Apoptosis Generates Stable Fragments of Human Type I Keratins*  

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Nam-On Ku‡, Jian Liao§, and M. Bishr Omary¶  

From the Veterans Administration Palo Alto Health Care System, Palo Alto, California 94304 and Digestive Disease Center, Stanford University School of Medicine, Palo Alto, California 94305, and §Clontech Laboratories Inc., Palo Alto, California 94303  

Type I and II keratins help maintain the structural integrity of epithelial cells. Since apoptosis involves progressive cell breakdown, we examined its effect on human keratin polypeptides 8, 18, and 19 (K8, K18, K19) that are expressed in simple-type epithelia as noncovalent type I (K18, K19) and type II (K8) heteropolymers. Apoptosis induces rapid hyperphosphorylation of most known K8/18 phosphorylation sites and delayed formation of K18 and K19 stable fragments. In contrast, K8 is resistant to proteolysis and remains associated with the K18 fragments. Transfection of phosphorylation/glycosylation-mutant K8 and K18 does not alter fragment formation. The protein domains of the keratin fragments were determined using epitope-defined antibodies, and microsequencing indicated that K18 cleavage occurs at a conserved caspase-specific aspartic acid. The fragments are found preferentially within the detergent-insoluble pool and can be generated, in a phosphorylation-independent manner, by incubating keratins with caspase-3 or with detergent lysates of apoptotic cells but not with lysates of nonapoptotic cells. Our results indicate that type I keratins are targets of apoptosis-activated caspases, which is a likely general feature of keratins in most if not all epithelial cells undergoing apoptosis. Keratin hyperphosphorylation occurs early but does not render the keratins better substrates of the downstream caspases.  

Intermediate filament (IF)* proteins encompass the nuclear lamins and a large family of tissue-specific cytoplasmic proteins that include keratins in epithelial cells, desmin in muscle, neurofilaments in neuronal cells, and vimentin in mesenchymal cells (reviewed in Refs. 1–3). Keratins are the largest IF protein subgroup and consist of more than 20 polypeptides (K1–K20) that are divided into relatively acidic type I (K9–K20) and basic type II (K1–K8) keratins (4, 5). All epithelial cells typically express at least one type I and one type II keratin, as noncovalent obligate heteropolymers, in an epithelial cell-type specific manner. For example, simple-type epithelia preferentially express K8/18 with various levels of K19 and K20 (6–10), keratinocytes express K1/10 and/or K5/14 depending on their differentiation state within the epidermal layer, and corneal epithelial cells express K3/12 (4). Although the full scope of keratin function is not known, one clear keratin function is to help maintain epithelial cell integrity particularly upon cell stress. This role is supported by several animal studies and a growing list of human epidermal, oral, and ocular diseases that result from keratin mutations (11–16).  

Keratins undergo several modifications that are likely involved in regulating their function (reviewed in Ref. 17), with phosphorylation being the most studied (reviewed in 18, 19). For K8/18, the known in vivo phosphorylation sites include Ser-52/Ser-33 of K18 (20) and Ser-23/Ser-431/Ser-73 of K8 (22, 23). Of note, keratin phosphorylation is highly dynamic (24–27) and is modulated during several physiological states, including mitosis and cell stress (17, 19). The use of phospho-epitope-specific antibodies has facilitated the study of IF protein phosphorylation (17, 28). For example, antibody LJ4, which recognizes Ser(P)/73 of K8, does not stain nondividing cells by immunofluorescence but exhibits a strong signal upon induction of apoptosis using anisomycin or etoposide or during mitosis as noted in cultured cells or in mouse liver after partial hepatectomy (23).  

