Inhibitor of Apoptosis Protein hILP Undergoes Caspase-mediated Cleavage during T Lymphocyte Apoptosis

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Abstract

Several endogenous or viral inhibitors of apoptosis, including Bcl-2, Bcl-xL, FLIP, p35, and CrmA, have been shown to be cleaved by caspases during apoptosis. In this study, we demonstrate that the endogenous inhibitor of apoptosis, hILP/XIAP, is also cleaved in apoptotic T lymphocytes, generating at least one prominent fragment of 29 kDa. This p29 cleaved fragment was detected in Jurkat cells induced to apoptosis by anti-Fas antibody, staurosporin, or VP-16. The cleavage of hILP appears to be caspase mediated because the production of the p29 protein was inhibited by the pan-caspase peptide inhibitor, Z-VAD.FMK. In Jurkat cells engineered to overexpress CrmA, cleavage of hILP in response to anti-Fas antibody or staurosporin was inhibited, whereas overexpression of Bcl-2 abrogated the cleavage in response to VP-16. Cleavage of hILP was also observed in cell-free reactions using in vitro translated hILP and recombinant caspase-3 or -7. Moreover, we found that the p29 hILP fragment retained the ability to bind caspase-3 and -7, as shown previously for full-length or BIR-2 hILP. The p29 cleavage product was also detected during T-cell receptor-mediated apoptosis in peripheral blood lymphocytes from normal donors. Furthermore, tumor-associated T lymphocytes purified from ascites of patients with ovarian cancer expressed fragmented hILP, which was not detected in control T cells purified from peripheral blood of normal donors. Our results suggest that the cleavage of hILP represents an important event in apoptosis of T lymphocytes in both normal and pathological in vivo settings.

Introduction

The execution of cellular apoptosis involves the activation of a cascade of intracellular proteases belonging to the caspase protease family (1, 2). Caspases are initially synthesized as inactive proenzymes, and activation involves processing to smaller active subunits. Activation of the apical proteases caspase-8 (3–7) after engagement of cell surface death receptors or caspase-9 (8, 9) after release of cytochrome c from mitochondria results in processing and activation of downstream executioner caspases including caspase-3 (8, 10). Executioner caspases cleave specific cellular substrate proteins, facilitating the demise of the cell (1, 2). A number of intracellular proteins that negatively regulate apoptosis execution, primarily by interfering with the caspase cascade, have been identified. Antia apoptotic members of the Bcl-2 protein family act to prevent release of cytochrome c from the mitochondria (11, 12) and can also bind and incapacitate Apaf-1 (13, 14), a critical cytoplasmic protein involved in cytochrome c-mediated activation of caspase-9 (8, 15). c-FLIP, a death effector domain-containing protein, prevents association of caspase-8 with cell surface death receptors, thereby blocking caspase-8 activation (16). More recently, it has been shown that members of the IAP (17) protein family bind and inhibit specific caspases (18–20).

Human IAP proteins, including hILP/XIAP (21, 22), c-IAP1, c-IAP2, NAIP, survivin, and Bruce, are characterized by the presence of one to three copies of a 70-amino acid motif, the BIR domain, which bears homology to sequences found in the baculovirus IAP proteins (reviewed in Ref. 17). The hILP, c-IAP1, and c-IAP2 proteins also contain COOH-terminal RING finger domains. hILP, c-IAP1, and c-IAP2 bind and inhibit the activated forms but not the proenzyme forms of caspase-3 and -7 (18–20). In addition, hILP, c-IAP1, and c-IAP2 bind procaspase-9, preventing processing and activation of this enzyme (18). These inhibitors also inhibit active caspase-9. However, despite demonstrations that hILP, c-IAP1, and c-IAP2 can bind and inhibit caspases, the molecular mechanism(s) of this inhibition remains unclear. Two caspase inhibitor proteins that are unrelated to IAPs, cowpox viral CrmA (23) and baculovirus p35 (24, 25), also bind directly to caspases (25). CrmA and p35 have been shown to be suicide inactivators of caspases (26–28). After binding, CrmA and p35 are proteolytically cleaved, and the cleaved products remain associated with the caspase to inhibit enzyme activity.

The hILP protein consists of three BIR domains and one RING finger domain and appears to be a more potent inhibitor of caspase-3 and -7 than c-IAP1 or c-IAP2 (19, 20). The RING finger domain is not essential for hILP binding to caspase-3 and -7, but it is important for binding to the cytoplasmic domain of bone morphogenetic protein type I receptor and may mediate functions of hILP unrelated to apoptosis (29). Of the three hILP BIR domains, the second BIR domain, but not the first or third domains, is sufficient for binding and inhibition of caspase-3 and -7 (30). Thus, only a portion of the molecule is needed for caspase inhibition and suppression of apoptosis.

