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Activation of NF-κB by XIAP, the X Chromosome-linked Inhibitor of Apoptosis, in Endothelial Cells Involves TAK1*

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Exposure of endothelial and many other cell types to tumor necrosis factor α generates both apoptotic and anti-apoptotic signals. The anti-apoptotic pathway leads to activation of the transcription factor NF-κB that regulates the expression of genes such as *A20* or members of the *IAP* gene family that protect cells from tumor necrosis factor α-mediated apoptosis. In turn, some anti-apoptotic genes have been shown to modulate NF-κB activity. Here we demonstrate that XIAP, a NF-κB-dependent member of the *IAP* gene family, is a strong stimulator of NF-κB. Expression of XIAP leads to increased nuclear translocation of the p65 subunit of NF-κB via a novel signaling pathway that involves the mitogen-activated protein kinase kinase kinase TAK1. We show that TAK1 physically interacts with NIK and with IKK2, and both XIAP or active TAK1 can stimulate IKK2 kinase activity. Thus, XIAP may be part of a system of regulatory loops that balance a cell's response to environmental stimuli.

Members of the nuclear factor κB (NF-κB)[1] family of transcription factors are ubiquitously expressed regulators of biological functions such as immune and inflammatory reactions, cell adhesion, proliferation, and survival. They are involved in the inducible expression of a wide variety of "stress response" genes encoding, *e.g.* cytokines, cell adhesion molecules, and anti-apoptotic genes. NF-κB is activated by proinflammatory cytokines (TNFα and IL-1), as well as by a number of other stimuli including lipopolysaccharide, viruses, or physical stress (for review, see Refs. 1–3). The NF-κB superfamily consists of transcriptional activators (p65/RelA, p50/NF-κB1, p52/NF-κB2, RelB, and c-Rel) that form homo- or heterodimers, as well as inhibitory subunits (IκBα, IκBβ, and IκBε) that function to retain the transcription factor in the cytoplasm. Stimuli that activate NF-κB act via phosphorylation of IκB molecules on two serine residues, 32 and 36, in IκBα and homologous phosphoacceptor sites in IκBβ, followed by ubiquitination and degradation by the 26 S proteasome (4). This phosphorylation is accomplished by specific IκB kinases, IKK1 (IKK-α) and IKK2 (IKK-β) that are contained within a 500–700 kDa molecular mass complex, termed signalosome (5–9). The signalosome contains additional proteins with modulatory function, such as NEMO/IKKγ (10, 11), IKAP (12), MEKK1 (6), the catalytic subunit of PKA (13), and possibly several other yet unidentified components. IκB kinases are activated mainly by the two upstream mitogen-activated protein kinase kinase kinases (MAP3K) NIK and MEKK1 (14, 15).

One major function of NF-κB is the regulation of programmed cell death (16, 17). Although in different cell types, *e.g.* B cells, and in pathophysiological situations different ways of initiation and execution of the apoptotic program exist (18, 19), NF-κB appears to play a protective role in the context of TNFα-mediated apoptosis by regulating the expression of anti-apoptotic genes. These anti-apoptotic genes include *A20, A1*, and *IEX-1* as well as *IAPs*, a gene family present in a variety of species ranging from insect viruses to humans (20–22). *IAPs* function to inhibit the terminal effector caspases 3 and 7 (23, 24) and block the cytochrome *c*-induced activation of caspase 9 (25).

Recently, we have reported on the NF-κB-dependent expression of XIAP and its functional role in protecting endothelial cells from TNFα-induced apoptosis (26). However, there is also an emerging role for anti-apoptotic genes in regulating NF-κB. Two members of the *IAP* gene family (c-IAP1 and c-IAP2) have been shown to be involved in TNFα signaling through binding to the TRAF1-TRAF2 complex (27, 28), which is also the case for A20 (29, 30). Moreover, A20 appears to suppress, whereas c-IAP2 stimulates NF-κB activity (31, 32). Here we demonstrate a novel function for XIAP as a potent activator of NF-κB. The mechanism by which XIAP exerts its influence involves the MAP3K TAK1 by interfering with the NF-κB signaling pathway upstream of IKKL.