Apoptosis is associated with a number of ordered morphological and biochemical events that ultimately lead to cell death (29, 30). Central to this process is the family of “caspase” proteases that cleave at aspartic acid in the context of preferred motifs (31–33). The importance of caspases is highlighted by the ability of short peptides, whose sequences mimic the sequence context of caspase substrates, to selectively inhibit apoptosis. Identification of in vivo caspase substrates is highly relevant in terms of understanding apoptosis in general and determining the relative importance of these substrates. The list of the caspase substrates is growing and includes pro-caspases, poly(ADP-ribose) polymerase (PARP), nuclear lamins, protein kinase C-δ, MEKK-1, topoisomerases, and fodrin (31–35). In this report, we describe the apoptosis-associated biochemical events that involve keratins. We show that apoptosis is associated with early keratin hyperphosphorylation that is later followed by preferential caspase-mediated proteolysis of type I (K18 and K19) but not type II (K8) keratins. The apoptosis-induced keratin phosphorylation, which involves most but not all known K8/18 phosphorylation sites, does not affect keratin susceptibility to degradation by the downstream caspases. In addition, K8 and K18 that are mutated at several known phosphorylation and glycosylation sites are equally susceptible to degradation in transfected cells. Our results suggest that apoptosis-induced fragment formation of K18 and K19 is likely to occur in other cytoplasmic IF proteins that have similar predicted caspase-sensitive sequences.

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‡ To whom requests for reprints should be addressed: Palo Alto VA Medical Center, 154J, 3801 Miranda Ave., Palo Alto, CA 94304.

§ To whom other correspondence should be addressed: Palo Alto VA Medical Center, G.I. 111, 3801 Miranda Ave., Palo Alto, CA 94304.

¶ This abbreviations used are: IF, intermediate filament(s); An, anisomycin; Emp, Empigen BB; K, keratin; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; PARP, poly(ADP-ribose) polymerase; pS, phospho-serine (Ser(P)); PVDF, polyvinylidene difluoride.

2 N. O. Ku, J. Liao, and M. B. Omary, submitted for publication.
**EXPERIMENTAL PROCEDURES**

Reagents and Cells—The antibodies used were: rabbit antibodies 3055 (26) and 8250 2, which recognize Ser(P)-52 (pS52) K18 and pS33 K18, respectively; mouse mAb L2A1, which recognizes human K8/18 (20); mAb D5, which recognizes K18 and its fragment p23; mAb KA4, which recognizes K19 (kindly provided by Dr. Robert Webster, Ciba-Corning, Alameda, CA); mAbs 5B3 (22) and LJ4 (23), which recognize K8 pS431 and pS73, respectively. Anti-K19 mAbs B/A2 and 4.62, and anti-K8 and K18 mAb 8.13 were purchased from Sigma. The mAbs KA4 and B/A2 recognize regions covering K19 amino acids 145–227 (domain IB) and 311–335 (domain II), respectively (36). Other reagents used were anisomycin (An) (CalBiochem, La Jolla, CA), rabbit anti-PARP, and recombinant caspase-3 (i.e. CPP32) (Upstate Biotechnology Inc., Lake Placid, NY). Human colonic HT29 cells (American Type Culture Collection, Rockville, MD) were cultured as recommended by the supplier. Apoptosis was induced using An (10 μg/ml) (23) and verified by annexin V and propidium iodide staining (not shown). Floater and adherent cells were either mixed or separated prior to further processing.

Immunologic and Biochemical Methods—Immunoprecipitation of K8/18 or K9/19 was done using mAb L2A1 or 4.62, respectively. Cells were solubilized directly with 1% Nonidet P-40, and then by sequential fractionation into a detergent-free cytosolic, 1% Nonidet P-40, 1% Empigen BB (Emp) pools, and a residual pellet that was suspended in 2% SDS-containing sample buffer (37). The cytosolic fraction was obtained after nitrogen cavitation followed by centrifugation, and the pellet was then solubilized with 1% Nonidet P-40 to obtain the subsequent fraction upon repelleting (37). The post-Nonidet P-40 pellet was then solubilized with 1% Emp followed by pelleting to isolate the Emp fraction. Fractions were transferred to polyvinylidene difluoride (PVDF) membranes followed by immunoblotting (26), or were used for immunoprecipitation (except for the final 2% SDS-solubilized pellet). SDS-polyacrylamide gel electrophoresis (PAGE) was done using 12% acrylamide gels (38). Microsequencing was done as described previously (37).

