

Caspase-2 is not required for thymocyte or neuronal apoptosis even though cleavage of caspase-2 is dependent on both Apaf-1 and caspase-9

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Received 29.11.01; revised 28.1.02; accepted 8.2.02

Edited by SJ Martin

Abstract

We have generated rat monoclonal antibodies that specifically recognise caspase-2 from many species, including mouse, rat and humans. Using these antibodies, we have investigated caspase-2 expression, subcellular localisation and processing. We demonstrate that caspase-2 is expressed in most tissues and cell types. Cell fractionation and immunohistochemistry experiments show that caspase-2 is found in the nuclear and cytosolic fractions, including a significant portion present in the Golgi complex. We found that caspase-2 is processed in response to many apoptotic stimuli but experiments with caspase-2 deficient mice demonstrated that it is not required for apoptosis of thymocytes or dorsal root ganglia (DRG) neurons in response to a variety of cytotoxic stimuli. Caspase-2 processing does not occur in thymocytes lacking Apaf-1 or caspase-9, suggesting that in this cell type, activation of caspase-2 occurs downstream of apoptosome formation.

Cell Death and Differentiation (2002) 9, 832–841. doi:10.1038/sj.cdd.4401033

Keywords: caspase-2; monoclonal antibody; apoptosis; subcellular localisation; caspase-9; Apaf-1

Abbreviations: LM, light membrane; HM, heavy membrane; mAb, monoclonal antibody; NGF, Nerve Growth Factor; ECL, enhanced chemical luminescence; zVAD-fmk, benzyloxy-carbonyl-Val-Ala-Asp-fluoromethylketone; DEX, dexamethasone; DRG, dorsal root ganglion

Introduction

The *Caenorhabditis elegans* cysteine protease, CED-3, and its mammalian homologues, constitute the effector arm of the apoptotic machinery. These enzymes are produced as zymogens which undergo processing into two subunits of ~20 and ~10 kDa that are assembled into the active enzyme (reviewed in references^{1,2}). Mammalian cells have at least two distinct caspase activation pathways.³ Death receptors of the tumour necrosis factor receptor (TNFR) family recruit FADD, an adaptor molecule which in turn recruits pro-caspase-8 or -10 to the death inducing signalling complex (DISC).^{4,5} The recruitment of pro-caspase-8 or -10 to the DISC promotes caspase-8/-10 processing^{6,7} by a mechanism that is thought to involve proximity-induced autoactivation of the pro-caspases.⁸ The second apoptosis signalling pathway is regulated by the Bcl-2 family of proteins and is initiated by a different set of initiator caspases and their adaptors. Mammalian caspase-9 contains an N-terminal caspase recruitment domain (CARD), which mediates interaction with its adaptor Apaf-1.⁹ Once activated, Apaf-1 recruits pro-caspase-9 to form the apoptosome protein complex. In a manner analogous to pro-caspase-8 activation, recruitment of pro-caspase-9 via its adaptor serves to bring pro-caspase molecules into close proximity, precipitating their activation.^{9,10}

Structurally, mammalian caspase-2 closely resembles caspase-9 and CED-3^{11,12} and previous studies have implicated caspase-2 in a variety of cell death pathways.^{11,13} The question, however remains, at what stage in the apoptotic process does caspase-2 act, since it has features of both upstream caspases (CARD pro-domain) and effector caspases (DEXD substrate specificity).¹⁴ Caspase-2 CARD has been shown to interact with the CARD present in the adaptor protein RAIDD,¹⁵ which when overexpressed in mammalian cells induces apoptosis. It remains, however, unclear whether RAIDD is necessary for caspase-2 activation¹⁶ and whether it plays a role in the apoptotic process. In many cell types, caspase-2 is processed early during apoptosis.¹⁷ Furthermore, overexpressed procaspase-2 can homodimerise in a CARD dependent manner and autoprocess.^{18,19} However, other studies have indicated that caspase-2 functions further downstream as an effector caspase. For example, caspase-3 can efficiently process pro-caspase-2,²⁰ indicating that caspase-2 activation may occur downstream of this effector

caspase. One possible scenario is that initial activation of caspase-2 occurs by an autocatalytic mechanism, but once effector caspases, such as caspase-3, have been activated, they are responsible for the bulk of pro-caspase-2 processing, forming an amplification loop.

In order to address the issue of whether caspase-2 is an initiator or effector caspase, we generated highly specific monoclonal antibodies against this protease. Using thymocytes derived from Apaf-1^{-/-} and caspase-9^{-/-}-deficient mice, we demonstrate that at least in this cell type, caspase-9 and Apaf-1 are required for caspase-2 processing indicating that cleavage of caspase-2 lies downstream of the Apaf-1/caspase-9 apoptosome. Furthermore, we have generated caspase-2-deficient mice and show that caspase-2 is dispensible for apoptosis of thymocytes or DRG neuronal cells induced by cytokine withdrawal or several stress stimuli.

These antibodies allowed us to confirm and extend the expression pattern of caspase-2 in mouse tissues²¹ and an extensive array of cell lines and to determine its subcellular localisation by subcellular fractionation. We show that caspase-2 is expressed in most tissues and cell types and that a significant proportion of caspase-2 is in the nucleus and within the Golgi apparatus.

Results and discussion

Characterisation of monoclonal antibodies to Caspase-2

Monoclonal antibodies recognising native mouse caspase-2 were identified by immunofluorescence staining and flow

cytometric analysis using a previously described protocol.²² 293T cells transfected with a plasmid encoding a C-terminally hemagglutinin (HA)-tagged caspase-2 in which the active site cysteine was replaced by alanine were stained with hybridoma supernatants plus FITC-conjugated goat anti-rat IgG antibodies and analyzed in a FACScan. Antibodies specific to caspase-2 were revealed by a double immunofluorescence peak (Figure 1D–F) similar to the profile obtained by staining with the anti-HA epitope tag-specific antibody (Figure 1A). The peak with lower intensity represents background immunofluorescence of untransfected 293T cells and the higher intensity peak represents specific caspase-2 staining in the transfected HA-caspase-2 expressing 293T cells. From an initial screen of 2000 hybridoma cultures, 17 anti-caspase-2 antibody-secreting hybridomas were selected, expanded and subcloned.

