THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 36, Issue of September 5, pp. 34042–34050, 2003
Printed in U.S.A.

Human Caspase-7 Activity and Regulation by Its N-terminal Peptide*

Received for publication, May 15, 2003
Published, JBC Papers in Press, June 24, 2003, DOI 10.1074/jbc.M305110200

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Central to the execution phase of apoptosis are the two closely related caspase-3 and -7. They share common substrate specificity and structure, but differ completely in the sequence of their respective N-terminal regions including their N-peptides, a 23–28 residue segment that are removed during zymogen activation. We show that the N-peptide of caspase-7 plays no role in the fundamental activation or properties of the active protease *in vitro*. However, the N-peptide modifies the properties of caspase-7 *in vivo*. In ectopic expression experiments, caspase-7 constructs with no N-peptide are far more lethal than constructs that have an uncleavable peptide. Moreover, the N-peptide of caspase-7 must be removed before efficient activation of the zymogen can occur *in vivo*. These disparate requirements for the N-peptide argue that it serves to physically sequester the caspase-7 zymogen in a cytosolic location that prevents access by upstream activators (caspase-8, -9, and -10). The N-peptide must first be removed, probably by caspase-3, before efficient conversion and activation of the zymogen can occur *in vivo*.

Apoptosis is an orchestrated series of cellular events in which a doomed cell has its cellular component dismantled and packaged into smaller bodies more easily removed by neighboring cells or macrophages. At the core of the death process is a family of cytosolic cysteine proteases, caspases, with a specificity for aspartic acid residues (reviewed in Refs. 1–3). Stimuli from death receptor ligands (extrinsic pathway) or a variety of chemotherapeutic agents, drugs or cellular stresses (intrinsic pathway) cause the activation of apical (initiator) caspase-8, -9, or -10. Once activated, these initiators are able to process and directly activate downstream executioner caspase-3 and -7. The limited proteolytic activity of the executioners on a set of cellular protein substrates is responsible for the hallmark phenotype of apoptosis (reviewed in Ref. 2).

For the executioner caspases, proteolysis between the large and small subunit is thought to be the fundamental activating event (4–8). All caspases possess N-terminal extensions, and in the case of the in initiators these are required for recruitment to the respective activation complexes. In contrast, a consensus role for the short N-terminal extensions of the executioners (Fig. 1*A*) has yet to be established. Initial findings suggest that the N-terminal peptides of caspase-3 and -7 have no effect on activity or the ability to be activated *in vitro*. However, *in vivo* both caspases seem to require the removal of the N-peptides for efficient activation. Indeed, in some cells caspase-3 removes the N-peptide of caspase-7 before the latter is activated by an initiator granzyme B, a serine protease that activates caspases during T-cell-mediated killing (9).

Other roles attributed to the N-peptide are the silencing of caspase-3 (10) and caspase-6 (11) and prevention of nuclear import of *Xenopus* caspase-7 (12). The later hypothesis is appealing, but reports are conflicting regarding the subcellular localization of caspase-7. Although it is generally agreed that the zymogen of caspase-7 is cytosolic, subcellular fractionation experiments have suggested that active caspase-7 relocalizes to the nucleus (12), microsomes (13), or mitochondria (14) during apoptosis. Some of those discrepancies may be artifacts of the experimental procedure given the fact that the pI of caspase-7 lacking the N-peptide is very different than the one of the zymogen (pI = 8.0 *versus* 5.5) and may cause the protein to precipitate upon N-peptide removal. Secondly and most importantly, apoptosis is associated with pH and ionic strength changes as well as nuclear-cytoplasmic barrier disruption (15) and may result in mixing and/or relocalization of compartment specific markers used in subcellular fractionation experiments. Finally, usage of EGFP fusion protein as a tracker for caspase localization (12, 16) may also be a source of experimental variation because of potential dimerization of green fluorescent proteins, natural tendency to localize to the nucleus and increase in molecular weight of the caspase complex. In the present study we have tested a number of these hypotheses for the function of the enigmatic N-peptide of caspase-7.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—293A cells (QBI-293A, Quantum Biotechnology Inc., Montréal, Canada) and COS-7 cells (ATCC, Manassas, VA) were cultivated in DME medium supplemented with 10% heat-inactivated bovine serum (Irvine Scientific, Irvine, CA), 2 mM L-glutamine, penicillin/streptomycin (Invitrogen) and routinely passed every 3 days. For ectopic expression, cells at 40–60% confluence were transfected using FuGENE 6 (Roche Applied Science) as suggested by the manufacturer using 3 μl of transfectant reagent per 1.0 μg of DNA in 0.1 ml of DME medium. Cells were treated and harvested as indicated for each experiment.

Mammalian Expression Constructs—The cDNA for human caspase-7 (GenBank™ acc. no. NM_001227) was use as a template for all constructs. All pcDNA3 constructs were subcloned into *Kpn*I and *Xho*I sites by PCR with oligonucleotides adding the appropriate restriction sites flanking the initiating methionine codon at the 5′-end and the stop codon at the 3′-end. The FLAG epitope was added to the C terminus (Fig. 1*B*) of each construct by PCR with the following reverse oligonucleotide 5′-ctcgagctactgtcatcgtcgtccttgtagtcttgactgaagtagagttcc (FLAG coding sequence underlined). The ΔN deletion mutant was made by PCR with oligonucleotide 5′-cccaagcttggtaccgccatgaagccagaccggtcctcgttt that replaces residues 1–24 by a Kozak sequence (underlined). All mutations were made using QuickChange XL mutagenesis protocol (Stratagene, La Jolla, CA) with the following oligonucleotides and the corresponding reverse sequence: C186A (catalytic cysteine residue also known as 285 in the caspase-1 numbering

* This work was supported by Fonds pour la Recherche en Santé du Québec (to J.-B. D.) and National Institutes of Health Grant NS37878 (to G. S. S.). 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.

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system), 5′-cttcattcaggctgcccgagggacc; NC (D23A), 5′-gcaaatgaagattcagtggctgctaagccagaccgg; D198A, 5′-ggcatccaggccgctagcgggcccatcaatg; D206A, 5′-ccatcaatgacacggccgctaatcctc. All constructs were sequenced to ensure DNA sequence integrity.

Recombinant Protein Expression and Purification—Recombinant caspases were expressed in *Escherichia coli* using as C-terminal His-tagged fusion proteins using the pET expression system (Novagen, Madison, WI). All constructs were subcloned into *Nde*I and *Xho*I sites of pET-23b(+) using the same strategy as for pcDNA3 constructs. The initiating methionine was contained within the *Nde*I site used in the subcloning. Proteins were expressed in BL21(DE3) *E. coli* stain (Novagen) and purified by Ni^{2+}-affinity chromatography as previously described (17). The NC and C186A mutants were generated as described above and the M45A mutant was obtained with the oligonucleotide 5′-gaagaaaaatgtcaccgcgcgatccatcaag and its reverse sequence.

