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Antinuclear autoantibodies: probes for defining proteolytic events associated with apoptosis

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Abstract

Antinuclear autoantibodies (ANAs) derived from patients with systemic autoimmune diseases have proven to be powerful tools in cell and molecular biology. The availability of these autoantibodies has been instrumental in the identification and characterization of a wide range of intracellular proteins involved in essential cellular activities. Recently, these autoantibodies have been used in molecular studies of apoptosis, particularly in the identification of substrates cleaved by proteases of the ICE/CED-3 family during this cell death pathway. The identification of these substrates may help to understand the role of proteolysis in apoptosis. Examples of nuclear autoantigens whose cleavage during apoptosis have been defined using ANAs include the 70 kD protein of the U1 small nuclear ribonucleoprotein particle (U1-70 kD), the nuclear mitotic apparatus protein (NuMA), DNA topoisomerase I, the RNA polymerase I upstream binding factor (UBF), and the catalytic subunit of DNA-dependent protein kinase (DNA-PK_{cs}). The use of ANAs as probes for defining proteolytic events associated with apoptosis promises to yield important insights into the mechanisms driving this cell death pathway.

Abbreviations: ANAs – Antinuclear autoantibodies; DNA-PK_{cs} – DNA-dependent protein kinase; ICE – interleukin-1β (IL-1β)-converting enzyme; MARs or SARs – matrix or scaffold attachment regions; NuMA – nuclear mitotic apparatus protein; PARP – poly (ADP) ribose polymerase; snRNPs – small nuclear ribonucleoproteins; SLE – systemic lupus erythematosus; UBF – upstream binding factor.

Introduction

Systemic autoimmune diseases such as systemic lupus erythematosus (SLE), Sjogren's syndrome, and systemic sclerosis (scleroderma) are characterized by a humoral immune response manifested by the production of high titers of ANAs [1]. These autoantibodies have been used extensively to screen cDNA expression libraries, which has led to the rapid identification of the molecular structure of many autoantigens. The use of ANAs as probes in molecular and cell biology has significantly contributed to our understanding of important cellular processes such as mRNA splicing, rRNA biosynthesis and processing, DNA replication, cell division, and more recently, apoptosis [1–5]. Apoptosis is a physiological form of cell death defined by distinctive morphological features including cell shrinkage, cytoplasmic membrane blebbing, and chromatin condensation and segmentation [6]. At the molecular level, apoptosis is characterized by the activation of endonucleases that degrade DNA into nucleosome units and by proteolysis of specific substrates [7, 8]. The elucidation of proteolytic events associated with apoptosis is currently the focus of intensive investigation and ANAs are being used in several laboratories as probes for defining these events.

Proteolysis and apoptosis

Although the exact molecular mechanisms underlying apoptosis still remain unclear, the available evid-

Table 1. The ICE/CED-3 family of proteases

Protease	Active site	Known substrates
ICE	QACRG	pro-ICE, pro-IL-1β, PKC-δ^a, actin
CED-3 (*C. elegans*)	QACRG	pro-CED-3, pro-CPP32, PARP
CPP32 / Yama / Apopain	QACRG	PARP, DNA-PK$_{cs}$, U1-70 kD, SREBP-1^a, SREBP-2^a, D4-GDI^a
Mch-2 (CAP in hamster)	QACRG	PARP, lamin A, pro-CPP32, pro-ICE-LAP-3
Mch-3 / ICE-LAP-3 /CMH-1	QACRG	PARP, U1-70 kD
Mch-4	QACQG	pro-CPP32, pro-Mch-3
ICE $_{rel}$II / TX / ICH-2	QACRG	pro-ICE, pro-TX
ICE $_{rel}$III	QACRG	Unknown
ICE-LAP 6	QACGG	PARP
ICH-1 (NEDD-2 in mouse)	QACRG	Unknown
FLICE / MACH	QACQG	PARP

Based on references 8–28. ^{a}Abbreviations: PKC-δ: protein kinase Cδ, SREBP: sterol regulatory element-binding protein, D4-GDI: GDP dissociation inhibitor.