MATERIALS AND METHODS

Cell Culture and Transfections—Human umbilical vein endothelial cells (HUVEC) were isolated and cultured as described (33). For stimulation, human recombinant TNFα (Genzyme) was used at a final concentration of 100 units/ml. Transfections were done in 6-well plates using LipofectAMINE Plus (DNA/LipofectAMINE/Plus reagent: 1.5 μg/4 μl/8 μl per well for 2.5 h; Life Technologies, Inc.) according to the manufacturer's recommendations. Human 293 cells were obtained from ATCC and propagated in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, antibiotics, and glutamine. For transfections, a DNA/LipofectAMINE/Plus-reagent ratio of 1.0 μg/3 μl/4 μl per well was applied for 5 h.

Vector Constructs—The IL-8 and E-selectin reporter constructs contain 1470 and 1800 nucleotides of the respective human promoters in pGL2-basic or pUBT-luc (34, 35). The NF-κB-dependent minimal promoter constructs contain three copies of the NF-κB-binding sites from the respective human promoters (IL-8 or E-selectin) inserted upstream of a 37-base pair *herpes simplex* thymidine kinase minimal promoter in pUBT-luc. Expression vectors for p65-NF-κB and XIAP have been de-

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[1] The abbreviations used are: NF-κB, nuclear factor κB; dn, dominant-negative; EGFP, enhanced green fluorescent protein; GST, glutathione *S*-transferase; HUVEC, human umbilical vein endothelial cells; MAP3K, mitogen-activated protein kinase kinase kinase; wt, wild-type; TNFα, tumor necrosis factor α; IL, interleukin.

scribed (36, 26); Myc-IκBα contains a Myc tag fused to the NH_2 terminus of porcine IκBα (35) in a cytomegalovirus-based expression vector. *Escherichia coli*-expressed IκBα used as substrate for *in vitro* kinase assays contained the NH_2-terminal 54 amino acids of porcine IκBα fused to GST; in a corresponding construct that serves as control the two phosphoacceptor sites Ser-32 and -36 were mutated to Ala. Expression constructs for truncated XIAP that contain either the BIR or the RING finger motifs were generated by subcloning at the single *Eco*RV cleavage site. Expression vectors for active (ΔTAK1) and dominant-negative (dn) TAK1 (K63W), HA-tagged wild-type (wt), and dn NIK (NIKK429R), and dn MEKK1 (K432A) were kind gifts from J. Ninomiya-Tsuji, G. Natoli, and R. Davis, respectively (37–39). I-TRAF and IKK2 were obtained by reverse transcriptase-polymerase chain reaction using primers derived from published sequences. Dn (kinase-dead) IKK2 was generated by mutation of Lys-44 to Ala using polymerase chain reaction. All constructs generated by polymerase chain reaction were sequenced. Expression vectors for β-galactosidase contained cytomegalovirus or ubiquitin promoters.

Immunostaining—Paraformaldehyde-fixed, Triton X-100-permeabilized cells were stained for p65-NF-κB using the anti-NF-κB p65(A) antibody (Santa Cruz Biotechnology) at a dilution of 1:1000 and biotin-conjugated donkey anti-rabbit second antibody (1:200) followed by incubation with streptavidin-alkaline phosphatase (1:50) and visualization with ELF-97 phosphatase substrate (Molecular Probes). Expression of enhanced green fluorescent protein (EGFP) from the co-transfected vector pEGFP-C1 (Promega) was viewed at ex 488/em 512 nm and could be distinguished from the ELF-97 substrate by its different excitation wavelength (ex 350/em 530 nm).

Western Blotting—Cells were lysed in Laemmli buffer and proteins separated by SDS-polyacrylamide gel electrophoresis using 10% polyacrylamide gels. Myc-tagged IκBα was detected using peroxidase-conjugated anti-Myc antibody (InVitrogen) at a dilution of 1:1500 followed by ECL-Plus (Amersham Pharmacia Biotech) detection. The anti-XIAP antibody was from R&D systems and was used at a 1:1000 dilution.