In this report, we demonstrate that cellular hILP is cleaved by a caspase protease after treatment of cells with a variety of apoptotic stimuli. Similar hILP cleavage was seen in vitro using recombinant caspase-3 or -7 and in vitro translated hILP as a substrate. The cleavage products were found to remain associated with caspase-3 and -7 and were detected in peripheral blood T cells from healthy individuals stimulated to undergo AICD3 and detected in vivo in TALs. These findings demonstrate that hILP is a substrate of caspases and may act as a suicide inactivator of these enzymes.

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3 The abbreviations used are: AICD, activation-induced cell death; Ab, antibody; mAb, monoclonal antibody; PBL, peripheral blood lymphocyte; TAL, tumor-associated lymphocyte; PMA, phorbol 12-myristate 13-acetate; PVDF, polyvinylidene difluoride; OvCA, ovarian carcinoma.

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Materials and Methods

Reagents. Agonistic anti-Fas Ab (CH-11; IgM) was purchased from Upstate Biotechnology (Lake Placid, NY); Staurosporin, etoposide (VP-16), PMA, and ionomycin were purchased from Sigma (St. Louis, MO). Anti-αβ4 mAb (A9) was a generous gift from Dr. T. E. Carey (University of Michigan, Ann Arbor, MI). Inhibitors of apoptosis, including Z-VAD-FMK and Z-DEVD-FMK, were purchased from Enzyme Systems (Livermore, CA). Recombinant caspase-3 and caspase-7 were purchased from PharMingen (San Diego, CA). A mAb specific for hILP was purchased from Transduction Laboratories (San Diego, CA). Rabbit anti-caspase-3 Ab and murine anti-caspase-7 mAb were purchased from PharMingen. Anti-CD3 mAb was purchased from DAKO (Carpinteria, CA).

Cell Lines. Jurkat T leukemia cell line was obtained from American Type Culture Collection (Manassas, VA). Jurkat cells were grown in RPMI 1640 containing 10% FCS, 2 mM l-glutamine, and 100 units/ml each of penicillin and streptomycin (complete medium). The generation of stable cell lines expressing epitope-tagged CrmA or Bcl-2 proteins has been described previously (33). The production of stable cell lines expressing epitope-tagged CrmA or Bcl-2 proteins has been described previously (33). Transfected cell lines were maintained in complete medium supplemented with 0.5 mg/ml G418 (Life Technologies, Inc.).

Induction of Apoptosis. Jurkat cells plated at 0.5–1×10^6 cells/ml in complete medium were treated with VP-16 (20 μM), agonistic anti-Fas Ab (200 ng/ml), or staurosporin (0.5 μM) at 37°C for varying lengths of time, as indicated for each experiment. To induce AICD, 48-well plates were precoated with anti-CD3 (5 μg/ml) in 50 mM Tris (pH 9.0). PBL-T cells (1×10^6 cells/ml) were plated in wells precoated with anti-CD3 mAb in complete medium in the presence of PMA and ionomycin at concentrations of 50 ng/ml and 0.5 μg/ml, respectively (32).

Western Blot Analysis. To generate whole cell extracts, cells were lysed in 1% NP40, 20 mM Tris-base (pH 7.4), 137 mM NaCl, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin for 1 hour at 37°C with 4 μl of [35S]-labeled hILP in the presence or absence of recombinant caspases and peptide inhibitors of caspases in a total volume of 20 μl/reaction. Reactions were terminated by the addition of SDS loading buffer and boiling for 5 min. Products of the cleavage reactions were resolved on 15% SDS-PAGE, transferred to PVDF membranes, and detected by autoradiography. Alternatively, the reaction products were detected by Western blotting with anti-hILP mAb.

Patients. Ascitic fluids were obtained from patients with ovarian carcinoma at Magee Women’s Hospital, University of Pittsburgh Medical Center (Pittsburgh, PA). The ovary was the primary site of malignancy for all patients. The patients were untreated at the time of specimen collection. The study was approved by the Institutional Review Board for human use at the University of Pittsburgh Medical Center.