Immunoblot Analysis—Cell extracts were prepared in mRIPA buffer (50 mM Tris, pH 7.4, 100 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS) with general protease inhibitors (1 mM EDTA, 1 mM 1,10-phenanthroline, 50 μM 3,4-dichloroisocoumarin, 10 μM E-64, and 10 μM leupeptin). Polycaspase inhibitors were added when required (100 μM z-VAD-FMK[1] and 100 μM Ac-DEVD-CHO). Lysates were centrifuged at 18,000 $\times$ *g* for 15 min at 4 °C to remove cellular debris. Nuclear extracts were prepared from the insoluble material of the mRIPA extracts by boiling samples in 4 M urea and 1% SDS for 10 min. DNA was sheered by 10 passages through a 27-gauge needle. Samples from 2×10^5 cells were resolved on 8–18% acrylamide gradient gels, transfer to polyvinylidene difluoride membrane in 10 mM CAPS, pH 11, and 10% methanol at constant current (0.4 A for 40–60 min) as previously described (18). Ensuing blots were processed to immunoblotting with the various antibodies and corresponding HRP-conjugated secondary antibodies (1:3000, APBiotech, Piscataway, NJ) and SuperSignal detection reagents (Pierce Chem. Co., Rockford, IL). Cytosolic extracts for cytochrome *c* were obtained using an hypertonic buffer (250 mM sucrose, 70 mM KCl, 137 mM NaCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.2, 200 μg/ml digitonin, and protease inhibitors) as previously described (19). The following antibodies were used: caspase-7 (monoclonal, 1:1000), cytochrome *c* (monoclonal, 1:1000) and PARP (monoclonal, 1:1000, BD Pharmingen, San Diego, CA); hsp90 (monoclonal, 1:2500) and protein kinase C δ, (polyclonal, 1:2000, Santa Cruz, CA); lamin B_1, (monoclonal, 1:1000, Zymed Laboratories Inc., San Francisco, CA); anti-FLAG (M2, monoclonal, 5 μg/ml, Sigma).

Enzymatic Assays—Transfected 293A cells grown in 60-mm dishes were prepared in 50 μl of mRIPA buffer, left on ice for 10 min and centrifugation at 18,000 $\times$ *g* for 20 min. Protein concentration was determined using DC protein assay (Bio-Rad, Hercules, CA) using bovine serum albumin as a standard. Similar amount of protein were assayed for caspase activity using 100 μM of fluorogenic Ac-DEVD-AFC substrate (Bachem Biosciences Inc., King of Prussia, PA) in caspase buffer (10 mM Pipes, pH 7.2, 100 mM NaCl, 10% sucrose, 0.1% Chaps, 1 mM EDTA, and 10 mM dithiothreitol) (17). Amidolytic activity was measured on a f-max Molecular Device spectrofluorometer at 37 °C (EX_λ = 405 nm, EM_λ = 510 nm). Recombinant enzymes were titrated to determine the exact active site concentration as previously described (17) and assays were carried in caspase buffer using either Ac-DEVD-AFC or the equivalent chromogenic substrate Ac-DEVD-pNA (Abs. 405 nm). Enzymatic assays using recombinant or ^{35}S-labeled *in vitro*-translated proteins were performed in caspase buffer at 37 °C in 20–50 μl reaction volume. Laemmli loading buffer was added, and samples were resolved by SDS-PAGE, stained with GelCode Blue stain reagent (Pierce) and dried. *In vitro* translation was done using the TnT *in vitro* translation kit (Promega Corp., Madison, WI); an equivalent amount of radioactive substrate was used for each assay.

Affinity Capture Assay—Cells were transfected in 60-mm plates with the indicated constructs 24–36 h prior labeling. Media was replaced with warm media containing 1 μM biotinyl-VAD(OMe)-FMK (ICN Pharmaceutical inc., Irvine, CA) and incubated for 5 h at 37 °C. Cells were harvested, washed and lysed in 0.5 ml of mRIPA buffer with the protease inhibitor mixture described above. Samples were immunoprecipitated with anti-FLAG (M2, 10 μg/ml), anti-caspase-3 (1:250) or anti-caspase-6 (10 μl of crude serum, generous gift of Dr. S. Krajewski) antibodies at 4 °C for 16 h. Protein-A/G agarose beads (30 μl slurry, Santa Cruz Biotechnology) were added and incubated for 1 h at 4 °C. Immune complex was recovered by centrifugation and washed three times with mRIPA buffer. Samples were resolved on SDS-PAGE gels and blotted to polyvinylidene difluoride membrane as described above. Biotinylated proteins were revealed using streptavidin-HRP (0.2 μg/ml, Sigma) in i-Block buffer (Tropix Inc., Bedford, MA) and SuperSignal reagents.

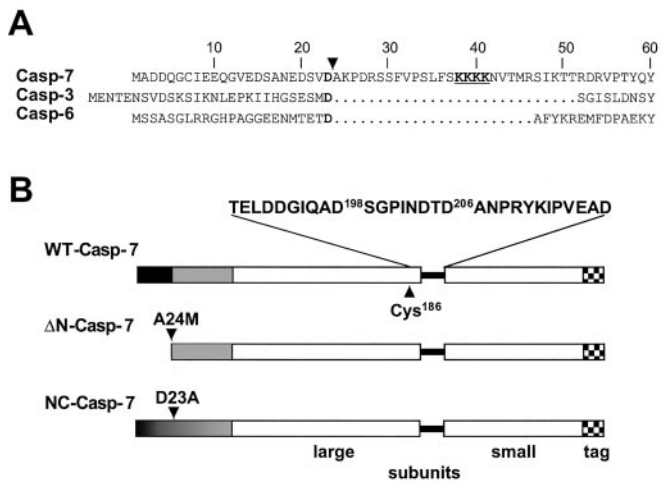

FIG. 1. *A*, N-terminal region of executioner caspases. Residues up to the conserved catalytic domain of caspases are presented. An *arrowhead* marks the N-peptide processing site; a potential nuclear localization signal is *underlined* in the caspase-7 sequence. *B*, schematic representation of caspase-7 constructs used in the present study. The epitope tag was either a FLAG (DYKDDDDK) for mammalian or a His_6 (ELHHHHHH) for *E. coli* expression. The sequence of the linker region is presented with the two processing sites identified by their residue number. Residue numbering is according to caspase-7.

DNA Fragmentation Assays—293A cells grown in 90-mm dishes were transfected, harvested, washed, and lysed in 0.2 ml of lysis buffer (20 mM Tris, pH 8.0, 10 mM EDTA, and 0.2% Triton X-100) and left on ice for 15 min. Lysate was clarified by centrifugation at 18,000 $\times$ *g* for 10 min and DNase-free RNase was added to 50 μg/ml and incubated at 37 °C for 1 h. SDS was added to 0.1% and proteinase K to 0.1 mg/ml and further incubated at 50 °C for 16 h. Samples were extracted twice with phenol:chloroform and ethanol-precipitated with 0.3 M sodium acetate. DNA was resuspended in water and analyzed on a 1.5% agarose gel containing ethidium bromide (20).

Fluorescence Microscopy—Cells were grown in slide chambers and transfected with the appropriate plasmid. Cells were washed twice with warm PBS and fixed with methanol for 2 min. Nonspecific binding sites were blocked with 5% nonfat dry milk in phosphate-buffered saline for 1 h at 37 °C, and samples were processed using anti-FLAG monoclonal antibody (M2, 10 μg/ml) and a FITC-conjugated secondary antibody (Molecular Probes, Eugene, OR). Samples were stained with DAPI (250 nM) and mounted with VectaShield (Burlingame, CA).

