Phosphorylation of PITSLRE p110Isoforms Accompanies Their Processing by Caspases during Fas-mediated Cell Death*

(Received for publication, January 22, 1998, and in revised form, April 6, 1998)

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A number of cellular proteins have been identified as caspase targets during cell death, including the PITSLRE protein kinases. These targets generally fall into one of three possible categories: 1) other caspases, 2) proteins that are inactivated during apoptosis, and 3) proteins that are required for execution of the cell death program. However, not all proteins are cleaved by caspases during apoptosis. Why only specific proteins are destined to be processed by caspases during cell death is currently not clear. Here we show that multiple caspase-like activities are involved in the processing of the PITSLRE p110 isoforms during Fas-induced apoptosis in Jurkat T-cells. Three p110 caspase cleavage sites have been mapped to the amino-terminal domain of p110 and verified by site-directed mutagenesis. Curiously, the mutagenesis studies revealed that cleavage of two juxtaposed caspase sites is necessary for the complete processing of this protein during cell death in vivo. Finally, we demonstrate that the PITSLRE p110 protein is rapidly phosphorylated during Fas-induced apoptosis in Jurkat cells and that phosphorylation of an amino-terminal portion of the protein may enhance caspase cleavage in this region.

It has been well established that a family of cellular cysteine proteases, collectively known as caspases, act in a cascade to elicit and potentiate a number of apoptotic responses (1, 2). Not all cellular proteins are targets of these caspases. Those proteins that are targets generally fall into three categories: 1) other caspases, 2) proteins whose function must be ablated for cell death to occur, and 3) proteins whose function is required for execution of the cell death program. More recently, interest has been focused on the possible relationship between phosphorylation and caspase cleavage (3). Serine phosphorylation of IκB-α was shown to inhibit its cleavage by caspase-3 in vitro, whereas the unphosphorylated form of the protein is readily cleaved. Thus, phosphorylation of certain caspase targets may be part of an important cellular mechanism for controlling apoptosis. Furthermore, a number of protein kinases have been identified as caspase substrates, and their processing by these proteases frequently generates active forms of these kinases by removing autoinhibitory domains (4–12). Ectopic expression of these activated protein kinases induces many of the biochemical and cellular changes associated with cell death, suggesting that they participate in regulating some aspect of apoptotic signaling (9, 10, 13–15). Based on these studies, it is tempting to speculate that caspase cleavage and activation of specific protein kinases/phosphatases during apoptosis facilitate caspase processing of certain protein targets.

The PITSLRE protein kinases constitute a large family of p34cdc2-related kinases whose function(s) appear to be somewhat diverse (4, 13, 16–18). The larger PITSLRE p110 isoforms may function to regulate some aspect of RNA splicing/transcription during the cell cycle (19), and they are cleaved by caspase-1 and caspase-3 during TNFα-induced cell death (20). In addition, ectopic expression of a PITSLRE p50 isoform that resembles the final caspase-modified product has been shown to induce apoptosis (13). Fas-mediated T-cell death has also been correlated with PITSLRE proteolysis and an increase in its histone H1 kinase activity (4). In the present study, we determined the in vivo caspase cleavage sites involved in PITSLRE p110 proteolysis during Fas-induced apoptosis and the possible relationship between the phosphorylation and proteolysis of these p110 isoforms. Intriguingly, phosphorylation of the amino-terminal domain of p110 during Fas-induced death appears to facilitate its processing by caspases in vivo. In addition, the p110 isoforms are rapidly serine-phosphorylated once Fas-induced apoptosis is initiated in Jurkat T-cells. Others have recently shown that phosphorylation of IκB-α by MEKK1-activated IκB-α kinase in vitro blocked its cleavage by caspase-3 (3). Taken together, these studies suggest that phosphorylation of specific proteins during Fas-induced apoptosis may help to regulate their processing by caspases in vivo.

EXPERIMENTAL PROCEDURES

Cell Lines, Expression Constructs, and Transfections—Human Jurkat cells and Jt-1 cells, Jurkat cells stably transfected with the rtTA transactivation plasmid pUHD172-1(21), were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum. For Fas-induced cell death analysis, cells at a density of 1.5 × 10⁶ were treated with an anti-Fas monoclonal antibody, CH-11 (Kamiya Biomedical Co.), at 100 ng/ml for 0, 2, 4, 6, and 8 h. Apoptotic cells were examined with a light microscope for the appearance of plasma membrane blebbing and by the TUNEL assay (22).

