RESEARCH COMMUNICATION

Activation of pro-caspase-7 by serine proteases includes a non-canonical specificity

Qiao ZHOU and Guy S. SALVESEN*

Program in Aging and Cell Death Research, The Burnham Institute, San Diego, CA 92037, U.S.A.

As a model to investigate the mechanism of caspase activation we have analysed the processing of pro-caspase-7 by serine proteases with varied specificities. The caspase-7 zymogen was rapidly activated by granzyme B and more slowly by subtilisin and cathepsin G, generating active enzymes with similar kinetic properties. Significantly, cathepsin G activated the zymogen by cleaving at a Gln–Ala bond, indicating that the canonical cleavage specificity at aspartic acid is not required for activation.

INTRODUCTION

Programmed cell death can be initiated by a variety of distinct signals such as specific receptor ligation, genotoxic damage, anti-cancer drugs and delivery of specific proteases into target cells [1,2]. Although these stimuli and the initial events that result from them are distinct, the morphology that ensues is usually identical and is termed apoptosis. Among the key mediators that execute the apoptotic programme are members of the caspase family of cysteine proteases [3]. Commitment to this execution phase of apoptosis is probably achieved by sequential activation of caspase zymogens [4,5]. How pro-caspase activation is achieved *in vivo* is not clear, but it has been demonstrated *in vitro* that activation requires limited cleavage within a conserved segment [6]. This segment, known as the linker segment, separates the regions of primary sequence that will become the large and small subunits of the catalytic unit of an active caspase [7–9].

In all cases that have been reported, whether spontaneous or driven by an active protease, formation of a catalytic unit results from cleavage at aspartic acid residues. Significantly, this explains the ability of granzyme B (GraB), a serine protease, to activate caspases (reviewed in [10]), since this protease shares the primary specificity of caspases [11]. Indeed, GraB probably exerts its physiological role following delivery from cytotoxic cells to target cells by triggering the apoptotic programme via activation of caspases.

A paradigm that emerges from these studies is that caspase zymogens need to be activated by proteolytic cleavage(s) after aspartic acid residues to generate the two-chain active enzyme. However, there is evidence that proteases with different substrate specificities can also cause cell death. For example, granzymes A and K are cytotoxic, though less efficient than GraB [12]. Trypsin, chymotrypsin, and proteinase K can all induce cell death with apoptotic morphology when artificially introduced into tumour cells [13]. If the caspase cascade is indeed the central part of the cell death pathway(s), these proteases may operate in one of two ways. They may start the cascade by cleaving and thus turning on an unidentified master-switch, a sensor of proteolysis, to activate the endogenous cascade. Alternatively, we hypothesize that they may directly cleave and activate caspase zymogens, meaning that the activation process would be less rigorous than had been expected. To test this hypothesis, we have investigated the processing of caspase-7 by several different serine proteases covering a cross-section of the specificity repertoire of proteolysis.

MATERIALS AND METHODS

Proteins and reagents

Granzymes A, B and K were gifts from Dr. Chris Froelich (Northwestern University Medical School, Evanston, IL, U.S.A.). Trypsin, chymotrypsin and subtilisin *Carlsberg* (SCARL) were purchased from Sigma (St. Louis, MO, U.S.A.). SCARL was further purified by ion-exchange chromatography [14]. Human neutrophil elastase and cathepsin G (CatG) were purified from neutrophils [15]. The GraB specific inhibitor, anti-GraB, was prepared and purified as described previously [16]. Eglin C was the kindly given by Dr. Hans-Peter Schnebli (Ciba–Geigy, Basel, Switzerland) and α_1-proteinase inhibitor was purified as described previously [17]. All other reagents were purchased from Sigma unless otherwise specified. The active-site concentrations of CatG and SCARL were determined by titration with standardized eglin C [18], and the active concentration of GraB was determined by titration with anti-GraB [16]. The chromogenic caspase substrate, *N*-acetyl-Asp-Glu-Val-Asp *p*-nitroanilide (Ac-DEVD-pNA), was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA, U.S.A.). The GraB substrate, succinyl-Ala-Ala-Pro-Asp-pNA, and the CatG substrate, succinyl-Ala-Ala-Pro-Phe-pNA, were from Sigma. The granzyme A substrate, tosyl-Gly-Pro-Arg-pNA, was purchased from Bachem U.S.A. (Torrance, CA, U.S.A.). All substrates were dissolved in DMSO as a 20 mM stock solution and stored at -20 °C.

