The Stability and Anti-apoptotic Function of A1 Are Controlled by Its C Terminus

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Marco J. Herold, Jonas Zeitz1, Christiane Pelzer1,2, Christa Kraus, Andrea Peters, Gisela Wohlleben, and Ingolf Berberich3

From the Institute for Virology and Immunobiology, University of Würzburg, Versbacher Strasse 7, 97078 Würzburg, Germany

Most Bcl-2 family members can localize to intracellular membranes via hydrophobic sequences within their C-terminal portion. We found that the C terminus of the anti-apoptotic family member A1 did not function as a membrane anchor. Instead, this stretch of the protein rendered A1 highly unstable by mediating its polyubiquitination and rapid proteasomal degradation. Moreover, the domain did not only function independently of its position within the A1 protein but when transferred could even destabilize unrelated proteins like enhanced green fluorescent protein and caspase-3. A1 was, however, much more stable in the presence of the Bcl-2 homology-only protein BimEL, suggesting that direct interaction of A1 with pro-apoptotic members of the Bcl-2 family strongly reduces its rate of turnover. We further show that the C-terminal end of A1 also contributes to the anti-apoptotic capacity of the protein. In conclusion, our data demonstrate that the C terminus serves a dual function by controlling the stability of A1 and by amplifying the capacity of the protein to protect cells against apoptosis.

Apoptosis plays a critical role in tissue development and homeostasis. This is witnessed by the fact that a number of mammalian pathologies, including cancers and autoimmune and degenerative disorders, arise as a consequence of apoptotic processes going awry (1). Many apoptotic pathways converge at the mitochondria where they induce permeabilization of the outer mitochondrial membrane and subsequently the release of death-inducing factors such as cytochrome c (2, 3). The stability of the outer mitochondrial membrane is controlled by members of the Bcl-2 family (4), which are characterized by the presence of between one and four conserved regions termed Bcl-2 homology (BH) domains (5). The Bcl-2 family consists of both pro- and anti-apoptotic members. The pro-apoptotic members can be further subdivided into those that possess multiple (two or three) BH domains, such as Bax and Bak, and those that possess only the short BH3 domain, such as Bim, Bad, Bid, and Noxa. The “BH3-only” proteins are thought to serve as sensors for different apoptotic stimuli and are stringently regulated at both the transcriptional and post-translational levels (6). The “multidomain” members, which are also referred to as “Bax/Bak-like” proteins, function downstream of their BH3-only relatives and are responsible for the formation of pores in the outer mitochondrial membrane (7). The anti-apoptotic Bcl-2 proteins, of which Bcl-2 is the prototype, appear to protect the integrity of the outer mitochondrial membrane by sequestering and thereby neutralizing the pro-apoptotic family members (8–10).

Current models of apoptosis posit that the fate of a cell exposed to an apoptotic stimulus will depend on the balance between the pro- and anti-apoptotic Bcl-2 family members (11). One well recognized mechanism for regulating protein levels is via ubiquitin-mediated proteasomal degradation (12). Levels of Bim, for example, are normally kept low as a consequence of constitutive ERK1/2-induced phosphorylation and ubiquitin-dependent degradation of the protein (13). Serum withdrawal, however, causes ERK1/2 activity to decline and levels of Bim to rise (14). In contrast, certain pathogens, including Chlamydia and Epstein-Barr virus, appear to promote cell survival by increasing the rate at which Bim is degraded by proteasomes (15, 16). There is mounting evidence that levels of the anti-apoptotic Bcl-2 family members are also regulated via the proteasome. Bcl-2 itself appears to be protected from proteasomal degradation by ERK1/2 phosphorylation (17). Recently, Mcl-1 levels were also shown to be regulated by the proteasome (18).

This study focuses on the anti-apoptotic factor A1. A1 and its human homolog Bfl-1 share the four conserved BH domains of Bcl-2 and Bcl-XL (19, 20). Overexpression studies in cell lines and mice have shown that it affords protection from various forms of apoptosis (21–24). In vitro studies have convincingly demonstrated that it can interact with Bim, Bid, Puma, and Noxa (25, 26). However, there are conflicting reports as to the subcellular distribution of A1 with independent studies locating it at the nucleus (27), at the mitochondrial membrane (28), and in the cytosol (25). Other anti-apoptotic Bcl-2 family members are targeted to intracellular membranes via a conserved stretch of hydrophobic amino acid residues in their C termini (29). The C terminus of A1 lacks this typical transmembrane domain, raising questions over the role the C terminus plays in its localization and, as a corollary, in its anti-apoptotic function. Here we show that the C terminus of A1 does not fulfill any specific targeting function but rather controls the short half-life of the protein. Interestingly, we observe an increased half-life of A1 in the presence of BimEL. Finally, we demonstrate that the C terminus of A1 also contributes to the anti-apoptotic potential of the protein.

