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The C-terminal Domain of A1/Bfl-1 Regulates its Anti-inflammatory Function in Human Endothelial Cells

Renata P. Guedes¹, Eduardo Rocha^{1,#}, Jerome Mahiou^{1,¶}, Herwig P. Moll¹, Maria B. Arvelo¹, Janis M. Taube^{1,‡}, Clayton R. Peterson¹, Elzbieta Kaczmarek¹, Christopher R. Longo^{1,‡}, Cleide G. da Silva¹, and Christiane Ferran^{1,2,*}

¹The Division of Vascular and Endovascular Surgery, the Center for Vascular Biology Research and the Transplant Institute, Department of Surgery, Boston MA 02215, USA

²Division of Nephrology Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston MA 02215, USA

Abstract

A1/Bfl-1 is a NF- κ B dependent, anti-apoptotic Bcl-2 family member that contains four Bcl-2 homology domains (BH) and an amphipathic C-terminal domain, and is expressed in endothelial cells (EC). Based on NF- κ B reporter assays in bovine aortic EC, we have previously demonstrated that A1, like Bcl-2 and Bcl-xL, inhibits NF- κ B activation. These results, however, do not fully translate when evaluating the cell's own NF- κ B machinery in human EC overexpressing A1 by means of recombinant adenovirus (rAd.) mediated gene transfer. Indeed, overexpression of full-length A1 in human umbilical vein EC (HUVEC), and human dermal microvascular EC (HDMEC) failed to inhibit NF- κ B activation. However, overexpression of a mutant lacking the C-terminal domain of A1 (A1 Δ C) demonstrated a potent NF- κ B inhibitory effect in these cells. Disparate effects of A1 and A1 Δ C on NF- κ B inhibition in human EC correlated with mitochondrial (A1) versus non-mitochondrial (A1 Δ C) localization. In contrast, both full-length A1 and A1 Δ C protected EC from staurosporine (STS)-induced cell death, indicating that mitochondrial localization was not necessary for A1's cytoprotective function in human EC. In conclusion, our data uncover a regulatory role for the C-terminal domain of A1 in human EC: anchoring A1 to the mitochondrion, which conserves but is not necessary for its cytoprotective function, or by its absence freeing A1 from the mitochondrion and uncovering an additional anti-inflammatory effect.

Keywords

A1/Bfl-1; Mitochondria; Nuclear Factor Kappa B; Apoptosis; C-terminal domain

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*Corresponding author: Christiane Ferran, MD, PhD, Beth Israel Deaconess Medical Center, Harvard Medical School, Division of Vascular and endovascular Surgery, Center for Vascular Biology Research and the Transplant Institute, RN #370 F/G, 99 Brookline Avenue, Boston MA 02215. Tel: 1-617-6670440; Fax: 1-617-6670445; cferran@bidmc.harvard.edu.

#Current addresses: ER Federal University of Rio de Janeiro, Brazil;

¶JM, Escoublac, Cambridge, MA;

‡JMT, Department of Pathology and Dermatology Johns Hopkins Hospital, Baltimore, MD;

‡CRL, Carolina Vascular Surgery, Raleigh, NC.

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1. Introduction

A1, also known as Bfl-1, is a Bcl-2 family member originally identified in hematopoietic cells, where it is abundantly expressed in response to treatment with granulocyte-macrophage colony stimulating factor [1]. Initially described as a hematopoietic-specific gene, A1 is also expressed in tumors including gastrointestinal and hematopoietic [2, 3]. Additionally, it is upregulated in response to inflammatory stimuli in numerous cell types such as endothelial cells (EC) [4, 5], renal proximal tubular epithelial cells (RPTEC) [6], and pancreatic beta cells [7].

Based on NF- κ B reporter assays in bovine aortic EC (BAEC), we have previously demonstrated that A1, like Bcl-2 and Bcl-xL, inhibits NF- κ B activation [5, 8], and hence could have anti-inflammatory functions in EC [9]. A1 is either not expressed or expressed at very low levels in EC at baseline, and becomes upregulated in response to pro-inflammatory stimuli in a NF- κ B-dependent manner. Accordingly, A1 Δ could possibly qualify as part of the anti-inflammatory and cytoprotective regulatory response of EC to injury [5, 10].

Homology between A1 and Bcl-2 was extensively studied. Currently, it is recognized that A1 Δ contains four Bcl-2 homology domains (BH1, BH2, BH3 and BH4) and an amphipathic tail anchoring peptide in its C-terminal domain that directs its binding to mitochondria, even if this domain is not a *bona fide* transmembrane domain like in other anti-apoptotic Bcl-2 family members [11–13]. This structural configuration, particularly the BH4 domain and the C-terminal mitochondria-anchoring domain, appears crucial for the anti-apoptotic function of Bcl-2 family members [8, 11, 12, 14–16]. In EC, we showed that the BH4 domain of Bcl-2 and Bcl-xL is also key for their NF- κ B inhibitory function [8]. However, the role of the C-terminal domain in supporting Bcl-2, Bcl-xL, or A1's NF- κ B inhibitory function in EC is still not fully elucidated.

The goals of this study were to evaluate the contribution of the C-terminal domain of A1 to its anti-inflammatory function in EC, and to extend our findings to human EC. Our results indicate that full-length A1 has no anti-inflammatory effect in human EC, contrasting with what we had reported in BAEC, when using transfection based NF- κ B reporter assays. However, the A1 deletion mutant lacking the C-terminal domain (A1 Δ C) showed NF- κ B inhibitory effect in human EC. We reproduced these data in rAd.A1 and rAd.A1 Δ C transduced BAEC, which indicated that discrepancies in results related to differences in experimental systems used. Both full-length A1 and A1 Δ C protected human EC from cell death, indicating that mitochondrial localization was not necessary for the cytoprotective function of this protein, at least in this context. Together these data resolve an important regulatory role for the C-terminal domain of A1 in EC, including in human primary EC cultures. It also cautions extrapolating results from reporter-based assays without evaluating the modulation of gene expression in the context of the cell's own signaling machinery and DNA/chromatin accessibility.

2. Material and Methods

2.1. Cell culture

Human umbilical vein EC (HUVEC) were purchased from Genlantis PrimaPure (San Diego, CA) and cultured according to manufacturer's instructions. Human dermal microvascular EC (HDMEC) and BAEC were isolated in our laboratory and cultured as described [17, 18]. The Burkitt lymphoma cell line, BJAB was a kind of Dr. R. Khosravi-Far, and the human hepatoma cell line HepG2 was purchased from ATCC (Manassas, VA). Cells were grown at 37°C in a 5% humidified CO₂ atmosphere. Experiments using EC were performed on confluent cells between passage 5 and 9 (BAEC), 5 and 7 (HDMEC), and 4 and 5 (HUVEC)

2.2. Expression plasmids

The N-terminal hemagglutinin A (HA)-tagged human A1 expression plasmid was previously constructed in our laboratory [5]. This construct was subcloned in the pAC.CMV-pLpASR+ (pAC) expression plasmid to generate recombinant adenovirus (rAd.), as previously described [19]. A1 deletion mutants were generated as follows: deletion of the C-terminal domain (A1 Δ C) was achieved, with HA tagged A1 Δ CDNA as template, by reverse transcription polymerase chain reaction (RT-PCR) using the following primers: sense: 5'-CGCGGGGTACCCCATGTATCCTTATGATGTT-3' and anti-sense: 5'-CCCCAAGCTTGGTTACATCCAGCCAGATTTAGG-3'. Deletion of the BH4 domain (A1 Δ BH4) was performed by site-directed mutagenesis based on overlap extension [20], using the following primers: RT-PCR reaction 1 - Sense A: 5'-CGCGGGGTACCCCATGTATCCTTATGATGTT-3' and Anti-sense B: 5'-TTCCACTTCTTTAGGTTGTGGTATCTGTAGGAC-3'; RT-PCR reaction 2 - Sense C: 5'-CCACAACCTAAAGAAGTGGAAAAGAATCTGAAG-3' and Anti-sense D: 5'-CCCCAAGCTTGGTCAACAGTATTGCTTCA-3'. Deletion of the C-terminal and BH4 domains (A1 $\Delta\Delta$) was achieved by RT-PCR using A1 Δ BH4 cDNA as template and the same primers previously employed to generate A1 Δ C RT-PCR products were verified by sequencing, and cloned in the CMV promoter driven pAC expression plasmid. Expression of HA-tagged transgenes was verified in transfected BAEC, as shown in the results section. All primers used for cloning were purchased from Gibco, Life Technologies (Grand Island, NY).

