Effect of Mutation and Phosphorylation of Type I Keratins on Their Caspase-mediated Degradation*

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Type I keratins K18 and K19 undergo caspase-mediated degradation during apoptosis. Two known K18 caspase cleavage sites are aspartates in the consensus sequences VEVDA and DALDS, located within the rod domain and tail domain, respectively. Several K14 (another type I keratin) mutations within the caspase cleavage motif have been described in patients with epidermolysis bullosa simplex. Here we use extensive mutational analysis to show that K19 and K14 are caspase substrates and that the ability to undergo caspase-mediated digestion of K18, K19, or K14 is highly dependent on the location and nature of the mutation within the caspase cleavage motif. Caspase cleavage of K14 occurs at the aspartate of VEMDA, a consensus sequence found in type I keratins K12–17 with similar but not identical sequences in K18 and K19. For K14, apoptosis-induced cleavage occurs sequentially, first at $\text{V}^{39}\text{D}^{39}\text{ALD}$ and then at $\text{V}^{234}\text{EVD}$. Hyperphosphorylation of K18 protects from caspase-3 in vitro digestion at $\text{V}^{234}\text{EVD}$ but not at $\text{V}^{39}\text{D}^{39}\text{ALD}$. Hence, keratins K12–17 are likely caspase substrates during apoptosis. Keratin hyperphosphorylation, which occurs early in apoptosis, protects from caspase-mediated K18 digestion in a cleavage site-specific manner. Mutations in epidermolysis bullosa simplex patients could interfere with K14 degradation during apoptosis, depending on their location.

Keratins are the cytoplasmic intermediate filament (IF) proteins of epithelial cells and consist of >20 separate gene products (1). The keratin subfamily of IF proteins is classified into two major groups, type I keratins (K9–20) and type II keratins (K1–8), which associate as noncovalent type I-II heteropolymers in an epithelial cell type-specific manner (1–4). Among cytoplasmic IF proteins, keratins and vimentin undergo caspase-mediated degradation as part of the cytoskeletal remodeling that takes place during apoptosis (5–8). The nuclear lamin IF proteins also undergo degradation during apoptosis and were the first IF proteins demonstrated to undergo apoptosis-associated digestion (9–11). The only keratins shown to undergo proteolysis during apoptosis are K18 and K19, whereas their type II partner (i.e. K8) manifests remarkable resistance to apoptotic degradation. Two known K18 caspase sites, VEVD and DALD, are located in the rod domain and tail domain, respectively (5–7). VEVD or similar consensus sequences are found in other IF proteins within the so-called linker 1–2 (L1–2) region of the rod domain (Fig. 1), whereas DALD is a unique caspase site that is found only in the K18 tail domain. The signals, if any, that target keratin degradation and the significance of this proteolysis are unknown. To that end, the only keratin-related apoptosis-associated change after an apoptotic signal is marked early keratin hyperphosphorylation. The significance of this early keratin hyperphosphorylation in association with apoptosis is not known, but amino acid substitution of the major K18 phosphorylation sites does not alter susceptibility to caspase digestion (6).

Understanding the significance and regulation of keratin (and other IF protein) degradation during apoptosis is important from a cell biological perspective and may also have pathophysiological relevance to human disease. For example, although most keratin mutations that have been identified in patients with epidermal blistering keratin diseases are located at the N-terminal region of the rod IA subdomain (12, 13), at least four K14 mutations have been described within the L1–2 region (14–17) in close proximity to the caspase recognition motif (VEMDA, also referred to herein as the caspase box). The cause of blister formation in these patients may be attributed to keratin filament assembly defects with resultant cell fragility. However, the proximity of these mutations to the caspase digestion site raises the possibility that the phenotype of the keratin disease in these instances may also be associated with perturbations in keratin degradation. If so, this could potentially impact the disease pathophysiology in patients with epidermolysis bullosa simplex (EBS), who harbor K14 L1–2 region mutations, and may offer more directed therapeutic strategies.

