SerpinB2 Protection of Retinoblastoma Protein from Calpain Enhances Tumor Cell Survival

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Abstract

The tumor suppressor retinoblastoma protein (Rb) plays a pivotal role in the regulation of cell proliferation and sensitivity to apoptosis through binding to E2F transcription factors. Loss of Rb in response to genotoxic stress or inflammatory cytokines can enhance cell death, in part, by eliminating Rb-mediated repression of proapoptotic gene transcription. Here we show that calpain cleavage of Rb facilitates Rb loss by proteasomal degradation and that this may occur during tumor necrosis factor α–induced apoptosis. The cytoprotective, Rb-binding protein SerpinB2 (plasminogen activator inhibitor type 2) protects Rb from calpain cleavage, increasing Rb levels and enhancing cell survival. Chromatin immunoprecipitation assays show that the increased Rb levels selectively enhance Rb repression of proapoptotic gene transcription. This cytoprotective role of SerpinB2 is illustrated by reduced susceptibility of SerpinB2-deficient mice to multistage skin carcinogenesis, where Rb-dependent cell proliferation competes with apoptosis during initiation of papilloma development. These data identify SerpinB2 as a cell survival factor that modulates Rb repression of proapoptotic signal transduction and define a new posttranslational mechanism for selective regulation of the intracellular levels of Rb. [Cancer Res 2008;68(14):5648–57]

Introduction

The retinoblastoma tumor suppressor protein (Rb), the product of the Rb1 susceptibility gene, was the first tumor suppressor gene to be identified and has emerged to play a central role in limiting cell cycle progression through regulation of the E2F family of transcription factors. In addition to its antiproliferative function, Rb possesses prosurvival activity, which is mediated by the ability of Rb to suppress apoptosis directly, independent of growth suppression (1, 2). In mice, genetic deletion of Rb1 results in excessive apoptosis associated with abnormal degeneration of neurons and lens fiber cells (3). Rb1 inactivation in epidermis causes increased apoptosis at an early stage of epidermal oncogenic progression, rendering mice less susceptible to skin carcinogenesis (4). Mechanisms implicated in mediating the antiapoptotic activities of Rb include its ability to repress E2F gene transcription (5) and its direct inhibition of proapoptotic signal transduction (1, 6). Rb ablation leads to up-regulation of E2F, which can sensitize cells to apoptosis (7). Gene expression profiling studies and direct transcription experiments show that up-regulation of E2F1 induces transcription of cell cycle genes, such as the G1-S cyclins, and also genes encoding proapoptotic cell-death machinery, including Apaf-1, p73, procaspase-3, and procaspase-7 (8, 9). Because Rb is capable of regulating the expression of cell cycle and apoptotic gene targets, additional factors in association with Rb likely contribute to ultimate cell fate decisions.

The activities of Rb are determined by the cellular proteins that interact with Rb and the functional consequences of these interactions. We recently identified the intracellular serine protease inhibitor SerpinB2 (also called plasminogen activator inhibitor type 2) as a Rb-binding protein that colocalizes with Rb in the nucleus and protects Rb from proteolytic degradation, resulting in enhanced Rb protein levels (10). SerpinB2 is a multifunctional protein of the serine protease inhibitor (serpin) family, which is synthesized by a variety of cells (11), and promotes cell survival (12–14). Transgenic overexpression of SerpinB2 in proliferating basal keratinocytes of mice inhibits apoptosis and promotes keratinocyte survival during skin carcinogenesis (15). SerpinB2 protects cells from the cytolytic effects of cytopathic viruses (16, 17) and mycobacterial infection (18, 19) and confers resistance to death induced by the inflammatory cytokine tumor necrosis factor α (TNFα). SerpinB2 biosynthesis is an acute-phase response to TNFα (20) and SerpinB2 inhibits the characteristic morphologic changes and DNA fragmentation patterns associated with TNFα-induced apoptosis (18). Although the cell survival activity of SerpinB2 is now well established, the molecular mechanism of SerpinB2 cytoprotection is not known. SerpinB2 protection from TNFα apoptosis cannot be explained by loss of TNFα receptors, impaired ability of TNFα to bind to receptors, impaired TNFα receptor signal transduction, or direct inhibition of caspases (18).

