DNA-dependent Protein Kinase Is One of a Subset of Autoantigens Specifically Cleaved Early during Apoptosis

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Summary

Proteolytic cleavage of key substrates appears to be 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 suggested. The identification of the relevant substrates of the ICE protease family during apoptosis therefore constitutes a major challenge. Using human autoantibodies, we demonstrate here that a subset of autoantigens is specifically cleaved early during apoptosis. One of these cleaved molecules is identified as the catalytic subunit of the DNA-dependent protein kinase. The time courses of all proteolytic cleavages are identical and coincide with the onset of morphologic apoptosis. Furthermore, all cleavages share the same inhibition characteristics, which implicate an ICE-like activity(ies). We propose that cleavage of these autoantigens targets these molecules for an autoimmune response by revealing immunocryptic fragments in a proimmune apoptotic setting. Study of the immunogenicity of these fragments may yield insights into the autoimmune targeting of molecules. Moreover, the autoantibodies described will be valuable tools for the elucidation of mechanistically important proteolytic steps along the apoptotic pathway.

The importance of specific proteolysis in mediating the apoptotic process has been underscored in genetic analysis of apoptosis in *Caenorhabditis elegans*, where the *ced-3* gene is required for execution of apoptosis (1). The protein encoded by *ced-3* is homologous to the IL-1β–converting enzyme (ICE)[1] family of cysteine proteases (2), especially in the substrate-binding and catalytic domains (3–5). This protease family is rapidly expanding and now includes at least three additional members (Nedd2/Ich-1 [6, 7], CPP32/prICE [8, 9], and TX [10]). All cloned members of this family have been shown to induce apoptosis when overexpressed in different cell types (6–8, 10, 11). It has recently been demonstrated that the active form of CPP32 is the enzyme responsible for the specific proteolytic cleavage of poly(ADP-ribose) polymerase (PARP) that occurs at the onset of apoptosis (12). Furthermore, specific inhibition of CPP32 activity attenuates apoptosis in vitro, strongly suggesting that proteolytic activity of this enzyme is mechanistically important in the apoptotic process (12). The observation that overexpression of crmA (a protease inhibitor of the serpin family that potently inhibits ICE) abolishes the ability of some ICE family members to induce apoptosis further supports the proposal that proteolysis of key substrates by ICE-like proteases mediates apoptosis (13). The identification of key substrates whose cleavage might mediate apoptosis therefore constitutes a major challenge. It is intriguing that the only two proteins demonstrated to date to be proteolytically cleaved by ICE-like enzymes during apoptosis (PARP and U1-70kDa) are both nuclear autoantigens (9, 14).

We have previously demonstrated that the autoantigens targeted in SLE are clustered in two discrete populations of surface structure on apoptotic cells: (*a*) apoptotic bodies that arise from the condensed, fragmented nucleus, and (*b*) numerous small surface blebs that arise from fragmented rough endoplasmic reticulum (ER) (15). 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 (15).

Only a minor fraction of all potential antigenic determinants on a molecule are presented on MHC class II molecules during natural processing of whole protein antigens (16). This immunodominance of a peptide appears to be determined by its intrinsic affinity for MHC class II, as well as by flanking regions that may influence its interactions with the binding groove (16). Several studies have suggested that self-tolerance is only induced to efficiently presented, dominant determinants, but not to cryptic determinants on self-molecules (17–19). Thus, potentially autoreactive T cells that recognize the cryptic regions of self-molecules still exist for many antigens (20–22). Furthermore, immuniza-

[1] *Abbreviations used in this paper:* DNA-PK, DNA-dependent protein kinase; IAA, iodoacetamide; ICE, IL-1β–converting enzyme; PARP, poly(ADP-ribose) polymerase; TLCK, tosyl-L-lysine chloromethylketone.

tion with a cryptic determinant can break tolerance to that area of the self-molecule (17, 22). If the whole autoantigen is also present, this autoreactive T cell response may subsequently diversify to other areas on the self-molecule to which the organism was previously tolerant (21, 22). Since limited proteolysis might reveal cryptic epitopes, it is possible that activation of apoptosis-specific proteases in apoptotic surface blebs may define the specific protease substrates at these sites as autoantigens.

In these studies, we have shown that a subset of infrequently targeted autoantigens is united by their specific proteolytic cleavage early during apoptosis. The kinetics and inhibition characteristics of these cleavages are identical and implicate an ICE-like protease(s). Further study of these apoptosis-specific proteolytic cleavages will yield new insights into the biochemical mechanisms underlying this cell death process. Furthermore, studies of the immunogenicity of the relevant fragments may provide a powerful system with which to explore the autoimmune targeting of self.

Materials and Methods

Cell Culture and Induction of Apoptosis. HeLa cells were passaged in 10% heat-inactivated calf serum using standard tissue culture procedures. Apoptosis was induced by irradiation with UVB using the conditions previously described (15). Briefly, confluent HeLa monolayers were washed twice with PBS (2.7 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl, and 8 mM Na_2HPO_4) and irradiated in PBS with 1,650 J/m^2 UVB. The cells were then incubated in growth medium in a 7.5% CO_2–humidified incubator for 0, 3, 6, or 9 h as indicated in the figure legends. At each of these times except 0 h after UVB irradiation, apoptotic cells were readily detectable morphologically; mixed populations of adherent cells (early apoptosis), floating blebbed cells (established apoptosis), and dispersed fragments (late apoptosis) were observed (14). Cell death by apoptosis was confirmed by visualization of internucleosomal DNA cleavage on agarose gels (data not shown).