RESULTS

Deletion of the N-Peptide Enhances the Ability of Caspase-7 to Kill—To obtain insight into the role of the N-peptide of caspase-7 we generated two mutants, one lacks the first 23 residues and corresponds to an N-peptide deletion (ΔN-caspase-7), and one contains a single amino acid mutation (D23A) making the N-peptide non-cleavable (NC-caspase-7, Fig. 1*B*). These were compared with wild-type caspase-7 for their ability to initiate death in recipient human 293A cells. Expression of FLAG-tagged caspase-7 in 293A cells is sufficient to cause some cell death but deletion of the N-peptide greatly increased the effect whereas prevention of N-peptide removal abrogates it (Fig. 2*A*). This differential effect is overcome at very high expression levels (3 μg plasmid/60-mm dishes) upon which all caspase-7 constructs were able to produce massive

[1] The abbreviations used are: z-VAD-FMK, benzyloxycarbonyl-VAD-fluoromethylketone; AFC, 7-amido-4-fluoromethylcoumarin; CHO, aldehyde; Chx, cycloheximide; Ac-, acetyl-; DAPI, 4′,6-diamidino-2-phenylindole; GrB, granzyme B; PARP, poly(ADP-ribose) polymerase; pNA, paranitroanilide; CAPS, 3-(cyclohexylamino)propanesulfonic acid; HRP, horseradish peroxidase; Pipes, 1,4-piperazinediethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; FITC, fluorescein isothiocyanate.

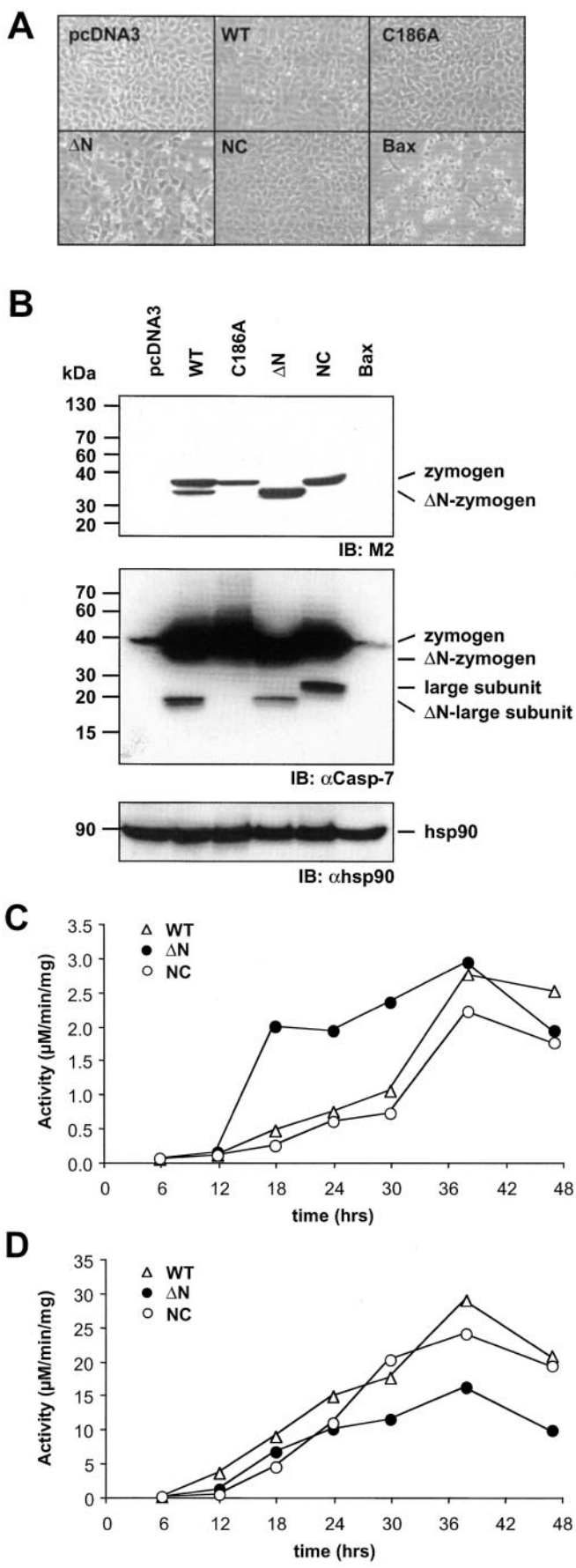

FIG. 2. **Deletion of the N-peptide increases cell death activity of caspase-7.** *A*, 293A cells were transfected with the indicated FLAG-tagged caspase-7 constructs, or Bax, in 6-well plates and visualized under brightfield microscopy. Routinely, ~90% of cells are transfected as judged by the survival of Bax-transfected cells. *B*, cell extracts were prepared in mRIPA buffer and probed with monoclonal anti-FLAG (M2, *top*), monoclonal caspase-7 (*middle*), and an hsp90 (*bottom*) antibodies. The blots reveal the processing status of each of the constructs. Time-course of caspase-7 activation in cells transfected with 0.5 μg (*C*) or 2.0 μg (*D*) of plasmid encoding WT (△), ΔN (●), or NC-caspase-7 (○) and harvested at the indicated time; the 0-h time point was when cells were transfected. Cell lysates was prepared in mRIPA buffer and assayed for caspase activity using Ac-DEVD-AFC substrate as described under "Experimental Procedures." Background activity (empty plasmid) never reached more than 0.2 μM/min/mg and is omitted for clarity.

cell death (not shown). In all transfection experiments the onset of cell death, measured by detachment from the dish, was sooner with ΔN-caspase-7 than wild type or NC-caspase-7. This is not due to disparity in protein levels since immunoblot analysis of FLAG-tagged caspase-7 constructs from cell extracts revealed equivalent expression levels (Fig. 2*B*, *top*). Furthermore, the amount of ΔN-caspase-7 present seems to be less as detected by a caspase-7 antibody (Fig. 2*B*, *middle*). It is noteworthy that substantial processing of the N-peptide of WT caspase-7 occurs during the expression period. The catalytic mutant, C186A, had no effect on cell viability and is not processed.

To quantify caspase activation induced by the constructs we performed a time-course analysis using different amounts of transfected DNA. Transfected cells were harvested at various time points and the caspase activity in cell extracts was measured using Ac-DEVD-AFC substrate (Fig. 2, *C* and *D*). In our hands saturation occurs when more than 2.5 μg/60-mm dish are used. We thus used DNA amounts up to 2.0 μg to detect changes in the activation profile and data for 0.5 and 2.0 μg are shown. Caspase activity was minimal at 12 h post-transfection for both conditions but increases afterward to reach a maximum at 38 h before declining. Most interestingly, at lower expression levels ΔN-caspase-7 activity increased dramatically at 18 h as compare with WT/NC-caspase-7 suggesting a faster activation rate (Fig. 2*C*); similar results were obtained using 0.25 μg of transfected DNA (data not shown). This difference was not apparent when higher amounts of DNA were used (Fig. 2*D* and data not shown). Even in the latter condition widespread cell death was delayed by ~6 h for wild-type and NC-caspase-7 as compared with ΔN-caspase-7 samples. This is despite the fact that no more DEVDase activity was observed, and suggests that removal of the N-peptide is important for the caspase-7 killing effect. As a measure of the amount of caspase (DEVDase) activity that is generated following caspase-7 transfection we compared the results with cell death induced by ectopic expression of the intrinsic pathway activator Bax. Transfection of caspase-7 results in a 10-fold increase of DEVDase activity in cell extracts as compared with the maximum activity obtained in Bax-transfected cells suggesting that the measured activity is mainly attributed to caspase-7 (data not shown).