Recombinant pro-caspase-7

The full-length caspase-7 cDNA was cloned into expression vector pET23b(+) (Novagen, Madison, WI, U.S.A.) with a C-terminal His_6 purification tag and expressed in *Escherichia coli* strain BL21(DE3)plysS as described previously [4]. Expression of the zymogen was optimal when induced with 0.4 mM isopropyl β-D-thiogalactoside at $A_{600} = 0.5$ for less than 3 h (up to $A_{600} = 1.5$) at 30 °C. The zymogen was purified by using immobilized

Abbreviations used: CatG, cathepsin G; GraB, granzyme B; SCARL, subtilisin *Carlsberg*; Ac-DEVD-pNA, *N*-acetyl-Asp-Glu-Val-Asp *p*-nitroanilide; pNA, *p*-nitroanilide.

* To whom correspondence should be addressed.

$NiSO_4$-charged chelating agarose (Pharmacia, Piscataway, NJ, U.S.A.). The concentration of caspase-7 was determined spectrophotometrically by using calculated absorption coefficients from the known amino acid compositions [19]. The yield of recombinant pro-caspase-7 was 1.8 mg per litre of culture.

Assay of pro-caspase-7 activation

Zymogen activation was followed by recording the release of *p*-nitroanilide (pNA) generated by cleavage of Ac-DEVD-pNA by active caspase-7 at 405 nm using a SpectraMAX 340 spectrophotometric plate reader (Molecular Devices, Sunnyvale, CA, U.S.A.) operating in the kinetic mode at 37 °C. The standard reaction mixture included the activators (2.0×10^{-11} M GraB, 2.6×10^{-9} M SCARL or 1.2×10^{-8} M CatG), 200 μM Ac-DEVD-pNA, various concentrations (2.0×10^{-9} M–3.5×10^{-7} M) of pro-caspase-7 and assay buffer [50 mM Hepes, 100 mM NaCl, 0.1 % (w/v) CHAPS, 1 mM dithiothreitol and 10 % (w/v) sucrose, pH 7.4] in a final volume of 100 μl. The assays were started by adding premixed activator and substrate to temperature-equilibrated pro-caspase-7 in buffer. Data were recorded every 2 s for various periods of time as appropriate for each assay.

Data analysis

The scheme for pro-caspases-7 activation can be described by:

$$A + Z \underset{}{\overset{K_{mZ}}{\rightleftharpoons}} AZ \xrightarrow{k_{catZ}} A + E$$

where A is the activating enzyme, Z is the caspase-7 zymogen, E is activated caspase-7, and k_{catZ} and K_{mZ} are the catalytic and Michaelis constants, respectively, for the activation process. The activated caspase is determined by cleavage of its synthetic substrate as a function of time. The rate of pNA production is exponential in the early phase, since it is a product of two linear rates (caspase activation and Ac-DEVD-pNA cleavage), and relaxes to a non-exponential function when the depletion of pro-caspase-7 becomes significant. When $Z \ll MK_{mZ}$ the first order rate constant k for the activation of pro-caspase-7 is determined according to a modification of the equation used to analyse the activation of plasminogen by streptokinase [20]:

$$A_{405} = \epsilon \cdot (M \cdot E_0 \cdot t + M \cdot Z \cdot t - M \cdot Z \cdot (1 - e^{-kt})/k) + A_0 \quad (1)$$

which is the integrated form of the Michaelis–Menten equation that allows determination of the rate constant by measuring cleavage of the reporter substrate as a function of time t. Here the constant M is $k_{cat}[S]/(K_m + [S])$ for the caspase-7 cleavage of the reporter substrate Ac-DEVD-pNA (with minimal substrate consumption), E_0 is the initial concentration of active caspase-7 at $t = 0$, A_{405} is the absorbance of pNA at 405 nm at time t, A_0 is the background absorbance at 405 nm, and ϵ is the absorbance coefficient of pNA, pre-determined for the standard assay condition. The parameter k ($= V_{max}/K_{mZ}$) which describes the linear activation of the caspase zymogen, is obtained by least-squares fit to data of time-dependent pNA generation, with the computer program DeltaGraph (Deltapoint, Monterey, CA, U.S.A.), using the Marquardt–Levenberg algorithm.

