD sequence to liberate the large and small subunits which comprise the active enzyme (Fernandes-Alnemri *et al.*, 1996). Interestingly, granzyme B has recently been shown to directly cleave several caspase substrates including DNA-PK_{CS} and NuMA, yielding cleavage products distinct

from those produced in response to treatment with caspases (Andrade *et al.*, 1998). Talanian and colleagues (1997b) have demonstrated that although granzyme B mediated apoptosis primarily operates via the activation of caspases, both growth arrest and cell death can occur in the absence of caspase activity. This suggests a mechanism whereby cytotoxic lymphocytes retain the ability to effectively kill target cells in the event of a block in the apoptotic pathway (for example due to the presence of viral or cellular caspase inhibitors). The ability of granzyme B to cleave and activate both caspases and caspase substrates potentiates an extremely efficient mechanism of initiating the death program in cells targeted by cytotoxic lymphocytes.

1.12 Regulation of Caspase Activity

1.12.1 Viral Inhibitors of Apoptosis

Viruses rely on cellular machinery to survive and reproduce and as such, it is to their advantage to prolong the lifespan of their host cells by inhibiting apoptosis. Many viruses carry proteins specifically for this function, some of which directly target caspases in order to prevent host cell apoptosis. While the previously mentioned cowpox virus encoded inhibitor protein CrmA has the highest affinity for caspases-1 and -8, the baculovirus protein P35 has a broader range of caspase targets (Bump *et al.*, 1995, Xue and Horvitz, 1995, Dorstyn and Kumar, 1997). P35 inhibits the apoptosis of baculovirus infected insect cells (Clem *et al.*, 1991) and the developmental apoptosis that occurs in *C. elegans* (Sugimoto *et al.*, 1994) and *Drosophila* (Hay *et al.*, 1994). P35 has also been shown to inhibit the apoptosis of a variety of mammalian cells induced to undergo apoptosis by factor deprivation (Rabizadeh *et al.*, 1993, Martinou *et al.*, 1995, Dorstyn and Kumar, 1997), γ -irradiation (Dorstyn and Kumar, 1997) and ligation of the Fas/APO-1 and TNF receptors (Beidler *et al.*, 1995). The mechanism of p35 caspase

inhibition was shown to reside in complex formation between the respective caspase and p35 due to the recognition of p35 as a substrate (Bump *et al.*, 1995). This was confirmed by Xue and Horvitz (1995) and Bertin and colleagues (1996) who showed that caspase-mediated cleavage of p35 between Asp-87 and Gly-88 was absolutely required for its inhibitory action. The baculoviral inhibitor of apoptosis (*iap*) gene was first identified in a screen for baculoviral genes that could complement a *p35* mutation (Clem *et al.*, 1991) and homologues of *iap* have subsequently been found in two other baculoviral strains (reviewed in Clem *et al.*, 1996). The baculovirus IAPs inhibit the apoptosis of insect, *Drosophila* and mammalian cells induced by a variety of stimuli (Clem *et al.*, 1996). It appears that IAPs inhibit apoptosis by preventing caspase activation; IAPs are not able to prevent apoptosis induced by already active caspases (Seshagiri and Miller, 1997b).

Recently, another class of virally encoded anti-apoptotic proteins has been discovered and termed v-FLIPs (viral FLICE-inhibitory proteins). These proteins have homology with the DEDs of FADD and caspase-8 (Bertin *et al.*, 1997, Thome *et al.*, 1997) and mediate the inhibition of apoptosis by binding to FADD and preventing subsequent caspase-8 recruitment and activation (Thome *et al.*, 1997). v-FLIPs therefore act at the apex of the apoptotic cascade induced by death receptors, preventing caspase activation.

1.12.2 Mammalian Inhibitors of Apoptosis

The activation of caspases in response to apoptotic stimuli is an integral component of the apoptotic pathway and as such, is likely to be under stringent control so that inappropriate caspase activation does not occur. A number of mammalian apoptosis inhibitors exist which regulate caspase activity. Mammalian homologues of both the

baculoviral inhibitors of apoptosis and v-FLIPs were identified subsequent to the initial description of their viral counterparts. Five human relatives of baculoviral IAPs have now been described; NAIP (Roy *et al.*, 1995, Liston *et al.*, 1996), cIAP1/HIAP-2/hMIHB, cIAP2/HIAP-1/hMIHC, XIAP/hILP/MIHA (Rothe *et al.*, 1995, Duckett *et al.*, 1996, Uren *et al.*, 1996) and survivin (Ambrosini *et al.*, 1997, Lu *et al.*, 1998). In a similar manner to their viral counterparts, mammalian IAPs are able to inhibit apoptosis induced by a variety of stimuli (Duckett *et al.*, 1996, Liston *et al.*, 1996, Dorstyn and Kumar, 1997). XIAP, cIAP1 and cIAP2 appear to exert their apoptosis inhibitory function by directly binding to and specifically inhibiting caspases-3, 7 and 9, but not caspases-1, 6, 8 or 10 (Devereaux *et al.*, 1997, Roy *et al.*, 1997, Devereaux *et al.*, 1998). These inhibitors could therefore conceivably be effective against a wide variety of apoptotic stimuli, which either involve caspases-3 and 7 as downstream, effector proteases, or which initiate an apoptotic cascade via caspase-9. NAIP (Neuronal apoptotic inhibitory protein) was initially characterised on the basis of its association with spinal muscular atrophy (Roy *et al.*, 1995), suggesting that loss of function of a neuronal apoptosis inhibitor could contribute to the inappropriate depletion of spinal cord motor neurons in this disease.

Mammalian FLIP (for **FLICE**-inhibitory protein) exists in a short (FLIP_s) and a long (FLIP_L) form, both of which inhibit apoptosis induced by all known human death receptors (Irmeler *et al.*, 1997). FLIP_s contains two death effector domains and is similar in structure to the v-FLIPs, while FLIP_L contains a caspase-like domain in addition to the death effector domains. Interestingly, the catalytic cysteine residue of caspase family members is replaced by a tyrosine residue in FLIP_L (Irmeler *et al.*, 1997) and additional divergence of other catalytically important caspase regions renders FLIP_L catalytically

inactive (Rasper *et al.*, 1998). FLIP_S and FLIP_L are both able to interact with FADD and caspase-8 to mediate inhibition of death receptor-induced apoptosis, with FLIP_L protein levels subject to regulation, both in normal tissue homeostasis and in disease (Irmeler *et al.*, 1997, Rasper *et al.*, 1998). A recent study demonstrates that the cellular levels of FLIP are upregulated in T lymphocytes in response to T cell activation via the novel cell surface signalling molecule Toso, thereby protecting them from inappropriate self-killing through death receptor mediated apoptosis (Hitoshi *et al.*, 1998). FLIP has been identified by a number of other groups in rapid succession and is also known as CASH, Casper, CLARP, FLAME-1, I-FLICE, MRIT and Usurpin (Goltsev *et al.*, 1997, Han *et al.*, 1997, Hu *et al.*, 1997, Inohara *et al.*, 1997, Shu *et al.*, 1997, Srinivasula *et al.*, 1997, Rasper *et al.*, 1998). A recently identified regulator of TRAIL-induced death receptor signalling located at the cell surface, is the decoy receptor (Pan *et al.*, 1997, Sheridan *et al.*, 1997). The decoy receptor TRID/DcR1, possesses an extracellular ligand binding domain and a transmembrane domain, but lacks an intracellular signalling domain. This molecule is therefore capable of binding the death ligand TRAIL, but does not transmit the resultant death signal. Decoy receptors may provide a mechanism of preventing the apoptosis of normal tissues while allowing the apoptosis of cells that have lost decoy receptor expression, such as transformed cells, to occur (Pan *et al.*, 1997, Sheridan *et al.*, 1997).

In an analogous manner to the protective action of CED-9 upstream of CED-3 and CED-4 in the *C. elegans* apoptotic pathway, Bcl-2 has been shown to act upstream of caspases to prevent their activation in the mammalian apoptotic pathway (Chinnaiyan *et al.*, 1996a, Erhardt and Cooper, 1996, Srinivasan *et al.*, 1996, Perry *et al.*, 1997, Estoppey *et al.*, 1997). The mechanism of caspase inhibition by Bcl-2 appears indirect;

Bcl-2 cannot inhibit caspase function post caspase activation (Perry *et al.*, 1997). While CED-9 appears to be the sole protein of its kind in *C. elegans*, there exists a family of Bcl-2-like molecules in mammals (reviewed in White, 1996). Some of these, including Bcl-2, Bcl-X_L, Mcl-1 and Bcl-W promote cell survival, while others such as Bax, Bcl-X_S, Bik, Bad and Bak promote cell death and are probably functional homologues of the previously mentioned *C. elegans* EGL-1 protein. All members of the Bcl-2 family share conserved domains which facilitate the formation of homo- and heterodimers which regulate their activity. As an example, the pro-apoptotic molecule Bax is able to heterodimerise with Bcl-2, thereby abrogating the ability of Bcl-2 to inhibit apoptosis (Oltvai *et al.*, 1993). The ratio of anti-apoptotic to pro-apoptotic Bcl-2 family members is therefore one stage at which the decision between the induction versus protection of apoptosis may be made (reviewed in Oltvai and Korsmeyer, 1994). As discussed earlier, recent evidence has shed light on the mechanism of apoptosis inhibition by anti-apoptotic Bcl-2 family members. Initial studies with the CED-9, CED-4 and CED-3 proteins demonstrated that physical interaction between CED-9 and CED-4 attenuates the CED-4-mediated activation of CED-3, thereby inhibiting CED-4-induced apoptosis (Chinaiyan *et al.*, 1997, Spector *et al.*, 1997, Wu *et al.*, 1997). Analogous studies with mammalian homologues Bcl-X_L, Apaf-1 and caspase-9 demonstrate that these proteins are also able to physically associate (Hu *et al.*, 1998, Pan *et al.*, 1998) and that Bcl-X_L inhibits Apaf-1-dependent activation of caspase-9 by preventing Apaf-1/caspase-9 interaction (Hu *et al.*, 1998). These studies suggest that Bcl-2 and its anti-apoptotic relatives inhibit caspase activation by binding and rendering unavailable the Apaf-1-like adaptor molecules required to initiate caspase activation. It has also been demonstrated that Bcl-2 functions to block the release of cytochrome *c* from mitochondria, a process which inhibits the cytochrome *c*-dependent activation of caspase-3 (Kluck *et al.*, 1997,

Yang *et al.*, 1997). The mechanism by which Bcl-2 inhibits apoptosis could therefore be a combination of preventing caspase activation by a) sequestering Apaf-1-like molecules and b) abrogating the mitochondrial release of cytochrome *c*.

1.13 Aims of this project

This project concentrates on the biochemical analysis of caspase-2 activity. At the time that this project was commenced, only the initial study describing the cloning, expression and apoptosis inducing activity of caspase-2 was published (Kumar *et al.*, 1994). The stage was therefore set to address the questions that ultimately contribute to defining the role that caspase-2 plays in the apoptotic pathway. The specific aims of this project were:-

1. To determine whether procaspase-2 is proteolytically processed into two subunits homologous to those which constitute the caspase-1 enzyme and to identify possible mediators of this process.
2. To investigate whether caspase-2 is activated *in vivo* in response to a range of apoptotic stimuli and to delineate at which stage of the apoptotic pathway this occurs.
3. To identify the amino acid residues at which procaspase-2 is cleaved to generate active caspase-2 and to analyse the effect that each site has on the catalytic activity of caspase-2.
4. To assess the role of the caspase-2 prodomain on the dimerisation, processing and activation of the proenzyme.

Chapter 2

Materials and Methods

2.1 Tissue Culture

2.1.1 Cell lines and culture conditions

The human megakaryoblastic cell line Mo7e (Avanzi *et al.*, 1988), obtained from Dr J.-P. Levesque (Hanson Centre for Cancer Research, Adelaide), was maintained in suspension culture in α MEM (GibcoBRL) supplemented with 10% foetal bovine serum (FBS) and recombinant human interleukin-3 (IL-3) at a final concentration of 2 ng/ml (Amgen). The human Jurkat T-lymphoblast leukaemia cell line was obtained from Prof A. Lopez (Hanson Centre for Cancer Research, Adelaide) and was maintained in suspension culture in RPMI 1640 (GibcoBRL) supplemented with 10% FBS. The adherent human HeLa cervical carcinoma cell line, obtained from Dr J. Carr (Division of Virology, IMVS, Adelaide), was cultured in Dulbecco's modified Eagle's medium (DMEM) (GibcoBRL) which was modified by the addition of 7.5% bi-carbonate and supplemented with 10% FBS.

The murine factor-dependent myeloid progenitor cell line FDC-P1 (Dexter *et al.*, 1980), obtained from Dr T. Gonda (Hanson Centre for Cancer Research, Adelaide), was cultured in DMEM supplemented with 10% FBS and 50 units/ml murine granulocyte-macrophage colony stimulating factor (GM-CSF) (kindly provided by Dr T. Gonda). The adherent murine fibroblast NIH-3T3 cell line was grown in DMEM supplemented with 10% FBS.

All cell lines were incubated at 37°C in a humidified atmosphere containing 5% CO₂. Cells were maintained in log phase ($1-10 \times 10^5$ cells/ml) at greater than 95% viability. Cell density was calculated using a haemocytometer and cell viability assessed by trypan blue exclusion. In the case of adherent cell line passage, medium was removed

by aspiration, cells were washed with phosphate-buffered-saline (PBS) and incubated briefly in trypsin (0.054%w/v trypsin (Difco), 0.54 mM EDTA in Hank's Balanced Salt Solution (HBSS)) to release them from the flask surface. Medium containing 10% FBS was then added to inactivate trypsin and cells were counted and subcultured.

2.1.2 Cryopreservation of cells

Cells were harvested in log phase and resuspended at $5-10 \times 10^6$ cells/ml in medium/10% FBS. An equal volume of cryoprotectant (30% heat-inactivated FBS, 20% dimethyl sulphoxide (DMSO) (BDH, Merck), 50% RPMI 1640) was added slowly and cell suspension dispensed in 1 ml aliquots into 1 ml cryotubes (Nunc). Cells were then frozen by controlled rate freezing and stored in liquid nitrogen-filled tanks.

2.1.3 Thawing cryopreserved cells

Cells were thawed rapidly in a 37 °C water bath, transferred to 10 ml conical tube and an equal volume of medium was added slowly, followed by a 5 min incubation. An equal volume of medium was added once more followed by a 5 min incubation, then the cells were washed three times in 10 ml medium. Cells were then seeded into an appropriately sized tissue culture flask in medium supplemented with 10% FBS.

2.2 DNA Manipulations

2.2.1 Restriction endonuclease digestion

DNA was digested with the desired restriction endonucleases (Pharmacia or New England Biolabs) in a 10-50 µl total volume comprising DNA, 1-5 units restriction enzyme/µg DNA, 1x digestion buffer (specific for each restriction enzyme) and sterile H₂O for 1-3 hours at 37 °C. Reactions were terminated by either heat inactivation at 65

°C or 85 °C or by the addition of DNA loading dye which was added immediately prior to analysis by gel electrophoresis.

2.2.2 Analysis and separation of DNA fragments

DNA was analysed by electrophoresis in agarose gels of the appropriate percentage to retain fragments of the expected size (0.8-2% agarose dissolved in Tris-Acetate-EDTA buffer (TAE, 40 mM Tris-Acetate, 1 mM EDTA)). Prior to electrophoresis, 10x DNA loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol) was added to a final concentration of 1x and the DNA then loaded onto gels immersed in TAE buffer. Routine electrophoresis was performed at 80 -100 Volts. Following electrophoresis, DNA was visualised by staining in ethidium bromide solution (2 µg/ml) and analysis under ultra-violet light.

2.2.3 Purification of DNA fragments from agarose or solution

A) BRESA-CLEAN™

In the case of DNA in solution, 3 volumes of BRESA-SALT™ solution was added and mixed well with DNA solution. In the case of DNA that was excised from agarose following ethidium bromide staining and low energy ultra violet irradiation, the weight of the excised band was measured and a volume of BRESA-SALT™ solution corresponding to 3 volumes of gel was added. The agarose was then dissolved at 55 °C for 5 min and mixed into solution.

Following vigorous vortexing of BRESA-BIND™, 5 µl plus 1 µl/ µg DNA was added to the DNA solution and incubated for 5 min at room temperature. Regular mixing of the solution was performed to maintain BRESA-BIND™ in suspension. The BRESA-

BIND™/DNA complex was then pelleted 10 sec and the supernatant removed. The pellet was washed with BRESA-WASH™ solution in a volume equivalent to that of the BRESA-SALT™ used in step 1 and then pelleted again for 10 sec. Supernatant was again aspirated and all traces of BRESA-WASH™ removed by air drying. DNA was recovered by resuspension of the BRESA-BIND™/DNA complex in a volume of sterile H₂O equivalent to twice the volume of the BRESA-BIND™ matrix used earlier, followed by incubation at 50 °C 5 min. BRESA-BIND™ was then pelleted for 1 min and the supernatant containing the DNA was removed.

B) Phenol/chloroform extraction

In the case of very small amounts of plasmid DNA or PCR product, 1 µl of glycogen (Boehringer Mannheim) was added prior to extraction to facilitate minimal DNA loss. Generally, the volume of DNA solution was increased to 200 µl and an equal volume of phenol/chloroform (1:1) was added and shaken vigorously. The aqueous and solvent layers were then allowed to disperse 3 min and were separated by centrifugation at 9000g 5 min. The upper, aqueous layer was then removed and extracted with an equal volume of chloroform as described above. DNA was precipitated by the addition of 0.1 volume 3M NaAc pH 4.6 and 2.5 volumes of 100% ethanol and incubation at -20°C for 60 min. DNA was recovered by centrifugation at 9000g, 15 min, 4 °C, then washed with 70% ethanol and resuspended in 10 µl - 30 µl sterile H₂O.

2.2.4 End-filling DNA fragments

Restriction digested DNA with 3' overhangs was end-filled to create blunt-ended DNA using Klenow (Pharmacia). Reactions were performed in solution containing DNA, 1x One-Phor-All Buffer Plus (Pharmacia), 0.2 mM dNTPs, 1 unit Klenow and sterile H₂O,

at 37 °C for 30 min. Following end-filling, DNA was purified by either phenol/chloroform extraction or BRESA-CLEAN™.

2.2.5 Kinase treatment of DNA

DNA fragments were phosphorylated by treatment with T4 polynucleotidekinase (T4 PNK) (Pharmacia). Reactions were performed in a solution containing DNA, 6 units T4 PNK, 0.5 mM ATP and sterile H₂O, at 37 °C for 30 min. Following kinase treatment, DNA was purified by either BRESA-CLEAN™ or phenol/chloroform extraction and ethanol precipitation.

2.2.6 Dephosphorylation of DNA

To remove 5' phosphate groups from restriction endonuclease digested DNA, DNA was treated with bovine intestinal phosphatase (BIP) (Boehringer Mannheim). Phosphatase treatment was performed in a 100 µl reaction comprising DNA, 1x OnePhorAll Buffer⁺ (Pharmacia), 2 µl bovine intestinal phosphatase and sterile H₂O. DNA was treated at 37 °C for 30 min and purified by phenol/chloroform extraction and ethanol precipitation.

2.2.7 Ligation of DNA into plasmid vectors

Ligation reactions were performed using a ratio of molecules of insert DNA:vector DNA 4:1. Ligation reactions were done in a 10 µl volume comprising vector DNA, insert DNA, 1x ligation buffer (50 mM Tris-Cl pH 7.6, 10 mM MgCl₂, 10 mM DTT, 50 µg/ml BSA, 1 mM ATP), 1 Weiss unit T4 ligase (Pharmacia) and sterile H₂O, at 4 °C overnight.

2.2.8 Transformation of competent bacterial cells

Chemically competent bacteria were thawed on ice for 20 - 30 min, transferred to 10 μ l ligation reactions, mixed gently and incubated a further 30 min on ice. Reactions were then heat shocked at 42 °C for 90 sec and returned to ice 3 min prior to the addition of 1 ml SOC medium (20 g bacto-tryptone, 5 g bacto-yeast extract, 0.5 g NaCl in 1000 ml sterile H₂O + 2.5 mM KCl, 10 mM MgCl₂, 20 mM glucose, pH 7.0). Cells were then incubated at 37 °C 60 min before plating an appropriate volume onto agar plates containing the required vector resistance antibiotics (ampicillin 100 μ g/ml or ampicillin 100 μ g/ml + kanamycin 25 μ g/ml). In the case of vectors encoding the genes required for α -complementation, isopropylthio- β -D-galactoside (IPTG) (4 μ l of 1M stock) and 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal) (40 μ l of 20 mg/ml stock), both from Progen, were spread onto plates prior to plating of the transformants. Plates were then incubated at 37 °C overnight.

2.2.9 Colony cracking

To analyse large numbers of putative positive transformants in vectors which did not allow for blue/white selection, colony cracking was employed to screen for colonies containing the desired clones. Transformant colonies were picked and touched onto a master plate before being dispersed into 15 μ l cracking solution (50 μ M NaOH, 0.5% SDS, 5 μ M EDTA, 0.1% bromophenol blue) and heated at 65 °C for 15 min. Reaction volumes were then loaded onto 1% agarose gels and electrophoresed at 40 Volts with the level of TAE just below the level of the wells until DNA had entered into the gel, after which the volume of TAE was increased to cover the gel and electrophoresis was performed at 100 Volts. Colonies containing positive clones were then further analysed.

2.2.10 Small scale plasmid purification

A) Boiling method

1 ml aliquots of putative positive cultures were chilled, pelleted, resuspended in 250 μ l lysis buffer (50 mM Tris HCl pH 7.5, 62.5 mM EDTA pH 8, 0.4% Triton X100, 2.5M LiCl) and incubated on ice 5 min. 20 μ l lysozyme (10 mg/ml) was then added and lysates were vortexed before being boiled 1 min. Lysates were then returned to ice 15 min, prior to centrifugation at 9000g, 4 °C, 20 min and supernatants were removed to new tubes. DNA was precipitated by the addition of 500 μ l 100% ethanol and pelleted at 9000g, 4 °C, 15 min. DNA was washed in 70% ethanol, air dried and resuspended in 30 μ l sterile H₂O. Plasmids were then analysed for the presence of insert DNA by restriction enzyme digestion.

B) QIAGEN method

1 ml aliquots of prospective positive bacterial cultures were chilled on ice, pelleted and resuspended in 150 μ l Buffer P1 (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 100 μ g/ml RNase A). 150 μ l Buffer P2 (200 mM NaOH, 1% SDS) was then added and mixed, followed by the addition of 150 μ l Buffer P3 (3M KAc pH 5.5). Lysates were then centrifuged at 9000g for 5 min and supernatants removed to new tubes. DNA was precipitated by the addition of 1 ml 100% ethanol and incubation on ice, 15 min. DNA was then pelleted at 9000g, 15 min, 4 °C, washed with 70% ethanol and resuspended in 100 μ l sterile H₂O.

C) *Alkaline Lysis Method*

1.5 ml aliquots of chilled bacterial cultures were pelleted at 9000g for 1 min and pellets were resuspended in 100 μ l Solution 1 (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl pH 8.0). 200 μ l freshly prepared Solution 2 (0.2 M NaOH, 1% SDS) was then added, mixed gently by inversion and incubated on ice 5 min. 150 μ l Solution 3 (3M KAc pH 4.8) was added, incubated on ice 5 min and lysates were then centrifuged 9000g, 10 min, 4 °C. RNase A was added to supernatants at a final concentration of 20 μ g/ml and incubated 20 min at 37 °C. DNA solution was then extracted twice with 200 μ l phenol/chloroform and DNA was precipitated by the addition of an equal volume of isopropanol and incubation on ice 15 min. DNA was pelleted at 9000g, 15 min, 4 °C, washed with 70 % ethanol and air dried.

To prepare DNA for sequencing, PEG precipitations were performed. In this case, DNA was dissolved in 32 μ l sterile H₂O and precipitated by the addition of 6 μ l 5M NaCl and 40 μ l 13% (w/v) polyethyleneglycol (PEG) (Sigma). After thorough mixing, DNA was incubated on ice 20 min and pelleted at 9000g, 15 min, 4 °C. DNA was then washed in 70% ethanol and resuspended in 10 μ l sterile H₂O.

2.2.11 Large scale plasmid DNA purification

20 ml aliquots of bacterial cultures were pelleted at 5000g and pellets were resuspended in 4 ml Buffer P1 (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 100 μ g/ml RNase A). 4 ml Buffer P2 (200 mM NaOH, 1% SDS) was then added, mixed gently and incubated at room temperature for 5 min. 4 ml chilled Buffer P3 (3M KAc pH 5.5) was added, mixed gently and incubated on ice 15 min. Lysates were then centrifuged at 20 000g, 30 min, 4

°C and supernatants removed and applied to QIAGEN-tip 100 cartridges which had been pre-equilibrated with 4 ml Buffer QBT (750 mM NaCl, 50 mM MOPS pH 7.0, 15% ethanol, 0.15% Triton X-100). Following flow through of the plasmid DNA solution, the columns were washed with 2x 10 ml Buffer QC (1M NaCl, 50 mM MOPS pH 7.0, 15% ethanol) and DNA was then eluted with 5 ml Buffer QF (1.25M NaCl, 50 mM Tris-HCl pH 8.5, 15% ethanol). 3.5 ml isopropanol was added to precipitate DNA and DNA was pelleted by centrifugation at 15 000g for 30 min at 4 °C. Following removal of the supernatant, DNA pellets were air-dried and resuspended in 400 µl sterile H₂O. DNA solution was then transferred to 1.5 ml eppendorf tubes and precipitated by the addition of 0.1 volume NaAc (3M pH 4.6) and 2.5 volumes ethanol and incubation at -20 °C for 60 min. DNA was centrifuged at 9000g, 15 min, 4 °C, washed with 70% ethanol and resuspended in 100 µl TE pH 8.0. To calculate the concentration of plasmid DNA in solution, the optical density at 280 nm was measured ($OD_{280} 1 = 50 \mu\text{g/ml}$).

2.2.12 First strand cDNA synthesis from RNA

The Pharmacia First Strand cDNA Synthesis Kit was used to generate first strand cDNA from total RNA. Synthesis reactions contained 10 µg RNA in a 40 µl volume in DEPC treated-sterile H₂O which was heated to 65 °C for 10 min and then chilled on ice. 2 µl pd(N)₆ random primer (random hexanucleotides at 0.2 µg/ml in aqueous solution), 22 µl reaction mix (cloned FPLCpure ®Murine Reverse Transcriptase, RNAGuard, RNase / DNase free BSA, dATP, dCTP, dGTP, dTTP, in aqueous buffer), 2 µl DTT (1 M) and sterile H₂O to a total volume of 66 µl were then added. Reactions were incubated at 37 °C for 2 h.

2.2.13 Polymerase Chain Reaction (PCR)

A) Primer Purification

Oligonucleotide primers were purified by extraction with butanol. 100 µl deprotected oligonucleotide solution was vortexed vigorously with 1 ml butanol, then centrifuged at 9000g, 1 min. After removal of the supernatant, DNA was air-dried and resuspended in 100 µl sterile H₂O. The optical density at 280 nm was then measured to calculate primer concentration ($OD_{280} 1 = 20 \mu\text{g/ml}$).

B) PCR Primers

p20 a 5' GAG AGA GGA TTC AAT GGT GAT GGT CCT 3'

p20 b 5' GAG AGA GGA TTC ATC TTG CTG GTC GAC 3'

p10 a 5' GAG AGA GGA TTC GCT GGC AAA GAG GAG 3'

p10 b 5' GAG AGA GGA TTC CGT GGG TGG GTA GCC 3'

D347 mut 5' TGC CAG CAG CAC TCT CCT 3'

primer A 5' CCG CTC GAG ATG GCG GCG CCG AGC GGG A 3'

primer G 5' CCG GAA TTC TGA TGG AGA ACA CTG AAA ACT CA 3'

primer H 5' GGT GGA TCC CGG TGA TAA AAA TAG AGT TCT TT 3'

primer I 5' CAG AAT TCC GGG AGG ACC ATC ACC ATT ATC 3'

primer J 5' CAG AAT TCC ATC TAA GGA GTG TTC CGT AGC 3'

primer K 5' CCG GAA TTC TGA TGG AGA ACA CTG AAA ACT CA 3'

primer M 5' CCG GAA TTC TGT CAA AAT CCA TTA AAA ATT TG 3'

CPP32CMUTF 5' ATT ATT CAG GCC GGC CGT GGT ACA G 3'

CPP32CMUTR 5' CTG TAC CAC GGC CGG CCT GAA TAA T 3'

hPARP1 5' CG GAA TCC TTC TAG GTC GTG CGT CGG 3'

hPARP2 5' GGA ATA TAC GGT CCT GCT 3'

The underlined bases in primers D347 mut, CPP32CMUTF and CPP32CMUTR correspond to the mutated bases. Other underlined sequences correspond to restriction endonuclease recognition sites:

GGATTC - *Bam* HI

GAATCC - *Eco* RI

TCTAGA - *Xba* I

CTCGAG - *Xho* I

C) *Taq* amplification

In standard polymerase chain reaction amplification, DNA was synthesised in a 100 µl volume comprising DNA template (5 µl first strand cDNA or 100-500 ng plasmid DNA), 1x amplification buffer, 2.5 mM MgCl₂, 500 ng each forward and reverse primer, 200 µM dNTPs, 0.5 µl AmpliTaq polymerase (Perkin Elmer) and sterile H₂O. After addition of all components to PCR tubes (Treff), light mineral oil was overlaid and the reaction was amplified according to the required conditions. Generally,

reactions were denatured at 95 °C for 7 min, then subjected to 25-30 cycles of denaturation at 94 °C 1 min, primer binding at 50 °C-55 °C (depending on primer composition) 1 min, and primer extension at 72 °C 1 min. A 10 min extension at 72 °C was then performed and reactions were held at 4 °C.

D) *Elongase amplification*

For amplification with slightly higher fidelity than *Taq* polymerase, *Elongase* (a blend of *Taq* polymerase and *Pyrococcus* GB-D polymerase) (Life Technologies) was used. DNA was synthesised in a 50 µl reaction comprising 20 µl of Mix #1 and 30 µl Mix #2. Mix #1 contained 200 µM dNTP mix, 400 nM of each forward and reverse primer, 100 ng template DNA and sterile H₂O to 20 µl. Mix #2 contained 5 µl 1x Buffer A and 5 µl 1x Buffer B (60 mM Tris-SO₄ pH 9.1, 18 mM (NH₄)₂SO₄, 1.5 mM MgSO₄). Following combination of Mixes #1 and #2, they were overlaid with light mineral oil and amplified according to the required conditions. Manufacturer's recommended amplification conditions were 1 cycle of denaturation at 94 °C for 30 sec, followed by 30-35 cycles of denaturation at 94 °C for 30 sec, primer annealing at 50-55 °C (depending on primer composition) for 30 sec and extension at 68 °C for 60 sec per kilobase of DNA. Reactions were then held at 4 °C.

E) *Amplification using Pfu polymerase*

Pfu DNA polymerase (Stratagene) was employed for amplification of DNA when high fidelity was required for subcloning. DNA was amplified in a 100 µl reaction comprising 200 µM dNTPs, 1 µM forward and reverse primers, 250 ng template DNA, 1x reaction buffer (20 mM Tris-Cl pH 8.75, 10 mM KCl, 10 mM (NH₄)₂ SO₄, 2 mM MgCl₂, 0.1% Triton X-100, 0.1 mg/ml BSA) and 2.5 units *Pfu* polymerase.

Amplification conditions were initial denaturation at 95 °C for 5 min, followed by 25-30 cycles of denaturation at 95 °C, primer annealing at 40-55 °C (dependent on primer composition) for 1.5 min and primer extension at 72 °C for 1 min per kilobase of DNA. Reactions were then held at 4 °C.

2.2.14 Site directed mutagenesis

A) Kunkel mutagenesis

Kunkel mutagenesis was performed essentially as described by Kunkel *et al.* (1987), with minor modifications. The plasmid DNA template for mutation was first transformed into the chemically competent bacterial strain CJ236 (*dut⁻ ung⁻ F'*) and transformants were grown overnight on ampicillin containing agar plates. Transformant colonies were then inoculated into 3 x 1.5 ml Luria's Broth cultures containing ampicillin (100 µg/ml) and were grown at 37 °C for 6 h with rapid agitation. 10^7 - 10^8 M13 phage were then added and cultures were grown a further 2 h at 37 °C with agitation prior to the addition of kanamycin to a final concentration of 70 µg/ml and overnight growth at 37 °C.

To recover phage, bacterial cultures were pooled and bacteria pelleted at 5000g, 4 °C, 15 min. 4 ml supernatant, containing phage, was then removed and phage were precipitated by the addition of 0.6 ml 20% PEG/2.5 M NaCl and incubation on ice 60 min. Phage were then pelleted at 5000g, 4 °C, 20 min and resuspended in 1 ml 0.3 M NaAc pH 6.0/1 mM EDTA. Single stranded DNA (ssDNA) was then purified from phage by phenol/chloroform extraction and ethanol precipitation. 1 ml phenol/chloroform was added, the solution vortexed and phases separated by centrifugation at 9000g, 5 min. The aqueous phase was removed and DNA was

precipitated by the addition of 0.1 volume 3M NaAc and 2.5 volumes of 100% ethanol. DNA was pelleted at 9000g, 4 °C, 20 min and resuspended in 100 µl TE pH 8.0. DNA recovery was analysed by gel electrophoresis.

500 ng ssDNA was hybridised to phosphorylated primer containing the required mutation in a 20 µl volume containing 1x EcoRI buffer (50 mM NaCl, 100 mM Tris-HCl, 10 mM MgCl₂, 0.025% Triton X-100), a 10-50 molar excess of phosphorylated primer and sterile H₂O. The reaction was heated to 70 °C and then cooled gradually to room temperature. Second strand DNA was then synthesised in a 100 µl reaction containing 20 µl annealing mix, 1 x reaction buffer (20 mM HEPES-NaOH pH7.8, 2 mM DTT, 10 mM MgCl₂), 200 µM dNTPs, 0.5 mM ATP, 0.5 µl Gene 32 protein (Pharmacia), 1 µl T4 DNA polymerase (Pharmacia), 1 unit T4 DNA ligase (Pharmacia) and sterile H₂O, at 37 °C for 60 min.

The mutated plasmid DNA was transformed into chemically competent XL1-Blue bacteria as previously described and colonies grown overnight at 37 °C on agar plates containing ampicillin (100 µg/ml). Putative positive transformants were then inoculated into 1.5 ml cultures containing ampicillin, grown overnight with vigorous agitation and plasmid DNA was isolated by alkaline lysis purification and PEG precipitation. DNA was then sequenced to confirm that the required mutation had been correctly incorporated.

B) *Quikchange site-directed mutagenesis*

The Quikchange Site-Directed Mutagenesis Kit (Stratagene) was used according to the manufacturer's protocol. Briefly, plasmid DNA template for mutagenesis was amplified in a 50 µl reaction comprising 1x reaction buffer (10 mM KCl, 10 mM (NH₄)₂SO₄, 20

mM Tris-HCl pH 8.8, 2 mM MgSO₄, 0.1% Triton X-100, 0.1 mg/ml nuclease-free bovine serum albumin (BSA)), 50 ng plasmid DNA template, 125 ng of each mutagenic primer, 2 mM dNTP mix, 2.5U *Pfu* DNA polymerase and sterile H₂O. Reactions were overlaid with sterile mineral oil and amplified according to the following protocol: one cycle of denaturation at 95 °C for 30 sec, followed by 12 cycles of denaturation at 95 °C, 30 sec, primer binding at 55 °C, 1 min and extension at 68 °C for 2 min/kb of plasmid length. Reactions were cooled prior to the addition of 1 µl *Dpn* I restriction enzyme and were then incubated at 37 °C for one hour. 5 µl of mutated DNA was then transformed into competent bacterial cells as described previously. Putative positive clones were grown in liquid culture prior to the extraction and sequencing of their plasmid DNA to confirm that the desired mutation had been incorporated.

2.2.15 Sequencing

A) T7 Superbase sequencing

The T7 Superbase Sequencing Kit (Bresatec) was used according to manufacturer's instructions. Briefly, to 2 µg plasmid DNA, 2 µl 2M NaOH was added and the volume increased to 10 µl with sterile H₂O. DNA was incubated at room temperature 10 min before being precipitated by the addition of 3 µl 3 M NaAc pH 4.6, 7 µl sterile H₂O and 60 µl 100% ethanol and incubation at -70 °C 15 min. DNA was pelleted at 9000g, 15 min, 4 °C, washed with 70% ethanol, dried and resuspended in 7 µl sterile H₂O. 2 µl 5x annealing buffer and 50 ng sequencing primer were then added and incubated at 37 °C for 30 min, then at room temperature for 20 min.