Cleavage within the linker region of caspase-7 is strictly required for its activation (4, 6, 21, 22). This region contains 2 cleavage sites that could each potentially result in activation of the zymogen (Fig. 1*B*). Therefore we generated Asp/Ala mutations at each site for expression in 293A cells (Fig. 3). Analysis using anti-caspase-7 and anti-FLAG antibodies shows that mutation of D198A abrogates cleavage of the linker region whereas mutation of D206A did not impede cleavage (Fig. 3, *A* and *B*). It is noteworthy that removal of the N-peptide was observed for all constructs probably owing the to ability of caspase-7 overexpression to activate endogenous caspase-7 (data not shown). Albeit weak, a cleavage fragment was observed with the D198A mutant that prompted us to test whether or not this species was active or simply an aberrant

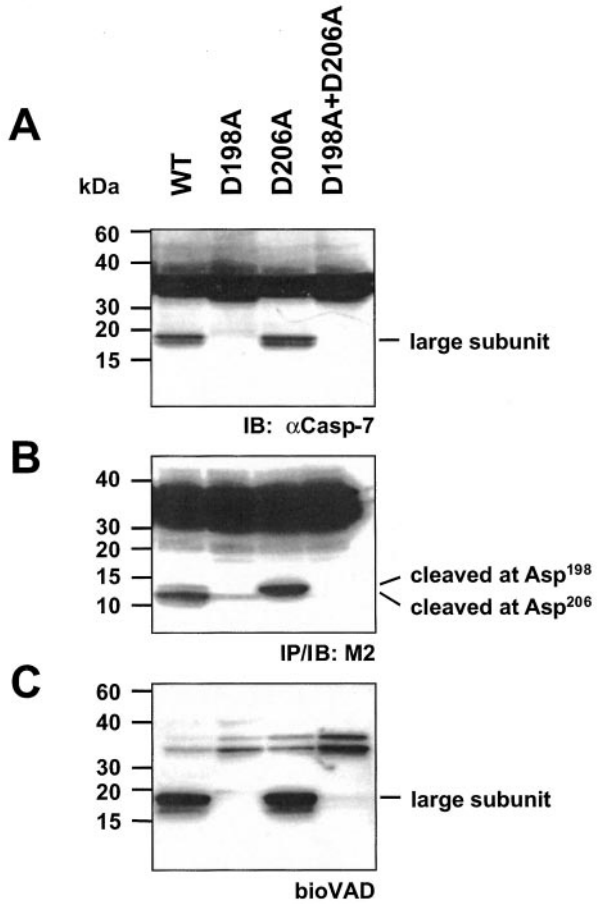

FIG. 3. **Cleavage of the linker region of caspase-7.** *A*, wild type and linker region mutants of FLAG-tagged caspase-7 were expressed in 293A cells. Extracts were prepared in mRIPA buffer and were analyzed by immunoblotting using caspase-7 antibodies. *B*, in an identical series of transfections, FLAG-tagged proteins were immunoprecipitated and immunoblotted with anti-FLAG antibody to better detect the small subunit. *C*, active caspase-7 was revealed by incubating transfected cells with 1 μM of the cell permeable irreversible inhibitor biotinyl-VAD(OMe)-FMK for 5 h. Cell extracts were prepared in mRIPA and immunoprecipitated with anti-FLAG. Biotinylated proteins were detected using streptavidin-HRP.

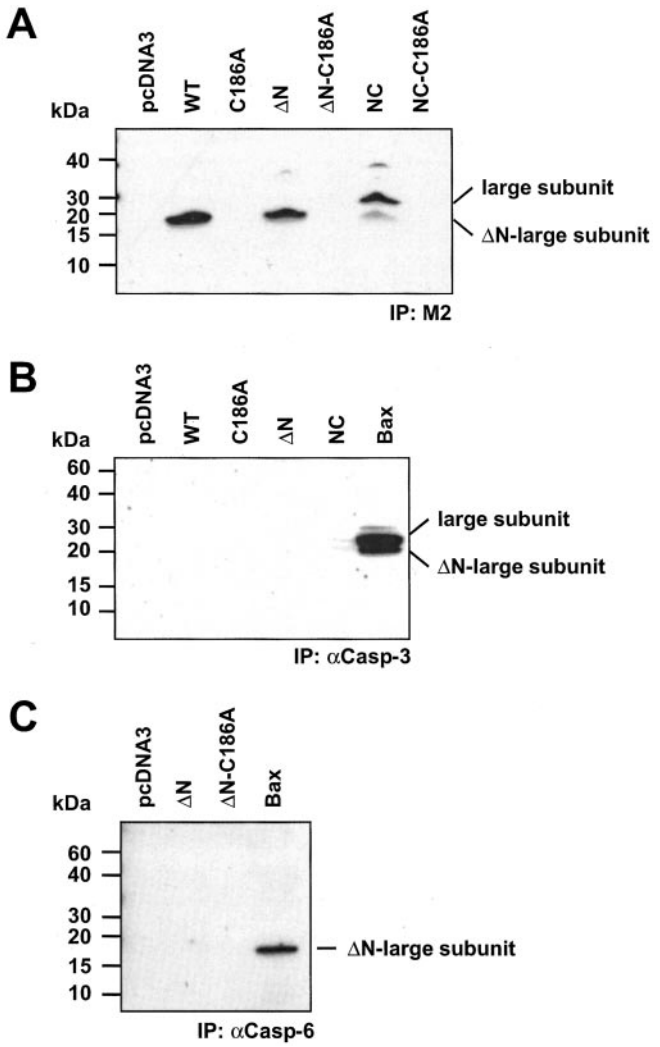

FIG. 4. **Caspase-7 does not activate other caspases.** Cells transfected with the indicated caspase-7 constructs were incubated with 1 μM of the cell permeable irreversible inhibitor biotinyl-VAD(OMe)-FMK for 5 h. Cell extracts were prepared in mRIPA and immunoprecipitated with anti-FLAG (*A*), anti-caspase-3 (*B*), or anti-caspase-6 (*C*). After separation by SDS-PAGE, biotinylated proteins were revealed with streptavidin-HRP. Cells transfected with the catalytic mutant of the corresponding constructs were used as a negative control and cells transfected to express human Bax were used as a control for caspase activation.

cleavage product. To do this we employed an affinity-labeling strategy to capture active caspases (22). Transfected cells were incubated with the biotinylated cell permeable irreversible polycaspase inhibitor, biotinyl-VAD(OMe)-FMK. Caspase-7 was then immunoprecipitated with anti-FLAG antibody and biotinylated proteins were detected with streptavidin-HRP (Fig. 3*C*). It is only when Asp^{198} was available for cleavage that a robust labeling of caspase-7 was observed confirming that caspase-7 activation as a consequence of overexpression is a sequential event requiring processing of Asp^{198} without prior to cleavage at Asp^{206}.

