THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 42, Issue of October 16, pp. 27084–27090, 1998

Printed in U.S.A.

Pro-caspase-3 Is a Major Physiologic Target of Caspase-8*

(Received for publication, July 6, 1998, and in revised form, August 8, 1998)

Henning R. Stennicke‡, Juliane M. Jürgensmeier§, Hwain Shin, Quinn Deveraux, Beni B. Wolf¶, Xiaohe Yang‖, Qiao Zhou, H. Michael Ellerby, Lisa M. Ellerby, Dale Bredesen, Douglas R. Green¶, John C. Reed, Christopher J. Froelich‖, and Guy S. Salvesen**

From The Program for Apoptosis & Cell Death Research, The Burnham Institute, La Jolla, California 92037, the ‖Department of Medicine, Evanston Hospital, Northwestern University, Evanston, Illinois 60201, and the ¶La Jolla Institute for Allergy and Immunology, San Diego, California 92110

The apoptotic signal triggered by ligation of members of the death receptor family is promoted by sequential activation of caspase zymogens. We show here that in a purified system, the initiator caspases-8 and -10 directly process the executioner pro-caspase-3 with activation rates (k_{cat}/K_m) of 8.7×10^5 and 2.8×10^5 M^{-1} s^{-1}, respectively. These rates are of sufficient magnitude to indicate direct processing *in vivo*. Differentially processed forms of caspase-3 that accumulate during its activation have similar rates of activation, activities, and specificities. The pattern and rate of caspase-8 induced activation of pro-caspase-3 in cytosolic extracts was the same as in a purified system. Moreover, immunodepletion of a putative intermediary in the pathway to activation, pro-caspase-9, was without consequence. Taken together these data demonstrate that the initiator caspase-8 can directly activate pro-caspase-3 without the requirement for an accelerator. The *in vitro* data thus help to deconvolute previous *in vivo* transfection studies which have debated the role of a direct *versus* indirect transmission of the apoptotic signal generated by ligation of death receptors.

Regulation of apoptosis is vital to the development and long term survival of metazoan animals. Apoptosis is required to maintain the balance between cell proliferation and cell death and, therefore, disruptions in the apoptotic program are associated with pathologies such as cancer, where there is too little cell death, and degenerative diseases, where there is too much cell death. Apoptosis can be initiated by at least three types of signals: (i) specific ligation of members on the tumor necrosis factor receptor (TNFR-1)[1] family, which includes Fas/Apo-1/CD95; (ii) cellular stress, which includes genotoxic damage and anti-neoplastic drugs; and (iii) delivery of granule-associated serine proteases from cytotoxic lymphocytes into target cells. Key mediators that initiate and execute the apoptotic program are members of the caspase family of cysteine proteases whose activation is believed to be essential for virtually all forms of apoptosis (1–3). Caspases-3, -6, and -7 are involved in the execution of cells in response to many apoptotic stimuli including ligation of death receptors of the TNFR-1 receptor family, resulting in cleavage of a number of proteins whose limited proteolysis is definitive of apoptosis. However, these executioner caspases are not directly activated by receptor ligation, but rely on the proteolytic activity of upstream initiator caspases-8 and -10 (4–6). In the case of caspase-8 the activation occurs by recruitment of the zymogen to the cytosolic face of the death receptor, such that the initial proteolytic signal originates by autoprocessing of the clustered zymogen (7, 8).

At the execution phase, caspase-3 seems to be upstream of caspases-6 and -7 and, therefore, its activation represents a key point in transmission of the proteolytic signal (9). However, the exact mechanism of how the death signal is conveyed from caspase-8 to caspase-3 remains unresolved. Is the apoptotic signal transmitted by direct activation of the executioners by the initiators, thus constituting a minimal two-step cascade that serves to mediate the apoptotic signals, or is the signal further amplified by the presence of additional factors as suggested by Scaffidi *et al.* (10).

To address these issues we have performed a detailed kinetic study of the activation of pro-caspase-3 by caspases-8 and -10 using recombinant zymogens and active proteases in a defined system, and compared this to the activation of the zymogen by caspases-8 and -10 in cytosolic extracts. This allows us to predict the sequence of events that results in transmission of the proteolytic death signal originating from the initiator caspases to the executioners, and on the basis of *in vitro* observations, test the hypothesis that additional amplifiers and regulators of the apoptotic signals are required *in vivo*.

EXPERIMENTAL PROCEDURES

Materials—Active caspase-3, -8, and -10 were expressed in *Escherichia coli* and isolated as described previously (4, 6, 11). The expression constructs for caspase-3 contained a His-6 tag at the C terminus of the full-length protein, while caspases-8 and -10 contained N-terminal His tags. Pro-caspase-3 was obtained by reducing the induction time in the presence of 0.2 mM isopropyl-1-thio-β-D-galactopyranoside to 30 min. The concentrations of the purified caspase-3 zymogen was determined from the absorbance at 280 nm based on the molar absorption coefficient calculated from the Edelhoch relationship (12): caspase-3 (ϵ_{280} = 26000 M^{-1} cm^{-1}). The concentration of caspases-8 and -10 were determined by active site titration using carbobenzoxy-Val-Ala-Asp-fluorom-

* The work was supported in part by National Institutes of Health Grants HL51399, NS37878, CA72994, AI40646, CA69381, NS33376, and CA69381 and the Illinois chapter of the National Arthritis Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by Danish Natural Science Foundation Grant 9600412.

§ Supported by the Deutche Forschungsgemeinschaft. Present address: Institut für Immunologie der Christian-Albrechts-Universität, Kiel, Germany.

** To whom correspondence should be addressed: The Burnham Institute, 10901 North Torrey Pines Rd., La Jolla, CA 92037. Tel.: 619-646-3114; Fax: 619-646-3190; E-mail: gsalvesen@burnham-inst.org.