Two independent mAbs, 10C6 and 11B4, that recognise mouse, human, rat and monkey caspase-2 in immunofluorescence staining, Western blotting and immunoprecipitation (Figure 1G–I), were chosen for further experiments. In tissues from normal mice both mAbs detected by Western blotting a single protein of ~51 kDa, corresponding to endogenous full length pro-caspase-2 (Figure 1G). The 10C6 and 11B4 (Figure 1G) antibodies were highly specific for caspase-2 since no crossreactivity to other proteins was observed by Western blotting and no protein band was detected in tissues made from caspase-2^{-/-} mice (Figures 1G, 2). These results document that the monoclonal antibodies 10C6 and 11B4 are suitable reagents to investigate caspase-2 expression, localisation and processing.

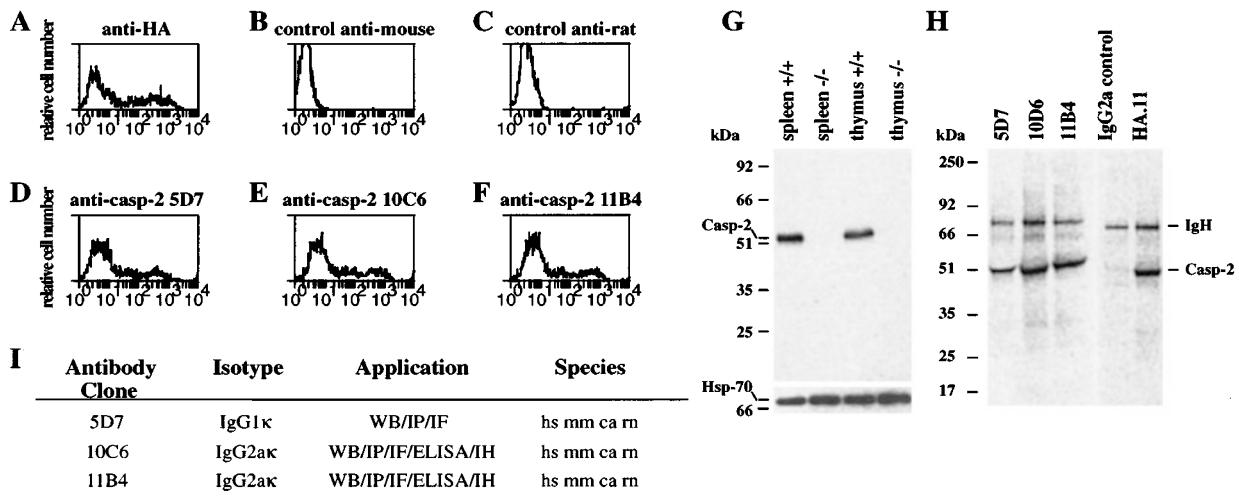


Figure 1 Screening for caspase-2 specific monoclonal antibodies (mAbs). 293T cells were transiently transfected with a construct encoding a haemagglutinin (HA) epitope tagged caspase-2 mutant in which the active site cysteine had been replaced by alanine. Transfected cells were fixed, permeabilised and stained with mouse anti-HA mAb (A, positive control), with secondary antibody alone (B and C, negative controls), or the rat anti-caspase-2 mAbs 5D7 (D), 10C6 (E) or 11B4 (F). Staining was visualised by FITC-conjugated goat anti-mouse IgG antibodies (A, B) or FITC-conjugated goat anti-rat IgG antibodies (C–F). (G) Western blotting with anti-caspase-2 mAb 11B4 revealed caspase-2 protein in lysates from wild type (wt) spleens and thymi but not in tissues from caspase-2^{-/-} mice (upper panel) (similar results were obtained with anti-caspase-2 clone 10C6) and probing with an anti-Hsp70 mAb was used as loading control (lower panel). (H) Immunoprecipitation with anti-caspase-2 mAbs 5D7, 10C6, 11B4, an isotype-matched control antibody or the anti-HA mAb of ³⁵S-labelled 293T cells transfected with mutant caspase-2 HA. (I) Summary of the characteristics of the anti-caspase-2 mAbs 5D7, 10C6 and 11B4. All antibodies recognise human (hs), mouse (mm), monkey (ca) and rat (rn) caspase-2 protein by immunofluorescence staining (IF), immunoprecipitation (IP), immunohistochemistry (IH), enzyme linked immunoadsorbent assay (ELISA) and Western blotting (WB)

Caspase-2 activation during apoptosis

In cells undergoing apoptosis, both anti-caspase-2 mAbs detected not only the 51 kDa zymogen, but also of the products of caspase-2 processing, namely the 32/33 kDa doublet, and the 19 kDa subunit. These cleavage products were observed in BAF-3, FDC-P1 and HeLa cells upon apoptosis induction by a variety of stress conditions, such as IL-3 deprivation (Figure 3 and data not shown) or treatment with staurosporine or UV-irradiation (not shown). In all cases, processing of caspase-2 was rapid. For example, caspase-2 cleavage products became visible within 4 h of IL-3 withdrawal from BAF-3 cells. Interestingly, treatment of IL-3 deprived BAF-3 cells with the broad spectrum caspase inhibitor zVAD-fmk prevented the generation of p19 subunits, but had no impact on the appearance of the 32/33 kDa polypeptides, which probably represent prodomain-less caspase-2. As caspase-2 is insensitive to zVAD-fmk,²³ this observation may indicate that the intermediate product in these cells is generated by autocatalytic activation of caspase-2, whereas, generation of the mature subunit, p19, may require a zVAD-fmk sensitive caspase, such as caspase-3. Alternatively, the first cleavage step may be mediated by an initiator caspase that is insensitive to zVAD-fmk or not easily accessible to zVAD-fmk perhaps due to sequestration inside an organelle.