EXPERIMENTAL PROCEDURES

Reagents—Cycloheximide, DTT, protease inhibitor mixture (P8340), and lipopolysaccharide (Pseudomonas aeruginosa) were purchased from Sigma. Etoposide and proteasome inhibitor II were purchased from Calbiochem.

Cell Culture—WEHI 231 cells (murine B cell lymphoma line), Daudi cells (human Burkitt lymphoma line), and 293T cells (human embryonic kidney cell line 293 expressing SV40 T antigen) were obtained from American Type Culture Collection. High density primary B cells (≥1.807 g/ml)

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**FIGURE 1.** The C terminus of A1 fails to target EGFP to specific subcellular locations. A, amino acid sequence comparison between mouse A1-a (used for all analysis in this study) and human Bfl-1. The arrows indicate the truncations of the A1 protein used in this study. The four C-terminal lysine residues are depicted in red and the potential phosphorylation sites in blue. The C terminus of A1 used for protein domain shuffling experiments is underlined. B, confocal microscopy of 293T cells transfused with either EGFP or EGFP tagged with the C terminus of various anti-apoptotic proteins (EGFP-A1ct, EGFP-Bcl-2ct, or EGFP-Bcl-Xlct). Mitotracker Red CMXRos was used for staining mitochondria. The yellow color shows the co-localization of EGFP-Bcl-2ct and EGFP-Bcl-Xlct with mitochondria.

Western Blot Analysis and Immunoprecipitation—Whole-cell lysates were prepared by lysing 3 × 10^6 cells in 25 μl of 6× Laemmli buffer. Proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane (Millipore), and analyzed by Western blotting. Polyclonal anti-caspase-3 (catalog number 9662) antibody was obtained from New England Biolabs. The monoclonal mouse anti-FLAG (M2) antibody was purchased from Sigma. The monoclonal anti Bcl-2 (C-2) antibody was obtained from Santa Cruz Biotechnology. The monoclonal green fluorescent protein (catalog number 632375) antibody was purchased from BD Biosciences. The monoclonal anti-HA (12CA5) antibody was obtained from Roche Applied Science. Detection was achieved using horseradish peroxidase-coupled secondary antibodies (Dianova) and the ECL system (Amersham Biosciences).

To identify ubiquitinated forms of A1, 293T cells were transiently transfected with various expression constructs (as indicated) in combination with an HA-tagged ubiquitin construct. 48 h after transfection cells were incubated with the proteasome inhibitor II (10 mM) for 1 h. Cells were lysed in RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1:100 protease inhibitor mixture, and 1 mM DTT), and proteins were subjected to immunoprecipitation with an anti-HA antibody or the anti-A1 antisem.

As a control for the expression of A1 protein, Western blot analysis was performed with an anti-FLAG antibody or the anti-A1 antisem.

For immunoprecipitation assays, 293T cells were transiently transfected with various constructs of A1 and N-terminal HA-tagged BimEL. Cells were lysed in 0.2% Nonidet P-40 buffer (150 mM KCl, 1 mM EGTA, 50 mM HEPES [pH 7.5], 5 mM MgCl₂, 1:100 protease inhibitor mixture, and 1 mM DTT), and proteins were subjected to immunoprecipitation with the anti-FLAG antibody. Immunoprecipitates were resolved by SDS-PAGE, and Western blot analysis was carried out with an anti-FLAG or an anti-HA antibody.

**Stability of Proteins in Vivo—**3 × 10^6 WEHI 231 wild type or 3 × 10^7 A1-transduced WEHI 231 cells were starved in Dulbecco’s modified Eagle’s medium without methionine and cysteine for 1 h. During metabolic labeling with [35S]methionine and [35S]cysteine (Amersham Biosciences), cells were kept at a density of 3 × 10^7/ml for 2 h. The cells were then chased in nonradioactive medium for the time periods indicated.
cated. Cells were lysed in RIPA buffer at 4 °C for 20 min. Samples containing equal amounts of protein were immunoprecipitated, collected with immobilized protein G-Sepharose (Amersham Biosciences), and resolved by SDS-PAGE. The gel was dried, and proteins were visualized by autoradiography.