2.3. Recombinant adenoviruses

Recombinant adenoviral vectors encoding hemagglutinin A (HA)-tagged human A1 (rAd.A1) and A1 Δ C (rAd.A1 Δ C) were generated in our laboratory, as described [19]. The control adenoviral vector encoding nuclear β -galactosidase (rAd. β gal) was a kind gift of Dr. R. Gerard (University of Texas, Southwestern Medical Center). Recombinant adenoviruses were produced in the human embryonic 293 kidney cell line (HEK 293, American Type Culture Collection, Rockland, MD), extracted and purified using the Adenopure kit (Puresyn, Inc., Malvern, PA) and tittered by limiting dilution on 293 cells, as described [19]. Pre-confluent (80–90%) human EC were transduced with a multiplicity of infection (MOI) of 200 plaque-forming units per cell (pfu/cell), while BAEC were transduced at a MOI of 1,000 pfu/cell that we have shown to achieve transgene expression in more than 95% of EC, without causing toxicity, and within 48 h of transduction [19].

2.4. Immunofluorescence staining

Human EC cultured on glass coverslips were transduced with rAd. β gal, rAd.A1 or rAd.A1 Δ C. Forty-eight hours after transduction, cells were labeled with 100 nM Mitotracker Red CMXRos, following the manufacturer's instruction (Invitrogen, Molecular Probes, Carlsbad, CA), then incubated with rat monoclonal anti-HA-fluorescein antibody (Roche Diagnostics, Indianapolis, IN) O/N at 4°C. Cells were then washed and mounted on glass slides. Images were obtained using an Olympus BX41 microscope connected to a DP72 digital camera, using 100X oil immersion objective.

2.5. Protein extraction and Western blot analysis

Total protein cell lysates were recovered from EC before, and at different times after treatment with human recombinant tumor necrosis factor (TNF) (R&D Systems, Minneapolis, MN). Protein cell lysates were analyzed by Western blot (WB), as described [21], using the following primary antibodies: rabbit anti-I κ B- α IgG, mouse anti- β -actin IgG₁ (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rat anti-hemagglutinin A (HA) monoclonal antibody (Roche Molecular Biochemicals, Indianapolis, IN). Donkey anti-

rabbit- or goat anti-mouse-HRP-conjugated antibodies (Thermo Scientific, Rockford, IL) were used as secondary antibodies. Protein bands were detected with enhanced chemiluminescence kit (ECL) (PerkinElmer Life Science, Waltham, MA), followed by exposure to autoradiography films.

2.6. Analysis of intercellular adhesion molecule-1 (ICAM-1) expression by fluorescence-activated cell sorting (FACS)

Forty-eight hours after rAd. transduction, EC were treated with TNF (200 U/ml) overnight, then recovered and incubated for 30 min in FACS buffer (0.5% BSA in PBS), followed by 1 h incubation with FITC-conjugated mouse anti-human ICAM-1 IgG (eBioscience, Inc., San Diego, CA) or a control isotype IgG₁ (BD Pharmingen, San Jose, CA) at room temperature in the dark. Cells were then washed, fixed in 2% paraformaldehyde, and evaluated for surface expression of ICAM-1 by flow cytometry (FACSCanto; BD, Franklin Lakes, NJ). Results were analyzed using FlowJo software (Tree Star Inc., Ashland, OR) and presented as percentage of median fluorescence intensity, considering β gal-transduced cells as control (100%).

2.7. Analysis of cell death

To induce cell death, we treated HUVEC and HDMEC, 48 h after rAd. transduction, with 100 nM staurosporine (Sigma-Aldrich, St Louis, MO) for 6 h. Cells were then collected, washed, fixed in cold 70% ethanol, and stained with propidium iodide buffer (200 μ L PBS, 0.1% Triton X, 0.1 mmol/L EDTA, 50 μ g/mL RNase and 50 μ g/mL propidium iodide) for 1 h in the dark. DNA content analysis was then conducted using a FACScan bench top model (Becton Dickinson, San Jose, CA) with Cellquest acquisition and analysis software (Becton Dickinson, San Jose, CA). Cells with a normal DNA content (at least diploid [2N]) were scored as viable, whereas cells with a hyploidiploid DNA content (lower than 2N) were scored as nonviable.

2.8. NF- κ B luciferase reporter assay

BAEC were co-transfected with the different expression plasmids encoding for A1, its mutants or the empty control plasmid, pAC (0.7 μ g/well), together with a NF- κ B luciferase reporter plasmid (0.6 μ g/well) comprising 4 NF- κ B binding sites derived from the pig E-selectin promoter, as described [17]. Rous Sarcoma Virus (RSV)- β -galactosidase reporter plasmid (RSV- β gal, 0.3 μ g/well) was co-transfected with the two other plasmids to correct for transfection efficiency. Transfection of BAEC was achieved using cationic liposome-mediated gene transfer (Lipofectamine, Gibco, Life Technologies, Grand Island, NY), as described [17]. BAEC were stimulated 48 h after transfection with TNF (200 U/mL), harvested 7 h later and assayed for β -galactosidase and luciferase activities, as described [17]. Luciferase activity was normalized for β gal by using the formula: luciferase activity/ β gal activity \times 1,000. Normalized luciferase activity is given in relative light units (RLU).

2.9. Statistical analysis

Statistical analysis was performed on Prism 5 for Mac (GraphPad software, Inc., La Jolla, CA, USA). Data were analyzed by one-way analysis of variance (ANOVA) followed by post-hoc Tukey test. Differences between groups were rated significant at a probability error (P) of less than 0.05. Results are presented as means \pm standard error of mean (SEM).

3. Results

3.1. Generation of A1 deletion mutants

In order to perform structure/function analysis of A1 in EC, we generated A1 mutants with deletions of the C-terminal domain (pAC.A1 Δ C), the BH4 domain (pAC.A1 Δ BH4) or both (pAC.A1 $\Delta\Delta$) (Fig. 1A). All A1 mutants were HA-tagged and cloned in the pAC expression plasmid, allowing for subsequent generation of recombinant adenoviruses (rAd.) [19]. All mutants were confirmed by DNA sequencing (Core Laboratory of Boston University). We subsequently transfected BAEC with 0.7 μ g of full length A1 or the above-described A1-deletion mutant plasmids, and evaluated expression of the different transgenes by Western blot (WB) analysis using anti-HA antibody. Our data showed that all mutants were expressed and migrated at their expected molecular size (Fig. 1B). Maximal transgene expression occurred 48h after transfection (data not shown).

3.2. Subcellular localization of A1 and A1 Δ C in human EC using recombinant adenovirus-mediated gene transfer

To evaluate the role of A1 and its mutant harboring a deletion in the C-terminal domain in human EC, we generated rAd. expressing A1 and A1 Δ C. rAd-mediated gene transfer usually achieves significant expression of the transgene in over 95% of transduced EC, and therefore overcomes the limitation of inefficient transfection systems in these cells. Following verification of rAd.A1 and rAd.A1 Δ C by sequencing, we evaluated the expression of these transgenes in human EC cultures by immunohistochemistry (IHC), using anti-HA antibody. Our results confirmed that the different transgenes were expressed in >95% of transduced EC (data not shown). Since subcellular localization of proteins is difficult by IHC, we performed co-immunofluorescence staining and showed that full-length A1 mostly co-localizes with mitotracker red in both HUVEC and HDMEC, indicating that it associated with mitochondria (Figs. 2A & 2B). This supports previous data reporting mitochondrial localization of A1 in different cell types [22, 23]. In contrast, A1 Δ C did not co-localize with mitotracker red, instead it showed diffuse cytosolic distribution and some nuclear staining.