Expression of caspase-2 in cell lines and mouse tissues

Caspase-2 expression in cell lines was determined by Western blotting. Examples of such Western blots are shown in Figure 4A and the overall results are summarised in Table 1. Readily detectable levels of caspase-2 were found in cell lines of lymphoid (B and T), myeloid, erythroid, fibroblast and epithelial origin. A broad survey of normal adult mouse tissues by Western blotting readily detected caspase-2 in the brain (E15), thymus, spleen, lymph nodes, colon, small intestine and testes (Figure 4B). Low levels of caspase-2 were found in the kidney, salivary gland and heart, but little or no caspase-2 expression could be detected in pancreas or liver (Figure 4B). This contrasts with the ubiquitous expression of caspase-2 mRNA observed during embryogenesis.¹¹ This may indicate that caspase-2 expression differs between embryo and adult tissues. These results confirm previous observations on the tissue expression of caspase-2¹¹ and extend them by demonstrating that caspase-2 is also expressed at high levels in the testes and colon, is barely detectable in the salivary gland and absent from pancreas. Overall these results indicate a

possible role for caspase-2 during development and in hematopoietic cells in the adult.

The smaller ~35 kDa band (*) observed in Western blots in some tissues, such as the lung, kidney, spleen (E15) and brain (E15) is most likely a processing intermediate (see above). It is, however, also possible that

Table 1 Summary of Western blot analysis of caspase-2 protein expression in cell lines

Cell line	Origin	Species	caspase-2
ABLS 8.1	pre-B lymphoma	mouse	+
7 OX/3	B lymphoma	mouse	+
CH1	B lymphoma	mouse	+
Sp2/0	plasmacytoma	mouse	+
NS-1	plasmacytoma	mouse	+
K052 DA.20	T lymphoma	mouse	+
WEHI 7.1	T lymphoma	mouse	+
WEHI 703*	T lymphoma	mouse	+
EL-4.1	T lymphoma	mouse	+
B6.2.16.BW2#	T lymphoma	mouse	+
Jurkat	T lymphoma	human	+
P388D1	Macrophage	mouse	+
RAW 2645.7	Macrophage	mouse	+
J774	Macrophage	mouse	+
F4N	erythroleukaemia	mouse	+
TS5	erythroleukaemia	mouse	+
CP16	erythroleukaemia	mouse	+
34.6MyI	granulocyte	mouse	+
P-815X-2.1	mastocytoma	mouse	+
416B MEG~	megakaryocyte	mouse	+
416B	myeloid	mouse	+
FDC-1	myeloid	mouse	+
BAF-3	myeloid	mouse	+
NIH/3T3	fibroblast	mouse	+
Rat1	fibroblast	rat	+
L-929	fibroblast	mouse	+
WEHI 11	fibrosarcoma	mouse	+
WEHI 164	fibrosarcoma	mouse	+
S17	stromal	mouse	+
MDCK	kidney	dog	+
MCF-7	breast carcinoma (epithelial)	human	+
HK-2	kidney	human	+
293T	embryonic kidney	human	+
G-401	Wilm's tumour (kidney)	human	+
TCMK-1	kidney	mouse	+
Cosm6	kidney (fibroblast)	monkey	+
MH134	hepatoma	mouse	+
HepG2	liver (epithelial)	human	+
SW480	colon carcinoma	human	+
EB-3	colon carcinoma	human	+
HeLa	cervical carcinoma	human	+
caspase 2-/-	thymus	mouse	-
caspase-2-/-	spleen	mouse	-
caspase-2-/-	brain	mouse	-

*Activated N-Ras transgenic mouse thymic T cell lymphoma. #Mouse T hybridoma expressing the same receptor as anti-HY TCR transgenic cells. ~Megakaryocyte differentiation induced by GATA-1 expression

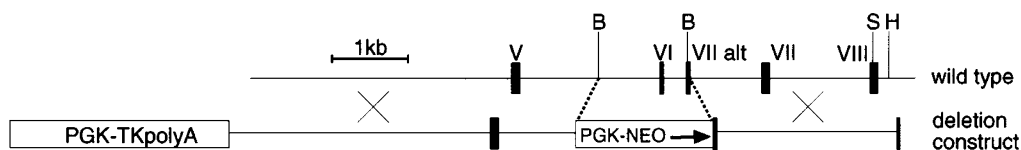


Figure 2 Targeted disruption of the mouse caspase-2 gene. Roman numerals denote exons. *Bam*HI, *Sal*I and *Hind*III sites are denoted 'B', 'S' and 'H', respectively. Exon VI which encodes for residues including the catalytic cysteine residue of caspase -2 and exon VII were all replaced by the PGK-NEO cassette

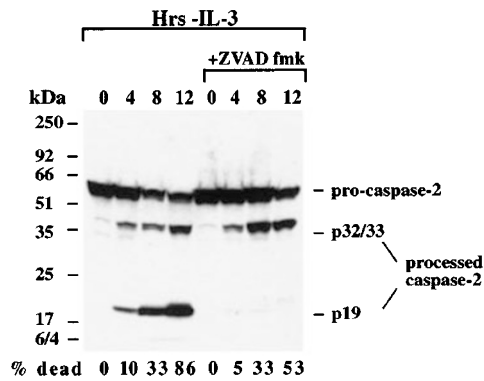


Figure 3 Caspase-2 mAbs detect the products of caspase-2 processing during apoptosis induction. Cell death was induced in BAF-3 cells by withdrawal of IL-3 and cell lysates were prepared 0, 4, 8 and 12h after apoptosis induction. Caspase-2 expression and processing was assessed by Western blotting using anti-caspase-2 mAb 11B4 and detected by ECL. Figures below the blot indicate the percentage of dead cells as assessed by trypan blue exclusion

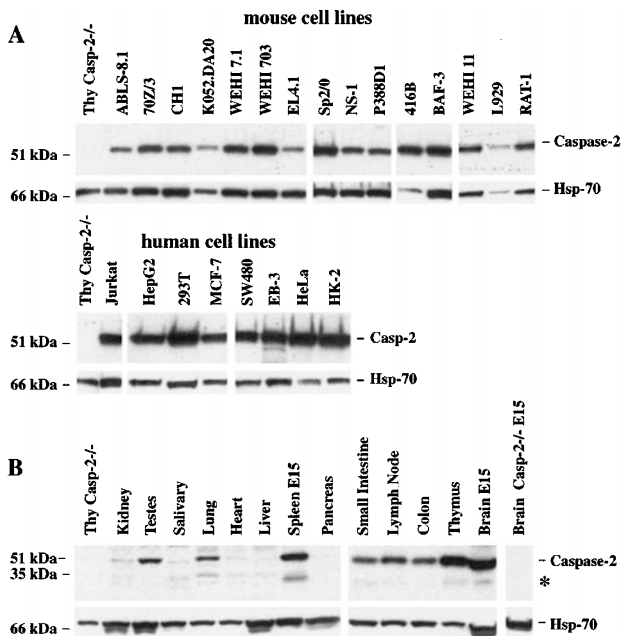


Figure 4 (A) Caspase-2 expression in cultured mouse and human cell lines. Caspase-2 protein was revealed in lysates from 2×10^5 mouse and human cell lines by Western blotting with anti-caspase-2 mAb 11B4 and detection by ECL. (B) Expression of caspase-2 by Western blot analysis in tissue lysates from normal and caspase-2^{-/-} mice (250 μ g total protein/lane). Probing with an anti-Hsp70 mAb was used as loading control for all blots. (*) may represent caspase-2 (short) or processed caspase-2