N-terminal sequencing of cleavage products

Activators (2.0×10^{-10} M GraB, 1.3×10^{-7} M SCARL or 1.2×10^{-6} M CatG) were incubated with 5×10^{-6} M pro-caspase-7 in assay buffer at 37 °C for 15 min. Portions were removed to determine the extent of activation and the reaction was stopped by adding boiling SDS sample-loading buffer. In the case of SCARL the reaction was stopped by adding PMSF to a final concentration of 1 mM and incubating for 20 min to avoid extensive degradation of the caspase by SCARL in the presence of SDS. Proteins were resolved by 5–15 % linear gradient SDS/PAGE, electroblotted on to an Immobilon-P transfer membrane (Millipore, Bedford, MA, U.S.A.) and briefly stained with Coomassie Brilliant Blue R250 [21]. After destaining and washing extensively with water, appropriate bands were cut out and subjected to N-terminal sequencing on a 476A protein sequencer (Applied Biosystems, Foster City, CA, U.S.A.).

RESULTS AND DISCUSSION

Characterization of the caspase-7 zymogen

The isolated zymogen expressed in *E. coli* was judged to be at least 95 % pure by SDS/PAGE. A small amount of processed two-chain forms was present in the preparations (less than 2 %), which correlated with the background activity in the absence of zymogen activators. If the cells were harvested after an 18 h induction period the yield of protein was higher, but was obtained in the completely processed, active, two-chain form as described previously [4]. The zymogen lacked the N-terminal 23 amino acids of the full length precursor encoded in the expression construct. Presumably this segment is removed by an *E. coli* protease or by the small amount of active caspase-7 in the zymogen preparation. Processing at this site is not required for the generation of catalytic activity [22], and it is generally assumed that the essential activation event is cleavage between the large and small subunits at Asp^{297} (caspase-1 numbering), which is conserved in most caspases.

Activation of pro-caspase-7 by serine proteases

None of the serine proteases tested showed any detectable activity with Ac-DEVD-pNA, so all increases in substrate hydrolysis above background are due to activation of pro-caspase-7. A survey of several proteases revealed that GraB, SCARL and CatG all activated the zymogen (Figure 1). Trypsin activity was minimal and no activation was observed for chymotrypsin, neutrophil elastase or granzyme K, and they were not studied in more detail.

At high concentrations, granzyme A showed an appreciable ability to activate the caspase-7 zymogen. However, this activity

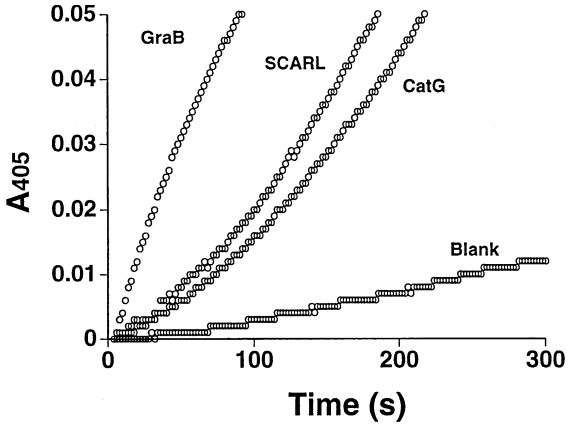

Figure 1 Activation of pro-caspase-7

Portions of 1 μl of each of the indicated proteases were added to the assay buffer containing 10^{-7} M pro-caspase-7 and 0.1 mM Ac-DEVD-pNA in a total volume of 100 μl. Activation of the caspase zymogen is shown by an increase over the background activity of the blank sample.