Isolation of TAL-T or PBL-T Cells. Ascitic fluid cells were washed twice in RPMI 1640, placed on Ficoll-Hypaque discontinuous density gradients, and centrifuged to harvest TAL and tumor cells as described previously (34). To select for T lymphocytes, TALs were incubated in the presence of anti-CD14, anti-CD16, anti-CD19 (10 μg/ml; DAKO) and anti-αβ4 mAbs (50 μg/ml) for 30 min at 0°C. The cells were then washed twice and incubated with magnetic beads coated with goat antimouse immunoglobulins (1 cell:30 beads; PerSep Diagnostics, Cambridge, MA) for 30 min at 0°C. After each of two successive incubations with magnetic beads, a magnet was used to collect beads containing attached cells. T cells from peripheral blood on normal donors were purified by a similar procedure. As assessed by flow cytometry, the negatively selected T cells were 99% CD3 positive.

Results and Discussion

Cleavage of hILP in Apoptotic Jurkat Cells. To determine the fate of cellular hILP protein during apoptotic execution, Jurkat T leukemic cells were stimulated with 200 ng/ml agonistic anti-Fas Ab for varying lengths of time. After stimulation, whole cell lysates were prepared and analyzed by immunoblotting using mAb raised against amino acids 268–426 of the human hILP protein. As expected, full-length hILP was detected as a 57-kDa protein (Fig. 1A). After 16 h of stimulation with anti-Fas Ab, the level of full-length hILP was

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Fig. 1. Cleavage of hILP protein during apoptosis induced by anti-Fas Ab, VP-16, or staurosporin. Jurkat T cells were treated 200 ng/ml agonistic anti-Fas mAb, 20 μM VP-16, or 0.5 μM staurosporin at 37°C for varying lengths of time. After treatment, whole cell lysates were prepared. Proteins (25 μg/lane) were resolved on 15% SDS-PAGE gels and transferred to PVDF membranes. In A, the membranes were probed with anti-hILP mAb. In B, the membranes were stripped and reprobed with polyclonal anti-caspase-3 Ab.
Inhibition of hILP Cleavage by Z-VAD.FMK. Jurkat cells were treated with anti-Fas Ab (200 ng/ml), staurosporin (0.5 μM), or VP-16 (20 μM) in the presence or absence of Z-VAD.FMK (50 μM). Whole cell lysates were electrophoresed on 15% SDS gels and analyzed by immunoblotting using anti-hILP mAb.

To further examine the involvement of caspases in hILP cleavage, we studied the effects of two inhibitors of caspase activation, Bcl-2 and CrmA. Bcl-2 inhibits caspase activation by blocking release of cytochrome c from the mitochondria and may also interact with Apaf-1, disrupting caspase-9 activation (11–14). Because Bcl-2 blocks cytochrome c release, it is a potent inhibitor of stimuli that primarily use the mitochondrial pathway of apoptosis, such as chemotherapeutic agents. By contrast, Bcl-2 is much less efficient at inhibiting Fas-mediated apoptosis, which is not dependent on the mitochondrial pathway. The CrmA protein binds and potently inhibits caspase-8, the apical caspase in Fas-mediated signaling (25). Thus, CrmA strongly inhibits anti-Fas-induced apoptosis. On the other hand, chemotherapy-induced caspase activation and apoptosis are only modestly inhibited by CrmA.

Jurkat cells engineered to overexpress Bcl-2 or CrmA (31) were treated with anti-Fas Ab, staurosporin, or VP-16, followed by immunoblot analysis with anti-hILP (Fig. 3). As a control, cells transfected with vector alone (Neo) were also analyzed. Bcl-2 overexpression completely abrogated hILP cleavage in response to VP-16 treatment. Considerably less protection was seen in Bcl-2/Jurkat cells treated with anti-Fas or staurosporin. CrmA, on the other hand, dramatically inhibited hILP cleavage in the anti-Fas- and staurosporin-treated cells but had little impact in VP-16-treated cells. Taken together, these results support the conclusion that cleavage of hILP in apoptotic cells is mediated by a caspase protease.

Cleavage of hILP by Recombinant Caspases. To identify caspases involved in hILP cleavage, in vitro translated 35S-labeled hILP protein was prepared and incubated with active, recombinant caspase-3 or -7. As assessed by autoradiography, a 29-kDa fragment was generated by each of the two caspases (Fig. 4, A and B). Production of the cleavage fragments was inhibited by the caspase inhibitor Z-DEVD.FMK. It appears that in vitro translated hILP is more accessible to recombinant caspases than the endogenous hILP is to endogenous caspases because additional proteolytic fragments were detected in vitro. However, like the endogenous fragment, the 29-kDa cleavage fragment generated from in vitro translated hILP was detected by anti-hILP mAb (Fig. 4, C and D).

Cleavage of hILP to a 29-kDa fragment was also observed after treatment with 20 μM VP-16 or 0.5 μM staurosporin (Fig. 1A). The 29-kDa fragment was first detected after 4 h of treatment with VP-16 and after 2 h of treatment with staurosporin. Processing of caspase-3 coincided with production of the hILP fragment.