Apoptotic Lysates. Apoptotic lysates, consisting of the combined adherent and floating populations, were prepared at the appropriate times after UVB irradiation as follows. Culture dishes were chilled to 4°C, and the medium was separated from adherent cells by gentle aspiration of the former. The adherent cells were washed twice with PBS, and then lysed in buffer A (1% NP-40, 20 mM Tris, pH 7.4, 150 mM NaCl, and 1 mM EDTA containing the following protease inhibitors: pepstatin A, leupeptin, antipain, chymostatin, and PMSF). The aspirated medium was centrifuged at 16,000 *g*, and the resulting pellets were washed once with PBS before adding to the lysates of their adherent counterparts. Control, unirradiated HeLa cultures were processed similarly, except that the medium was discarded because it contained no floating cellular material (that is, these lysates consisted only of adherent cells). Protein assays were performed on all lysates (Bio-Rad Laboratories, Richmond, CA) with gamma-globulin as a standard.

Proteins and Protease Inhibitors. Purified DNA-dependent protein kinase (DNA-PK) was obtained from Promega (Madison, WI). Protease inhibitor stock solutions were made up in distilled deionized water as follows: 20 mM tosyl-L-lysine chloromethyl ketone (TLCK, Boehringer Mannheim Corp., Indianapolis, IN); 10 mM iodoacetamide (IAA; Sigma Chemical Co., St. Louis, MO).

Gel Electrophoresis and Immunoblotting. Electrophoresis was performed on 10% SDS-polyacrylamide gels containing 0.087% bisacrylamide. Proteins were subsequently transferred to nitrocellulose (23), and immunoblotting was performed using enhanced chemiluminescence according to the manufacturers' directions (Amersham Corp., Arlington Heights, IL). Scanning of autoradiograms was performed on a scanner (Scanmaker IIXE; Microtek, Redondo Beach, CA) before quantitation (Sigmascan/Image software; Jandel Scientific, San Rafael, CA). All autoradiographic bands scanned were within the linear range of the x-ray film.

Antibodies and Antibody Screening Procedures. Reactivity of human autoimmune sera with saline soluble extracts of human spleen and rabbit thymus was determined by Ouchterlony double immunodiffusion using standard reference sera to Jo-1, Ro, La, Sm, U1-70kDa, and Scl-70 (topoisomerase 1) (24). 260 precipitin-positive sera, including those against unidentified antigens, were further screened by immunoblotting against HeLa lysates, using the primary antibodies at a minimum dilution of 1:5,000. Only sera that gave strong autoradiographic bands (i.e., bands that were readily detected with enhanced chemiluminescence after <15 s exposure of the x-ray film) were further evaluated. Sera from healthy controls did not immunoblot any proteins using identical screening conditions (data not shown). The following reference sera were also used in immunoblotting experiments: PARP, fibrillarin, topoisomerase II, calreticulin, ribosomal protein P, and p70/p80 Ku.

Affinity Purification of Antibodies from Human Sera. HeLa cells were lysed in buffer A containing protease inhibitors, subjected to electrophoresis on preparative gels, and transferred to Immobilon (Millipore Corp., Bedford, MA). The immobilized proteins were used to affinity purify antibodies from RW and AG sera as described (15).

Immunofluorescence Microscopy. Immunofluorescence microscopy was performed on HeLa cells grown on No. 1 glass coverslips. Cells were fixed in 4% paraformaldehyde (5 min, 4°C) before permeabilization in acetone (30 s, 4°C) and staining with the standard monospecific serum recognizing PARP (diluted 1:160 in PBS) (25). Bound antibodies were visualized with fluorescein-conjugated goat anti–human F(ab')2 (Organon Teknika Corp./Cappel, Durham, NC). After washing in PBS, cells were counterstained with propidium iodide (5 μg/ml in PBS; Molecular Probes, Inc., Eugene, OR) for 30 s. Coverslips were mounted on glass slides with Permafluor (Lipshaw, Pittsburgh, PA) and viewed on a scanning confocal microscope system (model MRC600; Bio-Rad Laboratories).

Results

Several Different Autoantigens Are Specifically Cleaved during Apoptosis. Two autoantigens (U1-70kDa and PARP) have previously been shown to be cleaved by proteases with ICE-like features in the apoptotic setting, generating fragments of 40 and 85 kD, respectively (Fig. 1, lanes *1–4*) (9, 14). To address whether other autoantigens might be similarly cleaved during apoptosis, 260 sera containing high titers of autoantibodies were screened by immunoblotting against lysates of apoptotic and control HeLa cells. Several autoimmune sera were identified that recognized proteins that were specifically cleaved during apoptosis (Fig. 1). Sera HM and JE recognized 170- and 75-kD proteins, respectively, which gave rise to fragments of 155 and 60 kD in apoptotic cells (Fig. 1, lanes *5–8*). Serum RW immunoblotted three prom-