Ablation of Rb through either inactivation or protease-induced degradation is associated with apoptosis initiated by genotoxic cell stress or TNFα (6). To date, only a few proteolytic mechanisms targeting Rb degradation have been identified. Rb may be targeted by viral oncoproteins, including the human papillomavirus E7, which accelerate Rb degradation by proteasomes (21, 22). Alternatively, in response to death receptor signals, Rb may be cleaved and inactivated through the action of caspases (23). Cleavage of Rb at the major caspase consensus DEAB-NH2–G site within the COOH terminus of Rb, and its subsequent degradation, occurs during TNF receptor 1–induced apoptosis (24). Mice engineered to express the caspase-resistant Rb mutant (Rb-MI) show resistance to TNFα-induced apoptosis in several tissues (25). Notably, caspase-resistant Rb could be eliminated under certain

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experimental conditions, suggesting the existence of additional intracellular proteolytic mechanisms for eliminating Rb.

Calpains are Ca\(^{2+}\) dependent, nucleo-cytoplasmic cysteine proteases that have emerged to play important roles in cell death signaling via the cleavage and/or degradation of a number of regulatory proteins and transcription factors (26, 27). The calpain family includes the ubiquitously expressed calpain-1 (μ-calpain) and calpain-2 (m-calpain), as well as a number of tissue-specific calpains (26). Both calpain-1 and calpain-2 are heterodimers containing a large ~80kDa subunit, encoded by the genes capn1 and capn2, respectively, and a common 28kDa regulatory small subunit, encoded by capn4. Here we report that Rb is a calpain substrate. Calpain cleavage of Rb precedes proteasome degradation and participates in the regulation of Rb turnover. We identify the calpain cleavage site within the COOH-terminal domain of Rb, which overlaps the SerpinB2 binding site contained within this domain (10). SerpinB2 blocks calpain cleavage of Rb, enhancing Rb survival. We conclude that SerpinB2 functions as a cytoprotective factor that influences cell death signaling pathways through Rb protection.

Materials and Methods

Cell culture. HeLa cells and the HeLa cell line stably expressing SerpinB2 (Sta) have previously been described (18). SV40-transformed CAPN4\(^{-/-}\) and CAPN4\(^{+/+}\) mouse embryonic fibroblasts (MEF) were provided by Dr. John SerpinB2 (Biotech Australia, Pty. Ltd.). PD150606 (Calbiochem), or preincubated with recombinant human DTT for 15 min at 30°C to protect. factor that influences cell death signaling pathways through Rb survival. We previously reported that SerpinB2 increases Rb protection.

Calpain activity assays. Calpain zymography was done on cell lysates using calpain zymograms incorporating 0.2% casein in the gel as described (29). Assay of calpain activity using the fluorogenic peptide substrate (Suc-Leu-Tyr-AMC) is described in Supplementary data.

Transfection. Human SerpinB2 in pRc/CMV (CMV) has been described previously (12, 18). The mammalian expression vector encoding full-length CAPN4 was purchased from ATCC (IMAGE clone no. 8578411 cloned into pCMV-SPORT6). The Rb-810 W mutant was generated from pcDNA3.1-Rb by site-directed mutagenesis. Primer sequences are given in Supplementary data. Transient transfections in CAPN4\(^{-/-}\) cells were done with the Amaxa Nucleofector Kit R (Program U-30). HeLa cells were transfected using Lipofectamine 2000 (Invitrogen). Stable clonal cell lines were selected with hygromycin B (Invitrogen).

TNFα-induced apoptosis. Cells were treated with 10 ng/ml TNFα (R&D Systems) in the presence of 10 μg/ml cycloheximide or with cycloheximide alone. Cell death was assessed after the indicated time by counting live cells using trypan blue exclusion, by flow cytometry after propidium iodide staining, or by immunoblotting for procaspase-3 activation and PARP cleavage.

Chromatin immunoprecipitation assays. Chromatin immunoprecipitation was done using the Chromatin Immunoprecipitation Assay Kit (Upstate). After stimulation, cells were fixed in formaldehyde at a final concentration of 1%. Chromatin was sheared by sonication (4 × 10 s at 30% maximum potency). Immunoprecipitations were done at 4°C overnight with anti-Rb mAb (G3-245). Immune complexes were collected with protein A and protein-DNA cross-links were reverted by heating at 65°C for 6 h. Immunoprecipitated DNA was isolated and used for PCR amplification. The sequences of the promoter-specific primers used are given in Supplementary data.