3.3. A1 Δ C, but not full length A1, exerts an anti-inflammatory effect in human EC

To evaluate the effect of A1 and A1 Δ C on NF- κ B activation in human EC cultures, we stimulated rAd.A1 and rAd.A1 Δ C-transduced HUVEC and HDMEC with 200 U/mL of TNF for 15 min and 2 h, and used WB to check for I κ B α degradation, a surrogate for NF- κ B activation. Non-transduced (NT) and rAd. β gal-transduced EC served as controls. Overexpression of full length A1 in both HUVEC and HDMEC failed to inhibit TNF-mediated I κ B α degradation. However, overexpression of the A1 Δ C mutant substantially reduced degradation of I κ B α in these cells 15 min after addition of TNF (Fig. 3A & 3B). To ascertain that overexpression of A1 Δ C was not simply delaying I κ B α degradation, we performed an extensive time course analysis of I κ B α protein levels, ranging 15 to 90 min. following TNF stimulation, in HUVEC. Our data confirmed that overexpression of A1 Δ C in EC inhibits, not simply delays TNF-mediated I κ B α degradation, and even allows faster recovery of baseline I κ B α protein levels (Supplementary Fig. 1).

Downstream of I κ B α degradation, we checked by flow cytometry for the expression of NF- κ B-dependent intercellular adhesion molecule 1 (ICAM-1), 16 h following treatment with TNF. Our results indicated that overexpression of A1 Δ C, but not full length A1, in HUVEC and HDMEC significantly inhibit TNF-induced upregulation of ICAM-1 (Fig. 4). Altogether, our data unveil a novel role for A1's C-terminus in regulating the anti-inflammatory function of this protein in human EC, i.e. the C-terminal domain that anchors

A1 to the mitochondria interferes with its ability to inhibit NF- κ B activation in human EC. These results contradict our previous data showing that full length human A1 inhibits TNF-mediated activation of a NF- κ B luciferase reporter in BAEC [5]. Given this discrepancy, we wanted to confirm our previous data using the same BAEC based co-transfection assay and evaluate in this system the effect of A1 Δ C, and also A1 Δ BH4, and A1 $\Delta\Delta$. Accordingly, we co-transfected BAEC with 0.7 μ g of pAC empty plasmid or pAC encoding full length A1 or A1 deletion mutants (A1 Δ C, A1 Δ BH4, or A1 $\Delta\Delta$, together with a NF- κ B luciferase reporter (0.7 μ g) and a RSV- β gal reporter (0.3 μ g), used to correct for transfection efficiency. Forty-eight hours following transfection, we checked for luciferase and β -gal activities in control non-treated cells, and 7 h after treatment with 200 U/ml of TNF. Our results confirmed that overexpression of full-length A1 inhibits TNF-induced activation of the NF- κ B reporter in BAEC (Supplementary Fig. 2A). Inhibition of TNF-induced activation of the NF- κ B reporter was maintained in BAEC transfected with A1 Δ C, indicating that this mutant has similar propensity to inhibit NF- κ B reporter activity in bovine EC and I κ B α degradation in human EC transduced with rAd. A1 Δ C (Supplementary Fig. 2A). In contrast, deletion of the BH4 domain from either full length A1 or the A1 Δ C mutant abolished A1's NF- κ B inhibitory effect (Supplementary Fig. 2A), which implies that, akin to Bcl-2, the BH4 domain is essential for A1's anti-inflammatory function, at least in reporter assay based evaluation of NF- κ B activity in BAEC. To further explore the mechanism(s) behind the discrepancies in A1's ability to inhibit NF- κ B luciferase reporter in BAEC transfected with an A1 plasmid, but not I κ B α degradation/NF- κ B activation in human EC transduced with rAd.A1, we used the same rAd.based transduction protocol to overexpress A1 and A1 Δ C in BAEC, then checked for TNF-induced I κ B α degradation. Interestingly, our results fully reproduced our data in human primary EC. i.e. overexpression A1 Δ C but not A1 significantly precluded TNF-induced I κ B α degradation (Supplementary Fig. 2B). We excluded differences in expression levels of the A1 transgene as the reason for discrepancy between transduced (rAd.A1) and transfected (A1 plasmid) in terms of NF- κ B activation. Indeed, either expression method resulted in comparable levels of HA-tagged A1 protein as shown by Western blot analysis (Fig. 1 and supplementary Fig. 2B).

In gaining insight into the physiologic role of A1 in EC's inflammatory response, we performed loss of function experiments by transducing HUVEC with A1-specific siRNA, achieving >80% knockdown of A1 mRNA (Supplementary Fig. 3A). A1 knockdown in HUVEC was particularly significant when compared to cells transfected with control AllStar negative siRNA that showed a 2 to 3 fold increase in A1 mRNA, a likely consequence of the usual toxicity of the transfection reagents triggering a survival response. None of the commercially available A1 antibodies was able to detect endogenous A1 protein levels in HUVEC. However, given the documented short half-life of the A1 protein (15 min), we believe that A1's protein knockdown should be at least commensurate to its mRNA knockdown, even if we can not formally prove it [24]. On the sole basis of mRNA levels, A1mRNA knockdown did not impact I κ B α degradation following TNF (Supplementary Fig. 3B), which could suggest that A1 is not a physiologic regulator of NF- κ B activation. This contrasts with knockdown of the NF- κ B inhibitory gene A20 that causes prolonged NF- κ B activation with delayed recovery of I κ B α protein levels in different cell types [25], including EC (data not shown). These data support our gain of function experiments in rAd.A1 transduced EC, as they indicate that full length A1 does not have a significant physiologic role in down-regulating NF- κ B activation in these cells.

3.4. Full length A1 and A1 Δ C equally protect human EC from Staurosporine-induced cell death

Classically, A1 is described as an anti-apoptotic Bcl-2 family member. However, several reports challenge this statement, demonstrating that A1 Δ could be anti-apoptotic, or

proapoptotic, depending on cell type and apoptotic stimulus [26–28]. In a microvascular endothelial cell line, A1 predominantly localized at the mitochondrial membrane, where it functioned to inhibit Cytochrome *c* release and Caspase 9 activation, maintaining mitochondrial transmembrane potential and promoting temporary survival of these cells in response to TNF [22]. To examine the contribution of A1 mitochondrial anchoring to its cytoprotective properties in human EC derived from primary cell cultures, we checked for the effect of A1 and A1 Δ C overexpression in HUVEC and HDMEC on STS-induced cell death. Our results showed that both A1 and A1 Δ C significantly protect HUVEC and HDMEC from STS-induced cell death, compared to control NT and rAd. β gal transduced EC (Fig. 5; n=3). These results indicate that localization of A1 to mitochondria is not required for its cytoprotective function in primary human EC cultures. These results are completely concordant with our data in BAEC transfected with A1 and A1 mutants expression plasmids (Supplementary Fig. 2C).

4. Discussion

A1/Bfl-1 is classically described as a TNF-inducible, NF- κ B-dependent, anti-apoptotic Bcl-2 family member in EC [4, 5, 22]. We had previously shown that A1 also exerts anti-inflammatory effects in BAEC by inhibiting NF- κ B activation [5]. In this study, we performed a structure-function analysis of A1 in order to delineate the role of its C-terminal domain upon its NF- κ B inhibitory and cytoprotective effects in EC, and extended this study to human EC.

Using co-immunofluorescence staining, we showed that full length A1 mostly localizes to mitochondria, which confirms that the amphipathic C-terminus of A1 drives its localization to this organelle, tempering some of the controversy about A1's cellular localization [11]. This result is consistent with data reporting that A1 and other anti-apoptotic Bcl-2 family members preferentially localize to mitochondria, where they directly (Bcl-2, Bcl-xL), or indirectly (A1/Bfl-1 through binding to Bid and tBid) [29] inhibit the activation of pro-apoptotic Bcl-2 family members (Bax) [30, 31].