**In Vitro Protolysis of K8/18 Immunoprecipitates with CPP32 or with Detergent Cell Lysates**—Cells were cultured in the presence or absence of An (16 h), followed by solubilization with 1% Nonidet P-40 or 1% Empigen BB (Emp) pools, and a residual pellet that was suspended in 2% SDS-containing sample buffer (37). The cytosolic fraction was obtained after nitrogen cavitation followed by centrifugation, and the pellet was then solubilized with 1% Nonidet P-40 to obtain the subsequent fraction upon repelleting (37). The post-Nonidet P-40 pellet was then solubilized with 1% Emp followed by pelleting to isolate the Emp fraction. Fractions were transferred to polyvinylidene difluoride (PVDF) membranes followed by immunoblotting (26), or were used for immunoprecipitation (except for the final 2% SDS-solubilized pellet). SDS-polyacrylamide gel electrophoresis (PAGE) was done using 12% acrylamide gels (38). Microsequencing was done as described previously (37).

**Fig. 1. Characterization of apoptosis-induced and transfection-generated K18 and K19 fragments.** Panel A, HT29 cells were cultured in the presence or absence of An. Cells (adherent and floating) were solubilized with 1% Nonidet P-40 followed by immunoprecipitation of K8/18 using mAb L2A1. An treatment is associated with K8 Ser-73 phosphorylation, which generates a distinct K8 species termed HK8 (23). Panels B and C, K8/18 immunoprecipitates were obtained from BHK cells (B) that were co-transfected with K8 and K18 and from An-treated HT29 cells (H) and then analyzed by SDS-PAGE and Coomassie staining (panel B). Identical precipitates were transferred to PVDF membranes and then blotted with antibodies that specifically recognize the indicated K8 or K18 phosphorylation sites (panel C). Arrow points to immunoglobulin band under nonreducing conditions. Panel D, total cell lysates were obtained from HT29 (– An) and K8/18-transfected (+) or untransfected (–) BHK cells followed by analysis by SDS-PAGE, transfer to a PVDF membrane, and then immunoblotting with anti-PARP antibody. Panel E, K8/18 immunoprecipitates were obtained from HT29 cells (– An). No floaters cells were present in – An cells, while the + An cells were separated into floating (lane 3) and adherent (lane 2) cells prior to solubilization and immunoprecipitation. Panel F, K8/18 (lanes 1), K8/19 (lanes 2), or control agarose-protein A (lane 3) immunoprecipitates were obtained from An-treated HT29 cells followed by SDS-PAGE analysis. Duplicate samples were also transferred to PVDF membranes followed by blotting with anti-K19 mAb KA4 or B/A2.
Apoptosis and Keratin Fragments

**RESULTS**

**Apoptosis Induces Fragmentation of K18**—Previously, we showed that treatment of HT29 cells with An induces apoptosis (23). Immunoprecipitation of K8/18 from An-treated HT29 cells co-precipitates two major bands of 29 and 23 kDa (termed p29 and p23), which were also noted upon cotransfection of BHK cells with K8 and K18 cDNA (Fig. 1, A and B). Purification of p29 and p23 from An-treated HT29 cells or from K8/18-transfected BHK cells followed by SDS-PAGE analysis, transfer to PVDF membranes, Coomassie Blue staining, and then direct N-terminal sequencing of p29 and p23. Alternatively, gel pieces containing individual p29 or p23 bands were digested with Lys-C protease, followed by HPLC separation of the released peptides, and then microsequencing of internal peptides. All derived sequences had 100% identity with human K18. Panel B, IF proteins share the structural features of N- and C-terminal globular “head” and “tail” domains, respectively, that are separated by an α-helical “rod” domain. The rod domain is divided into subdomains that are separated by short “linker” (L) regions. The K18 amino acid location of the domains, the location of Asp-234VEVD, and the internally sequenced peptides of p29/p23 are indicated.