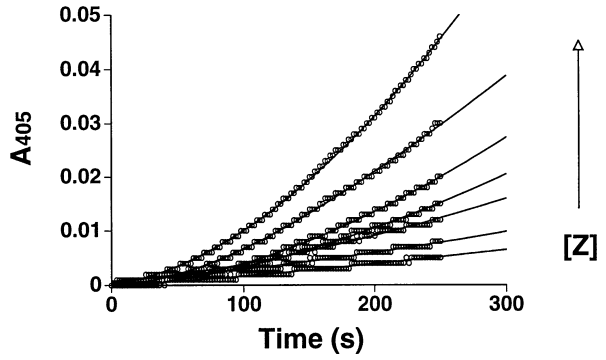

Figure 2 Progress curve analysis of pro-caspase-7 activation by GraB

A 1 μl portion of GraB (2×10^{-11} M final concentration) was added to 99 μl of assay buffer, pre-warmed to 37 °C, containing 0.1 mM Ac-DEVD-pNA, and pro-caspase-7 ([Z]) ranging from 2.5×10^{-9} M to 3.5×10^{-8} M. The change in absorbance at 405 nm was monitored continuously over the time period. Data points are given by the circles, and the curves are from data fitting according to eqn. (1).

was apparently caused by the presence of a tiny amount of GraB, tentatively estimated to be less than 0.1 %, since it was prevented by anti-GraB, a specific inhibitor of GraB [16]. Similar experiments were performed to check the CatG preparation for GraB contamination. In this case, anti-GraB had no effect on the activity, but activity was completely abolished by α_1-proteinase inhibitor, an excellent CatG inactivator [23]. Thus the observed activation by the CatG preparation is confirmed to be due to CatG.

Rates of pNA production were exponential in the initial phases, as expected for a simple zymogen activation. There was a possibility that the serine proteases were not activating the zymogen directly, but rather generating a small amount of active caspase which could then activate its own precursor. However, caspase-7 which had been maximally activated by GraB, CatG or SCARL was not able to activate the caspase-7 zymogen. Moreover, we observed no activity above background when 10^{-6} M pro-caspase-7 was incubated in the absence of an activator for up to 60 min or when a small amount of active caspase-7 was added. Thus we conclude that caspase-7 does not activate its own zymogen under the conditions of this experiment, but the zymogen is rapidly activated by three serine proteases. This is interesting since it has been shown that caspase-1 can activate its own precursor [24]. It is not clear how important this presumed feedback mechanism of caspase processing is *in vivo*, but at least in the case of caspase-7, it is probably very slow.

The activation of pro-caspase-7 by the three serine proteases was observed by using a constant concentration of the activator with various concentrations of pro-caspase-7, and monitoring caspase-7 activity via cleavage of Ac-DEVD-pNA. An example, in this case using GraB as an activator, is shown in Figure 2. The best estimates of the first-order rate constant k from curve fitting are listed in Table 1. The second-order rate constant for the activation was calculated by dividing k by the activator concentrations and was expressed as k/[Activator] in Table 1. This is equivalent to k_{catZ}/K_{mZ} for the activation of pro-caspase-7, assuming standard Michaelis–Menten kinetics.

Characterization of activated caspase-7

Pro-caspase-7 at 5×10^{-8} M was incubated with 4×10^{-8} M GraB, 2×10^{-7} M SCARL or 2×10^{-6} M CatG (designated as caspase7_{GraB}, caspase7_{SCARL} and caspase7_{CatG} respectively) for 5–10 min to achieve maximal activation and then assayed using Ac-DEVD-pNA to compare their enzymic properties. Observed

Table 1 Kinetics of the activation of pro-caspase-7

The following parameters describe the rate of activation of the caspase-7 zymogen by each of the serine proteases, determined according to eqn (1).