[1] The abbreviations used are: TNFR-1, tumor necrosis factor receptor 1; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; AFC, 7-amino-4-trifluoromethyl coumarin; pNA, *p*-nitroanilide; PAGE, polyacrylamide gel electrophoresis; PARP, bovine poly(ADP-ribose)-polymerase; TNF, tumor necrosis factor; Z-DEVD-AFC, carbobenzoxy-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin; Z-IETD-AFC, carbobenzoxy-Ile-Glu-Thr-Asp-7-amino-4-trifluoromethyl coumarin; Ac, acetyl; Pipes, 1,4-piperazinediethanesulfonic acid; PCR, polymerase chain reaction.

ethyl ketone from Bachem AG, Switzerland. Carbobenzoxy-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (Z-DEVD-AFC) and carbobenzoxy-Ile-Glu-Thr-Asp-7-amino-4-trifluoromethyl coumarin (Z-IETD-AFC) were purchased from Enzyme System Products (Dublin, CA). Dithiothreitol was from Diagnostic Chemicals Limited (Oxford, CT). Sucrose was from Mallickrodt (Paris, KY). All other chemicals were from Sigma. Mouse monoclonal anti-caspase-3 was purchased from Signal Transduction Laboratories. All SDS-PAGE were performed using 8–18% acrylamide gels in the 2-amino-2-methyl-1,3-propandiol/glycine discontinuous buffer system as described in Ref. 13. After electrophoresis the gels were either stained using Gel-Code (Pierce) according to the manufacture's protocol or Western blotted to Immobilon-P (Millipore) according to the procedure of Matsudaira (14). Western blot analysis were performed using the ECL kit (Amersham) according to the manufacture's protocol using rabbit anti-caspase-3, anti-caspase-9, or anti-PARP (Biomol). Rabbit anti-caspase-9 was generated from animals immunized with purified recombinant caspase-9 that had been expressed in *E. coli*, essentially as described by (15). Rabbit anti-sera for caspase-3 was prepared as described previously (16). Granzyme B was purified as described previously (17). Dr. Guy Porier kindly provided purified bovine poly(ADP-ribose)polymerase (PARP). The baculovirus caspase inhibitor p35 was obtained as a recombinant protein purified following expression in *E. coli* (18).

Design of Pro-peptide Mutants of Caspase-3—The D9A and D28A single mutants were prepared by PCR using the High Fidelity DNA polymerase (Boehringer) as described previously (19). PCR reactions were performed using the full-length gene encoding caspase-3 in pET-23b (Novagen) and the primers D9A (5′-ACTGAAAACTCAGTG**GcTag-c**AAATCCATTAAAAAT-3′) and D28A (5′-CATGGAAGCGAATCAATG-GcC**TCcGGA**ATATCCCTG-3′) which introduces a *Nhe*I and a BspEI restriction site (bold), respectively, combined with standard primers matching the regions flanking the polylinker of pET-23b. Mutated nucleotides are indicated in lowercase. The truncated form of caspase-3 was generated in a standard PCR reaction by introducing a start codon at Ser^{29} using the primer S29M (5′-CATGGAAGCGAATCAATG**catatg**GGAATATCCCTG-3′) which introduces a *Nde*I site at the new start codon, combined with a standard reverse primer matching the region downstream of the polylinker in pET-23b. The resulting PCR products were all introduced directly into pET-23b (caspase-3) expression vector as a *Nde*I-*Hin*dIII fragment. The D9A/D28A double mutant were prepared using the D9A primer combined with a standard reverse primer matching the region downstream of the polylinker in pET-23b and the D28A single mutant as template for the PCR reaction. The resulting PCR products were all introduced directly into pET-23b (caspase-3 D9A) expression vector as a *Nhe*I-*Hin*dIII fragment, thus, utilizing the restriction site introduced during the first round of mutagenesis. The sequence of the mutated DNA was verified using the Model 373A DNA sequencing system and the Dye terminator cycle sequencing kit from Applied Biosystems Inc.

In Vitro Activation of Caspases 3 by Granzyme B, Caspase-8, and Caspase-10—The activation of the caspases-3 and -7 was investigated using an on-line activation assay as described previously (20). All reactions were performed in caspase buffer (20 mM Pipes, 100 mM KCl, 10 mM dithiothreitol, 1 mM EDTA, 0.1% CHAPS, 10% sucrose, pH 7.2) (21). The reactions were carried out in a Molecular Devices SpectraMax 340 plate reader thermostated at 37 °C, operating in the kinetic mode. Rates of caspase activation were determined as described previously (20). Briefly, various zymogen concentrations in the range (69–690 nM) were warmed at 37 °C in caspase buffer containing 0.2 mM Ac-DEVD-pNA, followed by addition of caspase-8 to a final concentration of 18 nM. Substrate hydrolysis was followed at 5-s intervals and the resulting activation curve fitted by non-linear regression to the equation,

$$A_{405} = \epsilon \cdot (M \cdot Z \cdot t - M \cdot Z \cdot (1 - e^{-kt})/k) + A_0 \qquad \text{(Eq. 1)}$$

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 equals $k_{cat}[S]/(K_m + [S])$ for the caspase cleavage of the reporter substrate Ac-DEVD-pNA (under conditions where $[S] = [S]_0$), Z equals the initial pro-caspase concentration, 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 background activity of the activator on the reporter substrate is subtracted prior to analysis of the progress curve. The parameter k (= observed $k_{cat}/K_{m,z}$) which describes the linear activation of the caspase zymogen, is obtained by fitting to the time-dependent pNA generation using the Grafit program (22). The average k for the activation was determined from the slope of $[Z]k$ *versus* $[Z]$, which was linear and thus allows calculation of $k_{cat}/K_{m,Z}$. The resulting value was corrected for substrate competition due to hydrolysis of the reporter substrate by the activator according to the equation,

$$(k_{cat}/K_M)_Z = (k_{cat}/K_M)_{z,obs} \cdot (1 + ([S]/K_s)) \qquad \text{(Eq. 2)}$$

which describes simple substrate competition obeying a Michaelis mechanism under conditions where the concentration of the reporter substrate $[S] = [S]_0$ and K_s is the apparent binding constant of the reporter substrate to the activator. The K_s values for the hydrolysis of Ac-DEVD-pNA by caspases-8 and -10 were determined to 42 and 50 μM, respectively.

Culture and Transfection of MCF-7 Cells—MCF cells were maintained in RPMI 1640 with 10% heat-inactivated fetal calf serum supplemented with 2 mM L-glutamine, 100 units/ml penicillin, and 50 μg/ml streptomycin. The caspase-3 deficient MCF-7 line was kindly provided by J. Boothman (University of Wisconsin) (23). The absence of detectable caspase-3 was verified by immunoblotting with anti-caspase-3 rabbit polyclonal antibodies. The absence of caspase-3 is due to a 47-base pair deletion within exon 3 of the CASP-3 gene. This deletion results in the skipping of exon 3 during pre-mRNA splicing, thereby abrogating translation of the caspase-3 mRNA (24). MCF-7 cells were transfected with caspase-3 in a retroviral vector (pBabe-puro) (provided by Dr. T. Sladek, Chicago Medical School). After the selection with puromycin, stable expression of the caspase was verified by immunoblotting. MCF-7 control cell lines were generated by transfecting with empty pBabe-puro vector. Stable expression of caspase-3 was verified by immunoblotting after selection.

