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Macromolecular Substrates for the ICE-Like Proteases During Apoptosis

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Abstract The interleukin-1β-converting enzyme (ICE) family of proteases is an important component of the mechanism of the apoptotic process, but the physiologic roles of the different homologs during apoptosis remain unclear. Significant information about the roles of proteolysis in apoptosis will be gained through identification of the distal substrates through which these proteases achieve their pro-apoptotic effects. Identification of these substrates therefore remains an important challenge. A subset of autoantibodies from patients with systemic lupus erythematosus (SLE) recognize molecules that are specifically cleaved early during apoptosis. Several of the identified autoantigens are nuclear proteins (PARP, U1-70 kDa, and DNA-PK_{cs}) that are substrates for CPP32 in vitro and in apoptotic cells. Of note, these substrates are catalytic proteins involved in homeostatic pathways, suggesting that abolition of homeostasis is one fundamental feature ensuring the rapid irreversibility of the apoptotic process. Identification of the other substrates for this protease family will provide the tools to assess the roles of the different proteases in apoptotic death. J. Cell. Biochem. 64:50–54.

Proteolytic cleavage of key substrates is an important biochemical mechanism underlying the apoptotic process, and the centrality of interleukin-1β-converting enzyme (ICE)-like proteases as mediators of apoptosis has been emphasized [Kumar, 1995; Martin and Green, 1995]. Although at least six different mammalian ICE homologs have been published to date, identification of the downstream substrates cleaved by the different ICE family members remains a major challenge. The crystal structure of ICE has defined those residues in the protease that interact with the P_1-P_4 substrate amino acids, which contain most of the information required for specific recognition and cleavage by ICE [Thornberry and Molineaux, 1995]. In all ICE family members identified to date, there is absolute conservation of the residues involved with catalysis, as well as those involved in binding the carboxylate side-chain of the substrate P_1 aspartic acid [Nicholson et al., 1995]. The substrate specificity of the ICE-like proteases appears to be determined primarily by the P_1 and P_4 positions, and liberal substitutions in the P_2 and P_3 positions are tolerated [Thornberry and Molineaux, 1995]. Early studies with two well-characterized homologs indicate that the stringency of substrate recognition varies in different homologs, resulting in marked specificity of some homologs for their substrates (e.g., CPP32), and broader specificity and promiscuity of others (e.g., ICE) [Casciola-Rosen et al., 1996]. Elucidating the substrates cleaved by this enzyme family, ascribing physiologic cleavage to particular family members, and understanding the effects of cleavage on substrate function in apoptosis are of central importance in understanding the apoptotic process and its regulation.

Experimentally, the proteolytic events occurring during apoptosis may be grouped into two broad categories [Casciola-Rosen et al., 1995]: (1) early events, which occur prior to or coincident with the onset of apoptotic morphology, and prior to loss of cell integrity in in vitro models; and (2) later events, observed after the onset of apoptotic morphology. Since clearance of apoptotic cells within tissues is rapid and efficient [Arends and Wyllie, 1991], the biological significance of these delayed proteolytic events seen in in vitro systems is uncertain. Only early cleavages, which are most likely to be of mechanistic significance in the apoptotic process, will be dealt with in this review.

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PROTEOLYSIS DURING APOPTOSIS: IDENTIFICATION OF SUBSTRATES

The first protein fortuitously noted to be degraded to specific fragments in apoptotic cells was poly(ADP-ribose)polymerase (PARP) [Smith et al., 1992], an enzyme that is involved in genome surveillance and DNA repair [Satoh and Lindahl, 1992]. PARP was subsequently shown to be cleaved by the ICE homolog CPP32 [Tewari et al., 1995; Nicholson et al., 1995]. Several different approaches have since been successfully used to identify other substrates for the ICE family of proteases during apoptosis.

Lupus Autoantibodies Recognize Molecules Specifically Cleaved Early During Apoptosis

We previously demonstrated that the autoantigens targeted in SLE are clustered in structures at the surface of apoptotic cells [Casciola-Rosen et al., 1994a]. We proposed that this "clustered targeting" of unique autoantigens by the immune system might reflect the susceptibility of these particular molecules to common biochemical modifications in these apoptotic microenvironments [Casciola-Rosen et al., 1994a]. We subsequently showed that a subset of infrequently targeted autoantigens was united by their specific proteolytic cleavage early during apoptosis [Casciola-Rosen et al., 1995]. All cleavages (1) were specific to apoptosis, and were not observed in necrosis or other forms of cell damage or lysis; (2) occurred early during apoptosis, prior to or coincident with the onset of morphologic changes; (3) resulted in specific fragments which were not further processed; and (4) were abolished by iodoacetamide or inhibitors of the ICE family of enzymes. These features suggested that all cleavages were downstream of an ICE-like protease(s), either directly or via activation of other intervening proteases. One of the targeted molecules recognized by this approach was PARP, a known autoantigen and target for the ICE-like enzymes during apoptosis (see below). Several other cleaved molecules were identified by this approach. These included U1-70 kDa (essential for the splicing of precursor mRNA) and the catalytic subunit of the DNA-dependent protein kinase (DNA-PK_{cs}) [which functions in DNA double-strand break repair and V(D)J recombination]. In addition, eight proteins that are cleaved with identical characteristics remain unidentified; these include proteins of 380, 320, 220, 190, 170, 130, and 80 kDa. Identification of these other substrates, and characterization of the consequences of cleavage on their function, is likely to provide further insights into the role of proteolysis in apoptosis. Definition of the cleavage sites in each of these substrates and identification of the proteases responsible will clarify whether a protease cascade exists during apoptosis, and whether non-ICE family proteases have a role in the apoptotic process.