**Determination of Cell Viability**—Cell survival was determined by staining the cells with annexin V-Cy5 (BD Biosciences) following the manufacturer’s instructions and analyzed by flow cytometry (FACSCalibur; BD Biosciences).

**RESULTS**

The C Terminus of A1 Is Not Sufficient to Target Proteins to Intracellular Membranes—Most Bcl-2 family members can be anchored to intracellular membranes by hydrophobic amino acid residues in their C termini (33). However, the accumulation of hydrophobic residues at the C-terminal end of A1 is interrupted by hydrophilic amino acids that most likely interfere with efficient membrane targeting (Fig. 1A). To directly test the potential of the C-terminal end of A1 to anchor a protein to intracellular membranes, we fused the last 35 amino acids of A1 to the C terminus of the enhanced green fluorescent protein (EGFP-A1ct). We chose this approach because Kaufmann et al. (29) previously showed that attaching the C termini of Bcl-2 or Bcl-X, to EGFP is sufficient to induce membrane localization of the chimeric proteins. We transduced 293T cells using recombinant retroviruses and analyzed the distribution of the fluorescent proteins by confocal microscopy (Fig. 1B). As expected, the C-terminal residues of A1 were not sufficient to directly target the chimeric protein EGFP-A1ct to intracellular membranes, i.e. like the wild type EGFP, EGFP-A1ct was evenly distributed throughout the cell. In contrast, EGFP-Bcl2ct was found at several intracellular membranes, although EGFP-Bcl-Xct was mainly localized at mitochondria (Fig. 1B). Thus, our results indicate that the C terminus of A1 has no membrane targeting function and does not direct a specific localization within the cell.

**A1 Is a Short Lived Protein**—Next, we addressed the question of whether the C terminus of A1 is redundant for the protein. To this end, we generated a mutant of A1 lacking 17 amino acids at the C terminus. Because no commercial antibodies were available to detect A1 at that time, we used N-terminally FLAG-tagged versions of the proteins (fA1 and fA1-155). Initially, we expressed the proteins fA1 and fA1-155 by transducing the murine B cell lymphoma line WEHI 231 with recombinant retroviruses encoding these proteins. Surprisingly, levels of fA1-155 were much higher than that of fA1 (Fig. 2A, upper panel), although both populations expressed the internal ribosomal entry site-linked selection marker (a chimeric protein of EGFP and the Zeocin resistance protein) at identical levels (Fig. 2A, lower panel). One explanation for this difference could be that the full-length protein is turned over much more rapidly than the truncated version. To test this possibility, we generated a population of WEHI 231 cells co-expressing both proteins, and we analyzed their stability after inhibiting de novo protein synthesis with cycloheximide (Fig. 2B). As predicted, fA1 disappeared much more rapidly than fA1-155. The half-life of ~15 min for the full-length protein was extended to almost 60 min for the truncated protein. To further strengthen this finding, we constructed two more mutants lacking either 12 (fA1–160) or 21 (fA1-151) amino acids at the C-terminal end, and we tested their stability in WEHI 231 cells. Indeed, the more amino acids we removed at the end of the protein, the more stable the mutant A1 proteins were (Fig. 2C). However, a mutant lacking the final 27 amino acids, including part of the BH2 domain, was not stable, possibly because it was not properly folded (data not shown). Finally, we confirmed the rapid turnover of fA1 in a pulse/chase experiment (Fig. 2D).

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Together, these findings indicate that A1 is a very unstable protein and that the instability is mediated by a domain located within the last 20 amino acids.

By using a newly generated anti-A1 antisera, which cross-reacts with mouse and human A1/Bfl-1, but not mouse Bcl-2 (data not shown), we were also able to determine the stability of endogenous A1 in a pulse/chase experiment. The loss of the metabolically 35S-labeled endogenous A1 followed the same time course as that of the overexpressed (unflagged) protein (Fig. 2E) thus confirming the earlier studies with FLAG-tagged A1.