Processing of Caspases in MCF-7—MCF-7 cells (1×10^6/ml) were treated with 50 ng/ml recombinant tumor necrosis factor (TNF) (Pharmingen, Inc) in 1-ml microcentrifuge tubes containing RMPI supplemented with 0.5% bovine serum albumin. Detection of processed caspase-9 was performed as described previously for detection of other processed caspases in whole cells (24). Treated cells (10^6/ml) were lysed, resolved by SDS-PAGE (10%), and transferred to nitrocellulose. Rabbit antisera were used at a dilution of 1:1000 followed by incubation with anti-rabbit Ig-horseradish peroxidase (Amersham) at a dilution of 1:10,000. Signal was visualized with the ECL kit (Amersham).

Preparation of Cytosolic Extracts—For preparation of cell free lysates we used the procedure of Ellerby *et al.* (25) with minor modifications, the most important of which was omission of exogenous protease inhibitors. Human 293 cells from 12 10-cm Petri dishes were harvested by gentle scraping into phosphate-buffered saline at 4 °C, pelleted by centrifugation for 5 min at $200 \times g$, and subsequently washed once in the same buffer. The cell pellet was resuspended in HEB (20 mM Pipes, 10 mM KCl, 5 mM EDTA, 2 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.4) at 4 °C and pelleted by centrifugation at $1000 \times g$. The cell pellet was then resuspended in an equal volume of HEB and allowed to swell on ice for 30 min. The cells were then cracked by passing through a 24-gauge needle and pelleted by centrifugation at $16{,}000 \times g$ for 30 min, and the supernatant (cytosolic extract) recovered. The quality of the lysates were determined by the ability to support rapid generation of caspase activity by addition of cytochrome *c* and dATP as well as the inability to generate caspase activity upon addition of dATP alone.

Depletion of Caspases from Cell Free Extracts—Lysates from 293 cells were depleted of caspase-3 or caspase-9 by incubating 200 μl of cytosolic lysate with 15 μl of monoclonal anti-caspase-3 antibody, rabbit anti-caspase-9 antiserum, or as a control anti-NFκB antibody on ice for 30 min. The antibody/lysate mixtures were then added onto drained protein G beads (15 μl of packed beads) previously washed with HEB buffer containing 2 mM dithiothreitol and incubated on a rotator for 3 h at 4 °C. The protein G beads were removed by centrifugation to allow recovery of the specifically depleted extracts. The depleted extracts were stored in appropriate portions at −80 °C.

RESULTS

Activation Kinetics of Pro-caspase-3 by Caspases-8, -10, and Granzyme B—To determine the relative efficiency of activation of pro-caspase-3 by physiologic activators, we produced the zymogen in *E. coli* by reducing the induction times normally used for expression of the mature enzyme to 30 min. Pro-caspase-3 was obtained as a full-length precursor under these conditions (Fig. 1), and is fully activable by the apical caspases-8 and -10, and also by the cytotoxic lymphocytes serine protease granzyme B (Table I). Thus, we are able to deter-

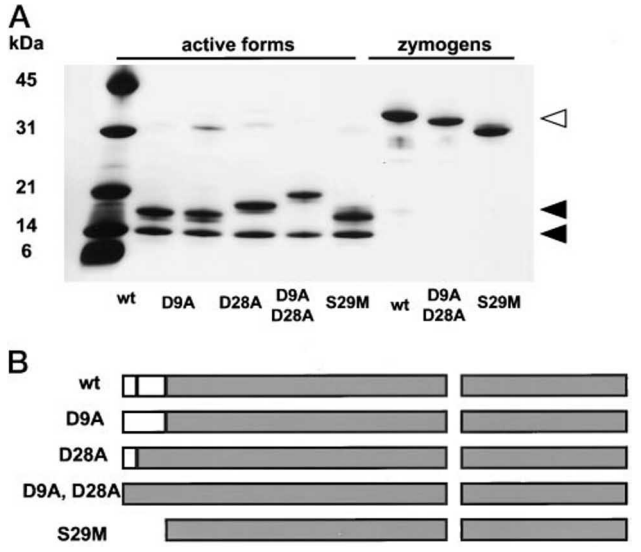

FIG. 1. **Recombinant caspase mutants and zymogens.** The indicated caspase-3 constructs were expressed and purified from *E. coli*, run in SDS-PAGE, and the gel was stained with Coomassie Blue (*panel A*). Active forms are composed of the characteristic large and small subunits (*closed triangles*), and the zymogens (*open triangle*) are single chains. The faster migration of the D9A/D28A zymogen is presumably due to the two Asp-Ala substitutions, since nucleotide sequencing and N-terminal Edman degradation confirmed the expected sequence. *Panel B* shows the fragments of the N-peptide removed during maturation.

TABLE I
Apparent k_{cat}/K_M values for the activation of caspase zymogens

	Caspase-8	Caspase-10	Granzyme B
		$M^{-1}\ s^{-1}$	
Pro-caspase-3	0.87×10^6	0.28×10^6	4.8×10^6
Pro-caspase-3 (S29M)	0.38×10^6	0.14×10^6	1.1×10^6
Pro-caspase-3 (D9A/D28A)	0.61×10^6	0.26×10^6	2.2×10^6

mine the intrinsic activation kinetics of the zymogen under defined conditions. Zymogen at varied concentrations was equilibrated at 37 °C in caspase buffer, followed by addition of an activator and analysis of activation rate using the reporter substrate Ac-DEVD-pNa (see "Experimental Procedures"). Background hydrolysis of the reporter substrate by caspases-8 and -10 was subtracted to allow direct analysis of the rate of zymogen activation. The observed rates of activation of caspase-3 depended on the activator. Granzyme B is by far the most efficient ($k_{cat}/K_m = 4.8 \times 10^6\ M^{-1}\ s^{-1}$), capable of activating the caspase zymogen more than 5.5-fold faster than caspase-8 ($k_{cat}/K_m = 0.87 \times 10^6\ M^{-1}\ s^{-1}$), which in turn catalyzes the activation about 3-fold faster than caspase-10 ($k_{cat}/K_m = 0.28 \times 10^6\ M^{-1}\ s^{-1}$).

Kinetics of Activation of Caspase-3 in Cytosolic Extracts—On the basis of the reaction kinetics calculated above, the kinetics of activation in more complex systems may be predicted. Therefore, this information served as a framework for the analysis of cytosolic extracts of 293 cells. First it was necessary to determine the concentration of the substrate, pro-caspase-3, in the extract. This was performed by quantitative Western blot analysis using dilutions of known concentrations of the recombinant zymogen in comparison with the lysate used throughout this study. The approximate concentration of caspases-3 was found to be 100 nM. We focused on the apical caspase-8 and asked whether the kinetics of pro-caspase-3 processing is the same in the cytosolic extract. To address these questions we compared the rate of processing of purified caspase-3 zymogen and endogenous zymogen in the 293 extracts. As shown in Fig. 2, the processing pattern is essentially identical, irrespective of the source of the caspase zymogen, natural or recombinant. Thus the processing of pro-caspase-3 by caspase-8 in the cytosolic extracts may be recapitulated in the purified system indicating that no accelerator or inhibitor of the activation process is required.