An) were similarly solubilized with 1 mM dithiothreitol in buffer A, and this lysate was used as a protease source after pelleting. Immunoprecipitates were then incubated (3 h, 37 °C) with CPP32 buffer (25 mM Hepes, 1 mM dithiothreitol, pH 7.5) in the presence or absence of CPP32, or with the 1% Nonidet P-40 detergent lysates that were obtained from An-treated cells, followed by analysis by SDS-PAGE and then staining with Coomassie Blue. Duplicate samples were transferred to a PVDF membrane followed by immunoblotting with anti-keratin antibodies.

**Apoptosis Also Induces Fragmentation of K19**—Apoptosis-induced digestion of K18 prompted us to test if similar digestion occurs in K19, which is also expressed in HT29 cells and has a similar 233SVEVD potential caspase-cleavable sequence within the L1–2 domain. Comparison of K8/18 and K8/19 immunoprecipitates, that were obtained from An-treated HT29 cells, showed several unique polypeptides that are preferentially associated with K8/19 precipitates (Fig. 1F). Of these, two major polypeptides (p28 and p20) corresponded to K19 fragments as determined using the epitope-defined anti-K19 antibodies KA4 and B/A2 (Fig. 1F) which recognize K19 domains IB and II, respectively (see Fig. 2B for keratin domains). The KA4 mAb recognized p28 (Fig. 1F, double asterisk), and mAb B/A2 recognized p20 (and K19) while neither antibody recognized the p29/p23 K18 fragments (Fig. 1F). Similarly, a panel of antibodies that recognize K8 and/or K18 did not recognize K19 or its p28/p20 fragments (not shown). The band that is indicated by a single asterisk (Fig. 1F), which was recognized by KA4 and B/A2 antibodies, was not investigated and may correspond to an overlapping fragment or may be nonspecific since it was not seen in blots of total cell lysates (Fig. 3, panel c). Similar K19 fragments were also noted after transfection of BHK cells with K8/19 and were confirmed by blotting with mAbs KA4 and B/A2 (not shown).

**Relationship of Keratin Phosphorylation to Caspase-mediated Keratin Digestion**—We compared the time course of apoptosis-induced keratin proteolysis and hyperphosphorylation. Immunoblotting of total cell lysates with epitope-specific anti-phosphokeratin antibodies showed that K8 (Ser-73/341) and K18 (Ser-52) hyperphosphorylation occurs within 30 min of An exposure, whereas K18 Ser-33 phosphorylation is not significantly altered (Fig. 3, panels d–g). In contrast, K18 and K19 fragments begin to appear, as analyzed in the total cell lysate or in association with K8/18 immunoprecipitates, after 4 h of An treatment (Fig. 3, panels a–c). This separation of keratin hyperphosphorylation and digestion suggests that if keratin phosphorylation plays a role in its caspase-de-
ated digestion then it may be by signaling downstream events and/or by improving the ability of keratins to act as caspase substrates.

The role of keratin phosphorylation in its caspase-mediated digestion was tested by subjecting K8/18 precipitates that were obtained from nonapoptotic or apoptotic cells (i.e. endogenous basal and hyperphosphorylated keratins, respectively) to \textit{in vitro} digestion by CPP32 or by cell lysates that were obtained from apoptotic or nonapoptotic cells. As shown in Fig. 4A, cell lysates from nonapoptotic cells do not cleave K18 (lanes 5 and 5') while cell lysates from apoptotic cells cleave K18 regardless of its phosphorylation state (lanes 6 and 6'). Also, CPP32 digested K18 equally well (Fig. 4A, lanes 3 and 3') regardless of its source. As expected, control precipitates that were obtained from An-treated cells (Fig. 4A, lanes 2' and 4') or that were treated with a nonapoptotic cell lysate (lane 5') had basally associated p29/p23. The role of keratin phosphorylation in keratin fragmentation was also examined in the BHK transfection cell system by transfecting a variety of K8 and K18 mutants that correspond to known phosphorylation and glycosylation sites (20, 22, 23, 40) followed by testing for the presence of the p29/p23 fragments. As shown in Fig. 4B, transfection with several phosphorylation and glycosylation K8 and K18 mutants afforded similar K18 fragments to those noted upon transfection of wild-type K8/18.