Caspase-7 Is the Most Downstream Caspase—Although caspase-7 is generally accepted to be a downstream (executioner) caspase (9, 23) its overexpression might lead to the activation of other caspases, in which case cell death would be a combination of multiple executioner (caspase-3, -6, and -7) activity and not solely that of caspase-7. Furthermore most caspases, and particularly caspase-3, exert overlapping substrate specificity with caspase-7 (24, 25) making the simple readout of DEVDase activity unreliable in diagnosis. Immunoblot analysis failed to reveal any processed caspase-3 or -6 in cell extracts of transfected cells (data not shown). However, because active caspase amounts undetectable by standard Western analysis may still be significant in cell death (26), we used affinity capture of caspases as a more sensitive approach. Using this method all three catalytically competent caspase-7 constructs were readily labeled (Fig. 4*A*). However, when caspase-3 (Fig. 4*B*) and caspase-6 (Fig. 4*C*) were selectively immunoprecipitated from transfected cell lysates no labeling was detected. The intensity of the labeled caspase-3 and -6 in a positive control extract, programmed to undergo caspase activation by Bax-transfection, suggests that none of those caspases were activated by the expressed caspase-7. Importantly, the lack of caspase-3 and -6 activation following overexpression of caspase-7 indicates that none of the apical caspases (caspase-8, -9, or -10) able to activate executioner zymogens had become activated.

Cell Death by Caspase-7—Having established that caspase-7 overexpression does not lead to the activation of other caspases, we looked for processing of indicator death substrates known to be caspase-3 or -7 substrates. We choose PARP (27) as a nuclear substrate and PKCδ (28) as a cytosolic substrate. Expression of caspase-7 results in cleavage of PARP to the diagnostic 86-kDa fragment, and a 36-kDa fragment characteristic of

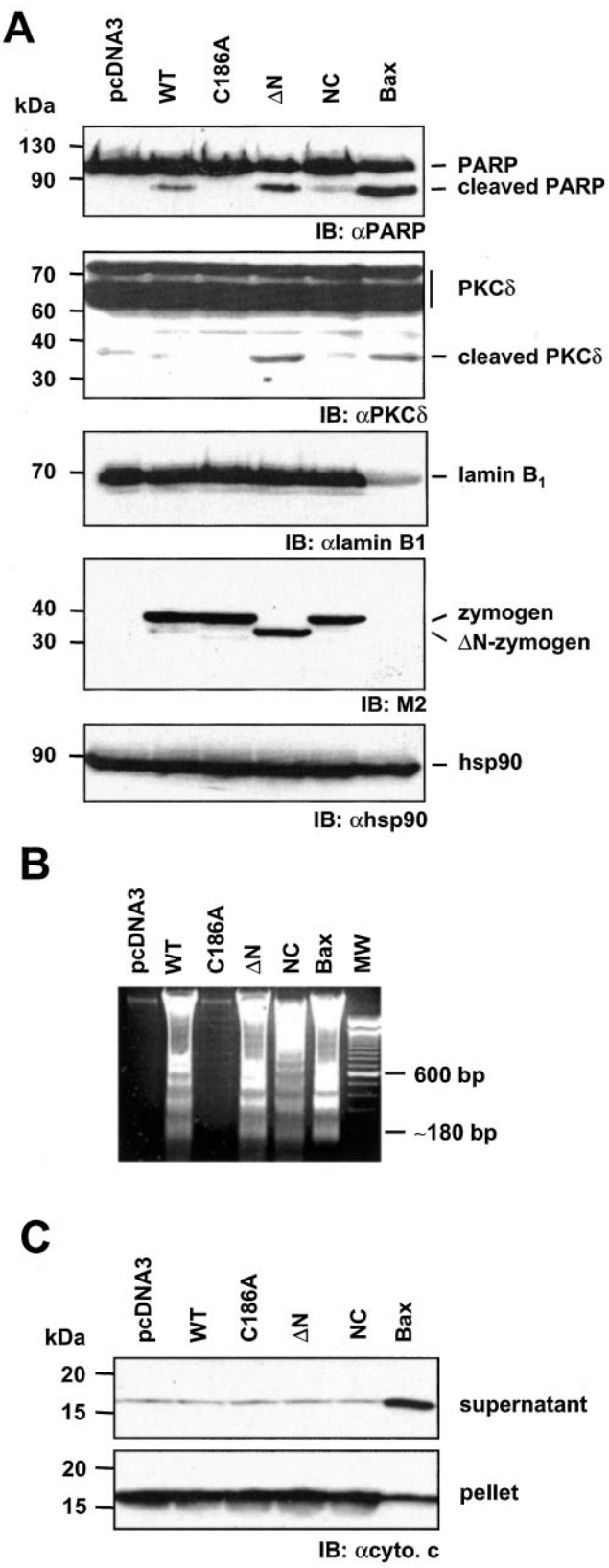

FIG. 5. **Caspase-7 expression induces hallmarks of apoptosis.** *A*, extracts from transfected cells were analyzed by immunoblot for cleavage of PARP, PKCδ, and lamin B_1. Samples were prepared in mRIPA buffer containing general protease inhibitors and 100 μM zVAD-FMK and 100 μM Ac-DEVD-CHO to prevent any adventitious caspase activation or death substrate cleavage post lysis. Immunoblotting with anti-FLAG (M2) and hsp90 antibodies are used as caspase-7 expression and protein loading controls respectively. *B*, fragmented genomic DNA from a 90-mm plate of transfected cells was extracted and analyzed on a 1.5% ethidium bromide agarose gel. *C*, cytochrome *c* is not released by caspase-7 overexpression. Cytoplasmic fraction of cells transfected with the indicated constructs were prepared as described under "Experimental Procedures" and analyzed by immunoblot with a cytochrome *c* antibody. *Top panel* shows the cytosolic fraction and *bottom panel* shows the mitochondria-containing pellet.

apoptosis is generated from full-length PKCδ (Fig. 5). In both cases, ΔN-caspase-7 was more effective in cleaving the substrates. It is noteworthy that Bax overexpression results in more PARP cleavage than simple ectopic expression of ΔN-caspase-7. This suggests that whereas caspase-7 can cleave PARP *in vivo*, a collaborating caspase facilitates access to PARP, possibly by enhancing nuclear entry. We examined lamin B_1, a known substrate of caspase-6 (29, 30), and found no cleavage whereas almost the entire pool of lamin B_1 is cleaved in Bax-transfected cells. This demonstrates that caspase-7 overexpression does not generate excessive nonspecific proteolysis.

We further examined DNA fragmentation (Fig. 5*B*), a hallmark of apoptosis (31). The extent of DNA fragmentation was comparable to Bax-transfected cells with wild-type and ΔN-caspase-7, slightly reduced with NC-caspase-7, and absent from cells transfected with an empty plasmid or caspase-7 catalytic mutant. To determine whether apoptosis induced by caspase-7 implicates the mitochondrial pathway we also checked for release of pro-apoptotic mediators from mitochondria in transfected cells. No increase in cytosolic cytochrome *c* (Fig. 5*C*) or SMAC/DIABLO (data not shown) was detected as compared with positive control cells transfected with Bax, ruling out participation of mitochondria in cell death mediated by caspase-7.

Human Caspase-7 Is Not a Nuclear Caspase—Shortly after the N-peptide of caspase-7 is a stretch of basic residues (K^{38}KKK, Fig. 1*B*) reminiscent of a nuclear localization signal. Removal of the N-peptide during apoptosis could expose this signal and cause relocation of caspase-7 as suggested for the *Xenopus* ortholog (12). We thus examined the subcellular localization of FLAG-tagged caspase-7. However, because cell death, for example by caspase-7 overexpression, is accompanied by important morphologic changes we examined the subcellular localization of the respective catalytic mutants in COS-7 cells (Fig. 6*A*). No difference in staining pattern was found for any of the full-length, ΔN, or NC-caspase-7 constructs; all were mainly excluded from the nucleus. This is consistent with subcellular fractionation experiments showing that both ΔN and NC-caspase-7 catalytic mutant were fully cytosolic (data not shown). Our results do not exclude that a small portion of caspase-7 may find its way to apoptotic nuclei, but they rule out the possibility that removal of the N-peptide allows an active transport or accumulation of human caspase-7 in the nuclei.