this ~35 kDa band could be the putative short form of caspase-2 (Nedd2s/lch-1s, 343 aa), which has been reported to be expressed at the mRNA level in similar tissues, such as the brain, skeletal muscle and to a lesser extent the spleen, lung, gut, testis and kidney.²⁴ The function of Nedd2s/lch-1s remains poorly defined, although it has been suggested to inhibit apoptosis in certain neuronal populations.^{21,25}

Subcellular localisation of caspase-2

The subcellular localisation of pro-caspase-2 remains controversial. Some of us have previously shown that GFP-tagged pro-caspase-2 localises to the cytoplasm and the nucleus and can be processed in both compartments.¹⁹ Subsequent studies using subcellular fractionation and a polyclonal anti-caspase-2 antibody²⁶ showed that pro-caspase-2 was located in the cytosol, nucleus and mitochondria.²⁷ Recent studies by Mancini²⁸ demonstrated that caspase-2 is predominantly located in the Golgi complex and nucleus.

To determine the localisation of pro-caspase-2, we performed sub-cellular fractionation and confocal microscopy analyses on cells expressing endogenous caspase-2. We chose to mechanically disrupt cells by Dounce homogenisation, rather than lysing them with non-ionic detergents, because these reagents have been shown to cause artefactual changes in localisation of some cell death regulators.²⁹ Subcellular fractions of healthy Jurkat cells were analyzed by immunoblotting with mAbs 10C6 and 11B4 (Figure 5A). Endogenous pro-caspase-2 protein was predominantly associated with the cytosolic fraction, the latter includes most of the Golgi apparatus (detected by anti- β -COP). A small but significant proportion of pro-caspase-2 protein was present in the nuclear fraction, which contained the nuclear protein PARP. Further fractionation of the cytoplasmic compartment of HeLa cells to obtain a light membrane (LM) and soluble fraction (S) revealed that caspase-2 was barely detectable in the LM fraction but present in the S fraction (Figure 5B). The presence of the large pro-caspase-2 pool in the soluble fraction may be due to weak attachment of pro-caspase-2 to intracellular membranes, which is easily disrupted during fractionation. Alternatively, some pro-caspase-2 might escape from nuclei during fractionation. To explore this further, we studied endogenous caspase-2 localisation by immunofluorescence staining and confocal microscopy in HeLa cells. Caspase-2 in these analyses was found in the nucleus since it co-localises with DAPI (Figure 5C) and in a cytoplasmic structure that corresponds to the Golgi apparatus since it co-localises with the anti- β -COP antibody (Figure 5C). Neither the fact that the cytosolic fraction contains most of the Golgi apparatus nor the immunofluorescence staining constitute definitive proof for the association of caspase-2 with this organelle, but they are highly suggestive.

Caspase-2 is not essential for apoptosis

It has been speculated that in response to many stress signals, caspase-2 may act as either a positive or negative regulator of cell death. Decreasing caspase-2 levels by anti-sense technology was reported to delay cell death induced by growth factor deprivation in FDC-P1 cells³⁰ and PC12 cells.¹³ Furthermore, caspase-2 deficient oocytes were shown to be resistant to apoptosis induced by chemotherapeutic drugs.²¹ However, oocytes may be unique in this regard, since, oocytes appeared to be the only cell types affected by lack

of caspase-2,²¹ whereas caspase-2^{-/-} lymphocytes were shown to be normally sensitive to chemotherapeutic drugs. We also generated caspase-2^{-/-} mice (Figure 2) and found

that in thymocytes, the absence of caspase-2 does not alter the kinetics of cell death or the dose response to a variety of cell death stimuli, including anti-Fas Ab (1-1000 ng/mL), the

