xIAP Induces Cell-Cycle Arrest and Activates Nuclear Factor-κB : New Survival Pathways Disabled by Caspase-Mediated Cleavage During Apoptosis of Human Endothelial Cells

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Abstract—Survival of human vascular endothelial cells depends on their ability to activate the transcription factor nuclear factor-κB (NF-κB), a regulator of antiapoptotic genes, such as the X chromosome–linked inhibitor of apoptosis protein (xIAP). In the present study, we demonstrated expression of xIAP in the endothelial lining of normal human arteries and veins and elevated levels in highly malignant human endothelial tumors. Using retroviral infection of human endothelial cells, we identified two novel survival mechanisms mediated by xIAP in endothelial cells. First, xIAP can activate the transcription factor NF-κB, a known survival factor for human endothelial cells. This positive feedback loop induced by xIAP is mediated via phosphorylation and sustained degradation of inhibitor (I) κBα. Second, xIAP can inhibit cell proliferation via downregulation of cyclins A and D1 and induction of the cyclin-dependent kinase inhibitors $p21^{Cip1/Waf1}$ and $p27^{Kip1}$. Cleavage of xIAP by caspases during endothelial cell apoptosis disables both of these biological functions of xIAP. Thus, caspase-mediated cleavage of xIAP interrupts a positive regulatory cytoprotective loop between NF-κB and xIAP and increases the vulnerability of the cell to apoptosis by releasing it from an xIAP-mediated quiescent state. **(*Circ Res*. 2001;88:282-290.)**

Key Words: apoptosis ■ caspases ■ cleavage ■ retrovirus

The inhibitor of apoptosis (IAP) family of proteins protects against apoptosis in several experimental systems.[1,2] First identified in baculovirus, IAPs are highly conserved between insects and mammals and contain at least one copy of the characteristic baculovirus IAP repeat (BIR) domain. The antiapoptotic function of the IAPs has been linked to the ability of the BIR domains to bind and directly inhibit caspases. However, despite their high degree of homology, not all BIR domains are equally efficient in inhibiting caspases. For example, the BIR2 domain of xIAP can inhibit caspase-3 but not its BIR1 or BIR3 domains.[3] At the same time, individual IAPs differ in their specificity toward different caspases. For example, xIAP potently inhibits caspases-3 and -7 and the activation of caspase-9 but is inefficient toward caspases-1, -6, and -8.[4–6]

Little is known about the transcriptional regulation of the IAPs. The expression of survivin in HeLa cells is upregulated during the G2/M phase of the cell cycle via G1 transcriptional repressor elements in its promoter.[7] Internal ribosomal entry site (IRES)–mediated translational upregulation of endogenous xIAP mRNA has been demonstrated in response to several forms of cellular stress, including growth factor (GF) deprivation.[8] Nuclear factor-κB (NF-κB) has been shown to mediate the transcription of the c-IAP1, c-IAP2, and xIAP genes by tumor necrosis factor-α (TNF-α).[9,10] NF-κB also has been identified as a crucial protective factor against apoptosis in several experimental systems[11,12] and has been proposed to inhibit apoptosis by the induction of antiapoptotic genes, such as IAPs and the Bcl-2 homologs A1 and Bcl-x_L.[13–15] These genes differ in their protective potency depending on the apoptotic stimulus, and some can modulate NF-κB in a positive or negative manner.[9,16] We and others have shown that activation of endogenous NF-κB is required for endothelial cell survival after withdrawal of GF, loss of extracellular matrix adhesion, and exposure to TNF-α/cycloheximide.[17–19]

Because the NF-κB–regulated protective genes are unknown in our system, we examined IAP family members as possible candidates to mediate survival of human endothelial cells in response to GF deprivation. In this study, we identified two novel biological functions of xIAP: its ability to activate NF-κB and its ability to mediate cell-cycle arrest

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via regulation of cyclins and cyclin-dependent kinase inhibitors (CDKIs). Additionally, we showed that xIAP is specifically cleaved by caspases during apoptosis of normal human endothelial cells and that cleavage of xIAP disables its functions. We propose a positive regulatory survival mechanism between xIAP, NF-κB, and cell-cycle regulatory elements, where intact but not cleaved xIAP activates NF-κB, suppresses cell-cycle progression, and protects endothelial cells against apoptosis.

Materials and Methods

Cell Culture and Reagents

Cultured human umbilical vein endothelial cells (HUVECs) were isolated and grown as previously described.[17,20] The following antibodies were used: c-IAP1 and caspase-9 (PharMingen); c-IAP2 (Santa Cruz Biotechnology); xIAP (Transduction Laboratories); survivin (Alpha Diagnostics); NF-κB subunits, as described elsewhere[17]; IκBα and IκBβ (Santa Cruz Biotechnology) and phospho-IκBα (New England Biolabs); and cyclins and CKIs, as described previously.[20]

Tissue Analysis

Immunostaining of paraformaldehyde-fixed tissue was performed using standard protocols.

Protein Analysis and Fluorogenic Caspase Assays

Protein analysis was performed on cell lysates, as previously described[17] (for details, see the online data supplement available at http://www.circresaha.org). The caspase assays were carried out with recombinant caspases, glutathione-*S*-transferase (GST) fusion proteins, and 10 μmol/L caspase-3 substrate (Ac-Asp-Glu-Val-Asp-amino-methylcoumarin [DEVD-AMC], Alexis) in reaction buffer (25 mmol/L HEPES, pH 7.4, 1 mmol/L EDTA, 0.1% CHAPS, 10% sucrose, and 3 mmol/L DTT) at room temperature, and production of fluorescent AMC was monitored continuously (excitation 380 nm and emission 460 nm) by use of a fluorescent plate reader (FL 500; Biotek).