In contrast, A1 Δ C was excluded from mitochondria, showing a nuclear and cytosolic pattern, similar to that described in MCF-7 cells overexpressing a Bfl-1 deletion mutant lacking the C-terminal domain [29]. While we expected cytosolic expression for a protein devoid of its C-terminus, we questioned the nuclear staining of A1 Δ C. By Western blot analysis, we demonstrated that only a negligible fraction of A1 Δ C is detected within the pool of soluble nuclear proteins, suggesting that A1 Δ C was mainly cytoplasmic. Even though, a fraction of the A1 Δ C protein may still be attached to the inner nuclear membrane, which would explain the peri-nuclear distribution that we detect by IF (Supplementary Fig. 4A). In fact, deletion of the A1 Δ C-terminus should not result in a nuclear localization sequence, contrasting with the short splice variant of human A1 (Bfl-1S), that in addition to lacking the C-terminus domain, incorporates a 56-bp exon-II into its sequence that creates a nuclear localization sequence [32]. Using Bfl-1 S specific primers, we demonstrated the presence of mRNA encoding for this short variant of A1 in HUVEC, although its levels were less abundant at baseline than that of full length A1, but alike A1 increased after addition of TNF (Supplementary Fig. 4B).

Next, we evaluated the effects of A1 and A1 Δ C on NF- κ B activation in human EC, using TNF-mediated I κ B α degradation and ICAM-1 upregulation as surrogates. Surprisingly, full length A1 did not maintain its NF- κ B inhibitory function in human EC, similar to our data in human RPTEC [6]. Furthermore, A1 silencing, at least as determined by mRNA knockdown, did not amplify TNF-induced pro-inflammatory responses of EC, suggesting that full length A1 does not influence inflammatory responses in EC. This is consistent with

the absence of a pro-inflammatory phenotype in mice with a knockdown of the mouse A1a isoform, as these mice only suffer from enhanced neutrophil apoptosis [33]. In striking contrast, A1 Δ C showed an anti-inflammatory function in HUVEC and HDMEC, similar to what we had reported in HUVEC transduced with a rAd.Bcl-2 adenovirus lacking its transmembrane domain [8, 34]. Inhibition of NF- κ B signaling only happens when A1 loses its amphipathic C-terminal domain and/or gets freed from the mitochondria. Additional experiments are underway to test this hypothesis. Intriguingly, these results partially disagree with our NF- κ B reporter assays in BAEC, where full length A1 and A1 Δ C were as effective in inhibiting TNF-induced NF- κ B reporter activity. Discrepancy between results obtained in BAEC and human EC were not due to differences in the expression level of the transgene but are likely related to differences in experimental systems used (reporter assays vs. direct probing of components of the NF- κ B signaling pathway in rAd. transduced EC), since rAd.A1 and rAd.A1 Δ C transduced BAEC gave similar results to what we reported in primary EC (Supplementary Figure 2B).

On the basis of NF- κ B reporter assays, we also have evidence that the BH4 domain of A1/Bfl-1 is responsible for its NF- κ B inhibitory effect. This result agrees with what we had already described for the prototypic Bcl-2 and Bcl-xL family members [8]. Future work in the laboratory aims to confirm this finding in human EC, and in systems evaluating the EC's own NF- κ B machinery.

In addition, we showed that full-length A1 protected EC from cell death triggered by the pan-protein kinase inhibitor, staurosporine. This added to previously published data showing that A1 protects EC from CHX/TNF-induced apoptosis [22, 35] emphasizes a broader anti-apoptotic effect of this protein in EC. This result is important because the cytoprotective effect of A1 is far from ubiquitous. Indeed, while A1 maintains its anti-apoptotic function in skeletal and cardiac myocytes and in several cancer cells [36–38], it promotes TNF-induced apoptosis of spinal cord motor neurons [28], B cells [26] and even 293T cells [27]. Surprisingly “anti-inflammatory” A1 Δ C equally protected EC from cell death, proving that mitochondrial localization is not required for the pro-survival function of A1 in EC. This result is analogous to data showing that C-terminus deletion mutants of human A1/Bfl-1 (A1 Δ C and $\Delta\alpha$ 9-Bfl-1) were still able to protect FL5.12 pro-B cells, and NIH-3T3 cells from STS and serum-starvation-induced apoptosis, respectively [11, 26]. However, this result contrasts with results obtained in the murine B cell lymphoma cell line, WEHI 231, where deletion or mutation of the C-terminus of A1 eliminated its ability to protect these cells from etoposide-induced cell death [24]. All these evidence stress cell type specific functions of full-length A1/Bfl-1 and its C-terminus-deletion mutant.

We, and others have shown that the anti-apoptotic function of A1 and prototypic Bcl-2 family members in EC and other cell types relies on intact BH1, BH2, and especially BH4 domains [8, 12, 14, 15]. In the case of A1/Bfl-1, an intact BH3 domain that associates with pro-apoptotic Bcl-2 family members, Bid and Bim, is also required [23, 24]. We established in BAEC transfected to express full length A1 or more importantly an A1 Δ C mutant where the BH4 domain was deleted (A1 Δ BH4, and A1 Δ C Δ BH4), that the BH4 domain of A1 and A1 Δ C was required for both their anti-inflammatory and anti-apoptotic functions. We presume that specific binding partners and/or functions associated with this domain support A1/A1 Δ C cytoprotective function without necessarily requiring its anchoring to the mitochondrial membrane. We recognize that this assumption challenges previously established concepts, at least in EC. Hence, future studies are underway to confirm these results in human EC, check their validity on the cell own NF- κ B machinery, and potentially discover their molecular basis.

In conclusion, our data exposes an important regulatory role for the amphipathic C-terminal domain of A1 in human EC. This domain is essential for A1 anchoring to the mitochondrion, but is not a pre-requisite for its cytoprotective function in EC. The C-terminal domain of A1, or alternatively mere anchoring of this protein at mitochondria interferes with its anti-inflammatory function in EC. This function only emerges when the C-terminus of A1/Bfl-1 is deleted, releasing it from the mitochondrion. Future work aims to determine whether this domain is ever cleaved under physiologic or pathologic conditions, in response to select EC activators, to transform A1 from a sole anti-apoptotic to a multi-functional anti-apoptotic and anti-inflammatory molecule. The fact that the C-terminal domain of A1 is also involved in its ubiquitination and protein turnover supports such an endeavor [24, 26] that could redefine A1's role in modulating EC homeostasis and phenotype. Ultimately, A1- Δ C-based EC therapies have the potential to treat vascular inflammation while providing a survival advantage.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

A1ΔBH4	Deletion of the BH4 domain
A1ΔC	mutant lacking the C-terminal domain of A1
A1$\Delta\Delta$	Deletion of the C-terminal and BH4 domains
ANOVA	analysis of variance
BAEC	bovine aortic EC
Bfl-1S	short form of A1/Bfl-1
BH	Bcl-2 homology domains
CHX	cycloheximide
EBM-2	endothelial basal medium-2
EC	endothelial cells
ECL	enhanced chemiluminescence kit
FACS	fluorescence-activated cell sorting
HA	hemagglutinin A
HDMEC	dermal microvascular EC
HEK 293	Human embryonic 293 kidney cell line
HUVEC	human umbilical vein EC

ICAM-1	intercellular adhesion molecule-1
IHC	immunohistochemistry
MOI	multiplicity of infection
NS	non-stimulated
NT	non-transduced
P	probability error
pAC.A1ΔBH4	plasmid encoding A1 mutant with deletion of the BH4 domain
pAC.A1ΔC	plasmid encoding A1 mutant with deletion of the C-terminal domain
pAC	pAC.CMV-pLpASR+ plasmid
pCA.A1ΔΔ	plasmid encoding A1 mutant with deletion of the C-terminal and BH4 domains
rAd.A1	Recombinant adenovirus human A1
rAd.A1ΔC	Recombinant adenovirus A1ΔC
rAd.βgal	recombinant adenovirus β-galactosidase
RLU	relative light units
RPTEC	renal proximal tubular epithelial cells
RSV	Rous Sarcoma Virus
RSV-βgal	Rous Sarcoma Virus-β-galactosidase
RT-PCR	reverse transcription polymerase chain reaction
SEM	standard error of mean
STS	staurosporine
TNF	tumor necrosis factor
VEGF	Vascular Endothelial Growth Factor
WB	Western blot