To the DNA/primer mix, 2 µl 1x labelling/extension mix, 1 µl 100 mM DTT, 1 µl sterile H₂O, 1 µl α-³²P-dATP and 2 units T7 DNA polymerase were added and

incubated at room temperature 5 min. 3.5 µl of each labelling/extension reaction was then transferred into 2.5 µl of each A, C, G, T termination mix (pre-warmed to 37 °C) and incubated at 37 °C for 5 min. 4 µl Stop/Loading buffer was added to each reaction and heated at 95 °C 5 min prior to electrophoresis on 8% polyacrylamide gels. Following electrophoresis, gels were dried under vacuum at 80 °C and exposed to X-ray film (Kodak) overnight.

B) *Dye Terminator sequencing*

The ABIPRISM Dye Terminator Cycle Sequencing Reaction Kit was used to sequence alkaline lysis purified plasmid DNA. In a sequencing reaction, 8 µl Terminator Ready Reaction Mix (A, C, G, T-Dye Terminator, dITP, dATP, dCTP, dTTP, Tris-HCl pH9.0, MgCl₂, thermal stable pyrophosphatase, AmpliTaq polymerase) was added to 500 ng template DNA and 3.2 pmol sequencing primer. The volume was then made up to 20 µl with sterile H₂O. The reaction was then cycled using a PERKIN ELMER GeneAmp PCR System 9600 according to the following program; [96 °C 10 sec, 50 °C 5 sec, 60 °C 4 min] x 25 cycles, followed by a 4 °C hold. Following cycle sequencing, DNA was precipitated by the addition of 2 µl 3M NaAc pH 4.6 and 50 µl 100% ethanol and incubation on ice 10 min. DNA was then pelleted at 9000g, 15 min, 4°C, washed in 70% ethanol and air dried. DNA was then sequenced using a Perkin Elmer automated sequencer.

2.2.16 *DNA constructs generated*

pGEXp33Caspase-2 lacking the prodomain was constructed by PCR amplification of the region of mouse *CASP-2* cDNA encoding p19 + p12 (amino acids 166-451). The primers used for amplification were p20a and p10b. The resulting PCR product was

purified, digested with *Bam* HI and cloned in frame downstream of GST in the *Bam* HI site of pGEX-2T (Pharmacia).

pBSCaspase-2 D347 was generated by Kunkel mutagenesis using MSN2.4 (Kumar *et al.*, 1994) as a template. Primer **D347 mut** was used to mutate Asp-347→Gly.

pCXN2D135, **pCXN2D326**, **pCXN2D330** and **pCXN2D333** were generated by releasing the 1.35 kb caspase-2 coding region from the respective caspase-2 mutants contained in the pSP73 vector (Allet *et al.*, 1996). *Cla* I and *Xho* I restriction enzymes were used to liberate the caspase-2 coding region, which was then purified, end filled using Klenow and ligated into PCXN2 (Niwa *et al.*, 1991), which had also been digested with *Xho* I, end filled using Klenow and dephosphorylated using BIP.

pCXN2D169 and **pCXN2D347** were generated by releasing the caspase-2 coding region of the D169 and D347 mutants contained in pBluescript (Stratagene) using *Xba* I and *Cla* I restriction enzymes. The resultant caspase-2 fragments were purified, end filled using Klenow and ligated into blunt pCXN2 (prepared as described above).

pCaspase-3-GFP was generated by PCR amplification of the coding region of caspase-3 from a pcDNA3 construct (kindly provided by Dr V. Dixit, Tewari *et al.*, 1995b) using primer G and primer H. The resulting product was purified and cloned into the *Eco* RI and *Bam* HI sites of pEGFP-N1 (Clontech).

pC32F-WT is a GFP-tagged fusion between the prodomain of caspase-2 and caspase-3. The prodomain of caspase-2 was amplified using pMSN2.4 as a template with primers A and I. Caspase-3 was amplified from pcDNA3YAMA using primers K and H. The amplified caspase-2 prodomain was digested with *Xho* I and *Eco* RI and caspase-3 was digested with *Eco* RI and *Bam* HI restriction enzymes. The resulting products were

cloned into the *Xho* I and *Bam* HI restriction sites of pEGFP-N1 in a three-way ligation reaction.

pC32F-Δ9 is a GFP-tagged fusion between the caspase-2 prodomain lacking C-terminal residues 167-169 and caspase-3 lacking the first 9 amino acid residues. This removes a potential aspartate cleavage sequence between the caspase-2 prodomain and caspase-3, ensuring that the caspase-2 prodomain can not be removed by caspase-mediated cleavage. The caspase-2 prodomain was amplified using from pMSN2.4 using primers A and J, while caspase-3 was amplified from pcDNA3YAMA using primers M and H. The resulting products were cloned into the *Xho* I and *Bam* HI restriction sites of pEGFP-N1 in a three-way ligation reaction.

pC32F-WT(C163G) and **pC32F-Δ9(C163G)** are catalytically inactive Cys→Gly mutants of the above mentioned fusion constructs and were generated from pC32F-WT and pC32F-Δ9 using the Stratagene Quikchange method of mutagenesis with primers CPP32CMUTF and CPP32CMUTR.

pBS hPARPt was generated by PCR amplification of nucleotides 93-1156 of human PARP using primers hPARP1 and hPARP2. The amplified product was cloned into the *Eco* RI site of pBluescript.

2.2.17 Isolation of chromosomal DNA from mammalian cells

Cell samples were pelleted at 1500g, 10 min, 4 °C and were washed twice in ice cold PBS before being resuspended in TE pH 8.0 at a concentration of 5×10^7 cells/ml. 500 μl extraction buffer (10mM Tris-Cl pH 8.0, 0.1 M EDTA pH 8.0, 20 μg/ml RNase A, 0.5% SDS) was added to each sample (10 ml extraction buffer per 1 ml cell suspension) and incubated at 37 °C for 1h. Proteinase K was added to a final concentration of 200

µg/ml and incubated at 50 °C for 2 h with frequent, gentle resuspension. Samples were cooled to room temperature and phenol/chloroform extracted by the addition of an equal volume of phenol/chloroform, thorough mixing and centrifugation at 9000g 3 min. The aqueous phase containing chromosomal DNA was then removed using wide-bore tips and transferred to new, 2 ml tubes (Eppendorf). DNA was precipitated by the addition of 0.2 volumes 10M NH₄Ac and 2 volumes 100% ethanol with gentle, thorough mixing. DNA was pelleted at 9000g, 5 min, washed with 70% ethanol and resuspended in 50 µl TE pH 8.0, overnight at 37 °C. An additional treatment with RNase A (20 µg/ml final) was then performed at 37 °C for 30 min. 10 µl chromosomal DNA samples were then analysed for endonucleosomal cleavage by electrophoresis on 1.8% agarose gels.

2.2.18 Transient transfection of mammalian adherent cells

A) Transfection using Superfect

The day prior to transfection, cells were plated out at 8-10 x 10⁵ cells/60 mm dish in 5 ml growth medium. On the day of transfection, 5 µg DNA was mixed with 150 µl of serum and antibiotic free medium and 20 µl-30 µl Superfect reagent was then added and mixed. DNA/Superfect complexes were then allowed to form during a 15 min incubation at room temperature. Medium was aspirated from cells and monolayers were washed with 5 ml PBS. 1 ml complete medium containing serum and antibiotics was then added to DNA/Superfect complexes, mixed and added immediately to cells. After incubating at 37 °C for 3 h, medium was aspirated, cells were washed with 5 ml PBS and were then refed with 3 ml fresh, complete medium. Cells were harvested using cell lifters (COSTAR) at the required time following transfection.

For cells plated out in 6-well trays, cells were seeded at a density of 2.5×10^5 cells/well in 3 ml growth medium. The day of transfection, 2.5 μ g DNA was added to 100 μ l of serum and antibiotic-free medium and 10 μ l Superfect reagent was then added, mixed and incubated 15 min. 600 μ l complete medium was then added to the transfection mix. DNA/Superfect complexes were added to cells immediately following a PBS wash of the monolayers and were incubated at 37 °C for 3 h. Cells were then washed with 2 ml PBS and refed with 3 ml complete medium.

B) *Transfection using FuGENE6*

The day prior to transfection, cells were plated out at 2×10^5 cells per 35 mm dish in 2 ml medium or $8-10 \times 10^5$ cells per 60 mm dish in 3 ml medium. On the day of transfection, 3 μ l or 6 μ l FuGENE 6 (Boehringer Mannheim) reagent was diluted into 100 μ l or 150 μ l respectively, of serum and antibiotic-free medium, and incubated for 5 min at room temperature. The diluted FuGENE 6 reagent was then added dropwise to 1 μ g or 2 μ g plasmid DNA and incubated for 15 min at room temperature. The FuGENE6/DNA mix was then added dropwise to cells with gentle mixing and incubated for 12-18 hours prior to expression analysis.

2.2.19 *Stable transfection of suspension cells*

3×10^5 cells/ml were aliquoted in 5 ml volumes into 60 mm tissue culture dishes. 5 μ g plasmid DNA was then diluted in 150 μ l serum and antibiotic-free medium and 20 μ l Superfect reagent was added and incubated at room temperature 15 min. DNA/Superfect complexes were then added dropwise to the cell suspension and mixed gently. 3 h post transfection, cells were replaced into fresh medium. 4 days post transfection, hygromycin B (Boehringer Mannheim) at a final concentration of 500 μ g/ml was added

to cells to enable selection of transfectants to occur. Cells were drug selected for at least two weeks and then maintained in growth medium containing hygromycin B (500 µg/ml).

2.3 RNA Manipulations

2.3.1 RNA extraction

Suspension cells were harvested, pelleted and resuspended in 200 µl RNazol™ B (BIOTECX LABORATORIES, INC.) per 10⁶ cells. 200 µl chloroform per 2 ml homogenate was then added and shaken vigorously for 15 sec prior to a 5 min incubation on ice. The suspension was then centrifuged at 9000g, 15 min, 4 °C and the aqueous phase containing RNA was removed. RNA was precipitated by the addition of an equal volume of isopropanol and incubation on ice for 15 min. RNA was pelleted at 9000g, 15 min, 4 °C, washed with 75% ethanol, air-dried and resuspended in diethylpyrocarbonate (DEPC) (Sigma) -treated sterile H₂O. Following analysis of RNA yield and integrity, RNA was precipitated by the addition of 0.1 volume 3M NaAc pH 4.6 and 2.5 volumes 100% ethanol and stored at -20 °C.

2.4 Protein Analysis

2.4.1 SDS-PAGE and protein transfer

Polyacrylamide gels of the appropriate percentage were cast and layered with 5% stacking gel. Gels were assembled into Hoefer gel tank apparatus and immersed in protein electrophoresis buffer (25 mM Tris, 250 mM glycine, 0.1% SDS). Samples were prepared for electrophoresis by the addition of an equal volume of protein loading dye (100 mM Tris-HCl pH 6.8, 200 mM DTT, 4% SDS, 0.2% bromophenol blue, 20%

glycerol) and were boiled for 5 min. Samples were then loaded and electrophoresed at 100 volts through the stacking gel and 200 volts through the resolving gel. Following electrophoresis, the gel was prepared for protein transfer by equilibration in protein transfer buffer (49 mM Tris, 39 mM glycine, 0.0375% SDS, 20% methanol). A sheet of polyvinylidene difluoride (PVDF) ("Polyscreen" Dupont) or nitrocellulose (Schleicher & Schuell) and 4 sheets of Whatman cut to the exact size of the gel were also equilibrated in protein transfer buffer. To assemble the gel for semi-dry transfer, 2 sheets of Whatman were placed on the cathode (+), followed by the PVDF filter, the gel and the final 2 sheets of Whatman. Protein was transferred at 130 mA for 1.5 h.

2.4.2 Coomassie staining

To visualise protein, gels were stained in Coomassie Brilliant Blue R-250 (Biorad) (0.25% w/v in 5:4:1 H₂O:MeOH:HAc). Staining was performed for 1 h, followed by destaining in 5:4:1 H₂O:MeOH:HAc for 1 h with 2 changes of destain solution. Gels were then dried under vacuum at 80 °C for 30 min.

2.4.3 Immunoblotting

In preparation for Immunoblotting, protein filters were blocked in 5% skim milk (Diploma) PBS-T (1x PBS, 0.05% Tween 20) solution for 1 h at room temperature or overnight at 4 °C. Primary antibodies and conditions used were as follows; Ich-1L rabbit polyclonal antibody (Santa Cruz Biotech. Inc.) 1/500 for 4 h, PARP rabbit polyclonal antibody (Boehringer Mannheim) 1/2000 for 1 h, Mch3 rabbit polyclonal antiserum (a kind gift of Dr G Cohen) 1/2000 for 2 h, Mch3 mouse monoclonal antibody (a kind gift of Dr Y Lazebnik) 1/1000 for 1 h, CPP32 mouse monoclonal antibody (Transduction Labs.) 1/1000 for 2 h, Bcl-2 mouse monoclonal antibody

(Transduction Labs.) 1/1000 for 2 h, GFP mouse monoclonal antibody (Boehringer Mannheim) 1/1000 for 4 h. Secondary antibodies used were anti-rabbit-IgG conjugated with horseradish peroxidase (HRP) (Amersham Corp.) to detect rabbit polyclonal antisera or anti-mouse-IgG conjugated with HRP (Amersham Corp.) to detect mouse monoclonal antibodies. Both secondary antibodies were used at a dilution of 1/2000 in 5% skim milk PBS-T solution and were incubated for 1 h at room temperature. All primary antibodies were diluted in 5% skim milk PBS-T solution. Following incubation in primary and secondary antibodies, filters were washed, 3 x 5 min and 2 x 10 min in PBS-T solution. Signals were visualised by using the enhanced chemiluminescence (ECL) system (Amersham Corp.). Briefly, filters were exposed to ECL reagents mixed in a 1:1 ratio for 1 min and then exposed to Hyperfilm ECL (Amersham Corp.) for the appropriate time.

2.4.4 Immunoblot stripping

To strip protein from Western Blots in preparation for re-probing, filters were washed 2 x 10 min in PBS-T and then incubated in stripping buffer (100 mM β -mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.8) for 10 min at 50 °C. Following stripping, filters were washed 2 x 10 min in PBS-T and blocked in 5% skim milk PBS-T solution as described above.

2.4.5 Protein Expression

A) Production of caspases in a bacterial expression system

The constructs pGEXp33Caspase-2 and pETNedd2 were both used to produce enzymatically active caspase-2 in a bacterial expression system. Overnight cultures of each construct were inoculated into fresh 10 ml LB cultures containing ampicillin (100

µg/ml). The pGEXp33Caspase-2 culture was inoculated at a 1/25 dilution, while the pETNedd2 culture was inoculated at a 1/10 dilution. pGEXp33Caspase-2 cultures were grown at 37 °C for 1 h prior to induction with 1 mM IPTG and a further 5 h growth at 37 °C with vigorous agitation. pETNedd2 cultures were grown at room temperature for 1 h prior to induction with 1 mM IPTG and a further 3 h growth at room temperature with vigorous agitation. Cultures were then pelleted at 1500g, 10 min, 4 °C and washed in 10 ml ice cold PBS. Bacterial pellets were resuspended in 0.5-1 ml Ice Assay Buffer (25 mM HEPES pH 7.4, 1 mM EDTA, 0.1% CHAPS, 10% sucrose, 5 mM DTT) or caspase-2 buffer (100 mM NaAc pH 6.2, 20% glycerol, 5 mM DTT, 0.5 mM EDTA) pH 5.5 (Talanian *et al.*, 1997) and sonicated on ice until lysis was observed. Lysates were centrifuged at 9000g, 10 min, 4 °C and clarified lysates were removed and stored at -70 °C.

B) Production of ³⁵S-Met labelled proteins by coupled in vitro transcription and translation

cDNAs were transcribed and translated *in vitro* using the Promega TNT™ Coupled Reticulocyte Lysate System. In a standard reaction, ³⁵S-Met labelled protein was generated in a 50 µl reaction volume comprising 25 µl TNT Rabbit Reticulocyte Lysate, 2 µl TNT Reaction Buffer, 1 µl T7 or SP6 polymerase, 1 µl amino acid mixture minus methionine, 4 µl ³⁵S-methionine, 1 µl RNasin Ribonuclease inhibitor, 1 µg DNA template and sterile H₂O. Reactions were incubated at 30 °C for 1.5 h and were used immediately or stored at -20 °C for no more than one week.

2.5 Apoptosis Assays

2.5.1 Transient transfection apoptosis assay

The day prior to transfection, NIH-3T3 cells were plated in 6 well tissue culture plates at a density of 2.5×10^5 cells per well. The following day, cells were co-transfected with 2 μ g Nedd2 expression constructs contained in the pCXN2 vector (Niwa *et al.*, 1991) and 0.5 μ g of the β -galactosidase expression vector (pEF- β gal) (Kumar *et al.*, 1994). The Superfect reagent was used for transfection according to the protocol described above. 24 h post-transfection, cells were analysed for β -galactosidase expression by rinsing with PBS, fixing with 2% formaldehyde, 0.2% glutaraldehyde in PBS for 5 min, rinsing twice with PBS and staining with 0.1% X-gal, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM $MgCl_2$ in PBS for 3 h. After rinsing in PBS, positively stained blue cells were analysed microscopically for apoptotic morphology and photographed. The extent of apoptosis cells was represented as the % of morphologically apoptotic cells among the total number of blue stained transfected cells.

2.5.2 Treatment of cells with apoptotic stimuli

Cells were treated with the following stimuli for various times to induce apoptosis; growth factor and/or serum withdrawal, etoposide (Calbiochem) at a final concentration of 40 μ M, γ -irradiation (30 Gy from a ^{137}Cs source), α -Fas antibody at a final concentration of 200 ng/ml (kindly provided by Dr Andreas Strasser) or 100 ng/ml (Upstate Biotechnology), tumour necrosis factor- α (TNF α) (R&D Systems) at a final concentration of 10 ng/ml together with 10 μ g/ml cycloheximide (Sigma).

2.5.3 Analysis of apoptotic morphology by DAPI staining

Cytosmears of control cells and of cells treated with apoptotic stimuli were prepared by centrifuging 5×10^4 cells per slide at 1500g, 5min and resuspending them in 100 μ l FBS per 5×10^4 cells. Cells were then centrifuged onto slides at 28g, 5 min, using a Cytospin 3 centrifuge (Shandon Scientific Ltd.). Cytosmears were stored at 4 °C prior to fixing and analysis. Cells were fixed in Carnoy's fixative (6:1 ethanol:acetic acid) for 10 min, washed 2x 5 min in PBS, rinsed in sterile H₂O and air dried. Cells were then stained in 4,6-diamino-2-phenylindole (DAPI) (8 μ M) for 1 min, rinsed 3 x in sterile H₂O, air dried, mounted in anti-fade medium (1% propylgalate, 86% glycerol) and visualised by fluorescence microscopy using an Olympus BH2-RFCA fluorescence microscope. Apoptotic cells were scored by the analysis of nuclear morphology which appears condensed and/or fragmented in cells committed to apoptosis.

2.5.4 Preparation of cytoplasmic extracts

Cytoplasmic extracts were prepared essentially as described by Martin *et al.* (1995a) with minor modifications. Following different apoptotic stimuli, cells were harvested at the appropriate timepoints, pelleted at 200g and washed twice in ice cold PBS. Cell pellets were resuspended in 100 μ l ice cold cell extraction buffer (50 mM 1,4-piperazinediethanesulfonic acid (PIPES) pH 7.0, 50 mM KCl, 5 mM EGTA, 2 mM MgCl₂, 1 mM dithiothreitol (DTT) and 0.5 mM phenylmethylsulfonyl fluoride (PMSF)) per 5×10^6 cells. Cells were allowed to swell on ice for 20 min and were then lysed by two cycles of freeze/thawing using dry ice and a chilled water bath. Cell lysis was confirmed by trypan blue uptake. Lysates were centrifuged at 200g for 5 min at 4 °C and supernatants then removed and centrifuged at 9000g for 15 min at 4 °C. The clarified cytosol was removed and either used immediately or stored at -70 °C.

2.5.5 Quantitation of protein concentration in cytoplasmic extracts

Protein concentration was assayed using the BCA Protein Assay Reagent (Pierce). Briefly, several dilutions of cytoplasmic extract were prepared, along with serial dilutions of the protein standard bovine serum albumin (BSA) ranging from 2 mg/ml-0.125 mg/ml. 10 μ l of each protein sample was then incubated with 200 μ l of the kit reagents A and B mixed together in a 50:1 ratio, for 30 min at 37 °C. The optical density at 562 nm for each protein sample was then measured and a standard curve constructed for the BSA measurements. The protein concentration of the cytoplasmic extracts was calculated by comparison with the standard curve.

2.5.6 Cleavage of fluorogenic caspase substrates

Cytoplasmic extracts (10 μ g total protein) or bacterially expressed caspase lysates were incubated with 100 μ M Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (DEVD-AFC), Tyr-Val-Ala-Asp-7-amino-4-trifluoromethyl coumarin (YVAD-AFC) (both from Enzyme Systems Inc.) or 10 μ M Val-Asp-Val-Ala-Asp-Amino-methylcoumaride (VDVAD-AMC) (California Peptide Research, Inc.) at 37 °C for 30 min. Reactions were performed in a final volume of 20 μ l in cleavage buffer (25 mM HEPES pH 7.4, 10% sucrose, 5 mM DTT, 1 mM EDTA, 0.1% CHAPS). The tetrapeptide inhibitors Asp-Glu-Val-Asp-CHO (DEVD-CHO) and Tyr-Val-Ala-Asp-CHO (YVAD-CHO) (both from Bachem) were used at a final concentration of 1 μ M and were pre-incubated with cytoplasmic extracts for 30 min at 37 °C in a 10 μ l volume prior to the addition of fluorogenic substrate. Following the completion of reactions, reaction volumes were transferred to cuvettes and 2 ml sterile H₂O was added to each cuvette. Fluorescence was quantified using an Aminco Bowman Luminescence Spectrophotometer set to excitation

wavelength 400 nm, emission wavelength 505 nm for the AFC-conjugated substrates and excitation wavelength 385 nm, emission wavelength 460 nm for the AMC-conjugated substrate.

2.5.7 Proteolysis assays

In a standard cleavage assay, 5 μ l of ^{35} S-Met (ICN) labelled *in vitro* translated protein was incubated for 3 h at 37 °C in the presence of either cytoplasmic extracts, purified proteases or bacterially expressed caspases. All assays were performed in cell extraction buffer in a final volume of 15 μ l or 20 μ l. Inhibitors were used at the following concentrations; iodoacetamide (Sigma) 50 μ M, leupeptin (Boehringer) 0.5 μ g/ml, aprotinin (Boehringer) 2 μ g/ml, N-tosyl-L-lysyl chloromethylketone (TLCK) (Boehringer) 50 μ M, N-tosyl-L-phenylalanyl chloromethylketone (TPCK) 25 μ M, trans-epoxysuccinyl-L-leucylamido(4-guanidino)-butane (E64) (Sigma) 2.5-100 μ M, YVAD-CMK (Bachem) 0.1 nM-50 μ M, YVAD-CHO (Bachem) 0.1 nM-10 μ M, DEVD-CHO (Bachem) 100 nM-10 μ M. Inhibitors were pre-incubated with cell extracts or isolated proteases for 30 min at 37 °C prior to the addition of *in vitro* translated protein. Reactions were terminated by the addition of an equal volume of 2x protein loading buffer comprising 100 mM Tris-HCl pH 6.8, 200 mM DTT, 4% SDS, 0.2% bromophenol blue and 20% glycerol. Samples were boiled for 5 min, centrifuged at 9000g for 5 min and analysed by SDS-PAGE electrophoresis. Following electrophoresis, proteins were transferred to PVDF (Dupont) or nitrocellulose (Schleicher & Schuell) membrane and visualised by autoradiography.

Chapter 3

Processing of the Caspase-2 precursor by caspases and Granzyme B

3.1 Introduction

The cloning of caspase-2 revealed significant homology with both CED-3 and caspase-1. At the protein sequence level, caspase-2 shares 29% identity (52% similarity) with caspase-1 and 31% identity (55% similarity) with CED-3 (Kumar *et al.*, 1994). Based on this homology, it was postulated that caspases may play an analogous role in the execution of mammalian cell death to that of CED-3 in *Caenorhabditis elegans*. Overexpression of caspase-1 in Rat-1 cells was shown to induce apoptosis (Miura *et al.*, 1993), confirming that caspase-1 may play a role in the apoptotic pathway in addition to its role in the processing of the interleukin-1 β precursor. Likewise, caspase-2 was shown to induce apoptosis when overexpressed in fibroblast and neuroblastoma cell lines (Kumar *et al.*, 1994, Wang *et al.*, 1994).

The initial biochemical characterisation of caspase-1 led to this caspase being the prototype for other members of the family. Caspase-1 is synthesised as a 45 kDa pro-enzyme that is cleaved by self-proteolysis at aspartate residues 103, 119, 297 and 316 to generate two subunits of 20 kDa (residues 120-297) and 10 kDa (residues 317-404) (Thornberry *et al.*, 1994). The mature form of the enzyme was identified by X-ray crystallography to be a tetramer of two (p20 + p10) heterodimers (Walker *et al.*, 1994, Wilson *et al.*, 1994). The shared sequence homology with caspase-1 suggested that caspase-2 may also be processed from its 51 kDa pro-enzyme into subunits of approximately 20 kDa and 10 kDa during the course of enzyme activation. Incubation of *in vitro* translated procaspase-2 at ambient temperature for up to 24 h does not result in detectable pro-enzyme cleavage (Kumar *et al.*, 1994), suggesting either that the correct conformation or concentration of caspase-2 for auto-cleavage is not achieved in this

environment, or that other members of the caspase family are required to mediate caspase-2 cleavage.

The unique substrate specificity of caspases cleaving carboxy-terminal to aspartate residues is shared only by granzyme B, a cytotoxic lymphocyte serine protease that is able to induce apoptosis in target cells in conjunction with the pore-forming protein, perforin (reviewed in Smyth and Trapani, 1995). The shared aspartate cleavage specificity of these two classes of proteases led to the postulation that granzyme B may initiate apoptosis by proteolytically processing and thereby activating members of the caspase family (Vaux *et al.*, 1994). Studies by Darmon and colleagues (1994) demonstrated that granzyme B is unable to activate caspase-1, leading us to investigate the possibility that granzyme B may cleave and activate caspase-2.

The aim of this study was to investigate whether caspase-2 is processed into subunits similar to those that constitute active caspase-1 and to identify possible mediators of this process. This study also aimed to identify whether granzyme B is able to activate caspase-2 to determine whether caspase-2 plays a possible effector role in cytotoxic lymphocyte-mediated cell death. At the time that these studies were commenced, only the initial study of caspase-1 processing was published and caspase-3 had recently been identified.

Results

3.2 Proteolysis of procaspase-2 to subunits 19 kDa and 12 kDa in size by cell extracts

To identify whether cells that are in the process of undergoing apoptosis contain an enzyme activity able to cleave caspase-2, *in vitro* translated, ³⁵S-Met labelled procaspase-2 was incubated with cytoplasmic extracts from healthy and apoptotic cells. Extracts were prepared from the growth factor dependent cell lines FDC-P1 (murine) and Mo7e (human) prior to, and then following, the removal of either GM-CSF, or IL-3 and serum, respectively, from growth medium. Removal of growth factors from the medium of these cells results in the induction of apoptosis. In the case of FDC-P1 cells the onset of apoptosis is rapid, while in Mo7e cells there is a substantial delay before the first signs of apoptosis are observed (N Harvey and S Kumar, unpublished observations). Cytoplasmic extracts from both healthy and growth factor-deprived apoptotic FDC-P1 and Mo7e cells had the ability to cleave procaspase-2 from its 51 kDa pro-enzyme form to subunits approximately 12 kDa and 19 kDa in size in a time and concentration dependent manner (Figure 3.1A, 3.1B, data not shown for Mo7e). Intermediate cleavage products of approximately 39 kDa and 37 kDa were also observed. Interestingly, while the magnitude of procaspase-2 cleavage by apoptotic cells was much greater than that of healthy cells in the case of Mo7e, the magnitude of procaspase-2 cleavage by healthy and apoptotic FDC-P1 cells was not significantly different (data not shown).

Comparison of the primary structure of caspase-2 with that of caspase-1 highlighted a number of candidate aspartate residues at which cleavage could possibly occur to generate two subunits of approximately 10 kDa and 20 kDa (Kumar *et al.*, 1994, Figure 3.1C). The possibility of cleavage at each of these sites was considered in the

interpretation of the following results. Time course studies showed that the first products to appear as a result of p51 procaspase-2 cleavage were approximately 39 kDa and 12 kDa in size (Figure 3.1A). This was followed by the appearance of 37 kDa and 19 kDa fragments. Over a 3 hour incubation time, most of the p51 precursor was cleaved to these smaller products. From this study, it appears that the 39 kDa band may be attributed to the initial removal of the 12 kDa subunit by cleavage at Asp-347 which would leave an intermediate of 39 kDa. The 37 kDa fragment may appear as a result of subsequent cleavage at Asp-333. Release of the 19 kDa large subunit from the 37 kDa intermediate by cleavage at Asp-169 would leave an 18 kDa prodomain fragment which may be further processed at amino terminal aspartate residues to liberate smaller peptides. Alternatively, the large intermediate bands could be generated as a result of cleavage at Asp-333, generating a 14 kDa fragment and a 37 kDa intermediate and cleavage at Asp-135, liberating a 15 kDa prodomain fragment and a 36 kDa intermediate (Figure 3.1B). A faint band of approximately 15 kDa which migrates just above the small fragment is visible in Figure 3.1A, but is more apparent in later experiments (Figure 3.4A, 3.6A, 3.6B). Release of the 19 kDa large subunit and the 12 kDa small subunit from the 36 kDa intermediate would then leave only small proteolytic fragments which would likely comigrate with the dye front. Subsequent studies on caspase-2 expressed in *E. coli* have determined that Asp-169, Asp-333 and Asp-347 are the residues cleaved in procaspase-2 to generate the 19 kDa and 12 kDa subunits which comprise the active enzyme (Xue *et al.*, 1996).

A large proportion of the processed caspase-2 is present as the 36-39 kDa intermediates in these experiments, even after 3 hour incubation with cytoplasmic extracts, indicating that the kinetics of procaspase-2 cleavage by cell extracts may be different at each

aspartate residue. The P₄-P₁ tetrapeptide sequence at the three determined cleavage sites differs; the sequence at Asp-347 is EESD³⁴⁷, at Asp-333 it is DQQD³³³ and at Asp-169 the tetrapeptide sequence is DN GD¹⁶⁹. The initial cleavage at EESD³⁴⁷ or DQQD³³³ giving rise to the small subunit appears to be the most efficient in these studies. The signal intensity of the 19 kDa fragment is relatively weak in these experiments, perhaps due to the presence of only two methionine residues available for labelling in this region compared to the six present in the 12 kDa subunit. The absence of a protein fragment corresponding to the amino-terminal 18 kDa pro-domain is probably due to further cleavage at one or more aspartate residues present in this region. As discussed above, a relatively weak band of approximately 15 kDa that migrates just above the 12 kDa small subunit could be due to cleavage of the prodomain at Asp-135 (Figure 3.1 and subsequent figures). This band is especially visible in caspase-3 treated samples (Figure 3.6), suggesting that caspase-3 has a high affinity for the cleavage site which gives rise to this proteolytic fragment. In all cases, small protein fragments appear to co-migrate with the dye front which may represent small amino terminal polypeptides.

3.3 Inhibition of procaspase-2 proteolysis by protease inhibitors

To further investigate which type of protease is responsible for procaspase-2 cleavage by cellular cytoplasmic extracts, the inhibitor profile of caspase-2 proteolysis was studied. As shown in Figure 2, procaspase-2 cleavage by apoptotic FDC-P1 extracts is inhibited by some serine and cysteine protease inhibitors as well as by peptide analogues of caspase substrates (Figure 3.2).

Procaspase-2 cleavage in these *in vitro* cleavage assay is inhibited by both *N*-tosyl-L-phenylalanyl chloromethyl ketone (TPCK) and *N*-tosyl-L-lysyl-chloromethyl ketone

(TLCK), which inhibit chymotrypsin-like and trypsin-like proteases respectively (Figure 3.2A). TPCK and TLCK have previously been shown to inhibit both apoptosis and cleavage of the caspase substrates PARP and nuclear lamins, in a number of *in vitro* studies by a number of groups (reviewed in Kumar and Harvey, 1995). Procaspase-2 cleavage was not inhibited by leupeptin or aprotinin which are more general serine protease inhibitors.

The cysteine protease inhibitor iodoacetamide inhibited proteolysis of caspase-2 by FDC-P1 cytoplasmic extracts. Iodoacetamide has previously been shown to inhibit the cleavage of PARP by caspases as well as the morphological changes of apoptosis induced by both drug treatment and γ -irradiation in isolated nuclei (reviewed in Kumar and Harvey, 1995). E64, which is known to inhibit certain cysteine proteases, but not caspases, did not significantly inhibit the proteolysis of procaspase-2. The caspase-1 tetrapeptide inhibitor YVAD-cmk blocked the cleavage of procaspase-2 at concentrations of more than 1 μ M (Figure 3.2B). At concentrations lower than 1 μ M, YVAD-cmk partially blocked proteolysis (Figure 3.2C). This indicates that the proteolysis of caspase-2 by FDC-P1 extracts may be due to the activity of caspases other than caspase-1, as YVAD-based tetrapeptide inhibitors have a K_i of approximately 1 nM for caspase-1 (Nicholson *et al.*, 1995).

3.4 Cleavage of procaspase-2 by apoptotic cell extracts

No significant difference was found between the procaspase-2 cleavage activity of healthy and apoptotic FDC-P1 cytoplasmic extracts, while apoptotic Mo7e cells displayed much higher cleavage activity than healthy cells (data not shown). This observation may indicate that FDC-P1 cells contain constitutively active caspases which

perform a function distinct from that of the execution of apoptosis and which does not result in apoptotic death of the cell. To investigate whether this phenomenon is apparent in other cell types, we assessed procaspase-2 cleavage activity of the NIH-3T3 cell line prior to and following serum starvation. We found that extracts from healthy cells showed no appreciable procaspase-2 cleavage activity (Figure 3.3). Interestingly, cytoplasmic extracts prepared from serum-starved, apoptotic cells showed significant procaspase-2 cleaving activity (Figure 3.3), which was completely inhibited by 1 μ M YVAD-cmk. The magnitude of procaspase-2 cleavage mediated by apoptotic NIH-3T3 cells was substantially lower than that observed with FDC-P1 cells. This may reflect differences in the relative levels of caspases in these two cell types, being much lower in NIH-3T3 cells. A greater magnitude of procaspase-2 cleavage was observed when higher concentrations of NIH-3T3 cytoplasmic extract were used (data not shown).

3.5 Caspase activity is constitutive in FDC-P1 cells, but is induced in Mo7e and NIH-3T3 cells

The level of caspase activity in healthy versus apoptotic cells was further examined in fluorogenic cleavage assays in which the tetrapeptide substrates DEVD-AFC and YVAD-AFC were incubated with cytoplasmic extracts from healthy and apoptotic FDC-P1 and Mo7e cells. Extracts from Mo7e cells that were subjected to serum and IL-3 withdrawal, or were treated with etoposide or γ -irradiation, displayed approximately 4-5 fold greater magnitude of DEVD-AFC cleavage than did untreated Mo7e extracts (Figure 3.4A). Conversely, the level of caspase activity in healthy, untreated FDC-P1 cells did not differ significantly from that of cells that were subjected to GM-CSF withdrawal or were treated with etoposide or γ -irradiation (Figure 3.4B). Several controls were performed to ensure that FDC-P1 cells were not being stimulated to

undergo apoptosis during the cell handling process. These included the preparation of cytoplasmic extracts from cells that were a) taken directly from culture and pelleted b) cells that were washed three times in PBS prior to pelleting and c) cells that were washed three times in PBS prior to returning to culture in medium containing GM-CSF. In all cases, including a), in which handling of the cells was minimal, there appeared to be constitutive caspase activity. It is therefore unlikely that the caspase activity observed in untreated cells is due to the induction of apoptosis during preparation and handling of the FDC-P1 cells. The analysis of DNA samples prepared from untreated FDC-P1 cells and FDC-P1 cells that were treated with a variety of apoptosis inducing stimuli confirm that untreated cells are not apoptotic (Figure 3.4C). Cells treated with apoptotic stimuli display characteristic DNA laddering, while untreated cells do not. The analysis of caspase activity in FDC-P1 cells using a cell permeable fluorogenic caspase substrate could potentially clarify these results. In the case of Mo7e cells, a low basal level of DEVD-AFC cleavage activity was routinely observed in untreated cells (eg. Figure 3.4B). This may reflect the activity of the low percentage of apoptotic cells in the normal population (approximately 3%), or alternatively may be a result of the ability of caspases to interact following their release from normal cellular regulatory mechanisms once cytoplasmic extracts are prepared. DEVD-AFC cleavage activity in both FDC-P1 and Mo7e cell extracts was completely blocked by prior incubation of cytoplasmic extracts with DEVD-CHO (Figure 3.4A, 3.4B). No significant activity on the YVAD-AFC substrate was observed by either cell line, confirming earlier observations which suggest that the caspases most related to caspase-3 are likely to be responsible for caspase-2 proteolysis in these *in vitro* cleavage experiments. It therefore appears that caspase-1 and its closest homologues are not activated in response to apoptotic stimuli in either FDC-P1 or Mo7e cells.