PITSLRE expression constructs were generated by insertion of appropriate cDNA fragments corresponding to the protein kinase into the Tet-inducible vector pUHD10–3. The PITSLRE ICE2 expression construct was made by polymerase chain reaction (PCR) amplification of the cDNA fragment containing amino acid residues 392–771 with a pair of primers containing a Kozak sequence at the N terminus and a FLAG tag at the C terminus. The amplified cDNA fragment was then inserted into the pUHD10–3 vector at EcoRI and XbaI sites, and its sequence

*This work was supported by National Institutes of Health Grant GM 44088 and Cancer Center Core Grant CA 21765 and by the American Lebanese Syrian Associated Charities. 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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† The abbreviations used are: TNFα, tumor necrosis factor α; PCR, polymerase chain reaction; FACS, fluorescence-activated cell sorter; PAGE, polyacrylamide gel electrophoresis; mAb, monoclonal antibody; TPCK, N-tosyl-L-phenylalanyl chloromethyl ketone; TUNEL, terminal deoxynucleotidyltransferase (TdT)-mediated dUTP nick end-labeling; ZFA-fmk, Ac-Phe-Ala-Fluoromethyl ketone; YYAD-fmk, Ac-Tyr-Val-Ala-Asp-fluoromethyl ketone; IETD-fmk, Ac-Ile-Glu-Thr-Asp-fluoromethyl ketone; DEVD-fmk, Ac-Asp-Glu-Val-Asp-fluoromethyl ketone.
integrity was confirmed by DNA sequence analysis. Using the same strategy, additional C-terminal FLAG-tagged expression constructs of PITSLRE, including the p58-FLAG construct containing amino acid residues 339–711, N419, residues 1–419), N370 (residues 1–370), N290 (residues 1–290) were generated.  

Transient transfection of Jurkat cells with PITSLRE expression constructs was performed by electroporation using Gene Pulser™ (Bio-Rad). Typically, 10⁶ cells were resuspended in 0.3 mL of cell growth medium containing 12 μg of the designated plasmid DNA were electroporated using a 4-mm gap cuvette at 0.25 kV and 960 microfarads. Cells were then cultured in 12 mL of growth medium for 48 h in the presence of 1 μg/ml of geneticin. The transfectants were assayed for transfection efficiency by SDS-PAGE and transferred to an Immunobilon-P membrane. Proteins were detected by autoradiography at ~80 °C with a pair of amplifying screens overnight. The area of the membrane containing phosphorylated PITSLRE p110 protein was then destained with a 4% SDS sample buffer. Samples were then subjected to SDS-PAGE separation followed by immunoblotting with the P2N100 antibody.  

**RESULTS**

**PITSLRE Kinases Are Processed by DEVD-, YVAD-, and IETD-sensitive Caspases during Fas-Induced Death**—We were initially interested in determining whether caspase cleavage of the PITSLRE p110 isoforms during Fas-mediated apoptosis resembled what was previously observed during TNFα-induced cell death (20). Mapping of these caspase cleavage sites was accomplished through analysis of cell lysate from Jurkat cells treated with various caspase inhibitors and transient expression of numerous PITSLRE expression constructs in these same cells. To determine whether Asp391 (Asp392 in the PITSLRE α2-1 cDNA used by Beyaert et al. (20)) cleaved dur-ing TNF-induced apoptosis was also cleaved during Fas-medi-ated death, we examined the proteolysis of exogenously expres-sed PITSLRE β1 (p58-FLAG) and a truncated form of the PITSLRE p110 protein that mimics the Asp392 cleavage (ICE2-FLAG) (Fig. 1A). Both of these exogenously expressed PITSLRE proteins were tagged at their carboxyl terminus with a FLAG epitope that is recognized by commercially available anti-FLAG polyclonal and monoclonal antibodies. As expected, the p58-FLAG protein containing the equivalent Asp393 was readily cleaved, whereas the ICE2-FLAG protein, which mimicked cleavage at this site, was not (Fig. 1A). Therefore, this caspase cleavage site must be localized to a region between residues 339 and 391 of the PITSLRE α2-2 p110 protein. Cell death was confirmed by monitoring the cells using the TUNEL assay (see “Experimental Procedures”). To help in determining the nature of this cleavage site, peptide inhibitors that block all or specific caspases (e.g. zVAD, DEVD, and YVAD (1, 25–29)) were used to treat the Jurkat Jr-1 cells prior to and during anti-Fas ab treatment. Two additional peptide inhibitors, ZFA and leupeptin, were used as controls. As expected, ZVAD effectively blocked cleavage (data not shown), and 50 μM DEVD-fmk, which preferentially inhibits caspase-3 (27), also inhibited the formation of the caspase-processed fragment (Fig. 1B). We also examined a D562N p58-FLAG mutant, which effectively disrupts kinase activity, to determine whether in addition to kinase activity influences the cleavage process. Caspase-processing of the D562N p58-FLAG protein and DEVD inhibition of its cleavage were identical to what was observed for the wild-type protein (Fig. 1B). Whereas neither of the control peptides inhibited caspase-directed cleavage at concentrations that were comparable to those of ZVAD and DEVD-fmk, high concentrations of ZFA (10–20-fold higher than either ZVAD or
cells. These cells either were left untreated or were treated with Me2SO containing a D562A mutation (4) were transiently expressed in Jt-1 FLAG proteins. Wild-type p58-FLAG and dominant negative p58-FLAG with an anti-FLAG monoclonal antibody (M2). The intact and caspase-