The "Candidate Substrate" Approach

Using this approach, several substrates for the apoptotic proteases have been identified based on the observed downstream biochemical and morphologic features that are characteristic of apoptosis. For example, the striking surface blebbing characteristic of the apoptotic cell suggested an alteration in the membrane skeleton, and led to the identification of fodrin (nonerythroid spectrin) cleavage as an early proteolytic event in apoptosis [Martin et al., 1995]. Similarly, lamin cleavage during apoptosis was demonstrated, explaining nuclear lamina disassembly during early apoptosis [Kaufmann, 1989]. The recent identification of cleavage of Gas2 at Asp^{279} by an as yet unidentified proteolytic activity seeks to explain some of the striking rearrangement of the actin cytoskeleton occurring during apoptosis [Brancolini et al., 1995]. In vitro ICE-mediated cleavage of actin itself has recently been demonstrated [Kayalar et al., 1996]. Since actin is an endogenous inhibitor of DNAse I, the authors have suggested that cleavage of actin during apoptosis may liberate active DNAse I, thereby contributing to the characteristic DNA degradation [Kayalar et al., 1996]. The candidate substrate approach might be fruitfully extended to other phenomena observed in apoptosis, including phosphatidylserine redistribution, cytosolic shrinkage, intracellular acidification, and nuclear scaffold disassembly.

PARP, U1-70 KDA, AND DNA-PK_{CS} ARE CLEAVED BY A PROTEASE(S) WITH CHARACTERISTICS OF CPP32 IN APOPTOTIC CELLS

Although at least 12 proteins have been shown to be specifically cleaved early during apoptosis, with inhibition characteristics typical of the ICE family of enzymes, only four

substrates have thus far been demonstrated to be directly cleaved by ICE homologs in vitro. These include PARP [Tewari et al., 1995; Nicholson et al., 1995], U1-70 kDa [Casciola-Rosen et al., 1994b], the catalytic subunit of DNA-dependent protein kinase (DNA-PK_{cs}) [Casciola-Rosen et al., 1995], and lamin B and lamin A [Lazebnik et al., 1995]. Since the expression of the different ICE homologs in different cells and tissues may vary, and since substrate specificity of the homologs may overlap (with varying degrees of promiscuity shown toward dissimilar substrates), great caution must be exercised when ascribing physiologic cleavage of substrates in apoptotic cells to a particular homolog. It is therefore essential to quantitatively determine for each substrate (1) the catalytic cleavage efficiencies by the different homologs in vitro, and (2) the IC_{50} values for inhibition of cleavage of each substrate by a profile of tetrapeptide aldehyde inhibitors in a permeable apoptotic cleavage assay. Using this approach, we have demonstrated that CPP32 efficiently cleaves PARP, U1-70 kDa, and DNA-PK_{cs} with similar k_{cat}/K_m values. Although ICE is also capable of cleaving these substrates, it does so 30-50-fold less efficiently [Casciola-Rosen et al., 1996]. The activity responsible for physiologic cleavage of PARP, U1-70 kDa, and DNA-PK_{cs} in apoptotic cells was investigated by determining the inhibition profile of the responsible activity in extracts of apoptotic cells incubated in vitro [Casciola-Rosen et al., 1996]. Furthermore, cleavage of U1-70 kDa, DNA-PK_{cs}, and PARP was abolished by nanomolar concentrations of Ac-DEVD-CHO (IC_{50} values of 0.2–1 nM), but only by >10 μM Ac-YVAD-CHO, confirming that the physiologic activity responsible for cleavage of these substrates during apoptosis is CPP32 or CPP32-like [Casciola-Rosen et al., 1996]. The recent identification by a number of laboratories of an ICE homolog that is highly homologous to CPP32 in the substrate-binding domain, and has a similar IC_{50} value for inhibition by Ac-DEVD-CHO, reinforces the fact that other homologs might also contribute to the cleavage of these substrates in apoptotic cells.

To date, all the proven substrates for the ICE-like enzymes in apoptosis are structural or catalytic nuclear proteins, whose cleavage fragments are found in apoptotic bodies [Casciola-Rosen et al., 1995], suggesting that cleavage is a nuclear event. Interestingly, ICE is found only in the cytosol, and no nuclear localization signal is present in members of the ICE family. However, the precise subcellular locations of the other ICE family members have not yet been conclusively demonstrated. It is likely that CPP32 may be activated within the nucleus, or that the active protease gains access to substrates at this site.