	[Activator] (M)	k (s^{-1})*	k/[Activator] ($M^{-1} \cdot s^{-1}$)	Relative k/[Activator]
GraB	2.0×10^{-11}	$(1.71 \pm 0.023) \times 10^{-3}$	8.6×10^{7}	269
SCARL	2.6×10^{-9}	$(1.93 \pm 0.63) \times 10^{-3}$	7.4×10^{5}	2.3
CatG	1.2×10^{-8}	$(3.85 \pm 0.77) \times 10^{-3}$	3.2×10^{5}	1

* Values are the means $\pm$ S.D. obtained from seven progress curves at various caspase-7 zymogen concentrations for each activator.

V_{max} and K_m values were listed in Table 2. The apparent catalytic constant, $k_{cat(app)}$, was calculated by assuming all zymogens (5×10^{-8} M) were converted into active forms. As shown in Table 2, caspase-7 activated by GraB, SCARL or CatG had very similar activities toward the synthetic substrate. The lower value of $k_{cat(app)}$ for SCARL is attributed to a lower concentration of caspase7_{SCARL}, since we observed that SCARL degraded caspase-7 by non-specific cleavages at the concentration required to fully activate the zymogen (see Figure 3, lower panel).

Specificity of the activation

All three serine proteases converted the single-chain caspase-7 zymogen to two-chain forms (Figure 3). All activators cleaved at the linker region between the large and small subunits, in correlation with the observed zymogen activation, and SCARL and CatG also cleaved near the N-terminus (Lys^{49} and Thr^{51} respectively). Cleavage at these latter sites did not affect activity, but cleavage at additional sites by SCARL (Met^{84} and Tyr^{223}) correlated with a decrease in activity consistent with proteolytic destruction of the activated caspase, whereas these cleavages were not observed at the SCARL concentrations used for analysis of the activation rate. Since activation by GraB occurs by a single cleavage, we conclude that the N-terminal sites recognized by SCARL and CatG are secondary cleavages, and that activation occurs by cleavage within the inter-chain linker segment. Significantly, activation by CatG resulted from cleavage at Gln^{196} in this segment.

It is apparent that GraB is the best activator of pro-caspase-7 among the serine proteases studied. The appearance of the unusual specificity for aspartic acid seems to be an evolutionary advantage achieved by GraB for the activation of caspases. GraB utilizes the same site that is cleaved during the processing of caspase-7 observed in material expressed in *E. coli* [4]. We suspect that this site is highly susceptible to cleavage, since it is so rapidly cleaved by GraB and because it is utilized by SCARL. Although little data are available on the ability of SCARL to bind aspartic acid in the primary specificity pocket [25], data on the closely related subtilisin BPN′ reveal that aspartic acid is among the least favoured substituents [26]. Therefore for this site

Table 2 Kinetic properties of caspase-7 produced by different activators

The following parameters describe the respective catalytic properties, with Ac-DEVD-pNA as the substrate, of maximally activated caspase-7 produced by each of the serine proteases.

	V_{max} ($M \cdot s^{-1}$)	K_m (M)	$k_{cat(app)}$ (s^{-1})	$k_{cat(app)}/K_m$ ($M^{-1} \cdot s^{-1}$)
Caspase-7_{GraB}	3.74×10^{-7}	5.5×10^{-5}	7.5	1.4×10^{5}
Caspase-7_{SCARL}	2.4×10^{-7}	6.5×10^{-5}	4.8	7.4×10^{4}
Caspase-7_{CatG}	3.6×10^{-7}	7.9×10^{-5}	7.2	9.1×10^{4}