Generation of xIAP Mutants, GST-Fusion Proteins, and In Vitro Cleavage

Details of the polymerase chain reaction amplification and site-directed mutagenesis strategies for xIAP and mutants are described in the online data supplement. GST-fusion proteins were generated in *Escherichia coli* BL21 (DE3) harboring pT-Trx (gift of T. Tamura, Medizinische Hochschule, Hannover, Germany) after induction with 0.2 mmol/L IPTG for 2 hours at 30°C and isolated using the Pharmacia GST purification module according to the manufacturer's instructions. In vitro transcription and translation and caspase incubations were performed as previously described.[17]

Generation of Retroviral Vectors, Retrovirus Production, and Infection of Endothelial Cells for Cell-Death/Cell-Survival Assays

All retroviral expression plasmids were constructed using the pBMN-IRES–enhanced green fluorescent protein (EGFP) and pBMN-IRES-Lyt2a retroviral vectors.[21] The cDNAs of the xIAP forms were cloned into the *Bam*HI/*Not*I sites of pBMN-IRES-EGFP and pBMN-IRES-Lyt2a. An NF-κB–responsive dEGFP reporter cassette (Clontech) was subcloned into a multiple cloning site of the retroviral vector pBM self-inactivating (SIN) (gift of G. Nolan, Stanford University, Stanford, Calif) to yield the pBM-SIN-κB-dEGFP reporter retroviral vector. High-titer retrovirus was prepared as previously described.[21] For infection, 3×10^5 HUVECs were seeded into 25-cm^2 tissue culture flasks 24 hours before infection and incubated with 3 to 5 mL virus stock for 10 to 12 hours in the presence of 4 μg/mL polybrene. After infection, retroviral supernatant was replaced with fresh medium, and cells were cultured for an additional 48 hours before use in subsequent experiments.

Flow Cytometry

The percentage of EGFP(+) cells was determined by flow cytometry using a FACScan (Becton Dickinson) flow cytometer and analyzed with CellQuest. For 2-color analysis of cells expressing the CD8 (Lyt2) and an NF-κB–dependent destabilized EGFP reporter gene (dEGFP), cells were stained after fixation with a phycoerythrin-conjugated anti-CD8 antibody (PharMingen). dEGFP expression (FL1) was then determined by flow cytometry after gating on subsets of retrovirally infected cells expressing different levels of the retroviral transgene, as monitored by CD8 (FL2) expression. dEGFP expression was quantified as the mean fluorescence intensity of >10 000 gated cells.

Electrophoretic Mobility-Shift Assays and Luciferase Reporter Assay

Gel-shift assays, transfections, and luciferase-reporter assays were performed as previously described[17] (see also the online data supplement).

An expanded Materials and Methods section can be found in an online data supplement available at http://www.circresaha.org.

Results

xIAP Is Expressed in Endothelial Cells and Is Cleaved During Endothelial Apoptosis

We find xIAP to be specifically expressed in the endothelial lining of normal human large and small arteries and veins in vivo (Figure 1A), and its levels are elevated in highly malignant human endothelial tumors, such as epithelioid angiosarcoma GIII, compared with benign endothelial hemangiomas (Figure 1A).

HUVECs undergo apoptosis when deprived of GFs. After 12 to 16 hours, ≈40% to 45% of the cells are apoptotic and appear in the culture supernatant as detached floating cells, whereas the adherent cells remain viable.[20,22] We have shown previously that the relative resistance of the viable cells to apoptosis depends on their ability to activate and maintain NF-κB–mediated transcription.[17] To characterize putative genes that mediate the protective effect of NF-κB in our system, we examined members of the IAP family of proteins.

The protein expression levels of c-IAP1, c-IAP2, and survivin are similar between GF-supplemented control cells and cells either surviving or apoptotic after GF deprivation (Figure 1B). However, full-length xIAP is completely absent from apoptotic cells, and a protein of ≈33 kDa cross-reacts with the antibody (Figure 1A). This protein, designated ΔxIAP, appears as early as 2 hours after induction of apoptosis and accumulates over time (Figure 1C). The kinetics of ΔxIAP appearance during apoptosis are slower than cleavage of poly-ADP-ribose polymerase (PARP), a known substrate for caspases, and correlate with caspase-9 (Figure 1C) and caspase-6 processing (data not shown).

xIAP Is a Substrate for Caspases in Endothelial Cells: Identification of the Cleavage Site and Generation of an Uncleavable Mutant

Because cleavage of xIAP by caspases has been previously described in transformed cell lines,[23] we tested whether caspases are involved in the generation of the apoptotic xIAP fragment in primary human endothelial cells. Induction of apoptosis in the presence of 50 μmol/L benzyloxycarbonyl-Val-Ala-Asp fluoromethyl ketone (ZVAD-fmk), a broad-

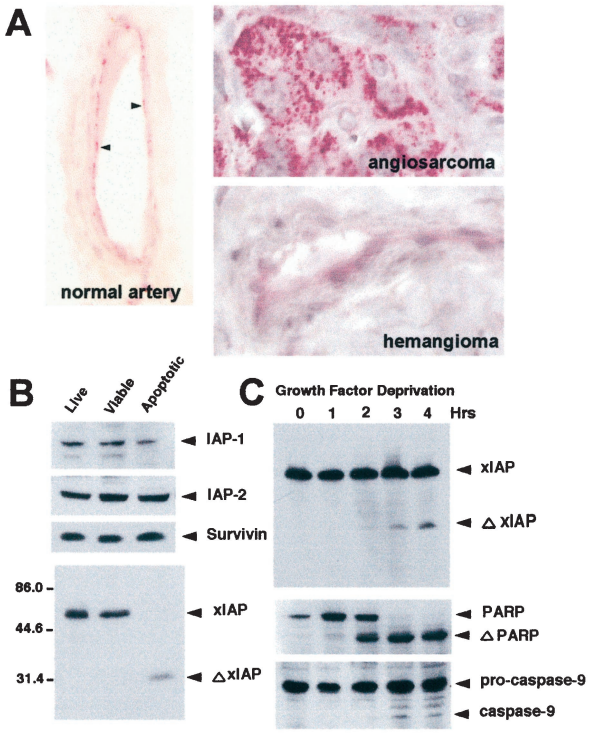

Figure 1. xIAP is cleaved during GF deprivation–induced apoptosis in human endothelial cells. A, Immunostaining for xIAP in a large human intramural myocardial artery (left), a benign hemangioma (bottom right), and a high-grade malignant human angiosarcoma (epithelioid angiosarcoma GIII, top right). B, Cell lysates from control HUVECs (live) and cells that were exposed to GF deprivation for 16 hours and either survived (viable) or underwent apoptosis (apoptotic) were subjected to Western blot analysis with antibodies to IAP-1, IAP-2, survivin, and xIAP. A protein of M_r ≈33 kDa (ΔxIAP) cross-reacts with the monoclonal antibody to xIAP. C, Cells were deprived of GFs for the indicated times, and lysates of total cell populations were immunoblotted for xIAP, PARP, and caspase-9.