To ensure that the fast turnover of A1 is autonomous and not limited to WEHI 231 cells, we also transduced primary mouse splenic B cells and the human embryonic kidney cell line 293T with fA1 (Fig. 3, A and B). In both cell types, we observed a loss of fA1 after cycloheximide treatment comparable with that seen in WEHI 231 cells (Fig. 3, A and B), i.e. fA1 disappeared with a half-life of less than 30 min.

The C-terminal ends of A1 and the human homolog Bfl-1 are highly conserved (Fig. 1A). Consistent with this, we observed a half-life of Bfl-1 in the human Burkitt lymphoma cell line Daudi that was comparable with the half-life of A1 (Fig. 3C). In summary, the high instability of A1 is not limited to a certain cell type and is conserved in the human A1 homolog Bfl-1.

**The C Terminus of A1 Regulates Its Stability**—Next, we addressed the question of whether the destabilization motif also functions independ-
ently of its position within the A1 protein by fusing the last 35 amino acids of A1 to the N terminus of the stabilized A1-155 (ctA1-155). Again, the protein was retrovirally expressed in WEHI 231 cells and compared with A1- and A1-155-expressing populations containing roughly the same number of proviral integrations. Western blot analysis showed that the expression of the newly generated protein was as low as that of the wild type protein. In addition, treating the cells with cycloheximide confirmed that it was as unstable as full-length A1 (Fig. 4A). Thus, the C-terminal residues of A1 mediate rapid degradation independently of their position within the protein.

To determine whether this domain could also destabilize other proteins, we generated several chimeric proteins containing the last 35 amino acids of A1. Fusing the C terminus of A1 to an enzymatically inactive mutant of caspase-3 (Casp3-A1ct) reduced its half-life compared with fusing the FLAG peptide at its C terminus (Casp3f) (Fig. 4B). Similarly, the exchange of the C terminus of Bcl-2 with that of A1 (Bcl2-A1ct) also resulted in a reduced half-life of the chimeric protein compared with wild type Bcl-2 (Fig. 4C). Finally, we transduced WEHI 231 cells with the chimeric proteins EGFP-A1ct or EGFP-Bcl-2ct, and we measured the fluorescence of the cells by flow cytometry. Regardless of the multiplicity of infection used, cells transduced with EGFP-A1ct showed a consistently lower mean fluorescence index than those transduced with EGFP-Bcl-2ct (Fig. 4D and data not shown). This difference became even more pronounced when de novo protein synthesis was blocked with cycloheximide. Taken together, all results show that the C terminus of A1 is not only necessary but also sufficient for mediating the instability of a protein.

A1 Is Degraded after Ubiquitination via the Proteasomes—It has been known for nearly 20 years that short lived proteins are degraded by proteasomes (34). To see whether A1 is also degraded via this pathway,
we analyzed the stability of fA1 and fA1-155 within the doubly transduced WEHI 231 population in the presence or absence of a proteasome inhibitor (proteasome inhibitor II). Blocking proteasomal degradation totally abolished the loss of the fA1 after inhibition of de novo protein synthesis with cycloheximide, although no change was observed in the level of the fA1-155 protein (Fig. 5A). In addition, we analyzed the protein stability of A1 after blocking the proteasomes in a pulse/chase experiment. Consistent with the cycloheximide data, A1 was almost entirely stabilized within the 2 h of observation in the presence of proteasome inhibitor II (Fig. 5B). This finding strongly suggests a proteasomal degradation pathway for A1.

As we have shown, EGFP becomes very unstable when fused with the C terminus of A1 (Fig. 4D). We then asked whether the rapid turnover of this fusion protein was also because of proteasomal degradation. Indeed, flow cytometric analysis demonstrated that blocking proteasomal activity with proteasome inhibitor II efficiently stabilized EGFP-A1ct (Fig. 5C, upper panel) but had no effect on levels of the stable EGFP-Bcl-2ct (Fig. 5C, lower panel). Similar results were obtained when we used a second proteasome inhibitor (MG-115) (data not shown). Taken together, these results imply that the proteasomal degradation of A1 is mediated via the C terminus of the protein.