duce cell death. Expressed proteins were detected by Western blotting with an agonistic anti-Fas monoclonal antibody for the times indicated to in-

392 of p110. FLAG-tagged p58 and ICE2 were expressed in Jt-1 cells as age site is located in the region between amino acid residues 339 and

PITSLRE p110 during Fas-induced apoptosis.

A,' wt

spond to the Asp391 site were observed (Fig. 2, upper panels). However, when the PITSLRE p110 isoforms were exam-

If only the Asp 391 site was utilized during Fas-induced ap-

A similar analysis was performed to identify the more aminoterminal caspase cleavage site(s). Careful examination of the amino-terminal portion of the PITSLRE p110 protein (16), from residues 1 to 391, revealed the presence of three possible caspase cleavage sites (Ser38-Asp-Asp-Arg-

mAb treatment, the p110, p64, and p56 bands were greatly diminished, and p14 was very prominent (data not shown). Once again, various peptide inhibitors of caspase activity were used in an effort to determine the nature of these cleavage events (Fig. 2, upper panels). The peptide inhibitor zVAD-fmk has been shown to inhibit all cellular caspase activity (25, 26). As expected, 1–5 µM zVAD effectively inhibited proteolysis of p110, but the cleavage sites involved in the generation of the p56 and p64 intermediates appeared to be more sensitive to zVAD than the cleavage site for p14 (Fig. 2, first upper panel). This differential sensitivity to caspase peptide inhibitors suggests that the p110 caspase cleavage sites involve multiple enzymes. A 10 µM concentration of IETD, a peptide inhibitor that preferentially blocks caspase-8 (33), was required to block the generation of p14, p56, and p64 (Fig. 2, first upper panel). Formation of the p14, p56, and p64 fragments was also blocked by 10–25 µM DEVD-fmk, but even at a 50 µM concentration of YVAD-fmk, a caspase-1-sensitive inhibitor (27), the formation of the p56 and p64 fragments was not completely inhibited (Fig. 2, second upper panel). These results suggest that more than one caspase-like activity is involved in the processing of p110 during Fas-mediated apoptosis.

Identification of the in Vivo Caspase Cleavage Sites of the PITSLRE p110 Kinase—Since Asp391 had previously been identified as a caspase-3/caspase-1-like p110 cleavage site in vitro during TNF-induced apoptosis (20), we used a p58-FLAG expression construct containing an Ala residue at this location (D391A) to examine caspase cleavage during Fas-induced cell death in vivo (Fig. 3A). The FLAG tag at the carboxyl terminus allowed us to clearly distinguish the exogenous and endoge-

N370-FLAG containing residues 1–370, revealed the presence of three possible amino-terminal portion of the PITSLRE-FLAG fusion proteins were detected by immunoblotting using the anti-FLAG M2 antibody.