ence strongly implicate the activation of a proteolytic cascade as an important event in the control of this cell death process [8–11]. The principal proteases involved in this cascade appear to be the members of the interleukin-1β (IL-1β)-converting enzyme (ICE) protease family. ICE is a mammalian cysteine protease responsible for the processing of pro-IL-1β to an active cytokine by proteolytic cleavage at aspartate residues. Members of the ICE family share sequence homology with CED-3, a cysteine protease involved in the control of cell death in the nematode *Caenorhabditis elegans* [12]. Table 1 summarizes the known ICE/CED-3 proteases. Some of these proteases have been identified simultaneously in different laboratories, which explains the multiplicity of names and suggests that the ICE/CED-3 family may not be very large. A key structural feature of these proteases is the presence of a conserved QACRG pentapeptide containing the active Cys residue. The role of ICE/CED-3 proteases in apoptosis has been established by the ability of some of these proteases to initiate cell death when overexpressed in mammalian cells and by their sensitivity to specific inhibitors that block apoptosis. Activation of the ICE/CED-3 proteases during apoptosis requires proteolytic processing to a two-subunit form possessing proteolytic activity, and can be mediated by granzyme B [13–17]. Non-ICE/CED-3 proteases such as calpains, serine proteases, and ubiquitin-dependent proteases have also been implicated in apoptosis [8].

Current challenges in apoptosis research include defining the mechanisms leading to the activation of the proteolytic cascade, characterizing the different proteases acting in this cascade, and identifying the substrates cleaved by these proteases. The identification of these substrates is important for understanding the exact role of proteolysis in apoptosis. Thus far, only a relatively small number of intracellular substrates have been shown conclusively to be cleaved during apoptosis. As reviewed below, these substrates include nuclear proteins targeted by autoantibodies present in sera from patients with systemic autoimmune diseases.

Selective proteolysis of nuclear autoantigens during apoptosis

Rosen and colleagues showed by confocal microscopy, using human autoantibodies as probes, that specific autoantigens targeted in SLE such as DNA, SSA/Ro, SSB/La, ribosomes, and the small nuclear ribonucleoproteins (snRNPs), were clustered on blebs in the surface of apoptotic cells [3, 18]. It was proposed that localization of these antigens in apoptotic blebs or bodies might make them vulnerable to biochemical modifications that could increase their immunogenicity [3]. These investigators also applied human autoantibodies to the analysis of proteolytic events associated with apoptosis. Using a highly specific human autoimmune serum they showed that the nuclear autoantigen U1-70 kD is specifically cleaved into a 40 kD fragment during apoptosis [19]. This cleavage was recently demonstrated to be mediated *in vitro* by apopain/CPP32, a member of the ICE/CED-3 family [20]. Autoantibodies have also been used by other investigators to demon-

strate the cleavage of U1-70 kD during apoptosis mediated by Fas, TNF, and granzyme B [14, 21]. In a more systematic study aimed at the identification of substrates cleaved during apoptosis, Casciola-Rosen et al. [4], evaluated 260 human autoimmune sera by immunoblotting of lysates from HeLa cells induced to undergo apoptosis by UVB irradiation. A subset of nuclear autoantigens was found to be cleaved by proteases. This subset was comprised of poly (ADP) ribose polymerase (PARP), U1-70 kD, DNA-PK_{cs}, and unidentified autoantigens of 195, 170, 130, and 75 kD. All these cleavages were shown to occur relatively early during the apoptotic process. The group of autoantigens that were not cleaved during apoptosis included SSA/Ro and SSB/La, calreticulin, ribosomal P proteins, Sm, Ku, fibrillarin, and Jo-1. This indicated that only a subset of autoantigens are cleaved by proteases during UVB-induced apoptotic cell death.

Recently, we used a panel of highly specific ANAs to well-characterized intracellular autoantigens to identify substrates cleaved by proteases during Fas-mediated apoptosis of Jurkat T cells [5]. A subset of the examined autoantigens, 7 of 33, underwent substantial cleavage during this cell death process. This subset was comprised of PARP, NuMA, lamin B, U1-70 kD, DNA topoisomerases I and II, and UBF (also known as the 90 kD antigen of the nucleolar organizer region or NOR-90). Table 2 lists the protein autoantigens that have been identified thus far as substrates cleaved during apoptosis. We observed that most of the frequently targeted autoantigens in systemic autoimmune diseases remained intact during Fas-mediated apoptosis [5]. Among these were SSA/Ro and SSB/La, the ribosomal P proteins, the subunit components of the Sm ribonucleoprotein particle, Ku, PM-Scl, fibrillarin, histone H2B, p80 coilin, proliferating cell nuclear antigen (PCNA), the major mitochondrial autoantigens, and Jo-1 (histidyl-tRNA synthetase). The selective cleavage of autoantigens during apoptosis suggests that the execution phase of this cell death pathway could be associated with inactivation of a number of important cell functions via proteolysis of key substrates.