spectrum caspase inhibitor that inhibits apoptosis in our system,[22] completely abolishes generation of the ΔxIAP fragment during apoptosis (Figure 2A). Incubation of in vitro–translated, [^{35}S]-labeled xIAP with recombinant active caspases-3, -6, or -7 generates a major xIAP fragment (Δ1) of ≈33 kDa (Figure 2B, left). This fragment corresponds to the ΔxIAP fragment detected in apoptotic cells, as shown by Western blot analysis of in vitro–cleaved xIAP adjacent to apoptotic cell lysates (Figure 2B, right). Caspase-3 seems to be the most effective enzyme. Using site-directed mutagenesis, we confirmed that the cleavage of xIAP we observed in endothelial cells occurred at the same position as identified in Jurkat cells,[23] because substitution of Asp^{242} with Glu completely abolished caspase-mediated cleavage of the in vitro–translated (Figure 2C) and retrovirally overexpressed mutant protein (Figure 3A).

Cleavage of xIAP Is Not Necessary for Inhibition of Caspases and Protection Against Apoptosis but Disables the Antiapoptotic Effect of xIAP

The mechanism of caspase inhibition by IAPs is presently unknown. Two potent caspase inhibitors, the cowpox virus protein CrmA and the p35 protein of baculovirus, are cleaved by caspases, and, in the case of p35, this cleavage is both necessary and sufficient for caspase inhibition.[24,25] To test whether xIAP inhibits caspases through a similar mechanism, we expressed wild-type (WT) xIAP and the uncleavable xIAP mutant (SESE) as recombinant GST-fusion proteins and compared their ability to inhibit active caspase-7 in vitro. In a fluorogenic caspase assay, both recombinant xIAP and the uncleavable SESE-xIAP mutant were equally efficient in inhibiting caspase-7 (Figure 2D).

To determine whether xIAP cleavage is necessary for protection against apoptosis and to test the effect of the apoptotic cleavage fragments on cell death, we used a retroviral single-transcript vector, pBM-IRES-EGFP,[21] to express WT-xIAP, SESE-xIAP, and two truncation fragments, T1-xIAP and T2-xIAP (amino acids 1–242 and 243–497, respectively), designed to simulate the two xIAP fragments generated by caspase cleavage of full-length xIAP (Figure 3A). This retroviral vector allows two cistrons to be translated independently from a single mRNA transcript after infection and integration of the retroviral cassette.[21] Using this system, we obtained high infection efficiencies (>95%) and excellent correlation between EGFP reporter gene ex-

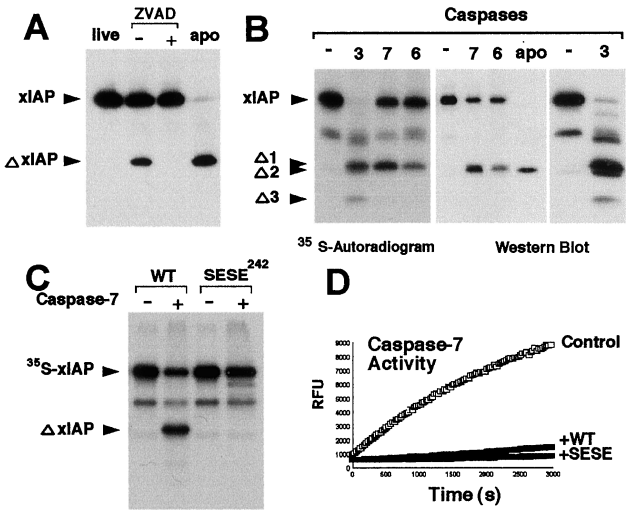

Figure 2. xIAP is a substrate for caspases in vitro and in vivo. Shown is identification of the cleavage site and caspase-inhibitory function. A, Cells were deprived of GFs with (+) or without (−) 50 μmol/L ZVAD-fmk, and cell lysates of total cell populations (16 hours) were immunoblotted for xIAP. B, xIAP was translated in vitro either with [^{35}S]-labeled or unlabeled methionine and incubated with reaction buffer alone (−) or with the indicated caspases at 37°C for 2 hours. The samples were subjected to 15% SDS-PAGE, and xIAP fragments were visualized by autoradiography (left) or Western blotting (right). Cell lysate from apoptotic cells was loaded on the same gel for comparison of in vitro– and in vivo–cleaved xIAP. C, In vitro–translated and [^{35}S]-methionine–labeled WT-xIAP and the xIAP substitution mutant SESE^{242} (substitution of aspartate 242 with a glutamate) were incubated with (+) or without (−) caspase-7 and analyzed by autoradiography as in panels A and B. D, Caspase-7 (250 pmol/L) was incubated with 10 μmol/L Ac-DEVD-AMC in the absence (control) or presence of 10 nmol/L GST-xIAP fusion protein encoding WT-xIAP and the SESE for the indicated time at room temperature. Production of fluorescent AMC was monitored continuously and expressed as random fluorescent units.