Removal of the N-Peptide Alters Self-activation of Caspase-7—The enhanced apoptosis attributable to N-peptide removal may reflect an increase in the inherent enzymatic activity of caspase-7. To test this we determined the kinetic parameters of recombinant proteins expressed in *E. coli*. Proteins were expressed as C-terminal His tag fusions and purified to near homogeneity by Ni^{2+}-affinity chromatography as described (17). Using active site-titrated enzymes, we established that ΔN-caspase-7 is enzymatically similar to wild type or NC-caspase-7. Indeed, K_m and k_{cat} values for ΔN-caspase-7 and NC-caspase-7 on the fluorogenic substrate Ac-DEVD-AFC and the chromogenic substrate Ac-DEVD-pNA were identical within experimental error (Table I). It is thus clear that any phenotypes observed in transfection experiments using the corresponding proteins are unlikely to be due to differences in catalytic activity but rather to some property of the N-peptide itself.

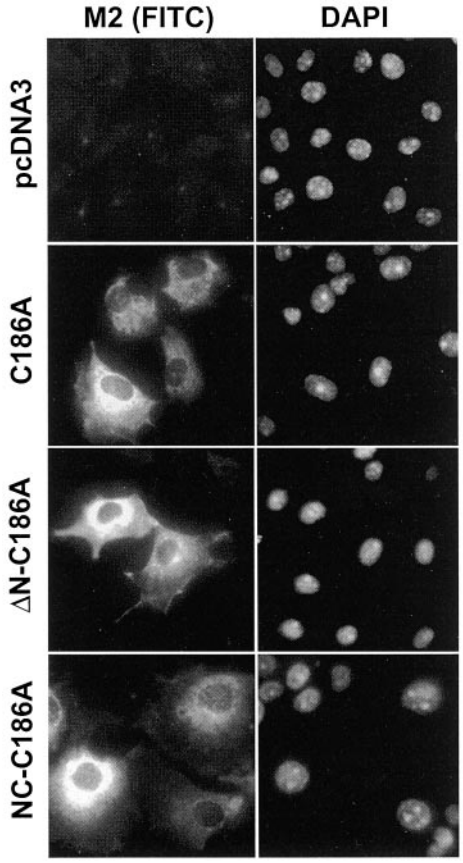

FIG. 6. **Human caspase-7 is not a nuclear caspase.** FLAG-tagged C186A mutants of the indicated constructs were transfected in COS-7 cells and analyzed by fluorescence microscopy with anti-FLAG antibody (*M2*) and a FITC-conjugated secondary antibody (*FITC*). Nuclei were stained with DAPI.

TABLE I
Kinetic parameters of caspase-7 constructs

Values were obtained in caspase buffer using active-site titrated enzyme (see "Experimental Procedures").

Substrate Enzyme	K_m[a]	k_{cat}	k_{cat}/K_m
	μM	s^{-1}	$M^{-1}\,s^{-1}$
Ac-DEVD-AFC[b]			
Caspase-7	60.5 ± 4.1	6.9 ± 0.2	1.1×10^5
ΔN-caspase-7	68.3 ± 3.8	9.5 ± 0.2	1.4×10^5
NC-caspase-7	68.7 ± 4.5	9.5 ± 0.2	1.4×10^5
Ac-DEVD-pNA[b]			
Caspase-7	64.6 ± 2.4	10.3 ± 0.2	1.6×10^5
ΔN-caspase-7	59.2 ± 2.1	13.3 ± 0.2	2.2×10^5
NC-caspase-7	59.6 ± 2.8	13.5 ± 0.3	2.3×10^5

[a] Values are averages of 2 different sets of experiments with the same lot of substrate; values from a different purification and/or different substrate lot were within 20% of the value listed here.

[b] Substrate stock concentration were standardized using complete hydrolysis of a given amount of substrate compared to a standard AFC or pNA stock with a known concentration as determined by the extinction coefficient of each compound.

We noticed that during purification of the various recombinant protein from *E. coli*, NC-caspase-7 was mainly present as a zymogen after 5 h of expression whereas ΔN-caspase-7 was mainly processed suggesting that the N-peptide may delay auto-catalytic activation of the zymogen. Time-course analysis of caspase-7 activation in *E. coli* allowed us to monitor the conversion of the zymogen that is induced by autolytic cleavage (Fig. 7*A*). Activation of ΔN-caspase-7 occurred concomitantly with cleavage of the inter-chain linker region at Asp^{198} (Fig. 1*B*), as determined by Edman degradation, as early as 3-h

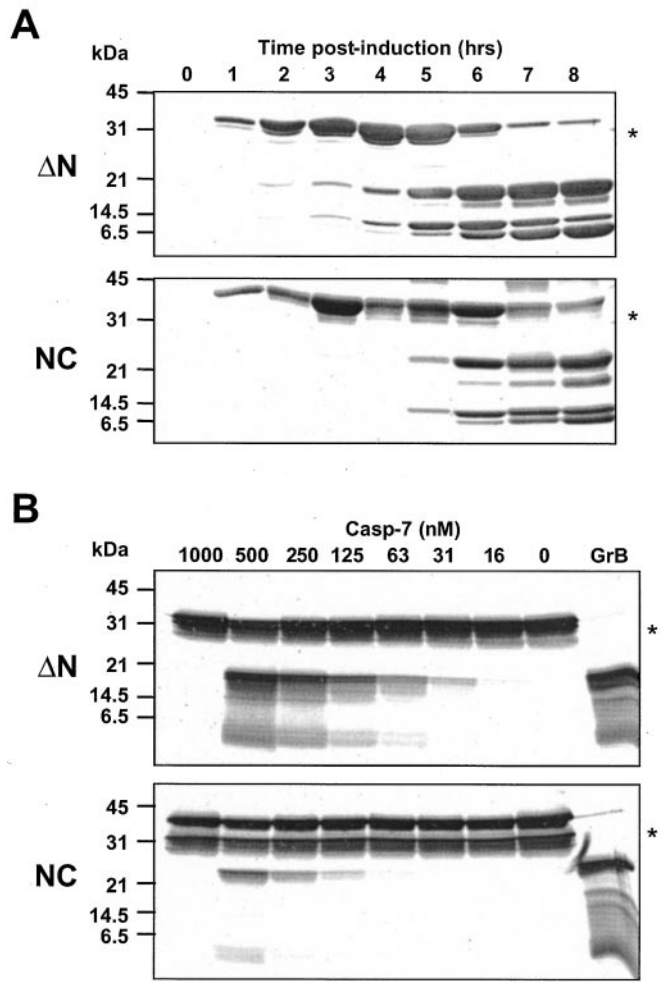

FIG. 7. **The N-peptide prevents self-activation of caspase-7.** *A*, caspase-7 zymogen activation in *E. coli*. ΔN (*top*) or NC (*bottom*)-caspase-7 expressing *E. coli* were harvested at the indicated time points after induction with 0.2 mM IPTG. Caspase-7 was purified using Ni^{2+}-resin, and analyzed by SDS-PAGE and GelCode blue staining. The *asterisk* marks a secondary translation product initiated at Met^{45}. *B*, processing of *in vitro* translated caspase-7. Caspase-7 ΔN (*top*)/NC (*bottom*) C186A were *in vitro*-translated in the presence of ^{35}S and incubated with serial dilution of active site-titrated recombinant caspase-7 at the indicated concentration for 18 h at 37 °C. Proteins were then resolved on SDS-PAGE. As a positive control, an aliquot of labeled protein was incubated with 50 nM granzyme B for 2 h at 37 °C to show that all of the *in vitro*-translated protein can be processed, and is therefore correctly folded.