Quantitative PCR. RNA was extracted with the RNeasy mini kit (Qiagen). For quantative real-time PCR, specific primers and fluorescence-labeled probes for cyclin A2 (Hs 00153138_m1), p73 (Hs 00232088_m1), thymidine kinase (Hs 00177406_m1), caspase-7 (Hs 00169152_m1), caspase-8 (Hs 00154256_m1), caspase-10 (Hs 00154268_m1), or p-actin (Hs 99999903_m1) were obtained from Applied Biosystems Assay-on-Demand Gene Expression products and assayed using the ABI PRISM 7900HT sequence detector system.

Chemical carcinogenesis. The SerpinB2\(^{-/-}\) mice on the C57BL/6 background (backcrossed seven generations) were provided by D. Ginsburg (Department of Internal Medicine and Howard Hughes Medical Institute, University of Michigan, Ann Arbor, MI; ref. 30). Both gender-matched wild-type littersmates from heterozygote crosses and C57BL/6 mice (The Jackson Laboratory) were used as controls. For the skin carcinogenesis experiments, the dorsal area of 8- to 14-wk-old mice was shaved and treated with a single application of 7,12-dimethylbenz(a)anthracene (DMBA; 25 μg) followed by promotion with phorbol-12-myristate-13-acetate (PMA; 12.5 μg), twice weekly (experiment 1) or thrice weekly for 3 wk and then twice weekly (experiment 2). Mice were monitored weekly for formation of papillomas (>1 mm). No papillomas formed as a result of treatment with vehicle alone. Animals were maintained and all experiments done under approved University of Maryland Institutional Animal Care and Use Committee protocols.

Immunohistochemistry on formalin-fixed tissues was done using standard protocols with antibodies specific for keratin 5 (AF-138, Covance) and murine SerpinB2 (protein G affinity-purified rabbit polyclonal generated as described; ref. 30). Apoptosis was monitored using the DeadEnd Colorimetric terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) system (Promega).

Statistical analyses. Student’s t test was used to compare averages of normally distributed data with equal variance. χ² analysis was used for analysis of frequency distributions. A threshold of P < 0.05 was considered significant.

Result. Proteolytic cleavage of Rb precedes Rb degradation by proteasomes. We previously reported that SerpinB2 increases Rb
protein levels, resulting from inhibition of Rb degradation (10). Whereas Rb is ultimately degraded via the proteasome pathway (22), a number of considerations indicate that it is unlikely that SerpinB2 is a direct inhibitor of the protease activity of the proteasome. Therefore, we postulated that proteasome-mediated Rb degradation may be preceded by a separate, SerpinB2-inhibitable, proteolytic cleavage event. If this was the case, then inhibition of proteasome activity could enable a normally transient Rb cleavage intermediate to be stabilized. Indeed, when Jurkat cells were treated with the proteasome inhibitors, lactacystin or MG-132, the accumulation of a faster-migrating Rb intermediate of ~95 kDa was detected, which was accompanied by the loss of full-length 110-kDa Rb (Fig. 1A). Rb proteolytic cleavage was assessed specifically by treatment of lysates with alkaline phosphatase to dephosphorylate Rb, thus eliminating bands due to multiphasphorylated Rb species (10). Detection of the ~95-kDa species required the presence of the caspase inhibitor Boc-D-FMK to inhibit caspases activated by proteasome inhibitors (31), which may also cleave and degrade Rb (23). This “trapped” 95-kDa Rb intermediate showed strong immunoreactivity with an NH2-terminal specific Rb mAb (Rb amino acids 1–240); however, immunoreactivity was lost with a COOH-terminal specific Rb mAb (C-15), indicating loss of a ~15- to 15-kDa COOH-terminal Rb peptide. These data suggested the occurrence of a COOH-terminal, caspase-independent proteolytic cleavage of the Rb protein, producing a 95-kDa Rb intermediate targeted for proteasomal degradation.