pression and that of genes cloned into the first cistron (data not shown), and levels of protein expression of WT-xIAP and SESE-xIAP are similar by Western blot analysis and significantly higher than endogenous xIAP (Figure 3A). With overexpression of SESE-xIAP and GF deprivation, we failed to detect cleavage of xIAP, demonstrating that the overexpression of the uncleavable mutant can prevent formation of the cleavage fragment within cells. However, even overexpression of WT-xIAP results in a small proportion of cleaved xIAP under apoptotic conditions (Figure 3A). Overexpression of both WT-xIAP and SESE-xIAP dramatically protects against GF deprivation–induced apoptosis (Figure 3B). Even after 36 hours, both forms potently and equally efficiently protect against apoptosis, with ≈40% of the cells still surviving, compared with <15% in the case of EGFP alone (Figure 3B, insert). Overexpression of T1-xIAP confers only modest protection after 12 and 16.5 hours of GF deprivation, which is lost after 21 and 36 hours (Figure 3B). Overexpression of T2-xIAP has no effect on survival compared with the control EGFP vector at any time measured (Figure 3B). The inhibition of apoptosis observed with overexpression of WT-xIAP and SESE-xIAP is similar to that observed with the pan-caspase inhibitor ZVAD-fmk (Figure 3C). To test whether coexpression of both xIAP cleavage fragments may reconstitute the protective effect of WT-xIAP, we superinfected cells with T1-xIAP and T2-xIAP and vice versa and compared survival with control superinfected cells (Figure 3D). We found that coexpression of the two xIAP cleavage fragments had no effect on survival compared with controls.

xIAP Activates NF-κB–Dependent Transcription Via Phosphorylation and Sustained Downregulation of IκBα

Some of the survival genes regulated by NF-κB, such as c-IAP2,[9] have been shown to positively or negatively modulate the activity of NF-κB. It was recently reported that xIAP induces NF-κB–dependent transcription, although the upstream regulatory mechanisms have been characterized in 293 cells and not in endothelial cells.[26] To examine whether retroviral overexpression of xIAP has an effect on NF-κB–dependent gene expression, we cloned the gene for destabilized EGFP (dEGFP) into the self-inactivating retroviral vector pBM-SIN (from G. Nolan) under the control of a promoter containing 4 tandem repeats of the κB-response element (κB-dEGFP). dEGFP has a half-life of ≈2 hours, which allows us to analyze NF-κB–dependent transcription over short time intervals, as demonstrated by the rapid stimulation of NF-κB in dEGFP-infected HUVECs 6 hours after treatment with lipopolysaccharide (LPS), a potent activator of NF-κB (Figure 4A, right).

To test the effect of xIAP expression on dEGFP expression, cells infected with the pBM-SIN-κB-dEGFP virus were superinfected with either control pBM-IRES-CD8 or pBM-WT-xIAP-IRES-CD8 retrovirus expressing truncated mouse CD8 as a cell-surface marker (Figure 4A, left). dEGFP levels were evaluated by flow cytometry in relationship to CD8 by gating on cells expressing low or high levels of CD8 and, thus, low and high levels of xIAP (Figure 4B). After 4.5 days, cells expressing high levels of xIAP showed a marked