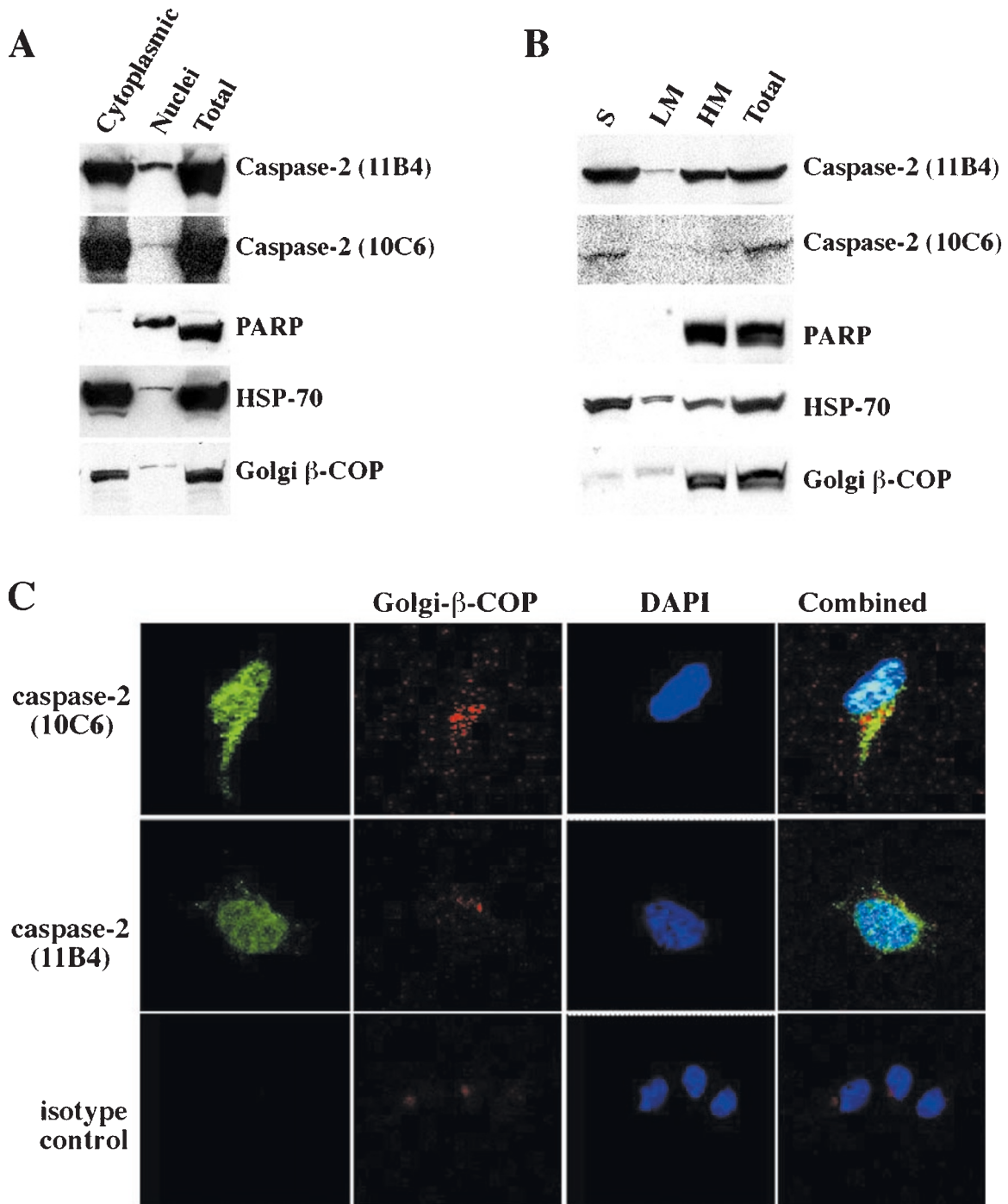


Figure 5 Analysis of caspase-2 localisation by subcellular fractionation and confocal microscopy. **(A)** Jurkat cell lysates prepared by Dounce homogenisation were separated into nuclear and cytoplasmic fractions. Proteins in fractions were size-fractionated by SDS-PAGE, electroblotted onto membranes and immunoblotting performed with mAbs specific for the proteins indicated. Caspase-2 protein, although found in both the nuclear and cytoplasmic fractions was predominantly expressed in the cytoplasmic fractions in extracts from healthy cells. Data shown are representative of three independent experiments. **(B)** HeLa cell lysates were prepared by Dounce homogenisation and separated into heavy membrane (HM) and supernatant fractions, the supernatant was further fractionated into light membrane (LM) and soluble fractions (S). Proteins in fractions were size-fractionated by SDS-PAGE, electroblotted onto membranes and immunoblotting performed with anti-caspase-2 mAbs (10C6 or 11B4) or with mAbs specific for the proteins indicated. Caspase-2 is localised in the nucleus and Golgi apparatus in HeLa cells. **(C)** HeLa cells were fixed, permeabilised, stained with anti-caspase-2 mAbs (10C6 or 11B4) plus FITC-conjugated goat anti-rat IgG antibodies (green), rabbit anti- β -COP plus biotinylated goat anti-rabbit and streptavidin Texas Red (red) and DAPI (blue) and examined by confocal microscopy

cytotoxic drug dexamethasone (1 μ M) or γ -irradiation (4-12 Gy) (Figure 6A–C). This indicates that activation of caspase-2 is not essential for thymocyte apoptosis induced by these stimuli.

Dorsal root ganglion (DRG) neurons from postnatal day 2 wt or caspase-2^{-/-} mice were cultured in the presence or absence of NGF and the percentages of live and dead cells at 24 and 48 h determined by using the MTT assay. DRG neurons from wt and caspase-2^{-/-} mice died at the same rate (Figure 7). This contrasts with previous studies, which reported that decreasing caspase-2 levels by anti-sense technology delayed apoptosis induced by trophic factor deprivation in sympathetic neurons.¹³ Our

observations are, however, in accordance with the observation that caspase-2 deficient sympathetic (SCG) neurons were normally sensitive to NGF withdrawal.²¹ Collectively, these studies indicate that apoptosis in thymocytes and neuronal cells can occur normally in the absence of caspase-2.

Caspase-2 processing in mouse thymocytes requires Apaf-1 and caspase-9

Previous studies have indicated that pro-caspase-2 can be activated by autocatalysis in a proximity-induced manner.^{18,19} However, caspase-2 can also be fully processed by caspase-3²⁰ and depletion of caspase-3 from cell extracts blocked caspase-2 processing *in vitro*.^{31,32} This prompted us to test whether caspase-2 activation can occur in cells lacking Apaf-1 or caspase-9, that constitute one of the main caspase-3 activation mechanisms.

We isolated wt, Apaf-1^{-/-} or caspase-9^{-/-} thymocytes from lethally irradiated mice, reconstituted with fetal liver cells derived from wt, Apaf-1^{-/-} or Caspase-9^{-/-} E14 embryos. CD4⁺8⁺ thymocytes were purified by immunofluorescent staining and flow cytometric cell sorting, and exposed to 5 Gy γ -irradiation. After 0, 4 or 8 h in culture, cells were harvested, lysed and Western blotting performed with anti-caspase-2 antibodies to follow the appearance of the p32/33 and p19 cleavage products and the disappearance of the p51 pro-form, as a sign of caspase-2 processing. The caspase-2 cleavage products were apparent after γ -irradiation only in wt thymocytes (Figure 8) but not in those of Apaf-1^{-/-} and caspase-9^{-/-} origin (Figure 8). These results demonstrate that in primary cells the cleavage of caspase-2 occurs downstream of the Apaf-1/caspase-9 apoptosome complex and are in agreement with the finding that depletion of caspase-9 from cell extracts *in vitro* abrogates cytochrome *c*-inducible activation of caspase-2.³¹ Thus, Apaf-1 and caspase-9 are required for caspase-2 activation.