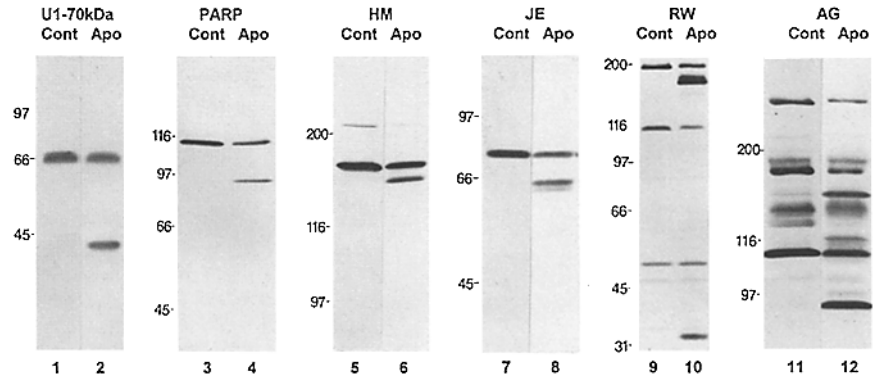

Figure 1. Several different autoantigens are cleaved during apoptosis. HeLa cells were induced to become apoptotic by irradiation with UVB followed by incubation at 37°C for 9 h (lanes *2*, *4*, *6*, *8*, and *10*) or 6 h (lane *12*). These cells, as well as control unirradiated HeLa cultures (lanes *1*, *3*, *5*, *7*, *9*, and *11*), were then lysed and immunoblotted with antisera from the following patients: HM (lanes *5* and *6*), JE (lanes 7 and *8*), RW (lanes *9* and *10*), AG (lanes *11* and *12*), or standard sera for U1-70kDa (lanes *1* and *2*) and PARP (lanes *3* and *4*). Note that for greater clarity of presentation of the complex blotting profile of AG, the 6 h after irradiation time was used (lane *12*). This demonstrates both intact and fragmented forms of the different antigens simultaneously, whereas the former are barely detectable in the AG profile seen at 9 h (compare Fig. 3, lanes *5* and *6*, *9* and *10*). Equal amounts of protein were loaded in both lanes of each pair, but varied between pairs (70–85 μg protein/lane) as did autoradiogram exposure times (2–15 s). Identical results were obtained in six to nine separate experiments performed with each serum. Migration positions of molecular weight standards are marked down the left side of each set.

inent proteins of 195, 112, and 50 kD (Jo-1) (Fig. 1, lane *9*). In apoptotic cells, the chemical amounts of the 195- and 112-kD proteins were decreased, and two new, discrete fragments of 160 and 35 kD were generated; in contrast, levels of Jo-1 remained constant (Fig. 1, lane *10*). The appearance of a large amount of the 160-kD protein in apoptotic cells could not be accounted for by a proportional decrease in the levels of the 195-kD protein, from which the 160-kD protein was presumed to arise. Furthermore, the source of the 35-kD fragment was not clear. To unequivocally determine the origin of these two apoptotic fragments, we affinity purified three antibodies from serum RW that recognized the 195-, 112-, and 50-kD antigens in control HeLa lysates. Antibodies purified against the 195-kD protein recognized both the 160- and 35-kD fragments in apoptotic cells (Fig. 2, lanes *3* and *4*). No fragments were recognized in apopotic cells by antibodies affinity purified against the 112- or 50-kD antigens, although the chemical amount of the 112-kD protein was clearly decreased (Fig. 2, lanes *5–8*). Antibodies affinity purified against the 160-kD protein from apoptotic cells recognized only the 160- and 195-kD forms of the protein, and failed to recognize the 35-kD fragment (data not shown). These data confirmed that, although the 160- and the 35-kD fragments are both derived from the intact 195-kD protein, they are recognized by distinct antibodies. Since the sizes of these two fragments together account for the entire 195-kD protein, it is likely that they are contiguous and arise from a single proteolytic cleavage.

In contrast to the five sera presented above in which only one of the proteins recognized by a particular autoimmune serum was cleaved in apoptotic cells, an extremely interesting serum (AG) was identified that recognized multiple proteins that were cleaved during apoptosis. This serum was derived from a patient with SLE and myositis, who also had high-titer antibodies to double-stranded DNA. In control HeLa lysates, this serum recognized five proteins of the following molecular masses: 350, 190, 140, 130, and 110 kD (Fig. 1, lane *11*). A protein of 195 kD was also intermittently recognized in some lysates of HeLa cells (Fig. 1, lane *11*). When serum AG was immunoblotted against lysates of apoptotic HeLa cells, the chemical amounts of four of these proteins (350, 190, 130, and 110 kD) were decreased compared with controls, whereas the amount of the 140-kD pro-

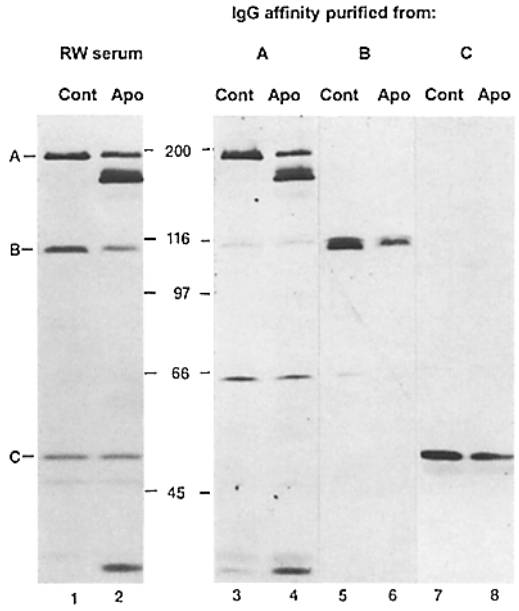

Figure 2. The 195-kD autoantigen recognized by RW undergoes a single cleavage in apoptotic cells to generate fragments of 160 and 35 kD. HeLa cells were induced to become apoptotic by irradiation with UVB followed by incubation at 37°C for 9 h (lanes *2*, *4*, *6*, and *8*). These cells, as well as control unirradiated cultures (lanes *1*, *3*, *5*, and *7*), were then immunoblotted with RW serum (lanes *1* and *2*) or IgG affinity purified against the three antigens recognized by serum RW: *A*, IgG affinity purified against the 195-kD antigen (lanes *3* and *4*); *B*, IgG affinity purified against the 112-kD antigen (lanes *5* and *6*); and *C*, IgG affinity purified against the 50-kD Jo-1 antigen (lanes 7 and *8*). 115 μg of protein was subjected to electrophoresis in each gel lane. Migration positions of molecular weight standards are marked between lanes *2* and *3*. The band migrating at 63 kD in lanes *3* and *4* is nonspecific and was detected in the other lanes at longer exposure times (data not shown).