In this study, we use a mutagenesis approach to confirm that Asp$^{396}$ in the K18 tail domain is a caspase cleavage site in vivo. In addition, we show that sequential K18 digestion occurs at the tail (DALDS) and then at the rod (VEVDA) domains and that keratin hyperphosphorylation protects against cleavage at the rod but not the tail motif. To address the significance of the caspase box residues in keratin degradation, we generated a battery of caspase box mutations that mimicked the K14 mutations described in EBS patients and examined parallel mutations in K18 and K19. The results show that: (i) K14 VEMDA→MEMDA or VEMDD has no effect on susceptibility to caspase digestion, (ii) K14 VEMDA→VERDA and equivalent K18 and K19 mutations altered the migration of the N-terminal fragment on gel analysis, and (iii) K14 VEMDA→VEMGA and the equivalent K18 mutation abolished caspase digestion (boldface letters indicate residues that are mutated). Hence, pathogenic K14 mutations within the L1–2 region in patients with EBS can prevent caspase-mediated...

* This work was supported by National Institutes of Health Grant DK47918 and VA Career Development Awards (to M. B. O.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

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The abbreviations used are: IF, intermediate filament; Ab, antibody; Aa, anisomycin; BOC, β-tosyl-L-arginine-β-methyl ester; EBS, epidermolysis bullosa simplex; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; WT, wild-type; L1–2, linker 1–2.
keratin degradation during apoptosis. In addition, the VEMDA caspase box, which is found in many type I keratins (K12–17) is a suitable caspase substrate, as shown here for K14.

**EXPERIMENTAL PROCEDURES**

**Reagents**—The primary monoclonal antibodies (mAbs) used (6) were L2A1 (anti-K8/K18), D5 (anti-K18), DC10 (anti-K18; Neomakers, Fremont, CA), M30 (anti-K18 Asp<sup>396</sup> fragment, Roche Molecular Biochemicals), and KA4 (anti-K19). Other reagents and antibodies used were rabbit anti-K14 (ICN Biomedicals, Inc., Aurora, OH), anisomycin (An), caspase inhibitor III (t-butoxycarbonyl-Asp(O-methyl)-fluoromethyl ketone (BOC)), human recombinant caspase-3 (Calbiochem, La Jolla, CA), and calf intestine alkaline phosphatase (Roche Molecular Biochemicals).

**Cell Culture and Transfection**—HT29 (human colon) and BHK-21 (hamster kidney) cells (American Type Culture Collection, Manassas, VA) were cultured in media as recommended by the supplier. HT29 cells express K8, K18, and K19, whereas BHK-21 cells do not express any easily detectable keratins (data not shown). Wild-type (WT) or mutant keratin constructs, generated using a Transformer<sup>™</sup> kit (CLONTECH), were transfected using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer’s instructions. Notably, this transfection method induces apoptosis and keratin degradation in BHK-21 cells as reported previously (6).

**Biochemical and Immunoblotting Analysis**—Keratin degradation and subsequent apoptosis were induced in HT29 cells by culturing the cells in the presence of An (10 μg/ml in Me<sub>2</sub>SO) for 0, 0.5, 1, 2, 4, 8, 12, or 16 h. Total cell lysates in 2% SDS-containing sample buffer were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) (18) and then transferred to polyvinylidene difluoride membranes, followed by immunoblotting (19). BHK-21 cells transiently transfected with vector alone (Control) or with WT K8 and one of the following K18 constructs: WT, D237E, D396E, or the double mutant D237/396E. The immunoprecipitates were analyzed by SDS-PAGE and then Coomassie Blue staining (A) or immunoblotted with: (i) anti-K18 N-terminal Ab (B) that recognizes intact K18 (data not shown), K18 a, or K18 a+b (see Fig. 1 for schematic representation of fragments); (ii) anti-K18 C-terminal Ab (C) that recognizes intact K18 (data not shown), K18 b+c, or K18 b (the K18 c fragment is too small to be detected by the gel conditions used); or (iii) mix of K18 N-terminal and C-terminal Abs (D). Limited amounts of K18 a or K18 b+c are noted upon mutation of K18 at D237E and/or D237/396E, which likely reflects insufficient digestion due to the mutation(s) and/or digestion of hamster K18 that may be expressed at low levels in BHK-21 cells.