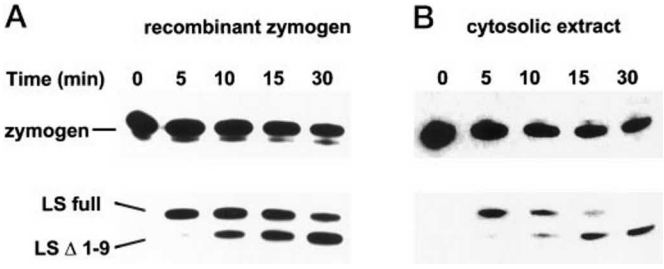

FIG. 2. **Western blot showing the rate of pro-caspases-3 processing by caspase-8.** Purified pro-caspase-3 (100 nM in caspase buffer) or 293 cytosolic extract was incubated for the indicated intervals at 37 °C with 16 nM caspase-8, and the reaction products separated by SDS-PAGE on a 8–18% gel. The reaction products were identified by probing with caspase-3 antibody. The processing patterns observed in *in vitro* and in the cell-free lysate are virtually identical. The large catalytic subunit (*LS*) contains the full-length N-peptide, and the *lower band* designated LSΔ1–9 lacks the first 9 residues.

Properties of Differentially Processed Caspase-3 N-peptide—A notable feature in the caspase-3 processing pattern, both with the recombinant and natural zymogens, is the appearance of two differently processed forms of the large subunit (Fig. 2), determined by Edman degradation to begin at either residue 1 or 10. Although not apparent in Fig. 2, eventually there is further processing at Asp^{28}. These differentially processed forms of the large subunit of caspase-3 have been observed many times in apoptotic cells and have been attributed to autoproteolytic cleavage at Asp^9 and Asp^{28} (26). Removal of an N-peptide (sometimes called the pro-peptide) is a classic way of activating proteases (27) and in the case of caspase-1 the removal of the N-peptide has been demonstrated to have significant influence on the properties of the resulting enzyme (28). We used purified recombinant caspase-3 zymogen to test the relevance of the cleavages in the caspase-3 N-peptide to the activation of the zymogen as well as the activity of the differentially processed forms of the enzyme. To address these issues we expressed and isolated the following caspase-3 derivatives: wild type caspase-3 enzyme, caspase-3 (S29M) which lacks the entire N-peptide, caspase-3 (D28A) which lacks the initial 9 amino acids of the N-peptide, and caspase-3 (D9A/D28A) where the N-peptide cannot be removed. Additionally, we expressed the enzyme caspase-3 (D9A), however, this mutation does not prevent the autocatalytic removal of the N-peptide and thus, the resulting enzyme is identical to the wild-type. Caspase-3 and derivatives thereof that were essentially fully processed between the large and small subunits (Asp^{297}) were obtained by expression in *E. coli* using a 3-h induction, whereas the zymogens were obtained by reduction of the expression time to 20 min (Fig. 1). The authenticity of the N-terminal sequences was confirmed by Edman degradation.

To test whether removal of the caspase-3 N-peptide modulated catalysis we determined the k_{cat}/K_m values for the hydrolysis of Ac-DEVD-pNA for the wild type, caspase-3 (S29M), caspase-3 (D28A), and caspase-3 (D9A/D28A). Since the k_{cat}/K_m for all the enzymes tested are essentially identical (see Table II), we rule out modulation of catalysis as a function for the N-peptide. Furthermore, comparison of the relative rates of hydrolysis of Ac-IETD-AFC and Ac-DEVD-AFC showed that the enzymes do not exhibit any significant differences in their substrate specificities (data not shown). Thus, the catalytic

TABLE II
Catalytic properties of caspase-3 mutants

Caspase-3 mutants constructed to retain portions of the pro-peptide were compared for activity *versus* a synthetic substrate and for inhibition by the natural inhibitor p35. Inhibitory data for the S29M mutant were not obtained since this is essentially identical to the wild type: it contains no pro-peptide.

Caspase-3	k_{cat}/K_M for DEVD-pNA	k_{obs} for p35
	$_M^{-1}\ s^{-1}$	s^{-1}
Wild type	48,600	2.4×10^{-3}
S29M	45,900	ND[a]
D28A	48,900	1.6×10^{-3}
D9A/D28A	46,700	2.2×10^{-3}

[a] ND, not determined.

apparatus does not appear to be sensitive to the presence/absence of the N-peptide.

It is formally possible that the presence of the N-peptide may to some degree restrict access to the active site, thereby imposing a discrimination toward protein substrates but not the short tetrapeptide substrates. To investigate this possibility we tested the ability of caspase-3, caspase-3 (D28A), and caspase-3 (D9A/D28A) to cleave the protein substrate PARP. Cleavage was monitored after addition of the caspase-3 constructs in concentration from 0.067 to 440 nM, a range which allowed a comparison of the amount of caspase required to cleave PARP in 30 min at 37 °C. Although caspase-3 containing the full-length N-peptide (D9A/D28A) cleaved PARP a bit more efficiently, the increase was considered marginal (Fig. 3). In addition, we tested the ability of the baculovirus caspase inactivator p35 to inhibit the different caspase derivatives. As it is evident from Table II the efficiency by which p35 inhibited the different caspase-3 derivatives was not affected to any significant degree and all showed comparable k_{obs} values at the same p35 concentration.

Since the N-peptide did not influence the activity of the matured enzymes, we considered that it may influence zymogen activation, either in a defined zymogen activator system, or in cytosolic extracts. Table I demonstrates a 2–4-fold decrease in activation rate with the zymogen lacking a pro-peptide (S29M), however, we do not consider this a dramatic difference when comparisons of the magnitudes of the rates are made. To determine whether a cytosolic factor could influence activation via the caspase-3 N-peptide the endogenous zymogen was depleted with a mouse anti-caspase-3 monoclonal and the recombinant pro-caspase-3 mutant zymogens were added to the depleted extracts. Western blotting of the immunodepleted cytosolic extracts confirmed removal of at least 95% of the endogenous pro-caspase-3.

Cytosolic extracts reconstituted with recombinant pro-caspase-3 mutant zymogens were treated with cytochrome *c* plus dATP, to achieve caspase activation (Fig. 4*A*) and activation followed by monitoring cleavage of the reporter substrate Ac-DEVD-pNa which, in a whole extract, measures a combination of the caspases present. The response of the reconstituted extracts was identical, with the exception of the activation of the S29M reconstituted extract, which showed an activation rate about 1.5-fold higher than the others. When the identical amount of purified zymogens were activated by granzyme B (Fig. 4*B*), there were no observable differences in rate of activation in agreement with the close similarity of the activation rates (Table I). Taken together these results argue that the N-peptide of caspase-3 only minimally influences activity of the enzyme, or its activation.

