

# Caspase-6 is the direct activator of caspase-8 in the cytochrome *c*-induced apoptosis pathway: absolute requirement for removal of caspase-6 prodomain

V Cowling<sup>1</sup> and J Downward<sup>\*1</sup>

<sup>1</sup> Signal Transduction Laboratory, Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London WC2A 3PX, UK.

\* Corresponding author: Julian Downward; Tel: +44 20 7269 3533; Fax: +44 20 7269 3094; E-mail: downward@icrf.icnet.uk

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## Abstract

**Caspase activation resulting from cytochrome *c* release from the mitochondria is an essential component of the mechanism of apoptosis initiated by a range of factors. The activation of Bid by caspase-8 in this pathway promotes further cytochrome *c* release, thereby completing a positive feedback loop of caspase activation. Although the identity of the caspases necessary for caspase-8 activation in this pathway are known, it is still unclear which protease directly cleaves caspase-8. In order to identify the factor responsible we undertook a biochemical purification of caspase-8 cleaving activity in cytosolic extracts to which cytochrome *c* had been added. Here we report that caspase-6 is the only soluble protease in cytochrome *c* activated Jurkat cell extracts that has significant caspase-8 cleaving activity. Furthermore the caspase-6 that we purified was sufficient to induce Bid dependent cytochrome *c* releasing activity in cell extracts. Inhibition of caspase-6 activity in cells significantly inhibited caspase-8 cleavage and apoptosis, therefore establishing caspase-6 as a major activator of caspase-8 *in vivo* and confirming that this pathway can have a critical role in promotion of apoptosis. We also show that caspase-6 is inactive until the short prodomain is removed. We suggest that the requirement for two distinct cleavage steps to activate an effector caspase may represent an effective mechanism for restriction of spontaneous caspase activation and aberrant entry into apoptosis.**

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**Keywords:** caspase; apoptosis; cytochrome *c*

**Abbreviations:** DISC, death inducing signalling complex; EKD, Biotin-z-Glu-Lys (Biotinyl)-Asp-CH<sup>s</sup>-DMB; IEF, iso-electric focusing

## Introduction

Much of the cellular reorganisation that occurs during programmed cell death is a result of the activity of the caspase family of cysteine proteases. The pro-apoptotic caspases are present as inactive zymogens in healthy cells, available to be rapidly activated by cleavage in response to apoptotic triggers.<sup>1</sup> Activation of caspases has been observed in response to all known triggers of apoptosis and is essential to achieve the defining characteristics of programmed cell death. Caspase-8 is activated both as a result of death receptor ligation and cytochrome *c* release from the mitochondria.<sup>2</sup> Activation of caspase-8 resulting from cytochrome *c* release is not well defined but is thought to constitute part of a feedback loop of caspase activation based around cytochrome *c* release from the mitochondria.<sup>3</sup>

Caspase-8 activation in the Fas Ligand triggered Death-Inducing Signalling Complex (DISC) has been well characterised. Ligation of Fas by Fas Ligand initiates recruitment of caspase-8 to the membrane bound DISC via the adaptor protein FADD.<sup>4,5</sup> Although the activity of procaspase-8 is thought to be limited it is sufficient to permit auto-cleaving activation when two or more zymogens are brought into close proximity in the DISC.<sup>6</sup> Active caspase-8, released from the DISC into the cytosol, initiates a chain of caspase activation and substrate cleavage which results in apoptosis.<sup>7</sup>

Activation of caspase-8 can also occur in the cytosol as part of the cytochrome *c* triggered caspase cascade, but in this case the mechanism is less well understood. A crucial event for many apoptotic triggers to be successful is the release of cytochrome *c* from the mitochondria into the cytosol.<sup>8</sup> This initiates the activation of a whole complement of cytosolic caspases starting with auto-activation of caspase-9 and activation of the other caspases by direct cleavage.<sup>9</sup> Biochemical studies have revealed that caspase activation is not a linear chain of events. Complex feedback mechanisms of processing occur – many activated caspases can cleave the caspase which activated them.<sup>10</sup> Caspase-8 processing in this system begins later than most other caspases and does not require the membrane bound DISC; the process can be reconstituted experimentally by the addition of cytochrome *c* to cytosol.<sup>11</sup> However, as discussed later, some experimental data suggests the involvement of FADD in cytochrome *c* triggered caspase-8 activation and, therefore, it is unclear by which mechanism caspase-8 is being activated, by auto-activation or by direct cleavage by another caspase.

Caspase-8 activation has an important role in the feedback loop of caspase activation and cytochrome *c* release. One of the substrates of caspase-8 is the pro-apoptotic Bcl-2 family member, Bid.<sup>12,13</sup> When caspase-8

cleaves Bid it becomes localised to the mitochondrial membrane where it promotes further cytochrome *c* release.

Valuable information revealing how caspase-8 is being activated has come from investigation using cells from caspase deficient mice and from immunodepletion studies. Mouse embryonic fibroblasts from caspase-9 and caspase-3 deficient mice are less able to activate caspase-8 in response to triggers that induce cytochrome *c* release.<sup>14</sup> However, interpretation of data from the caspase knock-out cells is limited because other caspases can compensate for the deleted caspase.<sup>15</sup> Detailed examination of the order of caspase activation has been carried out by immunodepletion of individual caspases from cell extracts followed by observation of which caspases remain able to be activated in response to cytochrome *c* addition.<sup>11</sup> In this system caspases-9, -3 and -6 were all found to be required for caspase-8 activation. The limitations of both of these types of investigation is that they can only indicate which caspases lie upstream of caspase-8 activation and cannot reveal which factors are directly processing it.

During apoptosis each active caspase is not a homogeneous population but a mixture of variants, with variable length prodomains, linker regions being present or absent and sometimes having other binding proteins.<sup>16,17</sup> Since in knock out mice and in immunodepletion studies all forms of each caspase are absent, the activity of these variants is not distinguished.

The aim of this work was to identify the protease which directly activates caspase-8 after cytochrome *c* release into the cytosol. In order to isolate the protease which directly interacts with and cleaves caspase-8 we undertook a biochemical purification of caspase-8 cleaving activity from cytochrome *c* activated Jurkat cell extracts using column chromatography. We found that caspase-6 is the major caspase-8 cleaving activity in this system and an essential requirement for caspase-6 to be active is that the prodomain must be removed.

## Results

Caspase-8 is cleaved in the cytosol following cytochrome *c* release by a protease that has not yet been identified. To characterise this mechanism of caspase-8 activation we recreated this process *in vitro* by incubating Jurkat cell cytosolic extracts with cytochrome *c* and dATP. This *in vitro* system imitates what happens in the cell in that the caspase-8 precursor is initially cleaved between the p18 and p10 domains resulting in fragments of 42 and 10 kDa; the p42 is further cleaved between the pro and p18 domains resulting in fragments of 25 and 18 kDa.<sup>4</sup> The mature active caspase consists of a heterotetramer of two p18 subunits and two p10 subunits.

### Caspase-8 is cleaved by transient interaction with a protease

We wanted to determine whether procaspase-8 is activated in this system by homo-dimerisation followed by auto-cleavage, as observed in the Fas induced DISC;<sup>4</sup> or whether it is

cleaved by stable or transient interaction with another protease.