The studies performed with NIH-3T3 cells, as well as subsequent studies with a range of cell types and apoptotic stimuli indicate that in all cases except that of FDC-P1 cells, caspase activity increases when cells are treated with apoptotic stimuli. This suggests that perhaps caspases play an additional, as yet unidentified role, in normal, healthy FDC-P1 cells, or that caspase activation occurs very rapidly in these cells upon lysis.

3.6 Processing of procaspase-2 by active recombinant caspase-2

The ability of caspase-1 to auto-process, together with the unique substrate specificity of caspases led us to investigate whether caspase-2 could be responsible for its own cleavage and whether procaspase-2 can be cleaved and activated by other members of the caspase family. By expressing the region of caspase-2 encompassing the 19 kDa and 12 kDa subunits fused to glutathione S-transferase (GST), active caspase-2 was generated in *Escherichia coli* as assessed by activity on the DEVD-AFC substrate. This suggests either that auto-cleavage of caspase-2 lacking the amino-terminal pro-domain can occur in bacterial cells, or that this molecule is able to adopt an active configuration without being proteolytically processed. Immunoblot analysis of this lysate with an antibody raised against the small subunit of caspase-2 demonstrated that caspase-2 lacking the prodomain was not processed in bacteria (Figure 3.5D), indicating that removal of the caspase-2 prodomain may allow caspase-2 to adopt an active configuration in the absence of proteolysis. Recombinant caspase-2 expressed in *E. coli* was further analysed for its ability to cleave *in vitro* translated 51 kDa procaspase-2 in our *in vitro* cleavage assay. As shown in Figure 3.5, procaspase-2 was processed to fragments approximately 39 kDa, 37 kDa, 24 kDa and 12 kDa in size by the bacterially expressed caspase-2, indicating that caspase-2 is capable of self-proteolysis. The pattern of proteolysis generated by caspase-2 was identical to that observed with cellular

cytoplasmic extracts with the exception of one band. The p19 band was not visible in caspase-2 treated samples, but was replaced by a protein fragment of approximately 24 kDa. The appearance of the 24 kDa fragment as opposed to the 19 kDa fragment that is generated by cell extracts indicates that bacterially expressed caspase-2 lacking the prodomain may have a slightly different substrate specificity to that of the caspases that are activated in the cytoplasmic extracts. It is possible that the 24 kDa band is an extension of the 19 kDa subunit and is generated by cleavage at a more amino terminal aspartate residue than Asp-169. These observations indicate that caspase-2 could be one of the proteases activated in apoptotic cells that contributes to *in vitro* procaspase-2 cleavage.

Subsequent to these experiments in which active caspase-2 was produced in a GST fusion lacking the prodomain, active caspase-2 was generated by expressing full length procaspase-2 fused to a carboxy-terminal 6 x His tag in the pET32a vector. Following the identification of VDVAD as the optimal substrate recognition sequence for caspase-2 (Talanian *et al.*, 1997), caspase-2 activity was compared on both the DEVD-AFC and VDVAD-AMC peptide substrates. Both pETprocaspase-2 and pGEXp33Caspase-2 displayed substantially higher activity (10-20 fold) on the VDVAD-AMC substrate compared to the DEVD-AFC substrate, confirming a preference for this pentapeptide sequence (N Harvey and S Kumar, unpublished observation). When utilised in assays to determine relative cleavage activity on *in vitro* translated procaspase-2, pETprocaspase-2 displayed significantly more efficient processing of procaspase-2 than did pGEXp33Caspase-2 (Figure 3.5C). Interestingly, although caspase-2 expressed in both constructs appeared to release the small subunit equally well, full length procaspase-2 liberated the proteolytic fragments corresponding to the p19 subunit and putative

prodomain (denoted by ★, Figure 3.5C) much more efficiently than did caspase-2 lacking the prodomain. The intensity of the 37 kDa intermediate was stronger in the pGEXp33Caspase-2 treated sample, suggesting a failure to further process this polypeptide. Correspondingly, only very weak p19 subunit and prodomain bands were visible in this lane, suggesting that the p19 and prodomain products are derived from the 37 kDa intermediate and that these cleavage sites may not be well recognised by caspase-2 lacking the prodomain. A difference in substrate specificity between the two recombinant caspase-2 enzymes was further confirmed in cleavage assays using *in vitro* translated RSP5 protein as a substrate. RSP5 is a yeast homologue of the mammalian ubiquitin protein ligase, NEDD4, and was observed in our laboratory to be selectively cleaved by pGEXp33Caspase-2, but not by other caspases (K Harvey, unpublished observation). While pGEXp33Caspase-2 cleaved RSP5 efficiently, full length pETprocaspase-2 was unable to cleave RSP5, even at high concentrations (data not shown). Furthermore, mutation analysis of putative caspase-2 cleavage sites in RSP5 demonstrated that the sequences recognised by pGEXp33Caspase-2 were not those most similar to the optimal substrate specificity of full length caspase-2 as described by Talanian and colleagues (1997) (N Harvey, unpublished observations).

To investigate whether full length procaspase-2 produced in our bacterial expression system is composed of subunits indicative of those that constitute the active enzyme, we performed immunoblot analysis on *E. coli* lysate expressing this construct with an antibody raised against the small subunit of caspase-2. In contrast to lysate expressing caspase-2 lacking the prodomain, *E. coli* lysate from cells transformed with pETprocaspase-2 contained the caspase-2 p12 subunit (Figure 3.5D). This observation suggests that p51 procaspase-2 is autoprocessed in bacteria to release p19 and p12

subunits. Intriguingly, even though caspase-2 lacking the prodomain is able to cleave both the VDVAD-AMC peptide substrate and *in vitro* translated procaspase-2, no enzyme processing, as detected by release of the p12 subunit was visible, even using high quantities of lysate (Figure 3.5D). This observation suggests that caspase-2 lacking the prodomain is able to adopt a partially active enzyme configuration in the absence of prior proteolytic processing. The difference in substrate cleavage efficiency between these two caspase-2 constructs may reflect an alteration in tertiary structure due to the absence of the prodomain and consequent lack of self-processing activity in the caspase-2-minus prodomain enzyme. These experiments imply that the caspase-2 prodomain plays an important role in both proteolytic self-processing and the optimal assembly and ultimate activity of the caspase-2 enzyme.

3.7 Cleavage of procaspase-2 by additional members of the caspase family

We further investigated the ability of other members of the caspase family to cleave *in vitro* translated procaspase-2. Cleavage studies revealed that purified recombinant caspase-1 was able to liberate the 19 kDa and 12 kDa subunits of caspase-2 that we observed when procaspase-2 was treated with cytoplasmic extracts from apoptotic cells (Figure 3.6A). This indicates that caspase-1 probably cleaves procaspase-2 at the same residues as the active caspases contained in apoptotic extracts and therefore may be one of the candidate caspases responsible for caspase-2 cleavage. Indeed, caspase-1 is expressed at low levels in FDC-P1 cells and at moderate levels in Mo7e cells (Kumar, 1995 and our unpublished results). However, because the inhibition of procaspase-2 processing requires concentrations of YVAD-cmk greater than that required to inhibit caspase-1 activity, we assessed the ability of several other members of the caspase family, which were identified during the course of this study, to cleave procaspase-2.

Caspases-3, -4, -6 and -7 were all produced in an *E. coli* expression system and tested for their activity on either the DEVD-AFC substrate (caspase-3, caspase-6 and caspase-7) or YVAD-AFC (caspase-4). Approximately equivalent amounts of each caspase (as calculated from activity on fluorogenic substrates) were then used in the *in vitro* caspase-2 cleavage assay. Caspase-3 cleaved caspase-2 very rapidly and efficiently, liberating the 19 kDa and 12 kDa subunits within 15 min of incubation at 37 °C (Figure 3.6B). Caspase-7 was able to cleave procaspase-2 with similar efficiency to caspase-3. All of the 51 kDa procaspase-2 was essentially cleaved by caspase-3 within 90 min (Figure 3.6B). At subsequent timepoints, the signal strength of the 12 kDa band remained constant, while a gradual increase in the strength of the 19 kDa band and decrease in strength of the 39 kDa and 37 kDa intermediates was observed. This observation confirms that the 19 kDa caspase-2 subunit is derived from cleavage of at least one of these intermediates.

The caspase-2 subunits released by caspase-3, -4, -6 and -7 cleavage were all identical in size (Figure 3.6A). In caspase-3 treated samples, a fragment slightly larger than the 12 kDa subunit was particularly obvious (Figure 3.6A, 3.6B, denoted by ★). As previously discussed, this band may represent the amino-terminal region of procaspase-2 generated by cleavage at a residue such as Asp-135, and may be most visible in caspase-3 treated samples due to the magnitude and efficiency of cleavage mediated by this caspase at this site. This band appears to be distinct from that which is generated by caspase-2 auto-cleavage, which is slightly larger than 15 kDa (Figure 3.6A, lane 10). From this study, it appears that caspase-2 itself, as well as the caspases most similar to caspase-3 in substrate specificity, are the most likely members of the caspase family to be responsible for cleaving procaspase-2 in apoptotic cell extracts.

It has been previously suggested that members of the caspase family may be activated in a hierarchy of sequential proteolysis (Kumar and Harvey, 1995, Nicholson and Thornberry, 1997). This model postulates that Class I caspases are initially activated in response to apoptotic signals and act in a uni-directional manner to cleave downstream substrates, including Class II caspases. The activation of these effector caspases then serves to cleave apoptotic substrates, mediating death of the cell. From *in vitro* studies, it appears that at least some Class II caspases are able to cleave Class I caspase family members, an effective mechanism possibly utilised for amplifying caspase activity. We sought to investigate whether caspase-2 possesses the ability to cleave and presumably activate caspase-3, to further delineate the position of caspase-2 in this hierarchy. Recombinant caspase-2 was unable to cleave *in vitro* translated caspase-3 at any concentration tested, even after incubation for 3 h (Figure 3.6C). This suggests either that caspase-3 lies upstream of caspase-2 in the caspase hierarchy, or that caspase-2 is upstream of caspase-3 in the sequence of caspase activation, but does not cleave and activate caspase-3. As discussed above, the ability of caspase-3 to cleave caspase-2 could contribute to the amplification of caspase activation, serving to mediate rapid apoptotic death.

3.8 Cleavage of procaspase-2 by human granzyme B

Cytotoxic lymphocytes mediate target cell killing by the delivery of their granule contents, which include perforin and a variety of proteases, to the target cell (reviewed in Smyth and Trapani, 1995). Perforin intercalates into the target cell membrane, forming pores through which the other granule proteins gain access to the cell. Cells killed by cytotoxic lymphocytes undergo characteristic apoptotic death. Granzyme B, a

serine protease abundantly expressed in the granules of cytotoxic lymphocytes, is the only identified protease which has a substrate specificity similar to that of caspases (Smyth and Trapani, 1995). We sought to identify whether procaspase-2 could also be cleaved by granzyme B, thereby implicating a role for caspase-2 activity in apoptosis induced by cytotoxic lymphocytes. Incubation of procaspase-2 with purified granzyme B resulted in the cleavage of the 51 kDa precursor molecule into 19 kDa and 12 kDa subunits, identical to those observed when caspase-2 was treated with cytoplasmic extracts and isolated caspases (Figure 3.6A). Under similar conditions where procaspase-2 was processed by granzyme B, caspase-1 was not cleaved (N Harvey and S Kumar, unpublished observation, Darmon *et al.*, 1994). Granzyme B mediated cleavage of caspase-2 was inhibited by TPCK (N Harvey and S Kumar, unpublished observation) and only partially inhibited by YVAD-cmk (N Harvey and S Kumar, unpublished observation), indicative of the fact that the substrate specificity of granzyme B differs significantly from that of caspase-1. The optimal substrate specificity of granzyme B, as determined using a positional scanning substrate combinatorial library, is I (or V) EPD (Thornberry *et al.*, 1997). The efficiency of granzyme B-mediated cleavage of caspase-2 appeared substantially lower than that mediated by caspase-3 which probably reflects the difference in P₄-P₂ substrate specificity between these two proteases. The activation of caspase-2 by granzyme B may therefore contribute to the initiation of the apoptotic pathway in cells targeted to die by cytotoxic lymphocyte-mediated apoptosis.

3.9 Discussion

The role of the caspase family of proteases as effectors of apoptosis is now firmly established (reviewed in Kumar and Lavin, 1996, Nicholson and Thornberry, 1997). Following the initial studies characterising caspase-1 structure and activation (Thornberry *et al.*, 1992, Walker *et al.*, 1994, Wilson *et al.*, 1994, Ramage *et al.*, 1995), it was envisaged that, by virtue of sequence homology, all caspase members would be similarly processed and assembled. The aim of this chapter was to investigate the mechanism of caspase-2 processing *in vitro* to establish whether caspase-2 was likely to be activated in response to apoptotic stimuli and to investigate possible mediators of this process.

In this chapter it has been shown that the caspase-2 precursor is processed *in vitro* into subunits analogous to the 10 kDa and 20 kDa subunits of caspase-1 and that this process can be mediated both by active caspases present in apoptotic cells and by several isolated caspases and granzyme B. By extrapolation from these results it is likely that caspase-2 is activated in cells that are committed to apoptosis by virtue of the fact that apoptotic cells contain active caspases that are capable of cleaving caspase-2. From our studies thus far, it is not possible to ascertain whether caspase-2 activation is likely to be an early, upstream, event or whether it is a downstream event mediated by caspases such as caspase-3. These questions will be addressed in later chapters.

Both procaspase-2 (51 kDa) and caspase-2 lacking the pro-domain fail to show detectable auto-cleavage when translated *in vitro*. However, caspase-2 expressed in *E. coli* either as a full length molecule, or lacking the prodomain, was proteolytically active when analysed on both the tetrapeptide substrates DEVD-AFC and VDVAD-AMC and

on *in vitro* translated procaspase-2. The lack of self-cleavage of *in vitro* translated procaspase-2 may be due to the relatively low concentrations of translated protein present in these reactions. Alternatively, the optimal conditions for caspase-2 processing may not have been provided in our *in vitro* reactions. As caspase-2 expression is usually low in adult, mammalian cells, it is unlikely in the normal situation that high enough local concentrations of procaspase-2 are reached to enable self-proteolysis and activation to occur. When caspase-2 is overexpressed in transient transfection experiments, apoptosis is initiated, presumably due to high enough concentrations of caspase-2 being reached to facilitate self-proteolysis. In an *in vivo* situation, it can be postulated that for caspase-2 auto-activation to occur in response to apoptotic stimuli, caspase-2 precursor molecules would need to be brought together in close proximity to facilitate localised high concentrations of the pro-enzyme and subsequent proteolysis.

From the *in vitro* cleavage studies with isolated caspases presented in this chapter, it appears that full length procaspase-2, caspase-3 and caspase-7 cleave *in vitro* translated procaspase-2 most efficiently of all the caspases we investigated and are possibly the proteases in the apoptotic cytoplasmic extracts we analysed that are responsible for mediating procaspase-2 cleavage. All of the caspases tested were able to cleave procaspase-2 into 12 kDa and 19 kDa subunits and 36-39 kDa intermediates identical to those released following procaspase-2 incubation with cellular cytoplasmic extracts, albeit with different efficiencies. Caspase-2 lacking the prodomain generated a cleavage profile slightly different from full length caspase-2, indicating that this caspase construct has an altered substrate specificity that is due to removal of the prodomain. Immunoblot analysis of both caspase-2 lacking the prodomain and full length procaspase-2 demonstrated that the small subunit of caspase-2 was not released from the

caspase-2 construct lacking the prodomain, indicating that this enzyme may not require proteolytic processing to adopt a partially active configuration. These results suggest an important role for the prodomain in directing assembly of the active enzyme.

During the course of these experiments, caspase-3 and caspase-7 were shown to be activated *in vivo* in response to apoptotic stimuli (Schlegel *et al.*, 1996, Duan *et al.*, 1996a). This observation suggested that these caspases were indeed likely to be active in the cytoplasmic extracts utilised in these experiments and may have been responsible for procaspase-2 cleavage by mammalian cell extracts *in vitro*. Although caspase-1 was capable of processing caspase-2, the fact that caspase-2 cleavage was inhibited only by high concentrations of YVAD-cmk together with the fact that cytoplasmic extracts from apoptotic cells did not show appreciable cleavage of the tetrapeptide YVAD-AFC, suggests that caspase-1 is not the protease in cytoplasmic extracts that was responsible for the caspase-2 cleavage observed in these experiments.

The ability of granzyme B to process procaspase-2 suggests that procaspase-2 may be one of the effector proteases which are activated following the perforin-mediated release of cytotoxic lymphocyte granules into target cells. Prior to this study, it had been shown that granzyme B could not activate caspase-1, but could activate caspase-3 (Darmon *et al.*, 1994, Darmon *et al.*, 1995), and subsequently granzyme B has been shown to also process caspases-6, 7, 8, 9, 10 and 11 (Gu *et al.*, 1996, Orth *et al.*, 1996, Fernandes-Alnemri *et al.*, 1996, Duan *et al.*, 1996, Srinivasula *et al.*, 1996, Wang *et al.*, 1996, Vincenz and Dixit, 1997). The specific role that caspase-2 plays in cytotoxic lymphocyte-mediated apoptosis has recently been revealed in studies of caspase-2 null mice (Bergeron *et al.*, 1998). The B-lymphoblasts of these mice are partially resistant to

granzyme B and perforin-induced apoptosis (Bergeron *et al.*, 1998), signifying that caspase-2 activation is important in this pathway. The ability of granzyme B to directly activate multiple caspases conceivably provides a rapid mechanism of initiating and amplifying the apoptotic pathway, which could presumably bypass the effect of certain viral or cellular caspase inhibitors.

In summary, the results in this chapter demonstrate that apoptotic signalling leads to the activation of caspases which can process procaspase-2 *in vitro* to subunits 12 kDa and 19 kDa in size. Experimental evidence presented here suggests that *in vivo*, procaspase-2 activation could be mediated either by self-proteolysis, or by the activity of a number of caspase family members with varying efficiency. The ability of granzyme B to cleave caspase-2 suggested that caspase-2 plays an important role in cytotoxic lymphocyte-mediated cell death, a suggestion that has subsequently been confirmed.

Figure 3.1. Cleavage of procaspase-2 by cytoplasmic extracts from apoptotic cells. A)

Time course of procaspase-2 cleavage by cytoplasmic extracts from apoptotic FDC-P1 cells. 5 μ l cytoplasmic extract (10 mg/ml total protein) was incubated with 5 μ l of ^{35} S-Met labelled procaspase-2 and cell extraction buffer in a total volume of 20 μ l. Reactions were stopped at the respective times by the addition of an equal volume of 2x protein loading buffer. At the completion of the timecourse, samples were analysed by electrophoresis on a 15% SDS/PAGE gel, transfer to PVDF and exposure to X-ray film overnight. There is concurrent appearance of the p12 subunit and the 39 kDa intermediate, followed by generation of the 37 kDa intermediate and the p19 subunit. Procaspase-2 cleavage is essentially complete after 3 h at 37 °C.

B) Concentration dependent cleavage of procaspase-2 by cytoplasmic extracts from apoptotic FDC-P1 cells. Increasing quantities of cytoplasmic extract ranging from 5 μ g to 100 μ g of total protein were incubated with 5 μ l ^{35} S-Met labelled procaspase-2 and cell extraction buffer in a total volume of 20 μ l, for 3 h at 37 °C. Samples were analysed as described above.

C) A model of procaspase-2 processing by cytoplasmic extracts from apoptotic cells.

The location of putative aspartate (D) residues at which cleavage could occur in procaspase-2 to generate the cleavage products observed in A) and B). Subsequent studies have demonstrated that caspase-2 expressed in *E. coli* is processed at D169, D333 and D347 to release 19 kDa and 12 kDa subunits (Xue *et al.*, 1996), confirming that cleavage occurs at the residues suggested in the model. A proteolytic fragment corresponding to the prodomain is not observed in these assays, possibly due to further cleavage at a number of aspartate residues in the prodomain region.

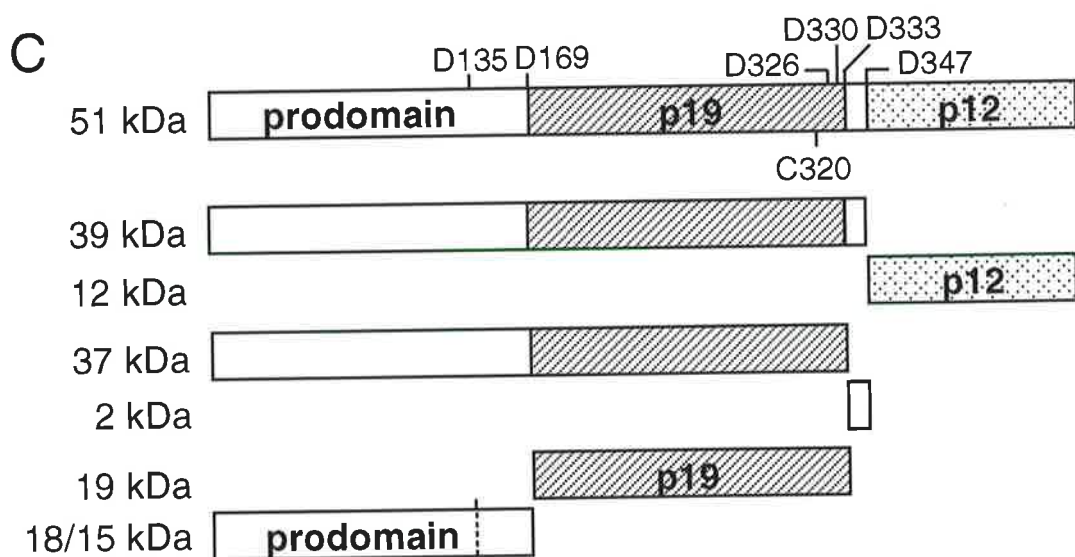
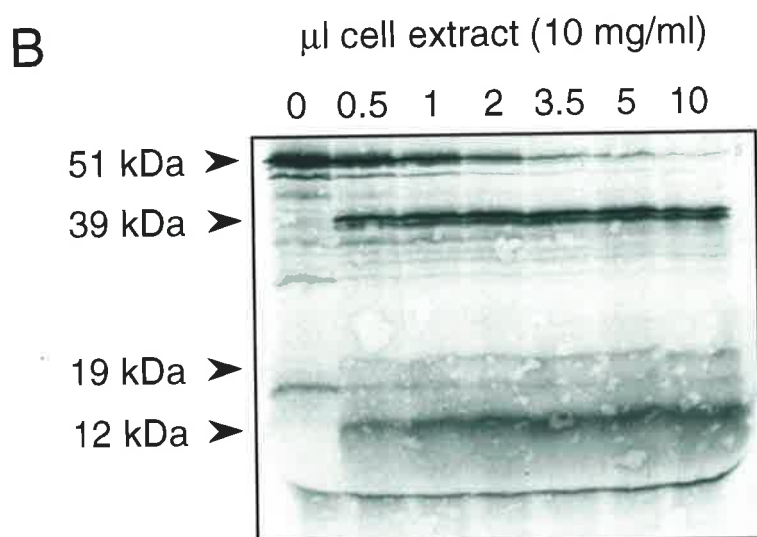
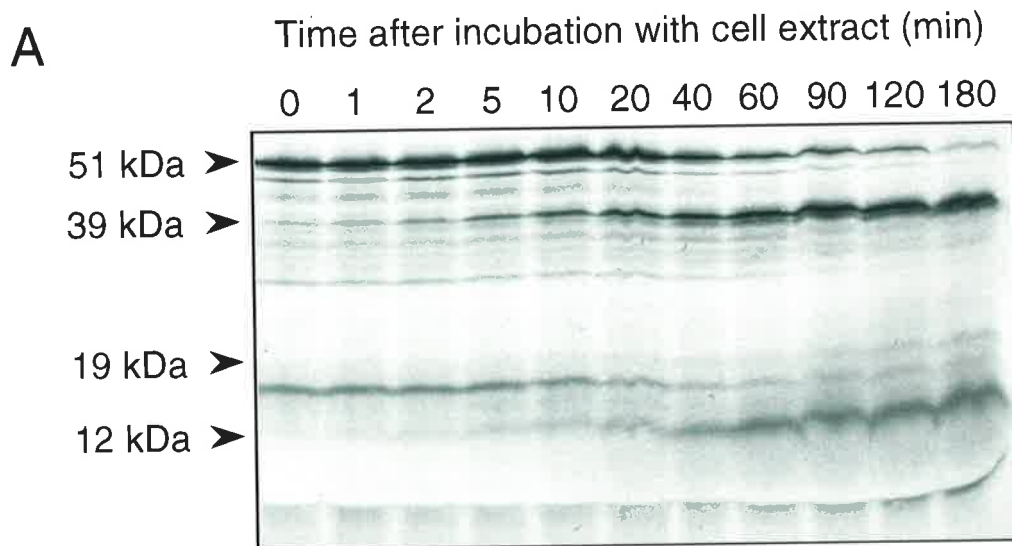


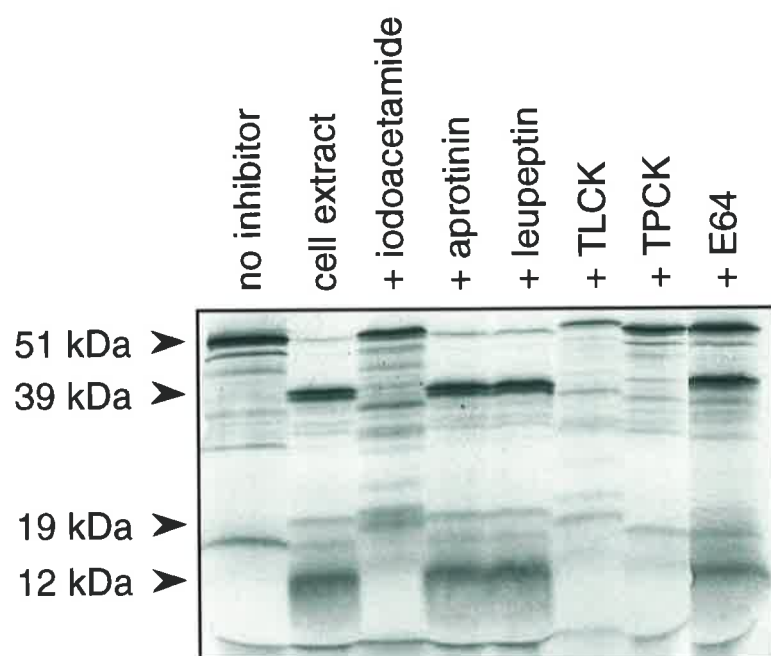
Figure 3.2. The effect of protease inhibitors on procaspase-2 cleavage by cytoplasmic extracts from apoptotic FDC-P1 cells.

A) The effect of various serine and cysteine protease inhibitors on procaspase-2 cleavage. 5 μ l of FDC-P1 cytoplasmic extract (10 mg/ml total protein) was preincubated with the respective inhibitors at the concentrations indicated in Materials and Methods (2.5.7), in a total volume of 20 μ l for 30 min at 37 °C. 5 μ l of ^{35} S-Met labelled procaspase-2 was then added and incubated a further 3 h at 37 °C. Samples were analysed as described in Figure 3.1 A.

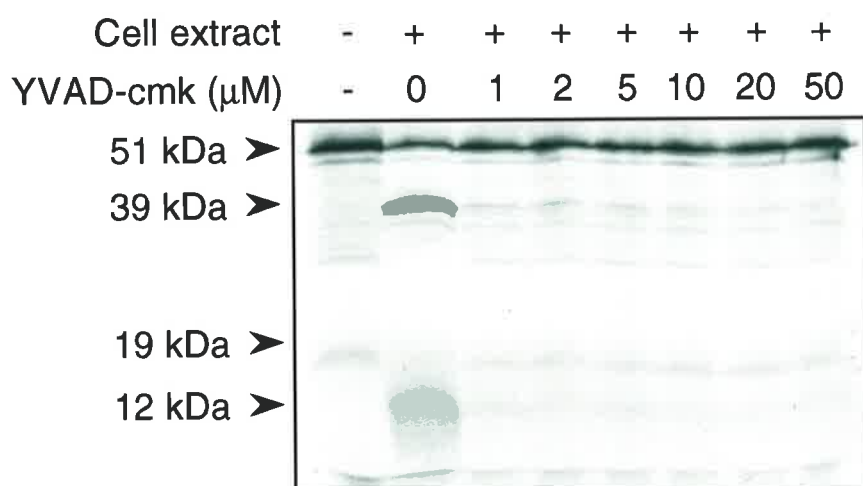
B) Inhibition of procaspase-2 cleavage by YVAD-cmk. 5 μ l of FDC-P1 cytoplasmic extract was preincubated with YVAD-cmk at a concentration ranging from 1-50 μ M for 30 min at 37 °C, in a total volume of 20 μ l. 5 μ l of ^{35} S-Met labelled procaspase-2 was then added and incubated for a further 3 h at 37 °C. Samples were analysed as described above.

C) Inhibition of procaspase-2 cleavage by YVAD-cmk. Experimental procedure was as described above, using YVAD-cmk at concentrations ranging from 0.1 nM to 20 nM. (The scale in this figure should read nM, 0, 0.1, 1, 10, 20, 100 not μ M, 0, 0.1, 2, 5, 10, 20.)

A



B



C

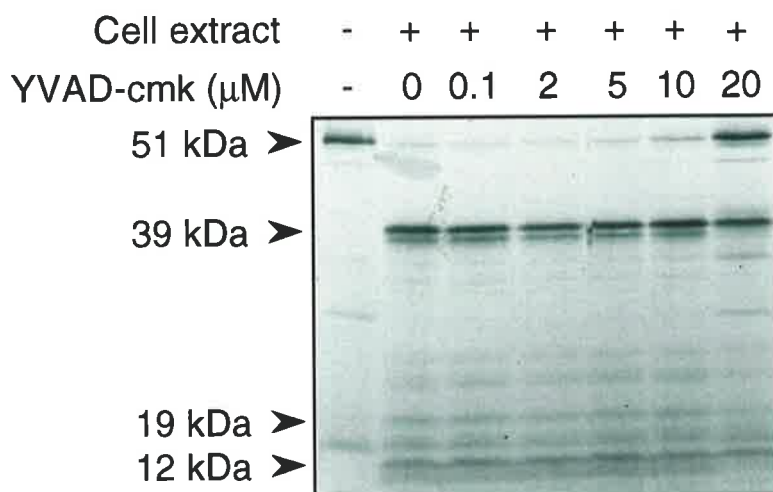


Figure 3.3. Cleavage of procaspase-2 by cytoplasmic extracts from apoptotic NIH-3T3 cells.

5 μ l of ^{35}S -Met labelled procaspase-2 was incubated with 10-50 μ g total protein from apoptotic (serum deprived) and non-apoptotic NIH-3T3 cells, in a final volume of 50 μ l, for 4 h at 37 $^{\circ}\text{C}$. Where indicated, NIH-3T3 extracts were preincubated with YVAD-cmk (1-1000 nM) for 30 min at 37 $^{\circ}\text{C}$, prior to the addition of ^{35}S -Met labelled procaspase-2. Reactions were terminated by the addition of 50 μ l 2x protein loading buffer and analysed as described previously.

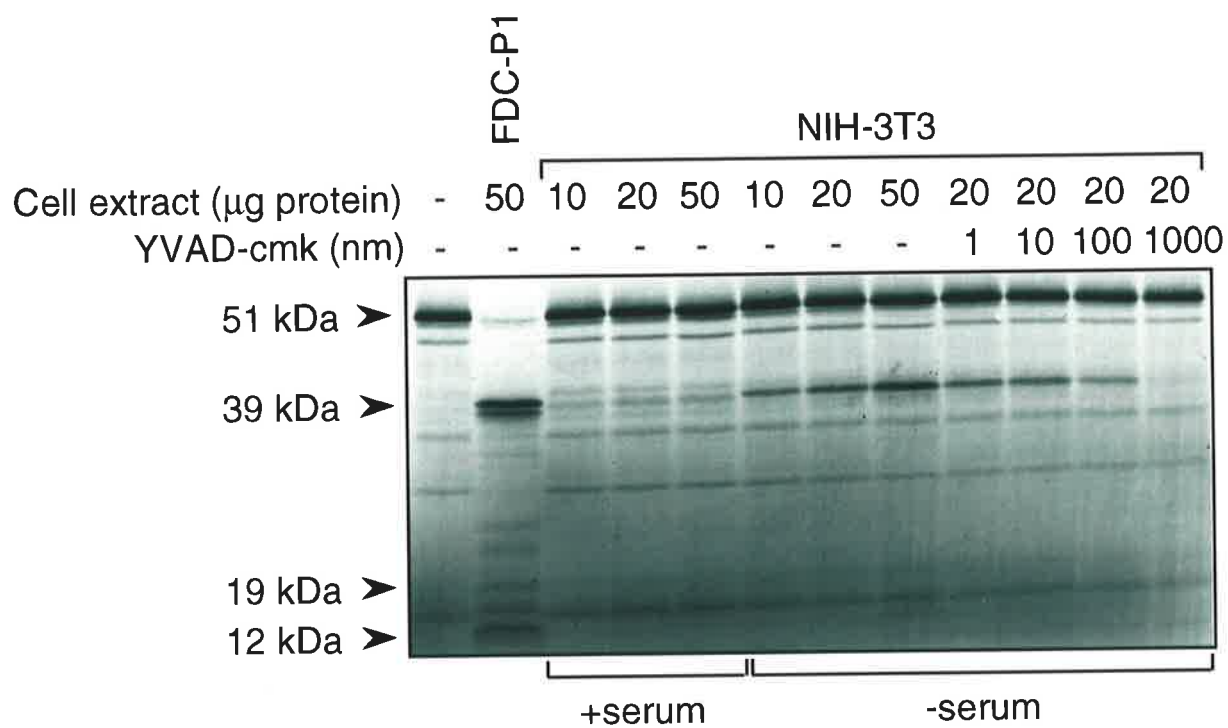


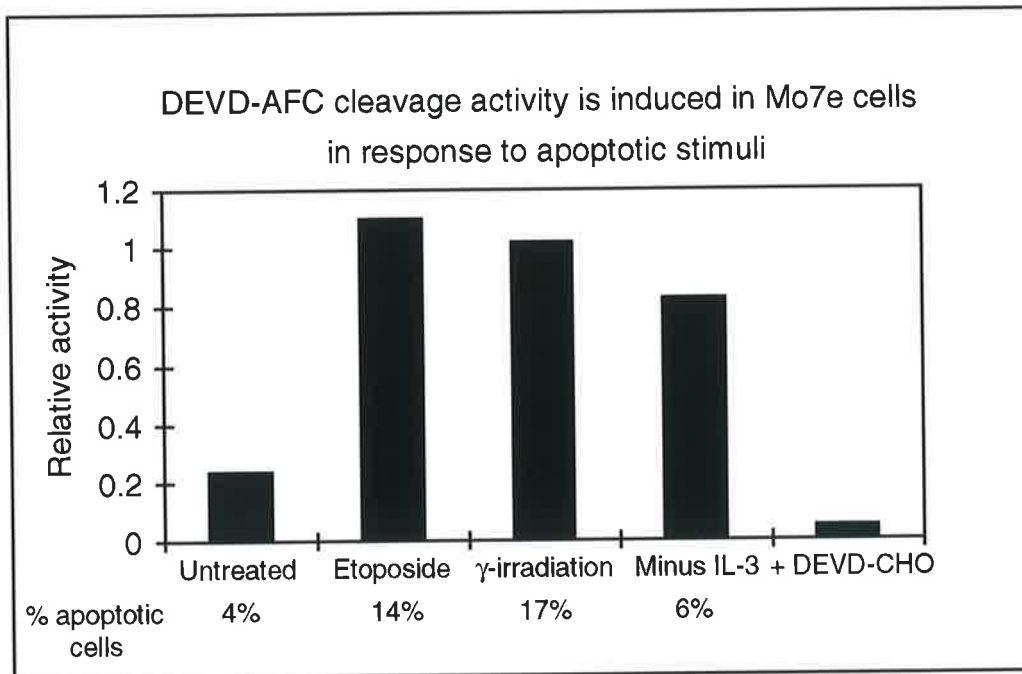
Figure 3.4. DEVD-AFC cleavage activity is induced in Mo7e cells, but is constitutive in FDC-P1 cells.