**In Vitro Caspase Digestion Assay**—HT29 cells were solubilized with...
Cleavage of Type I Keratins during Apoptosis

RESULTS

K18 Contains Two Caspase Recognition Sites That Are Sequentially Digested—Previous studies utilizing K18 D237A (5) or direct sequencing of apoptosis-generated K18 fragment b (Fig. 1 and Ref. 6) showed that K18 Asp237 is a major cleavage site in vivo. In addition, in vitro digestion of K18 using caspase-3 or caspase-7 indicated that a second caspase site is likely to be present in the tail domain of K18 (5). The second site was inferred to be K18 Asp396 by epitope mapping using mAb M30 (7), although this was not formally tested by mutational analysis. We mutated K18 D396E and K18 D237/396E and examined caspase digestion of the mutants as compared with WT K18-transfected BHK-21 cells undergoing apoptosis. K18 D396E generated a "new" 27-kDa band (K18 b+c fragment) that was recognized by a K18 C-terminal-specific Ab (Fig. 1A; Fig. 2, A and C), whereas K18 D237E accumulated a 43-kDa band (K18 a+b fragment; Fig. 1A; Fig. 2, B and D). The double mutant K18 D237/396E generated only one major undigested K18 species (Fig. 2A, lane 5). Hence, both K18 Asp237 and Asp396 are caspase digestion sites in vivo.

Generation of the K18 fragments a+b, a, or b was inhibited using the broad range caspase inhibitor BOC in cells transfected with WT K18 or with WT K8/K18 (Fig. 3A), thereby indicating that K18 fragmentation at Asp237 and Asp396 is caspase-mediated. Antibody specificity was confirmed by blotting K8-transfected cells (Fig. 3A; K8 expression was determined by blotting with anti-K8-specific Ab; data not shown). The M30 mAb (which recognizes an epitope that becomes exposed after K18 is cut at Asp396) does not detect K18 when mutated at D396 or D237 (Fig. 3A). Hence, both K18 Asp237 and Asp396 are caspase digestion sites in vivo.

FIG. 3. Blocking of K18 fragmentation by caspase inhibition and time course of K18 digestion. A, BHK-21 cells were transiently transfected with the indicated constructs. After 2 days, the transfected cells were treated with Me2SO (−) or BOC (+) as described under "Experimental Procedures." Total lysates were prepared from the transfected cells and then examined by immunoblotting with antibodies that recognize the indicated K18 fragments. Lysates from the transfected cells were prepared and then immunoblotted with antibodies as described in A. Note that the K18 p43 fragment (i.e. K18 a+b, which results upon cleavage at Asp396) accumulates in cells transfected with K18 D237E, but not in K18 D237/396E, because it cannot be further cleaved at Asp396 (see also Fig. 2A, arrowhead in lane 3 that also shows accumulation of p43). B, HT29 cells were treated with An (10 μg/ml) for the indicated times. Total lysates were then prepared and analyzed by immunoblotting with antibodies that recognize the indicated K18 fragments. Note that p43 (i.e. K18 a+b) was detected after 0.5 h of exposure to An, whereas p29 and p23 were detected after 2 h of exposure to An, thereby indicating that K18 caspase cleavage at Asp396 occurs earlier than cleavage at Asp237.