Processing of Pro-caspase-9—Although our data support a direct link between the initiator caspase-8 and the executioner caspase-3, others have suggested that the caspase-8 signal is indirectly passed on to pro-caspase-3 via a mitochondrial route (10) where caspase-9, the first caspase in the post-mitochondrial pathway (29), activates the caspase-3 zymogen. As shown in Fig. 5, caspase-9 processing occurs after the addition of caspase-8 to 293 cell extract. The zymogen of caspase-9 (48 kDa) was converted first to a 36-kDa form and then to one of 34 kDa. The first product is consistent with processing at Asp^{330} (caspase-9 numbering) and the second represents processing at Asp^{315}, previously demonstrated to be the cleavage utilized during processing of pro-caspase-9 (30). Cleavage at Asp^{330} is reported to be mediated by activated caspase-3, but the enzyme responsible for processing at Asp^{315} *in vivo* is unknown (30). Conceivably, either cut in caspase-9 could be due to the direct action of caspase-8.

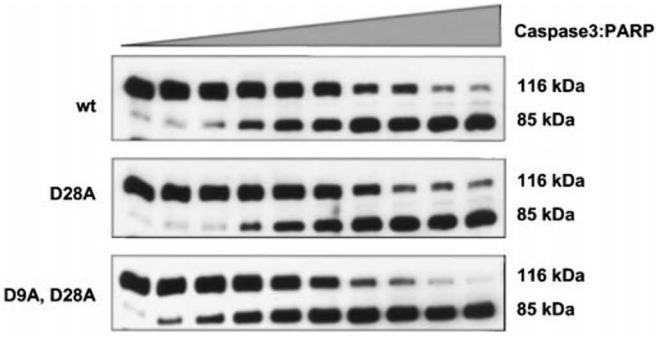

FIG. 3. **PARP cleavage by caspase-3 mutants.** The catalytic activities of caspase-3, caspase-3 (D28A), and caspase-3 (D9A/D28A) were compared for their ability to cleave purified bovine PARP. The figure shows a comparison of the influence of the caspase:PARP ratio on the amount of PARP converted in 30 min at 37 °C in caspase buffer. The caspase:PARP ratios ranges from 1:1 to 1:6561 as indicated by the *triangle above* the Western blots in steps of 3-fold dilutions of caspase-3. Cleavage was visualized by probing a Western blot with anti-PARP antiserum.

To determine whether caspase-8 directly activated pro-caspase-9 during death receptor-dependent apoptosis, and if so, whether activated caspase-9 accelerated the processing of caspase-3, we performed two different experiments. We first asked whether caspase-9 processing required the presence of caspase-3. To address this question, processing of caspase-9 was examined in MCF-7 cells which are known to be deficient in caspase-3 and in transfected MCF-7 cells that express this family member (24), but which still undergo apoptosis without DNA fragmentation (23). Following treatment with TNF, caspase-3 negative MCF-7 cells failed to show evidence of caspase-9 processing. In the caspase-3 positive transfectants, however, conversion of caspase-9 was apparent after 2 h (Fig. 5*B*), although it does not appear to result in generation of the fragments typically associated with caspase activation. Consequently, caspase-8 activation initiated by TNFR-1 ligation (5, 31) is not associated with caspase-9 processing unless caspase-3 is present. This indicates that caspase-8 does not directly activate caspase-9 in whole MCF-7 cells. However, since the capacity of caspase-8 to activate caspase-9 may not be operative in all cell types, we then evaluated this hypothesis using our model system where the cytosolic extract was depleted of caspase-9. Although the addition of cytochrome *c*/dATP did not result in detectable caspase-3 activation (data not shown), the extract was fully responsive to exogenous caspase-8 (Fig. 6). In combination, these data provide further evidence that the cleavage of caspase-9 is mediated initially as a feedback from caspase-3 (32). However, more importantly caspase-9 is not cleaved directly by caspase-8 *in vivo*.

DISCUSSION

Caspases are commonly divided into apical and executioner subsets (1, 33). There exist two well characterized points at

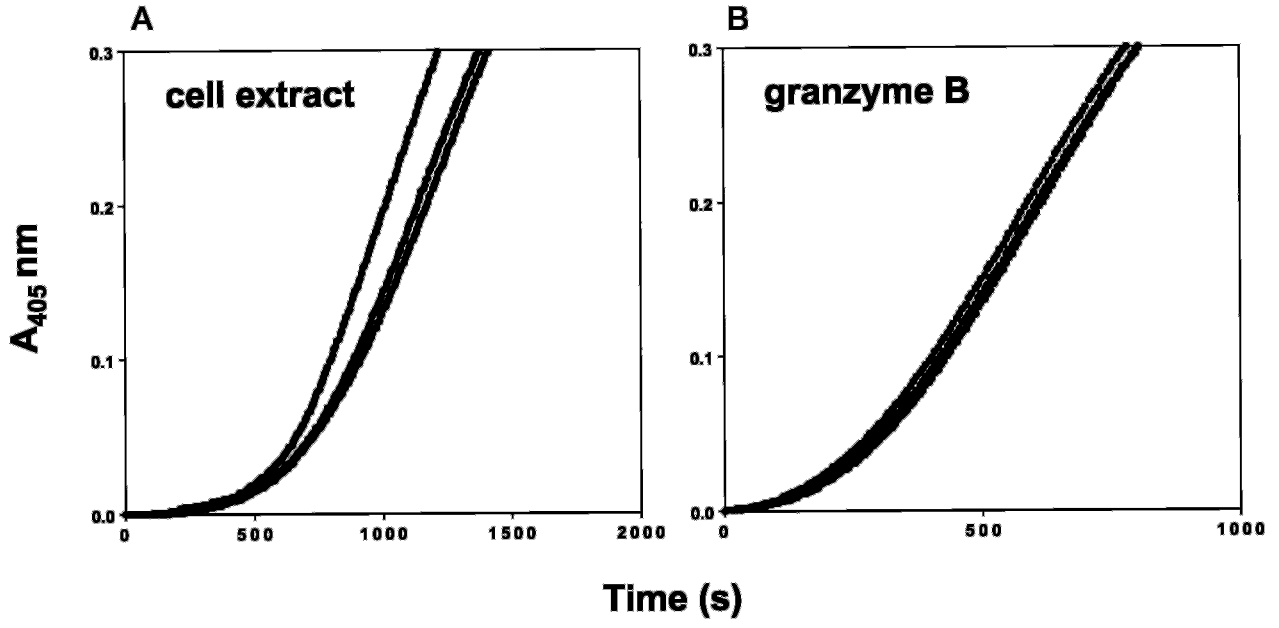

FIG. 4. **Activation of pro-caspase-3 mutants.** The figure shows the activation profile of pro-caspase-3, pro-caspase-3 (S29M), and pro-caspase-3 (D9A/D28A). In *panel A* the zymogens were reconstituted at 100 nM into 293 cytosolic extract that had been depleted of the endogenous zymogen. Activation was initiated by addition of cytochrome *c* (10 μM) and dATP (1 mM). *Panel B* shows activation of the purified zymogens by purified granzyme B. The activation profile of all the zymogens are superimposable, except that pro-caspase-3 (S29M) is activated slightly faster than the other two zymogens in the reconstituted extracts.