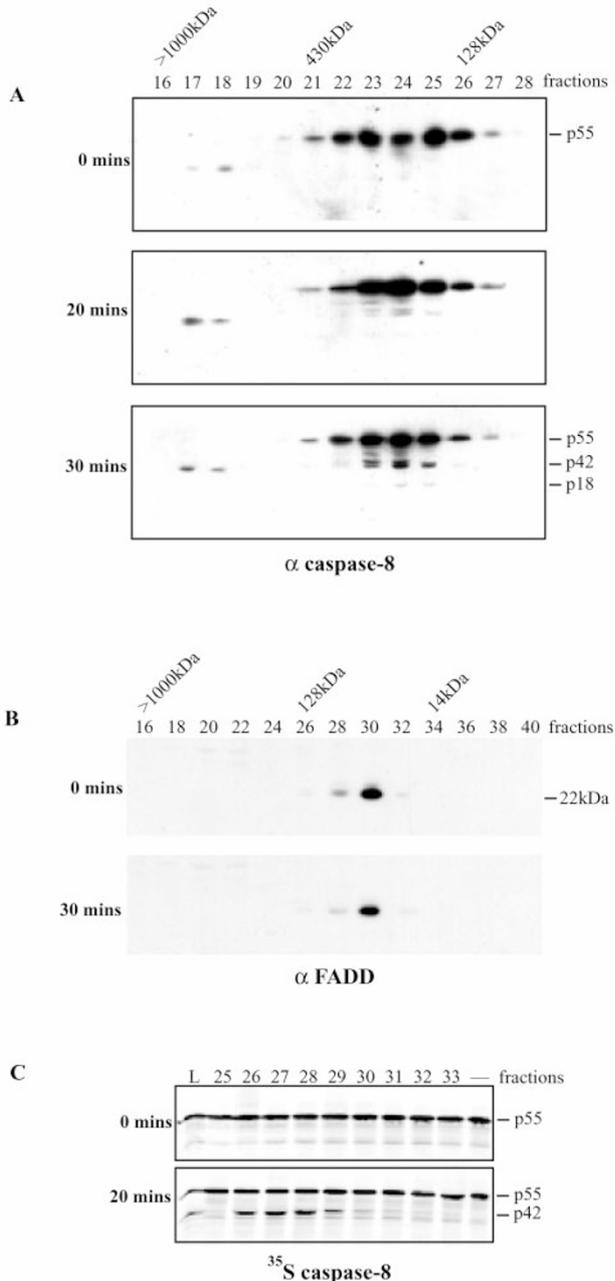
In our system, we observed that caspase-8 does not become part of a larger complex during activation. Post mitochondrial Jurkat extracts, activated by incubation with cytochrome *c* and dATP for 20 or 30 min, were loaded onto a Superdex 200 gel filtration column. Immunoblot analysis of the fractions collected using an antibody raised against the p18 domain revealed that caspase-8 elutes from the column in the same volume before and during cleavage (Figure 1A). In untreated extracts caspase-8 was un-cleaved, being visualised as a 55 kDa band which peaked at fraction 24. Following activation of extracts for 20 and 30 min, 42 and 18 kDa caspase-8 cleavage products were also observed, all of which peaked at fraction 24. Caspase-8, therefore, unlike caspases-9, -7 and -3, does not become part of a larger complex during activation. Since caspase-8 does not shift to a smaller fraction during cleavage, loss of an inhibitor binding to procaspase-8 is also unlikely to be a component of the mechanism of activation.

In order to distinguish whether caspase-8 was being cleaved by auto-activation or by transient interaction with another protease we determined the relative complex size of the caspase-8 cleaving activity using the gel filtration fractions (Figure 1C). Fractions from the Superdex 200 column which had been loaded with control or activated extracts were incubated with *in vitro* translated <sup>35</sup>S caspase-8 at 37°C for 30 min. To assess which fractions had the ability to cleave caspase-8 we looked for labelled caspase-8 cleavage products using SDS-PAGE and phosphorimaging. None of the fractions from the control extract contained caspase-8 cleaving activity; <sup>35</sup>S caspase-8 remained as a 55 kDa zymogen after incubation with all fractions. However fractions 26 to 29, separated from the activated extract, did cleave <sup>35</sup>S caspase-8 to reveal the 42 kDa subunit. Since the activity peaked at fraction 28 and endogenous caspase-8 peaks at fraction 24, we could conclude that in this system caspase-8 is being cleaved not by auto-activation but by transient interaction with a smaller protease.

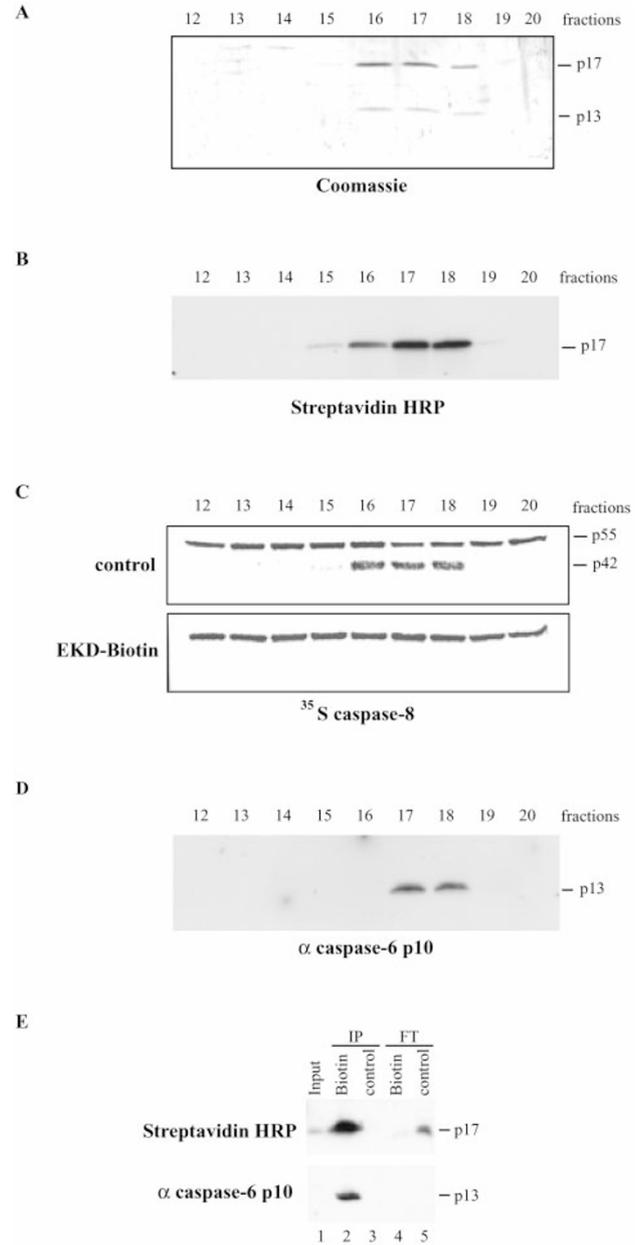
The adaptor protein FADD has been implicated in a number of studies to be involved in cytochrome *c* triggered caspase-8 activation.<sup>18</sup> Analyzing the activated extracts, which had been separated by gel filtration, we observed that FADD does not oligomerise during extract activation, peaking at fraction 30 before and during activation (Figure 1B). FADD was never found in the same fractions as caspase-8 and therefore does not appear to bind to the procaspase or the cleaved caspase in this system. In addition, FADD and caspase-8 could not be co-immunoprecipitated from control or cytochrome *c* activated extracts (data not shown). Although the FADD containing fractions and the fractions containing caspase-8 cleaving activity show some overlap, the quantity of FADD does not correlate with the quantity of caspase-8 cleaving activity. FADD is also not observed in the purified caspase-8 cleaving activity (Figure 2A). Therefore, in this system, FADD does not appear to have a role in caspase-8 activation.