A) DEVD-AFC cleavage activity is induced in Mo7e cells following treatment with apoptotic stimuli. 10 μ g (total protein) of cytoplasmic extract from Mo7e cells following the indicated treatments was incubated with 100 μ M DEVD-AFC for 30 min at 37 °C. Mo7e cells were treated with etoposide (50 μ M) and γ -irradiation (30 Gy) for 12 h, and medium devoid of serum and IL-3 for 24 h. Where indicated, cytoplasmic extracts were preincubated with DEVD-CHO at a final concentration of 1 μ M for 30 min at 37 °C prior to the addition of DEVD-AFC. Resultant fluorescence was quantified using an Aminco Bowman Luminescence Spectrophotometer set to excitation wavelength 400 nm, emission wavelength 505 nm. The percentage of apoptotic cells was calculated by trypan blue exclusion.

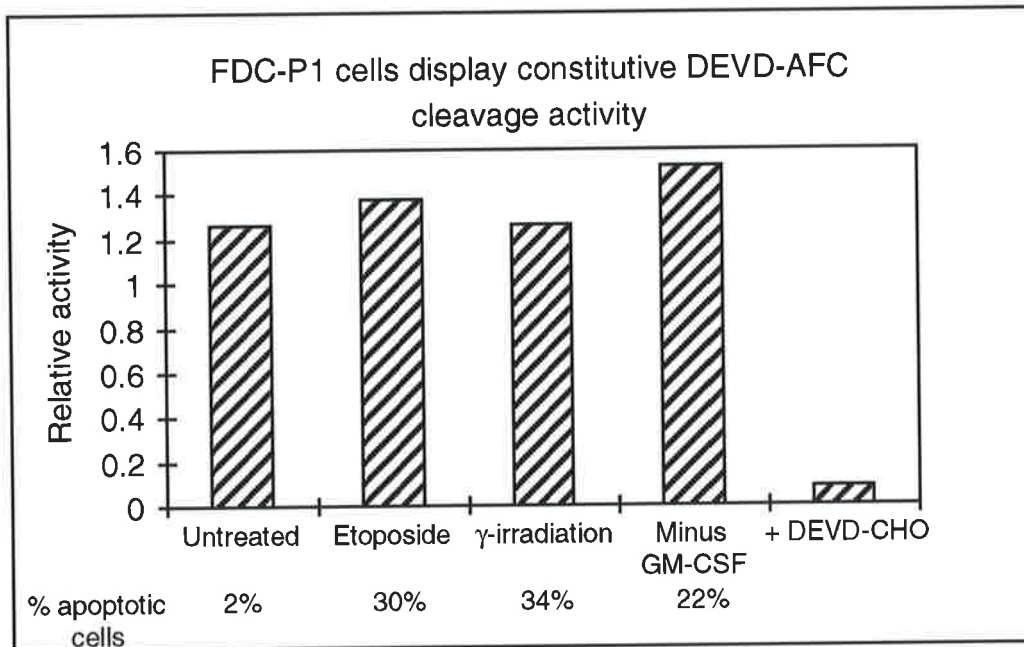
B) DEVD-AFC cleavage activity is constitutive in untreated FDC-P1 cells. 10 μ g (total protein) of cytoplasmic extract from FDC-P1 cells following the indicated treatments was incubated with 100 μ M DEVD-AFC for 30 min at 37 °C. FDC-P1 cells were treated with etoposide (50 μ M) and γ -irradiation (30 Gy) for 6 h and in medium devoid of GM-CSF for 8 h. Where indicated, cytoplasmic extracts were preincubated with DEVD-CHO at a final concentration of 1 μ M for 30 min at 37 °C prior to the addition of DEVD-AFC. Resultant fluorescence was quantified as detailed above. The percentage of apoptotic cells was calculated by trypan blue exclusion.

C) Untreated FDC-P1 cells do not exhibit the DNA laddering characteristic of apoptotic cells. Chromosomal DNA samples from untreated FDC-P1 cells and from cells treated for 6 h with either EGTA (5 mM), EtOH (5%), etoposide (50 μ M) or γ -irradiation (30 Gy), were prepared and analysed as described in Materials and Methods (2.2.18).

A



B



C



Figure 3.5. Caspase-2 is capable of self-proteolysis.

A) Concentration dependent cleavage of procaspase-2 by active recombinant caspase-

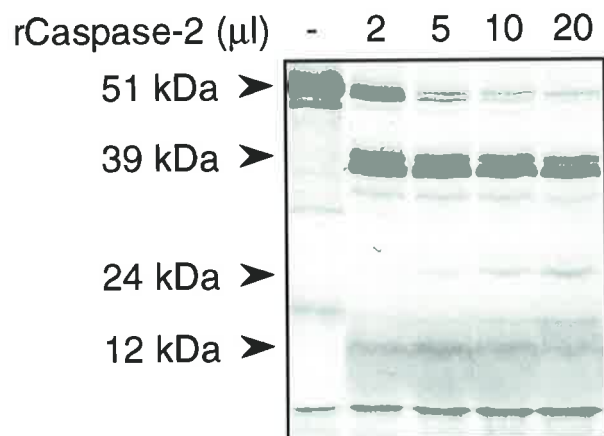
2. Increasing amounts of *E. coli* lysate expressing pGEXp33Caspase-2 were incubated with 5 μ l of ^{35} S-Met labelled procaspase-2 for 3 h at 37 °C in a total volume of 25 μ l. Samples were analysed as described in Figure 3.1 A

B) Time dependent cleavage of procaspase-2 by active recombinant caspase-2. 5 μ l of *E. coli* lysate expressing pGEXp33Caspase-2 was incubated with 5 μ l of ^{35} S-Met labelled procaspase-2 in a total volume of 25 μ l for the time indicated in the figure. Samples were analysed as described above.

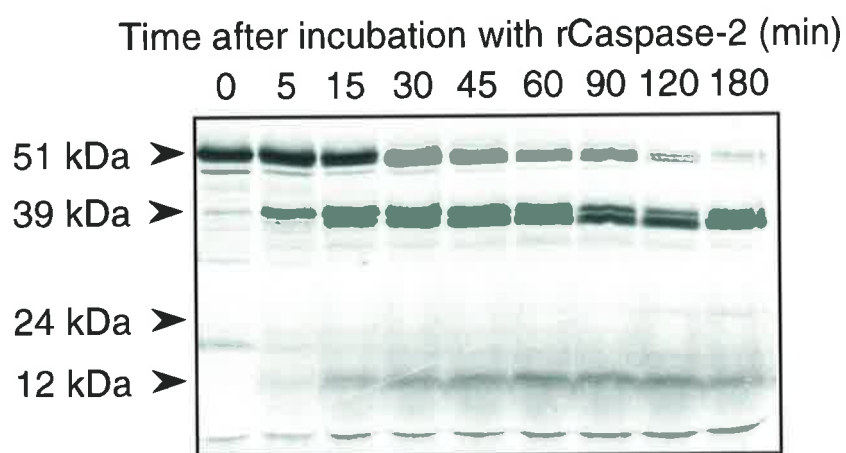
C) Cleavage of procaspase-2 by active recombinant full length caspase-2 and caspase-2 lacking the prodomain. 10 μ l of each pETprocaspase-2 and pGEXp33Caspase-2 lacking the prodomain was incubated with 5 μ l of ^{35} S-Met labelled procaspase-2 in a final volume of 20 μ l for 3 h at 37 °C. Samples were analysed as described above. The putative prodomain fragment is denoted by ★.

D) Immunoblot analysis of *E. coli* lysates expressing pGEXp33Caspase-2 and pETprocaspase-2. Immunoblot analysis using a polyclonal antiserum raised against the small subunit of caspase-2 was performed on a quantity of each *E. coli* lysate corresponding to an equivalent amount of DEVD-AFC cleavage activity. No p12 subunit was detected in pGEXp33Caspase-2 lysate, even when large quantities were analysed.

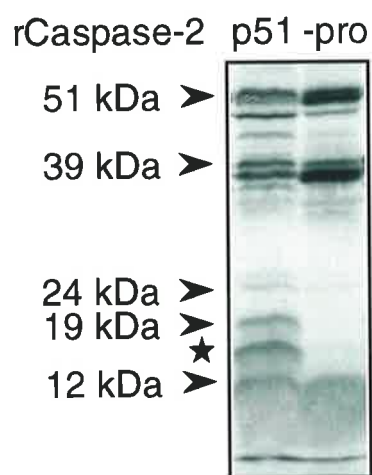
A



B



C



D

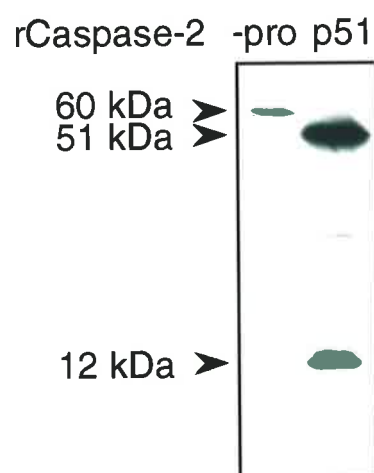


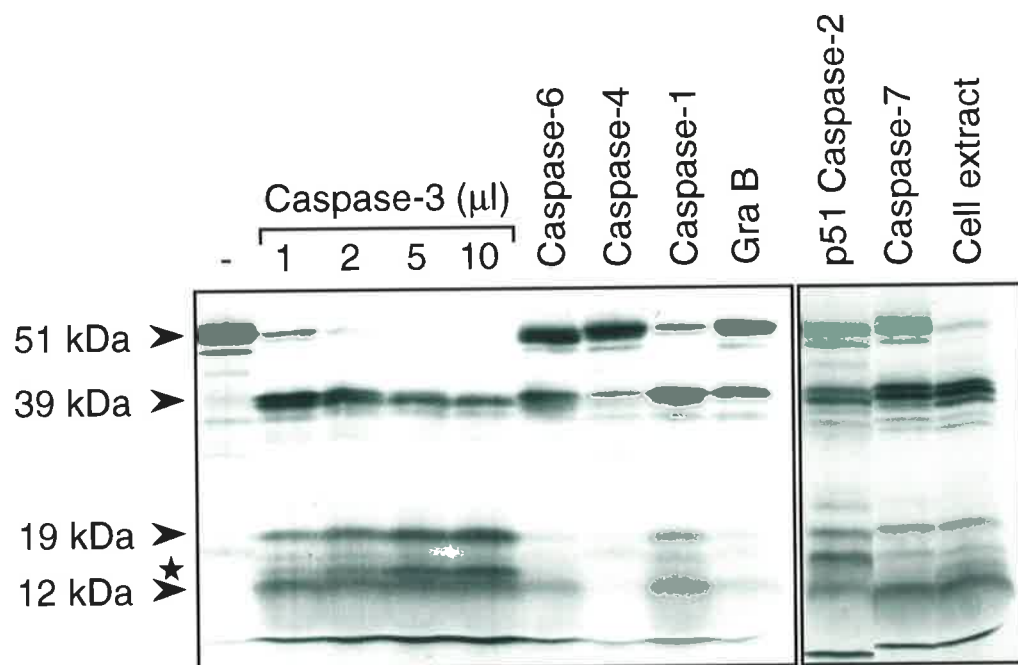
Figure 3.6. Cleavage of procaspase-2 by other caspase family members and granzyme B.

A) Cleavage of procaspase-2 by caspases-1, -2, -3, -4, -6 and -7, granzyme B and cytoplasmic extract from apoptotic Mo7e cells. 5 μ l ^{35} S-Met labelled procaspase-2 was incubated with increasing amounts of *E. coli* lysate expressing recombinant caspase-3 as indicated, or with 5 μ l of *E. coli* lysate expressing caspase-6 or caspase-7, 10 μ l *E. coli* lysate expressing caspase-4 or pETprocaspase-2, 5 μ l purified caspase-1 or purified granzyme B, or 20 μ g (total protein) of cytoplasmic extract from Mo7e cells treated with 30 Gy γ -irradiation. All reactions were performed in a total volume of 20 μ l for 3 h at 37 °C. Samples were analysed as described in Figure 3.1 A. The protein fragment denoted by ★ is liberated particularly efficiently by caspase-3 and may correspond to an amino terminal prodomain fragment.

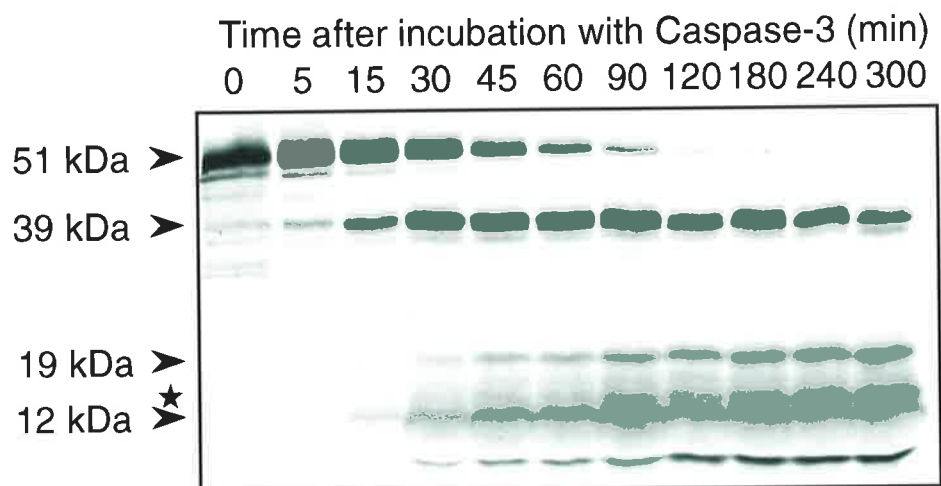
B) Time dependent cleavage of procaspase-2 by recombinant caspase-3. 5 μ l of *E. coli* lysate expressing recombinant caspase-3 was incubated with 5 μ l ^{35} S-Met labelled procaspase-2 in a total volume of 20 μ l over the indicated timecourse. Samples were analysed as described previously.

C) Caspase-2 is not able to cleave procaspase-3. Increasing quantities of *E. coli* lysate expressing recombinant pGEXp33Caspase-2 were incubated with ^{35} S-Met labelled procaspase-3 for 3 h at 37 °C. Samples were analysed as previously described.

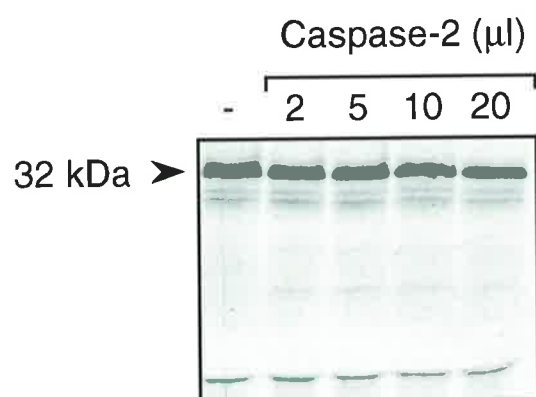
A



B



C



Chapter 4

***Functional activation of caspase-2 is an early process
in apoptosis***

4.1 Introduction

The identification of 13 caspase family members, all of which induce apoptosis to varying extents when overexpressed in mammalian cells, poses many questions as to the role that each plays in the execution of the apoptotic pathway. Clearly the situation is much more complex in mammals than in *C. elegans*, where the *ced-3* gene is so far the sole gene of the caspase family. The studies in this chapter aimed to elucidate the relative contribution that each caspase makes to apoptosis induced by various stimuli and the extent to which caspase family members show redundancy. Emerging evidence suggests that the caspases fall into two classes, the first (Class I) of which contain long pro-domains by virtue of which they are activated early in the apoptotic pathway and the second class (Class II) which have relatively short or absent pro-domains and are activated subsequently to "Class I" caspases.

At the commencement of this study, although many lines of evidence suggested that caspases play an important role in the apoptotic pathway, only three members of the family, caspases-3, -6 and -7, had been shown to be activated *in vivo* in response to apoptotic stimuli. Each of these caspases belong to "Class II"; they have pro-domains approximately 20 amino acids in length. The putative role of Class II caspases is to mediate the cleavage of cellular substrates that results in apoptotic death. We postulate that by virtue of its long, amino-terminal prodomain, caspase-2 belongs to the "Class I" caspases and may be activated early in response to apoptotic signalling. This may be facilitated by direct coupling of caspase-2 to a signalling complex via a signalling adaptor molecule, in an analogous fashion to the coupling of caspase-8 to the Fas/Apo1 signalling complex via MORT1/FADD (Boldin *et al.*, 1996, Muzio *et al.*, 1996). *In vitro* studies by Duan and Dixit (1997) have shown that caspase-2 associates via its pro-

domain with RAIDD, a death adaptor molecule thought to be involved in death signalling through the TNF-R1 receptor via its association with the RIP and TRADD death domain proteins. However, no *in vivo* association of these molecules has been shown to occur following this original observation and studies in our laboratory using the yeast two-hybrid system have failed to demonstrate association of caspase-2 with RAIDD (unpublished observation). The aim of this chapter was to investigate whether caspase-2 is activated *in vivo* in response to various stimuli and at which stage of the apoptotic pathway its activation occurs relative to that of other caspases.

Results

4.2 Induction of DEVD-specific cleavage activity in Mo7e cells following apoptotic stimuli

To investigate the magnitude and specificity of caspase activity induced by various apoptotic stimuli in the human megakaryoblastic cell line Mo7e, the fluorogenic tetrapeptide substrates DEVD-AFC and YVAD-AFC were utilised. The DEVD-AFC substrate mimics the cleavage site at which caspase-3 and caspase-3 relatives cleave PARP, while the YVAD-AFC substrate is specific for caspases most related to caspase-1. Cleavage of these substrates by cellular cytoplasmic extracts is thus indicative of *in vivo* proteolytic activation of these caspases in response to apoptotic signals. We initially studied caspase activation in the factor dependent haemopoietic cell line Mo7e, which undergoes apoptosis in response to the removal of IL-3 and serum from its growth medium. Following withdrawal of IL-3 and serum from Mo7e, there was a significant, time-dependent increase in the level of DEVD-AFC cleavage, indicating the progressive activation of caspase-3 and/or its close relatives in these cells (Figure 4.1A). This activation reflected the progressive decrease in viable cells as observed by trypan

blue exclusion (Figure 4.1A) coupled with an increase in the number of apoptotic cells as observed by DAPI staining (Figure 4.2B). The increase in DEVD-AFC activity preceded any significant morphological signs of apoptosis (Figure 4.1A). A progressive increase in the cleavage of chromosomal DNA into oligonucleosome sized fragments, a characteristic of apoptotic cell death, was also observed following IL-3 and serum withdrawal (Figure 4.3A). Cleavage of DEVD-AFC by cytoplasmic extracts from factor-deprived Mo7e cells was completely inhibited by pre-incubation of the cell extracts with the tetrapeptide aldehyde inhibitor of caspase-3 related proteases, DEVD-CHO (Figure 4.1A). No cleavage of YVAD-AFC was observed at any time after IL-3 and serum withdrawal, indicating that caspase-1 and its closest relatives are not activated in response to this stimulus in these cells (N Harvey and S Kumar, unpublished observation). A low level of DEVD-AFC cleavage activity was consistently observed in untreated Mo7e cells. This may be a consequence of the low percentage of apoptotic cells in the normal cell population (approximately 3%). Alternatively, it may be due to the release of caspases from their normal cellular localisation during the course of cytoplasmic extract preparation, enabling low level activation to occur in the absence of a death signal.

Exposure of Mo7e cells to 40 μ M etoposide or 30-Gy γ -irradiation also resulted in a progressive increase in caspase-3-like activity that correlated with the increase in the number of apoptotic cells as measured by DAPI staining (Figures 4.1B, 4.1C, 4.2B, 4.2C). These treatments also resulted in the formation of characteristic apoptotic DNA ladders (Figure 4.3B). As previously observed, a significant increase in substrate cleavage activity was evident before any morphological appearance of apoptosis (Figure 4.1B, 4.1C). The DEVD-AFC cleavage activity of cell extracts was inhibited by pre-

incubation of the extracts with 1 μ M DEVD-CHO. Once again, there was no evidence of caspase activity on the YVAD-AFC substrate. Similar studies in our laboratory were carried out on two isogenic Burkitt's lymphoma cell lines that are sensitive (BL30A) and resistant (BL30K) to the induction of apoptosis by genotoxic agents (Khanna *et al.*, 1996). In sensitive BL30A cells, we observed similar results as with Mo7e cells, but in the case of the resistant BL30K cells, no increase in caspase-3-like activity was observed following treatment with apoptotic agents (Harvey *et al.*, 1997). This correlates with the fact that these cells are resistant to apoptosis induced by etoposide and γ -irradiation. These observations confirm that the induction of caspase activity is restricted to cells undergoing apoptosis.

4.3 Cleavage of PARP during apoptosis in Mo7e cells

To further investigate the proteolytic activity in apoptotic and non-apoptotic extracts from Mo7e cells, we examined the cleavage of PARP, both *in vitro* and *in vivo*. It has been previously shown that caspases-3, 6 and 7 cleave PARP efficiently (Tewari *et al.*, 1995b, Nicholson *et al.*, 1995, Fernandes-Alnemri *et al.*, 1995a, Fernandes-Alnemri *et al.*, 1995b), whereas caspases-1, 2 and 4 do so poorly (Gu *et al.*, 1995). To facilitate the production of an easily translated PARP substrate *in vitro*, a truncated PARP construct was generated comprising the first 339 amino acids of human PARP (PARPt). The DEVD site at which caspase-3 cleaves PARP is located in this sequence at amino acid residues 211-214. Cytoplasmic extracts from Mo7e cells following factor withdrawal or treatment with etoposide or γ -irradiation cleaved *in vitro* translated, ^{35} S-methionine labelled, PARPt at the caspase-3-like recognition sequence DEVD, liberating 24 kDa and 14 kDa polypeptides from the 38 kDa PARPt precursor (Figure 4.4). Similarly, cytoplasmic extracts from BL30A cells following treatment with apoptosis inducing

stimuli contained PARP cleavage activity, while those prepared from BL30K cells did not (Harvey *et al.*, 1997). Consistent with results obtained in fluorogenic substrate cleavage assays (Figure 4.1), a marginal, baseline level of caspase-3-like activity was observed in untreated cells. These experiments confirm that activation of caspase-3-like proteases occurs rapidly in Mo7e and BL30A cells in response to apoptotic stimuli.

Western blot analysis indicated that in Mo7e cells, PARP was rapidly cleaved *in vivo* to 89 kDa and 24 kDa products in response to IL-3 withdrawal, etoposide treatment and γ -irradiation (Figures 4.5-4.7). The 115 kDa precursor was completely cleaved within the first 4 h following IL-3 withdrawal (Figure 4.5) and within the first 2 h following etoposide treatment or γ -irradiation (Figures 4.6, 4.7). The complete proteolysis of PARP significantly preceded the appearance of apoptotic cells, confirming that caspase activation occurs prior to the morphological changes of apoptotic cell death.

4.4 Activation of caspase-2 during apoptosis

Activation of caspase-2 requires the cleavage of the 51 kDa precursor molecule into subunits 19 kDa and 12 kDa in size (Harvey *et al.*, 1996, Xue *et al.*, 1996). Having established that the cytoplasmic extracts from cells treated with apoptotic stimuli contained caspase-3-like activity, we investigated whether procaspase-2 is processed *in vivo* to generate 19 kDa and 12 kDa subunits in response to apoptotic stimuli. We examined the activation of caspase-2 by Western blotting with an antiserum raised against the 12 kDa subunit of caspase-2 that recognises the 12 kDa small subunit and the 51 kDa caspase-2 precursor, but not the 19 kDa subunit. In Mo7e cells, IL-3 withdrawal, etoposide treatment and γ -irradiation resulted in the cleavage of procaspase-2 as observed by the appearance of the 12 kDa subunit (Figures 4.5-4.7). From time

course studies, the activation of caspase-2 appears to be rapid, detectable as early as 4 h after IL-3 and serum withdrawal (Figure 4.5) and as early as 2 h following exposure to etoposide or γ -irradiation (Figures 4.6, 4.7). This correlates well with the first significant increase in fluorogenic substrate cleavage (Figure 4.1) and PARP cleavage, both *in vitro* (Figure 4.4) and *in vivo* (Figures 4.5-4.7). In BL30A cells, but not in BL30K cells, progressive proteolysis of procaspase-2 over time was observed in response to treatment with both etoposide and γ -irradiation (Harvey *et al.*, 1997). Although several lines of evidence suggest a role for caspase-2 in the apoptotic pathway, this is the first observation of caspase-2 activation occurring during apoptosis.

Although the majority of activity on DEVD-AFC observed in our experiments is probably due to caspase-3 and those caspases most closely related to caspase-3, we and others have observed the ability of caspase-2 to cleave the DEVD substrate (Bump *et al.*, 1995, Results chapter 3), albeit with low efficiency. Therefore, it is possible that a small proportion of the DEVD-AFC cleavage apparent in Figure 1 may be due to activated caspase-2 in apoptotic cytoplasmic extracts.

4.5 Activation of caspase-2 occurs prior to that of caspase-3 during apoptosis in Mo7e cells and BL30A cells

To investigate at which stage in the apoptotic pathway caspase-2 activation occurs in relation to that of other caspases, we assessed the activation of caspase-3 by Western blotting using an anti-caspase-3 monoclonal antibody that detects both procaspase-3 and the 17 kDa large subunit. We did not observe significant cleavage of caspase-3 in Mo7e cells subjected to either IL-3 and serum withdrawal (Figure 4.5) or treated with etoposide (Figure 4.6) or γ -irradiation (Figure 4.7) at time points where procaspase-2

was completely cleaved (Figures 4.5-4.7). At later timepoints, some cleavage of procaspase-3 was evident (data not shown). These results indicate that in the apoptotic pathway in Mo7e cells, caspase-2 cleavage is an early event which occurs in response to various apoptotic stimuli, while caspase-3 is not significantly processed.

Activation of caspase-3 was clearly observed in BL30A cells treated with etoposide or γ -irradiation, but not in BL30K cells treated with these stimuli (Harvey *et al.*, 1997). In BL30A cells treated with both etoposide and γ -irradiation, the activation of caspase-3 was observed subsequent to that of caspase-2, confirming our observations in Mo7e cells which suggest that caspase-2 is activated prior to caspase-3. This lends weight to the hypothesis that caspase-2 is an upstream caspase that is activated early in response to apoptotic stimuli, prior to the activation of Class II caspases such as caspase-3.

4.6 Activation of caspase-7 in Mo7e cells following apoptotic stimuli

Because no rapid activation of caspase-3 was observed in Mo7e cells although cytoplasmic extracts from Mo7e contained DEVD-AFC cleavage activity, the activation of caspase-7, the closest homologue of caspase-3, was investigated. Western Blotting with a polyclonal antiserum to caspase-7 revealed that procaspase-7 is rapidly processed in Mo7e cells following the withdrawal of IL-3 and serum from growth medium (Figure 4.5) and following treatment with etoposide (Figure 4.6) or γ -irradiation (Figure 4.7). In these experiments, caspase-7 activation appears to occur concurrently with caspase-2 activation (Figures 4.5-4.7) and correlates with both the increase in DEVD-AFC cleavage activity (Figure 4.1) and the cleavage of PARP observed *in vitro* (Figure 4.4) and *in vivo* (Figure 4.5). These observations suggest that caspase-7 is likely to be the caspase in Mo7e cells that is responsible for the cleavage of DEVD-AFC and PARP.

4.7 Caspase-2 is activated in response to death receptor induced apoptosis

Caspase-8 was the first caspase to be directly associated with the cell death machinery, thereby physically linking the formation of the Fas/Apo-1 receptor death complex to the activation of a caspase following receptor ligation (Boldin *et al.*, 1996, Muzio *et al.*, 1996). The *in vitro* association of caspase-2 with RAIDD, mediating coupling via RIP and TRADD to the TNF-R1 death complex, suggested that caspase-2 may be activated as a result of death signalling through TNF-R1 (Duan and Dixit, 1997). By virtue of the ability of TRADD to interact with FADD (Hsu *et al.*, 1995), it is also possible that caspase-2 may be recruited to the Fas/Apo-1 receptor induced death complex during Fas/Apo-1 induced apoptosis. The following experiments investigate the activation of caspase-2 in response to apoptosis induced by both TNF- and Fas/APO-1 receptor ligation.

In TNF α responsive HeLa cells, caspase-2 was activated rapidly following treatment with TNF α (10 ng/ml) in conjunction with cycloheximide (10 μ g/ml) (Figure 4.8). The processing of procaspase-2 in these cells led to the liberation of a 14 kDa caspase-2 fragment prior to the appearance of the 12 kDa caspase-2 small subunit. This observation suggests that, in order to release the 12 kDa small subunit, procaspase-2 is first cleaved at Asp-333, generating a 14 kDa intermediate which is subsequently processed at Asp-347 to produce fully mature p12. The activation of caspase-3 appeared to occur concurrently with that of caspase-2 in these cells, with a decrease in the amount of procaspase-3 first evident 2 h following TNF α treatment (Figure 4.8). A timecourse analysing more timepoints immediately following TNF α treatment may delineate progressive activation of each caspase as we observed in other cell lines. In Jurkat cells

treated with α -Fas antibody (200 ng/ml), caspase-2 activation also occurred very rapidly, with the majority of procaspase-2 cleaved within 2 h of treatment (Figure 4.9). A smaller proportion of caspase-3 proenzyme was observed to be processed concurrently with caspase-2 activation in these cells (Figure 4.9). PARP cleavage appeared to be essentially complete by 2 h (Figure 4.9), the same timepoint at which the majority of procaspase-2 was processed, but the majority of caspase-3 cleavage was not yet completed (Figure 4.9). This suggests that PARP cleavage in response to Fas-induced apoptosis may be mediated by an early activated Class I caspase such as caspase-8 or caspase-10 or, alternatively, PARP cleavage could be mediated by caspase-7 as appears to be the case in Mo7e cells. Results presented here demonstrate that caspase-2 activation occurs rapidly in response to apoptosis induced by the death receptors Fas/APO-1 and TNF-R1 as well as to apoptotic stimuli induced by as yet undefined pathways such as drug treatment and γ -irradiation.

The effect of the cytotoxic drug etoposide was compared to the effect of α -Fas antibody on Jurkat cells. Treatment of Jurkat cells with α -Fas antibody (200 ng/ml) induced a greater proportion of apoptotic cell death than did treatment with 40 μ M etoposide at the same timepoints (Figures 4.10, 4.11). This corresponded to the more rapid induction of procaspase-2 and procaspase-3 processing in response to Fas/APO-1 receptor ligation (Figure 4.9) as compared to etoposide treatment (Figure 4.12). Processing of caspases-2 and -3 was observed 2 hours following treatment with α -Fas antibody, as was complete proteolysis of PARP (Figure 4.9). These processes were all delayed until 4 hours following etoposide treatment (Figure 4.12). Processing of procaspase-2 in Jurkat cells in response to both α -Fas antibody and etoposide resulted in the appearance of a 14 kDa

protein fragment that was liberated prior to release of the 12 kDa subunit, as was observed in HeLa cells treated with TNF α .

4.8 The effect of Bcl-2 on etoposide and Fas-induced apoptosis in Jurkat cells

To further investigate the location of caspase-2 in the caspase hierarchy, we analysed whether caspase-2 activation in response to various stimuli is able to be inhibited by Bcl-2. In *C. elegans*, the functional equivalent of Bcl-2, CED-9, functions upstream of both CED-3 and CED-4 to inhibit their activity (Hengartner *et al.*, 1992). In the mammalian pathway, the ability of Bcl-2 to inhibit caspase activity and subsequent apoptosis appears to be dependent on both cell type and death signal. Bcl-2 has been shown in a number of systems to function upstream of caspases to prevent their activation (Chinnaiyan *et al.*, 1996a, Erhardt and Cooper, 1996, Estoppey *et al.*, 1997, Perry *et al.*, 1997, Srinivasan *et al.*, 1996). However, Bcl-2 has been shown to afford no protection to lymphocytes in response to Fas-induced apoptosis (Strasser *et al.*, 1995), indicating that different death signals may utilise specific pathways to cell death which employ novel molecules for each pathway. To address the question of whether Bcl-2 is able to prevent procaspase-2 processing, we generated Jurkat cells stably transfected with Bcl-2. These cells were then treated with either etoposide (20 μ M) or α -Fas antibody (100 ng/ml) and samples were analysed over a 48 h timecourse. We found that Bcl-2 expression had no effect in preventing apoptotic death (Figure 4.13) and corresponding caspase-2 and caspase-3 activation in response to α -Fas treatment (data not shown). The level of Bcl-2 expressed in transfected cells was substantially higher than endogenous levels (Figure 4.14). In etoposide treated Jurkat/Bcl-2 transfectants, there appeared to be a partial protective effect against apoptotic cell death that was observed at late timepoints following etoposide treatment (Figure 4.13). Caspase-2

activation appeared to be slightly delayed by Bcl-2 expression in these cells compared to control cells (Figure 4.14). In addition, the magnitude of procaspase-2 cleavage was decreased in Bcl-2 transfected cells compared to control cells; more unprocessed pro-enzyme was still remaining at late timepoints following etoposide treatment (Figure 4.14). This was also the case with caspase-3 (Figure 4.14) and caspase-7 (Figure 4.14) processing; unprocessed pro-enzyme was still detected at all timepoints up to 48 h following etoposide treatment in *Bcl-2* transfectants, while the initial activation of each caspase was slightly delayed compared to control cells. The increased viability of *Bcl-2* transfectants at late timepoints following etoposide treatment correlates with the level of unprocessed pro-caspases still present in *Bcl-2* transfected cells at these times.

4.9 Discussion

Previous studies have documented the activation of caspases-3, 6 and 7 in response to various apoptotic stimuli (Chinnaiyan *et al.*, 1996a, Duan *et al.*, 1996, Orth *et al.*, 1996, Schlegel *et al.*, 1996). Experiments undertaken in this chapter sought to investigate whether caspase-2 is activated in response to various apoptotic stimuli and at which stage of the apoptotic pathway this occurs. Results presented here were the first to demonstrate that caspase-2 is activated rapidly in response to a variety of apoptotic stimuli in a variety of cell lines (Harvey *et al.*, 1997). Caspase-2 activation in response to apoptotic stimuli has subsequently been confirmed by additional investigators (MacFarlane *et al.*, 1997, Li *et al.*, 1997, Harvey *et al.*, 1998). In several of the cell lines we investigated, caspase-2 proteolysis was shown to occur prior to that of caspase-3, confirming that Class I caspases are the first to be activated in response to death signals.

Data from Mo7e cells suggest that caspase-3 may not be directly involved as a Class II caspase in the death pathway of these cells. Instead, the function of caspase-3 may be replaced by caspase-7, which was observed to be rapidly activated in response to apoptotic stimuli in Mo7e. Caspase-7 is probably the caspase responsible for the DEVD-AFC and PARP cleavage activity we observed in Mo7e cytoplasmic extracts. Knockout mice deficient in caspase-3 appear to be defective in neural development although apoptosis seems to proceed normally in other tissues, suggesting that caspase-3 is not critical to the execution of apoptosis in tissues other than the central nervous system (Kuida *et al.*, 1996). As caspase-7 is the closest relative of caspase-3, exhibiting similar activity in cleaving DEVD-AFC and PARP to that of caspase-3 (Fernandes-Alnemri *et al.*, 1995b), it is possible that this caspase plays a crucial effector role in the apoptotic pathway in tissues other than the central nervous system. It is not known whether caspase-2 is responsible for mediating the activation of caspase-7 in Mo7e cells or whether another Class I caspase such as caspase-8, 9 or 10 is also activated in response to the apoptotic stimuli we analysed which then proceeds to process caspase-7.

As shown in the previous chapter, caspase-2 is unable to process caspase-3 *in vitro* (Harvey *et al.*, 1996), which suggests that the caspase-3 activation we observed in BL30A cells treated with etoposide or γ -irradiation, Jurkat cells treated with α -Fas antibody or etoposide and HeLa cells treated with TNF may occur as a result of processing by a Class I caspase other than caspase-2. Alternatively, caspase-2 may activate another caspase family member which is able to effect caspase-3 activation. *In vitro* studies have shown that caspase-8 and caspase-10 are able to process all known caspases (Srinivasula *et al.*, 1996a, Muzio *et al.*, 1997), although whether these two caspases are able to mediate the same effect *in vivo* is not known. Both caspase-8 and

caspase-10 appear to play an important role in Fas and TNF-R1 induced apoptosis; catalytically inactive mutants of these two caspases inhibit apoptosis induced through either of these death receptors (Vincenz and Dixit, 1997). The relative contribution that caspase-2 activation makes in comparison to caspase-8 and caspase-10 during Fas and TNF-R1-induced apoptosis is not presently known. The fact that caspase-8 and caspase-10 differ with respect to their ability to be inhibited by CrmA (Srinivasula *et al.*, 1996a), suggests that at least two parallel pathways to receptor induced cell death exist that utilise the same downstream machinery, but are initiated by different upstream caspases.

The Class I caspases and associated molecules involved in the death pathway induced by other agents such as genotoxic drugs and γ -irradiation have not yet been defined. This study suggests that the activation of caspase-2 could be an important event in the initiation of etoposide and γ -irradiation-induced cell death. Recent studies in caspase-2 null mice show an important role for caspase-2 in the apoptosis of germ cells; female mice have an excess number of germ cells in their ovaries and oocytes from these mice are resistant to apoptosis induced by chemotherapeutic drugs (Bergeron *et al.*, 1998). This latter observation suggests an important role for caspase-2 in apoptosis mediated by cytotoxic drugs.