Literature Cited

1. Lin EY, Orlofsky A, Berger MS, Prystowsky MB. Characterization of A1, a novel hemopoietic-specific early-response gene with sequence similarity to bcl-2. *J Immunol.* 1993; 151:1979–1988. [PubMed: 8345191]
2. Choi SS, Park IC, Yun JW, Sung YC, Hong SI, Shin HS. A novel Bcl-2 related gene, Bfl-1, is overexpressed in stomach cancer and preferentially expressed in bone marrow. *Oncogene.* 1995; 11:1693–1698. [PubMed: 7478596]
3. Jourdan M, Reme T, Goldschmidt H, Fiol G, Pantesco V, De Vos J, Rossi JF, Hose D, Klein B. Gene expression of anti- and pro-apoptotic proteins in malignant and normal plasma cells. *Br J Haematol.* 2009; 145:45–58. [PubMed: 19183193]
4. Karsan A, Yee E, Kaushansky K, Harlan JM. Cloning of human Bcl-2 homologue: inflammatory cytokines induce human A1 in cultured endothelial cells. *Blood.* 1996; 87:3089–3096. [PubMed: 8605321]
5. Stroka DM, Badrichani AZ, Bach FH, Ferran C. Overexpression of A1, an NF-kappaB- inducible anti-apoptotic bcl gene, inhibits endothelial cell activation. *Blood.* 1999; 93:3803–3810. [PubMed: 10339487]

6. Kunter U, Daniel S, Arvelo MB, Choi J, Shukri T, Patel VI, Longo CR, Scali ST, Shrikhande G, Rocha E, Czismadia E, Mottley C, Grey ST, Floege J, Ferran C. Combined expression of A1 and A20 achieves optimal protection of renal proximal tubular epithelial cells. *Kidney Int.* 2005; 68:1520–1532. [PubMed: 16164629]
7. Sarkar SA, Kutlu B, Velmurugan K, Kizaka-Kondoh S, Lee CE, Wong R, Valentine A, Davidson HW, Hutton JC, Pugazhenti S. Cytokine-mediated induction of anti-apoptotic genes that are linked to nuclear factor kappa-B (NF-kappaB) signalling in human islets and in a mouse beta cell line. *Diabetologia.* 2009; 52:1092–1101. [PubMed: 19343319]
8. Badrichani AZ, Stroka DM, Bilbao G, Curiel DT, Bach FH, Ferran C. Bcl-2 and Bcl-XL serve an anti-inflammatory function in endothelial cells through inhibition of NF-kappaB. *J Clin Invest.* 1999; 103:543–553. [PubMed: 10021463]
9. Pober JS, Sessa WC. Evolving functions of endothelial cells in inflammation. *Nature Reviews Immunology.* 2007; 7:803–815.
10. Bach FH, Hancock WW, Ferran C. Protective genes expressed in endothelial cells: a regulatory response to injury. *Immunol Today.* 1997; 18:483–486. [PubMed: 9357140]
11. Brien G, Debaud AL, Robert X, Oliver L, Trescol-Biemont MC, Cauquil N, Geneste O, Aghajari N, Vallette FM, Haser R, Bonnefoy-Berard N. C-terminal residues regulate localization and function of the antiapoptotic protein Bfl-1. *J Biol Chem.* 2009; 284:30257–30263. [PubMed: 19759007]
12. D'Sa-Eipper C, Chinnadurai G. Functional dissection of Bfl-1, a Bcl-2 homolog: anti-apoptosis, oncogene-cooperation and cell proliferation activities. *Oncogene.* 1998; 16:3105–3114. [PubMed: 9671389]
13. Herman M, Nyman T, Welin M, Lehtio L, Flodin S, Tresaugues L, Kotenyova T, Flores A, Nordlund P. Completing the family portrait of the anti-apoptotic Bcl-2 proteins: Crystal structure of human Bfl-1 in complex with Bim. *FEBS Letters.* 2008; 582:3590–3594. [PubMed: 18812174]
14. Hirotani M, Zhang Y, Fujita N, Naito M, Tsuruo T. NH2-terminal BH4 domain of Bcl-2 is functional for heterodimerization with Bax and inhibition of apoptosis. *J. Biol. Chem.* 1999; 274:20415–20420. [PubMed: 10400666]
15. Huang DCS, Adams JM, Cory S. The conserved N-terminal BH4 domain of BCL-2 homologues is essential for inhibition of apoptosis and interaction with CED-4. *EMBO J.* 1998; 17:1029–1039. [PubMed: 9463381]
16. Lindsay J, Esposti MD, Gilmore AP. Bcl-2 proteins and mitochondria—Specificity in membrane targeting for death. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research.* 2010
17. Cooper JT, Stroka DM, Brostjan C, Palmethofer A, Bach FH, Ferran C. A20 blocks endothelial cell activation through a NF- κ B-dependent mechanism. *J. Biol. Chem.* 1996; 271:18068–18073. [PubMed: 8663499]
18. Richard L, Velasco P, Detmar M. A simple immunomagnetic protocol for the selective isolation and long-term culture of human dermal microvascular endothelial cells. *Exp Cell Res.* 1998; 240:1–6. [PubMed: 9570915]
19. Ferran C, Stroka DM, Badrichani AZ, Cooper JT, Wrighton CJ, Soares M, Grey ST, Bach FH. A20 inhibits NF-kappaB activation in endothelial cells without sensitizing to tumor necrosis factor-mediated apoptosis. *Blood.* 1998; 91:2249–2258. [PubMed: 9516122]
20. Aiyar A, Xiang Y, Leis J. Site-directed mutagenesis using overlap extension PCR. *Methods Mol Biol.* 1996; 57:177–191. [PubMed: 8850005]
21. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 1970; 227:680–685. [PubMed: 5432063]
22. Duriez PJ, Wong F, Dorovini-Zis K, Shahidi R, Karsan A. A1 functions at the mitochondria to delay endothelial apoptosis in response to tumor necrosis factor. *J Biol Chem.* 2000; 275:18099–18107. [PubMed: 10849436]
23. Werner AB. Bcl-2 Family Member Bfl-1/A1 Sequesters Truncated Bid to Inhibit Its Collaboration with Pro-apoptotic Bak or Bax. *Journal of Biological Chemistry.* 2002; 277:22781–22788. [PubMed: 11929871]
24. Herold MJ. The Stability and Anti-apoptotic Function of A1 Are Controlled by Its C Terminus. *Journal of Biological Chemistry.* 2006; 281:13663–13671. [PubMed: 16551634]