Co-immunoprecipitations and Immunocomplex Kinase Assays—293 cells were grown and transfected in 6-well plates, lysed, and immunoprecipitations performed as described (7) using anti-TAK1 antibody (Santa Cruz Biotechnology) and protein A-Sepharose (Roche Molecular Biochemicals) followed by Western blotting with peroxidase-conjugated anti-HA antibody. Flag-tagged IKK2 was precipitated with anti-FLAG M2 affinity matrix (Sigma). Immunocomplex kinase assay was performed as described (6) using an *E. coli* expressed NH_2-terminal fragment of IκBα fused to GST (GST-(N-term)IκBα).

RESULTS

XIAP Stimulates NF-κB-dependent Transcription—Previous observations describing a modulatory role of some anti-apoptotic genes toward NF-κB have prompted us to investigate a corresponding function for XIAP. Upon transfection of HUVEC with different NF-κB-dependent reporter constructs that contain NF-κB-binding sites derived from different genes cloned upstream of a minimal thymidine kinase promoter, a strong stimulation of the promoter activity was observed when XIAP was coexpressed (Fig. 1*A*). Likewise, XIAP stimulated transcription from the entire promoters of IL-8 and E-selectin that were shown to be dependent on NF-κB (Fig. 1*B*). When comparing different IAP gene family members, stimulation of the IL-8 promoter by XIAP was much more pronounced as compared with c-IAP1 and the published c-IAP2 (Fig. 1*C*). Activation required the entire XIAP protein containing both the COOH-terminal RING finger and the NH_2-terminal BIR repeats, as deletion of either motif resulted in loss of stimulation (Fig. 1*C*). This indicates that the caspase inhibitory function of XIAP, which can be exerted by the second of the NH_2-terminal BIR repeats alone (40), is not sufficient to augment NF-κB activity. To determine the effect of endogenous XIAP on TNFα-mediated NF-κB activation, an antisense XIAP construct was introduced into 293 cells that can be transfected with high efficiency, and NF-κB activity following TNFα stimulation was monitored. NF-κB activity in response to TNFα was partially reduced in antisense-XIAP expressing cells, as were the endogenous levels of XIAP determined by Western blotting (Fig. 1*D*).

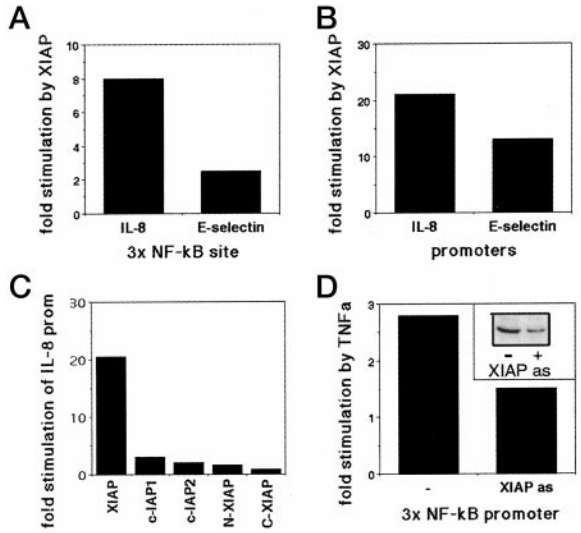

FIG. 1. **Stimulation of NF-κB-dependent transcription by XIAP.** *A,* HUVEC were transfected with luciferase reporter constructs containing multimerized NF-κB-binding sites derived from the IL-8 or E-selectin promoters together with an expression plasmid for XIAP or control vector. Values are expressed as -fold stimulation by co-transfected XIAP of luciferase relative light units normalized for β-gal expression used as internal control. *B,* HUVEC were transfected with reporter gene constructs for IL-8 and E-selectin promoters plus an expression vector for XIAP or empty vector as control. *C,* transfection of HUVEC with the IL-8 promoter-luciferase reporter construct plus expression vectors for XIAP, truncated XIAP containing either the NH_2-terminal BIR (N-XIAP) or the COOH-terminal RING finger (C-XIAP) motifs, c-IAP1, and c-IAP2. *D,* the minimal NF-κB reporter construct containing binding sites derived from the IL-8 promoter was transfected together with empty vector or antisense (*as*) XIAP expression vector into 293 cells. Two days later, cells were stimulated with TNFα for 6 h. Duplicate plates were analyzed by Western blotting for XIAP expression (*small insert*). Values are representative of at least three independent experiments.