The failure of Bcl-2 to inhibit Fas-induced caspase activation and subsequent apoptosis confirms results initially observed by Strasser and colleagues that demonstrate the inability of Bcl-2 to inhibit Fas-mediated apoptosis in lymphocytes (Strasser *et al.*, 1995). It is feasible to visualise that the inability of Bcl-2 to block the Fas-induced pathway of cell death may be due to the cellular localisation of Bcl-2 compared to the Fas death signalling complex. Bcl-2 is localised to the outer mitochondrial membrane ,

the nuclear membrane and the endoplasmic reticular membrane (Krajewski *et al.*, 1993, Lithgow *et al.*, 1994). Ligation of the Fas receptor results in the recruitment of caspase-8 and caspase-10, via the adaptor molecule FADD (Boldin *et al.*, 1996, Muzio *et al.*, 1996, Vincenz and Dixit, 1997), to the plasma membrane death complex, resulting in caspase activation. Although these two caspases are able to process substrates such as PARP directly, it is likely that they serve primarily to activate the Class II caspases which appear to be the main effectors of apoptotic substrate cleavage. Caspase-3 is localised to both the cytoplasm and the mitochondrial intermembrane space (Mancini *et al.*, 1998), while caspase-7 resides in the cytoplasm (Duan *et al.*, 1996a). It is possible that the activation of cytoplasmic Class II caspases by caspase-8 and/or caspase-10 bypasses Bcl-2 inhibition because Bcl-2 is localised at the mitochondrial membrane, serving to inhibit apoptosis that involves signalling pathways that converge on mitochondria. A recent study by Kuwana and colleagues (1998) confirms this suggestion. Although their studies demonstrated that caspase-8 induced apoptosis appears to promote the release of cytochrome *c* from mitochondria, thereby amplifying the apoptotic signal, Bcl-2 only inhibited apoptosis induced by very low concentrations of caspase-8. It can be envisaged that once a critical level of caspase-8 activation occurs, such as would be the case following Fas receptor ligation, this pathway would no longer be sensitive to inhibition by Bcl-2. Medema and colleagues (1998) have also recently shown that the Bcl-2 family member Bcl-X_L, acts downstream of caspase-8 activation in response to Fas receptor ligation. The ability of Bcl-2 to slightly delay caspase activation and apoptosis induced by etoposide treatment implies that etoposide death signalling converges on the mitochondrion in a pathway that is more sensitive to regulation by Bcl-2. Bcl-2 may function both to delay caspase activation and to reduce

the magnitude of cytochrome *c* dependent amplification of the apoptotic signal in this scenario.

In conclusion, we have shown that the activation of caspase-2 is an early event in the induction of apoptosis by multiple apoptotic stimuli in a variety of cell lines. Caspase-2 activation was shown to occur in response to both death receptor-induced apoptosis and apoptosis induced by as yet undefined pathways such as growth factor withdrawal, etoposide treatment and γ -irradiation. While the mechanism of Fas and TNF α -mediated death signalling is becoming more defined, the mechanism of activation and cellular localisation of signalling complexes that mediate death by other stimuli is still unclear. Our data suggest that caspase-2 may be a component of the death machinery in both types of death signalling. The activation of caspase-2 prior to that of caspase-3 in both Mo7e cells and Jurkat cells in response to a variety of stimuli confirms that Class I caspases are the first to be activated in response to apoptotic stimuli, probably by virtue of their direct recruitment to death signalling complexes.

Figure 4.1. Activation of DEVD-AFC cleavage activity in Mo7e cells in response to apoptotic stimuli.

Panels A), B) and C) display cell viability as measured by trypan blue exclusion (×), relative DEVD-AFC cleavage activity (●) and inhibition of DEVD-AFC cleavage by DEVD-CHO (○) for Mo7e cells following treatment with IL-3 and serum withdrawal (A), 40 μ M etoposide (B) or 30 Gy γ -irradiation (C). In DEVD-AFC cleavage assays, 10 μ g of cytoplasmic extract from Mo7e cells at the indicated times following treatment was incubated with 100 μ M DEVD-AFC for 30 min at 37 °C. Fluorescence resulting from DEVD-AFC cleavage was quantitated as previously described (Materials and Methods 2.5.6). In the case of DEVD-CHO treated samples, cytoplasmic extracts were preincubated with 1 μ M DEVD-CHO for 30 min at 37 °C prior to the addition of DEVD-AFC. The data shown represent the mean and standard error of triplicate samples.

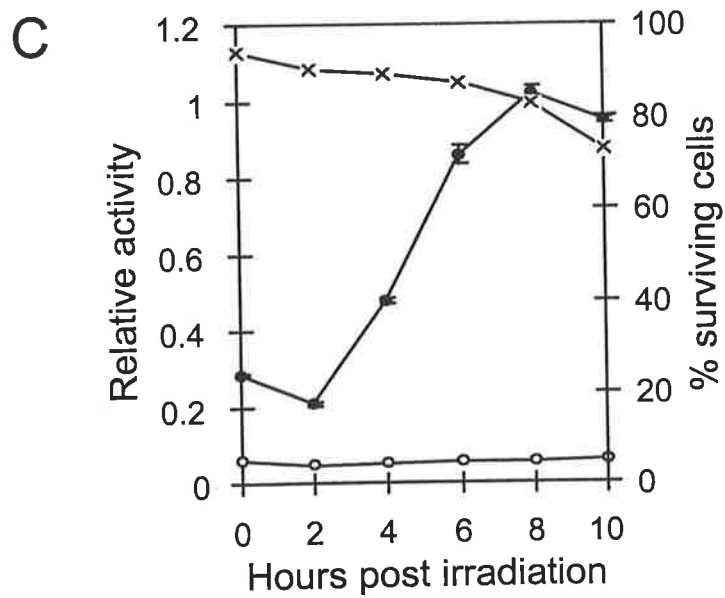
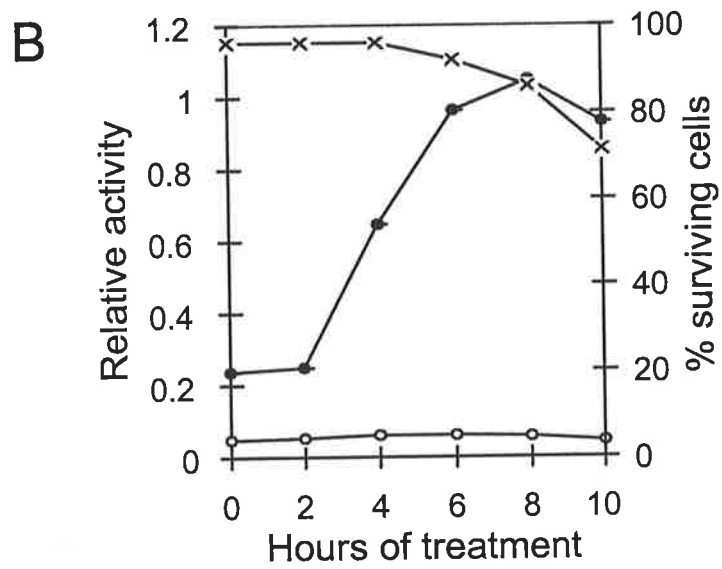
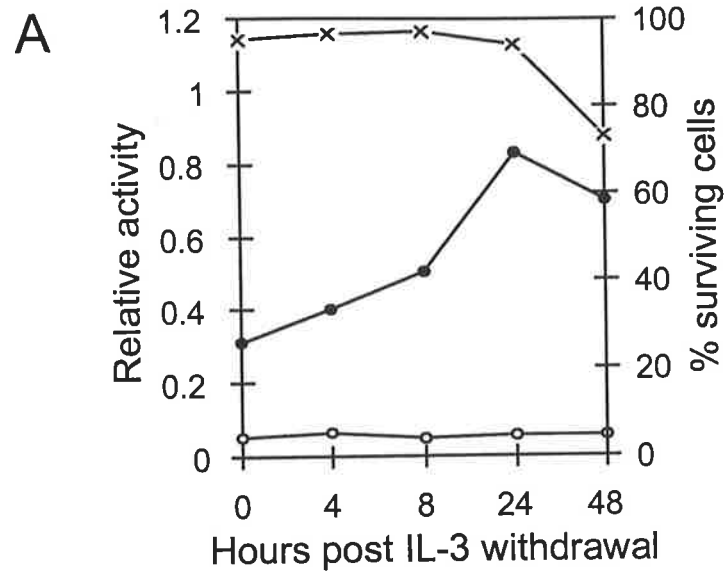


Figure 4.2. Apoptotic morphology of Mo7e cells following treatment with apoptotic stimuli.

Cytosmears of untreated Mo7e cells (A) and Mo7e cells 24 h following IL-3 and serum withdrawal (B), or 12 h following treatment with 40 μ M etoposide (C) or 30 Gy γ -irradiation (D) were stained with DAPI as described in Materials and Methods (2.5.3) and visualised by fluorescence microscopy. Cells were photographed under low magnification.

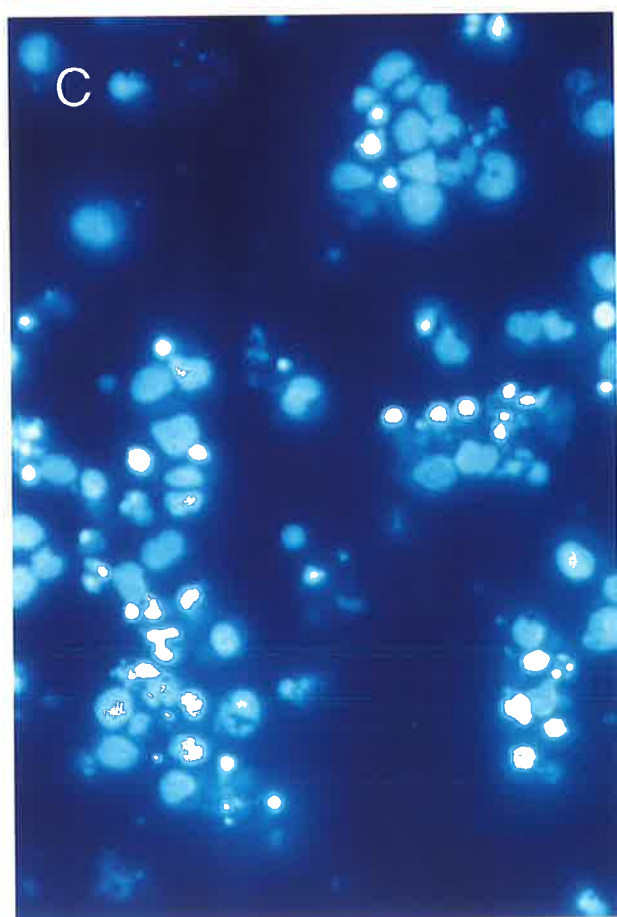
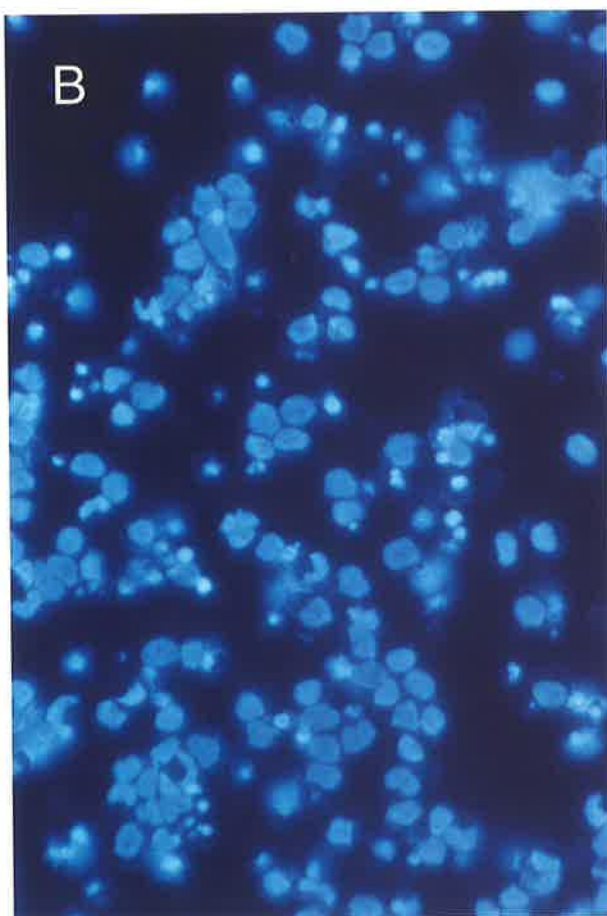
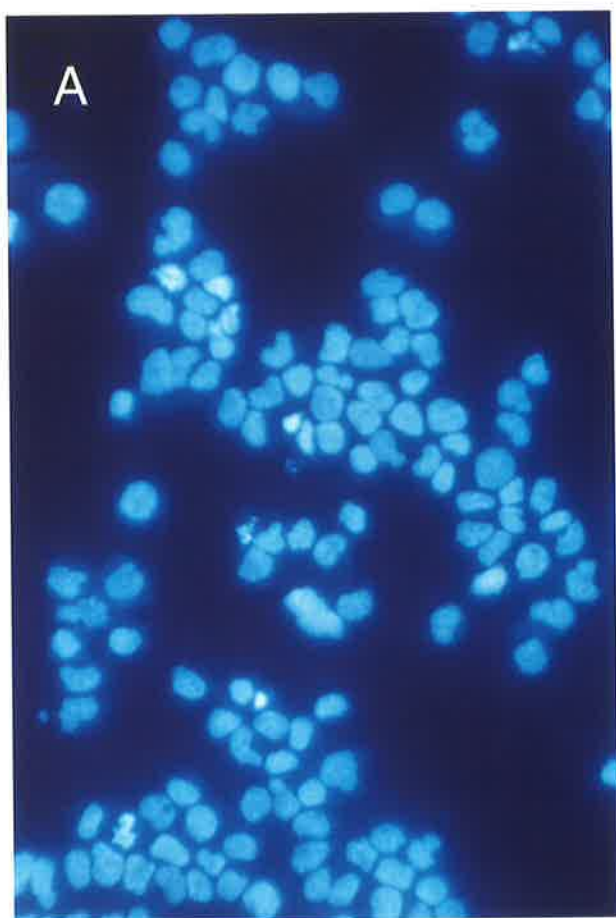


Figure 4.3. Mo7e cells treated with apoptotic stimuli exhibit DNA laddering that is characteristic of apoptosis.

Chromosomal DNA was extracted from untreated Mo7e cells and from cells following IL-3 and serum withdrawal (A) or treatment with EGTA (5 mM), EtOH (5%), etoposide (50 μ M) or γ -irradiation (30 Gy) for 12 h (B).

A



B

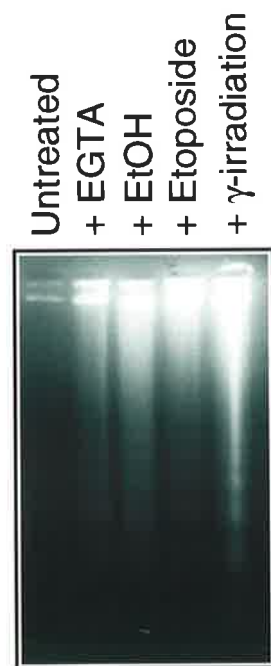


Figure 4.4. Cleavage of ^{35}S -Met labelled PARP by cytoplasmic extracts from apoptotic Mo7e cells.

In these assays, 5 μl ^{35}S -Met labelled truncated PARP (PARPt) was incubated for 60 min at 37 °C with 10 μg of cytoplasmic extract from Mo7e cells following withdrawal of IL-3 and serum (A), or following treatment with 40 μM etoposide (B) or 30 Gy γ -irradiation (C). Samples were analysed following electrophoresis on a 10 % SDS/PAGE gel, transfer to PVDF and autoradiography.

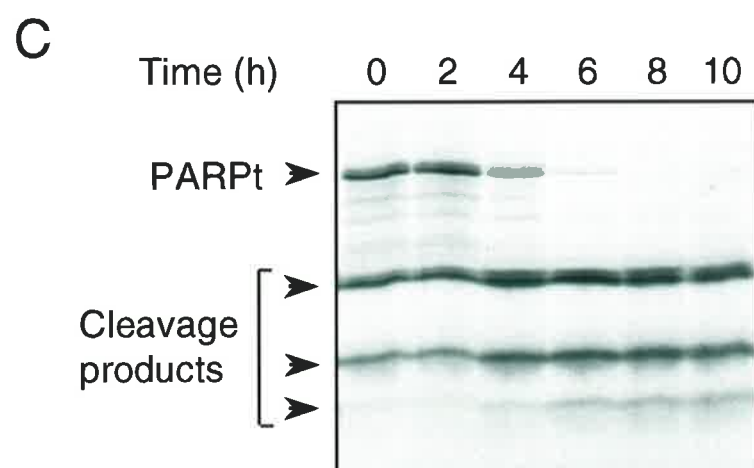
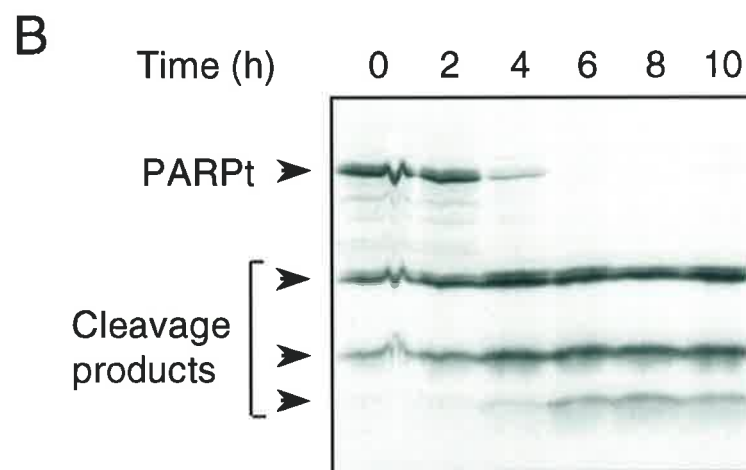
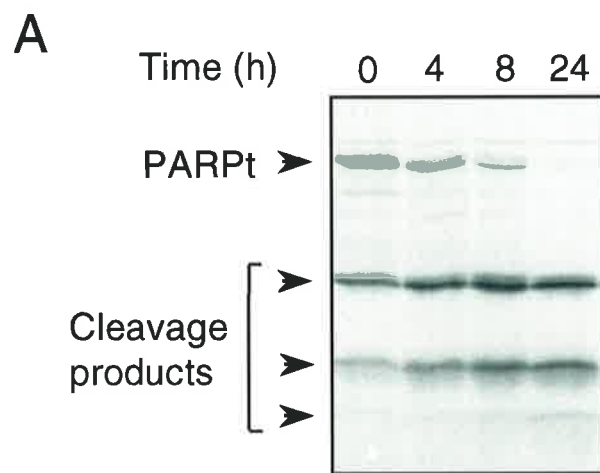


Figure 4.5. Activation of caspases and cleavage of the caspase substrate PARP in Mo7e cells following the withdrawal of IL-3 and serum from growth medium.

Immunoblot analysis of the activation of caspase-2, caspase-3 and caspase-7 and the *in vivo* cleavage of PARP in Mo7e cells over a timecourse of 24 h following IL-3 and serum withdrawal. Immunoblots were performed as described in Materials and Methods (2.4.3). Caspase activation is evident from the disappearance of caspase precursors and the appearance of the p20-like and/or p10-like subunits that comprise the active enzyme.

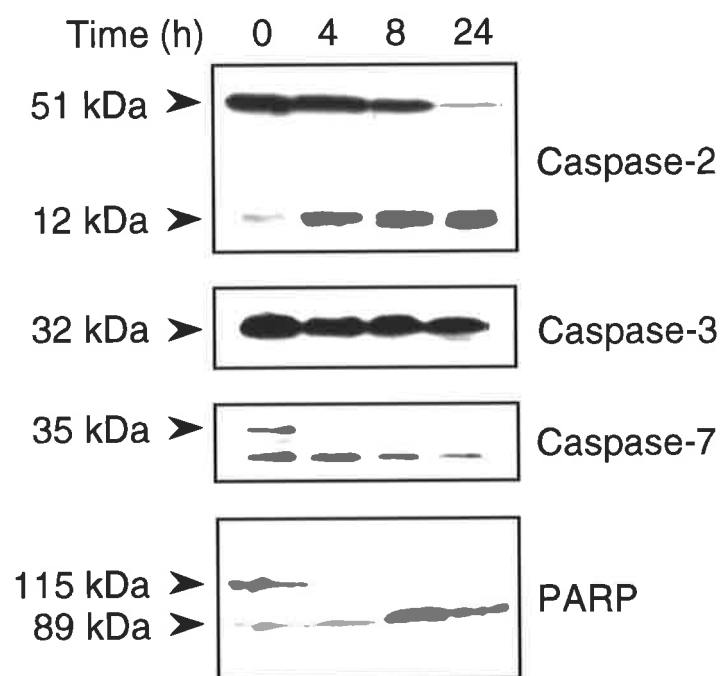


Figure 4.6. Activation of caspases and cleavage of the caspase substrate PARP in Mo7e cells following treatment with etoposide.

Immunoblot analysis of the activation of caspase-2, caspase-3 and caspase-7 and the *in vivo* cleavage of PARP in Mo7e cells over a 10 h timecourse following the addition of etoposide (40 μ M final concentration) to growth medium. Immunoblots were performed as described in Materials and Methods (2.4.3).

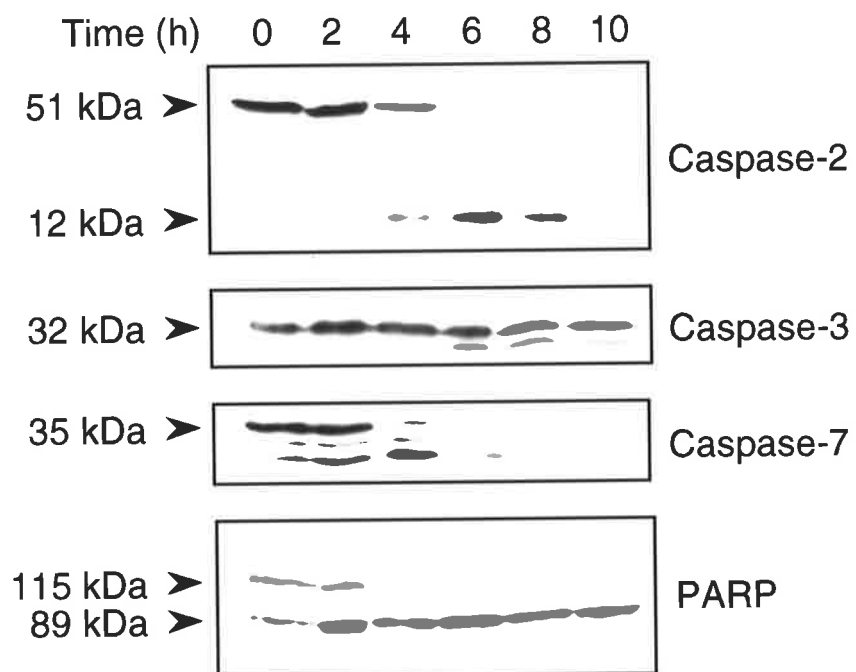


Figure 4.7. Activation of caspases and cleavage of the caspase substrate PARP in Mo7e cells following γ -irradiation.

Immunoblot analysis of the activation of caspase-2, caspase-3 and caspase-7 and the *in vivo* cleavage of PARP in Mo7e cells over a 10 h timecourse following 30 Gy γ -irradiation. Immunoblots were performed as described in Materials and Methods (2.4.3).

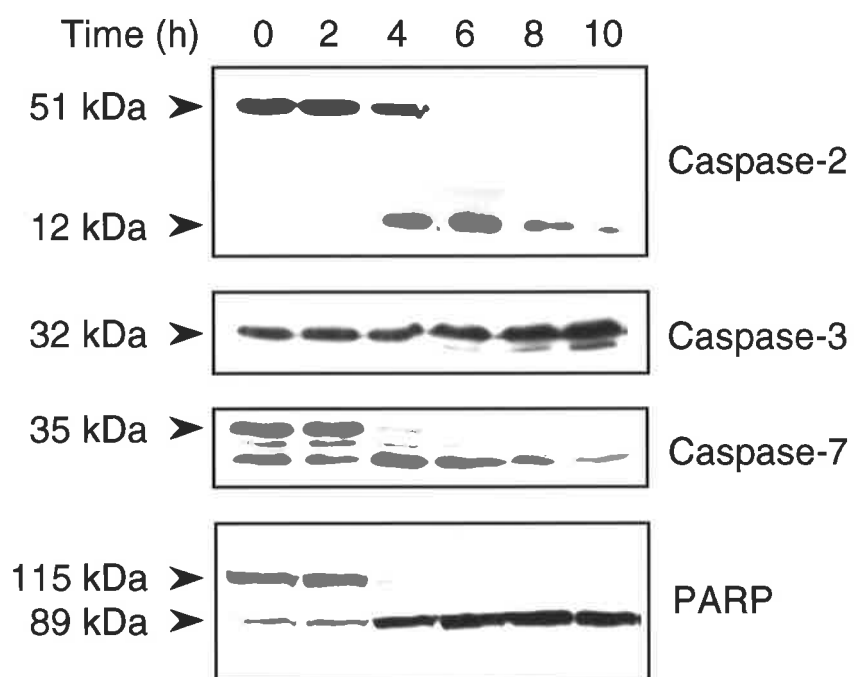


Figure 4.8. Activation of caspase-2 and caspase-3 in HeLa cells following treatment with TNF α .

Immunoblot analysis of caspase-2 and caspase-3 activation in HeLa cells over a 10 h timecourse following treatment with TNF α (10 ng/ml) in conjunction with cycloheximide (10 μ g/ml). Immunoblots were performed as described in Materials and Methods (2.4.3).

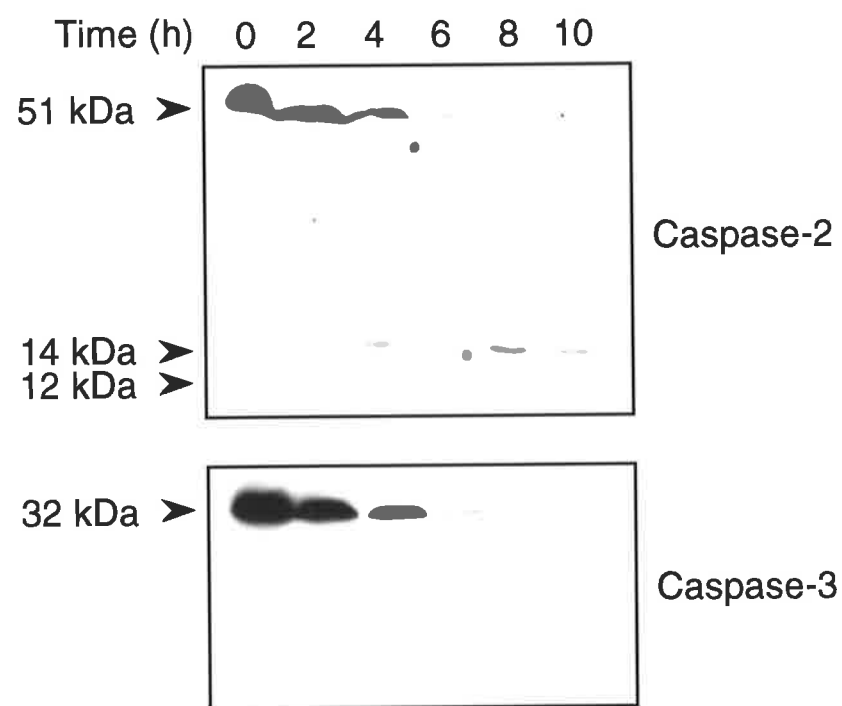


Figure 4.9. Activation of caspases and cleavage of the caspase substrate PARP in Jurkat cells following ligation of the Fas receptor by α -Fas antibody.

Immunoblot analysis of caspase-2 and caspase-3 activation and *in vivo* PARP cleavage in Jurkat cells over a timecourse of treatment with α -Fas antibody (200 ng/ml). Immunoblots were performed as described in Materials and Methods (2.4.3). The percentage of apoptotic cells was calculated following the morphological analysis of DAPI stained cytosmeared cells.

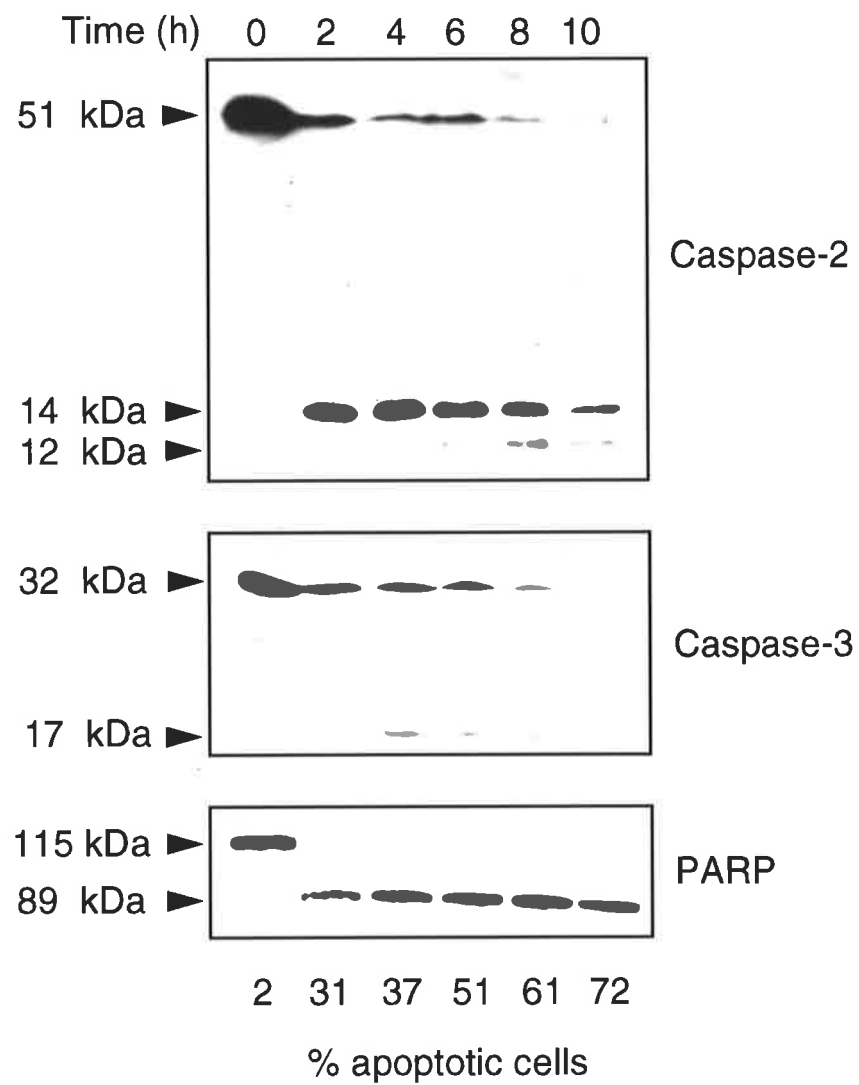


Figure 4.10. Apoptotic morphology of Jurkat cells following treatment with α -Fas antibody.

Cytosmears of Jurkat cells were prepared over a timecourse of treatment with α -Fas antibody (200 ng/ml). Smears were fixed and stained with DAPI as described in Materials and Methods (2.5.3) and analysed by fluorescence microscopy. Cells were photographed at low magnification. Apoptotic cells exhibit condensed and in some cases, fragmented, nuclei. The percentage of apoptotic cells at each timepoint, as calculated by analysis of nuclear morphology following DAPI staining, is documented in Figure 4.9.

- A) Untreated cells
- B) 2 h post treatment
- C) 4 h post treatment
- D) 6 h post treatment
- E) 8 h post treatment
- F) 10 h post treatment

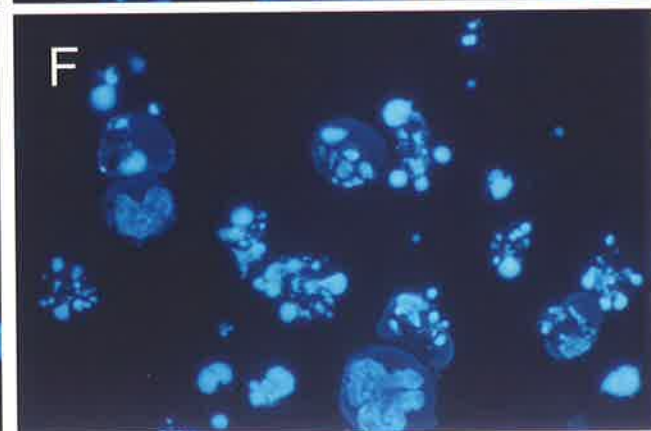
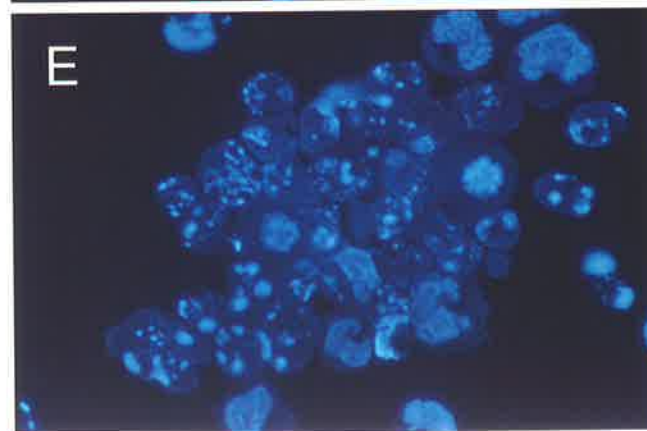
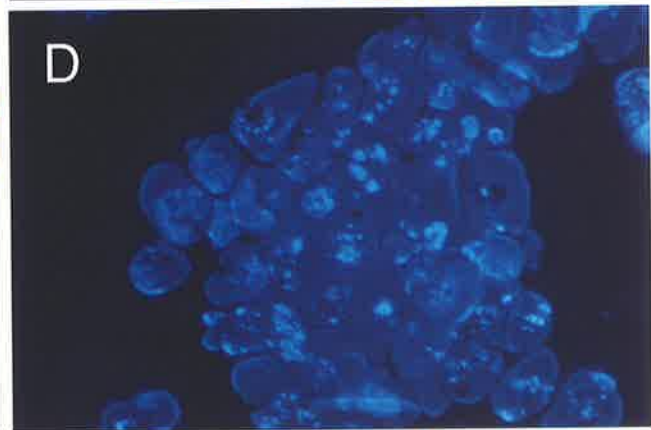
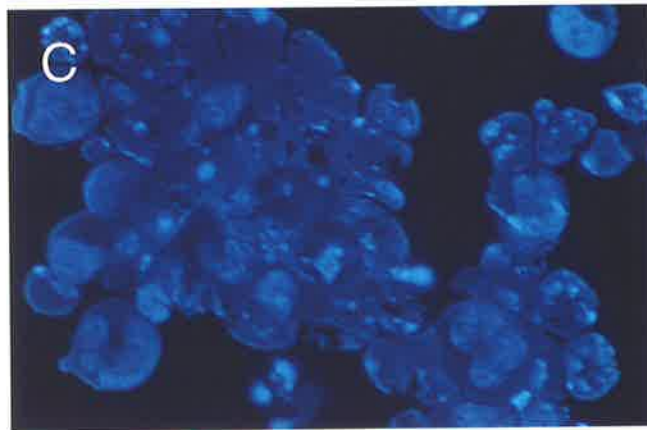
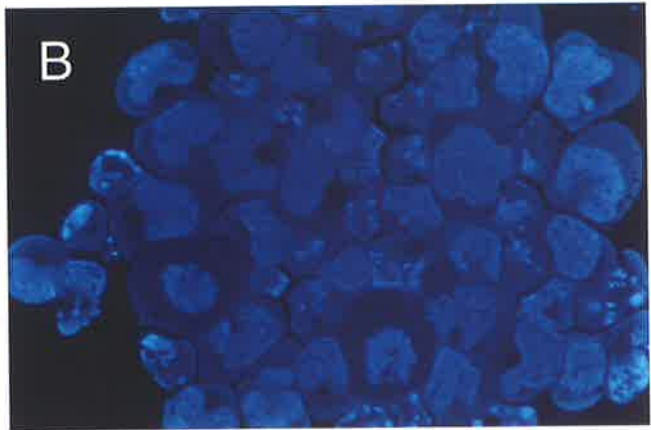
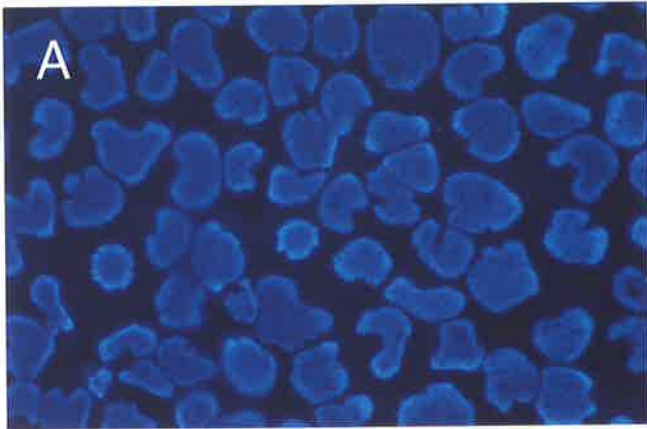


Figure 4.11. Apoptotic morphology of Jurkat cells following etoposide treatment.