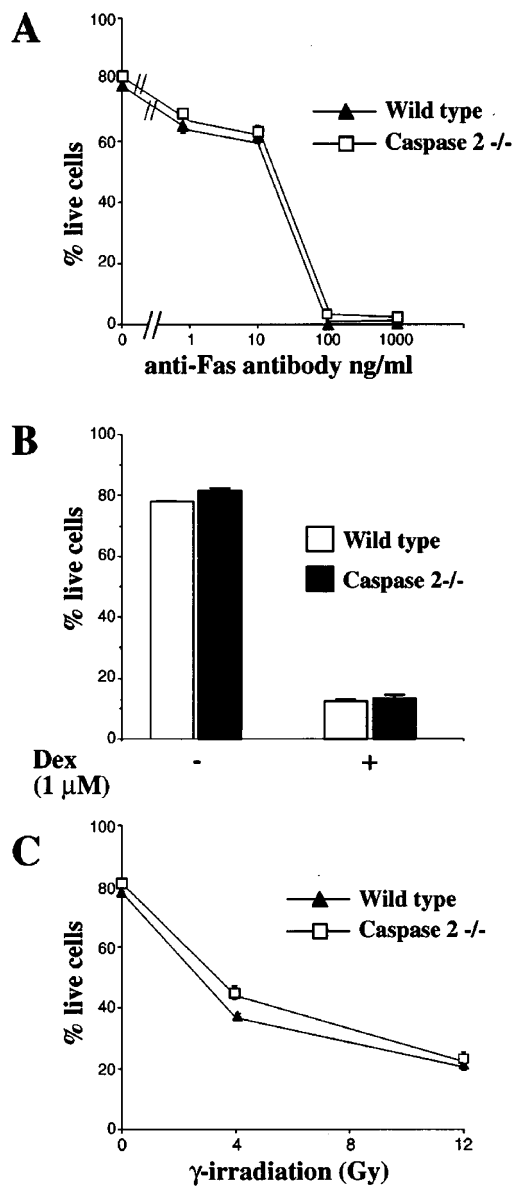


Figure 6 Caspase-2 is dispensable for apoptosis in thymocytes. Cell death was induced in wt and caspase-2^{-/-} thymocytes by treatment with 1–1000 ng/mL anti-Fas Ab (A), 1 μ M dexamethasone (B) or γ -irradiation (4–12 Gy) (C). Cell viability was determined by staining with propidium iodide (PI) followed by FACS analysis. Error bars are S.E.M. from three replicate plates

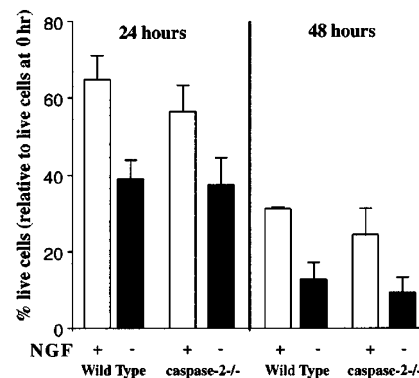


Figure 7 Caspase-2 is not required for trophic factor withdrawal induced apoptosis of DRG neurons from postnatal day 2 (P2) caspase-2^{-/-} mice or control (wt) C57BL/6 mice were cultured in the presence or absence of NGF. Cell viability was determined by the MTT assay and at each time point a minimum of five individual wells was counted. Data represent the mean of three separate experiments and at least four mice of each genotype were analyzed each time. The error bars represent \pm S.E.M.

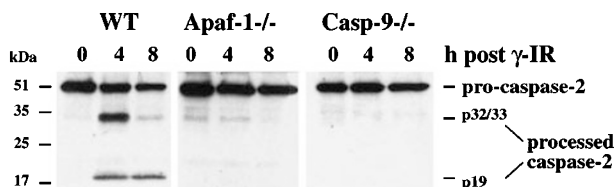


Figure 8 Caspase-2 processing is defective in thymocytes derived from Apaf-1 and caspase-9 deficient mice. Cell death was induced in WT, Apaf-1^{-/-} or caspase-9^{-/-} thymocytes by γ -irradiation (5 Gy) and cell lysates were prepared 0, 4 and 8 h after apoptosis induction. Caspase-2 processing was assessed by Western blotting using anti-caspase-2 mAb 11B4 and detected by ECL

Conclusions

Using monoclonal antibodies that recognize the p19 subunit of caspase-2 we have shown that caspase-2 is expressed in most tissues and cell types. Further, we demonstrate that caspase-2 is located in the nuclear and cytosolic compartments, in particular the Golgi apparatus. Although caspase-2 is activated in response to a broad range of apoptotic stimuli, it is not essential for apoptosis in many cell types, since thymocytes and DRG neuronal cells derived from caspase-2 mutant mice die normally. We also show, that caspase-2 processing during γ -irradiation induced cell death is dependent on the presence of Apaf-1 and caspase-9. This indicates that cleavage of caspase-2 occurs downstream of the Apaf-1/caspase-9 apoptosome and that caspase-2 processing may be dependent on other caspases, such as caspase-3. We have however seen that in MCF7 cells, that lack the caspase-3 gene,³³ caspase-2 activation can occur in response to various death stimuli (data not shown). This suggests that caspase-3 mediated proteolysis may be only one of the ways of caspase-2 activation and in the absence of caspase-3, either an autocatalytic mechanism, or other caspases, such as caspase-7 can still mediate pro-caspase-2 processing. Collectively these observations indicate that in many circumstances caspase-2 may act as an effector caspase. It remains, however, possible that in certain other cell types, such as neuronal cells, caspase-2 can also function as an initiator caspase, but does so in an overlapping manner with another initiator caspase, such as caspase-9.³⁴ To address the possible overlap between caspase-2 and caspase-9 function, it will therefore be interesting to generate mice lacking both of these enzymes.

Materials and Methods

Experimental animals

All experiments with animals were performed according to the guidelines laid down by the Royal Melbourne Hospital Research Foundation's Animal Ethics Committee. Wistar rats and C57BL/6 mice were obtained from The Walter and Eliza Hall Institute's breeding facility.