XIAP Augments Nuclear Translocation of NF-κB and Increases IκBα Degradation—As nuclear translocation is a prerequisite for NF-κB activity, we have analyzed the cellular distribution of p65-NF-κB, the subunit of major importance during the inflammatory response. HUVEC were transfected with XIAP (Fig. 2, *A* and *C*) or control vector (Fig. 2B, D) plus EGFP for identification of transfected cells. Expression of (endogenous) p65 was revealed using an anti-p65 antibody in combination with the ELF-97 phosphatase substrate that can be distinguished from EGFP fluorescence by its different excitation wavelength (Fig. 2, *A* and *B*). Transfected cells were identified by EGFP expression (Fig. 2, *C* and *D*). Fluorescence microscopy showed nuclear localization of endogenous p65-NF-κB in those cells transfected with the XIAP construct, but cytoplasmic localization in cells transfected with control vector.

To investigate whether nuclear translocation of NF-κB follows the classical pathway of IκBα degradation, we transfected 293 cells with Myc-tagged IκBα, which can be distinguished from the endogenous protein in non-transfected cells, in the presence or absence of XIAP. The next day, cells were treated with cycloheximide for various periods of time to prevent *de novo* protein synthesis. As determined by Western blotting, in XIAP transfected cells the levels of IκBα were significantly reduced in a time-dependent manner in comparison to control cells (Fig. 3). No significant IκBα degradation was seen in the absence of cycloheximide, probably due to strong resynthesis from the transfected construct (not shown). This is in line with our observations that IκBα is able to block the XIAP effect when overexpressed (see below) and also with the experiments described for c-IAP2 (32).

XIAP Acts on NF-κB Activation by Interfering Upstream of IKK2 and Downstream of the TNFα Receptor-associated Fac-

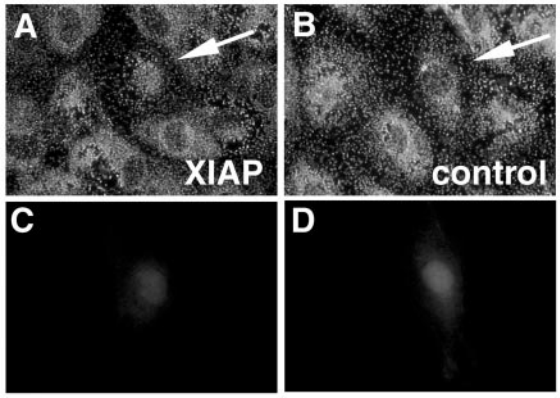

FIG. 2. **Nuclear translocation of p65-NF-κB.** HUVEC were transfected with expression vectors for XIAP (*A* and *C*) or with control vector (*B* and *D*). EGFP was included in all samples for identification of transfected cells. Cells were immunostained with anti-p65 antibody followed by detection with ELF-97 substrate and analyzed by fluorescence microscopy for p65 localization (*A* and *B*) and EGFP expression (*C* and *D*). A representative section is shown.

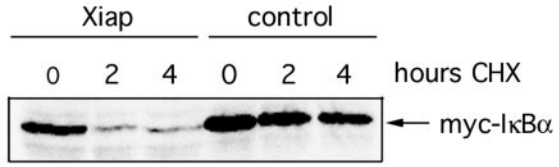

FIG. 3. **IκBα degradation in response to XIAP.** 293 cells were transfected with expression vectors for Myc-tagged IκBα together with either XIAP or control vector. After 2 days, cycloheximide (*CHX*) was added at a final concentration of 50 μg/ml, cells were lysed at the indicated time points, and analyzed by Western blotting. Equal loading was controlled by Ponceau S staining after blotting.