tein remained unchanged. In addition, three new discrete immmunoreactive fragments of 150, 118, and 85 kD were detected by serum AG in apoptotic lysates (Fig. 1, lanes *11* and *12*). To identify from which antigens these new fragments were derived, antibodies to each of the antigens recognized in control HeLa lysates were affinity purified and then used to blot apoptotic lysates. These studies demonstrated that the 150-kD fragment arose from the 350-kD antigen (Fig. 3, lanes *3* and *4*), the 118-kD fragment arose from the 130-kD antigen (Fig. 3, lanes 7 and *8*), and the 85-kD fragment arose from the 110-kD antigen (Fig. 3, lanes *9* and *10*). Although no autoantigens of 350 kD have previously been described, the 350-kD catalytic subunit of the DNA-PK forms a complex with its regulatory components, the p70/p86 Ku proteins, which are well-described autoantigens in human autoimmune disease (26–28). To confirm whether the 350-kD autoantigen was indeed the catalytic subunit of DNA-PK, purified HeLa cell DNA-PK was electrophoresed adjacent to lysates from control and apoptotic HeLa cells, before immunoblotting with antibodies affinity purified against purified DNA-PK, as well as an mAb against p350. Both these antibodies recognized the purified 350-kD protein and the comigrating protein in HeLa lysates (Fig. 4, lanes *1* and *2* and data not shown). Furthermore, the affinity-purified human antibodies recognized the 150-kD fragment in apoptotic lysates (Fig. 4, lane *3*), whereas the mAb recognized the complementary 220-kD

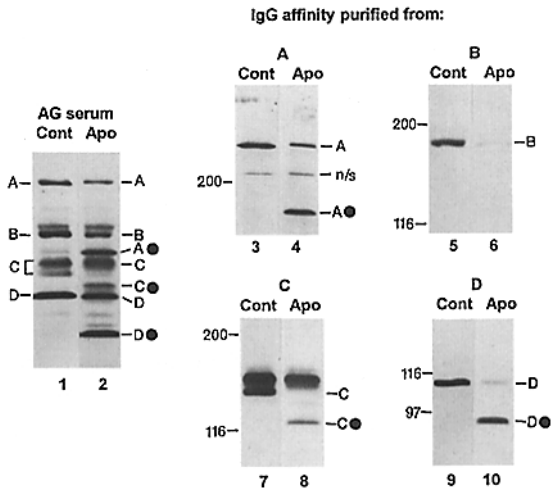

Figure 3. Three different autoantigens detected by patient serum AG are proteolytically cleaved in apoptosis. HeLa cells were induced to become apoptotic by irradiation with UVB followed by incubation at 37°C for 9 h (lanes *4*, *6*, *8*, and *10*) or 6 h (lane *2*). Lysates prepared from these cells, as well as control unirradiated cultures (lanes *1*, *3*, *5*, 7, and *9*), were immunoblotted with AG serum (lanes *1* and *2*) or were IgG affinity purified against the following antigens recognized by AG serum: *A*, 350 kD (lanes *3* and *4*); *B*, 190 kD (lanes *5* and *6*); *C*, 130 kD (lanes 7 and *8*); and *D*, 110 kD (lanes *9* and *10*). For greater clarity of presentation, the 6 h after irradiation time was used to show the AG serum blotting profile (lane *2*), since this demonstrates both intact and fragmented forms of the different antigens simultaneously. The solid dots adjacent to *A*, *B*, *C*, and *D* denote new fragments detected in apoptotic lysates. 120 μg of protein was loaded in each lane. Migration positions of molecular weight standards are indicated on the left side of *A–D*. *n/s*, nonspecific.

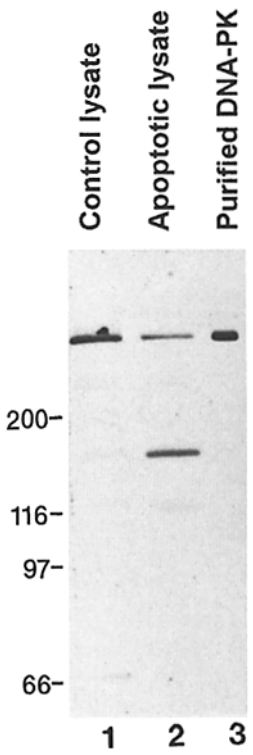

Figure 4. The 350-kD antigen recognized by serum AG is the catalytic subunit of DNA-PK. HeLa cells were induced to become apoptotic by irradiation with UVB, followed by incubation at 37°C for 9 h. Lysates from these cells (lane *2*) as well as those obtained from control, unirradiated cultures (lane *1*) were subjected to electrophoresis adjacent to DNA-PK purified from HeLa cells (lane *3*). The samples were immunoblotted with IgG affinity purified against DNA-PK (AG serum was used for the affinity purification). 115 μg of protein was subjected to electrophoresis in lanes *1* and *2*, and 0.7 μg in lane *3*. The upper and lower bands migrate at 350 and 150 kD, respectively. The experiment was performed on three separate occasions, with identical results.

fragment of p350 (data not shown). The 350-kD autoantigen is therefore the catalytic subunit of DNA-PK. These studies provide the first direct evidence that p350 DNA-PK (*a*) is an infrequently targeted autoantigen in autoimmune disease, and (*b*) is proteolytically cleaved during apoptosis. It is of note that serum AG did not recognize the p70/p86 Ku proteins by immunoblotting, nor did it coprecipitate this heterodimer from metabolically labeled HeLa cells lysed in a buffer containing 1% NP-40 (data not shown). Using reference sera to the Ku proteins, we established by immunoblotting that the p70/p86 proteins remained intact in apoptotic HeLa cell lysates (data not shown). These data are in partial contrast to a recent study that demonstrated decreased levels of both p350 and the Ku subunits in apoptotic PBLs and HL60 cells (29). That study is not directly comparable to the data reported here, because it did not address changes in Ku levels early after delivery of a synchronized proapoptotic stimulus, as was done here (see kinetics below).