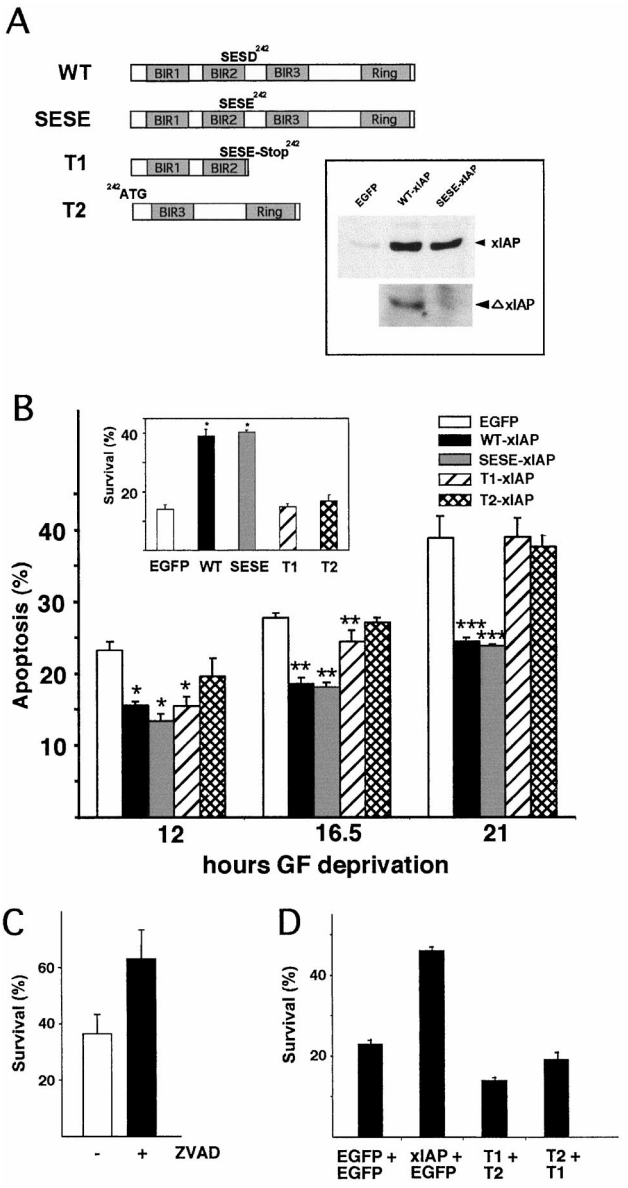

Figure 3. WT xIAP and the uncleavable SESE-xIAP mutant protect endothelial cells against GF deprivation–induced apoptosis, whereas xIAP cleavage fragments fail to protect. A, Diagram of xIAP showing the cleavage site in the native molecule and the uncleavable SESE-xIAP mutant as well as the two cleavage fragments, T1-xIAP and T2-xIAP. The expression levels of WT-xIAP and the uncleavable SESE-xIAP mutant were determined for apoptotic HUVECs (24 hours of GF deprivation) by Western blot analysis of infected cell lysates. B, Cells were infected with pBM-IRES-EGFP retrovirus encoding control vector, WT-xIAP, SESE-xIAP, T1-xIAP, and T2-xIAP mutants. Apoptosis during the first 21 hours is expressed as percentage apoptotic cells (floaters) from the total cell population. After 36 hours, cell survival is expressed as the percentage of cells remaining viable and attached (inset). C, HUVECs were deprived of GFs for 7 hours in the presence or absence of 50 μmol/L ZVAD-fmk, and survival is expressed as the percentage of cells from the total population that remain viable and attached. D, HUVECs were infected (first line) and superinfected (second line) with retrovirus carrying the indicated xIAP forms, and cell survival was measured as in panel B (inset) after 36 hours of GF deprivation. Data are reported as average±SD of triplicate samples *, **, ***$P<0.05$ vs control by Student's t test).

increase of ≈41% in dEGFP expression compared with controls (Figure 4C). Even cells expressing low levels of xIAP exhibited a detectable increase in dEGFP expression, suggesting that xIAP potently drives the expression of the NF-κB–dependent dEGFP gene.

The impact of individual cleavage fragments on NF-κB activity was evaluated by cotransfection of HUVECs with a κB-luciferase reporter construct together with WT-xIAP, T1-xIAP, T2-xIAP, or a control vector. The increase in κB-dependent luciferase activity was compared with that from a non–κB-dependent luciferase reporter transfected in parallel experiments (Figure 5A). WT-xIAP induced an ≈3-fold increase in κB-dependent transcription compared with vector controls, whereas neither of the xIAP cleavage fragments had any effect (Figure 5A). SESE-xIAP was as effective as WT-xIAP in activating NF-κB (data not shown). Electrophoretic mobility-shift assay (EMSA) for NF-κB showed that retroviral overexpression of WT-xIAP (Figure 5B), but not of T1-xIAP or T2-xIAP (data not shown), induced an increase in binding of NF-κB to DNA. Supershift analysis with antibodies to the p50 and p65 subunits of NF-κB demonstrates that the primary NF-κB complex induced by xIAP is a heterodimer of p50 and p65 (Figure 5B).

To determine whether the activation of NF-κB by xIAP is attributable to altered levels of the inhibitory proteins of NF-κB, the IκBs, we retrovirally overexpressed xIAP and analyzed the protein expression of IκBα and IκBβ. Over 7 days, a sustained decrease of IκBα was observed in xIAP-overexpressing cells compared with EGFP controls, whereas IκBβ levels were unchanged (Figure 5C). Because cytokine-induced NF-κB activation is mediated through site-specific phosphorylation and proteasomal degradation of IκBα,[27] we also probed the samples with a phospho-specific IκBα (Ser32) antibody. We observed increased phosphorylation of IκBα in xIAP-overexpressing HUVECs compared with EGFP controls, with an increase in the ratio of phospho-IκBα to total IκBα in xIAP-overexpressing cells (Figure 5C). To test whether xIAP induces NF-κB via an IκBα-dependent pathway, we superinfected cells with xIAP and IκBα or a dominant-negative IκBα mutant (IκBα Ser32, 36/Ala).[12] Gel-shift assays for NF-κB demonstrated that both IκBα and dnIκBα inhibit xIAP-induced NF-κB activation (Figure 5D).

xIAP Blocks Cell Proliferation in Endothelial Cells

During the xIAP overexpression experiments, we observed a decreased growth rate for cells infected with the WT-xIAP compared with the EGFP control retrovirus. Both WT-xIAP and SESE-xIAP inhibited proliferation with an ≈50% reduction in cell number 5 days after infection (Figure 6A). Although this inhibitory effect was already visible after 2 days, neither of the xIAP cleavage fragments showed any effect on proliferation. Additionally, after 3 days, xIAP overexpression caused a significant decrease in the percentage of cells in S phase and an increase in the percentage of cells in G0/G1 (Figure 6B). No abnormalities, such as polyploidy, nor an otherwise abnormal DNA content were observed.

Analysis of cell-cycle molecules demonstrated that cyclin A and cyclin D1 were downregulated in xIAP-overexpressing