1% Nonidet P-40 in phosphate-buffered saline containing a mixture of protease inhibitors (6). After 1 h, lysates were pelleted, and the supernatant was used for immunoprecipitation by incubation in the presence of Sepharose-protein A coupled to mAb L2A1. Two duplicate K8/K18 immunoprecipitates were either used as a control or incubated with calf intestine alkaline phosphatase (20 units) for 1 h at room temperature to obtain dephosphorylated K8/K18. Hyperphosphorylated K8/K18 immunoprecipitates were obtained from HT29 cells treated with 1 μg/ml okadaic acid for 2 h. The immunoprecipitates (control, dephosphorylated, or hyperphosphorylated K8/K18) were incubated with buffer alone or with buffer containing recombinant human caspase-3 for 0.5, 1.5, or 3 h. The samples were then separated by SDS-PAGE and analyzed by immunoblotting.
The VEVDA motif in the rod domain is found in K18 and K20, whereas the VEVDS motif is conserved nature of the rod domain motif (X1E/DX2DX3;X1-X3, the DALD motif in the tail domain is unique to K18. Given the present in other IF proteins including K14 (Fig. 1B), whereas the DALD motif in the tail domain is unique to K18. Given the conserved nature of the rod domain motif (X1E/DX2DX3;X1-X3) and the presence of K14 mutations at the aspartate of VEMD (D→G) and at X1 (V→M), X2 (M→R), and X3 (A→D) in patients with EBS, we asked whether these mutations have an effect on type I keratin fragmentation during apoptosis. To address this question, we generated several corresponding mutations in K14, K18, and K19 and tested the mutant constructs for susceptibility to caspase-mediated degradation in cell transfection systems. Mutation V236M in K18 or K19 to generate a WT K14-like caspase box (i.e. VEMD instead on VEV) had no effect on caspase-mediated degradation of K18 (Fig. 5C). Similarly, EBS-like mutations at the X1 or X2 positions of the caspase box of K14 (V270M or M272R, respectively) and of K19 (V234M or V236R, respectively) and at the X3 position of K18 (V236R) had no effect on keratin fragmentation upon apoptosis (Fig. 5; Table I). However, the X2 mutation of M (in K14) or V (in K18/19) to R generates an N-terminal fragment that migrates slightly faster on SDS-PAGE gels as compared with the equivalent N-terminal fragment generated with WT or other mutant K14, K18, or K19 fragments (Fig. 5, highlighted with an asterisk). It is unlikely that the altered migration is due to the valine to arginine substitution per se because the N-terminal fragment of K18 V220R migrates similarly to the WT K18 fragment (Fig. 5B, compare lanes 1 and 2). In addition, this faster-migrating N-terminal fragment does not appear to result from exposure of other potential cryptic caspase digestion sites (i.e. K18 Asp185 at 177VEND or K18 Asp183 at 188KVID that may be exposed after the Val→Arg mutation in K18 V236R). For example, the double mutants K18 D180E/V236R or K18 D189E/V236R generate fragments with migration similar to that of K18 V236R (data not shown). We then tested, using An-induced apoptosis, whether caspase digestion occurs sequentially or randomly at VEV and DALD. Immunoblotting of total lysates from HT29 cells treated with An for various time intervals with M30 showed that K18 a-b (i.e. digestion at DALD) begins to appear after 0.5 h, whereas K18 b (i.e. digestion at VEV) is detected after 2 h of An treatment (Fig. 3C). This indicates that upon An-induced apoptosis, Asp396 is initially cleaved followed by digestion at Asp397. Cleavage at Asp396 does not appear to be essential for cleavage at Asp397 because the K18 D396E mutant remains a caspase substrate at Asp397 (Fig. 2, compare lanes 2 and 4).

**Effect of Phosphorylation on Keratin Fragmentation in Vitro**—We showed previously that K8 (Ser73 and Ser431) and K18 (Ser75) but not K18 (Ser431) hyperphosphorylation occurred within 0.5 h after An treatment and that keratin Ser→Ala mutants at these sites remain comparable to WT K18 in terms of their susceptibility to caspase digestion (6). Here, we examined the effect of dephosphorylation or hyperphosphorylation on K18 fragmentation using in vitro digestion by caspase-3. K8/K18 immunoprecipitates that were isolated from okadaic acid-treated cells or treated with alkaline phosphatase were digested with caspase-3 and then analyzed for the formation of keratin fragments. As shown in Fig. 4A, K18 hyperphosphorylation inhibited digestion at K18 Asp397 without any significant effect on K18 digestion at Asp396. In contrast, dephosphorylation did not have a significant effect on K18 Asp397 or Asp396 digestion (Fig. 4A), which is consistent with our previous findings using phosphorylation-mutant keratins (6). The effect of okadaic acid and alkaline phosphatase on K8/K18 phosphorylation was confirmed by immunoblotting of the K5/K18 precipitates with anti-phospho-K8 and anti-phospho-K18 antibodies (Fig. 4B).