## Biochemical purification of caspase-8 cleaving activity

We used column chromatography to purify the cytosolic protease that, once activated by incubation of extracts with



**Figure 1** Caspase-8 is cleaved by transient interaction with a smaller protease. Post-mitochondrial Jurkat extracts were incubated with 0.1 mM cytochrome *c* and 0.01 mM dATP at 30°C for 0, 20 or 30 min, as indicated. Extracts were loaded onto a Superdex 200 gel filtration column and 0.5 ml fractions were collected, indicated by the numbers above the figures. 15  $\mu$ l of fractions were immunoblotted with, in (A) polyclonal anti-caspase-8 antibody and in panel (B) monoclonal anti-FADD antibody. 15  $\mu$ l of the gel filtration fractions were also incubated with 0.1  $\mu$ l *in vitro* translated,  $^{35}$ S caspase-8 for 30 min at 37°C. Fractions were subjected to SDS-PAGE and  $^{35}$ S caspase-8 fragments were visualised using a phosphorimager (C). In lanes 'L' and '—', 50  $\mu$ l of pre-load and buffer, respectively, were used in the same assay



**Figure 2** EKD-Biotin incubated with purified caspase-8 cleaving activity inhibits the protease and binds to a single 17 kDa band. Purified caspase-8 cleaving activity was incubated with 1  $\mu$ M EKD-Biotin at 37°C for 10 min prior to loading onto a Superdex 200 gel filtration column. 1 ml fractions collected were separated by SDS-PAGE and stained with Colloidal Coomassie, (A) or following electroblotting, EKD-Biotin bound proteins were visualised by Streptavidin-HRP, (B) and caspase-6 p13 was visualised by incubation with monoclonal anti-caspase-6 antibody, (D). In (C) 50  $\mu$ l of gel filtration fractions separated after pre-load preincubation without (a) or with (b) EKD-Biotin were incubated with 0.1  $\mu$ l *in vitro* translated,  $^{35}$ S caspase-8 for 30 min at 37°C. Fractions were subjected to SDS-PAGE and  $^{35}$ S caspase-8 fragments were visualised using a phosphorimager. In (E) 600  $\mu$ l EKD-Biotin bound material from fractions 16 to 18 was incubated with 25  $\mu$ l monoclonal anti-Biotin antibody bound agarose or 25  $\mu$ l Protein G Sepharose and 2.5  $\mu$ g isotype matched control antibody for 1 h at room temperature and protein was eluted from the beads into 50  $\mu$ l loading buffer. Protein was analyzed by immunoblotting with Streptavidin-HRP in the upper blot and monoclonal anti-caspase-6 antibody in the lower blot. In lane 1 is 15  $\mu$ l pre-load, lanes 2 and 3 are 20  $\mu$ l protein eluted from the anti-Biotin antibody and the control antibody, respectively and lanes 4 and 5 are 15  $\mu$ l protein left unbound after incubation the anti-Biotin antibody and the control antibody, respectively

cytochrome *c*, cleaves caspase-8. Throughout the purification caspase-8 cleaving activity was assayed by measuring the ability of fractions to cleave *in vitro* translated <sup>35</sup>S caspase-8, as described above. The isolation of caspase-8 cleaving activity from cytochrome *c* activated Jurkat cell extracts was carried out by the following sequential steps: Q-Sepharose anion exchange chromatography, SP-Sepharose cation exchange chromatography, Octyl-Sepharose hydrophobic interaction chromatography, Ammonium sulphate precipitation and Superdex 200 gel filtration. After gel filtration caspase-8 cleaving activity appeared in fractions 16 to 18 (Figure 2C – upper panel). The same fractions were separated by SDS–PAGE followed by colloidal Coomassie staining (Figure 2A). Two bands of 17 and 13 kDa were found specifically in fractions 16 to 18, co-eluting with caspase-8 cleaving activity.

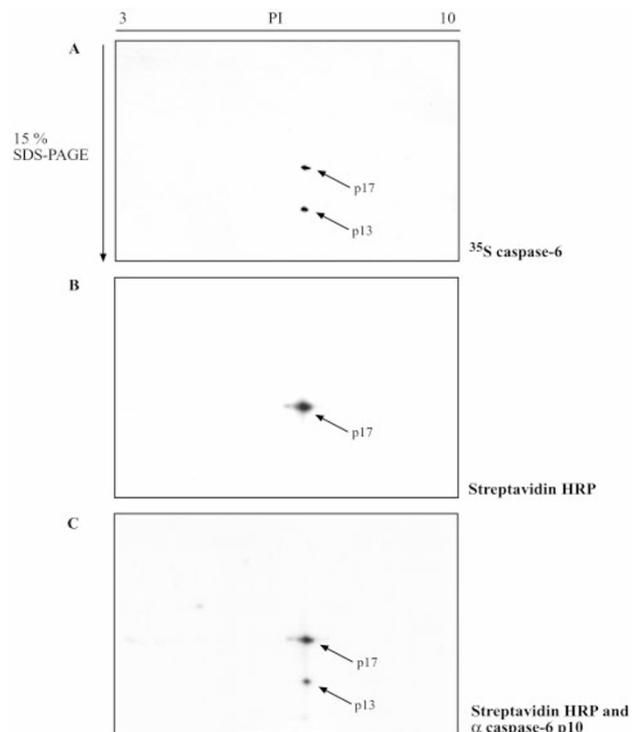
To identify whether these 17 and 13 kDa bands constituted the caspase-8 cleaving activity, the gel filtration fractions were incubated with a peptide substrate, z-Glu-Lys (Biotinyl)-Asp-CH<sub>2</sub>-DMB (EKD-Biotin), which mimics the recognition site between the p18 and p10 of caspase-8 – Glutamate, Valine, Aspartate or EVD.<sup>19</sup> The EKD-Biotin covalently modifies the p20 subunits of the caspases to which it binds, inhibiting their activity and also contains a Biotin group to allow detection. Following incubation with EKD-Biotin, the fractions were separated by SDS–PAGE, blotted onto PVDF and the Biotin bound proteins were detected by Streptavidin-HRP (Figure 2B). A 17 kDa band from fractions 16 to 18 bound to EKD-Biotin and resolved as a single spot on a 2D gel (Figure 3B). Following peptide binding, the fractions were desalted and assayed for caspase-8 cleaving activity (Figure 2C lower panel). Fractions 16 to 18 were no longer able to cleave <sup>35</sup>S caspase-8 confirming that the p17, EKD-Biotin binding species contained the catalytic site of the caspase-8 cleaving activity.