Rb is COOH-terminally cleaved by calpain, distinct from caspase cleavage. To investigate whether intracellular calpains cleave Rb, Jurkat cell extracts containing Rb were treated with calpain and immunoblotted for detection of Rb protein. Mild calpain treatment resulted in a decrease in full-length 110-kDa Rb and the appearance of a 95-kDa Rb cleavage product [Fig. 1B, Rb (G3-245)], which was identical to the Rb intermediate trapped by proteasome inhibition. An additional 100-kDa Rb band was also observed, but subsequent experiments showed that this cleavage product was generated only under cell-free conditions (data not shown), indicating that it was not relevant in vivo. The 95-kDa Rb was not immunoreactive with the Rb (C-15) antibody [Fig. 1B, Rb (C-15), lane 2], showing that proteolytic cleavage occurred near the Rb COOH terminus. Cleavage was calpain and Ca2+ dependent, being maximally effective during the first 4 to 8 hours after exposure to calpain inhibitors because cell viability did not change throughout the time period of the experiments (data not shown). Time course studies in Jurkat cells revealed that Rb stabilization was time dependent, being maximally effective during the first 4 to 8 hours after exposure to calpain inhibitors (Fig. 2A, right).

Calpain regulates Rb turnover directly through a mechanism inhibitable by SerpinB2. To further investigate the participation of calpain in the regulation of Rb turnover, we used calpain-deficient (CAPN4−/−) MEFs (28). Genetic deletion of the Capn4 gene abolishes both calpain-1 and calpain-2 activities (28). CAPN4−/− MEFs had substantially increased Rb protein levels compared with control CAPN4+/+ MEFs (Fig. 2B, lane 5). When recombinant SerpinB2 was added to the Jurkat cell extract before calpain addition, SerpinB2 inhibited the appearance of 95-kDa Rb [Fig. 1B, Rb (G3-245), lane 3 versus lane 2] and stabilized full-length 110-kDa Rb [Fig. 1B, Rb (C-15), lane 3 versus lane 2]. The cleavage of Rb by caspase was unaffected by SerpinB2 [Fig. 1B, lane 6]. These data suggested that SerpinB2 could protect Rb from COOH-terminal cleavage by calpain.

COOH-terminal Rb cleavage by calpain is inhibited by SerpinB2. To determine whether calpain cleaves Rb directly, we used a purified system in which purified recombinant Rb protein was treated with catalytic amounts of calpain-1. Multiple cleavage fragments were detected (Fig. 1C), showing that Rb is a direct calpain substrate. Generation of the 95-kDa Rb, recognized by the NH2-terminal Rb mAb (N-240; Fig. 1C, left and right), occurred very rapidly and was accompanied by the release of a ~10-kDa Rb fragment (Fig. 1C, left, arrow) shown to be COOH-terminal by immunoreactivity with the COOH-terminal specific Rb (C-15) antibody (Fig. 1C, middle, arrow). Experiments using calpain-2 generated similar Rb cleavage products (data not shown). When SerpinB2 was added to Rb before addition of calpain, the generation of the 95-kDa Rb was specifically abolished, showing that this calpain-mediated proteolytic cleavage was directly inhibitable by SerpinB2 (Fig. 1D, lane 5 versus lane 4). Whereas Rb was capable of cleaving Rb at multiple sites under these cell-free conditions, the 95-kDa Rb fragment was the only cleavage product observed in cultured cells in vivo (Fig. 1A and data not shown).

Identification of the SerpinB2-protected calpain cleavage site at Rb-Lys810. The identity of the calpain cleavage site within the COOH-terminal domain of Rb was determined by NH2-terminal sequence analysis of the purified 10-kDa calpain cleavage product (Fig. 1C, arrow). Ser811 was identified at the NH2 terminus of this peptide fragment, revealing the calpain cleavage site at P1, Lys810. This cleavage is consistent with calpain substrate preference for a basic amino acid at the P1 position (27, 32). Using fluorogenic peptide assays of calpain protease activity, SerpinB2 did not inhibit calpain protease activity directly (data not shown). Considering that Lys810 is centered within the SerpinB2-Rb binding domain previously mapped to Rb amino acids 768 to 785 and 825 to 840 (Fig. 1D; ref. 10), these data may suggest that SerpinB2-Rb binding interaction may interfere with accessibility of calpain to its cleavage site at Rb-Lys810.