The 110-kD protein recognized by serum AG was positively identified as PARP by the following criteria: (*a*) An 85-kD fragment was generated in apoptotic cells (9); (*b*) both the intact protein and the fragment comigrated on SDS-PAGE with proteins recognized by standard anti-PARP sera (data not shown); and (*c*) when control HeLa lysates were immunoprecipitated with standard antibodies to PARP, followed by immunoblotting with serum AG, the 110-kD PARP protein was specifically recognized (data not shown).

We confirmed that the fragments detected by the six different sera shown in Fig. 1 were not unique to HeLa cell apoptosis by examining lysates made from apoptotic human

keratinocytes, human umbilical vein endothelial cells, and a mouse pre–B cell line (103 cells). Identical apoptotic fragments were observed in all these cell types, except that p350 and its fragment were absent from the murine cell line, a finding consistent with previous observations (30 and data not shown). Generation of the various fragments was specific to apoptosis since similar fragments were never observed if necrosis was induced by heat shock or incubation in high concentrations of H_2O_2 (data not shown).

With the exception of U1-70kDa (to which high-titer antibodies were found in ~10% of screened sera), the autoantigens described in Fig. 1 were the infrequent target of a high-titer autoantibody response, found in <1% of sera screened. No other frequently targeted autoantigen in SLE, scleroderma, or polymyositis (including 52- and 60-kD Ro, ribosomal protein P, Sm, calreticulin, p70/p80 Ku, topoisomerase I, fibrillarin, and Jo-1) was cleaved during apoptosis (data not shown).

Kinetics of Autoantigen Cleavage during Apoptosis. The activation of ICE-like enzymes in cells undergoing apoptosis is a necessary step in the apoptotic process (12). Since mechanistically important proteolytic cleavages are likely to occur before or coincident with the onset of apoptotic morphology, we examined the kinetics of autoantigen cleavage in HeLa cells after UVB irradiation to address directly whether there was any correlation between these events. We found that the 150-kD DNA-PK fragment was clearly seen 3 h after irradiation (Fig. 5, *top panel*), coincident with the onset of detectable apoptotic morphology (data not shown). As incubation times after irradiation increased, the chemical amounts of intact p350 decreased, accompanied by a concomitant stoichiometric increase in the amount of the 150-kD fragment. The kinetics of cleavage of all the proteins shown in Fig. 1 were identical to each other and to p350; PARP, as well as sera JE and HM, is shown as a representative example (Fig. 5). In all instances, >50% of the intact antigen was cleaved into the fragment by 9 h after irradiation (Fig. 5).

Interestingly, we noted that La and topoisomerase I, two autoantigens previously found to be sensitive to proteolysis during purification (31, 32), were also cleaved in apoptotic cell populations. In contrast to the kinetics of the cleavages described above, the La and topoisomerase I cleavages only occurred several hours after the morphologic onset of apoptosis, and <10% of the total autoantigen was cleaved at 9 h (Fig. 5 and data not shown). Cleavage of the intact 100-kD topoisomerase I generated a 70-kD fragment (Fig. 5, *lowest panel*), whereas proteolysis of the intact 48-kD La molecule generated a 43-kD fragment as previously described (33 and data not shown). Furthermore, in contrast to the cleavages shown in Fig. 1, those of La and topoisomerase I had acidic pH optima and occurred even in lysates of nonapoptotic cells incubated in vitro (data not shown).

Inhibition Characteristics of Autoantigen Cleavage in Apoptotic Cells. Since the cleavage kinetics of the autoantigens shown in Fig. 1 were all identical to that of PARP, and since prICE/CPP-32 generates this specific PARP cleavage in apoptotic cells, we studied the inhibition characteristics of the activity(ies) responsible for these cleavages in apoptotic cells. HeLa cells were irradiated to induce apoptosis and then incubated in the absence or presence of various protease inhibitors, added individually. Leupeptin, antipain, pepstatin A, chymostatin, PMSF, E-64, and acetyl-leucyl-leucyl-normethional did not prevent cleavage of these autoantigens, nor did they alter the morphology of apoptosis as assessed by phase contrast microscopy (12 and data not shown). Fragmentation of PARP was entirely prevented by incubation of irradiated cells with 50 μM IAA (Fig. 6, *top panel*, lane *2*) or 1 mM TLCK (Fig. 6, *top panel*, lane *3*). Similar findings were made for sera recognizing JE and U1-70kDa (Fig. 6), as well as HM, RW, and AG (data not shown). Addition of 100 μM of the specific tetrapeptide ICE inhibitor *N*-(*N*-Ac-tyr-val-ala)-3-amino-4-oxobutanoic acid to the incubation medium resulted in only minimal inhibition of autoantigen cleavage (10–30% in different experiments) (data not shown). Furthermore, purified recombinant human ICE was unable to cleave these substrates in vitro when added to cell lysates (14 and data not shown), reinforcing that apoptotic ICE homologues, rather than ICE itself, might be responsible for the cleavage of these proteins in apoptosis (9, 12, 14, 34, 35). Since the substrate specificity of the two ICE ho-