### Caspase-8 cleaving activity is caspase-6

Since analysis of the human genome sequence has revealed that all close caspase-homologs have been cloned, we blotted the purified activity for known pro-apoptotic caspases. Active fractions from each stage of the purification were immunoblotted using a panel of antibodies raised against a range of caspases. The only antibody to recognise this material was a monoclonal antibody raised against the small subunit of caspase-6 which bound to a 13 kDa band in the active S200 fractions from the purification, 17 and 18 (Figure 2D). This caspase-6 p13 subunit could be co-immunoprecipitated with the EKD-Biotin binding p17 subunit from S200 fractions using a monoclonal anti-Biotin antibody (Figure 2E). Therefore, the caspase-8 cleaving activity contained both the p17 and the p13 bands seen in the Coomassie stained gel of the purified material.

Since the caspase-6 antibody was raised against a sequence that has high homology to other caspases, we sought to confirm that the caspase-8 cleaving activity was indeed caspase-6 by another approach. In order to follow caspase-6 through the biochemical purification using an

alternative detection method, *in vitro* translated, <sup>35</sup>S labelled procaspase-6 was added to the post mitochondrial extract prior to activation and purification. After purification by the modified protocol described in Materials and methods, the activity was labelled with EKD-Biotin and subjected to two dimensional gel analysis. The protein was focused on a 7 cm IEF strip with PI range 3–10 and the strip subjected to SDS–PAGE and blotted on to PVDF. Visualisation by phosphorimaging, revealed two <sup>35</sup>S caspase-6 spots of 17 and 13 kDa, both being between 6.5 and 7 PI units (Figure 3A). This corresponds to the p17 subunit of caspase-6 (amino acids 24–179) which has a predicted PI of 6.82 and the p13 subunit plus linker (amino acids 180–293) which has a predicted PI of 6.90. The blot was probed with Streptavidin-HRP which detected one spot that colocalised with the upper <sup>35</sup>S caspase-6 spot confirming that the catalytic domain of caspase-8 cleaving activity is the p17 subunit of caspase-6 (Figure 3B). The blot was then probed with the anti-caspase-6 p13 antibody which detected one additional spot that colocalised with the lower <sup>35</sup>S caspase-6 spot confirming that the 13 kDa



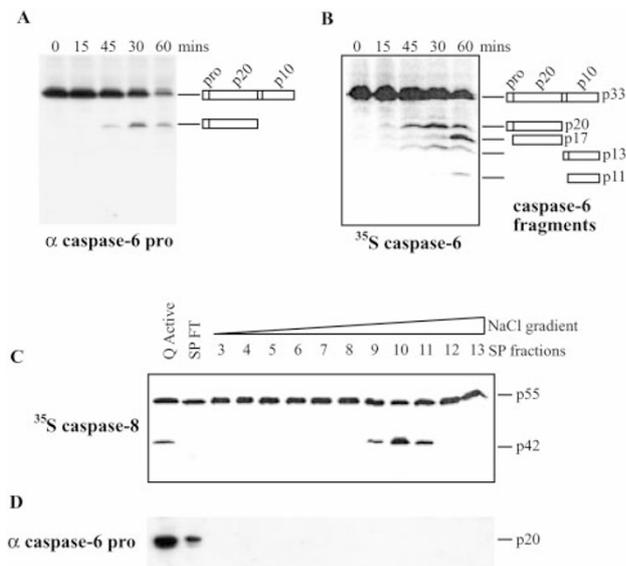
**Figure 3** Caspase-8 cleaving activity is caspase-6. Extracts from 2.5 l of Jurkat T cells were mixed with 160 μl of *in vitro* translated, <sup>35</sup>S caspase-6 prior to activation with 0.1 mM cytochrome *c* and 0.01 mM dATP. The activated material was partially purified before being incubated with 1 μM EKD-Biotin, precipitated and resuspended in 2-dimensional gel electrophoresis loading buffer. The sample was focussed on a 7 cm, pH 3–10 linear gradient isoelectric focussing strip followed by 15% SDS–PAGE and electroblotting onto PVDF. The labelled caspase-6 spots were visualised using the phosphorimager, (A). The same blot was probed with Streptavidin-HRP to visualise EKD-Biotin bound protein, (B) and a mixture of Streptavidin-HRP and monoclonal anti-caspase-6 antibody recognising the p13 subunit with the relevant secondary antibody, (C)

species that the antibody detects in the purified activity is caspase-6 p13 subunit (Figure 3C).

### The prodomain of caspase-6 must be removed to achieve caspase-8 cleaving activity

Procaspase-6 contains a 23 amino acid prodomain. We observed that this prodomain must be removed for caspase-6 to be able to cleave caspase-8.

During the activation process there is a heterogeneous population of caspase-6 species.<sup>17,20</sup> Analysis of the *in vitro* translated caspase-6 cleaved in cytochrome *c* activated extracts revealed that caspase-6 large subunit is present both with (p20) and without (p17) the prodomain (Figure 4B). This is most clearly visualised after 60 min of cytochrome *c* addition where five distinct caspase-6 species are observed; corresponding to the 33 kDa full length caspase, the 20 kDa prodomain plus large subunit, the 17 kDa large subunit alone, the 13 kDa small subunit plus linker and the 11 kDa small subunit alone. In Figure 4A the same gel was immunoblotted using an anti-caspase-6 antibody which recognises the prodomain only. In this blot only two bands are observed which correspond to the 33 kDa full length caspase and the 20 kDa prodomain plus large subunit.



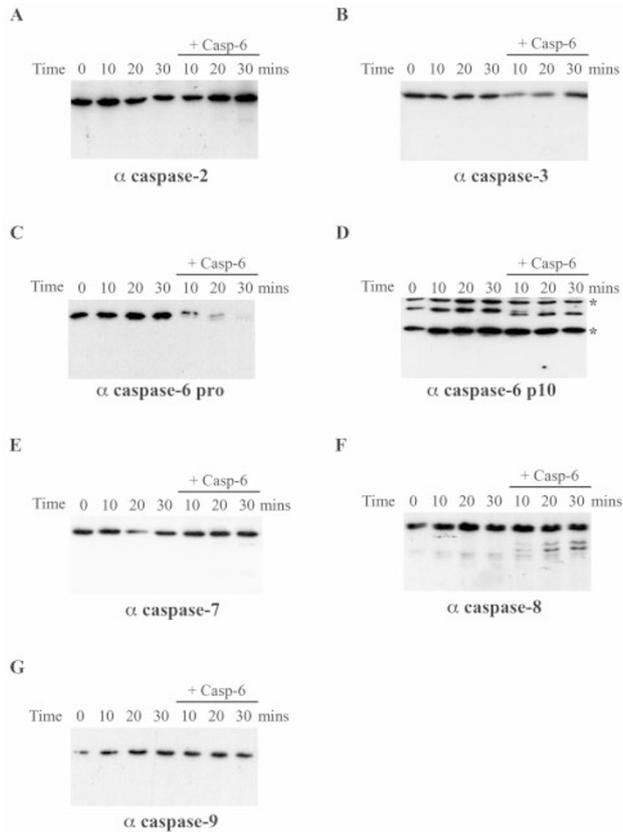
**Figure 4** The pro-domain of caspase-6 must be removed for caspase-8 cleavage. 5  $\mu$ l *in vitro* translated <sup>35</sup>S caspase-6 was mixed with 10  $\mu$ g Jurkat cell extract, 0.1 mM cytochrome *c* and 0.01 mM dATP and incubated at 37°C. 15  $\mu$ l aliquots incubated for the times indicated were analyzed by immunoblotting using a polyclonal anti-caspase-6 prodomain antibody, (A) or phosphorimaging, (B). The caspase-8 cleaving material from Q-Sepharose column was loaded on to the SP-Sepharose column and eluted with a linear gradient of 50 to 300 mM NaCl. In (C) 15  $\mu$ l aliquots of preload (Q Active), SP flow through (SP FT) and fractions collected from the gradient (indicated by the numbers above the blot) were incubated with <sup>35</sup>S caspase-8 for 30 min at 37°C and the resultant fragments were visualised by phosphorimaging. In (D) the same aliquots were immunoblotted using a polyclonal anti-caspase-6 prodomain antibody