Calpain inhibitors stabilize Rb, decreasing Rb turnover. Our data provided evidence for calpain cleavage of Rb in cell-free systems, which could control Rb levels by regulating Rb turnover. To determine whether inhibition of calpain affects Rb turnover in cells, we exposed several different cell types to a series of cell-permeable calpain inhibitors (Fig. 2A). Calpain inhibition specifically enhanced the accumulation of full-length Rb protein (Fig. 2A, left), which was not accompanied by changes in Rb mRNA levels (measured by Q-PCR; data not shown), consistent with posttranscriptional stabilization of Rb protein and inhibition of Rb turnover. The increased Rb was not a general stress response to calpain inhibitors because cell viability did not change throughout the time period of the experiments (data not shown). Time course studies in Jurkat cells revealed that Rb stabilization was time dependent, being maximally effective during the first 4 to 8 hours after exposure to calpain inhibitors (Fig. 2A, right).

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in a dramatic decrease in Rb protein levels [Fig. 2C, lane 2 versus lane 1, Rb (G3-245)], showing that calpain specifically mediates Rb turnover in these cells. Strikingly, when an expression plasmid encoding SerpinB2 (pCMV-SerpinB2) was cotransfected along with the pCMV-CAPN4 into CAPN4/C0/C0/C0 MEFs, Rb was stabilized and detected at a level indistinguishable from control transfected cells (Fig. 2C, Rb (G3-245), lane 4 versus lane 1), although calpain activity was rescued (Fig. 2C, lane 4, CAPN4 and Zymogram). SerpinB2 expression alone in the absence of calpain did not affect Rb levels [Fig. 2C, lane 3 versus lane 1, Rb (G3-245)]. Together, these data provide compelling evidence for regulation of Rb turnover through calpain cleavage by a mechanism inhibitable by SerpinB2.

**Rb cleavage by calpain occurs during TNFα-induced cell death.** We previously identified SerpinB2 as a cytoprotective factor...
that confers resistance to TNFα-induced apoptosis in HeLa cells (18). Calpains have been implicated in orchestrating the induction of apoptosis (33), and Rb loss is associated with enhanced susceptibility to apoptosis when cells are exposed to TNFα (6).

To determine whether SerpinB2 may confer resistance to apoptosis through the calpain-Rb pathway, we investigated the effect of SerpinB2 on Rb levels during apoptosis induced by TNFα in HeLa cells. Exposure of HeLa cells to TNFα and cycloheximide initiates cell death (18) as evidenced by activated caspase-3 and the appearance of the marker of caspase activation, PARP cleavage within 3 hours (Fig. 3A). The HeLa cell line expressing SerpinB2 (S1a) shows resistance to TNFα-induced death (18) and further showed delayed caspase activation (Fig. 3A). These TNFα-resistant S1a cells showed increased levels of COOH-terminally intact Rb [Fig. 3A, Rb (C-15)], suggesting that resistance to TNFα-induced death could be mediated by SerpinB2 inhibition of Rb loss via protection of Rb from calpain cleavage. In contrast, full-length Rb begins to disappear in HeLa cells after exposure to TNFα for 3 hours (Supplementary Fig. S1). To specifically investigate the role of calpain and SerpinB2 in protecting Rb during TNFα-induced apoptosis, we investigated calpain-deficient MEFs, CAPN4−/−MEFs, which have elevated Rb (Fig. 2B), show resistance to TNFα-induced cell death compared with CAPN4+/+ MEFs (Fig. 3B). When calpain activity was restored in CAPN4−/−MEFs by transfection of CAPN4, the sensitivity of CAPN4−/−MEFs to TNFα-induced death was rescued (Fig. 3B, CAPN4). Coexpression of SerpinB2 with CAPN4 could block calpain-dependent cell death, resulting in resistance to TNFα (Fig. 3B, CAPN4 + SerpinB2). These data show that calpain cleavage of Rb contributes to initiation of TNFα-induced death and that this activity can be blocked by SerpinB2.