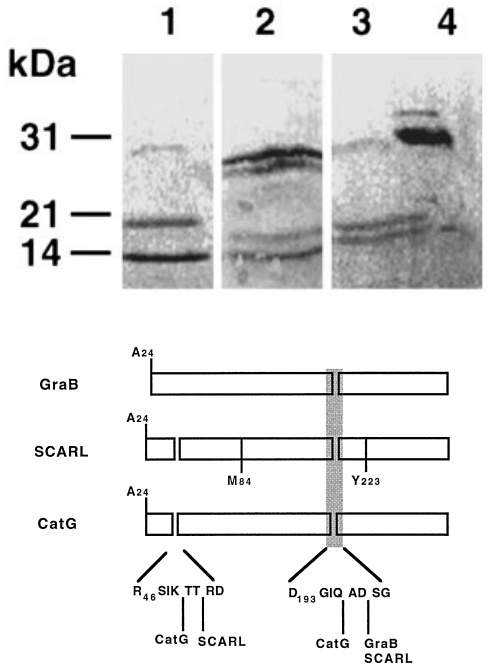

Figure 3 Activation of pro-caspase-7 results from specific limited proteolysis

Upper panel; samples of pro-caspase-7 (lane 4) were treated with GraB (lane 1), SCARL (lane 2) or CatG (lane 3) as described in the Materials and methods section. The products were resolved by SDS/PAGE. The panel is a composite of three gels aligned to demonstrate the relative size of the activation products (kDa). Lower panel; resolved bands were excised and submitted to Edman degradation to determine the cleavage sites that resulted in their generation, and these are depicted for each activator. The location of cleavage sites is shown by vertical bars, and the inter-domain connector region is shadowed. SCARL-mediated cleavages at positions 84 and 223 were observed only at enzyme concentrations higher than those used for the gel.

to be cleaved at all by SCARL requires a special combination of interactions with other subsites, or an unusual structure in the substrate. Perhaps the most surprising result of our study was the observation that CatG activates pro-caspase-7 by cleaving two residues upstream of the conserved Asp, at Gln^{196}. Recognition of glutamine by CatG is also extremely unusual, since this protease has a preference for aromatic side-chains and also lysine in its primary specificity pocket [27,28]. Taken together these observations suggest that the linker segment between the large and small subunits of pro-caspase-7 is an unusually susceptible inter-domain connector designed to be utilized for rapid proteolysis.

Physiological consequences

The rate of activation of pro-caspase-7 by GraB is one of the fastest zymogen activations yet recorded. Compare it with, for example, the following two physiologically important zymogen activations: plasminogen by tissue plasminogen activator in the presence of fibrin cofactor where $k_{cat}/K_m = 1.2 \times 10^7\ M^{-1} \cdot s^{-1}$ [29], and the activation of coagulation factor X by the full Xase activator complex where $k_{cat}/K_m = 3.7 \times 10^7\ M^{-1} \cdot s^{-1}$ [30]. Consequently one would expect rapid activation of pro-caspase-7 *in vivo* following delivery of this protease from cytotoxic cells to targets. This alone, however, would not account for the characteristic apoptosis caused by delivery of GraB, since caspase-7 by itself is not sufficient for full apoptotic morphology [4]. Other caspases are required and thus the role of GraB must include activation of multiple caspase zymogens.

The intriguing, and sometimes perplexing, finding that intracellular, seemingly non-specific proteolysis can lead to apoptosis [13] can now be readily explained within the framework of our findings. We do not expect that SCARL would be involved in apoptosis, nor CatG, since this enzyme has a major role in neutrophil phagocytosis. However, activation of pro-caspase-7 by SCARL and CatG provides the basis for a testable model wherein non-aspartic-acid selective proteases such as those from lysosomes or viruses could engage the apoptotic apparatus under pathological conditions.

We thank Scott Snipas and Annamarie Price for technical assistance, and Henning Stennicke and Edwin Madison for helpful discussions.