**Effect of Caspase Box Mutations on Susceptibility to Keratin Fragmentation during Apoptosis**—The VEVDA motif in the rod domain is found in K18 and K20, whereas the VEVDS motif is found in K19. Similar rod domain motifs, such as VEMDA, are present in other IF proteins including K14 (Fig. 1B), whereas the DALD motif in the tail domain is unique to K18. Given the conserved nature of the rod domain motif (X1E/DX2DX3;X1-X3) and the presence of K14 mutations at the aspartate of VEMD (D→G) and at X1 (V→M), X2 (M→R), and X3 (A→D) in patients with EBS, we asked whether these mutations have an effect on type I keratin fragmentation during apoptosis. To address this question, we generated several corresponding mutations in K14, K18, and K19 and tested the mutant constructs for susceptibility to caspase-mediated degradation in cell transfection systems. Mutation V236M in K18 or K19 to generate a WT K14-like caspase box (i.e. VEMD instead on VEV) had no effect on caspase-mediated degradation of K18 (Fig. 5C). Similarly, EBS-like mutations at the X1 or X2 positions of the caspase box of K14 (V270M or M272R, respectively) and of K19 (V234M or V236R, respectively) and at the X3 position of K18 (V236R) had no effect on keratin fragmentation upon apoptosis (Fig. 5; Table I). However, the X2 mutation of M (in K14) or V (in K18/19) to R generates an N-terminal fragment that migrates slightly faster on SDS-PAGE gels as compared with the equivalent N-terminal fragment generated with WT or other mutant K14, K18, or K19 fragments (Fig. 5, highlighted with an asterisk). It is unlikely that the altered migration is due to the valine to arginine substitution per se because the N-terminal fragment of K18 V220R migrates similarly to the WT K18 fragment (Fig. 5B, compare lanes 1 and 2). In addition, this faster-migrating N-terminal fragment does not appear to result from exposure of other potential cryptic caspase digestion sites (i.e. K18 Asp185 at 177VEND or K18 Asp183 at 188KVID that may be exposed after the Val→Arg mutation in K18 V236R). For example, the double mutants K18 D180E/V236R or K18 D189E/V236R generate fragments with migration similar to that of K18 V236R (data not shown). We then tested, using An-induced apoptosis, whether caspase digestion occurs sequentially or randomly at VEVD and DALD. Immunoblotting of total lysates from HT29 cells treated with An for various time intervals with M30 showed that K18 a-b (i.e. digestion at DALD) begins to appear after 0.5 h, whereas K18 b (i.e. digestion at VEV) is detected after 2 h of An treatment (Fig. 3C). This indicates that upon An-induced apoptosis, Asp396 is initially cleaved followed by digestion at Asp397. Cleavage at Asp396 does not appear to be essential for cleavage at Asp397 because the K18 D396E mutant remains a caspase substrate at Asp397 (Fig. 2, compare lanes 2 and 4).

**Effect of Phosphorylation on Keratin Fragmentation in Vitro**—We showed previously that K8 (Ser73 and Ser431) and K18 (Ser75) but not K18 (Ser431) hyperphosphorylation occurred within 0.5 h after An treatment and that keratin Ser→Ala mutants at these sites remain comparable to WT K18 in terms of their susceptibility to caspase digestion (6). Here, we examined the effect of dephosphorylation or hyperphosphorylation on K18 fragmentation using in vitro digestion by caspase-3. K8/K18 immunoprecipitates that were isolated from okadaic acid-treated cells or treated with alkaline phosphatase were digested with caspase-3 and then analyzed for the formation of keratin fragments. As shown in Fig. 4A, K18 hyperphosphorylation inhibited digestion at K18 Asp397 without any significant effect on K18 digestion at Asp396. In contrast, dephosphorylation did not have a significant effect on K18 Asp397 or Asp396 digestion (Fig. 4A), which is consistent with our previous findings using phosphorylation-mutant keratins (6). The effect of okadaic acid and alkaline phosphatase on K8/K18 phosphorylation was confirmed by immunoblotting of the K5/K18 precipitates with anti-phospho-K8 and anti-phospho-K18 antibodies (Fig. 4B).