tors—As the elucidation of NF-κB signaling has led to the delineation of an apparently consecutive queue of kinases and interacting factors that connect cell surface receptors, *e.g.* TNFα and IL-1 receptors, to NF-κB, we have attempted to determine if, and at which point XIAP stimulates the signaling pathway. XIAP was transfected into 293 cells together with dn mutants of IKK2, NIK, and MEKK1, as well as with I-TRAF (27) or IκBα, and cell lysates analyzed for activation of the NF-κB-dependent reporter gene. Expression vectors for the inhibitory proteins were used in amounts that resulted in significant reduction of TNFα stimulated NF-κB activation. Under these conditions, XIAP-mediated stimulation was inhibited by dnNIK, dnIKK2, and IκBα, but not by dnMEKK1 (Fig. 4). I-TRAF, a TNFα receptor-specific inhibitory protein, reduced TNFα stimulation partially, but was unable to block XIAP-mediated activation. The slight stimulation of NF-κB activity observed in the presence of XIAP plus I-TRAF may be explained by a situation where blockage of TNFα receptor signaling could favor access of the XIAP-TAK1 pathway to common signaling molecules, *e.g.* NIK or IKK2. Together, these results suggest that the point of interference of XIAP with the NF-κB signaling pathway is located upstream of IKK2 and downstream of the TNFα receptor-associated factors.

Activation of NF-κB by XIAP Is Mediated by TAK1—XIAP was recently identified as a bridging molecule that connects the bone morphogenetic protein receptor IA (BMPR-IA) to TAB1, a co-activator of TAK1 (41). TAK1 is an upstream MAP3K that activates the p38 pathway and has recently been implicated in wnt signaling during *Caenorhabditis elegans* development (42–44). We have therefore tested whether TAK1 might be involved in the XIAP-mediated activation of NF-κB. As shown in Fig. 5*A*, co-transfection of dnTAK1 inhibited the XIAP-mediated NF-κB stimulation in a dose-dependent manner. These findings are consistent with a recent observation that TAK1 may activate NF-κB; however, both NIK-dependent and -independent pathways have been described (45, 37).

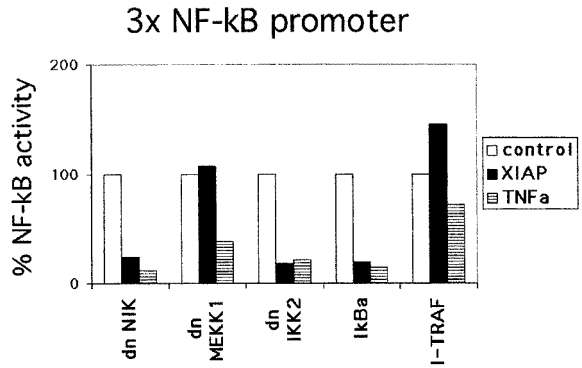

FIG. 4. **Effect of co-transfected dominant-negative mutant proteins on XIAP-stimulated NF-κB activity.** 293 cells were transfected with the minimal NF-κB reporter construct plus XIAP, together with expression vectors for dnNIK, dnMEKK1, dnIKK2, IκBα, or I-TRAF as indicated. Relative luciferase values are shown as percent of expression from the reporter gene as compared with XIAP alone (control = 100%). To control the effect of the inhibitory constructs, cells were transfected as above except that XIAP was omitted, but instead stimulated with TNFα for 16 h.

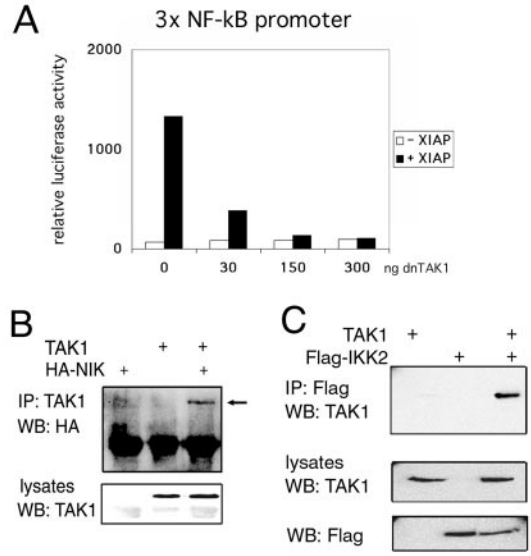

FIG. 5. **Involvement of TAK1 in XIAP-mediated NF-κB stimulation.** *A,* effect of dnTAK1 on NF-κB-dependent transcription. 293 cells were transfected with a NF-κB-dependent promoter-luciferase reporter gene together with XIAP as well as different amounts of dnTAK1 expression vector. *B,* coimmunoprecipitation of TAK1 with NIK. 293 cells were transfected with TAK1 and/or HA-NIK and immunoprecipitations (*IP*) performed using an anti-TAK1 antibody followed by Western blotting (*WB*) with anti-HA antibody. The NIK-specific band is indicated by an *arrow*. *C,* interaction of TAK1 with IKK2. 293 cells were transfected with TAK1 and/or Flag-IKK2 and immunoprecipitations performed using anti-Flag M2 Sepharose followed by Western blotting with anti-TAK1 antibody. Whole cell extracts were analyzed by Western blotting for expression of transfected TAK1 and IKK2.