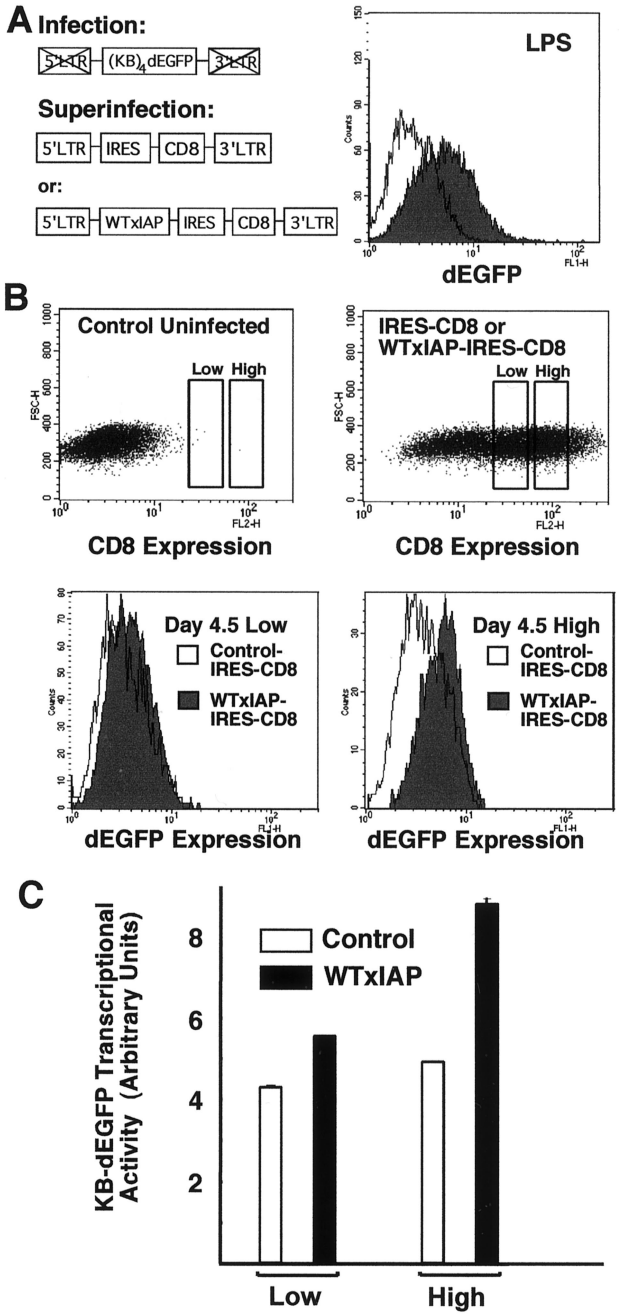

Figure 4. Overexpression of xIAP leads to increased transcription of a novel retroviral NF-κB–responsive EGFP-reporter gene. A, Diagram of the retroviral constructs used in the NF-κB–EGFP reporter gene assay (left). HUVECs were infected with pBM-SIN-NF-κB-dEGFP retrovirus, which encodes an NF-κB–dependent destabilized EGFP reporter gene expression cassette (right). Cells were stimulated with 500 ng/mL LPS for 6 hours, and dEGFP expression was determined by flow cytometry. B, Cells infected with the pBM-SIN-NF-κB-dEGFP reporter retrovirus were superinfected with IRES-CD8 or WT-xIAP-IRES-CD8 retrovirus, which expresses the murine cell-surface marker CD8 alone or together with WT-xIAP, respectively. Expression of the dEGFP reporter gene was determined by flow cytometry in cells expressing low and high cell-surface CD8 levels as a marker of expression levels of the integrated IRES-retroviral cassette (therefore, of xIAP levels) 4.5 days after infection (FL2 channel). C, Quantitation of dEGFP expression in the same samples on the basis of mean fluorescence intensity (FL1 channel) of triplicate samples (average±SD).

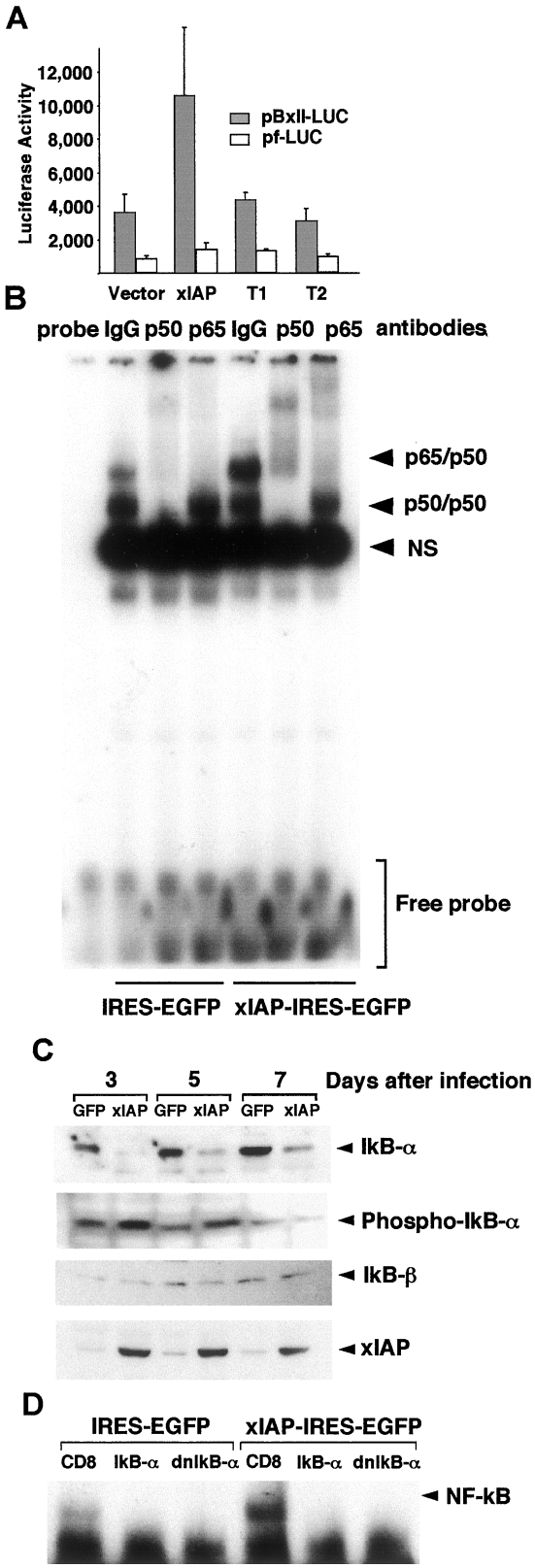

Figure 5. Full-length xIAP, but not its cleavage fragments, activates NF-κB–dependent transcription. A, HUVECs were cotransfected with an NF-κB–responsive (pBxII-LUC) or non–NF-κB–dependent (pf-LUC) luciferase reporter construct together with vector control DNA, WT-xIAP, or the T1-xIAP and T2-xIAP truncation mutants. The total amount of transfected DNA was kept constant (11 μg) by addition of an empty control vector (pcDNA3.1). Luciferase activity (arbitrary units) was determined 24 hours after transfection. Experiments were repeated 5 times. Data represent the average of triplicates±SD. B, Supershift EMSAs for NF-κB DNA-binding activity were performed with nuclear extracts collected from cells infected with the indicated constructs encoding control vector, IRES-EGFP, or xIAP-IRES-EGFP 5 days after infection. Arrows indicate the mobility of the p65/p50 and p50/p50 NF-κB complex indicated by the observed shift in mobility with specific antibodies. C, Western blot analysis of IκBα, phospho-IκBα (Ser 32), IκBβ, and xIAP in cells infected with pBM-IRES-EGFP and pBM-WT-xIAP-IRES-EGFP at 3, 5, and 7 days after infection. D, EMSAs for NF-κB were performed with nuclear extracts from cells infected with retrovirus encoding EGFP or xIAP and superinfected with retrovirus encoding IRES-CD8, IκBα-IRES-CD8, or dnIκBα-IRES-CD8.

cells, whereas cyclin E, cyclin-dependent kinase (CDK) 2, and CDK4 levels were unchanged (Figure 6C and data not shown). We also observed an induction of the CDK inhibitors $p21^{Cip1/Waf1}$ and $p27^{Kip1}$. These data suggest that xIAP suppresses cell-cycle progression at the G1/S boundary, resulting in a potent inhibition of proliferation.