**Effect of Caspase Box Mutations on Susceptibility to Keratin Fragmentation during Apoptosis**—The VEVDA motif in the rod domain is found in K18 and K20, whereas the VEVDS motif is...
shown). Interestingly, an arginine substitution at the X1 position of K18 (V234R) blocks K18 cleavage at Asp 237 (Fig. 5B, lane 3), thereby indicating that a basic residue substitution at the X1 caspase box position is likely to inhibit caspase enzyme-substrate recognition.

An EBS-like K14 VEMD→VEMG mutation and a similar K18 mutation (VEVD→VEVG) abolish caspase cleavage (Table I). As shown previously for K18 (5) and shown here for K19 (D237E) and K14 (D273E), D→E mutations in these keratins also block caspase cleavage (Fig. 5, C and D). In addition, K18 VEVD→VEVE generates the 43-kDa fragment (K18 a) due to caspase digestion at Asp396 in 393DALD, a site that is not found in other non-K18 type I keratins. Another EBS-like mutation at the X3 position in K14 (A274D) or K18 (A238D) does not affect susceptibility to caspase-mediated digestion (Table I), thereby indicating that the X3 position is insensitive to acidic charge perturbations.

**DISCUSSION**

**Apoptosis-associated Degradation of Keratins—K18 (5–7) and K19 (6, 8) are the only keratins that have previously been demonstrated to undergo degradation during apoptosis. Degradation occurs primarily in type I keratins, with marked relative sparing of type II keratins as determined for K8, which is the only type II keratin studied in this context (5, 6). Sparing of type II keratins may be related to differences of the context of the caspase box within the L1–2 region of the rod (Fig. 1B, note the E3S substitution in type II keratins within the VEVD sequence), but type II keratins do possess other potential caspase recognition sequences (e.g. 77LEVD and 253LDMD in K8) that do not appear to be prominently cleaved. One important finding herein is that K14 is also a caspase substrate in transfected cells. This indicates that the remaining keratins of K12–17, in addition to desmin and neurofilament-L, are also likely to be caspase substrates because they all share the same VEMD motif, which differs slightly from the K18–20 (VEVD) motif.

The type of keratin fragments generated during apoptosis may differ depending on the presence or absence of other caspase recognition motifs and their susceptibility to cleavage. In the case of K18, two well-defined cut sites occur as defined
immunologically (7) and molecularly (Fig. 2). These two sites undergo sequential caspase-mediated digestion (Fig. 6) with release of the small K18 tail fragment (397–429) from the K8/K18 complex, followed by cleavage at K18 Asp237 to generate two stable fragments (1–237 and 238–396) that remain associated with K8. This apoptosis-associated keratin cleavage is accompanied by significant reorganization of the keratin cytoskeletal network (5, 20–23). Transient transfection of the K18, K19, and K14 mutants with WT K8 did not have any significant effect on filament organization as determined by immunofluorescence staining (data not shown).

**Modulation of Caspase Cleavage by Phosphorylation and by Mutations within the Caspase Box Motif**—Keratin hyperphosphorylation occurs as an early event upon exposure of cells to an apoptotic signal (6, 24). Mutation of the major K8 and K18 phosphorylation sites did not affect the susceptibility of caspase-mediated cleavage of K18 at Asp237, thereby indicating that dephosphorylation did not affect keratin degradation during apoptosis (6). However, hyperphosphorylation does significantly inhibit caspase-3 *in vitro* digestibility of K18 at Asp237 (the second sequentially cut K18 site), but not at Asp396 (the first cut site) (Fig. 6). This raises the possibility that hyperphosphorylation of the remaining type I keratins, which are cleaved at the K18 Asp396-equivalent site (Fig. 1B), may also be protective. Several functional roles, acting alone or in concert, can be envisioned for keratin hyperphosphorylation during apoptosis: (i) a simple by-product of the apoptosis-associated activation of multiple kinases (e.g. Refs. 25 and 26); if so, this favors a role for keratins as a phosphate reservoir or sink (24), (ii) a facilitator, alone or in concert with keratin degradation, of keratin filament reorganization during apoptosis, or (iii) a mechanism that either protects from apoptosis-induced damage (in this case degradation of keratins) or allows for a graded sequence of apoptotic events.