To explore direct interactions between kinases, we have tested TAK1 in coimmunoprecipitations with NIK and with IKK2. 293 cells were transfected with expression vectors for TAK1, HA-NIK, or Flag-IKK2. As shown in Fig. 5*B*, the anti-TAK1 antibody coimmunoprecipitated NIK from cell lysates, supporting our data obtained from the reporter gene studies. In addition, direct binding of TAK1 to IKK2 could be demonstrated (Fig. 5*C*). In contrast, no association between TAK1 and MEKK1, which was also reported to activate the IKKs, could be observed (data not shown).

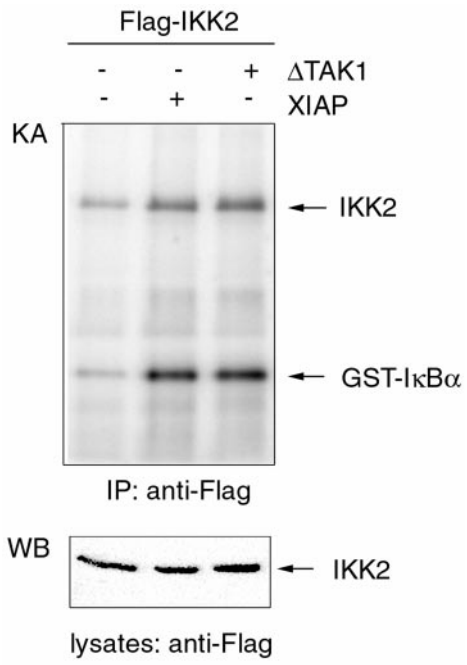

FIG. 6. **Activation of IKK2 kinase activity by XIAP and constitutively active TAK1 (ΔTAK1).** 293 cells were transfected with Flag-IKK2 plus XIAP, ΔTAK1, or control vector. IKK2 was immunoprecipitated using anti-Flag M2 Sepharose and immunocomplex kinase assay (*KA*) performed using GST-(N-term) IκBα as substrate. The positions of the phosphorylated IκBα and autophosphorylated IKK2 are indicated. A kinase-dead mutant of IKK2 as well as a mutant GST-(N-term) IκBα where the phosphoacceptor sites Ser-32 and -36 had been replaced by Ala were used as controls (not shown). Western blot (*WB*) of transfected IKK2 was performed to control for equal amounts of IKK2 in immunoprecipitates.

To further characterize the functional interaction between TAK1 and IKK2 we performed *in vitro* kinase assays. Cells were transfected with expression vectors for Flag-IKK2 in combinations with XIAP or constitutively active TAK1 (ΔTAK1). Following immunoprecipitation of IKK2, immunocomplex kinase assays were carried out using GST-(N-term) IκBα as substrate. As shown in Fig. 6, the presence of either the co-transfected XIAP or ΔTAK1 stimulated IKK2 activity toward IκBα. In addition, IKK2 autophosphorylation was stimulated. No phosphorylation of a mutant IκBα substrate, where Ser-32 and -36 were substituted by Ala was seen (data not shown). In summary, TAK1 physically interacts with NIK and IKK2, but not with MEKK1, and both XIAP and TAK1 are able to stimulate IKK2 kinase activity.