25. Lee EG, Boone DL, Chai S, Libby SL, Chein J, Lodolce JP, Ma A. Failure to regulate TNF-induced NF- κ B and cell death responses in A20-deficient mice. *Science*. 2000; 289:2350–2354. [PubMed: 11009421]
26. Kucharczak JF, Simmons MJ, Duckett CS, Gelinas C. Constitutive proteasomemediated turnover of Bfl-1/A1 and its processing in response to TNF receptor activation in FL5.12 pro-B cells convert it into a prodeath factor. *Cell Death Differ*. 2005; 12:1225–1239. [PubMed: 16094403]
27. Yang WS, Ko JK, Park SO, Choi HY, Kim YN, Kim CW. C-terminal region of Bfl-1 induces cell death that accompanies caspase activation when fused with GFP. *J Cell Biochem*. 2005; 94:1234–1247. [PubMed: 15696550]
28. Crosio C, Casciati A, Iaccarino C, Rotilio G, Carrì MT. Bcl2a1 serves as a switch in death of motor neurons in amyotrophic lateral sclerosis. *Cell Death and Differentiation*. 2006; 13:2150–2153. [PubMed: 16710367]
29. Simmons MJ, Fan G, Zong WX, Degenhardt K, White E, Gélinas C. Bfl-1/A1 functions, similar to Mcl-1, as a selective tBid and Bak antagonist. *Oncogene*. 2007; 27:1421–1428. [PubMed: 17724464]
30. Antonsson B, Conti F, Ciavatta AM, Montessuit S, Lewis S, Martinou I, Bernasconi L, Bernard A, Mermod JJ, Mazzei G, Maundrell K, Gambale F, Sadoul R, Martinou JC. Inhibition of Bax channel-forming activity by Bcl-2. *Science*. 1997;370. [PubMed: 9219694]
31. Boise LH, Gonzalez-Garcia M, Postema CE, Ding L, Lindsten T, Turka LA, Mao X, Nunez G, Thompson CB. bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. *Cell*. 1993; 74:597–608. [PubMed: 8358789]
32. Ko JK, Lee MJ, Cho SH, Cho JA, Lee BY, Koh JS, Lee SS, Shim YH, Kim CW. Bfl-1S, a novel alternative splice variant of Bfl-1, localizes in the nucleus via its C-terminus and prevents cell death. *Oncogene*. 2003; 22:2457–2465. [PubMed: 12717423]
33. Hamasaki A, Sendo F, Nakayama K, Ishida N, Negishi I, Hatakeyama S. Accelerated neutrophil apoptosis in mice lacking A1-a, a subtype of the bcl-2-related A1 gene. *The Journal of experimental medicine*. 1998; 188:1985–1992. [PubMed: 9841913]
34. Bilbao G, Contreras JL, Mikheeva G, Krasnykh V, Eckhoff DE, Thomas FT, Thomas J, Curiel DT. Genetic cytoprotection of human endothelial cells during preservation time with an adenoviral vector encoding the anti-apoptotic human Bcl-2 gene. *Transplantation proceedings*. 1999; 31:1012–1015. [PubMed: 10083450]
35. Karsan A, Yee E, Harlan JM. Endothelial cell death induced by tumor necrosis factor-alpha is inhibited by the Bcl-2 family member, A1. *J Biol Chem*. 1996; 271:27201–27204. [PubMed: 8910286]
36. Iwata A, Morgan-Stevenson V, Schwartz B, Liu L, Tupper J, Zhu X, Harlan J, Winn R. Extracellular BCL2 proteins are danger-associated molecular patterns that reduce tissue damage in murine models of ischemia-reperfusion injury. *PLoS One*. 2010; 5:e9103. [PubMed: 20161703]
37. Jenal M, Batliner J, Reddy VA, Haferlach T, Tobler A, Fey MF, Torbett BE, Tschan MP. The anti-apoptotic gene BCL2A1 is a novel transcriptional target of PU.1. *Leukemia*. 2010; 24:1073–1076. [PubMed: 20200559]
38. Olsson A, Norberg M, ökvist A, Derkow K, Choudhury A, Tobin G, Celsing F, österborg A, Rosenquist R, Jondal M, Osorio LM. Upregulation of bfl-1 is a potential mechanism of chemoresistance in B-cell chronic lymphocytic leukaemia. *British Journal of Cancer*. 2007; 97:769–777. [PubMed: 17726463]

Highlights

- Deletion of the amphipathic C-terminal domain of A1/Bfl1 (A1 Δ C) is performed.
- A1 Δ C frees A1 from mitochondrial anchorage.
- Both full-length A1 and A1 Δ C are cytoprotective in human endothelial cells.
- rAd. mediated overexpression of A1 Δ C but not A1 inhibits NF- κ B in human EC.

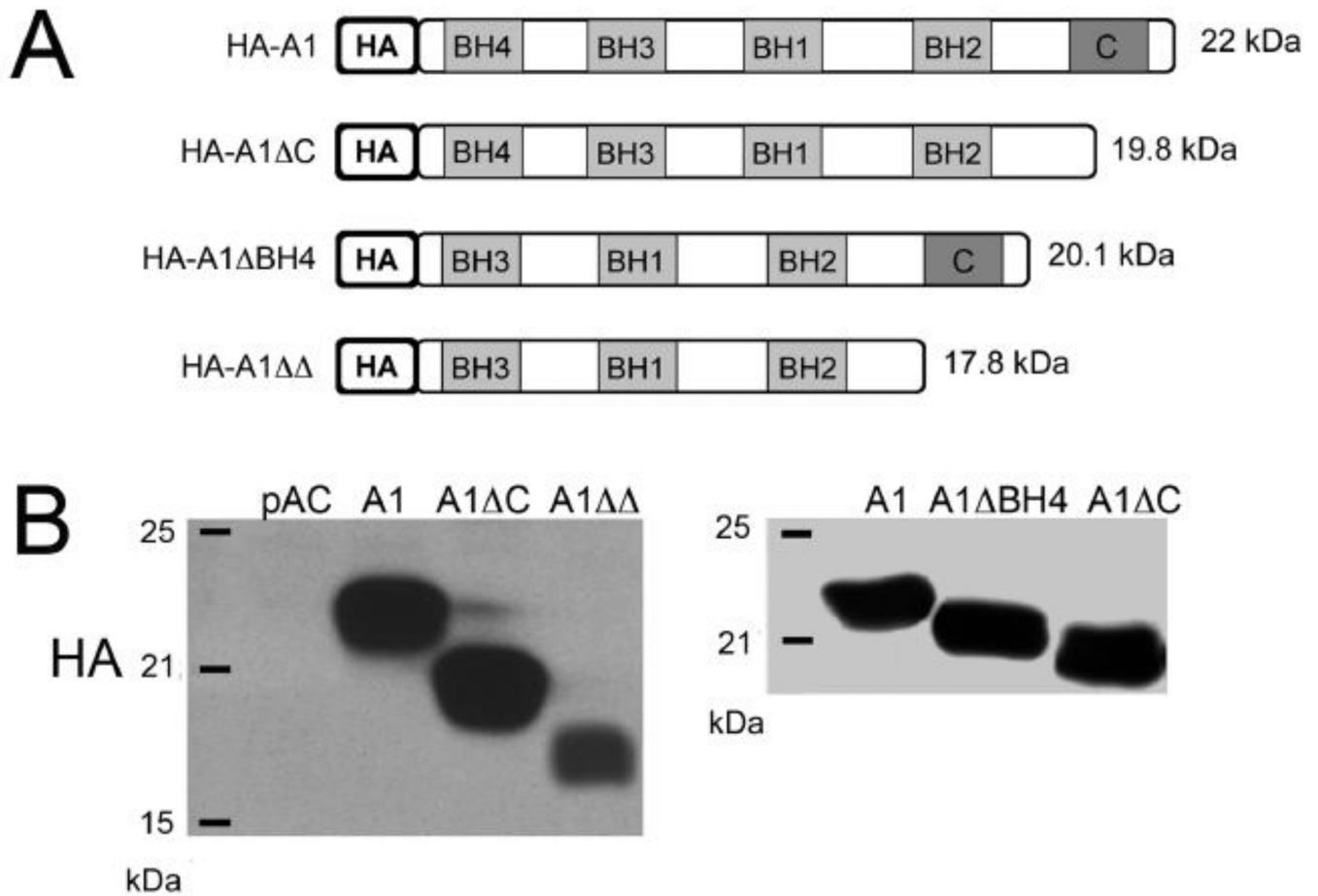


Fig. 1.
(A) Schematic representation of the sequences of HA-tagged full-length human A1 (22 kDa), A1ΔC (19.8 kDa) – A1 mutant with a deletion of the C-terminus domain, A1 BH4 (20.1 kDa) – A1 mutant with a deletion of the BH4 domain, A1 (17.8 kDa) – A1 mutant with a deletion of the C-terminus and the BH4 domains that were cloned in pAC plasmids.
(B) Expression of A1 and A1 mutants in bovine aortic endothelial cells (BAEC) following their transfection with the respective plasmids, as demonstrated by Western blot analysis using anti-HA antibody. Molecular weight markers are labeled on the left side of the figures.