Discussion

Consequences of Caspase Cleavage of a Potent Caspase Inhibitor, xIAP

In several experimental systems, xIAP is a potent inhibitor of apoptosis and can inhibit caspases-3, -7, and -9.[5,6] In the present study, we showed that xIAP itself is a target for caspases during GF deprivation–induced apoptosis of human vascular endothelial cells and that cleavage destroys its antiapoptotic function. Recently, an independent study observed xIAP cleavage during Fas-induced apoptosis in Jurkat cells but not during Bax-induced apoptosis in human embryonic kidney 293 cells.[23] Although the cleavage site is identical to the one we have identified, these investigators observed that the individual cleavage fragments differed in their antiapoptotic effect, dependent on the apoptotic stimulus. The complexity of molecular mechanisms contributing to GF deprivation–induced apoptosis, the stringent conditions used to access survival in our study, and the evaluation of normal cells (in our study) versus transformed cell lines may all contribute to the observed differences.

The equilibrium between caspase activation and caspase inhibition in a live cell prompts the following question: under what conditions in vivo do caspases cleave xIAP? Possibilities could include a molar excess of activated caspases after an overwhelming apoptotic stimulus or a preferential activation of caspases that cannot be inhibited by xIAP, such as caspases-6 or -8.[5] xIAP could be a bona fide target for such xIAP-insensitive caspases, as we have shown in vitro, which would then impair xIAP inhibition of its target caspases in vivo. In addition, the newly identified molecule Smac/DIABLO is released from the mitochondria after an apoptotic stimulus and, by associating with caspase-bound xIAP, frees the caspase from its inhibitor.[28–31] By associating with the Apaf-1 apoptosome and inhibiting xIAP, Smac/DIABLO promotes the activation of the caspase, which can then cleave the disabled xIAP.

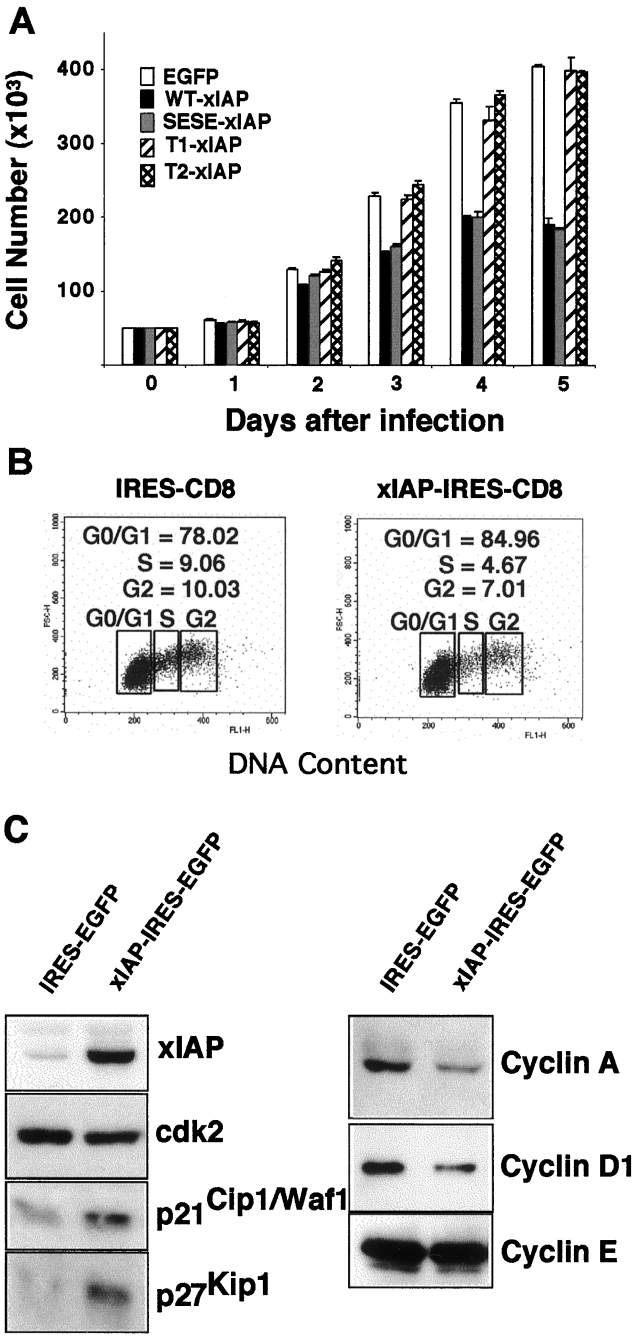

Figure 6. xIAP potently blocks proliferation of endothelial cells and differentially regulates cell-cycle elements. A, Cells infected with pBM-IRES-EGFP retrovirus encoding control vector, WT-xIAP, SESE-xIAP, T1-xIAP, and T2-xIAP mutants were grown for 5 days, and cell number was determined every 24 hours. B, DNA content of cells overexpressing IRES-EGFP and xIAP-IRES-EGFP was analyzed 3 days after infection with the DNA dye Sytox (Molecular Probes). Cells in G0/G1, S/G2, S, and G2 are expressed as percent of total cells. C, Cell-cycle elements in HUVECs infected with control pBM-IRES-EGFP and pBM-WT-xIAP-IRES-EGFP viruses 3 days after infection were analyzed by Western blotting after separation on 15% SDS-PAGE.