CPP32 CLEAVES REPAIR PROTEINS AT A DXXD MOTIF

The CPP32 cleavage sites within DNA-PK_{cs}, U1-70 kDa, and PARP are strikingly similar, and include DEVDN (DNA-PK_{cs}) [Casciola-Rosen et al., 1996], DGPDG (U1-70 kDa) [Casciola-Rosen et al., 1996], and DEVDG (PARP) [Lazebnik et al., 1994]. In all cases, aspartate is found in P_1 and P_4, suggesting the requirement of a DXXD motif for cleavage specificity by CPP32. A unifying feature of these CPP32-mediated cleavages is that important functional domains are separated from the remainder of the molecule. For example, CPP32-mediated cleavage of PARP results in the separation of the two zinc finger DNA-binding motifs (24 kDa) from the C-terminal catalytic domain (89 kDa) [Nicholson et al., 1995]. Similarly, cleavage of DNA-PK_{cs} separates the PI3 kinase-like domain (160 kDa) from the remainder of the molecule (250 kDa) [Casciola-Rosen et al., 1996], and decreases kinase activity [Casciola-Rosen et al., 1996]. CPP32-mediated cleavage of U1-70 kDa separates the N-terminal RNA recognition motif (40 kDa) from the second C-terminal RSD-rich domain (22 kDa), which potentially has a dominant negative effect on mRNA splicing [Romac and Keene, 1995]. All the CPP32 substrates identified to date function in homeostatic pathways: PARP and DNA-PK_{cs} are required for double-strand DNA break repair, and U1-70 kDa is essential in mRNA splicing. Since mRNA processing is essential for expression of the transcriptional homeostatic response, it is possible that the cleavage of U1-70 kDa abrogates this response. The rapid macromolecular degradative pathways initiated in apoptosis (e.g., specific proteolysis of cytoskeletal and membrane skeletal components, as well as internucleosomal DNA cleavage) may well be responsible for the stereotyped morphology of apoptosis, and imply that macromolecule degradation far outpaces repair. We propose that the focused crippling of homeostasis by CPP32 (and potentially other similar homologs) may be a

fundamental feature ensuring the rapid irreversibility of the apoptotic process.

ARE ACTIVE ICE FAMILY MEMBERS ALWAYS ASSOCIATED WITH APOPTOSIS?

Although all members of the ICE family have the capacity to induce apoptosis in different transfected systems, the mere presence of the active protease in vivo is not necessarily accompanied by apoptosis. For example, although ICE-mediated processing of pro-IL1β clearly can occur in apoptotic macrophages, normal processing and secretion of mature IL1β most frequently occurs in cells that show no features of apoptosis [Singer et al., 1995]. The ICE knockout mouse has demonstrated normal apoptosis in almost all developmental and induced systems, but a striking decrease in the secretion of both IL1α and IL1β [Li et al., 1995; Kuida et al., 1995]. This has suggested that ICE has a critical, though still undefined, role in the secretion of these cytokines, but little nonredundant role in apoptosis. It is likely that a number of the other described homologs will also have primary roles in normal, nonapoptotic cell functions; a clearer understanding will await the identification of physiologic substrates for these other homologs, and creation of knockout mouse models. The recent description of cleavage of sterol regulatory element–binding proteins (SREBPs) by the hamster homolog of CPP32 isolated from nonapoptotic liver cytosol initially raised the question of whether CPP32 might have physiologic roles in addition to apoptosis [Wang et al., 1995]. The subsequent studies have clearly demonstrated that SREBPs are cleaved by CPP32 during apoptosis, at a site distinct from that cleaved during cholesterol homeostasis [Wang et al., 1996]. The elucidation of the role of SREBP cleavage during apoptosis may provide significant insights into several aspects of the apoptotic process, including transcriptional activation, plasma membrane alterations, and possibly phagocytosis and clearance by surrounding cells [Wang et al., 1996].

PROTEOLYSIS AND APOPTOSIS: FUTURE DIRECTIONS

The identification of the downstream substrates for the ICE family, whose aggregated cleavage abolishes critical cell structures and functions, thereby generating apoptosis, remains a major challenge. Information about the sites and kinetics of cleavage, the responsible protease(s), and the functional consequences of cleavage on these substrates will reveal the roles of ICE family proteases in apoptosis. These studies will also indicate whether ICE (and non-ICE) family proteases are part of an apoptotic protease cascade. Studies to define the cell biology of activation of the proteases, as well as the mechanism of their access to nuclear substrates, are major priorities. At this early phase in the definition of the complex pathways of apoptotic proteolysis, care must be exercised not to attempt to oversimplify to a single protease–single substrate model. The existence of multiple ICE homologs, with potentially overlapping specificities and promiscuity, differential tissue expression, and potential for participation in a protease cascade, predicts that a large amount of quantitative data will need to be accumulated. The experiments are certain to define the proteolytic routes to cell death, thereby highlighting the essential cellular processes upon which continued life as a cell depends.

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