DEVD) appeared to inhibit the generation of the PITSLRE p14 fragment (Fig. 2). Furthermore, 10–40 μM ZFA inhibited caspase processing of poly(ADP-ribose) polymerase, whereas a 50 μM concentration did not. This rather curious effect of ZFA, a serine protease inhibitor, is similar to what has been reported when the chymotrypsin-like serine protease inhibitor TPCK was used to treat apoptotic T-cells (30). It was suggested that these effects are consistent with at least three distinct targets of TPCK, involving cell survival, internucleosomal cleavage of DNA, and modulation of apoptosis induced by different stimuli. In addition, others have shown that serine proteases (e.g. granzyme B and AP24) act both upstream and downstream of caspase-3 during apoptosis (31, 32), suggesting that ZFA may not be an appropriate negative control. When cells were treated with leupeptin, however, there was no discernible effect on caspase processing of PITSLRE p110 or poly(ADP-ribose) polymerase (Fig. 2). This is similar to what has been reported by another group studying caspase processing of protein kinase Cδ (5).

If only the Asp391 site was utilized during Fas-induced ap-


doned, and p14 was seen 1–2 h after anti-Fas mAb treatment (Fig. 2, upper panels). In these experiments, cell death was monitored by both TUNEL assay and caspase cleavage of poly(ADP-ribose) polymerase (Fig. 2, lower panels). By 6 h post anti-Fas

387, Tyr-Val-Pro-Asp391-Ser) revealed that an additional caspase cleavage site is located at Asp387. Whereas the Asp391 cleavage site most closely resembles the consensus caspase-1 recognition motif (1, 25), the Asp387 site resembles the consensus caspase-8 cleavage site (1, 25). The possible involvement of caspase-8-like activities in the processing of PITSLRE p110 is consistent with the peptide inhibitor studies described above. Therefore, we constructed p58-FLAG mutant expression constructs containing either the D387A or the D387A/D391A double mutation. The D387A mutant was much less sensitive to caspase cleavage than the D391A mutant during Fas-induced apoptosis, but a portion of the protein continued to be cleaved (Fig. 3A). When the D387A/D391A double mutant was used, all caspase processing was blocked (Fig. 3A).

A closer examination of the sequence of the p110 protein in this region (Leu-Thr-Glu-Gly-Asp387-Tyr-Val-Pro-Asp391-Ser) revealed that an additional caspase cleavage site is located at Asp387. Whereas the Asp391 cleavage site most closely resembles the consensus caspase-1 recognition motif (1, 25), the Asp387 site resembles the consensus caspase-8 cleavage site (1, 25). The possible involvement of caspase-8-like activities in the processing of PITSLRE p110 is consistent with the peptide inhibitor studies described above. Therefore, we constructed p58-FLAG mutant expression constructs containing either the D387A mutation or the D387A/D391A double mutation. The D387A mutant was much less sensitive to caspase cleavage than the D391A mutant during Fas-induced apoptosis, but a portion of the protein continued to be cleaved (Fig. 3A). When the D387A/D391A double mutant was used, all caspase processing was blocked (Fig. 3A).

A similar analysis was performed to identify the more aminoterminal caspase cleavage site(s). Careful examination of the amino-terminal portion of the PITSLRE p110 protein (16), from residues 1 to 391, revealed the presence of three possible caspase cleavage sites (Ser38-Asp-Asp-Arg-

Ser362-Asp-Leu-Gln-Asp366-Ile-Ser-Asp269-Ser). All three are consensus DXD caspase-3 cleavage sites (1). Three different amino-terminal PITSLRE-FLAG expression constructs were generated to examine this region of the protein: 1) N419-FLAG containing residues 1–419 fused to a FLAG epitope at its carboxyl terminus, 2) N370-FLAG containing residues 1–370, and 3) N290-FLAG containing residues 1–290. Only the N419-FLAG construct contains the Asp387 and Asp391 caspase sites, and cleavage at these sites should eliminate the ability to detect the FLAG-tagged polypeptide due to its very small size.
Indeed, this was the case, and no FLAG-tagged polypeptide was detected when the Jurkat cells expressing this fusion protein were treated with an agonistic anti-Fas mAb (data not shown). However, when the N370 and N290 expression constructs were used, fragments of 30 and 22 kDa were detected with the anti-FLAG antibody (data not shown). This suggested that only the most amino-terminal of the three possible caspase-3 sites is cleaved during Fas-mediated cell death in Jurkat cells. None of the conditions used resulted in the cleavage of the N370-FLAG fusion protein at either of the two possible caspase sites (data not shown).