For the generation of caspase-2^{-/-} mice the targeting construct (Figure 2) contained the gene for neomycin resistance under the control of the *pgk* promoter and a PGK-TK cassette for positive-

negative selection. The *neo* cassette replaced a 1.65 kb fragment of the caspase-2 gene containing exon 6 (including the catalytic cysteine) and part of exon 7. The construct was linearised and electroporated into w9.5 embryonic stem cells that were selected using gancyclovir and G148 (neomycin). Antibiotic-resistant ES cell clones were isolated, expanded and screened by Southern blot hybridisation using a probe situated at the 3' end of the deletion construct. ES cells targeted at the caspase-2 locus were confirmed to contain a single integration using a neomycin probe. These cells were used to derive chimaeric mice that were mated with C57BL/6 female mice and the heterozygous offsprings interbred to yield wild-type caspase-2^{+/+}, heterozygous caspase-2^{+/-}, and mutant caspase-2^{-/-} mice. Mouse genotypes were determined by Southern blot analysis of genomic DNA obtained from tail biopsies. Mice were maintained on a mixed 129/sv \times C57BL/6 background. The generation of Apaf-1 mutant and caspase-9 mice has been described.^{35,36} Apaf-1^{+/-} and caspase-9^{+/-} animals were backcrossed for >8 generations with C57BL/6 mice prior to mating for generation of Apaf-1^{-/-} and caspase-9^{-/-} embryos.

Production and purification of the hexa-His-tagged Caspase-2 p19 polypeptides

A region of mouse caspase-2 encoding the p19 subunit (amino acids 167 to 333) was PCR amplified from pMSN2.4¹¹ and cloned into pQE30 vector (Qiagen) containing an amino terminal 6 \times His tag to generate pQEp19. Overnight *E. coli* M15(pREP4) cultures harbouring pQEp19 were diluted 50-fold in fresh medium, grown for 2 h at 37°C and induced with 1 mM IPTG for a further 4 h. Bacterial pellets were lysed in 8.0 M urea, 0.1 M NaH₂PO₄, 0.01 M Tris HCl, pH 8.0 and 6 \times His-tagged protein purified using a TALON column (Clontech). The recombinant protein was dialysed against PBS, precipitated by cold acetone and redissolved in PBS at 1 mg/mL.

Immunisation, hybridoma fusion and screening for antibodies to caspase-2

Wistar rats were initially immunised by subcutaneous (s.c.) injection with 100 μ g purified recombinant 6 \times His tagged caspase-2 p19 subunit mixed with complete Freund's adjuvant (Difco). Two subsequent boosts of the immunogen, resuspended in incomplete Freund's adjuvant (Difco), were injected s.c. 3 and 6 weeks later. A final boost with p19 protein dissolved in PBS was given intravenously (i.v.) and intraperitoneally (i.p.) 4 weeks after this. Three days later, hybridomas were generated by fusing spleen cells from immunised rats with the SP2/0 myeloma cell line as previously described.²² Hybridomas producing monoclonal antibodies to caspase-2 were identified using a screening strategy that we have previously described.²² Briefly, 293T cells were transiently transfected with a C-terminally HA-tagged mutant of mouse caspase-2 in which the active site cysteine had been changed to alanine. Transfected cells were fixed in 1% paraformaldehyde, permeabilised with 0.3% saponin (Sigma) and stained with hybridoma supernatants. Bound antibodies were revealed with fluorescein-isothiocyanate (FITC)-conjugated goat anti-rat Ig antibodies (Southern Biotechnology) and analysed in a FACScan analyzer (Becton Dickinson). Hybridomas producing antibodies to caspase-2 were cloned twice and adapted for growth in medium containing low serum. For production of large amounts of antibodies, hybridomas were cultured for several weeks in the miniPERM classic 12.5 kDa production and nutrient module (Her-aeus). Antibodies were purified on a protein-G Sepharose column (Pharmacia).

Cell lines, tissue culture, cell death assays and transfection with expression constructs

The cell lines used for analysis of caspase-2 expression are indicated in Table 1. Details of these cell lines are available from the authors. The cells were cultured in the high glucose version of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS), 50 μ M 2-mercaptoethanol (2-ME), 13 μ M folic acid and 100 μ M L-asparagine or were grown in DME or RPMI medium with 10% FCS alone. Cultures of parental FDC-P1 and BAF-3 cells and their derivatives were supplemented with IL-3 (1000 U/mL). Granulocyte differentiation of 34.6 Myl cells was induced by the addition of 1.5% dimethyl sulfoxide.³⁷ Liposome (Lipofectamine, Gibco BRL) mediated transfection of 293T cells was performed as previously described.³⁸

Apaf-1^{-/-} and caspase-9^{-/-} thymocytes were isolated from C57BL/6-Ly5.1 mice, which had been lethally irradiated (2×5.5 Gy) and reconstituted with foetal liver cells from wild-type (wt), Apaf-1^{-/-} or Caspase-9^{-/-} E14 fetuses (all >8 generations C57BL/6-Ly5.2). These fetuses were generated by intercrosses of either Apaf-1^{+/-}³⁵ or Caspase-9^{+/-} mice.³⁶ Donor (Ly5.2⁺)-derived CD4⁺ thymocytes were purified by immunofluorescent staining with monoclonal antibodies to Ly5.2, CD4 and CD8, followed by cell sorting in a MoFlo (cytometry).³⁹ These thymocytes were left untreated or were exposed to 5 Gy γ -irradiation. After 4 or 8 h in culture, cells were harvested, lysed and Western blotting performed with anti-caspase-2 antibodies.

In BAF-3 and FDC-P1 cells, apoptosis was induced by withdrawal of IL-3, their essential growth factor. Cell viability was determined by counting cells stained with the vital dye Trypan Blue.

Thymocytes from wild-type (wt) or caspase-2^{-/-} mice were cultured in DMEM supplemented with 10% foetal calf serum (FCS), 50 μ M 2-mercaptoethanol (2-ME), 13 μ M folic acid and 100 μ M L-asparagine at 10^6 /ml. Cell death was induced by treatment with either 1–1000 ng/ml anti-Fas Ab (Jo2, Pharmingen), 1 μ M dexamethasone (Sigma) or γ -irradiation (4–12 Gy). Cell viability was determined after 24 h by propidium iodide (PI) staining and analysis in a FACScan.