REFERENCES

1 Raff, M. C. (1992) Nature (London) **356**, 397–400
2 Thompson, C. B. (1995) Science **267**, 1456–1462
3 Alnemri, E. S., Livingston, D. J., Nicholson, D. W., Salvesen, G., Thornberry, N. A., Wong, W. W. and Yuan, J. (1996) Cell **87**, 171
4 Orth, K., O'Rourke, K., Salvesen, G. S. and Dixit, V. M. (1996) J. Biol. Chem. **274**, 20977–20980
5 Enari, M., Talanian, R. V., Wong, W. W. and Nagata, S. (1996) Nature (London) **380**, 723–726
6 Thornberry, N. A. and Mollineaux, S. M. (1995) Protein Sci. **4**, 3–12
7 Walker, N. P. C., Talanian, R. V., Brady, K. D., Dang, L. C., Bump, N. J., Ferenz, C. R., Franklin, S., Ghayur, T., Hackett, M. C., Hammill, L. D. et al. (1994) Cell **78**, 343–352
8 Wilson, K. P., Black, J. A., Thomson, J. A., Kim, E. E., Griffith, J. P., Navia, M. A., Murcko, M. A., Chambers, S. P., Aldape, R. A., Raybuck, S. A. and Livingston, D. J. (1994) Nature (London) **370**, 270–275
9 Rotunda, J., Nicholson, D. W., Fazil, K. M., Gallant, M., Gareau, Y., Labelle, M., Peterson, E. P., Rasper, D. M., Tuel, R., Vaillancourt, J. P., Thornberry, N. A. and Becher, J. W. (1996) Nat. Struct. Biol. **3**, 619–625
10 Zhivotovsky, B., Burgess, D. H., Vanags, D. M. and Orrenius, S. (1997) Biochem. Biophys. Res. Commun. **230**, 481–488
11 Odake, S., Kam, C. M., Narasimhan, L., Poe, M., Blake, J. T., Krahenbuhl, O., Tschopp, J. and Powers, J. C. (1991) Biochemistry **30**, 2217–2227
12 Shi, L., Kam, C. M., Powers, J. C., Aebersold, R. and Greenberg, A. H. (1992) J. Exp. Med. **176**, 1521–1529
13 Williams, M. S. and Henkart, P. A. (1995) J. Immunol. **153**, 4247–4255
14 Komiyama, T., Gron, H., Pemberton, P. A. and Salvesen, G. S. (1996) Protein Sci. **5**, 874–882
15 Baugh, R. and Travis, J. (1976) Biochemistry **15**, 836–841
16 Quan, L. T., Tewari, M., O'Rourke, K., Dixit, V., Snipas, S. J., Poirier, G. G., Ray, C., Pickup, D. J. and Salvesen, G. (1996) Proc. Natl. Acad. Sci. U.S.A. **93**, 1972–1976
17 Pannell, R. D., Johnson, D. and Travis, J. (1974) Biochemistry **13**, 5439–5445
18 Salvesen, G. and Nagase, H. (1989) in Proteolytic Enzymes: A Practical Approach (Beynon, R. J. and Bond, J. S., eds.), pp. 83–104, IRL Press, Oxford
19 Edelhoch, H. (1967) Biochemistry **6**, 1948–1954
20 Chibber, B. A. K., Radek, J. T., Morris, J. P. and Castellino, F. J. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 1237–1241
21 Matsudaira, P. (1987) J. Biol. Chem. **262**, 10035–10038
22 Gu, Y., Sarnecki, C., Fleming, M. A., Lippke, J. A., Bleackley, R. C. and Su, M. S.-S. (1996) J. Biol. Chem. **271**, 10816–10820
23 Beatty, K., Bieth, J. and Travis, J. (1980) J. Biol. Chem. **255**, 3931–3934
24 Thornberry, N. A., Bull, H. G., Calaycay, J. R., Chapman, K. T., Howard, A. D., Kostura, M. J., Miller, D. K., Molineaux, S. M., Weidner, J. R., Aunins, J. et al. (1992) Nature (London) **356**, 768–774
25 Perona, J. J. and Craik, C. S. (1995) Protein Sci. **4**, 337–360
26 Grøn, H., Meldal, M. and Breddam, K. (1992) Biochemistry **31**, 6011–6018
27 Nakajima, K., Powers, J. C., Ashe, B. M. and Zimmerman, M. (1979) J. Biol. Chem. **254**, 4027–4032
28 Powers, J. C., Kam, C.-M., Narasimhan, L., Oleksyszyn, J., Hernandez, M. and Ueda, T. (1989) J. Cell. Biochem. **39**, 33–46
29 Madison, E. L., Coombs, G. S. and Corey, D. R. (1995) J. Biol. Chem. **270**, 7558–7562
30 Krishnaswamy, S., Field, K. A., Edgington, T. S., Morrissey, J. H. and Mann, K. G. (1992) J. Biol. Chem. **267**, 26110–26120

Received 10 March 1997/2 April 1997; accepted 11 April 1997