When a D42A N370 mutant was expressed under the same conditions, only the intact unprocessed 44-kDa (p44) FLAG-tagged fusion protein was detected (Fig. 3B). Expression of a D39A N370 mutant appeared to have minimal effect on caspase cleavage, whereas expression of a D39A/D42A N370 mutant (see Fig. 4A for a map of the constructs). Indeed, this was the case, and no FLAG-tagged polypeptide was detected when the Jurkat cells expressing this fusion protein were treated with an agonistic anti-Fas mAb (data not shown). However, when the N370 and N290 expression constructs were used, fragments of ~30 and ~22 kDa were detected with the anti-FLAG antibody (data not shown). This suggested that only the most amino-terminal of the three possible caspase-3 sites is cleaved during Fas-mediated cell death in Jurkat cells. None of the conditions used resulted in the cleavage of the N370-FLAG fusion protein at either of the two possible caspase sites (data not shown). When a D42A N370 mutant was expressed under the same conditions, only the intact unprocessed 44-kDa (p44) FLAG-tagged fusion protein was detected (Fig. 3B). Expression of a D39A N370 mutant appeared to have minimal effect on caspase cleavage, whereas expression of a D39A/D42A N370 mutant.
blocked caspase cleavage of this protein (Fig. 3B). We conclude that only the Asp 42 caspase cleavage site, and not Asp 266 or Asp 269, is utilized during Fas-induced cell death in Jurkat T-cells. Therefore, the p14 and p56 fragments detected by the P2N100 antiserum (Fig. 2, upper panels) most likely correspond to the p64 fragment detected by the same antibody. It should be noted that the p14 band apparently runs anomalously on SDS-PAGE, as its predicted size is 8–10 kDa. The locations of the caspase cleavage sites relative to other structural features of the PITSLRE p110 protein kinase are shown in Fig. 3C.

**Phosphorylation of PITSLRE p110 during Fas-induced Apoptosis**—While we were mapping the amino-terminal caspase cleavage sites using the FLAG-tagged N419, N370, and N290 fusion proteins, we noticed that, under appropriate gel conditions, the broad PITSLRE-FLAG fusion protein bands (Figs. 2 and 3) resolved into multiple species (Fig. 4A and B). Furthermore, when the Jurkat cells expressing any of these three different expression constructs were treated with the agonistic anti-Fas mAb, the upper fusion protein band was cleaved at a much faster rate than the lower band (Fig. 4A). It should be noted that the decreased intensity of the N290-FLAG protein signal (Fig. 4A), as detected by the anti-FLAG antibody, is apparently the result of difficulties in detecting the FLAG epitope due to the conformation of this particular fusion protein and is not due to decreased protein expression. When the same blot was probed with a PITSLRE-specific antibody, N290-FLAG protein levels were comparable to both N370-FLAG and N419-FLAG levels (data not shown). In addition, the p24 species detected by the anti-FLAG antibody in the N419-FLAG cells treated with the agonistic anti-Fas mAb most likely corresponds to a partial caspase cleavage product that does not undergo complete caspase cleavage at either Asp 387 or Asp 391, generated by proteolysis at Asp 266 and/or Asp 269. The predicted size of this peptide (24 kDa) corresponds closely to the 150–153 amino acids encoded between these Asp residues and Glu 119 and the end of the FLAG peptide. Neither of these sites is normally cleaved during Fas-mediated apoptosis, suggesting that the N419-FLAG polypeptide has an altered conformation that allows cleavage at one of these sites and inhibits cleavage.