Degradation of short lived proteins via proteasomes is frequently preceded by polyubiquitination of the targeted substrates (35). To test whether A1 is also a substrate for polyubiquitination, we transiently co-transfected 293T cells with an expression construct for fA1 and a construct coding for HA-tagged ubiquitin (haUbi). After precipitation of fA1, we analyzed the precipitate for haUbi by Western blotting. fA1 was strongly polyubiquitinated, whereas co-expression of fA1-155 with haUbi only resulted in weak ubiquitination (Fig. 5D, left panel). Importantly, substitution of all 11 lysines to alanine (A1(11A)) totally inhibited the conjugation of haUbi to A1 (Fig. 5D, right panel). In summary, these results suggest that A1 is first polyubiquitinated and then proteasomally degraded with the longer half-life of fA1-155 explained by its reduced ubiquitination.

It is known that the ubiquitin protein is covalently linked to lysine residues in the target protein by so-called E ligases and that mutation of these residues in short lived proteins usually increases the stability of the protein (35). We therefore investigated whether the mutation of all four lysine residues in the C terminus of A1 has an impact on its stability. We overexpressed the corresponding lysine to arginine mutant (fA1(4R)) in WEHI 231 cells. Most unexpectedly, we did not observe a substantial increase in the half-life of fA1(4R) compared with the fA1 (Fig. 6A). This was not because of structural modifications introduced by the arginine substitution, as replacing the four lysine residues with alanine also had no influence on the half-life of the A1 protein (data not shown). We also mutated the four lysine residues in the C terminus of the chimeric proteins fBcl-2-A1ct and fCasp3-A1ct to arginines (fBcl-2-A1ct(4R) and fCasp3-A1ct(4R), respectively). Consistent with the lysine mutant
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FIGURE 6. The C terminus of A1 mediates instability independently of its lysine residues. A, WEHI 231 cells, transduced with fA1 or fA1(4R), were treated with cycloheximide (Chx) (10 μg/ml) for the indicated periods, and protein lysates were analyzed for the various ubiquitinated caspase-3 proteins with an anti-FLAG antibody (upper panel). Bcl-2-A1ct or Bcl-2-A1ct(4R) expressing WEHI 231 cells were treated with cycloheximide (10 μg/ml) for the indicated periods. Total lysates were analyzed by Western blotting with an anti-Bcl-2 antibody (upper panel). Bcl-2 (endo) indicates endogenous Bcl-2. Equal protein loading was verified by anti-ERK staining (lower panels). C, 293T cells were transiently transfected with the indicated constructs. Two days later cells were treated for 1 h with proteasome inhibitor II (10 μM) and afterward lysed in RIPA buffer. Aliquots of the total lysates (TL) were analyzed by Western blotting for the expression of ubiquitin with an anti-HA antibody (upper panel) and the various FLAG-tagged caspase-3 proteins with an anti-FLAG antibody (lower panel). The rest of the lysates was subjected to immunoprecipitation with an anti-FLAG antibody. Precipitates (IP: FLAG) were analyzed for the various ubiquitinated caspase3 proteins with an anti-HA antibody by Western blotting (upper panel). Subsequent staining with an anti-FLAG antibody shows the precipitated caspase-3 proteins (lower panel).

of A1, neither fBcl-2-A1ct(4R) (Fig. 6B) nor fCasp3-A1(4R) (data not shown) showed any significant change in their stability.

Finally, we compared the level of polyubiquitination of fCasp3-A1ct and fCasp3-A1ct(4R) in 293T cells co-expressing haUbi (Fig. 6C). In line with the comparable instability of the two proteins, we did not observe a significant difference in polyubiquitination. These results indicate that the lysine residues in the C terminus of A1 are not necessary for the degradation of A1. Furthermore, these results suggest that the C terminus of A1 might serve as a docking site for E3 ligases to allow the assembly of polyubiquitin chains at “distant” lysine residues within the target proteins.

Bim Stabilizes A1 by Reducing Its Ubiquitination—BH3-only proteins can bind to A1 (25, 26). As both A1 and BimEL serve important functions in immune cells, we wondered whether BimEL has any influence on polyubiquitination and/or the fast turnover rate of A1 (19, 36). Therefore, we co-transduced WEHI 231 cells with HA-tagged BimEL (BimELha) and fA1. Intriguingly, we observed a dramatic stabilization of fA1 in the presence of BimELha as compared with fA1 alone (Fig. 7A, upper and middle panel), i.e. there was almost no loss of the A1 protein after 90 min of cycloheximide treatment when BimELha was present. As BimEL, like A1, can also be degraded by proteasomes (13), we had to exclude that the stabilization of A1 is because of an overload of the proteasomes. Therefore, we additionally co-expressed Casp3-A1ct, which, as we have shown (Fig. 6C), is also polyubiquitinated and rapidly degraded. We found that the short half-life of Casp3-A1ct did not change in the presence of BimELha and fA1 (Fig. 7A, lower panel). Therefore, we conclude that the increased stability of A1 in the presence of BimEL is not caused by a general inhibition of the proteasomes in these cells but is specific for the interaction with BimEL.