\textit{Distribution of K18 Fragments within the Cytosolic and Detergent-soluble and -insoluble Compartments}—We examined the distribution of p29/p23 K18 fragments in the cytosolic, Nonidet P-40, Emp, and remaining pellet fractions of apoptotic HT29 cells. The majority of the K8/18 pool is associated with the Emp and pellet fractions (Fig. 5, A and B) and so is p29/p23. Almost all pellet-associated K18 is degraded as detected by Coomassie staining and immunoblotting (Fig. 5, A and B), as is K18 in floater cells (Fig. 1E). In addition, p23 is distributed in
the Emp and pellet fractions (Fig. 5B, panel b), whereas p29 is found predominantly in the pellet fraction (Fig. 5B, panels a and d).

**DISCUSSION**

The findings of this report are summarized in Fig. 5C. Apoptosis results in selective caspase-mediated proteolysis of type I keratins, K18 and K19, while relatively sparing their partner type II keratin, K8. Although one K18 caspase site was defined (Asp-237) (Fig. 2), it is possible that other cut sites also occur with the potential presence of very small fragments. Given that similar caspase digestion motifs are found in other type I keratins and cytoplasmic IF proteins, it is likely that such proteolysis may be a generalized phenomenon. The time course of keratin degradation was similar to that of PARP (not shown). A significant fraction of K18 p29/p23 fragments remains associated with K8, and the majority of the K18 fragments are insoluble (Fig. 5), as normally found for K8/18 in nonapoptotic cells (17). However, although the cytosolic and Nonidet P-40 fractions have nearly equal amounts of p29/p23, the Emp and pellet fractions contain different proportions of p29 versus p23. This difference may reflect enhanced Emp extractibility of p23 versus p29 or may indicate an association of p23 with a cellular protein that is Emp-extractable.

The significance of keratin fragmentation remains to be determined. The apoptosis-associated morphological changes suggest that cytoskeletal reorganization is likely to be important in facilitating these changes. Most apoptotic cells (as determined by nuclear staining) manifested keratin filament disruption (not shown), although it remains to be determined if the disruption is due to keratin cleavage and/or hyperphosphorylation, particularly since both modalities can independently cause filament reorganization. For example, keratin filament reorganization may occur in association with a variety of hyperphosphorylated states (17, 19), or after adenovirus infection and subsequent cleavage of the K18 head domain at Met-73 by the viral L3 proteinase (41). Of note, a similar finding of K18 caspase-mediated fragmentation was noted in apoptotic human SNG-M endometrial and mouse HR-9 parietal cells (42). Deletion analysis coupled with the known lamin caspase cleavage site (43, 44) pointed to Asp-237 as a cleavage site that was verified by the inability of caspases to cleave K18 Asp-237 Glu (42). Interestingly, keratin fragments have been identified in sera of patients who harbor any one of a variety of epithelial tumors (45, 46) and may have diagnostic potential (47). Keratin hyperphosphorylation occurred early after induction of apoptosis while fragmentation was a late event. Increased keratin phosphorylation involved at least three of four known K8/18 sites (i.e. K8 S73/431 and K18 S52, but not K18 S33). The function of K8 pS73 is not known, but K18 pS52 is important for filament reorganization (20) and occurs with K8 pS431.