Analysis of the relative kinetics of autoantigen cleavage after induction of apoptosis in Jurkat cells with anti-Fas antibody revealed that autoantigens were targeted by proteases at different times during the cell death process [5]. PARP, NUMA, U1-70 kD, and lamin B were found to be targeted relatively early after induction of apoptosis, whereas substantial cleavage of DNA topoisomerases I and II and UBF/NOR-90 was observed only after the accumulation of an appreciable number of apoptotic cells. These differences in the autoantigen cleavage kinetics suggested either differential accessibility or activation of distinct proteases during the apoptotic cell death pathway. The identity of the protease(s) responsible for the cleavage of most of these autoantigens in intact cells remains unclear, although ICE/CED-3 proteases have been shown to cleave *in vitro* DNA-PK_{cs}, PARP, U1-70 kD, and the nuclear lamins [15, 16, 20–28]. A possible role for ICE/CED-3 proteases in the cleavage of DNA topoisomerase I into a 70 kD fragment is implied by the observation that this cleavage was blocked in Jurkat cells by Z-VAD-fluoromethylketone, a peptide inhibitor of ICE/CED-3 proteases, and by the presence of a conserved candidate ICE/CED-3 cleavage site, 'DEDDVD', in the N-terminal region of DNA topoisomerase I [5, 29]. Cleavage at this site should yield a carboxyl-terminal fragment of approximately 70 kD. The apoptotic cleavage of DNA topoisomerase I into a 70 kD form is of interest in the field of autoimmunity since this protein was previously termed Scl-70 (scleroderma- associated antigen of 70 kD) due to its migration as a 70 kD protein in immunoblots of nuclear extracts from different sources reacted with sera from patients with scleroderma [30]. It was subsequently established that the 70 kD protein was a proteolytic product of the 100 kD DNA topoisomerase I enzyme [31,32]. This 70 kD fragment was likely derived either from apoptotic populations present in the cell cultures or tissues employed to prepare nuclear extracts, or from accessibility of the antigen in the lysates to proteases that are normally activated during apoptosis.

Nuclear autoantigen cleavage and the collapse of nuclear structure during apoptosis

A central issue in apoptosis research concerns the role of nuclear protein cleavage in apoptotic cell death. Recent studies with anucleate cells (cytoplasts) indicate that apoptosis-associated changes in the structure of the cytoplasmic membrane can occur in the absence of the nucleus [33–35]. In addition, Fas-mediated apoptosis in anucleate cells involves activation of ICE/CED-3 proteases, with cleavage of fodrin, a cytoskeletal protein known to undergo proteolysis during apoptosis [36]. Moreover, it was shown, using a cell free-system, that mitochondria undergoing opening of permeability transition pores are capable of inducing the characteristic nuclear changes of apoptotic cell death,

Table 2. Nuclear protein autoantigens shown conclusively to be cleaved during apoptosis

Autoantigen	Known or suggested functions	Native protein	Proteolytic fragments
DNA-PK_{cs}	DNA repair	460 kD	120, 150, 240 kD
DNA topo I	Modification of DNA topology	100 kD	70 kD
DNA topo II (α/β)	Modification of DNA topology; anchoring chromatin to nuclear matrix	170/180 kD	multiple (125–160 kD)
Lamin A	Nuclear envelope formation; anchoring chromatin to nuclear matrix	70 kD	45 kD
Lamin B	Nuclear envelope formation; anchoring chromatin to nuclear matrix	68kD	45 kD
NuMA	Mitotic spindle formation; anchoring chromatin to nuclear matrix	210–240 kD	160, 180 kD
PARP	DNA repair; interacts with chromatin in the nuclear matrix	116 kD	85 kD
UBF	RNA pol I transcription; post-mitotic nucleolar organization	90 kD	multiple (24–55 kD)
U1-70 kD	Pre-mRNA splicing; nuclear matrix association (?)	70 kD	40 kD

Based on references 8–28.

including chromatin condensation and DNA fragmentation, possibly through the release of a putative soluble factor [37]. Consistent with these observations, a more recent study demonstrated that cytochrome c, a protein localized in the inner membrane of mitochondria, is released from cells undergoing apoptosis *in vivo*, and is involved, in the presence of dATP, in the activation of the apoptotic program [38]. These studies suggest that nuclear events associated with apoptosis, which include proteolysis of specific nuclear autoantigens, might be directed by cytoplasmic factors, some of which might be present in mitochondria. Alternatively, these events may occur simultaneously and perhaps in an independent fashion with events occurring in the cytoplasm.