One reason for the longer half-life of A1 in the presence of BimEL could be that binding of BimEL masks critical lysine residues within A1 and/or interferes with the binding of a putative E3 ligase. In both cases one might expect to find a reduction in the ubiquitination of A1. To test this, we transiently co-transfected 293T cells with constructs coding for unmodified A1, FLAG-tagged BimEL (BimELha), and haUbi. After precipitating A1 with the anti-A1 antiserum, we analyzed the degree of ubiquitination of A1 (Fig. 7B, upper panel). Even though the expression levels of haUbi were similar in total lysates and comparable amounts of A1 could be precipitated, the amount of polyubiquitinated A1 was dramatically reduced in the presence of BimELha. In summary, these experiments reveal that ubiquitination of A1 is strongly reduced in the presence of BimEL, providing an explanation for the longer half-life of A1 under these conditions.

The C Terminus of A1 Is Necessary for Its Anti-apoptotic Function—So far, our studies have established that the A1 C terminus is a decisive factor in the fast turnover rate of the protein via ubiquitin-mediated protein degradation. We next queried whether this domain also influences the anti-apoptotic function of the protein. For this purpose we transduced WEHI 231 cells with either A1 or A1-155 and induced apoptosis with the topoisomerase inhibitor etoposide. As others have shown (37), full-length A1 was able to protect cells from etoposide-induced apoptosis, even at very high doses (Fig. 8A). Interestingly, A1-155 had to be expressed at considerably higher levels in order to mediate the same level of protection as full-length A1 (Fig. 8A). Similar results were obtained when we compared the anti-apoptotic capacity of the even more stable mutant A1-151 with A1 after etoposide treatment (data not shown).

During our studies, we generated a mutant in which the three potential phosphorylation sites in the C-terminal domain of A1 were converted to glutamic acid (STT → EEE) to mimic phosphorylation. This mutant is slightly more stable than wild type A1 but is entirely incapable of protecting WEHI 231 cells against etoposide-induced apoptosis (Fig. 8B). Together, our experiments indicate that truncating or mutating the C terminus of A1 strongly interferes with the anti-apoptotic function of the protein.

It is generally believed that the anti-apoptotic Bcl-2 family members function through binding and thereby neutralizing their pro-apoptotic antagonists (10). Because the proteins A1-155 and A1-Emut showed reduced anti-apoptotic capacities, we tested if this could be due to reduced binding to pro-apoptotic Bcl-2 family members. Therefore we evaluated the interaction of the pro-apoptotic BimELha and different FLAG-tagged versions of A1 in 293T cells. We found no difference in the ability of the three A1 proteins to precipitate BimELha (Fig. 8C, upper panel), suggesting that the reduced anti-apoptotic potential of the
mutant A1 proteins A1-Emut and A1-155 is not because of reduced binding to pro-apoptotic members of this family.

DISCUSSION

In this study we have shown that the C terminus of A1 plays an important function for the protein by regulating its stability and supporting its anti-apoptotic potential. Many Bcl-2 family members can localize to intracellular membranes via hydrophobic sequences in their C-terminal portion (33). However, in A1 the hydrophobic cluster at the C-terminal end is interspersed with polar and charged amino acids, making it rather unlikely that the C terminus by itself is very efficient in anchoring the protein to intracellular membranes. Indeed, fusion of the last 35 amino acids of A1 to EGFP does not change its intracellular anchoring the protein to intracellular membranes. Rather, like EGFP-A1, EGFP-Bfl-1 appears to be randomly distributed in the cytosol. Given that the C terminus of A1 does not appear to play a role in intracellular targeting, we then questioned whether it was in fact functionally redundant. Surprisingly, our experiments demonstrate that the presence or absence of the C terminus strongly influences the stability of A1 by being responsible for the efficient ubiquitination and as a consequence for the rapid degradation of the protein by the proteasomal pathway. This domain does not only function independently of its position within the A1 protein but can even induce degradation of unrelated proteins like EGFP and caspase-3.