During the purification of caspase-6 we observed that the caspase 6 retaining the prodomain (p20) was separated from the caspase-8 cleaving activity (containing the mature caspase-6, p17) by the SP-Sepharose column. Figure 4C and D show the analysis of the SP-Sepharose column fractions; Figure 4C being the analysis of the caspase-8 cleaving activity and Figure 4D being an immunoblot using the anti-caspase-6 prodomain antibody. The preload (Q Active) contains the active material, caspase-6 p17 and caspase-6 p20 since they co-purify on the Q-Sepharose column. However the p20 is found solely in the flow through of the SP-Sepharose column (SPFT) which is inactive in the caspase-8 cleaving assay, whereas the active material which contains Caspase 6 p17 is bound to the column and elutes during the NaCl gradient (fractions 9–11). Since the SP flow through lacks caspase-8 cleaving activity, caspase-6 retaining the prodomain is inactive in this assay. One possible explanation of why the prodomain renders the p20 inactive is that it is bound to an inhibitor. However we found that the caspase-6 p17 and the p20 co-eluted on both Superdex 75 and Superdex 200 gel filtration columns and therefore if the prodomain is binding to an inhibitor it is not a significant size (data not shown).

### Purified caspase-6 activates caspases-6 and -8 in the cytosol leading to cytochrome *c* release from the mitochondria

Assessment of the activity of purified caspase-6 up to this point had been carried out using *in vitro* translated protein as a substrate. We wanted to observe whether the purified caspase-6 could cleave endogenous caspase-8 and subsequently activate Bid dependent cytochrome *c* releasing activity in the cytosol.

Purified caspase-6 from the sequential purification described above was incubated with post-mitochondrial Jurkat extracts at 30°C for 10, 20 and 30 min. Extracts were then immunoblotted for caspase-8 (Figure 5F) as well as caspases-2, -3, -6, -7 and -9 (Figure 5A–E,G). The purified caspase-6 was observed to activate caspase-8 in this system. Caspase-6 was also cleaved by the purified caspase-6. Whereas in cytochrome *c* activated extracts, caspase-6 is cleaved initially between the p20 and p10 (Figure 4A,B) by upstream caspases; mature caspase-6 cleaves full length caspase-6 between the prodomain and p17 only and not between the p13 and p11. This is observed in Figure 5C where immunoblotting with an antibody raised against the prodomain of caspase-6 shows loss of immunoreactive fragments after caspase-6 cleavage because the prodomain alone is too small to be separated on SDS-PAGE; and in Figure 5D where immunoblotting with an antibody raised against the p13 of caspase-6 shows loss of full length caspase-6, appearance of a 30 kDa band corresponding to the size of caspase-6 with the pro-domain removed and no appearance of the p13 or p11. We observed some processing of all caspases assayed in this system by 60 min (data not shown), including caspase-3 as has been reported previously.<sup>21</sup> However, the caspase-6 we purified clearly has a higher specificity for caspases-8 and -6.

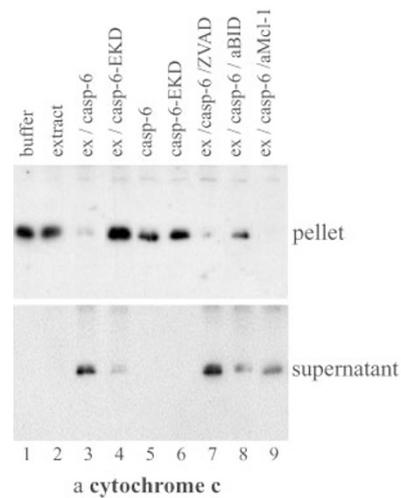


**Figure 5** Purified caspase-6 activates caspases-8 and 6. Post-mitochondrial Jurkat extracts (200  $\mu$ g) were incubated with or without purified caspase-6 (~5 ng), as indicated, in a final volume of 50  $\mu$ l at 30°C for the times indicated. Extracts were immunoblotted using antibodies raised against the caspases indicated in the figure (\* indicates non-specific band)

To assess whether cytochrome *c* releasing activity had been produced in the cytosol, the purified caspase-6 activated extracts were incubated with mitochondria isolated from mouse liver. The mitochondria and the cytosol were then separated by centrifugation and immunoblotted using monoclonal anti-cytochrome *c* antibodies (Figure 6). Neither the caspase-6 alone (lane 5) nor the extract incubated with EKD-inhibited caspase-6 (lane 4) could induce cytochrome *c* release from the mitochondria. Extracts preincubated with the purified caspase-6 induced cytochrome *c* release when added to the mitochondria (lane 3). This cytochrome *c* release could be partially inhibited by neutralising polyclonal anti-Bid antibodies (lane 8) but not by control antibodies (lane 9). We also found that the broad spectrum caspase inhibitor zVAD did not inhibit the cytochrome *c* releasing activity once generated (lane 7). The caspase-6 that we purified is thus sufficient to cleave caspase-8 and initiate Bid dependent cytochrome *c* releasing activity in the cytosol.

### Inhibition of caspase-6 in COS-7 cells results in inhibition of serum starvation induced caspase-8 activation and apoptosis

Using an *in vitro* system based on the soluble cytosolic proteins we had defined that caspase-6 cleaves caspase-8