Cytosmears of Jurkat cells were prepared over a timecourse of treatment with etoposide (40 μ M). Smears were fixed and stained with DAPI as described in Materials and Methods (2.5.3) and analysed by fluorescence microscopy. Cells were photographed at low magnification. Apoptotic cells exhibit condensed and in some cases, fragmented, nuclei. The percentage of apoptotic cells, as calculated by the analysis of nuclear morphology following DAPI staining, is documented in Figure 4.12.

- A) Untreated cells
- B) 2 h post treatment
- C) 4 h post treatment
- D) 6 h post treatment
- E) 8 h post treatment
- F) 10 h post treatment

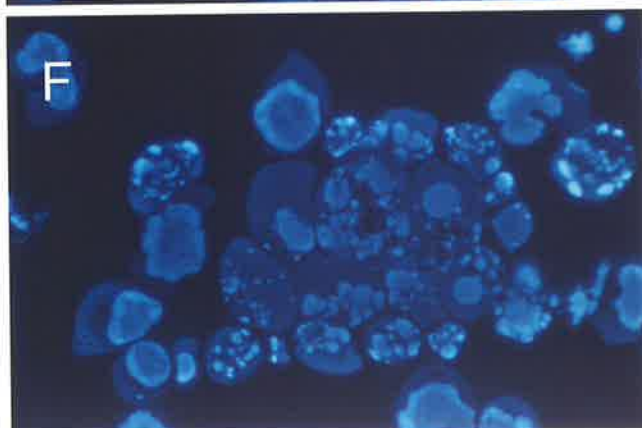
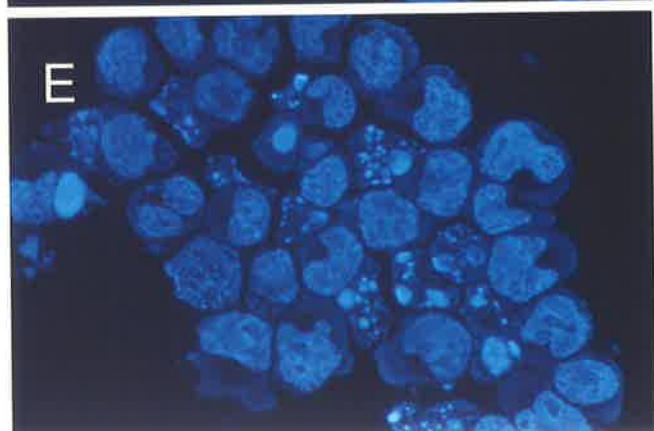
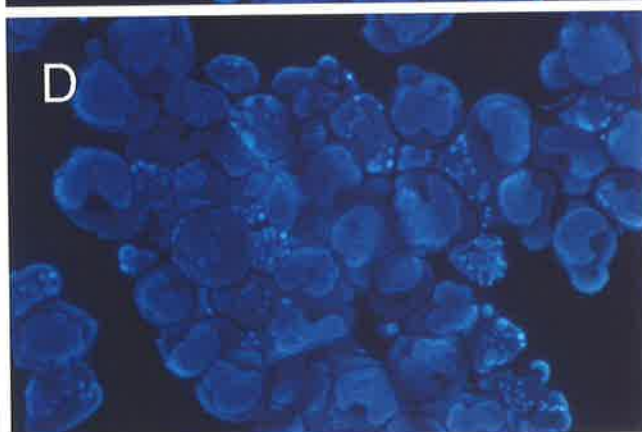
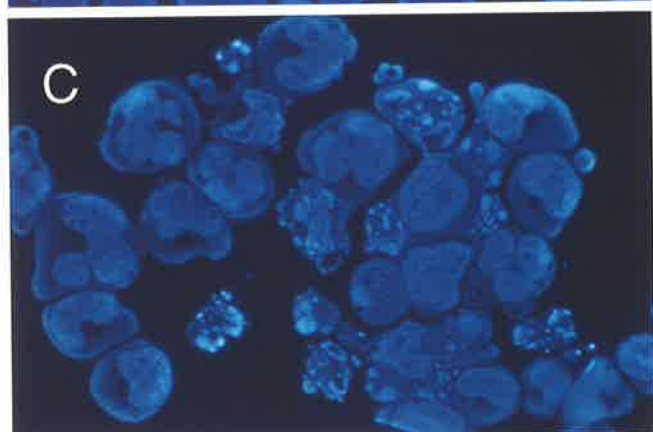
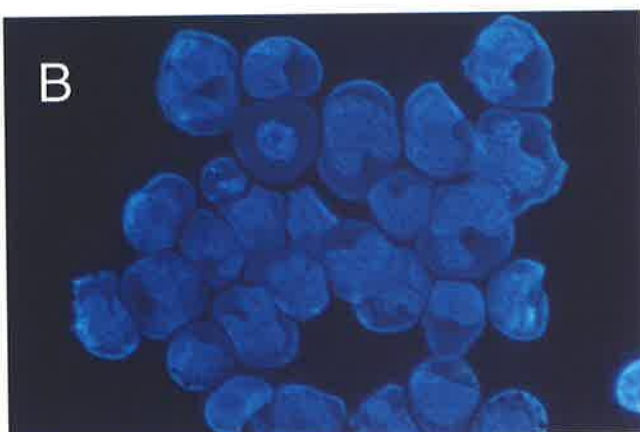
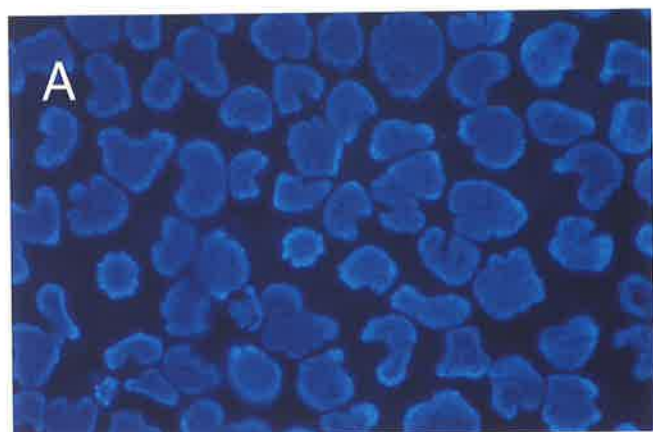


Figure 4.12. Activation of caspases and cleavage of the caspase substrate PARP in Jurkat cells following treatment with etoposide.

Immunoblot analysis of caspase-2 and caspase-3 activation and *in vivo* PARP cleavage in Jurkat cells over a timecourse of treatment with etoposide (40 μ M). Immunoblots were performed as described in Materials and Methods (2.4.3). The percentage of apoptotic cells was calculated following the morphological analysis of DAPI stained cytosmeared cells.

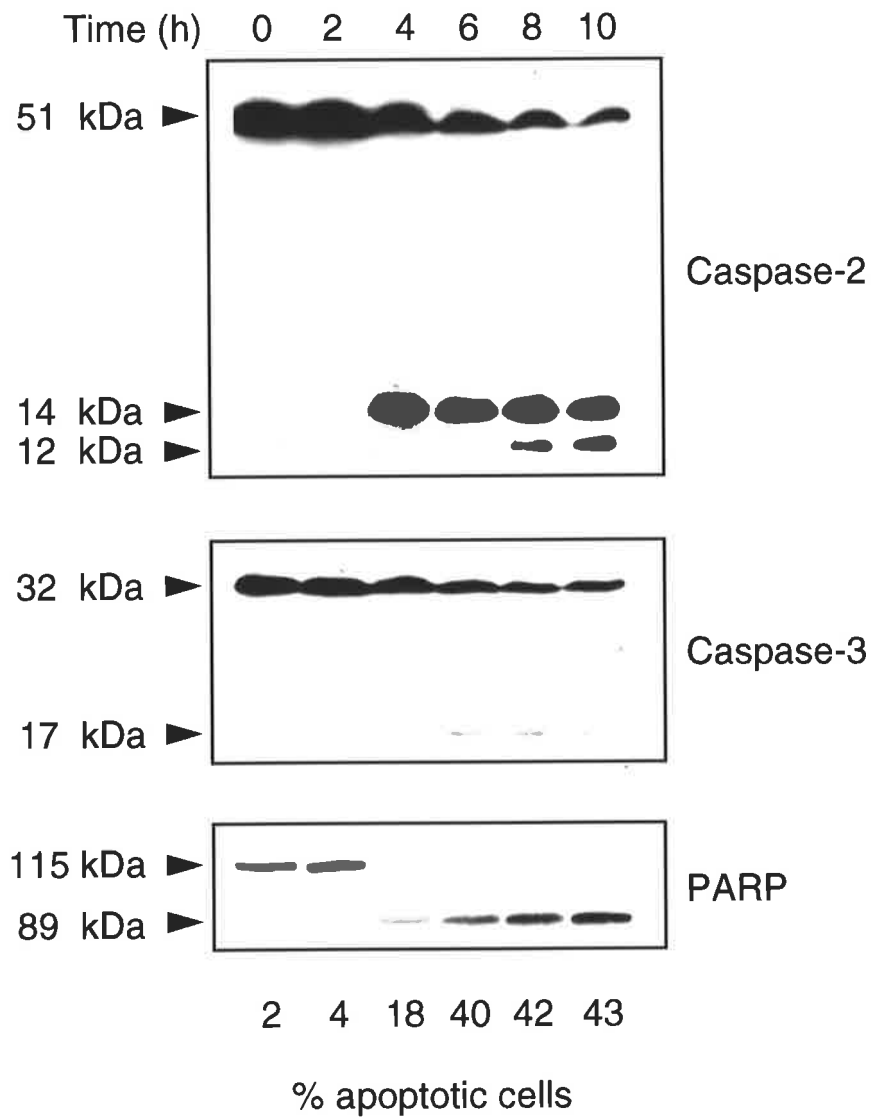


Figure 4.13. Bcl-2 partially protects Jurkat cells against etoposide induced apoptosis, but not against Fas-induced apoptosis.

Jurkat cells and Jurkat cells stably transfected with *bcl-2* were treated with α -Fas antibody (100 ng/ml) (A) and etoposide (20 μ M) (B). The percentage of apoptotic cells as calculated by trypan blue exclusion was analysed over a 48 h timecourse.

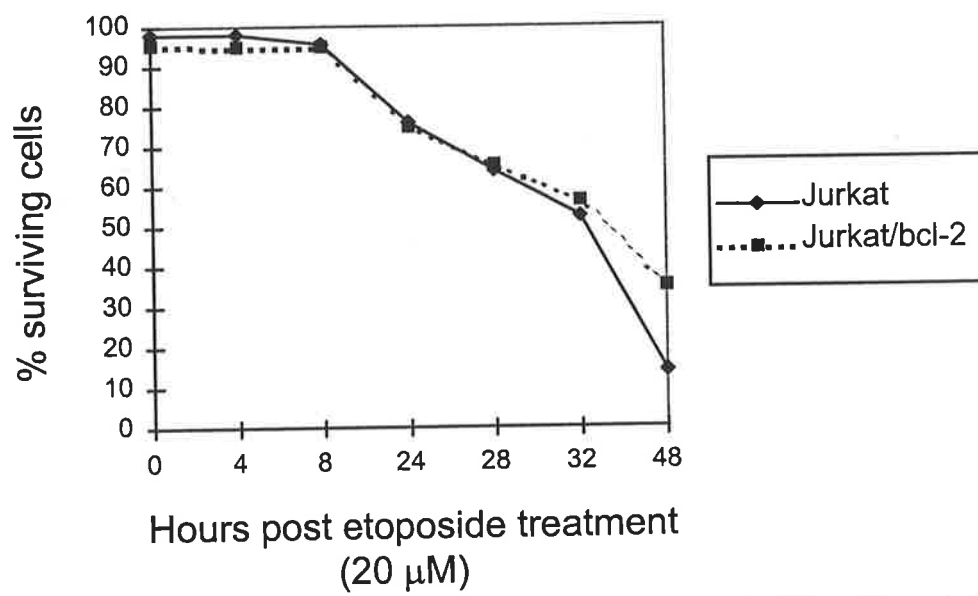
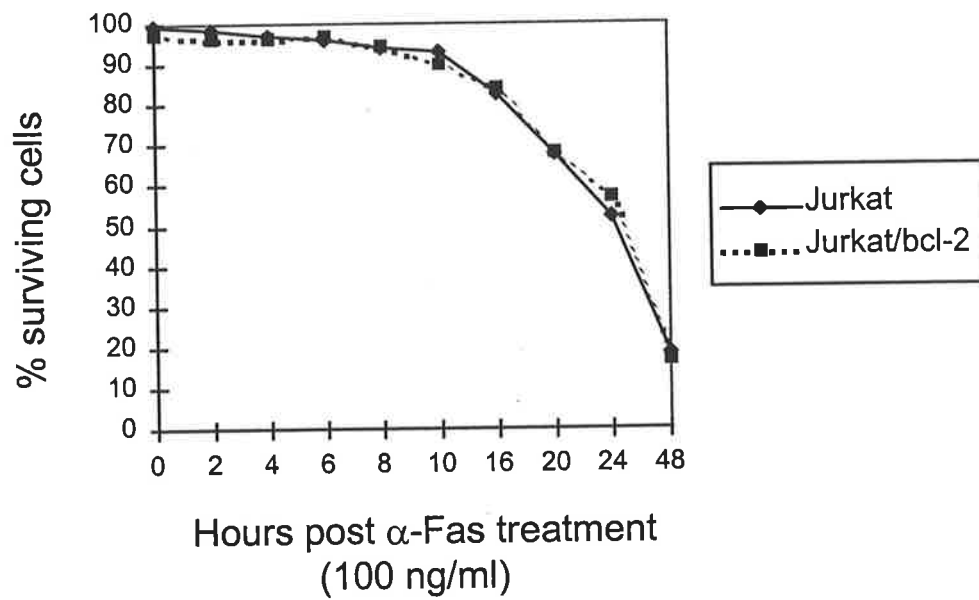
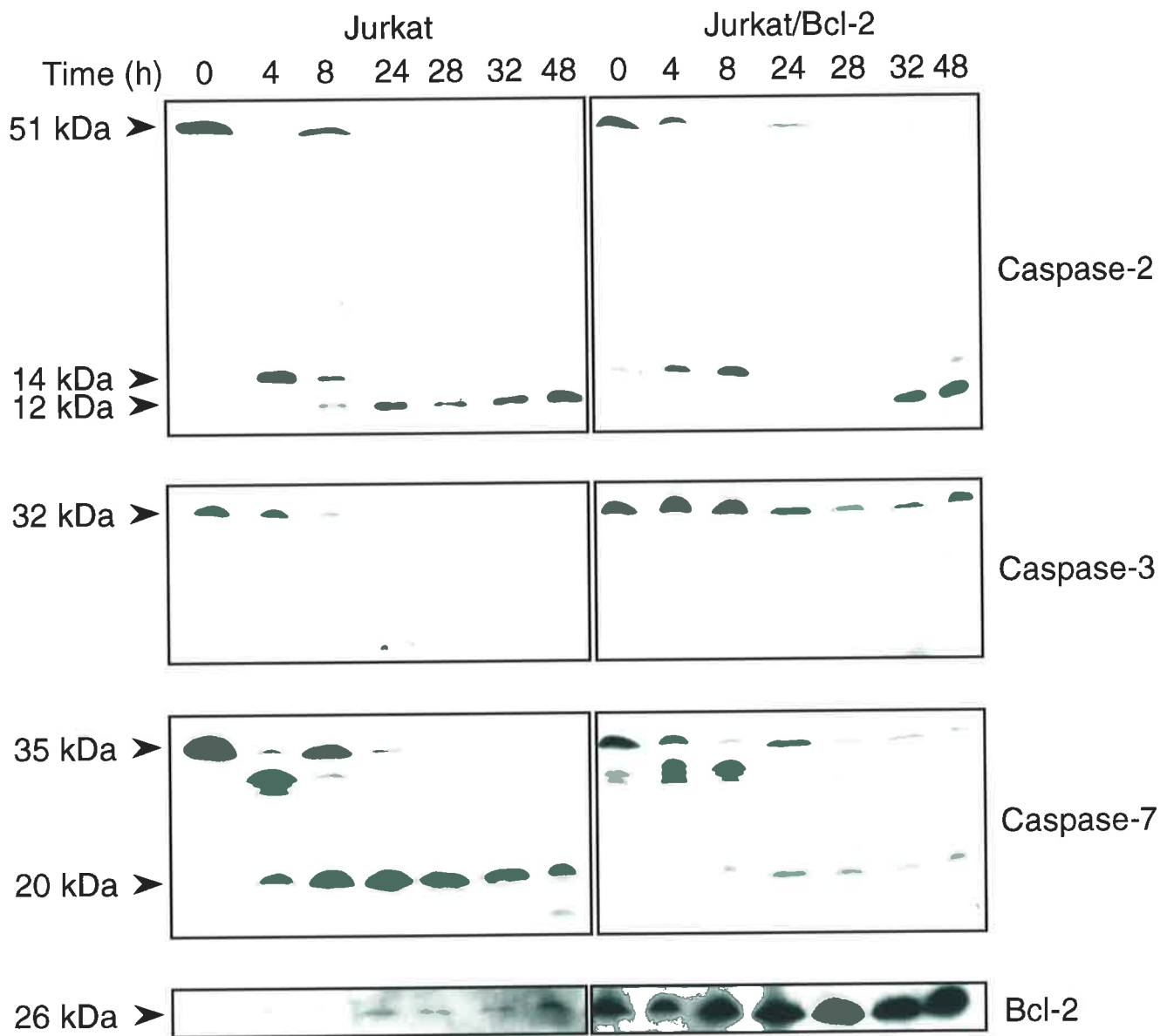


Figure 4.14. Activation of caspases and expression of Bcl-2 in Jurkat cells and Jurkat/Bcl-2 transfectants following etoposide treatment.

Jurkat cells and Jurkat/Bcl-2 transfectants were treated with 20 μ M etoposide over a 48 h timecourse. Immunoblots were performed as described in Materials and Methods (2.4.3). The initial activation of each caspase analysed appears to be slightly delayed in Jurkat/Bcl-2 transfectants as compared to untransfected Jurkat cells. In addition, elevated levels of unprocessed caspases are evident at late timepoints in Bcl-2 transfected cells which are not present in untransfected cells. The level of Bcl-2 expressed in transfected cells is significantly higher than in untransfected Jurkat cells.



Chapter 5

Mutation Analysis of Caspase-2 Proteolytic Processing and Apoptotic Activity

5.1 Introduction

Having established that caspase-2 is activated early in the apoptotic pathway in response to a variety of apoptotic signals and that caspase-2 is able to undergo self-proteolysis, the amino acid residues important for these processes were investigated. A previous study by Xue and colleagues demonstrated that the cleavage sites at which caspase-2 is processed in *E. coli* are DNKD¹⁶⁹, DQQD³³³ and EESD³⁴⁷, resulting in the liberation of 18 kDa, 13 kDa and 12 kDa polypeptides (Xue *et al.*, 1996). The aim of this study was to determine what effect mutation of these, and other candidate Asp cleavage sites in the close vicinity, have on both *in vitro* and *in vivo* caspase-2 processing and on the ability of caspase-2 to induce cell death. The residues chosen for mutation analysis were the previously determined cleavage sites Asp¹⁶⁹, Asp³³³ and Asp³⁴⁷, along with Asp¹³⁵, Asp³²⁶ and Asp³³⁰. The mutation of these sites should demonstrate the degree of site specificity at which caspase-2 is cleaved, as well as the relative importance of complete release of each the p12 and p19 subunits for enzyme activity. A recent study with caspase-1 determined that the prodomain of caspase-1 serves to mediate dimerisation and subsequent processing of the proenzyme (Van Crielinge *et al.*, 1996), suggesting a mechanism by which long prodomain-containing caspases can associate and undergo autoprocessing. A parallel study in our laboratory investigated whether caspase-2 is also capable of dimerisation and the effect that the above mentioned aspartate mutations have on caspase-2 dimerisation and autoprocessing (Butt *et al.*, 1998).

Results

5.2 *In Vitro* Processing of Procaspace-2 by Recombinant Caspase-2 and Caspase-3

We and others have shown that procaspase-2 is cleaved *in vitro* into two subunits 19 kDa and 12 kDa in size by several caspases including caspase-2 and caspase-3 (Results

chapter 3, Harvey *et al.*, 1996, Xue *et al.*, 1996). To ascertain whether mutations at various Asp residues can affect cleavage of procaspase-2, mutant proteins (Figure 5.1) were subjected to *in vitro* cleavage by recombinant caspase-2 (Figure 5.2). Wild-type and Asp³⁴⁷ proteins were efficiently processed by recombinant caspase-2, as apparent by the disappearance of 51 kDa procaspase-2 and appearance of the processed subunits (19 kDa and 12 kDa) and intermediates (Figure 5.2B). The processed products resulting from Asp³⁴⁷ cleavage are more visible in longer exposures. In the case of Asp¹³⁵ and Asp³²⁶, only marginal cleavage of the proenzyme was seen, most of the precursor remained intact. Mutations at Asp³³⁰ and Asp³³³ suppressed the release of p14, the intermediate preceding generation of p12 (as described in Results chapter 4). Asp¹⁶⁹ was partially processed by recombinant caspase-2, but the cleavage profile of this mutant was altered when compared to wild-type caspase-2.

As caspase-3 has previously been shown to mediate the cleavage of procaspase-2 (Harvey *et al.*, 1996, Xue *et al.*, 1996), we subjected the procaspase-2 Asp mutants to proteolysis by recombinant caspase-3 (Figure 5.2C). Interestingly, Asp¹³⁵ and Asp³²⁶ mutants which were poorly processed by caspase-2 were more efficiently cleaved by caspase-3. Caspase-3 cleavage of Asp³³⁰ and Asp³³³ (P₄ and P₁ positions respectively, in the D³³⁰QQD³³³ sequence in procaspase-2) mutants generated a fragment larger than the small subunit generated by cleavage of wild-type caspase-2, suggesting that caspase-3 normally cleaves wild-type caspase-2 at Asp³³³. Caspase-3 is possibly cleaving caspase-2 at an alternative, nearby site in the Asp³³⁰ and Asp³³³ mutants which results in the release of this unique fragment. These results suggest that the mechanisms of procaspase-2 processing by caspase-2 and caspase-3 are different and that these two caspases have different substrate specificities.

5.3 *In Vivo* Processing and Apoptotic Activity of Caspase-2 Mutants

The first biological assays to show a role for caspase-2 in apoptosis utilised procaspase-2 overexpression in transiently transfected cells (Kumar *et al.*, 1994, Wang *et al.*, 1994). These experiments clearly showed that overexpression of procaspase-2 induced apoptosis, but it was not clear how the cell death was mediated. Work from our laboratory and that of others has suggested that the overexpression of caspases can result in autoprocessing (Kumar *et al.*, 1994, Wang *et al.*, 1994, Allet *et al.*, 1996, Dorstyn and Kumar, 1997), perhaps in a concentration-dependent manner, but it has not been firmly established in the transient transfection system. To address this issue, the death-inducing activity and proteolytic processing of the various caspase-2 mutants were analysed in transiently transfected NIH-3T3 cells (Figures 5.3-5.5). Consistent with *in vitro* caspase-2 processing data, mutations at Asp¹⁶⁹ and Asp³⁴⁷ retained their apoptotic activity, while substitutions at Cys³²⁰, Asp¹³⁵, Asp³²⁶ and Asp³³³ completely abolished the apoptosis-inducing activity of caspase-2 (Figure 5.3). Asp³³⁰ retained some apoptotic activity (approximately 45%), suggesting that processing at the D³³⁰QQD³³³ site is absolutely dependent on Asp³³³, with a lesser requirement for Asp³³⁰.

The *in vivo* processing of caspase-2 aspartate mutants was analysed using an antibody raised against the p12 subunit of caspase-2. NIH-3T3 cells were transfected with various expression constructs and harvested 12 hours later. Cells were harvested at this timepoint following transfection to maximise the detection of expressed caspase-2 protein prior to the complete apoptotic demise of the majority of transfected cells. Immunoblotting of extracts from these cells showed that wild-type procaspase-2 and the Asp³⁴⁷ mutant were efficiently processed *in vivo*, however, in extracts transfected with the Asp³⁴⁷ mutant, p14, but not p12 was released, confirming that cleavage at Asp³⁴⁷

generates the mature p12 subunit. Asp³³⁰ and Asp³³³ show partial processing in transfected cells, with release of the prodomain generating a p19 + p14 intermediate that is further processed to some extent in the Asp³³⁰ mutant to generate p14 and p12 subunits. This observation correlates with the fact that Asp³³⁰, but not Asp³³³, retains some apoptotic activity. Asp¹³⁵ and Asp³²⁶ mutants were not processed (Figure 5.5), which is consistent with their inability to induce apoptosis. Some processing of the Cys³²⁰ mutant was also evident in transfected cells. Since this mutant is catalytically inert, we believe that partial processing of Cys³²⁰ in transfected cells may be mediated by other endogenous caspases rather than by autocatalysis.

Further results from our laboratory using yeast two-hybrid analysis demonstrated that catalytically inactive procaspase-2 is able to dimerise in yeast, suggesting that caspase-2 autoprocessing is facilitated by prior dimerisation of proenzyme molecules (Butt *et al.*, 1998). Analysis of the aspartate mutants for their ability to dimerise demonstrated that the dimerisation of pro-caspase-2 was only able to occur in mutants that lacked autoprocessing activity, consistent with the assumption that dimerisation occurs prior to procaspase-2 processing. In addition, removal of either the prodomain or the C terminus of procaspase-2 abolished dimerisation, indicating that both the prodomain and carboxy-terminal residues are required for dimerisation to occur (Butt *et al.*, 1998).

5.4 Discussion

This study has highlighted several residues which are important for the processing and apoptotic activity of caspase-2. As described by Allet and colleagues (1996), mutation of Asp¹³⁵, Asp³²⁶ or Asp³³³ abolished the apoptosis-inducing activity and autoprocessing of caspase-2 when expressed in mammalian cells. Interestingly, in this study, mutation

of Asp³³⁰ retained partial apoptosis-inducing and autoprocessing activity, indicating that it is the contribution that this residue makes to the D³³⁰QQD³³³ site that is important for caspase-2 processing, rather than its function as a unique cleavage site. Based on microsequencing of the active enzyme subunits resulting from the expression of procaspase-2 in *E. coli*, previous work has defined the cleavage sites in procaspase-2 as DNKD¹⁶⁹, DQQD³³³ and EESD³⁴⁷ (Xue *et al.*, 1996). Our results suggest that to generate the small subunit of the mature enzyme, the first cleavage occurs at Asp³³³ followed by subsequent cleavage at Asp³⁴⁷. Although cleavage at Asp³⁴⁷ generates the mature p12 subunit, this cleavage is not crucial for catalytic activity of caspase-2; the Asp³⁴⁷ mutant is still able to be processed at Asp³³³ and retains apoptosis-inducing activity similar to that of wild-type caspase-2. In immunoblot analysis of caspase-2 following the treatment of Jurkat cells with Fas antibody and etoposide and treatment of HeLa cells with TNF α , a caspase-2 proteolytic product of 14 kDa always precedes the appearance of p12 (Results, chapter 4). It seems likely then that the 14 kDa intermediate is able to be incorporated into the processed caspase tetramer to produce active enzyme. Mutation at Asp¹⁶⁹, at which the cleavage that liberates the p19 subunit from the prodomain occurs, also has no effect on the apoptosis inducing activity of caspase-2. This suggests that either cleavage is able to occur at an amino terminal site other than Asp¹⁶⁹, or that removal of the prodomain is not essential for the production of active enzyme.

Using a Semliki virus expression system, Allet and colleagues showed that Asp¹³⁵ is unable to be processed in mammalian cells and was also unable to induce apoptosis when microinjected into sympathetic neurons (Allet *et al.*, 1996). Our results are consistent with these observations. Since the Asp¹³⁵ mutant was not efficiently cleaved by recombinant caspase-2, even at Asp residues at the C-terminus of procaspase-2, it

appears that the Asp¹³⁵ residue, which lies in the prodomain region, may be important in maintaining the overall structure of the proenzyme. Mutation at this residue may alter the conformation of the molecule such that other cleavage sites are no longer accessible to caspase-2 cleavage. Interestingly, despite the fact that Asp¹³⁵ is not processed, unlike Cys³²⁰, it is unable to either homodimerise or heterodimerise with the Cys³²⁰ mutant in yeast (Butt *et al.*, 1998). This observation suggests that Asp¹³⁵ alters the conformation of the prodomain region in such a way that the dimerisation of procaspase-2 molecules is prevented, consequently preventing caspase-2 processing and apoptosis inducing activity. Mutation of Asp³²⁶ also inhibited processing and apoptosis inducing activity of caspase-2, suggesting that this residue may also be important for maintaining the secondary structure of caspase-2.

The results presented in this chapter, together with the yeast two-hybrid dimerisation studies, suggest that procaspase-2 homodimerises prior to processing and that dimerisation of procaspase molecules is essential for autoprocessing and subsequent proapoptotic activity. The recruitment of caspase-2 via adaptor molecules such as RAIDD to specific death complexes upon the receipt of a death signal is likely to achieve the high, localised concentration of procaspase-2 that is required for dimerisation, autoprocessing and subsequent apoptosis to occur. These experiments have highlighted the importance of several aspartate residues in procaspase-2 which are crucial for autoprocessing and apoptosis inducing activity.

Figure 5.1. Schematic representation of caspase-2 demonstrating the location of aspartate residues which were mutated and analysed in the following studies.

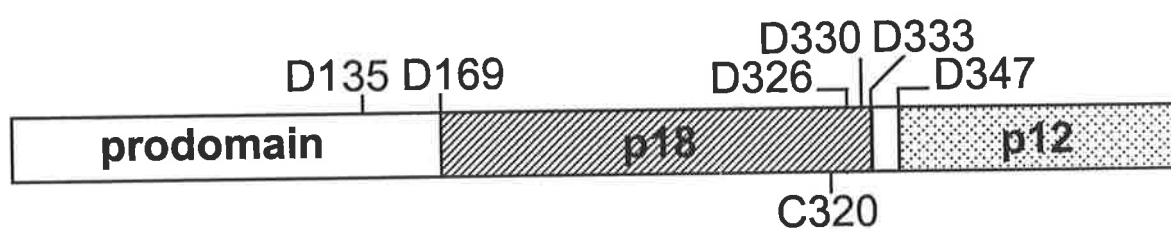


Figure 5.2. *In Vitro* processing of wild-type and mutant caspase-2 proteins by active recombinant caspase-2 and caspase-3.

³⁵S-Met labelled caspase-2 wild-type and aspartate mutant proteins were incubated alone (A), or with recombinant caspase-2 (pETprocaspase-2) (B) or caspase-3 (C) for 3 h at 37 °C. Samples were analysed following electrophoresis, transfer and autoradiography as previously described. In these experiments, it was not possible to distinguish between the p14 and p12 subunits. Although the signal from mutant D347 is weak in panel (C), longer exposures reveal that the pattern of D347 proteolysis by caspase-3 appears to be the same as that of wild-type caspase-2.

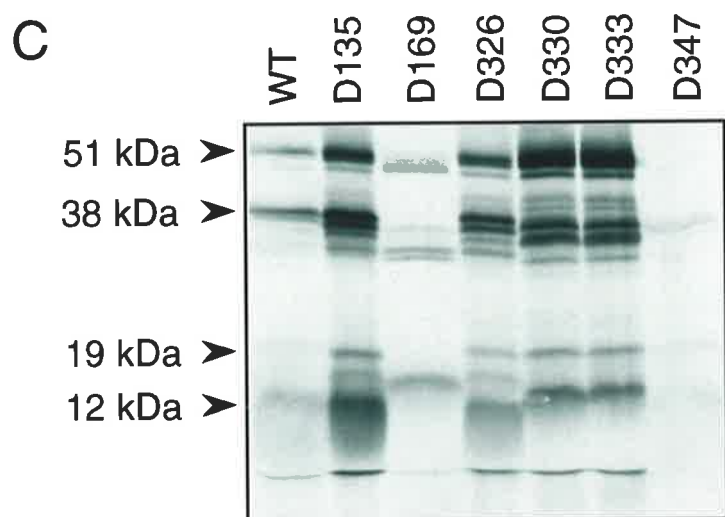
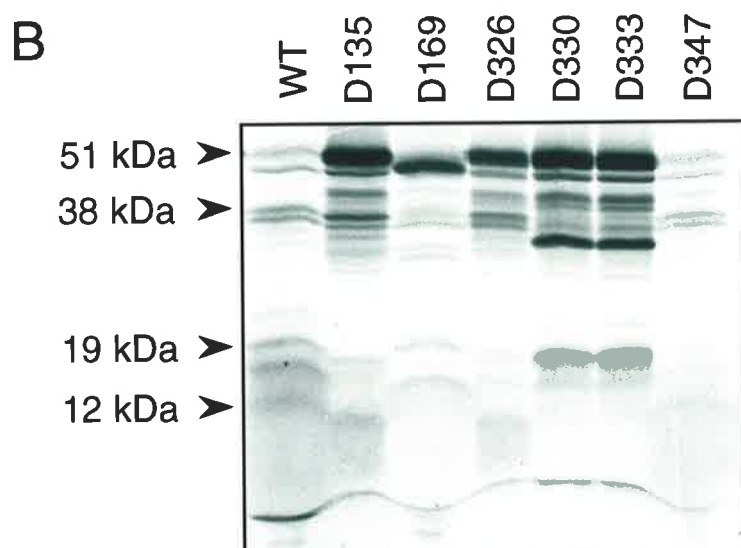
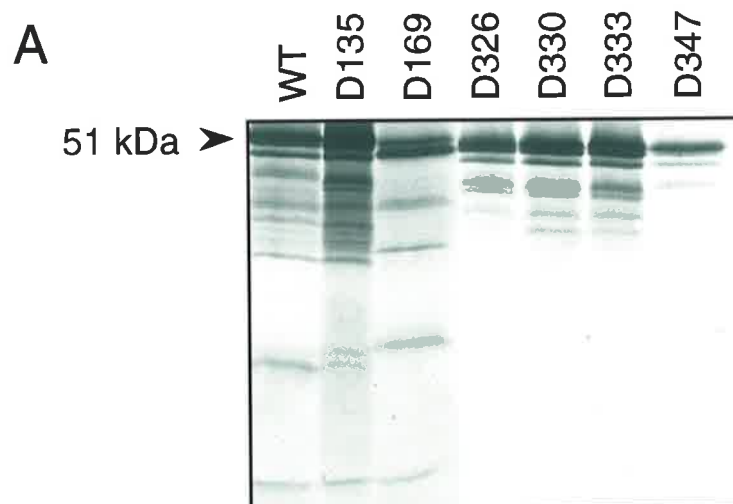


Figure 5.3. Proapoptotic activities of various caspase-2 mutants in NIH-3T3 cells.

Wild-type and mutant caspase-2 cDNAs cloned into the mammalian expression vector pCXN2 were cotransfected with a β -galactosidase expression vector into NIH-3T3 cells. 24 h post-transfection, cells were fixed and stained with X-gal as described in Materials and Methods (2.5.1). Apoptotic and non-apoptotic β -galactosidase-positive blue cells were scored by microscopic examination. Data shown represent average values from two separate experiments performed in duplicate.