**Fig. 4.** Phosphorylation of the amino-terminal region of PITSLRE p110 increases its apparent sensitivity to caspase cleavage. In A, the right panel shows the map corresponding to the FLAG-tagged amino-terminal fragments of PITSLRE p110. The region of the p110 protein used to generate the P2N100 antibody is also indicated, and the vertical arrows denote in vivo caspase cleavage sites determined by mutagenesis. The sizes of the expected proteins are shown under the map. The left panel shows an anti-FLAG M2 antibody immunoblot of the three different PITSLRE-FLAG fusion proteins from Jurkat cells that were either untreated or treated with the anti-Fas CH-11 mAb for 4 h at 100 ng/ml. The positions of the fusion proteins are indicated on the left. In B, the N370 protein was immunoprecipitated from Jurkat cell lysates, 30 min following agonistic anti-Fas mAb treatment, with the anti-FLAG M2 antibody, and the immunoprecipitated proteins were left untreated (first lane), mock-treated (second lane), or treated with increasing amounts of calf intestine alkaline phosphatase as described under “Experimental Procedures.” Immunoblotting was performed using the PITSLRE-specific P2N100 antibody. A series of three bands, indicated by the arrows on the left, were resolved from the exogenously expressed N370-p44 protein. The upper band was lost when the protein was treated with calf intestine alkaline phosphatase, leaving two tightly migrating lower bands, as indicated by the arrows on the right. In C, Jurkat cells were labeled with 32P-Pi, and these cells were treated with the agonistic anti-Fas mAb, as described under “Experimental Procedures,” for the times indicated. Cellular proteins were immunoprecipitated (IP) using either a PITSLRE (P2N100) antiserum or a preimmune (pre) control serum. After separation by SDS-PAGE, the protein was transferred onto an Immobilon membrane. The phosphorylated PITSLRE p110 isoforms were detected by autoradiography. In D, the phosphoamino acid composition of the phosphorylated 110-kDa PITSLRE protein from the 1-h anti-Fas mAb time point was determined.
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at either Asp^{387} or Asp^{391}. Because these sites are not normally cleaved in the p110 polypeptide or the N290-FLAG or N370-FLAG fusion protein in \textit{vivo}, this observation was not pursued further at this time.

Our results suggested that a post-translationally modified form of the p110 amino-terminal region is cleaved by caspases preferentially. To determine whether this modification involves phosphorylation, the N370-FLAG fusion protein was immunoprecipitated with an anti-FLAG mAb from Jurkat cell lysates 30 min after agonistic anti-Fas mAb treatment. These immunoprecipitates were either mock-treated or treated with increasing amounts of alkaline phosphatase (Fig. 4B). The samples were resolved by SDS-PAGE, and the proteins were detected with the P2N100 antibody (which will detect both the exogenous FLAG-tagged p44 protein species and endogenous p110 proteins). In the lanes containing either untreated or mock-treated samples, three distinct bands were detected (Fig. 4B). When these samples were treated with increasing amounts of alkaline phosphatase before SDS-PAGE analysis, the top band collapsed (Fig. 4B). This indicates that the N370-FLAG fusion protein is phosphorylated \textit{in vivo}.

These experiments prompted us to further examine the phosphorylation status of the PITSLRE p110 isoforms during Fas-induced cell death. Jurkat T-cells were grown in the presence of \textsuperscript{32}P and cell lysate from either the untreated or anti-Fas mAb-treated cells immunoprecipitated with a preimmune control or affinity-purified P2N100 antibody (Fig. 4C). Phosphorylation of the p110 isoforms was induced within 30 min of anti-Fas mAb treatment, and by 1 h, they were quite heavily phosphorylated. Within 2 h of anti-Fas mAb treatment, phosphorylation of the PITSLRE p110 isoforms had been reduced substantially, possibly due to the cleavage of this protein by caspases. Finally, phosphoamino acid analysis of the phosphorylated p110 protein 1 h after anti-Fas mAb treatment revealed that the majority of the protein was serine-phosphorylated (Fig. 4D).

DISCUSSION

In this report, we provide evidence that PITSLRE p110 protein kinase isoforms are cleaved \textit{in vivo} by multiple caspases during Fas-mediated cell death at several sites within the amino-terminal domain of p110, that these p110 isoforms are rapidly serine-phosphorylated, and that the ability of the most amino-terminal caspase site to be cleaved appears to be affected by phosphorylation. Specifically, we have shown that at least two different caspases, possibly corresponding to two different caspases, possibly corresponding to amino-terminal caspase site to be cleaved appears to be altered by phosphorylation. One obvious possibility is that phosphorylation modifies the structure of the protein, making an otherwise inaccessible site for caspase processing available for caspase activation. Whatever the mechanism(s) responsible for this difference, the results from this study and those examining the role of phosphorylation and caspase-3 processing of I\textsubscript{x}B-\alpha (3) suggest that regions near certain caspase target sites may be modified prior to their cleavage during apoptosis.

Acknowledgment—We thank Dr. J. Lahti for helpful discussions and critical evaluation of this manuscript.

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