DISCUSSION

An apparently consecutive queue of signaling molecules has been identified that connects TNFα and IL-1 receptors to the transcription factor NF-κB. However, the (partial) characterization of the high molecular weight signalosome suggests that several additional molecules exist that may be important for the multistep process of NF-κB activation. In addition, besides a central signaling pathway, under certain circumstances both positive and negative autoregulatory loops may modulate the activation of a transcription factor. In the case of NF-κB, a negative regulatory loop acting through NF-κB-dependent expression of its own inhibitor IκBα has been established (35). Furthermore, the apoptosis inhibitory protein A20 has been shown to interfere with NF-κB activation in EC in response to TNFα (30, 32). Here we provide evidence that a different, positive regulatory loop involving the anti-apoptotic protein XIAP can augment NF-κB activity.

Based on our initial observations that in transient transfections XIAP stimulates NF-κB-dependent transcription, and that increased nuclear translocation of the transcription factor subunit p65 could be detected in response to XIAP expression, we sought to determine the point where XIAP engages into the cognate pathway of NF-κB activation. Nuclear translocation of NF-κB is governed by its release from the inhibitory subunits IκBα and/or IκBβ, which are phorphorylated by IκB kinases. In XIAP-transfected cells, increased turnover of IκBα was observed, indicating that NF-κB translocation follows the cognate scheme. In addition, the effect of XIAP on NF-κB could readily be blocked by dn mutants of the upstream activators of IκBα, IKK2, and NIK, but not by MEKK1, for which divergent findings have been reported in regard to its role in NF-κB activation (for discussion, see Ref. 3). Therefore, in the context of XIAP signaling, MEKK1 is not involved. Taken together, XIAP appears to stimulate the NF-κB signaling pathway upstream of the point where signals from the TNFα and IL-1 receptors converge.

Two other members of the *IAP* gene family, c-IAP1 and c-IAP2, interact with components of the TNFα signaling pathway via association with TRAF-1 and TRAF-2, and the latter has been reported to stimulate NF-κB activity (31). We were not able to demonstrate interaction of XIAP with any of the TRAF family members (TRAF1 to 6) in a yeast two-hybrid system,[2] nor have others reported interaction of XIAP with cognate components of the cytoplasmic TNFα or IL-1 receptor complexes. However, XIAP was recently identified as a bridging protein between TAB1 and the cytoplasmic part of the bone-morphogenetic protein receptor IA (41). TAB1 in turn functions as an activator of TAK1, a type MAP3K originally described to be involved in TGF-β signaling, as well as an upstream activator of the p38 MAP kinase pathway (42). Co-transfection of TAK1 with its coactivator TAB1 has been shown to induce NF-κB (45). In the context of IL-1 signaling, TAK1 is capable of associating with TRAF6 and NIK (37), however, also a NIK-independent pathway for NF-κB activation has been reported (45).

The involvement of TAK1 in XIAP-mediated NF-κB activation is supported by the following experiments. First, dnTAK1 could inhibit XIAP stimulated NF-κB activation in a dose-dependent manner (Fig. 5*A*). Second, TAK1 interacts with NIK and with IKK2 in coimmunoprecipitations (Fig. 5, *B* and *C*); co-transfection of NIK did not interfere with the TAK1-IKK2 interaction (not shown). In contrast, TAK1 binding to IKK1 occurs only in the presence of (or via) NIK (37). Third, active TAK1 stimulates IKK2 kinase activity in an immunocomplex kinase assay. It might well be that the heterodimeric IKK1 and IKK2 protein complex associates with NIK-TAK1, whereby NIK preferentially activates IKK1 and TAK1 activates IKK2. These observations are consistent with the existence of an at least trimolecular complex consisting of NIK, TAK1, and IKK2, although the precise sequence of activation as well as the possible location of the kinases within the signalosome remains to be elucidated.

Our findings of a mutual regulation of NF-κB and XIAP support the hypothesis that after a decision between apoptosis and survival in response to TNFα has been taken, cellular mechanisms become operative to amplify those signaling pathways that substantiate the decision and make it irreversible. A different example is the caspase-mediated cleavage of p65-NF-κB to yield a transcriptionally inactive molecule that acts as a dominant negative inhibitor of NF-κB (46). Likewise, and also during the process of apoptosis, IκBα is truncated at the amino terminus to generate a non-phosphorylatable super-

[2] J. A. Schmid, unpublished data.

repressor of NF-κB (47). In the case of survival, expression of XIAP would, besides inhibition of caspases, lead to augmented NF-κB activity and to an amplification of the anti-apoptotic response.

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