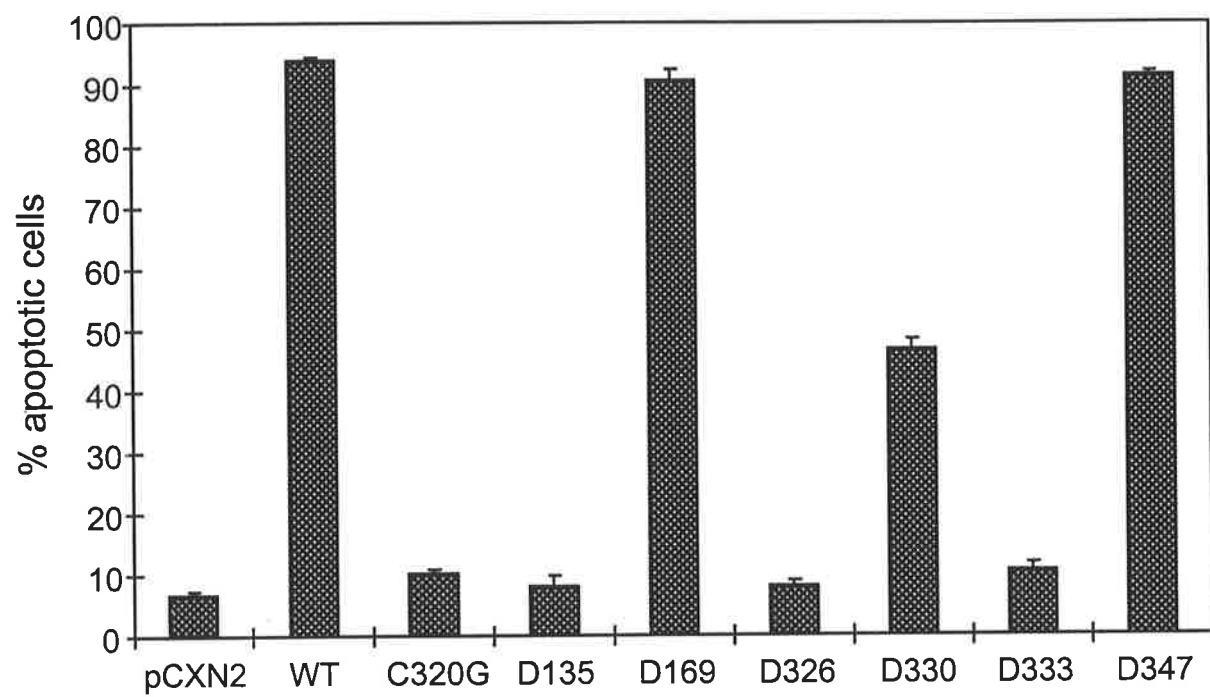


Figure 5.4. Morphology of NIH-3T3 cells transfected with various caspase-2 mutants.

Wild-type and mutant caspase-2 cDNAs cloned into the mammalian expression vector pCXN2 were cotransfected with a β -galactosidase expression vector into NIH-3T3 cells. 24 h post-transfection, cells were fixed and stained with X-gal as described in Materials and Methods (2.5.1). Apoptotic β -galactosidase-positive blue cells appear rounded and condensed, while non-apoptotic cells exhibit normal, flat morphology.

A) pCXN2

B) pCXN2Caspase-2

C) pCXN2Caspase-2(C320G)

D) pCXN2D135

E) pCXN2D169

F) pCXN2D326

G) pCXN2D330

H) pCXN2D333

I) pCXN2D347

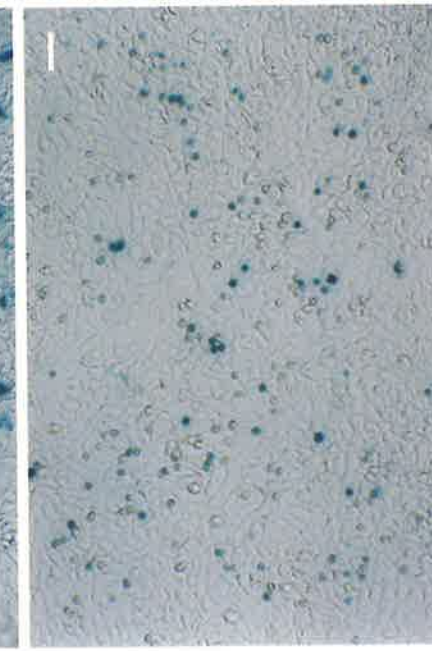
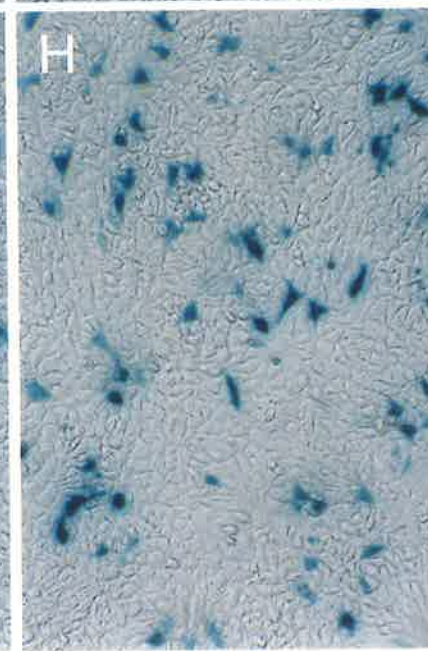
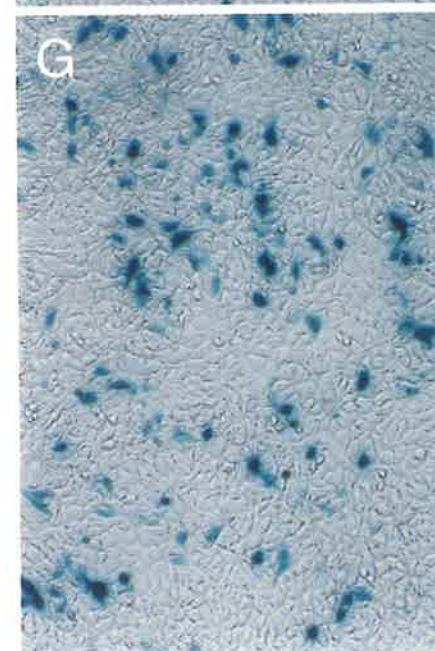
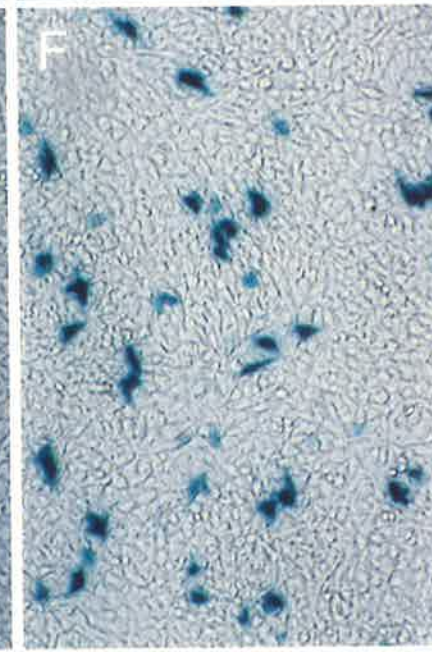
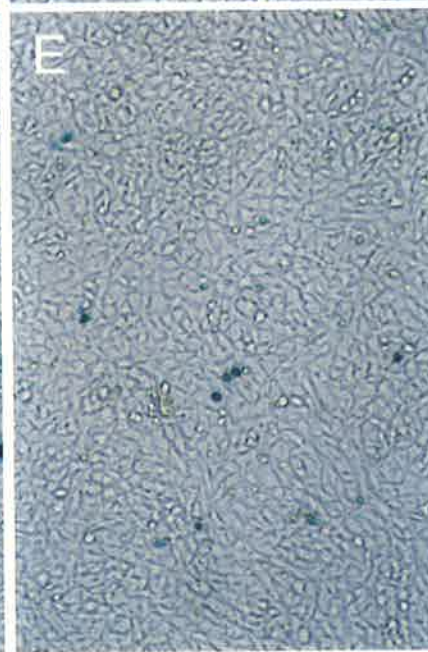
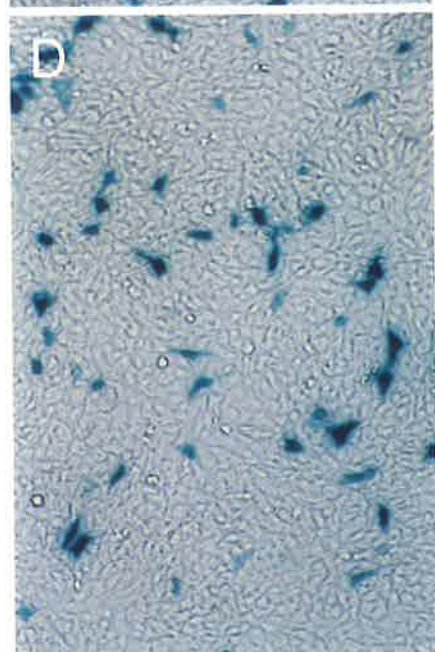
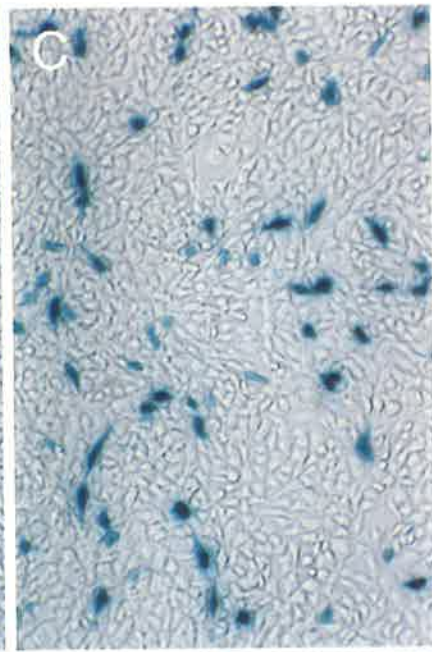
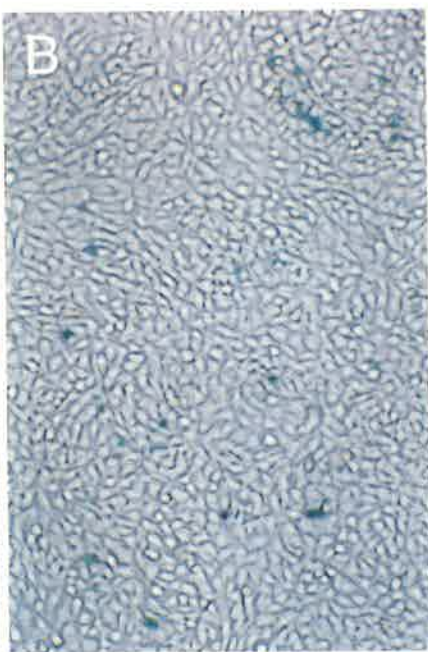
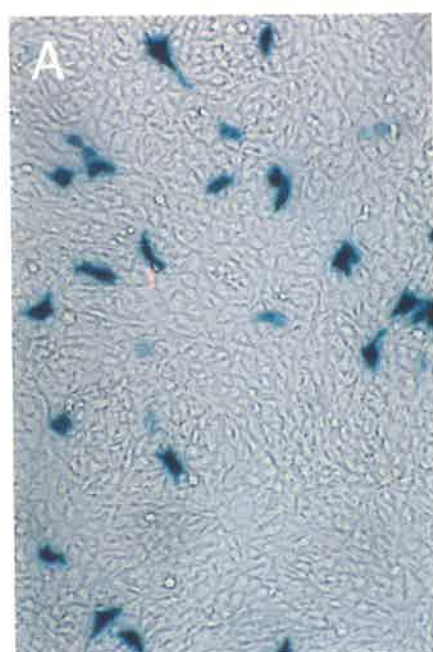
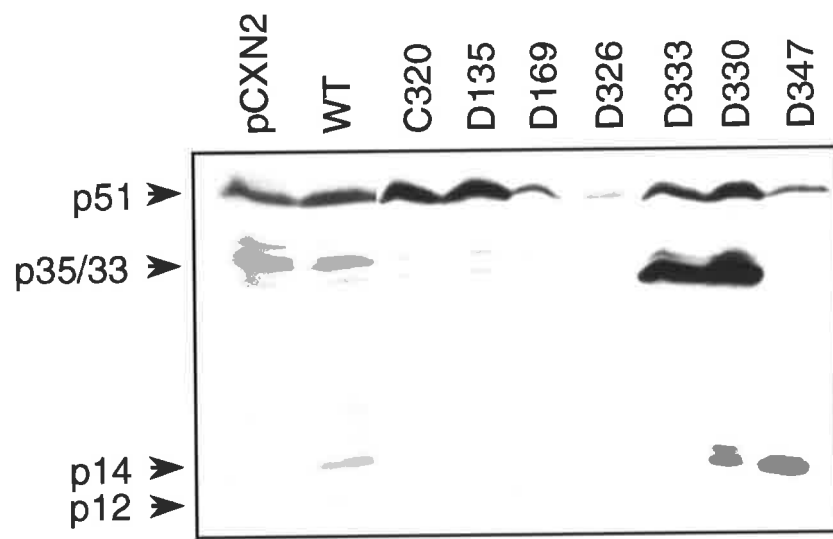


Figure 5.5. Proteolytic processing of caspase-2 mutants in transiently transfected NIH-3T3 cells.

NIH-3T3 cells were transfected with various caspase-2 constructs in the pCXN2 mammalian expression vector. Cells were harvested 12 h post-transfection and cell lysates were analysed by immunoblotting with a polyclonal antiserum raised against the small subunit of caspase-2. The 51 kDa procaspase-2 band in NIH-3T3 cells transfected with vector alone represents the endogenous level of caspase-2 protein present in these cells. The length of exposure to film for the first two lanes is different from the remainder of the figure. No processing of D326 is evident even following long exposure times. Although the transfection efficiency of the D326 mutant was similar to that of the other catalytically inactive mutants such as Cys320 and D135, weak signals for D326 protein were seen in all experiments performed. This may reflect relative instability of this protein in transfected cells. WT = wild-type.



Chapter 6

Conversion of Procaspase-3 to an Autoactivating Caspase by Fusion to the Caspase-2 Prodomain

6.1 Introduction

As documented in earlier chapters, many studies have shown that the activation of various caspases occurs in response to apoptotic stimuli and that this process is an integral step in the apoptotic pathway. The mechanism by which activation of the apical caspase in a particular apoptotic pathway is mediated is however, poorly understood to date. Recent studies including those from our laboratory demonstrate that dimerisation/oligomerisation of Class I procaspase molecules mediated either by their prodomains (Butt *et al.*, 1998) or by artificial sequestration (Muzio *et al.*, 1998, Yang *et al.*, 1998) results in their proteolytic activation. These observations suggest that the primary role of adaptor molecules such as FADD and Apaf-1, which bind the prodomains of specific Class I caspases, may be to bring procaspase molecules into close proximity with one another, thereby enabling dimerisation and subsequent autocatalysis. The afore-mentioned study by Muzio and colleagues (1998) demonstrated that caspase-8 possesses intrinsic, low enzymatic activity that enables autoproteolysis to occur when procaspase-8 molecules are induced to dimerise artificially. This is presumably the situation that is created *in vivo* when FADD recruits caspase-8 to the Fas/Apo-1 receptor, resulting in caspase-8 activation. We envisage this to be a possible mechanism for the activation of all apical Class I caspases. Several *in vitro* cleavage studies suggest that Class II caspase activation is induced by Class I caspase-mediated proteolysis (reviewed in Kumar and Lavin, 1996, Nicholson and Thornberry, 1997) and Class II caspases then serve as the effector caspases of cell death, cleaving cellular substrates.

We have recently demonstrated that caspase-2, a Class I caspase, can dimerise and autoprocess in *Saccharomyces cerevisiae* and mammalian cells (Butt *et al.*, 1998). The



prodomain of caspase-2 is essential for dimerisation of the precursor molecule and caspase-2 lacking the prodomain is poorly processed in *S. cerevisiae* cells. Overexpression of procaspase-2 in mammalian cells induces apoptosis (Kumar *et al.*, 1994, Wang *et al.*, 1994, Kumar *et al.*, 1997, Dorstyn and Kumar, 1997) due to its autoprocessing activity (Results chapter 3, Butt *et al.*, 1998). Caspase-3, a Class II effector protease, lacks a long prodomain (Tewari *et al.*, 1995b, Nicholson *et al.*, 1995). Unlike caspase-2, caspase-3 is a poor inducer of apoptosis when overexpressed in mammalian cells (Tewari *et al.*, 1995, Nicholson *et al.*, 1995, Dorstyn and Kumar, 1997, Colussi *et al.*, 1998b), presumably due to its inability to autoactivate. We reasoned that if the prodomain of a Class I caspase is essential for procaspase activation, fusion of a Class I caspase prodomain onto a Class II caspase would generate a procaspase molecule able to dimerise and mediate autocatalytic processing. The aim of this chapter was to determine whether amino terminal fusion of the caspase-2 prodomain (C2P) to procaspase-3 affects the ability of caspase-3 to dimerise, autoprocess and induce apoptosis.

Results

6.2 Fusion of the Caspase-2 Prodomain to Procaspase-3 Augments Caspase-3 Killing Activity

Overexpression of procaspase-2 results in apoptosis in many cell types while overexpression of procaspase-3 leads to a relatively low proportion of cell death (Kumar *et al.*, 1994, Wang *et al.*, 1994, Tewari *et al.*, 1995b, Nicholson *et al.*, 1995, Kumar *et al.*, 1997, Dorstyn and Kumar, 1997, Colussi *et al.*, 1998b). To determine whether a Class I prodomain fused to procaspase-3 can enhance the apoptotic activity of caspase-3, expression constructs were generated in which the caspase-2 prodomain (C2P) was

fused in frame at the amino terminus of procaspase-3 (Figure 6.1A). Two chimeric expression constructs were produced in which either full length procaspase-3 or procaspase-3 lacking the first 9 amino terminal amino acids ($\Delta 9$) was fused to the prodomain of caspase-2. Since one of the cleavage sites in procaspase-3 occurs following Asp-9 (Xue and Horvitz, 1995, Fernandes-Alnemri et al., 1996), the small prodomain region of caspase-3 and 3 amino acid residues at the carboxyl terminus of the caspase-2 prodomain were deleted in the caspase-3 $\Delta 9$ construct to remove a potential aspartate cleavage site and thereby avoid removal of the caspase-2 prodomain upon proteolytic processing in transfected cells. To aid direct visualisation of transfected cells, the coding region of green fluorescence protein (GFP) was fused at the carboxyl terminus of C2P-caspase-3 chimeric molecules (Figure 6.1A). All control and chimeric expression constructs were transfected into NIH-3T3 cells and visualised by fluorescence microscopy 18 hours later. Essentially all cells transfected with the control GFP vector displayed normal flat morphology (Figures 6.1B, 6.2A). As expected, procaspase-2-GFP expression killed almost all transfected cells (>95%) (Figures 6.1B, 6.2B), while caspase-3-GFP induced apoptosis in around 15% of cells (Figures 6.1B, 6.2D). Most of the caspase-3 cells showed normal morphology and as reported previously (Colussi *et al.*, 1998b), the caspase-3-GFP fusion protein localised largely to the cytoplasm (Figure 2D). The cells transfected with the procaspase-2-GFP construct showed very weak fluorescence, presumably due to the proteolytic destruction of GFP in apoptotic cells (Figure 6.2B). Although caspase-3 induced apoptosis in a small proportion of transfected cells, apoptotic cells retained bright GFP fluorescence, suggesting that these cells represent early apoptotic cells as opposed to the late stage apoptotic cells transfected with caspase-2. The chimeric C2P-caspase-3-GFP molecules were both potent killers of transfected NIH-3T3 cells, displaying an apoptotic activity

comparable to that of caspase-2 (Figures 6.1B, 6.2F, 6.2H). C2P-caspase-3(C163G)-GFP and C2P-caspase-3 Δ 9(C163G)-GFP, catalytically inactive mutants of caspase-3 fused to the caspase-2 prodomain, were unable to induce apoptosis in transfected cells (Figure 6.1B, 6.2G, 6.2I), demonstrating that autocatalytic activity of C2P-caspase-3 chimeras is absolutely required for their apoptosis inducing activity.

As documented in a recent study from our laboratory (Colussi *et al.*, 1998a), the C2P-caspase-3(C163G)-GFP mutant chimeric protein localised to discreet “dot-like” structures within the cell (Figure 6.2G, 6.2I). These results suggest that the caspase-2 prodomain, when fused to procaspase-3, in addition to conferring strong apoptosis-inducing activity to caspase-3, shifts the cellular distribution of caspase-3 from the cytoplasm to largely nucleus-associated structures where caspase-2 has been shown to localise (Colussi *et al.*, 1998a). The nuclear localisation of caspase-2 is dependent on the prodomain; caspase-2 lacking the prodomain localises largely to the cytoplasm (Colussi *et al.*, 1998a). It can be postulated that the congregation of caspase molecules in specific “dot-like” regions mediates their apoptotic activity by promoting oligomerisation and processing.

6.3 Inhibition of Chimeric Caspase-3 Activity by MIHA and P35

Many viral and cellular proteins have distinct inhibitory effects on the biochemical activity of caspases (Kumar and Lavin, 1996, Nicholson and Thornberry, 1997). We therefore decided to investigate whether fusion of the caspase-2 prodomain to procaspase-3 would affect the interaction of caspase-3 with viral and cellular inhibitors of apoptosis. We compared the inhibitory effects of three direct caspase inhibitors (Crma, P35 and MIHA/XIAP) and Bcl-2 on apoptosis induced by caspase-2-GFP and

the C2P-caspase-3-GFP chimera. Caspase expression constructs were co-transfected with expression constructs containing one of the inhibitors at a ratio of 1:3 and transfected cells were analysed for morphology by GFP expression 18 hours later. As shown in Figure 6.3, only P35 significantly inhibited apoptosis induced by caspase-2 (by approximately 20%). Interestingly, cells co-transfected with caspase-2-GFP and MIHA, and to a lesser extent with CrmA, although apoptotic, appeared to be “caught” at an earlier morphological stage than apoptotic cells transfected with caspase-2 alone (Figure 6.4C, 6.4E). These earlier cells appeared to have brighter GFP fluorescence and a more blebbed phenotype than did caspase-2 transfectants, and were similar in appearance to the caspase-3 transfected apoptotic cells.

In contrast, C2P-caspase-3-GFP induced apoptosis was strongly inhibited by MIHA and P35, and partially inhibited by Bcl-2 and CrmA (Figures 6.3, 6.4). The observation that C2P-caspase-3-GFP-mediated apoptosis is suppressed by P35 and MIHA, but not by CrmA, is consistent with the previously reported inhibitory profile of caspase-3 (Nicholson *et al.*, 1995, Tewari *et al.*, 1995b, Xue and Horvitz, 1995, Devereaux *et al.*, 1997, Dorstyn and Kumar, 1997), suggesting that the caspase-2 prodomain fusion does not alter the basic kinetic characteristics and substrate specificity of caspase-3.

6.4 Chimeric Prodomain-Caspase-3 Undergoes Autoproteolytic Processing *In Vivo*

To determine whether the addition of the caspase-2 prodomain to caspase-3 can confer the ability of the chimeric molecule to undergo processing *in vivo*, we carried out immunoblot analysis on extracts from transfected cells. As shown in Figure 6.5, caspase-3-GFP (59 kDa) does not show appreciable processing in transfected NIH-3T3 cells. However, in cells transfected with C2P-caspase-3-GFP, no full length chimeric

precursor protein (77 kDa) was detected. Instead, a band of 39 kDa, representing processed p12-GFP protein was present (Figure 6.5), demonstrating that the chimeric C2P-caspase-3-GFP molecule was much more readily processed than wild-type caspase-3. Conversely, catalytically inactive C2P-caspase-3(C163G)-GFP was detected as a single, unprocessed band of 77 kDa (Figure 6.5), demonstrating that autocatalytic activity of caspase-3 is required for processing of the chimeric molecule. Interestingly, in all transfection experiments, the intensity of the processed protein band (39 kDa) was always much lower than that of the unprocessed protein, suggesting that processed caspases may be very unstable. This may explain, at least in part, why the GFP fluorescence is very weak in apoptotic NIH-3T3 cells transfected with caspase-2-GFP and C2P-caspase-3-GFP (Figure 6.2). In NIH-3T3 cells co-transfected with C2P-caspase-3-GFP and either MIHA or P35, but not Bcl-2 or CrmA, almost complete suppression of chimeric caspase molecule processing was seen (Figure 6.5), suggesting that MIHA and P35 inhibit C2P-caspase-3-GFP-induced apoptosis by inhibiting procaspase processing. Bcl-2 is known to act upstream of caspase-3 in the apoptotic pathway and has been shown to inhibit the activation of procaspase-3 by inhibiting processing of the Class I caspase/s that are required for subsequent caspase-3 proteolysis (Chinnaiyan *et al.*, 1996a). The lack of inhibition of C2P-caspase-3-GFP processing by Bcl-2 suggests that the chimeric molecule, like caspase-3, is not directly inhibited by Bcl-2.

The effect of Bcl-2, CrmA, MIHA and P35 on caspase-2-GFP processing was also analysed. Consistent with cell killing experiments, none of these molecules significantly blocked procaspase-2 processing; a processed doublet of 39/41 kDa, representing p12-GFP/p14-GFP was seen in all cases (Figure 6.5). The inability of Bcl-2 to inhibit

caspase-2 processing and apoptosis inducing activity confirms the lack of inhibition of C2P-caspase-3-GFP processing and cell killing activity by Bcl-2. Even though Bcl-2 was not expected to inhibit the catalytic activity of caspase-3 directly, it was feasible that it may have inhibited the activity of the chimeric molecule had caspase-2 been subject to regulation by Bcl-2 in a prodomain dependent manner. As expected, caspase-2(C320G)-GFP protein was not significantly processed in transfected cells. These results provide compelling evidence that the addition of a Class I prodomain to a Class II caspase can confer autoprocessing ability to Class II caspases.

6.5 Discussion

Studies performed in our laboratory in conjunction with the results presented in this chapter analysed the dimerisation potential of the chimeric molecules generated in this study. Yeast two-hybrid analysis of the C2P-caspase-3 chimeric molecules demonstrated that the catalytically inactive mutant chimeras, C2P-caspase-3(C163G) and C2P-caspase-3 Δ 9(C163G), were able to homodimerise, while wild-type caspase-3 was unable to homodimerise (Table 1, taken from Colussi *et al.*, 1998b). These observations demonstrate that the addition of the caspase-2 prodomain to procaspase-3 can greatly enhance the ability of caspase-3 molecules to dimerise. Taken together, these results provide strong support for the idea that the primary function of the prodomain in Class I caspases is to promote dimerisation of procaspase molecules and that dimerisation is necessary and sufficient for autoprocessing. These results also support the idea that recruitment of Class I caspases through adaptor molecules mediates dimerisation and activation of Class I caspases in response to apoptotic signals. A recently published study shows that artificially induced procaspase-3 oligomerisation

was sufficient for its activation (MacCorkle *et al.*, 1998), supporting the results reported here.

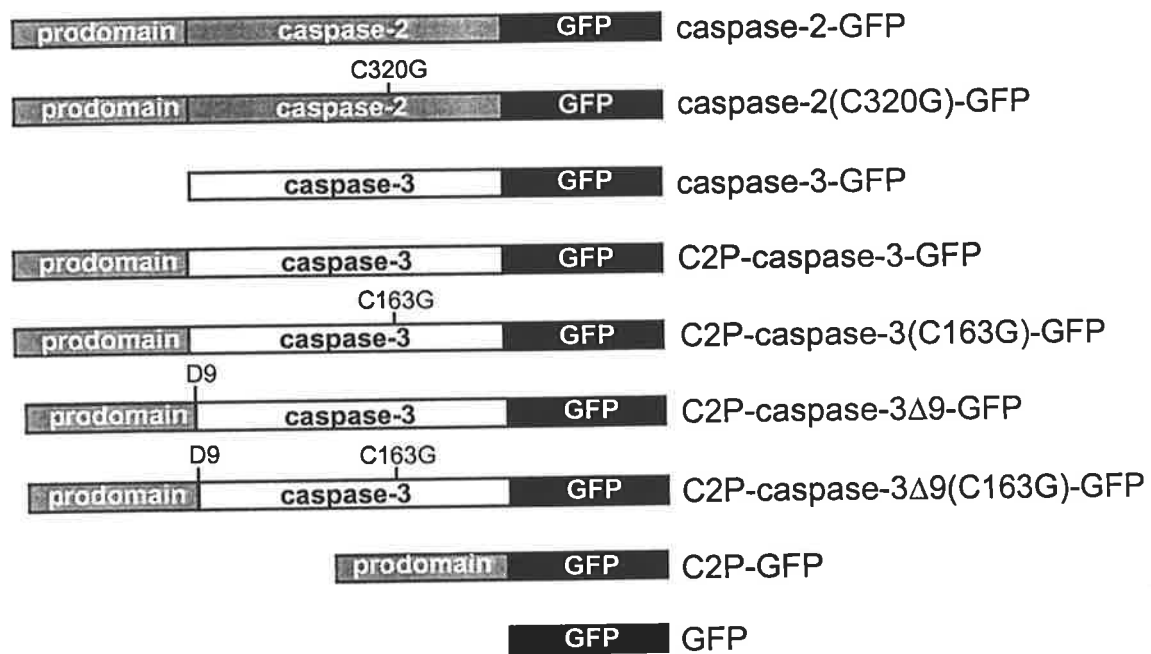
As previously discussed, procaspase-8 has been shown to possess low level intrinsic protease activity that probably mediates its autoprocessing (Muzio *et al.*, 1998). Since procaspase-3 is not processed in transfected cells, the intrinsic protease activity of procaspase-3 may be responsible for its weak apoptosis-inducing activity when overexpressed in mammalian cells. Since most cells express moderate levels of many procaspases, intrinsic procaspase activity is unlikely to have any deleterious effect until an apoptotic signal is received by the cell. In this scenario, death signal induced dimerisation would facilitate the recruitment of a critical level of Class I procaspase molecules, enabling the processing and generation of fully activated caspases to occur (Figure 6.6, modified from Kumar, 1998). In conclusion, our results provide direct evidence of a role for Class I caspase prodomains in autoactivation and processing and establish a basis for functional hierarchy among the two classes of caspases.

Figure 6.1. Fusion of the caspase-2 prodomain (C2P) onto caspase-3 greatly enhances the cell killing activity of caspase-3.

A) Structure of the various GFP-caspase fusion proteins used in this study. All constructs were generated by fusing GFP at the carboxyl terminus of caspase molecules in the mammalian expression vector pEGFP-N1. The $\Delta 9$ chimeric constructs lack the 3 carboxy-terminal residues of the caspase-2 prodomain (C2P) and the first 9 amino-terminal residues of procaspase-3. This deletes potential caspase cleavage sites between C2P and caspase-3.

B) Apoptosis-inducing activity of various GFP-fusion constructs in NIH-3T3 cells. GFP-fusion constructs were transiently transfected into NIH-3T3 cells. 18 h post-transfection, cells were fixed and analysed for apoptotic morphology by fluorescence microscopy. Data, shown as the percentage of morphologically apoptotic cells among all GFP-positive cells, are mean values from two experiments, each performed in duplicate.

A



B

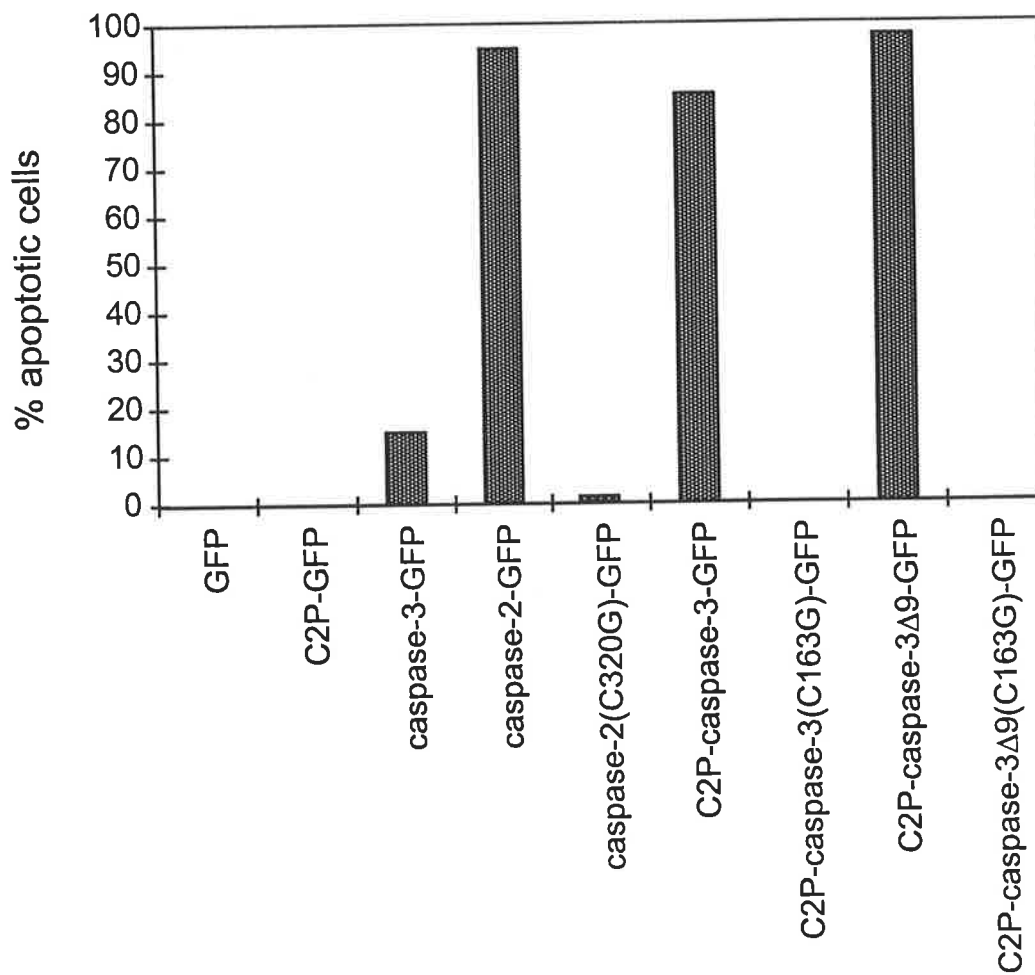


Figure 6.2. Immunofluorescence microscopy demonstrating the morphology of various caspase-GFP fusion transfected NIH-3T3 cells.

NIH-3T3 cells were transiently transfected with various expression constructs. 18 h post-transfection, cells were fixed and photographed using a fluorescence microscope.

- A) GFP
- B) caspase-2-GFP
- C) caspase-2(C320G)-GFP
- D) caspase-3-GFP
- E) C2P-GFP
- F) C2P-caspase-3-GFP
- G) C2P-caspase-3(C163G)-GFP
- H) C2P-caspase-3 Δ 9-GFP
- I) C2P-caspase-3 Δ 9(C163G)-GFP

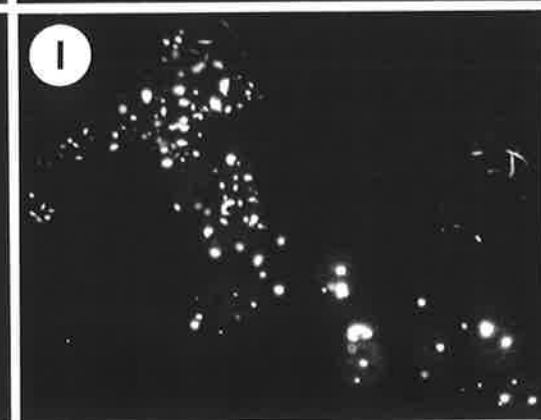
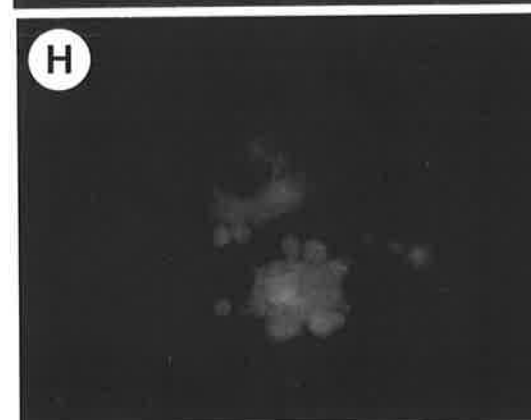
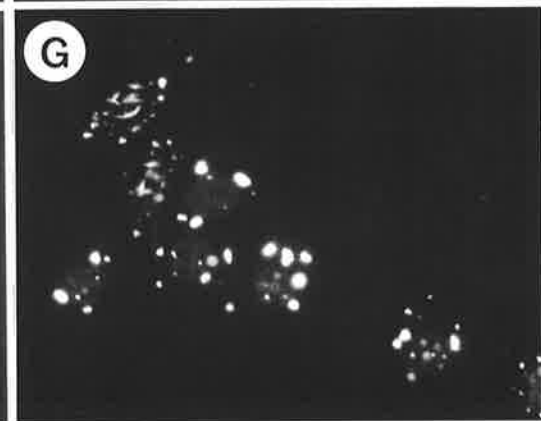
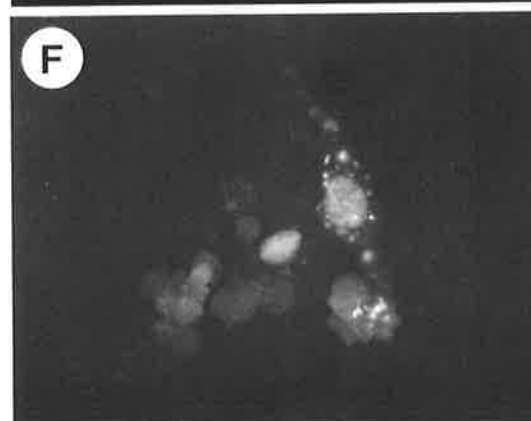
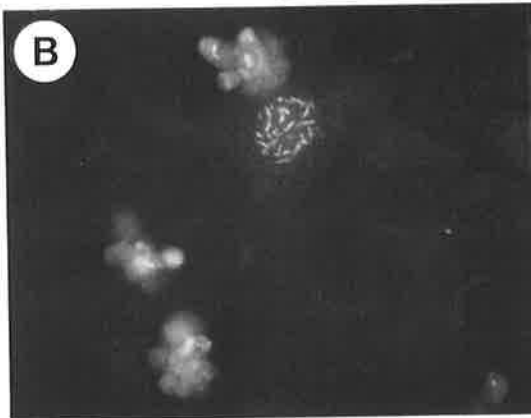
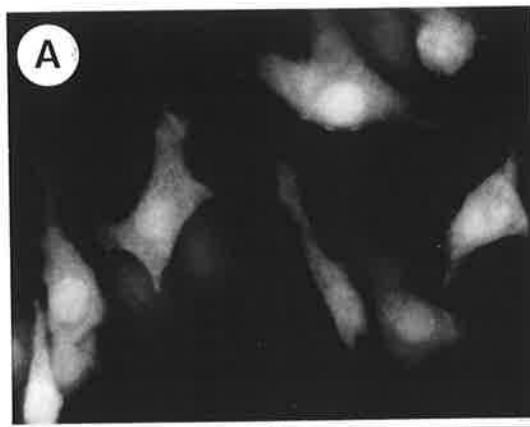


Figure 6.3. The different inhibitory profiles of P35 and MIHA on apoptosis induced by caspase-2 and chimeric C2P-caspase-3.