Inhibition of hILP Cleavage by Bcl-2 and CrmA. Clonal Jurkat cell lines engineered to overexpress Bcl-2 or CrmA (31) were treated as described in the Fig. 2 legend. Jurkat cells transfected with vector alone (Neo) served as control. After treatment, whole cell extracts were resolved by SDS-PAGE, transferred to PVDF membranes, and probed with anti-hILP mAb.
Because hILP has been reported to bind caspase-3 and caspase-7 (20, 30), we examined whether the p29 fragment maintains its ability to physically associate with caspases. To this end, in vitro translated $^{35}$S-labeled hILP was treated with recombinant caspase-3 or caspase-7. The caspases were then immunoprecipitated by a specific anti-caspase Ab and resolved on SDS gels. The $^{35}$S-labeled coimmunoprecipitated proteins were examined by autoradiography. As shown in Fig. 5, the p29 fragment was not only immunoprecipitated by anti-hILP Ab but was also coimmunoprecipitated with either caspase-3 or caspase-7. These results demonstrate that although hILP is cleaved by caspases, the p29 product remains associated with the cleaving enzyme. Thus, hILP may function in a fashion similar to that of CrmA and p35, with the cleaved products remaining bound to the enzyme and inhibiting the caspase activity. Alternatively, hILP may behave like Bcl-2, losing its antiapoptotic activity after cleavage and perhaps even becoming proapoptotic (37).

Cleavage of hILP in T-cell Receptor-mediated Apoptosis. To investigate the relevance of hILP cleavage in normal T lymphocytes, peripheral blood T cells from healthy individuals were stimulated to undergo AICD by incubation with immobilized anti-CD3 mAb in the presence of PMA and ionomycin for 14 h at 37°C (32). As assessed by Western blot analyses performed on whole cell extracts, the p29 fragment was detected in T cells activated via the T-cell receptor (Fig. 6A, Lane 1), but not in control cells stimulated with only PMA and ionomycin (Fig. 6A, Lane 4). Interestingly, in human peripheral blood T cells, an additional p45 hILP protein was detected in cells treated with anti-CD3, VP-16, or staurosporin, suggesting that hILP may be subjected to cleavage before production of the p29 fragment. In T lymphocytes pretreated with the pan-caspase inhibitor Z-VAD.FMK before stimulation of AICD, no cleavage products of hILP were detected (data not shown).

Detection of p29 hILP in OvCA Ascitic TALs. To further investigate the physiological significance of hILP cleavage, we examined T cells purified from either OvCA ascitic TALs or T cells from periph-

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**Fig. 4.** In vitro translated $^{35}$S-labeled hILP is cleaved by recombinant caspase-3 or caspase-7. In vitro translated $^{35}$S-labeled hILP was treated for 1 h with recombinant caspase-3 (A) or caspase-7 (B; 0.2 μg enzyme/reaction) in the presence or absence of Z-DEVD.FMK (50 μM). The reaction products were resolved by SDS-PAGE and transferred to PVDF membranes. The membranes were analyzed by autoradiography (A, B, and D) or by Western blotting with anti-hILP mAb (C).

**Fig. 5.** Association of the p29 cleavage product of $^{35}$S-labeled hILP with recombinant active subunits of caspase-3 or caspase-7. In vitro translated $^{35}$S-labeled hILP was treated for 1 h at 30°C with recombinant caspase-3 (top) or recombinant caspase-7 (bottom). After treatment, immunoprecipitating Abs specific for hILP, caspase-3, or caspase-7 were added. Immune complexes were selected using either protein G (for anti-hILP or anti-caspase-3 mAb) or protein A (for anti-caspase-3 Ab). The immune complexes were resolved on 15% SDS gels, transferred to PVDF membranes, and examined by autoradiography. As controls, the in vitro-translated $^{35}$S-labeled hILP was subjected to no immunoprecipitation or immunoprecipitated with anti-hILP, mouse IgG, or rabbit IgG. The arrow indicates the p29 cleavage product immunoprecipitated by anti-hILP or coimmunoprecipitated with either anti-caspase-3 or anti-caspase-7.
blood T cells undergoing AICD and also in TALs demonstrate that hILP proteolysis occurs in vivo. Thus, hILP cleavage may be fundamentally important to the process of apoptosis in both normal and pathological in vivo settings.

**Note Added in Proof:** While this manuscript was under review, a similar pattern of caspase-mediated hILP/XIAP cleavage was reported by Deveraux et al. (Q. L. Deveraux et al., EMBO J., 18: 5242–5251, 1999).

**References**


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