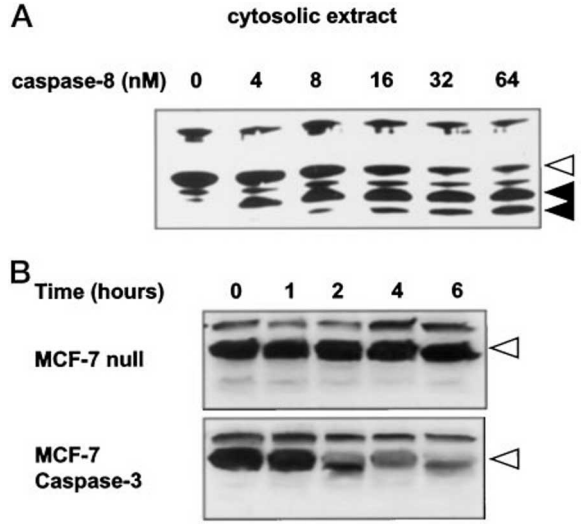

FIG. 5. **Processing of pro-caspase-9.** In *panel A*, a range of caspase-8 concentrations (4–64 nM) were incubated with cytosolic extract for 15 min at 37 °C. Samples were analyzed by SDS-PAGE and Western blots were probed with the caspase-9 antiserum. The processing of the zymogen (*open triangle*) occurs first to give a band of approximately 36 kDa (*upper closed triangle*), followed by processing to a 34-kDa band (*lower closed triangle*). Two nonspecific bands are seen, one above the zymogen and one just below. The identity of the processing intermediates is explained in the text. In *panel B*, extracts of MCF-7 cells that had been treated with TNF for the indicated periods were run in SDS-PAGE and Western blotted with the caspase-9 antiserum. MCF-7 null cells are the parental line of the MCF-7-Casp-3 transfectants.

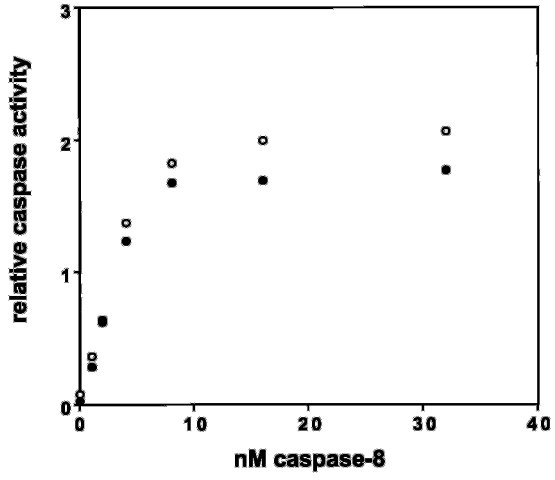

FIG. 6. **Caspase-9 depletion does not prevent caspase activation.** A 100-μl portion of 293 cytosolic extract was immunodepleted of pro-caspase-9. Depletion had a minimal effect (<5%) on the amount of total latent caspase activity judged by granzyme B activation. The figure shows the amount of caspase activity in 3-μl portions of cytosolic extracts obtained after a 1-h incubation of the indicated caspase-8 concentrations at 37 °C in complete cytosolic extract (*open circles*) and caspase-9 depleted cytosolic extract (*solid circles*). The background activity due to the added caspase-8 has been subtracted from all rates. As it is evident from the graph, no substantial influence of the presence of pro-caspase-9 on the rate of activation was observed.

which apical caspases initiate apoptotic signals. One is at the cell surface where members of the TNFR-1 family of death receptors transmit a signal across the cell membrane following receptor clustering. The second point of initiation follows the release of mitochondrial factors (34, 35), and although this post-mitochondrial pathway is well documented it is unclear how the mitochondrion perceives the apoptotic signal. Nevertheless, anti-neoplastic drugs, genotoxic damage, and inhibition of cellular signal transduction pathways all seem to converge on the mitochondrial route (36). At each point of initiation the first recognizable biochemical event is specific caspase activation, and each initiation point utilizes distinct caspases. In the death receptor pathway(s) the apical caspase-8 (and possibly 10) transmits a proteolytic signal following autoactivation at the cytosolic face of the receptor (4–6, 37, 38). The signal for activation appears to be local clustering of pro-caspase-8 that possesses enough activity in its zymogen to achieve autolytic proteolytic maturation (7, 8, 39). In the mitochondrial route specific or nonspecific delivery of cytochrome *c* (29, 40–42) to the protein Apaf-1 results in recruitment and activation of caspase-9 (29). Both caspase-8 and caspase-9 have

been demonstrated to act on *in vitro* translated pro-caspases-3 and -7, the executioner caspases whose activation correlates with apoptosis. Thus there are two potential routes to activate the executioner caspases, and in this context both caspases-8 and -9 can be thought of as initiators whose pathways converge at the execution phase of apoptosis.

Although caspases-8 and -10 can activate pro-caspase-3 *in vitro*, it has proven difficult to determine whether the apical caspases perform this function directly or indirectly, because previous studies have relied on *in vitro* translated zymogens or cytosolic extracts (6, 31, 37, 43). We here demonstrate that caspase-8 and -10 can rapidly activate caspase-3. More importantly, focusing on caspase-8 we observe that activation proceeds with the same rate in a 293 cytosolic extract, eliminating a requirement for an intermediary component. Additionally, depletion of pro-caspase-9 from the extract has no impact on caspase activation by caspase-8, and MCF-7 cells deficient in caspase-3 failed to support processing of pro-caspase-9. Therefore, processing of pro-caspase-9 in death receptor-mediated apoptosis requires the presence and activation of caspase-3 which makes it a downstream event unlikely to play a major role in caspase activation.