Most short lived proteins are degraded by proteasomes (39). Proteasomal degradation is usually dependent on the polyubiquitination of the target protein (35). In this process, three different enzymes (ubiquitin-activating enzyme, ubiquitin-conjugating enzyme, and E3) activate and finally conjugate the small protein ubiquitin to one or more lysine residues of the target protein. The following observations argue that the instability of A1 is also because of degradation via this enzymatic pathway.
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machinery. 1) A1 is polyubiquitinated. 2) A1 is stabilized by proteasomal inhibitors. 3) Mutating all 11 lysines within A1 interferes with polyubiquitination of A1. During the initial phase of this study, the four lysines at the C terminus of A1 appeared to be the obvious candidates for the assembly of the polyubiquitin chain(s) on the protein. However, mutation of all four of these lysines within A1 and within the two chimeric proteins Bcl-2A1ct and iCasps-3A1ct did not stabilize and/or inhibit polyubiquitination of the proteins. This is consistent with a recent finding showing that mutating the corresponding lysines in the C terminus of Bfl-1 is not sufficient to fully stabilize or completely abrogate ubiquitination of the protein (40). Thus, these lysines are not the targets, or at least not the only targets, for the conjugation of ubiquitin. Systematic mutation of N-terminal lysines in the fA1(4R) construct failed to significantly stabilize or reduce ubiquitination of the protein. This suggests that multiple lysines can function as acceptor sites for polyubiquitination. This interpretation is in line with the results by Petroski and Deshaies (41) showing that any of three pairs of closely spaced lysines in the N-terminal region of the cyclin-dependent kinase inhibitor Sicl is sufficient to sustain degradation of the protein.

Successful deletion of the C terminus of A1 led to increasing stabilization of the protein. As exemplified for A1-155, the stability of the protein correlates with reduced polyubiquitination. In addition, attaching the C terminus of A1 is sufficient to induce conjugation of ubiquitin to the chimeric proteins iCasps-3-A1ct and iCasps-3-A1ct(4R). Together these results strongly suggest that an unknown E3 ligase recognizes a certain amino acid sequence within the last 20 amino acids. What sequences could be responsible for mediating this instability? Sequence analysis indicated a similarity between the sequence KKfepKSGwTmT within the C terminus of A1 and the destruction motif of IκB, β-catenin, and Vpu (KXnDSGXSS) (42, 43). Upon phosphorylation of the two serines in the destruction motif, the Skip1-Cullin-F-box (SCF) protein complex b-TrCP binds via the F-box protein to the substrate and attaches polyubiquitin chains to nearby lysines. Thereafter, the proteins are recognized by the proteasomes and degraded. Mutating the serine residues into alanines, i.e. interfering with the phosphorylation of the substrate, renders these proteins stable (44). We tested the involvement of a phosphorylation-dependent E3-ligase complex for the degradation of A1 by mutating the respective serine and threonines into alanines (data not shown). We could not observe a significant stabilization of A1 arguing against the involvement of an SCF-like protein complex in the polyubiquitination and degradation of A1.

The C terminus of A1 is highly conserved in evolution, with only one conservative substitution between the mouse and rat sequences. Mutating the serine residues into alanines, i.e. interfering with the phosphorylation of the substrate, renders these proteins stable (44). We tested the involvement of a phosphorylation-dependent E3-ligase complex for the degradation of A1 by mutating the respective serine and threonines into alanines (data not shown). We could not observe a significant stabilization of A1 arguing against the involvement of an SCF-like protein complex in the polyubiquitination and degradation of A1.

The C terminus of A1 is highly conserved in evolution, with only one conservative substitution between the mouse and rat sequences. The human counterpart Bfl-1 shows only two nonconservative substitutions within the last 27 amino acids of the protein. We analyzed Bfl-1 with respect to its stability in human cell lines (Fig. 3C and data not shown). The protein is slightly more stable than A1 but is still highly unstable. Our result is consistent with the recent observation by Kucharczak et al. (40) showing that Bfl-1 can be polyubiquitinated and rapidly degraded. We further analyzed the stability of Bfl-1 in murine cell lines (data not shown). Notably, we found no remarkable difference between the cell lines of the two species. Thus, the E3 ligase responsible for the degradation complex of A1/Bfl-1 seems to be highly conserved.