Resistance to TNFα-induced cell death in CAPN4−/−MEFs was associated with delayed caspase activation, evidenced by delayed PARP cleavage in CAPN4−/−MEFs compared with CAPN4+/+MEFs on exposure to TNFα (Fig. 3C, left, lane 6 versus lane 3). In a similar way, pretreatment of TNFα-sensitive HeLa cells (18) with calpain inhibitor III to inhibit calpain activity stabilized Rb and delayed caspase activation in response to TNFα (Fig. 3C, right, lane 5 versus lane 4).

These data implicate regulation of Rb by calpain during induction of the TNFα-induced cell death response. To explore this directly, a mutant Rb (Rb-810W) was generated, which, when expressed in HeLa cells (Supplementary Fig. S2), showed resistance to cleavage by exogenous calpain at Rb-Lys810 compared with wild-type Rb (Fig. 3D). When calpain activity was restored in CAPN4−/−MEFs by transfection of CAPN4, the sensitivity of CAPN4−/−MEFs to TNFα-induced death was rescued (Fig. 3B, CAPN4). Coexpression of SerpinB2 with CAPN4 could block calpain-dependent cell death, resulting in resistance to TNFα (Fig. 3B, CAPN4 + SerpinB2). These data show that calpain cleavage of Rb contributes to initiation of TNFα-induced death and that this activity can be blocked by SerpinB2.

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wild-type Rb (Fig. 3D, left). HeLa cells transfected with Rb-810W were more resistant to caspase activation after exposure to TNFα, compared with HeLa cells transfected with wild-type Rb or parental HeLa cells, evidenced by significantly less PARP cleavage and severalfold less active caspase-3 (Fig. 3D, right). These studies suggest that regulation of Rb by calpain contributes to induction of TNFα-induced apoptotic responses and that inhibition or blocking of calpain cleavage of Rb can delay caspase activation and confer resistance to TNFα-induced death.

SerpinB2 sustains Rb-mediated repression of proapoptotic genes. How does SerpinB2 stabilization of Rb cause antiapoptotic activity? Increased stabilization of Rb might be expected to mediate antiapoptotic activity through repression of the transcription of proapoptotic E2F target genes. A Rb-containing signal transduction module present on selective E2F-regulated proapoptotic gene promoters (e.g., Apaf-1, p21, p73, and several caspases) has been associated with repression of apoptotic cell death (9, 34). To determine whether SerpinB2 stabilization of Rb contributes to Rb-mediated repression of proapoptotic genes, HeLa and S1a cells were compared for differential Rb binding to promoters of E2F-regulated genes during induction of TNFα-induced death using chromatin immunoprecipitation assays. Rb binding to the promoters of proapoptotic genes p73, caspase-7, and p21 was substantially decreased after exposure of HeLa cells to TNFα.
Constitutive Rb-mediated repression of S1a cells. Rb inactivation in epidermal keratinocytes renders mice less capable of activating a cell death response, thereby eliminating any surveillance mechanism whereby cells that have lost Rb function would be eliminated. The mechanism of SerpinB2 protection involves inhibition of calpain cleavage of Rb and subsequent proteasome degradation, resulting in delayed onset of papilloma development. We speculate that TNFα induced by PMA during skin carcinogenesis, cell proliferation competes with apoptosis (36), and Rb plays a critical role (35). Quantitation of apoptotic cells in hyperplastic lesions of SerpinB2−/− mice revealed a marked increase in apoptotic index (Fig. 5C), analogous to enhanced apoptosis detected in hyperplastic lesions of keratinocyte-targeted Rb−/− phenotype (35), with genetic loss of Rb or SerpinB2 leading to fewer and smaller papillomas.

SerpinB2 deficiency is associated with increased apoptosis. In the initiation stages of tumor development during DMBA/PMA skin carcinogenesis, cell proliferation competes with apoptosis (36), and Rb plays a critical role (35). Quantitation of apoptotic cells in hyperplastic lesions of SerpinB2−/− mice revealed a marked increase in apoptotic index (Fig. 5C), analogous to enhanced apoptosis detected in hyperplastic lesions of keratinocyte-targeted Rb−/− mice (4). These data suggest that SerpinB2 deficiency decreases keratinocyte survival early during the transformation process, resulting in delayed onset of papilloma development. We can speculate that TNFα induced by PMA during skin carcinogenesis (37) could initiate a calpain-dependent pathway to eliminate Rb, which would be blocked in cells expressing SerpinB2. These findings suggest that sustained SerpinB2 expression is important for the continued survival of basal keratinocytes and acts by reducing their sensitivity to apoptosis during stress, thereby enabling better survival and, paradoxically, promoting tumor development.