It has been proposed that mitochondria are required to transmit the apoptotic signal generated by treatment of cells by agonistic Fas antibodies, but only in a small selection of cell lines (10). This would imply that caspase-8, the apical caspase of the Fas pathway, initiates a mitochondrial signal. In support of this, the generation of caspase activity initiated by addition of human caspase-8 to cytosolic extracts of *Xenopus* eggs is accelerated in the presence of mitochondria (44). However, the relevance of this to homologous systems is unclear, since it is not known whether *Xenopus* has a caspase-8, and the kinetics of activation of a putative *Xenopus* caspase-3 ortholog, or whether the caspase activity in *Xenopus* extracts is due to such an ortholog, have not been determined. The data presented above, in contrast, do not suggest any requirement for an intermediary between caspase-8 and caspase-3. Therefore, the question is whether the mitochondrial acceleration occurs *in vivo*, and whether caspase-8 must transmit its signal via mitochondria to the executioners *in vivo*. Currently the most valuable evidence for a role of mitochondria in apoptosis triggered by death receptor ligation comes from several studies investigating expression of ectopic or transgenic Bcl-2, which is hypothesized to operate by blocking mitochondrial-dependent apoptosis (reviewed in Ref. 36). Most investigators agree that Bcl-2 prevents apoptosis triggered by genotoxic damage, glucocorticoids, and chemotherapeutic drugs, but there are inconsistencies in the data describing the protective effect of Bcl-2 against apoptosis induced by death receptors (10, 33, 45, 46). In a survey of several cell lines, Scaffidi *et al.* (10) noted that most are not protected by Bcl-2 from apoptosis triggered by agonist Fas antibodies. However, the use of agonist antibodies and immortalized cell lines may not be the best way to determine a role for Bcl-2. More significantly, T-cell apoptosis *in vivo*, which is dependent on physiologic Fas ligation, is unaffected in Bcl-2 transgenic mice (46). In contrast, death following injection of agonist Fas antibodies in whole mice was significantly retarded in Bcl-2 transgenic mice (47). These somewhat contradictory studies can be reconciled if some cells support direct transmission of caspase-8 to caspase-3, while others require a mitochondrial accelerator (10).

Since pro-caspase-9 is not processed by caspase-8, the mitochondrial accelerator must act upstream of the caspase-9 activator complex and, therefore, presumably upstream of the mitochondrion. Possibly the pro-apoptotic mitochondrial signal is activated by the action of caspase-8 on mitochondria, or a latent protein that activates mitochondria for apoptosis. The importance of the mitochondrial route in Fas death is not clear, since evidence suggests that only a minority of cell lines require mitochondria to transmit the caspase-8 signal. If direct transmission of the signal from caspase-8 to the executioner caspase-3 occurs in most cell lines, what is the advantage of a mitochondrial intermediate? Usually, levels of complexity are added to allow additional regulation points, as clearly evidenced by the evolution of the vertebrate blood coagulation cascade. It is not immediately clear what advantage cells would achieve by adding a level of regulation to the Fas-triggered death signal, but future studies will doubtlessly focus on this issue. Regardless, it would appear that those cells in the body that are primed to undergo apoptosis during education of the immune system have evolved death receptors, caspase-8, and the caspase-8 activator complex to allow rapid direct transmission of the death signal, bypassing any mitochondrial requirement, and that pro-caspase-3 is a major physiologic substrate of caspase-8 (Fig. 7).

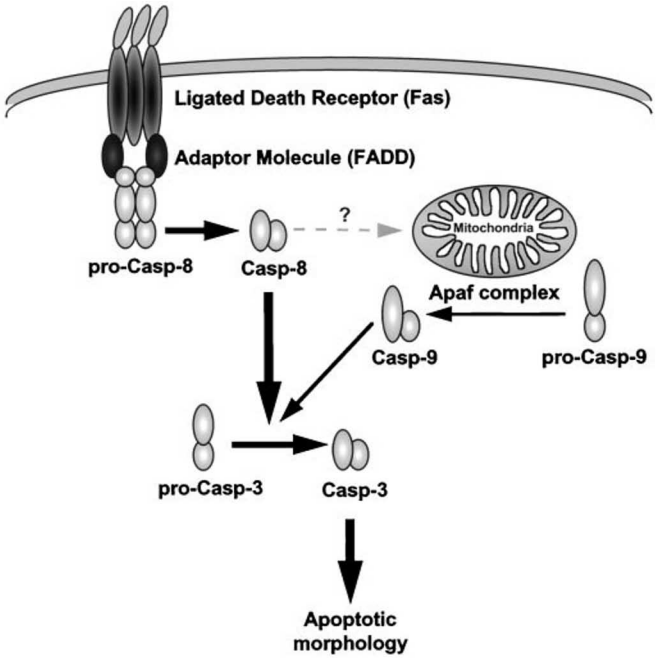

FIG. 7. **Pro-caspase-3 activation.** Currently there exist two recognized points at which apical caspases are activated to initiate apoptosis. Following TNFR-1 or Fas ligation, the initiator caspase-8 is activated by adapter-mediated recruitment to the receptor's cytosolic face (7, 8). Alternatively, the initiator caspase-9 is activated following release of mitochondrial components to form the Apaf complex (34, 35). Both activated initiators converge on the proteolytic activation of caspase-3. In death receptor-triggered apoptosis the main pathway (*bold arrows*) is direct activation of pro-caspase-3 by caspase-8. In some cell types an additional pathway (*light arrow*) may operate by caspase-mediated delivery of a signal (*question mark*) to mitochondria. The importance of the mitochondrial pathway in death receptor-triggered apoptosis is unknown, but apparently subordinate to the dominant, direct pathway in most cell types. This model predicts that deficiency of caspase-9 would not affect death receptor apoptosis that has been triggered by caspase-8 activation.

Acknowledgments—We thank Dr. Vishva Dixit for helpful discussion, and Scott Snipas and Annamarie Price for technical assistance.

REFERENCES

1. Salvesen, G. S., and Dixit, V. M. (1997) *Cell* **91,** 443–446
2. Cohen, G. M. (1997) *Biochem. J.* **326,** 1–16
3. Nicholson, D. W., and Thornberry, N. A. (1997) *Trends Biochem. Sci.* **22,** 299–306
4. Muzio, M., Chinnaiyan, A. M., Kischkel, F. C., O'Rourke, K., Shevchenko, A., Ni, J., Scaffidi, C., Bretz, J. D., Zhang, M., Gentz, R., Mann, M., Krammer, P. H., Peter, M. E., and Dixit, V. M. (1996) *Cell* **85,** 817–827
5. Boldin, M. P., Goncharov, T. M., Goltsev, Y. V., and Wallach, D. (1996) *Cell* **85,**