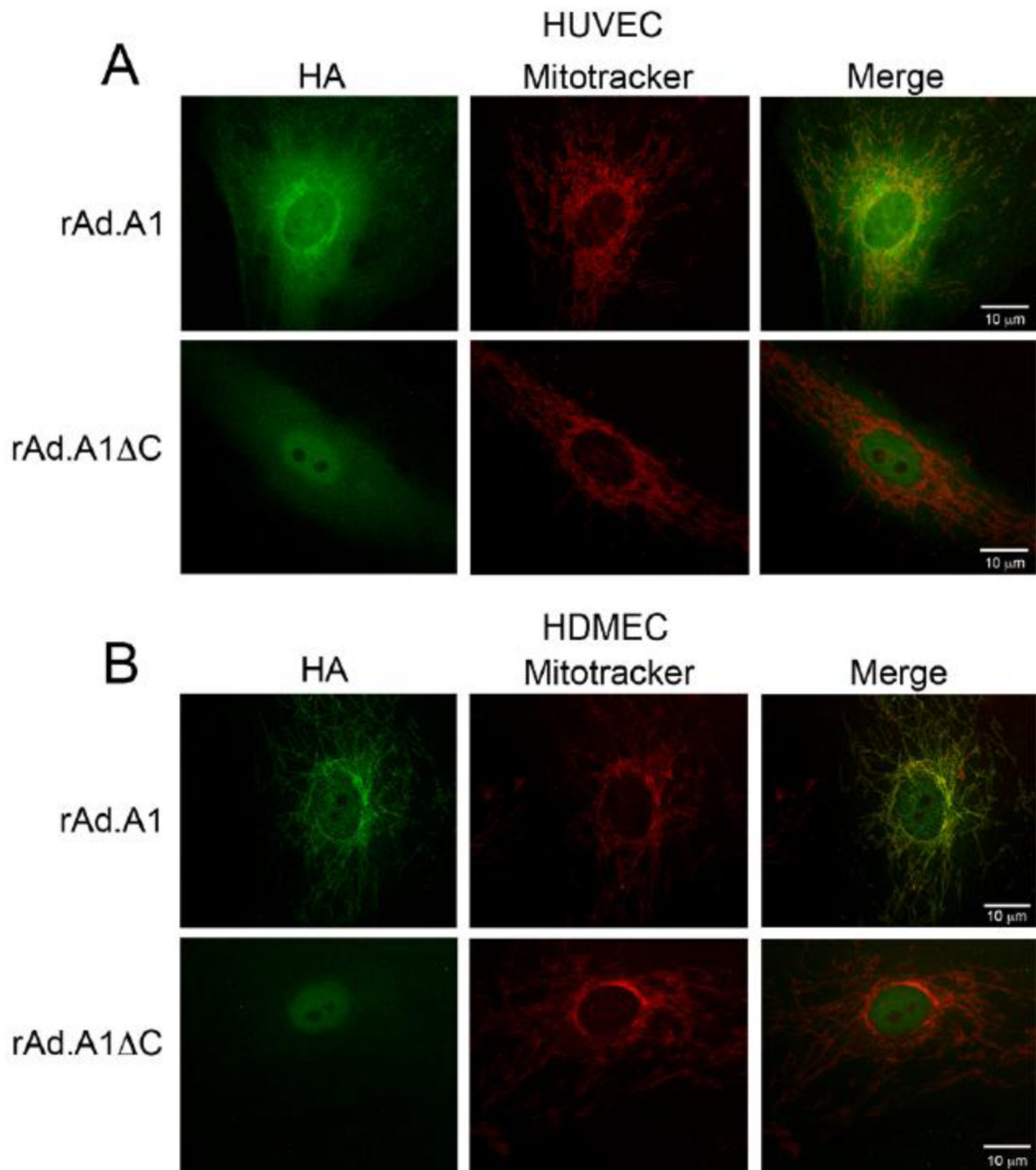


Fig. 2. Subcellular localization of A1 and A1ΔC overexpressed transgenes in human EC. **(A)** HUVEC and **(B)** HDMEC transduced with rAd.A1 and rAd.A1ΔC were double-labeled with HA-FITC antibody and the mitochondrial probe Mitotracker Red and analyzed by immunofluorescence microscopy with overlay of the different fluorescence colors. Results show that A1, but not A1ΔC, co-localizes with Mitotracker Red, indicating that the C-terminal domain of A1 is required to anchor it to mitochondria. In contrast, A1ΔC fluorescence shows a cytosolic and nuclear pattern. Representative micrographs of 3 (HUVEC) and 2 (HDMEC) different experiments are shown. Original magnification 1000X.

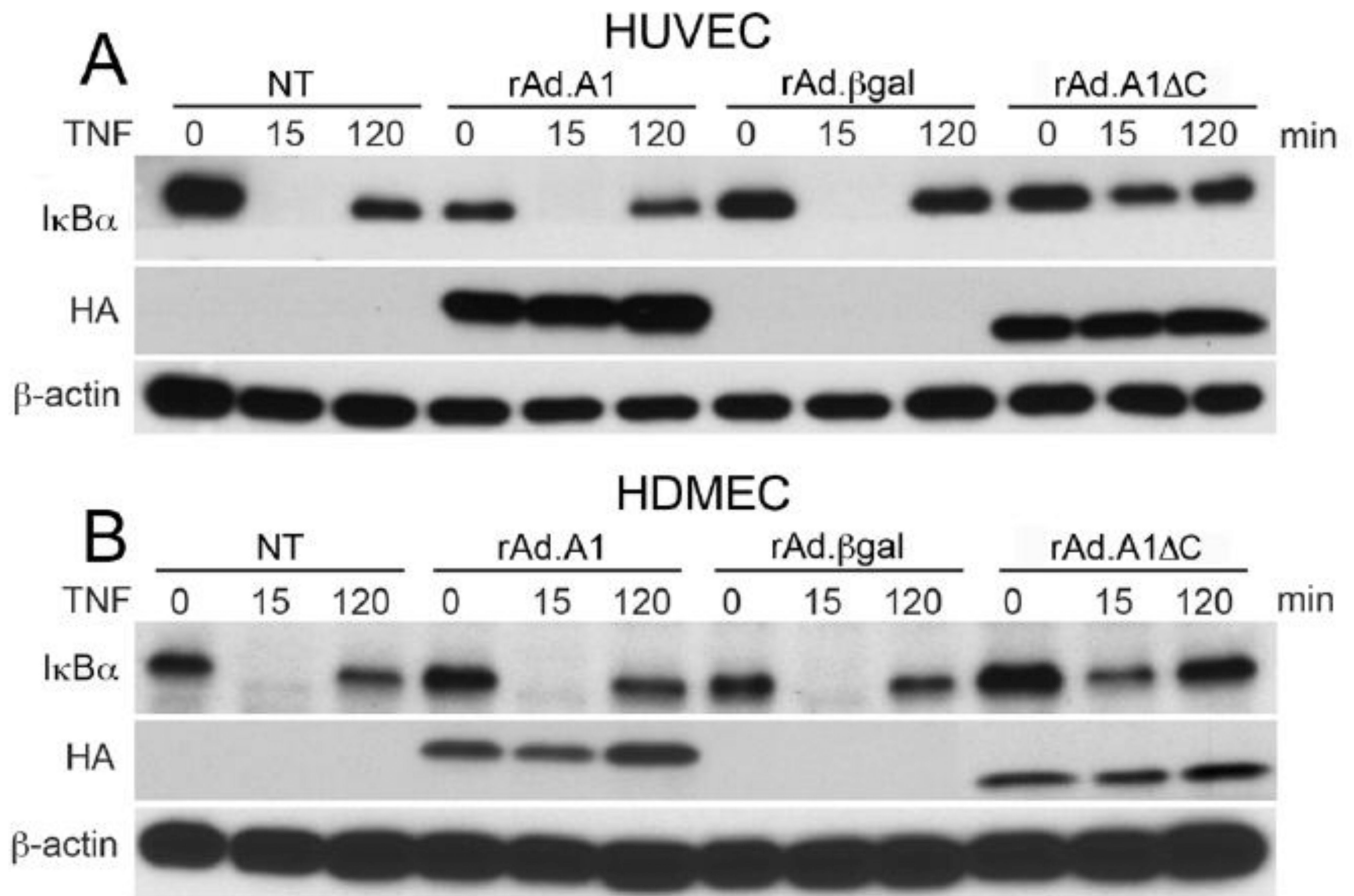
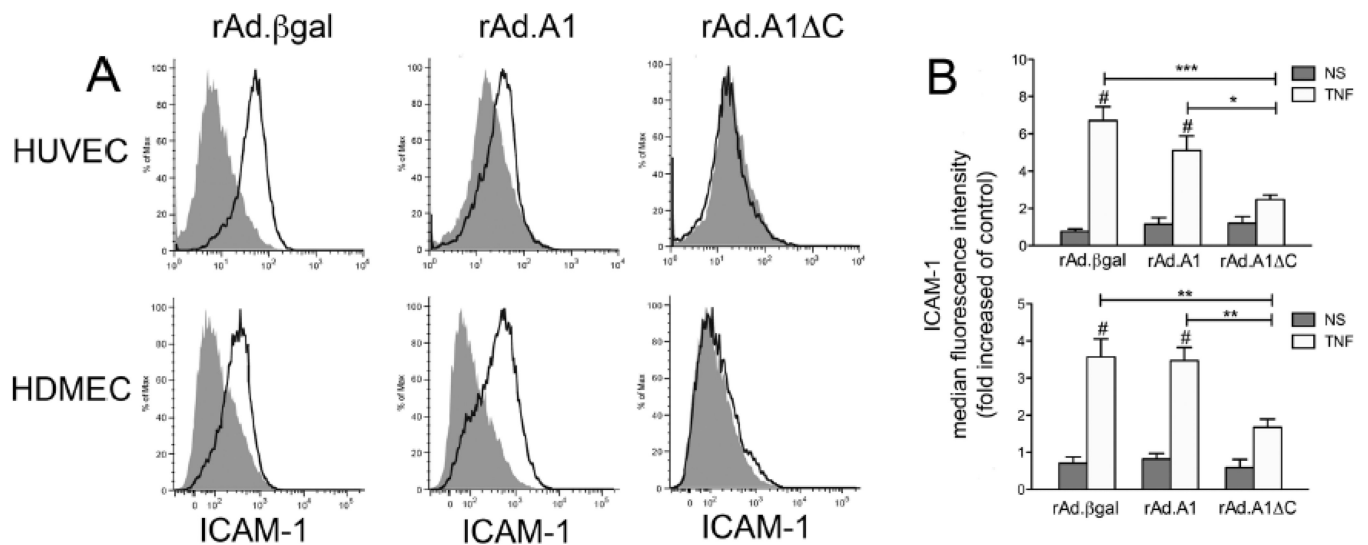