The caspase box motif that is found within the L1–2 region of the rod domain of cytoplasmic IF proteins is a prototype caspase recognition motif, represented in type I keratins by X1E/DX2DX3 (with site of cleavage occurring at the D between X2 and X3; X1, X2, X3, hydrophobic residues). Our results showed that replacement of X1 by an Arg prevents caspase-mediated degradation (Table I), which suggests that basic residue substitutions at X1 are likely to be incompatible with substrate-enzyme recognition. In contrast, the X2 or X3 positions were not affected by basic residue substitutions in that M/V

### Table I

| Keratin constructs and their degradation phenotypes |
|-----------------------------------------------|
| Type I keratin | Caspase box sequence | Construct mutation | Cleavage in rod domain |
|----------------|----------------------|--------------------|------------------------|
| K14            | VEMD/A               | None (wild-type)   | Yes                    |
|                | MEMD/A               | V270M (EBS)        | Yes                    |
|                | VERD/A               | M272R (EBS)        | Yes                    |
|                | VEMG/A               | D273G (EBS)        | No                     |
|                | VEME/A               | D273E              | No                     |
|                | VEMD/D               | A274D (EBS)        | Yes                    |
| K18            | VEV/A                | None (wild-type)   | Yes                    |
|                | VEAM/D               | V236M (K14-like)   | Yes                    |
|                | VERD/A               | V236R (EBS-like)   | Yes                    |
|                | VEMG/A               | D237G (EBS-like)   | No                     |
|                | VEME/A               | D237E              | No                     |
|                | VEVD/A               | A238D (EBS-like)   | Yes                    |
|                | REVD/A               | V234R              | No                     |
| K19            | VEV/A                | None (wild-type)   | Yes                    |
|                | MYVD/S               | V234M (EBS-like)   | Yes                    |
|                | VEMD/S               | V236M (K14-like)   | Yes                    |
|                | VERD/S               | V236R (EBS-like)   | Yes                    |
|                | VEME/S               | D237E              | No                     |
|                | VEVA/S               | D237A              | No                     |

*Altered fragment migration in SDS-PAGE gels.*

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**Fig. 6. Schematic of the fate of K18 during apoptosis.** Exposure of epithelial cells to an apoptotic signal results in rapid hyperphosphorylation (P) of K18 with subsequent caspase-mediated cleavage of K18 at Asp396. The fate of K18 397–429 is unknown, but it is likely to be released from the K8/K18 complex because it is not detected upon SDS-PAGE analysis of K8/K18 immunoprecipitates after apoptosis (data not shown). The remaining larger K18 fragment (1–396), which remains associated with K8 (e.g. Fig. 2A, lane 3), undergoes a subsequent cleavage step to generate two major K18 fragments (1–237, termed p29 or K18a; and 238–396, termed p23 or K18b; see also Fig. 1A). These two fragments also remain for the most part associated with K8 (e.g. Fig. 2A, lanes 2 and 4).
ments during apoptosis has potential clinical utility because
detection of such fragments has been used in a number of
studies as diagnostic and prognostic markers (30–35). These
include the so-called tissue polypeptide antigen and tissue
polypeptide-specific antigen, which are related to K8, K18, and
K19 (36) and to a C-terminal K18 fragment (37), respectively.
However, the molecular mechanisms for generating tissue
polypeptide antigen and tissue polypeptide-specific-like frag-
ments are unknown, although caspase-mediated degradation
and/or other protease activation are likely mechanisms. Hence,
understanding the precise molecular changes that occur to
keratins during apoptosis is an important first step in deter-
ing the significance of caspase-mediated keratin fragment
formation and release in tumors.

The presence of keratin mutations within the caspase box
in patients with epidermal diseases raises the possibility that
alterations in susceptibility to caspase-mediated cleavage could
impact disease pathogenesis or alter susceptibility to other
skin diseases. For example, apoptosis (and presumably subse-
quently keratin degradation) is a feature of several skin diseases
(38, 39). Identification of the keratin cleavage sites during apoptosis will allow subsequent in vivo

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J. Biol. Chem. 2001, 276:26792-26798.
doi: 10.1074/jbc.M103315200 originally published online May 16, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M103315200

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