803–815
6. Srinivasula, S. M., Ahmad, M., Fernandes-Alnemri, T., Litwack, G., and Alnemri, E. S. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 14486–14491
7. Muzio, M., Stockwell, B. R., Stennicke, H. R., Salvesen, G. S., and Dixit, V. M. (1998) *J. Biol. Chem.* **273,** 2926–2930
8. Yang, X., Chang, H. Y., and Baltimore, D. (1998) *Mol Cell* **1,** 319–325
9. Hirata, H., Takahashi, A., Kobayashi, S., Yonehara, S., Sawai, H., Okazaki, T., Yamamoto, K., and Sasada, M. (1998) *J. Exp. Med.* **187,** 587–600
10. Scaffidi, C., Fulda, S., Srinivasan, A., Friesen, C., Li, F., Tomaselli, K. J., Debatin, K. M., Krammer, P. H., and Peter, M. E. (1998) *EMBO J.* **17,** 1675–1687
11. Orth, K., O'Rourke, K., Salvesen, G. S., and Dixit, V. M. (1996) *J. Biol. Chem.* **271,** 20977–20980
12. Edelhoch, H. (1967) *Biochemistry* **6,** 1948–1954
13. Bury, A. (1981) *J. Chromatog.* **213,** 491–500
14. Matsudaira, P. (1987) *J. Biol. Chem.* **262,** 10035–10038
15. Duan, H., Orth, K., Chinnaiyan, A. M., Poirier, G. G., Froelich, C. J., He, W.-W., and Dixit, V. M. (1996) *J. Biol. Chem.* **271,** 16720–16724
16. Krajewska, M., Wang, H.-G., Krajewski, S., Zapata, J., Shabaik, A., Gascoyne, R., and Reed, J. C. (1997) *Cancer Res.* **57,** 1605–1613
17. Hanna, W. L., Zhang, X., Turbov, J., Winkler, U., Hudig, D., and Froelich, C. J. (1993) *Protein Exp. Purif.* **4,** 398–404
18. Zhou, Q., Krebs, J. F., Snipas, S. J., Price, A., Alnemri, E. S., Tomaselli, K. J., and Salvesen, G. S. (1998) *Biochemistry* **37,** 10757–10765
19. Higuchi, R. (1989) in *PCR Technology: Principles and Applications for DNA Amplification* (Erlich, H. A., ed) pp. 61–70, Stockton, New York
20. Zhou, Q., and Salvesen, G. S. (1997) *Biochem. J.* **324,** 361–364
21. Stennicke, H. R., and Salvesen, G. S. (1997) *J. Biol. Chem.* **272,** 25719–25723
22. Leatherbarrow, R. J. (1993) *U. K. Erithacus Software,* U. K. Erithacus Software Ltd., Staines, United Kingdom
23. Talanian, R. V., Yang, X., Turbov, J., Seth, P., Ghayur, T., Casiano, C. A., Orth, K., and Froelich, C. J. (1997) *J. Exp. Med.* **186,** 1323–1331
24. Janicke, R. U., Sprengart, M. L., Wati, M. R., and Porter, A. G. (1998) *J. Biol. Chem.* **273,** 9357–9360
25. Ellerby, H. M., Martin, S. J., Ellerby, L. M., Naiem, S. S., Rabizadeh, S., Salvesen, G. S., Casiano, C. A., Cashman, N. R., Green, D. R., and Bredesen, D. E. (1997) *J. Neurosci.* **17,** 6165–6178
26. Martin, S. J., Amarante-Mendez, G. P., Shi, L., Chuang, T.-S., Casiano, C. A., O'Brien, G. A., Fitzgerald, P., Tan, E. M., Bokoch, G. M., Greenberg, A. H., and Green, D. R. (1996) *EMBO J.* **15,** 2407–2416
27. Neurath, H. (1989) *Trends Biochem. Sci.* **14,** 268–271
28. Yamin, T.-T., Ayala, J. M., and Miller, D. K. (1996) *J. Biol. Chem.* **271,** 13273–13282
29. Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., and Wang, X. (1997) *Cell* **91,** 479–489
30. Srinivasula, S. M., Fernandes-Alnemri, T., Zangrilli, J., Robertson, N., Armstrong, R. C., Wang, L., Trapani, J. A., Tomaselli, K. J., Litwack, G., and Alnemri, E. S. (1996) *J. Biol. Chem.* **271,** 27099–27106
31. Muzio, M., Salvesen, G. S., and Dixit, V. M. (1997) *J. Biol. Chem.* **272,** 2952–2956
32. Srinivasula, S. M., Ahmad, M., Fernandes-Alnemri, T., and Alnemri, E. S. (1998) *Mol. Cell.* **1,** 949–957
33. Chinnaiyan, A. M., and Dixit, V. M. (1996) *Curr. Biol.* **6,** 555–562
34. Liu, X., Kim, C. N., Yang, J., Jemmerson, R., and Wang, X. (1996) *Cell* **86,** 147–157
35. Kroemer, G., Zamzami, N., and Susin, S. A. (1997) *Immunol. Today* **18,** 44–51
36. Reed, J. C. (1997) *Cell* **91,** 559–562
37. Fernandes-Alnemri, T., Armstrong, R., Krebs, J., Srinivasula, S. M., Wang, L., Bullrich, F., Fritz, L., Trapani, J. A., Croce, C. M., Tomaselli, K. J., Litwack, G., and Alnemri, E. S. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 7464–7469
38. Vincenz, C., and Dixit, V. M. (1997) *J. Biol. Chem.* **272,** 6578–6583
39. Martin, D. A., Siegel, R. M., Zheng, L., and Lenardo, M. J. (1998) *J. Biol. Chem.* **273,** 4345–4349
40. Zamzami, N., Susin, S. A., Marchetti, P., Hirsch, T., Gomez-Monterrey, I. M. C., and Kroemer, G. (1996) *J. Exp. Med.* **183,** 1533–1544
41. Kluck, R. M., Bossy-Wetzel, E., Green, D. R., and Newmeyer, D. D. (1997) *Science* **275,** 1132–1136
42. Zou, H., Henzel, W. J., Liu, X., Lutschg, A., and Wang, X. (1997) *Cell* **90,** 405–413
43. Orth, K., Chinnaiyan, A. M., Garg, M., Froelich, C. J., and Dixit, V. M. (1996) *J. Biol. Chem.* **271,** 16443–16446
44. Kuwana, T., Smith, J. J., Muzio, M., Dixit, V., Newmeyer, D. D., and Kornbluth, S. (1998) *J. Biol. Chem.* **273,** 16589–16594
45. Srinivasan, A., Li, F., Wong, A., Kodandapani, L., Smidt, R., Jr., Krebs, J. F., Fritz, L. C., Wu, J. C., and Tomaselli, K. J. (1998) *J. Biol. Chem.* **273,** 4523–4529
46. Strasser, A., Harris, A. W., Huang, D. C., Krammer, P. H., and Cory, S. (1995) *EMBO J* **14,** 6136–6147
47. Lacronique, V., Mignon, A., Fabre, M., Viollet, B., Rouquet, N., Molina, T., Porteu, A., Henrion, A., Bouscary, D., Varlet, P., Joulin, V., and Kahn, A. (1996) *Nat. Med.* **2,** 80–86