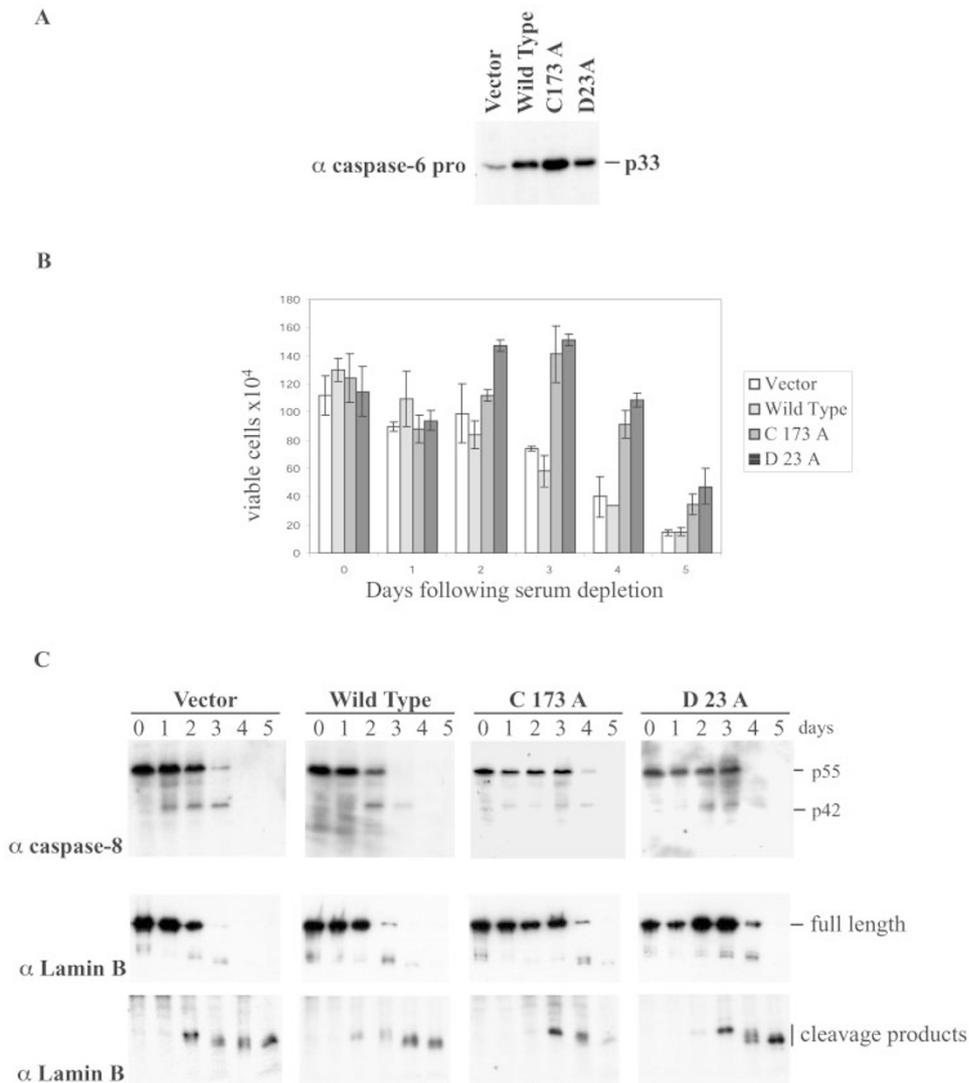


**Figure 6** Purified caspase-6 activates cytochrome *c* releasing factor in lysates. Purified mitochondria (30  $\mu$ g) were incubated for 60 min at 37°C with pre-treated Jurkat extracts (50  $\mu$ g) in a final volume of 25  $\mu$ l. Mitochondria were incubated with buffer (lane 1), untreated extracts (lane 2), extracts pre-incubated with ~5 ng purified caspase-6 at 30°C for 30 min (lane 3), extracts pre-incubated with purified ~5 ng caspase-6 inactivated with EKD-Biotin at 30°C for 30 min (lane 4), with purified caspase-6 (lane 5), with purified caspase-6 inactivated with EKD-Biotin (lane 6). Following preincubation with purified caspase-6 for 30 min extracts were mixed with 50  $\mu$ l zVADfmk (lane 7), 0.5  $\mu$ g polyclonal anti-Bid antibody (lane 8) or 0.5  $\mu$ g polyclonal anti-Mcl-1 antibody (lane 9) prior to incubation with mitochondria. Mitochondria (upper panel) and supernatants (lower panel) were separated by centrifugation, both resuspended in loading buffer in 50  $\mu$ l final volume and 10  $\mu$ l aliquots immunoblotted with monoclonal anti-cytochrome *c* antibodies

and that this cleavage was dependent on removal of the prodomain of caspase-6. We wanted to determine whether in an intact cell, where the complexity of signalling pathways is much greater, caspase-6 activation was necessary for caspase-8 cleavage and apoptosis.

We made two point mutants of caspase-6 both of which we predicted might inhibit caspase-8 activation. In the first, we mutated the critical cysteine 173 residue in the active site to alanine to make a catalytically inactive caspase-6 C173A. In the second, we mutated the aspartate 23 residue to alanine, D23A, which has been shown to prevent cleavage between the prodomain and p17 of caspase-6.<sup>20</sup> Caspase-6 D23A might inhibit caspase-8 activation in cells because it can compete with endogenous caspase-6 for activation by other caspases but will not be able to activate caspase-8. We transfected these constructs in addition to vector control and wild-type caspase-6 into COS-7 cells and drug selected pools of cells. None of the constructs were toxic to the cells (data not shown) and expression of the caspase-6 variants in the pools was confirmed by immunoblot using polyclonal anti-caspase-6 antibody (Figure 7A).

We induced apoptosis in the cell lines by serum deprivation (0.1% FCS). We wanted to use a gentle apoptotic trigger which we hoped might induce slow cytochrome *c* release thereby promoting the putative positive feedback loop of caspase activation based around cytochrome *c* release from the mitochondria. Each day viable cell number was assessed by counting Trypan Blue



**Figure 7** Serum deprivation induced caspase-8 activation and apoptosis is delayed by inhibiting production of mature active caspase-6. **(A)** Pools of COS-7 cells stably expressing pCDNA 3.1+ (V), caspase-6 (WT), caspase-6 C173A (C173A) and caspase-6 D23A (D23A) were lysed in 0.1% NP40 and 10 g of each were immunoblotted with polyclonal anti-caspase-6 antibody.  $0.5 \times 10^6$  of each cell type, as indicated, were serum deprived (0.1% FCS) for 0–5 days as indicated. **(B)** Number of viable cells were assessed by Trypan blue exclusion (error bars indicate S.E.M.  $n=2$ ). **(C)** Cytoplasmic fractions were separated from the rest of the cell pellet using the digitonin method (see Materials and Methods). Cytoplasmic fractions were immunoblotted for caspase-8 (upper panel), rest of cell pellet fractions were immunoblotted for Lamin B (middle and lower panels)

negative cells (Figure 7B). Cytoplasmic extracts were separated from the cell pellet using the digitonin method and used for looking at caspase-8 activation with the remaining cell pellet used for looking at Lamin B cleavage by immunoblot (Figure 7C).

Cells expressing the vector control and wild-type caspase-6 died more rapidly than either cells expressing catalytically inactive caspase-6 (C 173 A) or the non-cleavable prodomain mutant caspase-6 (D 23 A). Caspase-8 activation and Lamin B cleavage were both retarded in the caspase-6 mutant compared to the wild-type and vector control cells. Although the cleavage of these proteins is delayed only by 1 day this delay was observed repeatedly and correlates with the loss of cell viability. Both inhibition of caspase-6 activity and

caspase-6 prodomain removal therefore inhibit caspase-8 activation and this correlates with an inhibition of apoptosis.

## Discussion

We investigated how caspase-8 was being activated in the cytosol following cytochrome *c* release from the mitochondria. By activating Bid, caspase-8 catalyses further cytochrome *c* release and thus has a crucial role in reinforcing caspase activation by promoting an amplification loop. We found that the only soluble cytosolic caspase which could cleave caspase-8 is caspase-6. Furthermore, we found that the short prodomain of caspase-6 had to be removed for processing of caspase-8 to occur.