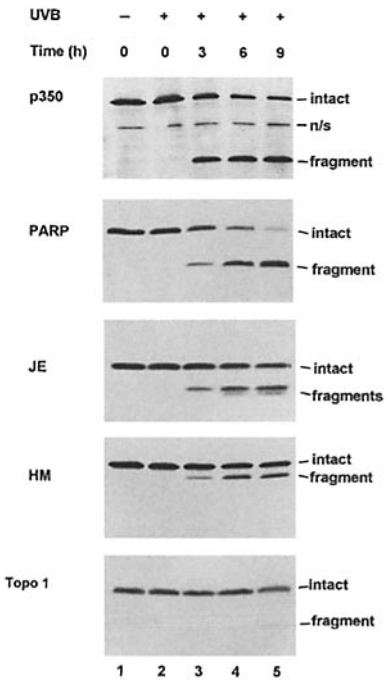

Figure 5. Kinetics of apoptotic fragment generation. HeLa cells were induced to become apoptotic by irradiation with UVB and were then incubated at 37°C for 0 (lane *2*), 3 (lane *3*), 6 (lane *4*), or 9 h (lane *5*). Control cultures (lane *1*) were not irradiated with UVB. At the indicated times, cultures were lysed. Equal protein aliquots (110 μg) were subjected to electrophoresis and immunoblotted with the following sera: p350 (*top panel*), PARP (*second panel*), JE (*third panel*), HM (*fourth panel*), and topoisomerase I (*lowest panel*). Migration positions of the intact protein and any fragment(s) generated are marked and were as follows: p350, 350 (intact) and 150 kD (fragment); PARP, 110 (intact) and 85 kD (fragment); JE, 75 (intact) and 60 and 57 kD (fragments); HM, 170 (intact) and 155 kD (fragment); and topoisomerase I, 100 (intact) and 70 kD (fragment). Identical results were obtained in two separate experiments with each serum.

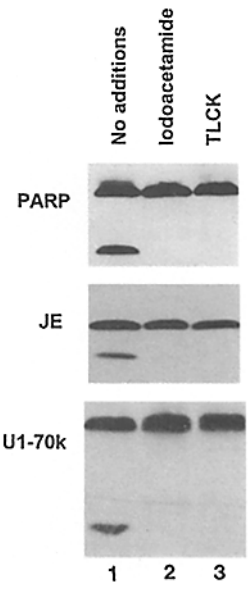

Figure 6. Effect of protease inhibitors in vivo on apoptotic fragment generation. HeLa cells were irradiated with UVB and then incubated for 3 h in growth medium containing the following protease inhibitors: no inhibitors (lane *1*), 50 μM IAA (lane *2*), or 1 mM TLCK (lane *3*). The cells were then lysed and immunoblotted with standard sera against PARP (*top panel*), U1-70kDa (*lowest panel*), or patient serum JE (*middle panel*). 120 μg of protein was subjected to electrophoresis in each lane. Migration positions of the intact protein and the fragment were as follows: PARP, 110 (intact) and 85 kD (fragment); JE, 75 (intact) and 60 kD (fragment) (note that no 57-kD fragment is detected 3 h after irradiation; see Fig. 4); and U1-70kDa, 67 (intact) and 40 kD (fragment). Identical results were obtained in two separate experiments with each serum.

mologues described to date are different, these proteases are thus most effectively inhibited by different tetrapeptide aldehydes (12). Specific inhibitor studies on the substrates identified here must therefore await the sequencing of intact autoantigens and identification of their cleavage sites.

Interestingly, degradation of the 190- and 112-kD proteins recognized by sera AG and RW, respectively, which produce no detectable fragments in apoptotic cells, was not prevented by IAA or TLCK (data not shown). Since these cleavages occur early during apoptosis but do not share the inhibition characteristics of the other antigens described, it is likely that they are substrates for another family of proteases active during apoptosis. Indeed, activity of other protease families (e.g., calpain) during apoptosis has recently been suggested (36).

Nuclear Autoantigens Are Redistributed during Apoptosis. Although the ICE-like proteases purified to date have all been isolated from the cytosol, PARP is nuclear in distribution (25). Interestingly, U1-70kDa and p350 DNA-PK are also nuclear proteins, as is the unidentified 75-kD antigen recognized by serum JE. We have previously demonstrated that U1-70kDa is redistributed in apoptotic cells, appearing first as a rim around the condensing chromatin and subsequently around apoptotic bodies at the cell surface as the nucleus fragments (15). A similar morphologic sequence was demonstrated for PARP in apoptotic cells (Fig. 7), using a human serum that was monospecific by immunoblotting and immunoprecipitation (25 and Fig. 1, lane *3*). PARP stained exclusively in the nucleus of HeLa cells before irradiation, with a homogeneous pattern that excluded nucleoli (Fig. 7 *A*). At all stages of apoptosis, PARP staining was associated only with the condensed nucleus or its fragments and was never detected in the cytosol (Fig. 7, *B–D*). These results were confirmed biochemically by immunoblotting NP-40 HeLa lysates that were separated into nuclear pellet and postnuclear supernatant (>90% of both PARP and its fragment were present in the nuclear pellet; data not shown).

Discussion

A Subset of Infrequently Targeted Autoantigens Is Cleaved Early during Apoptosis. These studies have defined a group of autoantigens that is specifically cleaved early during apoptosis. Members of this group include the 350-kD catalytic subunit of DNA-PK, PARP, and U1-70kDa, as well as unidentified autoantigens of 195, 170, 130, and 75 kD. All seven cleavages generate discrete fragments that are not further processed. In all cases, >50% of the total protein is degraded to the fragment in apoptotic cells. Affinity-purified antibodies recognize both the intact and cleaved forms of the molecules. The cleavages are observed when apoptosis is induced in multiple different cell types and are never detected after lysis of nonapoptotic control cells, or in cells rendered necrotic by a variety of insults. These early, apoptosis-specific cleavages may be of potential mechanistic significance in this cell death process. They are to be contrasted with the cleavage of topoisomerase I and La, which is minimal, late, and not specific to apoptotic cells.