Dorsal root ganglion (DRG) neuronal cells from wt and caspase-2^{-/-} mice were prepared as described⁴⁰ from post-natal day 2 mice. Briefly, DRGs were dissected free of surrounding tissue and incubated in HEPES-buffered Eagles medium (HEM), 0.025% trypsin, 0.001% DNase for 30 min. FCS was added (10%) and cells centrifuged at $300 \times g$ for 5 min, resuspended in HEM, 10% FCS, 0.01% DNase and triturated through 18 to 24 gauge needles to obtain a single cell suspension. Cells were washed in HEM, 10% FCS, 0.01% DNase and resuspended in Monomed, 10% FCS. To assess DRG neuron survival, single cell suspensions were resuspended at a density of 10^4 cells/mL with or without added nerve growth factor (2.5S NGF, 10 ng/mL, Sigma). Cells were plated on fibronectin (Sigma) coated Terasaki plates at approximately 100 cells/well. At each time point, at least five wells were stained with MTT (0.5 mg/mL 3-(4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide, Sigma) for 1–2 h at 37°C and viable (blue stained cells) counted. This number was expressed as a percentage of the mean number of surviving cells at 0 h.

Western blotting

Primary cells, cell lines or transfected 293T cells were harvested, washed twice in cold PBS and lysed in lysis buffer (20 mM Tris/HCl, pH 8.0, 125 mM NaCl, 1 mM EGTA, 1% Triton X-100, 10% glycerol, 0.5 μ g/mL Pefabloc, 1 μ g/mL of each of leupeptin, aprotinin, soybean trypsin inhibitor and pepstatin, 5 mM NaF and 2 mM Na₃VO₄; all reagents from Sigma or Roche Diagnostics). For preparation of tissue

lysates, organs were excised, washed in PBS, immediately frozen in isopentane on dry ice, and were later homogenised at 4°C in lysis buffer as described before.⁴¹

Proteins in cell lysates were size-fractionated by SDS-PAGE and electroblotted onto nitrocellulose membranes (Amersham Life-Science). Prior to immunoblotting, non-specific binding of antibodies to membranes was blocked by incubation overnight in 5% skimmed milk, 1% casein, 0.05% Tween-20. Membranes were probed with 10C6 or 11B4 anti-caspase-2 mAbs (1 μ g/mL) followed by goat anti-rat IgG antibodies conjugated to HRP (Southern Biotechnology) and detection by enhanced chemiluminescence (ECL; Amersham Pharmacia). To control for the concentration and integrity of proteins in the tissue lysates, blots were probed with mouse anti-HSP70 mAb N6 (a gift from Dr R Anderson, Peter MacCallum Cancer Institute, Melbourne, Australia), followed by HRP-conjugated sheep anti-mouse Ig antibodies (Silenus) and detection by ECL.

Subcellular fractionation

Jurkat or HeLa cells were resuspended in hypotonic buffer (either 0.01 M NaCl, 1.5 mM MgCl₂, 0.01 M Tris-HCl, pH 7.4 or 10 mM HEPES pH 7.4, 38 mM NaCl) and allowed to swell on ice for 10 min at 4°C. Cells were lysed using a Dounce homogeniser (12–36 strokes with a type 'B' pestle; Konte Glassware Corporation).⁴² After 3 min centrifugation at $1000 \times g$ at 4°C, followed by two washes in hypotonic buffer, the pelleted nuclei or heavy membranes (HM) were resuspended in the lysis buffer used for Western blotting (see above). The resulting supernatant was fractionated further by centrifugation at $130\,000 \times g$ for 1 h (or 50 000 r.p.m. for 30 min in a TLA 100.3 Beckman benchtop rotor) to generate the soluble (S) and light membrane (LM) fractions. The LM pellet was resuspended in RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% deoxycholic acid, 50 mM Tris-HCl pH 8.0 plus protease inhibitors). Lysates from equivalent numbers of cells were analyzed by immunoblotting with the rat anti-caspase-2 mAbs 10C6 or 11B4, the mouse mAb anti-PARP (Calbiochem-Novabiochem Corp), rabbit anti-Golgi β -COP (Affinity Bioreagents Inc) or mouse anti-HSP-70 mAb. Bound antibodies were revealed with appropriate HRP-conjugated anti-mouse, anti-rat or anti-rabbit Ig antibodies and ECL detection.

Immunofluorescence staining and confocal microscopy

For immunofluorescence staining with anti-caspase-2 mAbs, HeLa cells were grown in chamber slides (Becton Dickinson). Cells were attached using Cell Tak (Becton Dickinson), fixed and permeabilized with PBS/4% paraformaldehyde, containing 0.18% Triton X-100 for 10 min at RT. The fixed cells were stained overnight at 4°C with the rat anti-caspase-2 10C6 or 11B4 mAbs in PBS containing 10% FCS, washed with PBS and then incubated with FITC-conjugated goat anti-rat Ig antibodies (Southern Biotechnology). The slides were then re-blocked with normal goat serum and stained rabbit anti-beta-Coatamer protein (β -COP) antibody (Affinity Bioreagents, Inc) in PBS containing 10% FCS, followed by biotinylated goat anti-rabbit IgG antibodies (Vector) in PBS containing 10% FCS. Staining was detected by Texas Red conjugated streptavidin (Vector) containing DAPI (2 μ g/mL, Molecular Probes). Slides were mounted in fluorescent mounting medium (Dako) containing 100 μ g/mL DIABCO (Sigma). Controls included staining with an isotype matched rat IgG2a antibody (Pharmingen) or with the primary or secondary antibodies alone. Samples were analysed with a Leica confocal scanning microscope using SCANware software (Leica Lasertechnik).

Acknowledgements

We are grateful to Prof. Peter Gruss (Max Plank Institute, Goettingen, Germany) for supplying the Apaf-1^{+/-} mice. We thank S Novakovic, G Hausmann, F Battye, D Kaminaris, J Parker, V Lapatis, C Tarlinton, A Milligan and J Merryfull for help with various aspects of this study. This work was supported by grants and fellowships from, the Leukemia and Lymphoma Society of America (New York), the Anti-Cancer Foundation of South Australia, the Anti-Cancer Council of Victoria, the National Health and Medical Research Council, the Sylvia and Charles Viertel Charitable Foundation, the Dr. Josef Steiner Cancer Research Foundation (Bern, Switzerland) and The Murdoch Children's Research Institute.

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