Deletion of the C terminus renders Bcl-2 cytosolic and impairs its ability to prevent apoptotic death (45). Even though the C terminus of A1 does not direct it to membranes, deletion of this domain significantly reduced the potential of A1 to prevent etoposide-induced cell death. The reduced anti-apoptotic potential of A1-155 cannot be explained by compromised binding with pro-apoptotic members of the Bcl-2 family as it was as efficient as wild type A1 in precipitating BimEL. Furthermore, A1-Emut, which carries three point mutations in its C terminus, almost entirely lost its anti-apoptotic potential, although its binding to BimEL was also not impaired (Fig. 8, B and C). Our finding that the C terminus is important for the full anti-apoptotic activity of A1 conflicts with a recent publication showing that truncating Bfl-1 did not affect cell survival (40). The reasons for this discrepancy might include the different apoptotic signals and cell types employed or the fact that Kucharczak et al. (40) analyzed single cell clones and not large populations of transduced cells as in this study. Perhaps more significantly, Kucharczak et al. (40) expressed Bfl-1 as a chimeric protein with EGFP which, for reasons unknown, significantly enhanced the half-life of Bfl-1. In contrast, the half-life of ectopically expressed A1 or FLAG-tagged A1 was much shorter and, more importantly, was comparable with endogenous A1 (Fig. 2E).

All our data indicate that A1 is a very unstable protein. However, we have shown that A1 is dramatically stabilized in the presence of the BH3-only protein BimEL. A similar phenomenon was recently reported to occur between the anti-apoptotic protein Mcl-1 and PUMA (46). The BH3 domain of PUMA was required to stabilize Mcl-1, which is consistent with our finding that the Casp-3A1ct mutant, which lacks a BH3-interaction domain, remains unstable in the presence of Bim, although F1 was stabilized (Fig. 7A).

The fact that A1 can interact with several other pro-apoptotic members (25, 26) raises the possibility that BimEL is not the only factor that can stabilize A1. Nevertheless, it is important to ask how stabilization of A1 is achieved by Bim. Possible scenarios include BimEL displacing an E3-ligase complex, which is constitutively bound to the C terminus of A1. Alternatively, BimEL might mask critical lysine residues on A1 preventing its ubiquitination. Regardless of the exact molecular details, we envisage a mechanism whereby the interaction of A1 with pro-apoptotic members of the Bcl-2 family is sufficient for its stabilization. However, when we analyzed the stability of ectopically expressed A1 after inducing apoptosis by etoposide, we did not observe increased stabilization. This is most likely because the level of pro-apoptotic proteins activated by etoposide at a certain point in time was too low to induce a significant change in the stability of the large pool of overexpressed A1.

As the experiments with the proteins A1-155 and A1-Emut in etoposide-induced apoptosis demonstrate, tampering with the C terminus of A1 destroys a lot of the anti-apoptotic capacity of the protein. Even though we do not know how the C terminus of the protein boosts the anti-apoptotic potential of the protein, several conclusions might be drawn from our experiments. 1) The neutralization of pro-apoptotic members of the Bcl-2 family by mere binding to A1 is rather inefficient and might not allow cell survival when A1 is expressed at physiological levels. 2) The dual functions of the C terminus, namely degradation of the protein and the anti-apoptotic efficacy, can be dissociated based on studies with the A1-Emut, which is unstable like the wild type protein but has almost entirely lost its anti-apoptotic potential, although its binding to pro-apoptotic members of the Bcl-2 family is sufficient for its stabilization. However, when we analyzed the stability of ectopically expressed A1 after inducing apoptosis by etoposide, we did not observe increased stabilization. This is most likely because the level of pro-apoptotic proteins activated by etoposide at a certain point in time was too low to induce a significant change in the stability of the large pool of overexpressed A1.

In conclusion, our data demonstrate that the C terminus of A1 serves a dual function by controlling the stability and by amplifying the capacity of the protein to protect cells against apoptosis. Because of its high

5 M. Langer and I. Berberich, unpublished data.
turnover rate paired with its ability to become stabilized in the presence of BH3-only proteins. A1 seems to be an ideal rapid response protein whose levels can be easily modulated depending on the actual need of a cell. Because most of the protein does not seem to be anchored to intracellular membranes but to be primarily localized within the cytosol, A1 appears to have evolved as a first line defense mechanism to “mop up” pro-apoptotic Bcl-2 proteins before they reach critical sites for apoptosis induction, like the mitochondria.

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