Previous analysis of cytochrome *c* triggered caspase activation using the Jurkat cell extract system has revealed valuable information concerning caspase-8 activation.<sup>11</sup> Immunodepletion of caspases-9, -3 or -6 from the cytosol inhibited subsequent cytochrome *c* triggered caspase-8 activation. However, in that study, uncleaved inactive caspases were depleted prior to activation by complex feedback loops, so it was left unresolved which of these caspases is directly involved in caspase-8 cleavage or if another, yet unknown factor was responsible. The feedback loops of caspase activation produce caspase processing variants which may have different activities. This phenomenon has been described in detail with respect to caspase-9. Caspase-9 cleaved at Asp 315 and caspase-9 cleaved at Asp 330 have a significant difference in that the latter can bind to XIAP resulting in its inhibition whereas the former cannot.<sup>22</sup> Techniques that deplete or remove caspases prior to activation cannot distinguish which member of a caspase activation feedback loop is directly responsible for substrate cleavage and also cannot distinguish the individual activities of a group of processing variants of the same caspase. We decided that a biochemical approach would identify the caspase directly responsible for caspase-8 activation and which processing variant of this caspase was active.

In this paper we purified caspase-6 as the protease which directly cleaves caspase-8 in cytochrome *c* activated extracts. Closer examination of the purified active caspase-6 revealed that the 23 amino acid prodomain was removed. In the same extracts caspase-6 retaining the prodomain was observed too but was found to be completely inactive in our assays. By expressing caspase-6 D23A in cells, a mutant which cannot have its prodomain removed, we could inhibit serum starvation induced caspase 8-activation. The explanation of why cleaved caspase-6 retaining the prodomain is inactive is not yet resolved. One explanation is that an inhibitor may be binding to the prodomain. We find this unlikely because if only a small fraction of the purified cleaved caspase-6 retaining the prodomain had lost its inhibitor and therefore became active it would then have the capability to cleave off caspase-6 prodomains, so setting up a positive feedback of caspase-6 activation which would result in caspase-8 cleaving activity. However, we never found a trace of activity in fractions containing purified cleaved caspase-6 retaining the prodomain. Once this protein was separated from other caspases it was found to be quite stable. In addition the retention volume of caspase-6 on a range of gel filtration columns did not alter during its activation indicating that caspase-6 was not becoming significantly smaller during activation. We more favour that caspase-6 with the prodomain cannot interact with its substrates because we see a dramatic change in the physical properties of caspase-6 following prodomain removal. Only when the prodomain is removed is caspase-6 retained on the SP-Sephadex column. We also observed that the presence or absence of the linker found between p17 and p13 domains of the zymogen did not have an effect on the activity of the processed caspase (data not shown).

Recent work is beginning to suggest that the effector caspase prodomain, far from being an inconsequential peptide has, in fact, a role in regulating activity. For

example, wild-type caspase-3 has been found to be less effective at killing HeLa cells than caspase-3 with the prodomain removed.<sup>23</sup> Our work shows for the first time that an effector caspase requires its prodomain to be removed in order to cleave a substrate. Caspase-6, along with caspase-3, are the most abundant caspases and we suggest that inhibition of the protease by its own prodomain may be a mechanism which blocks any residual activity of the uncleaved caspase in a healthy cell.<sup>17</sup> The fact that at least two points of cleavage are required for effector caspase activation may also represent a point of regulation of commitment to apoptosis.

FADD, the adaptor protein that recruits caspase-8 to the Fas receptor, has been implicated in many studies also to be involved in caspase-8 activation in the cytosol following cytochrome *c* release. Expression of the death domain of FADD (FADD DN), has been found to inhibit death induced by some triggers which might act independently of the death receptors.<sup>24–26</sup> Expression of FADD DN was found to offer some protection against death induced by microinjection of cytochrome *c*.<sup>27</sup> In addition, overexpression of FADD induced caspase-8 activation probably via an interaction of their death effector domains.<sup>28,29</sup> In our system however we found that FADD does not bind to caspase-8 either before or during activation following cytochrome *c* addition. A transient interaction between FADD and caspase-8 was also not necessary for activation since the purified caspase-8 cleaving activity did not contain FADD. Therefore FADD did not seem to have a role in cytochrome *c* induced caspase-8 activation in this system. However, it may have an indirect influence in intact cells.

At the outset of this work we were open to the possibility of there being more than one activator of caspase-8 because the preferred peptide substrates of other caspases active in this system are homologous to the cleavage sites found within caspase-8.<sup>19</sup> In addition we expected redundancy in the system. We were surprised to find that only caspase-6 could efficiently activate caspase-8. We followed caspases 1 to 10 by immunoblot through the purification but found that only caspase-6 and none of the other caspases were active in cleaving caspase-8. In addition, caspase-8 did not auto activate. Thus a stringent specificity of the caspase substrate interactions was revealed using the biochemical purification. Never the less, it is unlikely that caspase-8 is activated only by one other protease downstream of cytochrome *c* release in intact cells and we may expect to find other activators. Even if this proves to be the case we suggest that caspase-6 is the major activator of caspase-8 in the intact cell since cells expressing catalytically inactive caspase-6 strongly inhibit caspase-8 activation.

## Materials and Methods

### Reagents

Polyclonal antibodies (Pabs) directed against caspase-9 amino acids 1–134 and monoclonal antibodies (Mabs) directed against Lamin B (Ab-1) were obtained from Calbiochem. Pabs directed against caspase-8 p20 were obtained from Santa Cruz. Mabs directed against

caspase-7 (clone B94-1) were obtained from Stressgen. Mabs directed against caspase-3 (clone 5F6.H7) and Pabs directed against caspase-6 amino acids 163–176 were obtained from Oncogene Research Products. Mabs directed against caspase-2 (clone G310-1248), cytochrome *c* (clone 7H8.2C12) and caspase-6 p10 (clone G3-4) were obtained from Pharmingen. Mabs directed against FADD were obtained from Transduction Labs. Pabs directed against caspase-6 amino acids 16–32 were obtained from Upstate Biotechnologies. Mabs directed against Biotin and immobilised on agarose were obtained from Sigma. PABs directed against Bid and Mcl-1 were obtained from R&D Systems. Mabs directed against PARP (clone C2-10) were obtained from Alexis.

z-EKD-Biotin (z-Glu-Lys (Biotinyl) Asp-CH<sub>2</sub>-DMB) was obtained from Peptide Institute Inc. Bovine cytochrome *c* and Brilliant Blue G Colloidal concentrate were obtained from Sigma. dATP and Promix were obtained from APB. ZVADfmk was obtained from Enzyme System Products.

### Cell culture

The human leukemic T cell line, Jurkat E-61, was maintained in RPMI 1640 medium supplemented with 5% fetal calf serum. The COS-7 cell line was maintained in E4 medium supplemented with 10% fetal calf serum.

COS-7 cells were transfected using Gene PORTER (Gene Therapy Systems, Inc.) and stable pools were selected using 0.5 mg/ml Geneticin (Gibco).

### Coupled *in vitro* transcription/translation

<sup>35</sup>S Methionine-labelled caspases were transcribed and translated using the TNT kit according to manufacturers instructions (Promega).