The mechanism by which IAPs inhibit caspases is presently unknown. Two caspase inhibitors that are structurally different from the IAPs, the baculovirus antiapoptotic protein p35 and a member of the serpin family, CrmA, are cleaved by caspases with which they interact.[24,25] Cleavage of p35 has been found to be both necessary and sufficient for inhibition of caspases.[24] However, in our studies, both recombinant WT-xIAP and the uncleavable SESE-xIAP mutant inhibit caspase-7 with comparable efficiencies in vitro, and both confer similar protection against GF deprivation–induced apoptosis. Thus, cleavage of xIAP does not seem to be necessary for either its antiapoptotic or caspase-inhibitory functions. Possibly, IAPs may function as competitive inhibitors that bind and occupy the catalytic groove of the caspase they inhibit.[2] However, the unique cleavage of xIAP, and none of the other IAPs examined, suggests that xIAP may have antiapoptotic activities beyond caspase inhibition.

xIAP Has Two Novel Biological Functions That May Contribute to Its Antiapoptotic Actions: Activation of NF-κB and Inhibition of Cell Proliferation

In the present study, we showed that overexpression of xIAP, but not caspase-cleavage fragments of xIAP, induces transcriptional activation of NF-κB in human endothelial cells. A role of IAPs in NF-κB activation has been previously reported for c-IAP2 in Jurkat cells[11] and, more recently, for xIAP in HUVECs.[26] However, in the latter study, the mechanisms of NF-κB activation were characterized in 293 cells, where the transforming GFβ–activated kinase 1 was implicated as a mediator of xIAP-induced NF-κB activation via stimulation of IκB kinases (IKKs).

In endothelial cells, we demonstrated that overexpression of xIAP leads to site-specific phosphorylation of IκBα at Ser32 and its sustained downregulation. We showed additionally that IκB degradation is involved in xIAP-induced NF-κB activation, because the effect of xIAP can be inhibited by overexpression of IκB and the mutant IκBα[12] that prevents phosphorylation by IKKs.[27] Activation of IKK2 kinase by xIAP[26] provides additional evidence for IκBα phosphorylation and degradation as primary mechanisms of NF-κB activation by xIAP.

A second novel biological function of xIAP that we have identified is its role as an active repressor of the cell cycle. Overexpression of xIAP in human endothelial cells inhibits proliferation at the G1/S phase of the cell cycle. Another member of the IAP family, survivin, has been implicated recently in the regulation of cell division after DNA replication (cytokinesis). Survivin is highly expressed in the G2/M phase and localizes to the mitotic spindle microtubules of dividing cells. Interference with survivin function leads to hyperploidy and multinucleation.[7,32] In contrast, xIAP seems to exhibit its antiproliferative effect in the G1/S phase of the cell cycle. Overexpression of xIAP leads to downregulation of cyclins A and D1 and upregulation of two CDKIs, $p21^{Cip1/Waf1}$ and $p27^{Kip1}$.

The mechanism by which xIAP inhibits proliferation is not yet known. One possibility is that caspase activity is necessary for normal cell-cycle progression. Antiapoptotic members of the Bcl-2 family of proteins have been shown to suppress proliferation,[33] and recently caspases have been found necessary for proliferation of primary human T cells.[34,35] Although the physiological significance of the

reported physical interaction between caspases and cell-cycle elements (caspase-3 associates with $p21^{Cip1/Waf1}$ and survivin with microtubules and CDK4) in nonapoptotic cells is unknown,[32,36] these observations suggest that caspases are more than simply death proteases. This possibility is supported by the role demonstrated for caspases in lens fiber differentiation and interleukin-2 release from activated T lymphocytes.[37,38] xIAP may also suppress proliferation through its recently identified ubiquitin ligase activity.[39] The ubiquitin-proteasome system is a key control mechanism of the cell cycle through temporal degradation of cyclins, CDKIs, and upstream CDK regulators.[40,41] It is also possible that IAPs as selective ubiquitin ligases[42] of xIAP may target individual cell-cycle progression elements, such as cyclin A and D1, for degradation, whereas other cyclins, such as cyclin E and the inhibitory proteins $p21^{Cip1/Waf1}$ and $p27^{Kip1}$, are left unaffected. Our studies identify xIAP as a novel cell-cycle regulator under nonapoptotic conditions, which actively represses the cell-cycle machinery in normal diploid cells.

Cleavage of xIAP Disables Multiple Survival Pathways and Activates Proapoptotic Mechanisms

How do the cleavage of xIAP and alteration of its biological functions fit into the apoptotic and survival programs activated after GF deprivation in HUVECs? Endogenous activation of NF-κB is a cellular defense mechanism[17] that protects cells by inducing survival genes, such as xIAP and $Bclx_L$.[10,15] Besides its function as a caspase inhibitor, xIAP induces NF-κB, thus defining a new positive regulatory survival mechanism. When GF deprivation persists, caspases are activated and target both NF-κB and its regulator xIAP. By disabling both the transcriptional potential of NF-κB[17] and the activating function of xIAP on NF-κB, caspases interrupt the NF-κB survival pathway at multiple levels. However, caspases disable another antiapoptotic function of xIAP as well: its ability to suppress the cell cycle and thus prevent the cell from exiting G0/G1 phase, where endothelial cells are less vulnerable to apoptosis induced by many stimuli.[43,44] Cleavage of xIAP together with the cleavage of CDKIs would disable cell-cycle arrest mechanisms and promote proapoptotic CDKs.[20] Activated CDK2 can additionally suppress NF-κB activation via the transcriptional regulator p300/CBP.[45] These positive and negative regulatory mechanisms interact to amplify each other and together causally contribute to the commitment of the endothelial cell to apoptosis after GF deprivation.

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