**Fig. 4.** In vitro reconstitution of K18 proteolysis and effect of K8/18 phosphorylation mutants on keratin proteolysis. Panel **A**, K8/18 precipitates and detergent lysates (1% Nonidet P-40) were obtained from An+ and An− cells as described under “Experimental Procedures.” K8/18 precipitates were incubated with CPP32 buffer (lanes 2 and 2'), buffer + CPP32 (lanes 3 and 3'), no additions while keeping over ice (lanes 4 and 4'), or with detergent lysates from -An-treated (lanes 5 and 5') or +An-treated (lanes 6 and 6') cells. Lane 1 shows CPP32 alone. After 3 h (37 °C), equivalent fractions were separated by SDS-PAGE and Coomassie stained or were blotted with antipS33 K18 (anti-K18/p29) or with mAb D5 (anti-K18/p23). Note that CPP32 also cleaves K18 into a slightly faster migrating species than undigested K18 (lanes 3 and 3'). Panel **B**, BHK cells were transiently transfected with vector alone, wild-type K8/18 or the indicated K8 and K18 mutant constructs. After 3 days, cells were solubilized, followed by immunoprecipitation of K8/18, analysis by SDS-PAGE, and then Coomassie staining. The glycosylation sites of K18 (Ser-29, -30, and -48) have been previously described (40).
Apoptosis and Keratin Fragments

During mitosis and growth factor receptor activation (22, 26), hyperphosphorylation did not involve K18 Ser-33, which regulates binding to 14-3-3 proteins (37), and no binding of 14-3-3 to K18 has been reported. The lack of K18 hyperphosphorylation in K8/18 digestion appears independent of keratin phosphorylation. In addition, phosphorylation at these sites is not essential for K8/18 digestion. In BHK cells, phosphorylation is obligate, important, or a bystander phenomenon. The fate of K8/18/19 after induction of apoptosis remains to be determined if keratin degradation and hyperphosphorylation are obligate, important, or a bystander phenomenon. Transfection of K8/18 phosphorylation mutants into BHK cells did not decrease fragment formation (Fig. 4B), which indicates that phosphorylation of these sites is not essential for proteolysis. In addition, in vitro reconstitution of caspase-mediated K8/18 digestion appears independent of keratin phosphorylation (Fig. 4A), which indicates that keratin hyperphosphorylation does not increase keratin susceptibility to caspase digestion.

Although the most common locations of human keratin mutations that have been identified to date are at the beginning and end of the rod domain (11–14) (termed helix initiation and termination domains, respectively, Fig. 2B), mutations within the L1–2 domain of K14 have been described in a few patients. Such mutations are in immediate proximity to the potential K14 caspase cleavage site, based on sequence similarity of this region (48) with K18 and K19 (e.g. K18 and K19 have 234/VEVD while K14 has 270/VEVD sequences). For example, Met-272 → Arg (49) and Val-270 → Met (50) mutations have been reported in patients with the Koebner and Weber-Cockayne syndromes, respectively, of epidermolysis bullosa simplex. Our results raise the questions of whether K14 undergoes caspase-mediated proteolysis and do the K14 mutations interfere with such proteolysis. One clear relevance of these questions is that a decrease in keratin degradation could play a role in the pathophysiology of these mutations.

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**FIG. 5.** Subcellular distribution of K18 fragments and summary of K8/18/19 fate upon apoptosis. Panel A, cytosolic (C), Nonidet P-40 (NP), Emp and remaining pellet (P) fractions were sequentially obtained from An-treated HT29 cells as described under “Experimental Procedures.” Also, K8/18 precipitates were obtained from the C, NP, and Emp fractions. Aliquots of each were examined by SDS-PAGE and then Coomassie stained. Panel B, samples equivalent to those shown in panel A, lanes 1–4, were transferred to PVDF membranes and then blotted with anti-K18 pS52 (rabbit Ab 3055), anti-K18 p23 (mAb D5), anti-K8 pS73 (mAb LJ4), and anti-K8/18 pS33 (rabbit Ab B250) and anti-K8 pS431 (mAb 5B3) had similar blotting patterns to anti-K18 pS52 and anti-K8 pS73, respectively (not shown). Panel C, schematic shows qualitative relative levels of K8, K18, p29, and p23 K18 fragments within the C, NP, Emp, and remaining pellet and summarizes the fate of K8/18/19 after induction of apoptosis.
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