### 2-Dimensional gel electrophoresis

Samples were focussed on 7 cm, pH 3-10 linear iso-electric focussing strips (APB) using a IGPhor (APB) according to manufacturers instructions before being loaded on to a 15% precast Tris-glycine gels (Biorad).

### Isolation of mouse liver mitochondria

The entire procedure was carried out on ice. One mouse liver was homogenised with 15 strokes of a loose pestle in 30 ml HSB (0.3 M sucrose, 10 mM HEPES-KOH pH 7.4). The material was centrifuged twice at 600 *g* for 5 min and the supernatant was centrifuged at 8700 *g* for 10 min to pellet the mitochondrial fraction. The pellet was resuspended in 10 ml HSB/1 mM PMSF and centrifuged at 8700 *g* for 10 min. The pellet was resuspended at 10 mg/ml in MSB (0.4 M Mannitol, 10 mM NaH<sub>2</sub>PO<sub>4</sub>, 20 mM HEPES-KOH pH 7.4, 0.5 mM EGTA, 5 mg/ml BSA) and kept on ice prior to use.

### Rapid preparation of cytosolic fraction

Digitonin method according to Rytoma *et al.*<sup>30</sup>

### Assay for cytochrome *c* release from mitochondria

Thirty µg mitochondria were incubated with 10 µl samples at 37°C for 60 min in a final volume of 25 µl in MRB (250 mM Mannitol, 100 mM sucrose, 20 mM HEPES-KOH pH 7.4, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF). After

centrifugation at 9000 *g* for 10 min at 4°C, the pellet and supernatant were resuspended in 30 µl and 10 µl, respectively, of SDS–PAGE loading buffer.

### Generation of caspase-6 mutants

Substitution of residues in pcDNA3-Mch2 $\alpha$  was achieved using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) with the oligo 5'-GATATTTATCATCCAGGCAGCTCGGGGAAACCAGC-3' for the substitution of Asp-23 residue with Ala and the oligo 5'-GGAAGAAA-CATGACAGAAAACAGCCGCTTCTATAAAAGAGAAATG-3' for the substitution of Cys-263 with Ala. The resulting PCR products were introduced into pcDNA3.1+ as *EcoRI*–*XhoI* fragments. The sequences of the above constructs was verified by DNA sequencing using the ABI Prism Sequencing system and the dRhodamine Terminator Cycle sequencing ready reaction kit (Perkin-Elmer).

### Assay for caspase-8 activating protease

Fifty µl fractions were incubated with 0.1 µl *in vitro* translated, <sup>35</sup>S Methionine labelled caspase-8 for 30 min at 37°C. Fractions were then subjected to SDS–PAGE followed by analysis using a Storm Phosphorimager (Molecular Dynamics).

### Purification of caspase-8 cleaving activity

All purification steps were carried out at 4°C. All the chromatography steps were carried out using an AKTA Fast Protein Liquid Chromatography Station (Amersham Pharmacia Biotech Inc.).

*Step 1: Preparation of post-mitochondrial extracts* Fifty litres of Jurkat T lymphoblastoid cells were wash once in cold phosphate buffered saline and once in cold SCEB (50 mM HEPES pH 7.4, 0.3 M Sucrose, 5 mM MgCl<sub>2</sub>, 0.1 mM EGTA, 1 mM PMSF, 5 mM DTT). Cells were lysed in a ratio of 4 vol of SCEB buffer to 1 vol cell pellet using 20 strokes of a dounce homogeniser (Glas-Col). The homogenates were centrifuged at 18 000 *g*. The resultant supernatant was incubated with 0.1 mM bovine cytochrome *c* (Sigma) and 0.01 mM dATP (Amersham) for 90 min at 30°C. The extracts were centrifuged again as before.

*Step 2: Q-sepharose chromatography* The extracts from step 1 (2 g protein) were loaded in two halves on to a Q-Sepharose column (50 ml bed vol) equilibrated with 10 mM HEPES, 5 mM NaCl and 5 mM DTT (buffer A). After loading the column was washed with five column volumes of buffer A and bound proteins were eluted with a five column volume linear gradient of buffer A to buffer A containing 0.3 M NaCl. Fifteen ml fractions were collected and assayed for caspase-8 cleaving activity.

*Step 3: SP-sepharose chromatography* Active fractions (0.1 g protein) were loaded onto a SP-Sepharose column (20 ml bed vol) equilibrated in buffer A (now with 50 mM NaCl). After loading the column was washed with 10 column volumes of buffer A and the bound proteins were eluted with a 10 column volume linear gradient of buffer A to buffer A containing 0.3 M NaCl. Ten ml fractions were collected and assayed for caspase-8 cleaving activity.

*Step 4: Octyl-sepharose chromatography* Ammonium sulphate was added directly to active fractions from step 2 (10 mg protein) to 1 M and the solution was centrifuged for 30 min at 13K r.p.m. and loaded on to a Octyl-Sepharose column (1 ml bed vol.) equilibrated in buffer A with 1 M ammonium sulphate. After loading the column was washed in

10 column volumes of buffer A containing 0.8 M ammonium sulphate. Bound protein was step eluted in buffer A containing 0.5 M ammonium sulphate.

At this stage the activity was, when appropriate, incubated with 1  $\mu$ M EKD-Biotin at 37°C for 10 min.

**Step 5: Ammonium sulphate precipitation** Ammonium sulphate was added directly to protein from step elution (2.5 mg) up to 50% saturation with shaking at 4°C. Protein was centrifuged for 30 min at 13K r.p.m. Protein pellet was resuspended in 1ml buffer A and assayed for caspase-8 cleaving activity or bound biotin.

**Step 6: Superdex 75 or Superdex 200 gel filtration chromatography** Active or biotinylated protein from step 5 was loaded in two halves (500  $\mu$ l) on to a Superdex 75 or Superdex 200 column (23 ml bed vol) equilibrated in buffer A with 50 mM NaCl. The column was eluted with the same buffer with 0.5 ml fractions being collected and assayed for caspase-8 cleaving activity or bound biotin.

### Purification of caspase-8 cleaving activity from extracts spiked with <sup>35</sup>S caspase-6

The purification was carried out as above with the following modifications. Extracts from 2.5l Jurkat cells were mixed with 160  $\mu$ l *in vitro* translated <sup>35</sup>S caspase-6 which had been desalted on a microspin column (Biorad). Purification was carried out up to step 5 including incubation with EKD-Biotin (step 4). Following Ammonium Sulphate precipitation, the pellet was resuspended in 2 ml water, acetone precipitated and resuspended in 750  $\mu$ l 2-D gel loading buffer.

### Immunoprecipitation

Six hundred  $\mu$ l EKD-Biotin bound material from step 6 of the purification was incubated with either 25  $\mu$ l monoclonal anti-Biotin antibody immobilised on agarose or 25  $\mu$ l Protein G Sepharose and 2.5  $\mu$ g isotype matched control monoclonal antibody in IPB (10 mM HEPES pH 7.4, 0.1% NP40 1% BSA, 1 mM DTT) rotating for 1 h at room temperature. Beads were pelleted, washed three times with IPB and protein was eluted with 50  $\mu$ l SDS – PAGE loading buffer. Twenty  $\mu$ l aliquots of pre-load and unbound protein was resuspended in loading buffer and eluted protein were analyzed on 15% SDS – PAGE followed by immunoblot.

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