Kinetics and Inhibition Characteristics of Cleavage Indicate the Involvement of an ICE-like Protease(s). The formation of such discrete protein fragments in apoptotic cells and their lack of further processing strongly suggest (*a*) the presence of highly restricted cutting sites in the cleaved proteins, and (*b*) the activity of a limited number of proteases in this setting. Furthermore, the inhibition characteristics of all the cleavages described in Fig. 1 are quite striking; like PARP, they are completely inhibited by IAA and TLCK treatment of intact cells (37), but not by other inhibitors of serine and cysteine proteases, including leupeptin, PMSF, chymostatin, antipain, acetyl-leucyl-leucyl-normethional, and E-64. These mixed inhibition characteristics are identical to those observed for PARP cleavage (37), which is catalyzed by the apoptotic ICE homologue, CPP32 (12). They strongly suggest the involvement of member(s) of the ICE family, either directly (as for PARP), or perhaps upstream of these cleavages in an apoptotic protease cascade. Sequencing studies to determine the specific cutting site for each autoantigen will be essential to resolve these possibilities, since ICE family–mediated cleavages are absolute in their requirement of an Asp in the P1 position (38).

ICE-like Enzymes and Apoptosis. A growing number of genes that have similarity to the ICE family of cysteine proteases have recently been identified by sequence homology studies (3, 6, 7, 10). The respective substrates for these potential proteases, however, are not yet known (3, 6, 7, 10). Since the proteolytic activity of the ICE family members studied to date appears to be highly restricted (ICE cleaves only pro–IL-1β, and not PARP, and CPP32 cleaves PARP and not pro–IL-1β [9, 12]), it is likely that these other ICE family members will be active against a limited group of substrates. We propose that the infrequently targeted autoantigens described here are some of the possible substrates

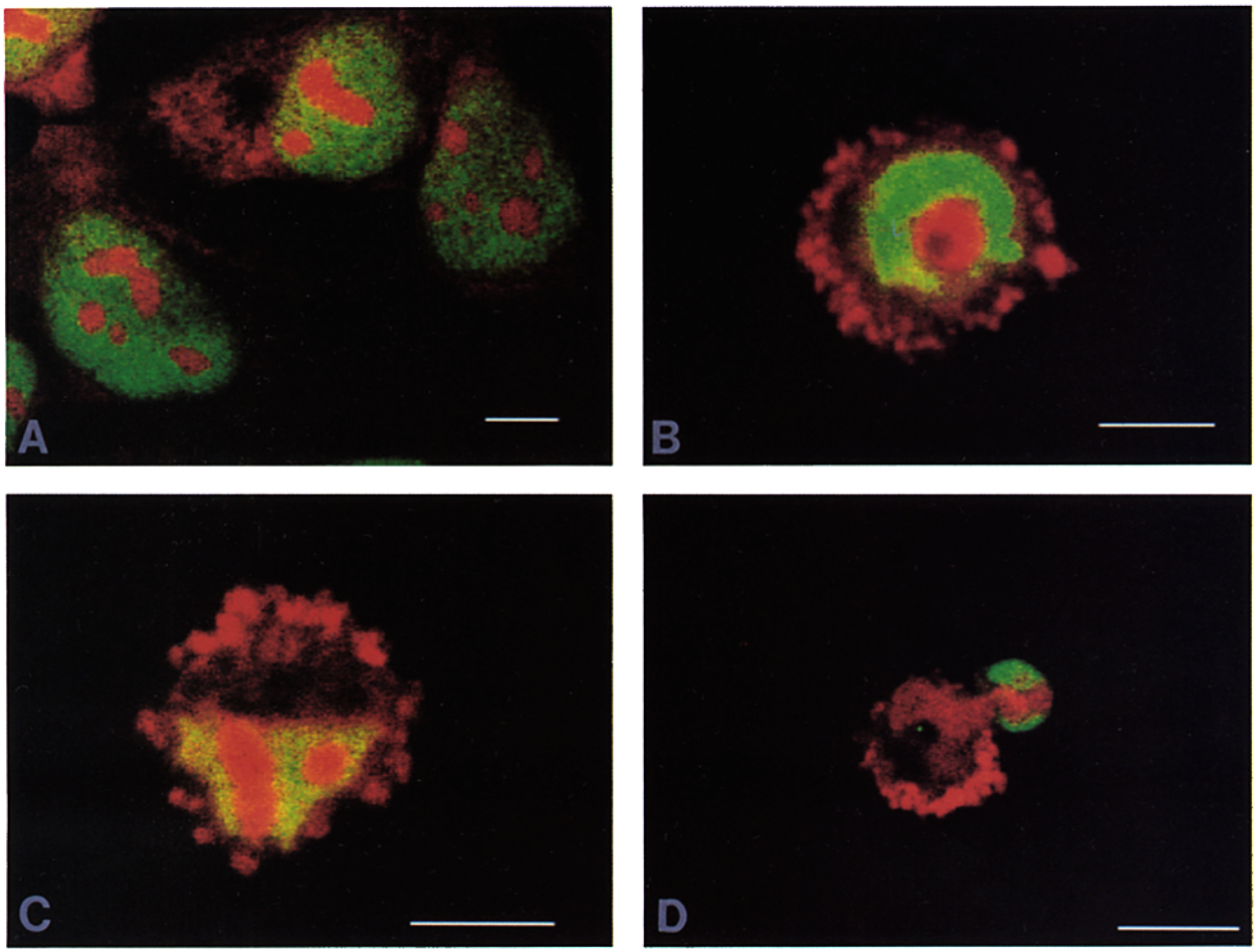

Figure 7. PARP stains the rim of the condensing apoptotic nucleus and is found in apoptotic bodies at the cell surface. Unirradiated (*A*) and irradiated (*B–D*) HeLa cells were double stained with a monospecific human PARP standard serum and with propidium iodide, and were examined by confocal fluorescence microscopy. PARP antibodies were visualized with FITC–goat anti–human antibodies and assigned the color green, whereas propidium iodide staining was assigned as red. In unirradiated cells (*A*), PARP stained diffusely throughout the nucleus, excluding nucleoli. In apoptotic cells (*B–D*), propidium iodide staining of small surface blebs and apoptotic bodies was prominent. PARP staining was condensed in the nucleus of early apoptotic cells (*B* and *C*) and surrounded apoptotic bodies after nuclear fragmentation (*D*). No staining in the FITC channel was seen if primary antibody was omitted. Duplicate coverslips were prepared, and at least 50 apoptotic cells per coverslip were examined; representative cells are presented. The experiment was repeated on three separate occasions, with similar results. Bars, 5 μm.