The cleavage of specific nuclear substrates might facilitate the collapse of nuclear structure during the execution phase of apoptosis. It is noteworthy that several of the nuclear autoantigens that are cleaved during apoptosis are involved in either DNA repair or in maintaining the proper conformation of DNA in the nucleus through interaction with the nuclear matrix (Table 2). PARP and DNA-PK_{cs}, for instance, are cleaved very early during apoptosis, and it is presumed that their cleavage may render cells unable to repair DNA damage efficiently, irreversibly committing them to apoptosis [4, 20]. It has also been suggested that cleavage of PARP permits the ADP- deribosylation of certain endonucleases, which consequently leads to activation of these enzymes and degradation of DNA [25].

The early proteolytic cleavage of the nuclear lamins during apoptosis, which leads to nuclear envelope breakdown, also appears to contribute to the collapse of nuclear structure [27]. Lamins play a key role in the organization of the nucleus by anchoring chromatin loops around the nuclear periphery through interaction with specialized DNA sequences, termed MARs or SARs (for matrix or scaffold attachment regions) [39, 40]. Using a cell-free apoptosis system, Lazebnik et al. [27], demonstrated that lamin cleavage during apoptosis is not required for chromatin condensation and DNA degradation but is required for fragmentation of the nucleus into multiple apoptotic bodies. Lamin cleavage may disrupt lamin-chromatin interactions, thereby freeing the chromatin at the nuclear periphery to be packaged into apoptotic bodies [27].

The autoantigen NuMA also plays a very important role in maintaining nuclear structure and is required for reformation of the nucleus after mitosis [41]. Like lamin B, this protein binds specifically to MARs, which suggests a role in the organization of chromatin loops [39]. We and others have demonstrated that NuMA is targeted by proteases early during apoptosis [5, 42, 43]. In non-apoptotic cells, NuMA appears uniformly distributed in the nucleoplasm, most likely in association with chromatin, whereas in apoptotic cells the protein is concentrated in patches dissociated from the chro-

matin [42]. This suggests that the association of NuMA with chromatin is significantly altered during apoptosis. Furthermore, a 180 kD truncated form of NuMA produced during apoptosis lacked a tail domain that is important for interactions with other macromolecules [43]. Cleavage of NuMA into this 180 kD form may increase its solubility, thereby facilitating detachment of chromatin loops from the nuclear matrix and subsequent micronucleation [43]. Whether NuMA cleavage facilitates chromatin degradation remains to be established, although it was shown that the appearance of NuMA cleavage products correlated with DNA fragmentation [42, 43].

The apoptosis-associated cleavage of DNA topoisomerases I and II is also intriguing given the fact that these enzymes maintain DNA topology in the nucleus [44]. At the present time, however, there is no information available regarding whether the cleavage of these autoantigens during apoptosis might block or alter their function in the nucleus, consequently leading to disruption of chromatin structure. The observation that these enzymes appear to be targeted by proteases relatively late during apoptosis [5] suggests that they may not be responsible for DNA degradation into oligonucleosomal units. It is conceivable, however, that these cleavages may facilitate DNA degradation in the later stages of apoptosis. The contribution of U1-70 kD cleavage to apoptosis also remains unclear. Since this protein is associated with snRNPs involved in pre-mRNA splicing, it has been suggested that its cleavage may play a role in regulating mRNA splicing during apoptosis [19]. The contribution of UBF/NOR-90 cleavage to apoptosis also remains to be explored, although it could be speculated that this cleavage may play a role in down-regulating ribosomal RNA synthesis during cell death. Clearly, further studies are necessary to fully define the significance of autoantigen proteolysis to apoptosis.

Concluding remarks

The availability of highly specific human autoantibodies to a wide range of intracellular proteins is advantageous for studies on the molecular mechanisms underlying apoptosis. Particularly, these autoantibodies have proven to be powerful tools for defining important proteolytic events along this cell death pathway. The value of these reagents is enhanced by their reactivity with multiple and often conformational epitopes within an autoantigen, which facilitates the characterization of cleavage fragments that may escape detection by immunoblotting when linear sequence-targeting antibodies are used. In addition, autoantibodies are also valuable probes to investigate, using immunofluorescence microscopic techniques, changes in the intracellular localization of autoantigens during apoptosis. Further studies on the fate of intracellular autoantigens during apoptosis should also provide clues as to whether cell death plays an important role in the genesis of autoantibodies in the systemic autoimmune diseases.

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