post-induction to produce large and a small subunits (Fig. 7*A*, *bottom*). In comparison, autolytic conversion of NC-caspase-7 was delayed by about 2 h. In both cases a second cleavage occurs resulting in the trimming of the small subunit N terminus at Asp^{206}. These data indicate that the N-peptide plays a role in stabilizing the zymogen, at least *in vitro* under the abnormally high concentrations obtained during expression in *E. coli*. However, it is possible that differences in expression level or onset of expression influenced activation, so we designed an experiment in which the processing of pro-caspase-7 by added caspase-7 could be more carefully controlled. We used the catalytic mutant (C186A) forms of ΔN and NC-caspase-7 to abrogate any complications from additional activity of the processed zymogens. These were *in vitro* translated in the presence of [^{35}S]methionine and used to assay the ability of recombinant caspase-7 to process the zymogens. The presence of a secondary translation product initiated at Met^{45} was observed (marked by the asterisk); its mutation to alanine removed the secondary translation product but did not change the outcome

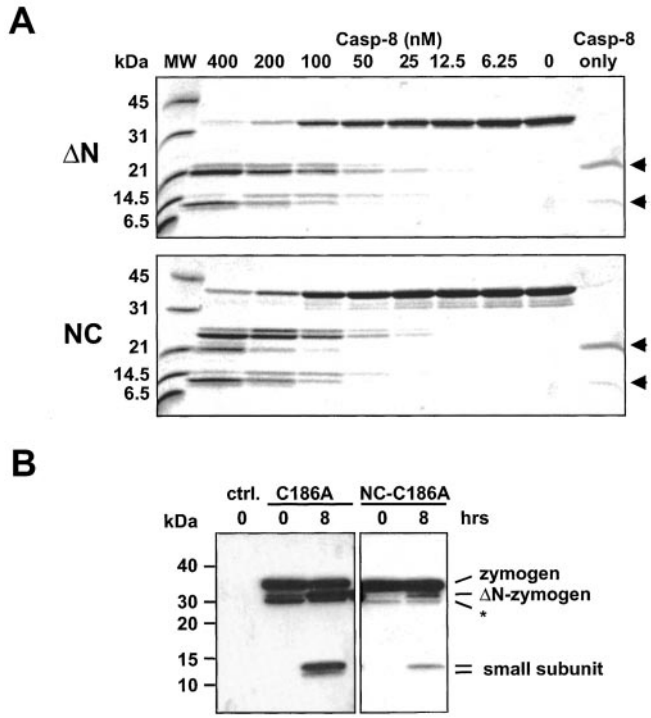

FIG. 8. **The N-peptide prevents activation of caspase-7 by an apical caspase *in vivo* but not *in vitro*.** *A*, caspase-7 processing by caspase-8 *in vitro*. Recombinant zymogen ΔN (*top*)/NC (*bottom*)-caspase-7(M45A/C186A, 2.5 μM) was incubated with the indicated concentration of active site-titrated recombinant caspase-8 for 18 h at 37 °C. Caspase-8 alone at the highest concentration used was loaded in the last lane as a reference. *Arrowheads* indicate caspase-8 large and small subunit bands. *B*, cleavage of the linker region is impeded by the N-peptide. Cells were treated for 0 or 8 h with TNFα (10 ng/ml) and Chx (10 μg/ml) and cell extracts were prepared in mRIPA containing protease inhibitors, immunoprecipitated with anti-FLAG (M2) and visualized by immunoblotting to reveal the processed small subunit. The *asterisk* marks a secondary translation product initiated at Met^{45}. NC-caspase-7 C186A tends to reveal aberrant cleavage surrounding the N-peptide when the NC mutation is introduced.

of the results in the experiments described below.

We observed that processing of the labeled ΔN-caspase-7 zymogen was more efficient than for the NC-caspase-7 zymogen for the same catalytic amount of added active recombinant caspase-7 (Fig. 7*B*). This suggests that the N-peptide interferes with conversion of the zymogen and could partially explain why the onset of cell death is delayed in 293A cells for WT/NC-caspase-7 (Fig. 2). We conclude that removal of the N-peptide does not affect the intrinsic enzymatic activity of the enzyme, but increases the rate of zymogen processing. Consequently, it is pro-caspase-7 acting as a substrate, not caspase-7 acting as an enzyme, that is influenced by the N-peptide.

The N-peptide of Caspase-7 Prevents Activation in Vivo but Not in Vitro—Self-processing is not thought to be the endogenous pathway to caspase-7 activation used in mammalian cells induced to undergo apoptosis. Indeed this is thought to be the role of apical caspase-8 and -9 (reviewed in Refs. 1–3). We thus investigated the role of the N-peptide in the activation of pro-caspase-7 by caspase-8 *in vitro* (Fig. 8*A*). Serial dilutions of recombinant caspase-8 were used to process recombinant catalytic mutant versions of ΔN-caspase-7 and NC-caspase-7. We introduced the M45A mutation to eliminate a secondary translation product frequently observed when the proteins are expressed in *E. coli*. This mutation is not expected to alter any properties of caspase-7 since it is not conserved in mouse or in the hamster orthologs (both have an alanine at this position). Cleavage of caspase-7 zymogen by caspase-8 generates two large and two small subunit derivatives corresponding to proteolysis of the linker region at the two activation sites (see above and Fig. 1*B*). Significantly, no difference was observed in the rates of cleavage of the two forms of caspase-7 (Fig. 8*A*). A similar conclusion was drawn using caspase-9 as an activator although 10-fold less enzyme was required to process the same amount of zymogen (data not shown). Thus the N-peptide does not alter the ability of apical caspases to activate pro-caspase-7 *in vitro*.

To test whether the N-peptide influences zymogen activation *in vivo* we expressed catalytic mutant caspase-7 constructs in 293A cells, where they act as a reporter of processing without themselves participating in the process. To analyze cleavage products, FLAG-tagged proteins were immunoprecipitated and revealed with anti-FLAG antibody (which results in clearer detection of the analyzed protein). Small subunit related bands produced from the pro-caspase-7 (Fig. 8*C*) are similar to the ones observed in the *in vitro* assay and should represent caspase-8-mediated processing. This processing was greatly reduced for NC-caspase-7. NC-caspase-7 is processed, and therefore activated, far more slowly than WT-caspase-7. We conclude that the N-peptide regulates the activation of caspase-7 by the extrinsic pathway (caspase-8) *in vivo*, but not *in vitro*. The reasons for this are discussed below.