NIH-3T3 cells were cotransfected with caspase-2-GFP or C2P-caspase-3-GFP and either a Bcl-2, CrmA, P35 or MIHA expression vector at a 1:3 ratio. Cells were examined for apoptotic morphology using a fluorescence microscope 18 h post-transfection. Results represent mean values from two experiments, each performed in duplicate.

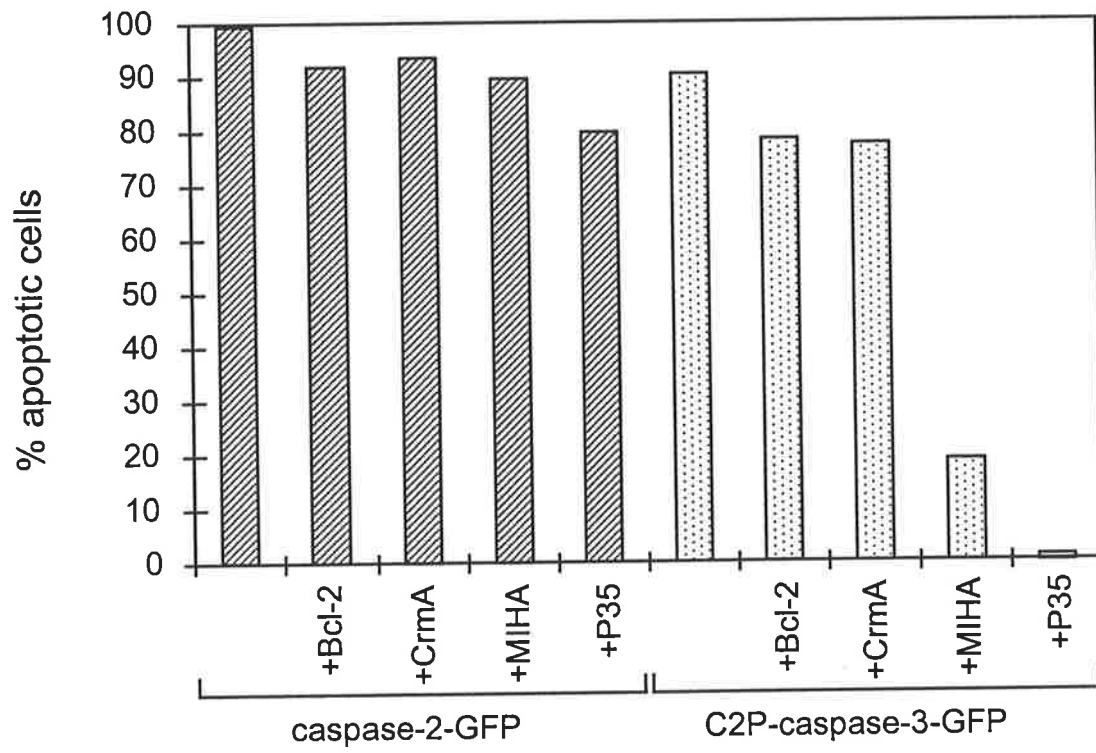


Figure 6.4. Immunofluorescence microscopy of caspase-2-GFP and C2P-caspase-3-GFP + inhibitor cotransfectants.

NIH-3T3 cells were transfected as described in Figure 6.3. 18 h post-transfection, cells were fixed and photographed using a fluorescence microscope.

- A) caspase-2-GFP + Bcl-2
- C) caspase-2-GFP + CrmA
- E) caspase-2-GFP + MIHA
- G) caspase-2-GFP + P35
- B) C2P-caspase-3-GFP + Bcl-2
- D) C2P-caspase-3-GFP + CrmA
- F) C2P-caspase-3-GFP + MIHA
- H) C2P-caspase-3-GFP + P35

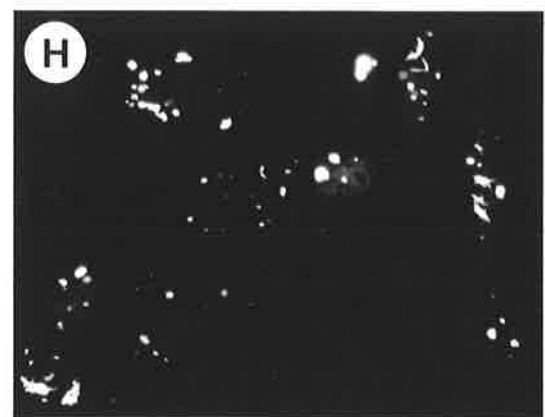
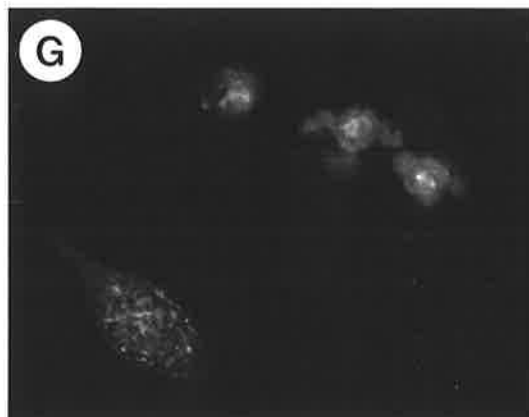
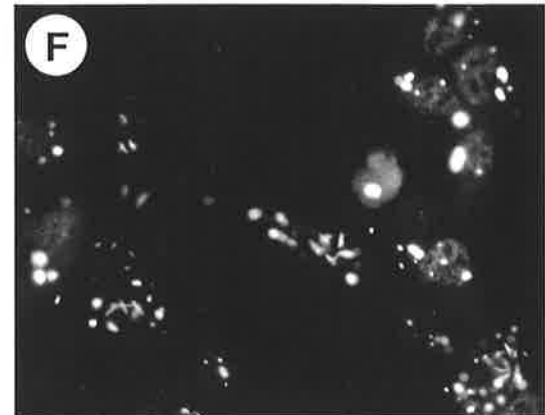
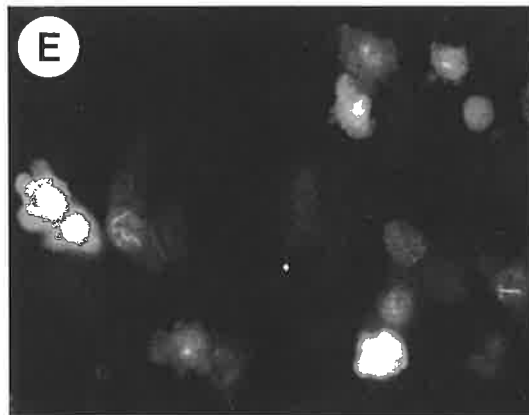
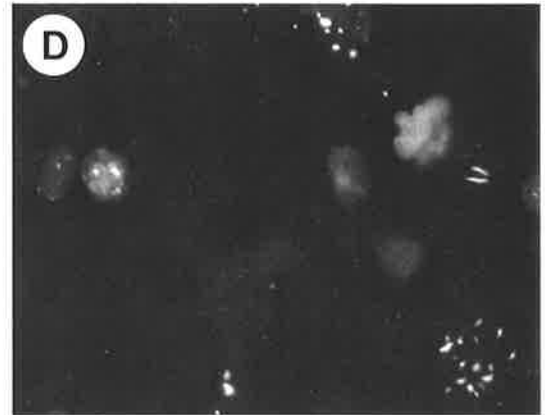
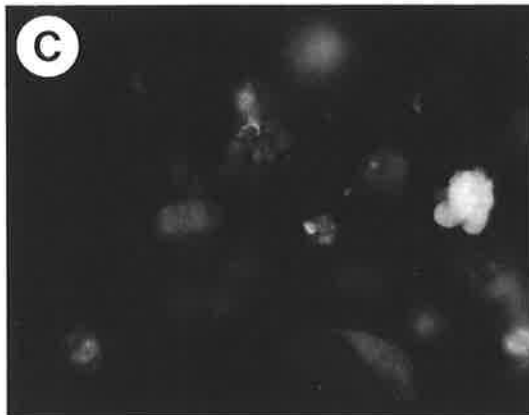


Figure 6.5. Chimeric C2P-caspase-3 protein is efficiently processed in transfected NIH-3T3 cells.

NIH-3T3 cells were transfected with caspase-2-GFP or C2P-caspase-3-GFP along with the indicated inhibitor construct as described in Figure 6.3. 18 h post-transfection, cells were harvested and lysates were analysed by immunoblotting with an anti-GFP antibody. GFP transfected cells express the 27 kDa GFP protein. Caspase-2-GFP is essentially completely processed in transfected cells, with only a doublet corresponding to p14 + GFP/p12 + GFP visible in this lane. Caspase-2-GFP (77 kDa) does not show significant processing in transfected cells. None of the inhibitors used in this assay significantly inhibited caspase-2-GFP processing, corresponding to the fact that these inhibitors do not significantly protect against caspase-2-induced apoptosis. While caspase-3-GFP (59 kDa) is not significantly processed in transfected cells, C2P-caspase-3-GFP is completely processed, with only a 39 kDa band representing p12 + GFP present in this lane. C2P-caspase-3(C163G)-GFP is not processed in transfected cells, appearing as a 77 kDa band which corresponds to caspase-3 + C2P + GFP. MIHA and P35, which both inhibit C2P-caspase-3-GFP-induced apoptosis, inhibit C2P-caspase-3-GFP processing. The caspase-3-GFP and caspase-2(C320G)-GFP lanes in this figure were derived from different exposure times to the remainder of the figure.

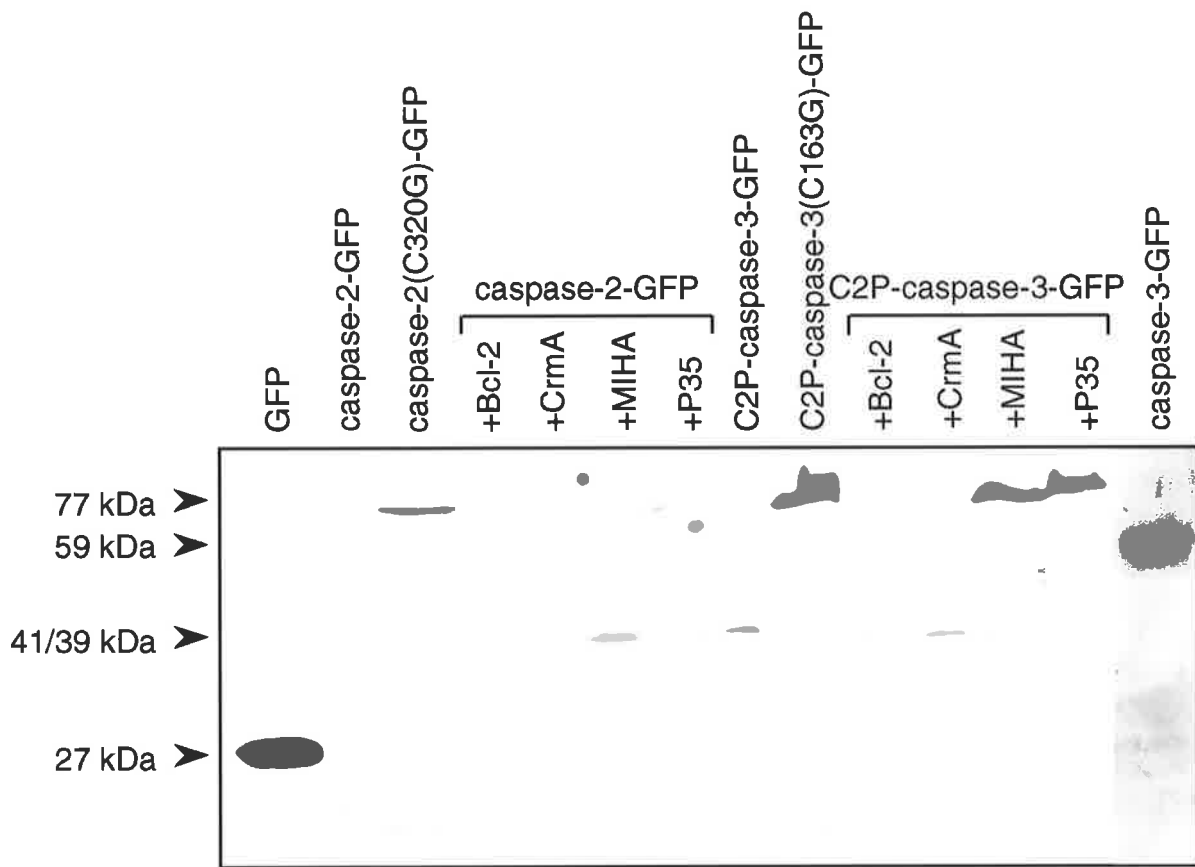


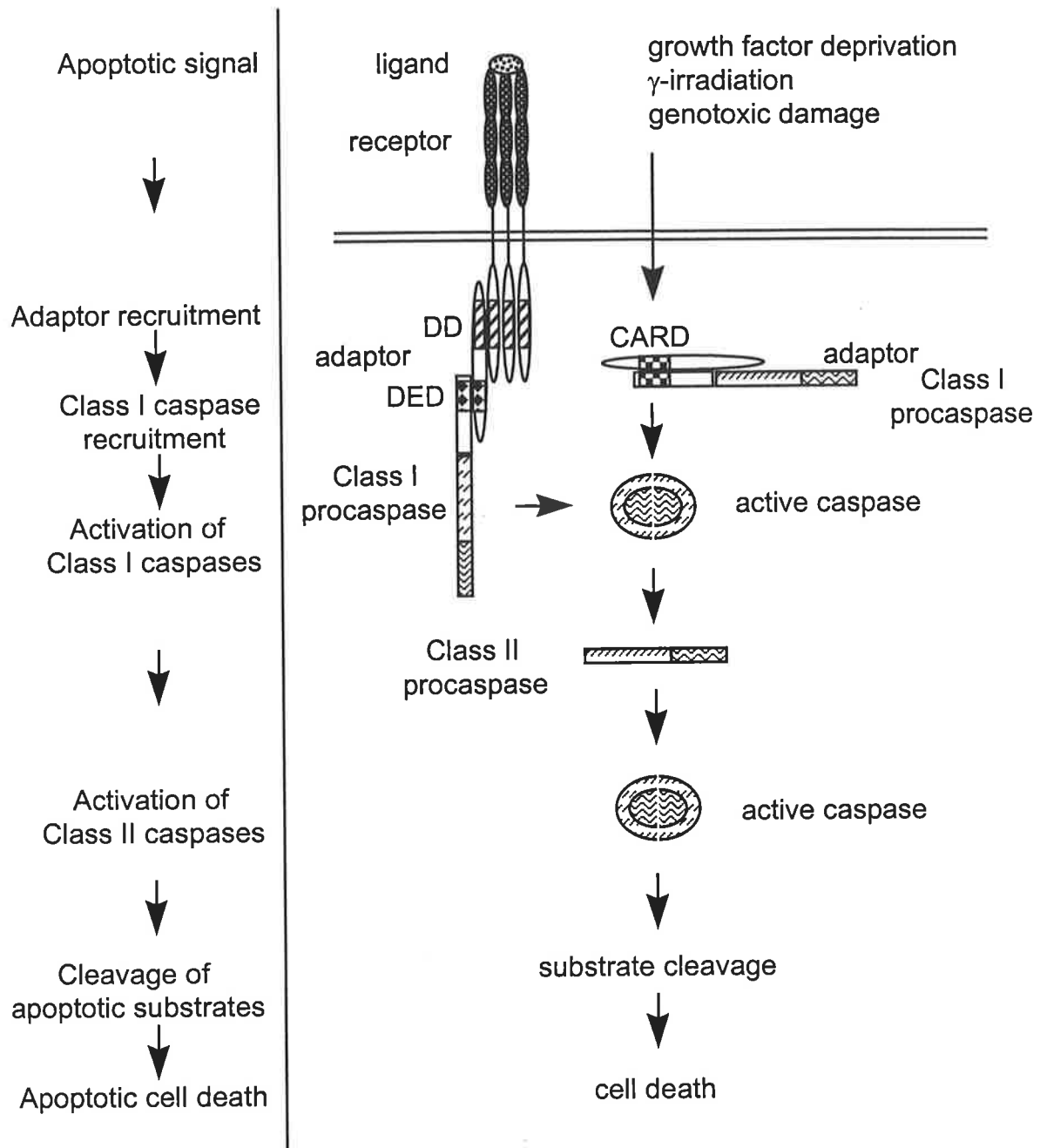
Table 6.1 Chimeric C2P-caspase-3 proteins homodimerise in *S. cerevisiae*.

Various caspase-3 and C2P-caspase-3 constructs in Gal4BD and Gal4AD vectors were cotransformed into *S. cerevisiae* Y190 strain. Cotransformation of p53 and SV40 large T in Gal4BD and Gal4AD vectors, respectively, served as a positive control. Colonies were assayed for β -galactosidase activity by colony lift filter assay using X-gal as a substrate. In caspase-3(C163G) transformed cells, less than 10% of colonies were positive for β -galactosidase (shown as -/+) as compared to 70% for C2P-caspase-3 Δ 9, and >95% for C2P, C2P-caspase-3(C163G) and C2P-caspase-3 Δ 9(C163G).

Gal4BD construct in	Gal4AD construct in	β -galactosidase activity
pAS2.1 vector	pACT2 vector	
empty vector	empty vector	-
p53	SV40 large T	+
caspase-3	caspase-3	-
caspase-3(C163G)	caspase-3(C163G)	-/+
C2P	C2P	+
C2P	empty vector	-
empty vector	C2P	-
C2P-caspase-3 Δ 9	C2P-caspase-3 Δ 9	+
C2P-caspase-3 Δ 9	empty vector	-
empty vector	C2P-caspase-3 Δ 9	-
C2P-caspase-3(C163G)	C2P-caspase-3(C163G)	+
C2P-caspase-3(C163G)	empty vector	-
empty vector	C2P-caspase-3(C163G)	-
C2P-caspase-3 Δ 9(C163G)	C2P-caspase-3 Δ 9(C163G)	+
C2P-caspase-3 Δ 9(C163G)	empty vector	-
empty vector	C2P-caspase-3 Δ 9(C163G)	-

Figure 6.6. A model of caspase activation in response to apoptotic stimuli

This model illustrates the mechanism of caspase activation in response to apoptosis induced via death receptors and via pathways which are not yet fully characterised. Activation of death signalling through death receptors such as Fas/APO-1 and TNFR1 results in the recruitment of adaptor molecules such as FADD and TRADD, via death domains (DD) located in both the cytoplasmic tail of the receptor and the adaptor molecule. This results in the recruitment of Class I caspases (caspase-8 and caspase-10 in the case of Fas and TNFR1-induced apoptosis) to the death complex via death effector domains (DED) located in both the caspase prodomain and the adaptor molecule. The adaptor molecule-mediated oligomerisation of Class I caspases results in caspase activation via self-proteolysis. Activated Class I caspases are then capable of processing downstream Class II caspases which are responsible for cleaving the cellular substrates which execute apoptotic cell death. The sequence of events leading to Class I caspase activation in response to apoptotic signalling by stimuli such as growth factor deprivation, genotoxic drugs and γ -irradiation has not yet been defined. However, the activation of caspase-9 has been documented to occur in response to many apoptotic stimuli and is reliant on oligomerisation induced by the adaptor molecule Apaf-1 in the presence of cytochrome *c* and dATP. The exact cellular location of this death complex is not known, although it is thought to be in close proximity to the mitochondria. The association of caspase-9 with Apaf-1 is mediated by a caspase recruitment domain (CARD) located in the prodomain of caspase-9 and in Apaf-1. In a similar manner, a CARD domain mediates the *in vitro* association of caspase-2 with RAIDD. Once activated, caspase-9 processes downstream Class II caspases which execute apoptotic cell death.



Chapter 7

General Discussion

Discussion

The 1993 cloning of the *C. elegans ced-3* cell death gene which revealed homology to the interleukin-1 β converting enzyme (Yuan *et al.*, 1993) was a seminal discovery in the field of apoptosis research. For the first time a component of the cellular pro-apoptotic machinery was defined and characterised at the molecular level. The molecular mechanisms of apoptotic cell death have since been intensively studied in an effort to fully characterise the events that proceed in the apoptotic pathway, from the receipt of a death stimulus to the engulfment of apoptotic bodies. At the commencement of the studies presented in this thesis, caspase-1, caspase-2 and caspase-3 were the only identified members of the caspase family, with the remaining family members identified in rapid succession during the course of the following months. The initial description of caspase-2 cloning, expression and apoptosis inducing activity (Kumar *et al.*, 1994) established caspase-2 as a protease likely to play an important role in the mammalian apoptotic pathway. This project aimed to address a number of questions regarding the mechanism of caspase-2 processing and activation in order to assess the potential role of caspase-2 in the execution of mammalian cell death.

Initial studies presented in chapter 3 demonstrated that procaspase-2 is proteolytically processed *in vitro* to release two subunits of 19 kDa and 12 kDa and that this event can be mediated by cytoplasmic extracts from apoptotic cells as well as by other caspase family members and granzyme B. These experiments demonstrated that caspase-2 is likely to be activated *in vivo* in apoptotic cells by virtue of the fact that apoptotic cell extracts contain active caspases which were shown to cleave caspase-2 *in vitro*. Subsequent experiments presented in chapter 4 confirmed that caspase-2 is activated *in vivo* in various cell types in response to a variety of apoptotic signals. The caspase-2

cleavage studies presented here were the first to show that granzyme B can proteolytically process caspase-2 *in vitro*, suggesting that caspase-2 activation may play a role in the induction of apoptosis mediated by cytotoxic lymphocytes. Recent studies in caspase-2 null mice have confirmed this suggestion (Bergeron *et al.*, 1998). B lymphoblasts from these mice are resistant to apoptosis induced by perforin and granzyme B, but remain sensitive to apoptosis induced by anti-Fas antibody, doxorubicin, etoposide, γ -irradiation and staurosporine. These results indicate that caspase-2 mediates a very specific function in the granzyme B induced apoptotic pathway of these cells. Caspase-2 null mice also display excess numbers of germ cells in the ovaries of female mice and their oocytes are resistant to doxorubicin-induced apoptosis. These observations demonstrate that caspase-2 is essential for the normal execution of apoptosis in female germ cells and also for the apoptotic pathway induced by the chemotherapeutic agent doxorubicin (Bergeron *et al.*, 1998). The role of caspase-2 in apoptosis therefore appears to be non-redundant in tissues such as oocytes and B lymphoblasts, but is possibly redundant in other cell lineages.

The ability of caspase-2 to undergo self-proteolysis indicated that caspase-2 may not require the prior activation of other caspase family members for its own activation. Studies presented here (Chapters 3, 5, and 6) along with additional studies performed in our laboratory demonstrate that self-proteolysis is likely to be the primary mechanism of caspase-2 activation *in vivo*. This also appears to be the mechanism of activation of other Class I caspases (Muzio *et al.*, 1998, Yang *et al.*, 1998, Srinivasula *et al.*, 1998). The first evidence that caspase-2 is capable of self-proteolysis came from experiments detailed in Chapter 3, in which active recombinant caspase-2 was produced in *E. coli*. These experiments demonstrated that caspase-2 is capable of self-proteolysis, at least

when present at high concentrations. Two different constructs were used to generate active recombinant caspase-2; full length procaspase-2 in the pET32 vector and caspase-2 lacking the prodomain in the pGEX-2T vector. Analysis of the activity and substrate specificity of caspase-2 expressed in each construct revealed differences in both the mode of activity and optimal specificity of each enzyme. Interestingly, although both full length caspase-2 and caspase-2 lacking the prodomain cleaved the peptide substrates DEVD-AFC and VDVAD-AFC and *in vitro* translated procaspase-2, they did so with different efficiencies (Chapter 3). Full length caspase-2 processes *in vitro* translated caspase-2 more efficiently, particularly at selected sites, than does caspase-2 lacking the prodomain. In addition, caspase-2 lacking the prodomain recognises and cleaves sites in the yeast protein RSP5 that are not processed by full length caspase-2 (Chapter 3). These observations suggest that removal of the prodomain alters the substrate specificity of caspase-2, perhaps by altering the secondary structure of the enzyme. Evidence lending weight to this hypothesis was revealed in immunoblot analysis of lysate expressing each caspase-2 construct, which demonstrated that the p12 subunit of caspase-2 was present only in lysate expressing full length caspase-2. This observation suggested that caspase-2 lacking the prodomain is likely to adopt a partially active conformation in the absence of proteolytic processing. It is therefore reasonable to conclude that the caspase-2 prodomain is required for optimal processing of procaspase-2 to generate an enzyme with optimal catalytic function.

Further studies in our laboratory have analysed the role of the caspase-2 prodomain in self-proteolysis. We have shown that procaspase-2 molecules are able to dimerise in a prodomain dependent manner and that dimerisation is required for subsequent caspase-2 self-processing (Butt *et al.*, 1998). This perhaps explains why caspase-2 lacking the

prodomain is not capable of self-proteolysis. Dimerisation of Class I caspases *in vivo* has been shown to occur via adaptor protein-mediated recruitment of Class I procaspase molecules to cellular death signalling complexes (Vincenz and Dixit, 1997, Srinivasula *et al.*, 1998). Motifs contained in the prodomains of Class I caspases are essential for the recruitment process by mediating the physical association between Class I caspases and their respective adaptor proteins. In the case of caspases-8 and -10, FADD mediates the recruitment of procaspase molecules via death effector domain (DED) and death domain (DD)-mediated coupling, to the Fas/APO-1 and TNFR1 signalling complexes, resulting in caspase activation (Boldin *et al.*, 1996, Muzio *et al.*, 1996, Vincenz and Dixit, 1997). In the case of caspase-9, Apaf-1, in the presence of cytochrome *c* and dATP, facilitates oligomerisation and activation of caspase-9 via recruitment to an intracellular death complex (Li *et al.*, 1997, Srinivasula *et al.*, 1998). Caspase-2 has been shown to associate *in vitro* with RAIDD/CRADD, an association which has been postulated to link caspase-2 to the TNFR1 and Fas signalling complexes (Duan and Dixit, 1997, Ahmad *et al.*, 1997). Although results presented here demonstrate that caspase-2 is activated rapidly in response to both Fas and TNF receptor ligation, it has not been shown *in vivo* that RAIDD/CRADD is responsible for mediating this event. Caspase-2 is also activated in response to apoptotic stimuli such as growth factor withdrawal, treatment with chemotherapeutic agents and γ -irradiation, which are not mediated by death receptors. The identity and cellular location of the death complexes which mediate signalling by these stimuli is presently unknown. It remains to be seen whether caspase-2 is an apical caspase in initiating the death signal in response to these stimuli, or whether caspase-2 activation occurs as a result of proteolysis by downstream caspases such as caspase-3, serving to mediate amplification of the caspase cascade.

A lack of inhibition of caspase-2 activation by Bcl-2 in response to Fas/APO-1 ligation in Jurkat cells confirms results previously reported by Strasser and colleagues (1995). As previously discussed, the inability of Bcl-2 to inhibit Fas-mediated apoptosis may reflect the difference in cellular distribution of the Class I caspases, caspase-8 and caspase-10, compared to Bcl-2. Caspases-8 and -10 are recruited to surface membrane associated death receptor complexes where they are activated in response to Fas/APO-1 and TNFR1 death signalling, while Bcl-2 resides mainly at the outer mitochondrial membrane, as well as membranes of the nucleus and endoplasmic reticula (Krajewski *et al.*, 1993, Lithgow *et al.*, 1994). Although caspase-8 activation results in the release of cytochrome *c* from mitochondria, which amplifys the apoptotic signal, mitochondrial involvement is not required for the initiation of caspase-8 activation, rendering Fas-induced apoptosis refractory to inhibition by Bcl-2 (Kuwana *et al.*, 1998). The partial inhibition of caspase processing in Jurkat cells in response to etoposide treatment suggests that this death stimulus may directly involve the mitochondria, where Bcl-2 family members may function to hold Class I caspases in an inactive complex until disrupted by a death signal. It is possible that caspase-2 may be present in such a complex at the mitochondrial membrane, or alternatively, that caspase-2 is activated downstream of mitochondrial involvement as part of an amplification of the etoposide-induced death signal. Further evidence for mitochondrial involvement in apoptotic signalling is documented in a recent study by Marzo and colleagues (1998), which demonstrates that the addition of recombinant caspases, including caspase-2, to purified mitochondria induces a permeability transition *in vitro*, resulting in the release of mitochondrial cytochrome *c*. This presumably mediates the activation of caspase-9 and subsequent downstream caspases, which would facilitate amplification of the apoptotic signal and rapid cell death.

Mutation analysis of selected residues in caspase-2 demonstrated that residues additional to those at which cleavage occurs to generate the active protease are critical for caspase-2 self-processing and apoptosis-inducing activity (Results, Chapter 5). Aspartate residues 135 and 326 which lie in the prodomain and near the active site, respectively, are both critical for caspase-2 processing and death inducing activity. In addition, the Asp-135 mutant is unable to dimerise. The mutation of prodomain residue Asp-135 may result in an alteration in secondary structure such that procaspase-2 molecules can no longer dimerise, thereby abolishing self-processing. The structure of the caspase-2 caspase recruitment domain (CARD) that lies in the first 100 amino acids of the caspase-2 prodomain, has recently been modelled based on the solution structure of the RAIDD CARD (Chou *et al.*, 1998). The CARD consists of six tightly packed helices arranged in a similar organisation to the Fas death domain (Huang *et al.*, 1996), and contains a basic and an acidic region on opposite sides of the structure (Chou *et al.*, 1998). Interaction between these basic and acidic regions is thought to mediate interaction between the RAIDD and caspase-2 CARDS, and is therefore likely to mediate homodimerisation between procaspase-2 molecules. Mutations which disrupt the CARD domain structure are likely to abolish caspase-2 self-proteolysis and apoptosis-inducing activity by preventing caspase-2 dimerisation. Mutation of Asp-326 may abolish caspase-2 processing and apoptosis inducing activity by virtue of its proximity to the active site of caspase-2. Of the mutations at aspartate residues at which processing has previously been documented to occur, only the Asp-333 mutant abolished caspase-2 apoptosis inducing activity, suggesting that this is the critical cleavage site for generation of the active enzyme. Mutations at cleavage sites Asp-169 and Asp-347 retain the ability to induce apoptosis. This observation suggests that

liberation of the prodomain from the p19 subunit, and release of the stuffer fragment from the p12 subunit, are not events which are absolutely required for the assembly of catalytically active caspase-2.

The studies presented in Chapter 6 analysing the role of the caspase-2 prodomain demonstrate that fusion of the caspase-2 prodomain to caspase-3 confers to caspase-3 the properties of a Class I caspase. The caspase-2 prodomain, when fused to caspase-3, enables caspase-3 to dimerise, autoprocess and induce apoptosis in transfected cells, all processes which are not efficiently mediated by caspase-3 alone. The shift in distribution from the normal diffuse cytoplasmic localisation of caspase-3 to the discrete dot-like structures associated with caspase-2 may facilitate the dimerisation of procaspase molecules, thereby promoting their autocatalysis. Recently, the DEDs of FADD and caspase-8 have been shown to form filamentous structures when overexpressed in transfected cells (Perez and White, 1998, Siegel *et al.*, 1998). These structures are thought to represent oligomerised DED containing proteins and have been postulated to mediate the recruitment and autoactivation of DED-containing caspases. It is not currently known whether the localisation of caspases into these structures is representative of the *in vivo* situation initiated in response to apoptotic signals, or whether it occurs as a result of overexpression.

Chimeric caspase molecules may have potential therapeutic application in the treatment of drug and radiation resistant cancers. Accumulating evidence suggests that drug and radiation resistant cancer cells fail to activate caspases in response to apoptotic stimuli. In many cases, drug resistance has been ascribed to overexpression of Bcl-2 and Bcl-X_L (Strasser *et al.*, 1997), which act upstream of caspase-9 to prevent its activation. The

targeted introduction of self-activating caspase molecules such as the chimeric molecules generated here (Chapter 6) could potentially restore the ability of drug resistant cells to undergo apoptosis. Future studies in our laboratory will address the efficiency of inducing apoptosis in drug and radiation resistant cancer cells by the introduction of chimeric caspase molecules.

A cellular substrate of caspase-2 has so far remained elusive, despite the identification of the optimal peptide substrate of caspase-2. Caspase-2 does not cleave caspase-3 substrates such as PARP, hnRNP-C1 and C2 or NEDD4 (Gu *et al.*, 1995, Waterhouse *et al.*, 1997, Harvey *et al.*, 1998), implying that its substrate specificity is quite divergent from caspase-3 and its closest homologues. The recent demonstration that caspase-2 localises to the nucleus in addition to the cytoplasm (Colussi *et al.*, 1998a), suggests that caspase-2 may have a novel nuclear substrate. While the association of caspase-2 with RAIDD *in vitro* implies that caspase-2 could be recruited to the Fas- and TNFR1 death complexes located at the plasma membrane, the identity and location of other death complexes in which caspase-2 is involved is not currently established. The search for a caspase-2 substrate will no doubt continue in order to further delineate the function that caspase-2 performs once activated in the apoptotic pathway.

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Thesis Amendments

Chapter 3

Page 67, line 13; Fig 3.4B should read Fig. 3.4A

Figure 3.2A; Although some processed procaspase-2 is visible in the TLCK, TPCK and E64-treated samples, inhibition of the processing of the 51 kDa precursor is clearly visible.

Figure 3.2C; The scale in the figure is incorrect and was adjusted in the figure legend. From the amended scale, it is obvious that 100 nM and to a lesser extent, 20 nM, YVAD-cmk partially block procaspase-2 proteolysis.

Figure 3.5; Cleavage at Asp-135 by bacterially expressed caspase-2 lacking the prodomain could generate a fragment of 24 kDa.

Chapter 4

Figure 4.4; A baseline level of DEVD-AFC cleavage activity was routinely observed in untreated Mo7e extracts. Possible reasons for this are discussed on pages 67 and 81.

Figure 4.5; Caspase-3 activation in Mo7e cells was also analysed 48 h post serum deprivation, and as discussed, was minimal in magnitude.

Figure 4.8; A very faint 12 kDa fragment is visible in this figure, the resolution of this fragment has been lost in photographic reproduction.

Figure 4.8; At the time these experiments were performed, caspase-8 activation was not analysed because we did not have access to a caspase-8 antibody.

Chapter 5

Figure 5.2; The level of p19 generated in this experiment as a percentage of 51 kDa precursor is minimal and may be attributed to a very low level of cleavage at D333 in the D330 mutant and vice versa.

Page 95, paragraph 2; Caspase-3 could be cleaving caspase-2 at Asp-326 in the Asp-330 and Asp-333 mutants which would generate the unique fragment observed in these lanes.

Figure 5.4; Counts from Figure 5.4G and duplicate plates were used to generate the data displayed in Fig 5.3 and were consistently observed.

Chapter 6

Figure 6.2E; The caspase-2 prodomain localisation observed in this figure is the subject of our recent paper; Colussi PA, Harvey NL and Kumar S (1998a) Prodomain-dependent Nuclear Localisation of the Caspase-2 (Nedd2) Precursor. *J. Biol. Chem.* **273**:24535-24542.

Figure 6.4; The data in this figure clearly show the difference between apoptotic morphology (eg. Fig 6.4 A and C) and healthy morphology (eg. 6.4 F and H) and mirror the results observed in figures 6.3 and 6.5.