Fig. 3. Overexpression of A1ΔC but not full length A1 prevents TNF-induced IκBα degradation in HUVEC and HDMEC. Cell lysates from non-transduced (NT), rAd.A1, rAd.A1ΔC or rAd.βgal-transduced cells (**A**) HUVEC, and (**B**) HDMEC treated with TNF (200 U/ml) for 15 and 120 min were immunoblotted with anti-IκBα antibody. Results demonstrate that overexpression of A1 does not affect TNF-induced degradation and loss of IκBα in all EC tested. However, overexpression of A1ΔC inhibits TNF-induced IκBα degradation in both HUVEC and HDMEC. Immunoblotting with anti-HA and anti-β-actin antibodies was used to confirm transgene expression and correct for loading, respectively. Data shown are representative of 3 independent experiments for each endothelial cell type.

**Fig. 4.**

Overexpression of A1ΔC but not full length A1 prevents TNF-induced ICAM-1 upregulation in HUVEC and HDMEC. **(A)** FACS analysis of ICAM-1 surface expression, rAd.A1, rAd.A1ΔC, or rAd.βgal-transduced HUVEC, and HDMEC 16 h following treatment with TNF (200 U/ml). Our results demonstrate that overexpression of A1 does not affect TNF-induced upregulation of ICAM-1 in all EC tested. However, overexpression of A1ΔC significantly inhibits TNF-induced ICAM-1 upregulation in HDMEC and HUVEC. Histograms shown are representative of 3 different experiments. Gray shaded areas represent baseline ICAM-1 expression in non-stimulated (NS) EC, while empty black-lined areas indicate the shift in ICAM-1 surface expression following TNF stimulation. **(B)** Bar charts depicting fold increase of median fluorescence intensity SEM ± of 3 experiments performed. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ indicate significance among different rAd. treatment groups, whereas # $p < 0.001$ indicate significance between NS and TNF-treated cells within the same experimental group.

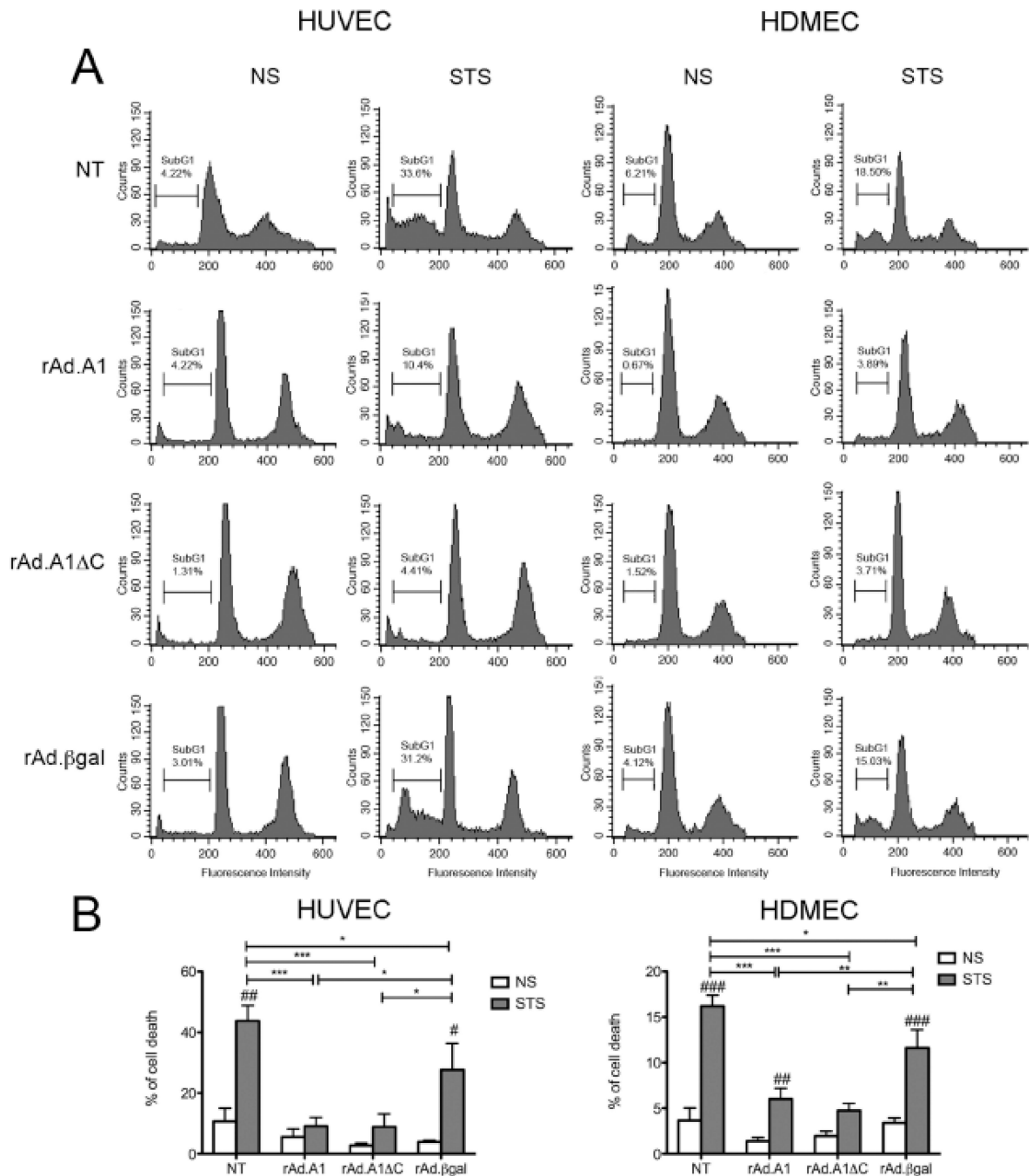


Fig. 5. Full-length A1 and A1ΔC mutant protect HUVEC and HDMEC from cell death induced by staurosporine (STS). Non-transduced (NT), rAd.A1, rAd.A1ΔC, and rAd.βgal-transduced (A) HUVEC and HDMEC were treated with STS (100 nM) for 6 h, then labeled with propidium iodide. Apoptosis was evaluated by FACS analysis of DNA content. Data shown are representative of 3 independent experiments. The percentage of cells undergoing cell death, as evidenced by DNA<2N (subG1 phase) is reported. (B) Bar chart representation of mean percentage of cell death ± SEM of the 3 experiments performed. *p<0.05, **p<0.01, ***p<0.001 indicate significance among different treatment groups, i.e. non transduced (NT)

EC and EC transduced with different rAd. #p<0.05, ##p<0.01, ###p<0.001 indicate significance between non stimulated (NS) and STS-treated HUVEC and HDMEC within the same experimental group.