DISCUSSION

The presumptive role of caspase-7 as an executioner caspase, involved in the multiple cleavage of apoptotic substrates, is based primarily on its close relationship with caspase-3. Perhaps the most important similarity from a functional perspective is that both have almost indistinguishable specificity on small synthetic substrates (25), although the overall rates of catalysis are lower for caspase-7 (24). Moreover, they are the only proteases known to be specifically inhibited by the second BIR repeat of the endogenous caspase inhibitor XIAP (reviewed in Refs. 32 and 33). Why are two apparently similar proteins retained during vertebrate radiation? Given the close relationship, the simplest answer for the existence of both is that they constitute a redundant pair, so that loss of one is compensated by presence of the other. Arguing against this is the delayed apoptotic phenotype observed in mice ablated in the caspase-3 gene. $casp3^{-/-}$ mice on a 129X1/SvJ genetic background demonstrate neuronal hyperplasia and disorganized cell deployment with neonatal lethality (34). However, $casp3^{-/-}$ mice on a C57BL/6J genetic background have a much less severe, almost unnoticeable developmental phenotype (35) indicating that the latter genotype contains suppressors of caspase-3 ablation. Conceivably, caspase-7 is such a suppressor. Furthermore, the human tumor cell line MCF-7 that lacks caspase-3 (36) still undergoes apoptosis albeit slowly, displaying activation of caspase-7 (37). On the other hand, silencing of caspase-3 expression causes compensatory increases in other caspases, including caspase-7 (38). However, the most significant difference between the proteins resides in the composition of their N-peptides and the short sequence that follows the N-peptide cleavage site before the beginning of the catalytic domain. These may program differential activity of caspase-3 and -7 *in vivo*.

The size and nature of the N-terminal peptides defines different caspase groups. Initiators such as caspase-8, -9, and -10 contain relatively long peptides that encompass distinct recognized folding domains (death effector domains or caspase recruitment domains) required for their recruitment to activator complexes. In these complexes the apical caspases attain catalytic activity, becoming able to activate executioner caspase-3 and -7 by direct limited proteolysis. The zymogens of the executioners contain relatively short N-peptides without a recog-

nizable folding pattern, and the function of these N-peptides is elusive. The caspase-3 and -7 N-peptides are removed during apoptosis and they are highly conserved from frogs to man. For example, although human and *Xenopus* pro-caspase-7 share limited amino acid identity within their respective N-terminal domain, both contain the DSVD ↓ A cleavage site for the N-peptide and the KKKK sequence described in Fig. 1. This likely reflects a function preserved in all vertebrate orthologs of caspase-7.

One proposal for the N-peptide of caspase-7 is that it covers a partial nuclear localization signal comprising the KKKK segment (12). While this may be the case with *Xenopus* caspase-7, with which the forgoing study was conducted, it does not seem to be the case for human caspase-7, since we were unable to observe any nuclear accumulation of full-length or N-peptide deleted caspase-7 in COS-7 cells. Massive overexpression of caspase-7 can result in some nuclear translocation, but this is likely to be an artifact of forced overexpression. Consequently, the postulated function of the N-peptide in regulating nuclear translocation has not been conserved in vertebrates.

Another role that has been proposed for the N-peptide is the silencing of the zymogen form of caspase-3 (10). The general conclusion from this paper was that the caspase-3 N-peptide retains the protease in an inactive state. At first glance it seems that a similar function could be ascribed to the caspase-7 N-peptide, since its deletion renders recipient mammalian cells more sensitive to death by caspase-7 transfection. However, we are not aware of instances where apoptosis is triggered at the effector stage, where caspase-7 resides, without participation of upstream initiator caspases and our results on death mediated by ectopic expression of caspase-7 do not signify that this is a route *in vivo*. They simply reveal that the N-peptide of caspase-7 alters the properties of pro-caspase-7 as a substrate of caspase-7, but importantly not as a substrate of the initiator caspase-8 or -9.

Our data suggest that differences in the rate of activation of wt, ΔN and NC pro-caspase-7 are due to the zymogens acting as substrates for processing mediated by caspase-7, but significantly not the physiologic activators caspase-8 and -9 *in vitro*. This suggests that the presence of the N-peptide either hinders access of caspase-7 to the zymogen linker, or acts as a weak inhibitor of caspase-7 itself. The latter possibility is ruled out by our kinetic data that show essentially identical catalytic parameters for each of the active forms of caspase-7. The former possibility suggests that the N-peptide alters the conformation of the zymogen in a subtle manner. However, the atomic resolution structures available for full-length caspase-7 or pro-caspase-7 do not reveal density for residues prior to Ser^{47}, either because there is no defined structure or because of mobility with respect to the fixed bulk of the catalytic domains in the crystals (4, 6, 39). In addition, there are no contacts visible between the N-peptide and the catalytic domain. This observation weakens the hypothesis that the N-peptide can directly modulate caspase-7 structure, activity or activatability, and so another mechanism must be considered for the function of the N-peptide.

There is a major difference in the processing pattern of caspase-3 and -7 that sheds light on the differential function of their respective N-peptides. Caspase-3 activation, both in recombinant and natural settings, occurs by inter-domain cleavage followed by N-peptide removal (40). In stark contrast, in the same settings the activation of caspase-7 occurs first by N-peptide removal, and it has been shown that caspase-3 removes the N-peptide of caspase-7 before it is activated by the cascade initiator granzyme B in MCF-7 cells (9). Here we show that it is also true for triggering by caspase-8, since prevention of N-peptide removal diminishes caspase-7 interdomain processing *in vivo*. More importantly, we also show that initial removal of the N-peptide is essential for the efficient ultimate activation of caspase-7 in cell transfection experiments, and therefore presumably also *in vivo*.

There is still no clear evidence of the reason for the requirement of N-peptide removal in caspase-7 activation. However, the data are consistent with a physical sequestration of the zymogen from its apical activators. It seems that this sequestration is overcome when the N-peptide is removed from pro-caspase-7, after which it can be activated by the normal route of interdomain cleavage. Part of the mechanism may reside in the inherent properties of the N-peptide; it is a highly negatively charged region. Indeed, the isoelectric point of the full-length zymogen is much lower than the one of the protein lacking the N-peptide. Classical protein sequestration involves binding to a structural protein or organelle. The highly charged N-peptide and the following region could serve such a purpose. However, most subcellular fractionation and cell-free studies report endogenous caspase-7 as primarily cytosolic in healthy cells (13, 14), so it is not clear where the sequestering partner resides. But the results of our study underscore the importance of subcellular localization in the apoptotic process. *In vitro*, including in hypotonic cytosolic extracts (9) pro-caspase-7 is activated by caspase-8 and granzyme B in an N-peptide independent manner, but in whole mammalian cells these cascade initiators are hindered in their access until the N-peptide is removed, presumably by caspase-3.

The study presented here shows that caspase-7 is not simply a redundant executioner. It appears to require a two-step activation mechanism *in vivo*, though not *in vitro* (9). It is fully capable of conducting most of the apoptotic program, but is highly dependent on the activity of caspase-3 to remove the block due to the N-peptide before the canonical activation process begins. Clearly further studies are required to identify the molecular mechanism(s) by which the N-peptide prevents self-activation and sequestration of caspase-7. Undoubtedly, determining the structure of the N-peptide will shed light on both issues and provide insights on the role of caspase-7 in the apoptotic program.

Acknowledgments—We thank Scott Snipas and Annamarie Price for outstanding technical support and Henning Stennicke for helpful advice.

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Enzyme Catalysis and Regulation:

Human Caspase-7 Activity and Regulation by Its N-terminal Peptide

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J. Biol. Chem. 2003, 278:34042-34050.
doi: 10.1074/jbc.M305110200 originally published online June 24, 2003