for these proteases, and the autoantibodies that recognize the intact proteins and their fragments will provide the tools to further elucidate the roles of the ICE family of proteases during apoptosis.

Although it is still uncertain how cleavage of specific molecules might mediate the apoptotic process, it is of great interest that two of the cleaved proteins, PARP and DNA-PK, have roles in DNA break repair (39–42). The specific cleavage of these two enzymes is tantalizing, since a hallmark biochemical feature of apoptosis is the generation of large numbers of internucleosomal DNA breaks. These breaks are a potent stimulus for activation of DNA repair systems. The cleavage of PARP and DNA-PK is therefore physiologically consistent in apoptosis (to terminate DNA repair in a cell that has already become committed to die), but it remains unclear whether cleavage of these substrates is mechanistically critical for the apoptotic pathway. It will therefore be important to determine the effects of proteolysis on the function of these enzymes in their respective pathways. Furthermore, elucidating the identities of the other autoantigens described in this study and their cellular functions might yield new insights into the mechanisms and control of this important form of cell death.

Although both ICE family members purified to date have been derived from cytosolic extracts (2, 12), PARP is found exclusively in the nucleus (Fig. 7; 25). Furthermore, two other proteins described in this paper (U1-70kDa and p350 DNA-PK) whose cleavage kinetics and inhibition characteristics are identical to those of PARP are also catalytic nuclear proteins. These three proteins are immunostained in apoptotic bodies, as are the autoantigens recognized by serum HM and JE (15 and data not shown). Even late in apoptosis, PARP remains associated with the nucleus and its fragments and is never found in the cytoplasm.

This predicts that the responsible ICE family members or downstream proteases have access to the nucleus or its fragments in apoptotic cells.

Novel Fragmentation of Autoantigens and Immunogenicity. It has been proposed that some autoreactive T cells escape tolerance induction because they are directed against cryptic determinants on self-antigens that are not efficiently generated during antigen processing (for review see reference 16). These autoreactive cells may become pathogenic if that cryptic determinant is subsequently revealed. It is possible that the generation of novel protein fragments by apoptosis-specific proteases might be a mechanism to reveal this cryptic structure. In all the sera presented here, the proteolytic fragments in apoptotic cells are recognized by the autoantibody response, an observation that is consistent with this mechanism. The enhanced recognition by the autoantibody response of proteolytic fragments of certain autoantigens in apoptotic cells (e.g., as is seen with the 160-kD RW fragment) further supports this hypothesis.

Serum AG is of great significance because three of the five proteins targeted by this patient serum were cleaved during apoptosis. The kinetics and inhibition features of all cleavages were identical. The limited proteolysis of three infrequently targeted autoantigens in a single patient argues persuasively for the important role of cleavage in defining these molecules as autoantigens. Further study of the immunogenicity of these autoantigen fragments in in vitro and in vivo models will be essential to test this hypothesis.

Although thymocyte apoptosis during positive and negative selection has recently been persuasively demonstrated in situ by demonstrating DNA strand breaks (43), it is not yet clear whether the other biochemical mechanisms of thymic apoptosis and clearance of apoptotic material are identical to those found at peripheral sites. It will be essential to evaluate whether these specific autoantigen cleavages occur during the apoptosis of thymocyte selection, since the failure to generate these fragments in the thymus might deprive the organism of necessary toleragens and allow the persistence of potentially autoreactive T cells.

Model of Autoimmunity. We have previously demonstrated that apoptosis induces the physical clustering of autoantigens in two distinct populations of cell surface structures (15). We proposed that this clustered targeting of autoantigens by the immune system might reflect the susceptibility of these molecules to a common biochemical modification in this apoptotic microenvironment (15). In these studies, we have shown that a distinct subset of infrequently targeted autoantigens is united by their specific proteolytic cleavage early during apoptosis. We therefore propose that cleavage of these autoantigens targets them for an autoimmune response, perhaps by revealing previously immunocryptic fragments. This will only occur in individuals who possess class II MHC molecules that can bind to and present these cryptic determinants to potentially autoreactive T cells (16). The lack of proteolytic cleavage of the ribonucleoprotein and deoxyribonucleoprotein autoantigen complexes more frequently targeted in individuals with autoimmune diseases suggests that other biochemical mechanisms (e.g., nuclease activity, generation of reactive oxygen species) may be operative in apoptotic cell surface blebs that render components of these complexes immunogenic. Altered structure of clustered autoantigen molecules, when occurring in novel proimmune contexts (e.g., viral infection [44]) or antigen-presenting microenvironments (e.g., Langerhans cell–rich epidermis), may be the proximate mechanism underlying the autoimmune targeting of self.

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Note added in proof: Recently, a report that CPP32 mediates the specific cleavage of PARP during apoptosis has been published (Tewari, M., L. T. Quan, K. O'Rourke, S. Desnoyers, Z. Zeng, D. R. Beidler, G. G. Poirier, G. S. Salvesen, and V. M. Dixit. 1995. *Cell.* 81:801–809.).

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