Discussion
The elimination of Rb is a cellular response to stress induced by genotoxic agents and TNFα (6). Depending on the biological context, Rb loss can precipitate induction of a cell death response and apoptotic cell death. Here we show that the cytoprotective protein SerpinB2 protects cells from Rb loss and delays cell death. The mechanism of SerpinB2 protection involves inhibition of calpain cleavage of Rb and subsequent proteasome degradation, thus promoting elevated Rb levels and enhancing Rb-mediated...
repression of E2F-regulated proapoptotic genes. Loss of this cytoprotective pathway, such as occurs with SerpinB2 deficiency, increases susceptibility to cell death. These data define a novel pathway for rapid and selective modulation of Rb that affects cell survival pathways. Rb degradation is required for TNFα receptor 1 to signal apoptosis (25). Our findings that Rb is a calpain substrate and that calpain regulates Rb levels provide an additional mechanism, in addition to caspase cleavage of Rb (25), for eliminating Rb. The role of calpains in cell death, however, is clearly complex. Calpains are reported to play both proapoptotic and antiapoptotic roles in several different cell systems and in response to a wide range of stimuli (38). Some of the complexity in calpain functions may be due to cell-specific regulatory factors such as the presence of SerpinB2. Our data show that calpain, likely activated as a consequence of stress-induced signaling, contributes to elimination of Rb and subsequent proapoptotic signaling events through loss of Rb-mediated transcriptional repression.

Calpain cleaves Rb within the COOH-terminal domain (Rb-Lys1050) and the calpain-cleaved Rb1–810 intermediate is targeted for proteasomal degradation. The Rb COOH-terminal domain is recognized to contribute to Rb-mediated growth suppression through binding to transcriptional regulators such as c-Abl (39) and E2F1 (40). It may be that differential protein-protein interactions associated with this domain potentially dictate the diversity of Rb functions in transcription, chromatin remodeling, differentiation, and cell survival. Further studies are required to determine whether calpain cleavage of Rb affects binding of transcriptional regulators that associate with Rb and subsequent downstream signaling events.
SerpinB2 protects Rb from calpain cleavage. SerpinB2 is a member of the intracellular clade B or ov-serpin subfamily (41). Many cells involved in the innate immune response produce clade B serpins. SerpinB2 is expressed in a limited number of cells, placental trophoblasts, monocytes/macrophages, and keratinocytes; however, it may be strongly up-regulated in multiple cell types following exposure to inflammatory and cellular stress mediators including cytokines, growth factors, viruses, and bacterial endotoxin (11). Several clade B members protect cells from exogenous and endogenous proteinase-mediated injury triggered by various death-inducing stimuli using a wide range of mechanisms (42–47). In addition, clade B serpins are implicated in direct inhibition of the activity of specific prolytic enzymes, mostly serine and/or papain-like cysteine proteases. We did not find that SerpinB2 inhibited calpain protease activity directly, suggesting a unique mechanism of inhibition that likely involves Rb binding via the PENF motif within the C-D interhelical loop region of SerpinB2 (10).

The induction of cytoprotective SerpinB2 could affect cell survival for multiple physiologic stress–induced pathways but, in addition, would likely be restricted to cells or situations where Rb is a determinant of the death response. DMBA/HPA–induced skin carcinogenesis is an Rb-dependent process (4, 37, 48). Transgenic overexpression of SerpinB2 in mouse skin increases survival of transformed keratinocytes during DMBA/HPA skin carcinogenesis (15), and we show here that SerpinB2 deficiency decreases their survival by reducing their susceptibility to transformation. PMA tumor promotion during skin carcinogenesis induces TNFa (37), which may initiate a calpain-dependent pathway to eliminate Rb and thus initiate the activation of a cell death response unless protective factors such as SerpinB2 are present. These data provide new insight into cell-specific and serpin-dependent mechanisms by which cell death versus prosurvival cell fates may be determined.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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