IκB-α, the NF-κB Inhibitory Subunit, Interacts with ANT, the Mitochondrial ATP/ADP Translocator*

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The transcription factor NF-κB regulates a wide set of genes involved in the establishment of many cellular processes that control cell activation, proliferation, and apoptosis. IκB inhibitory subunits integrate NF-κB activation signals through phosphorylation and ubiquitination of its N-terminal domain. Using the two-hybrid system in yeast, we searched for IκB-α N-terminal domain interactors and therefore potential NF-κB regulators. An interaction of IκB-α with the mitochondrial ATP/ADP translocator ANT was detected in yeast and confirmed in glutathione S-transferase pull-down assays and co-precipitation experiments in transfected cells. Subcellular cell fractionation, resistance to proteinase K treatment, and electron microscopy experiments demonstrated the presence of IκB-α and associated p65 NF-κB in the mitochondrial intermembrane space. IκB-α/NF-κB appeared to be released from mitochondria upon the induction of apoptosis by engagement of the Fas receptor. These data suggest that the mitochondrial IκB-α/NF-κB pool participates in the regulation of apoptosis.

Rel/NF-κB transcription factors are ubiquitously expressed and respond to more than 150 stimuli to regulate an equally wide array of genes (1). While NF-κB was well known to participate in the control of immune (2) and inflammatory responses (3), it recently appeared as an important player in regulating the balance between cell survival and apoptosis (4, 5). NF-κB is maintained inactive by inhibitory subunits of the IκB family such as IκB-α. IκB-α interacts with NF-κB via its ankyrin motifs, masking nuclear localization signals on NF-κB subunits. The release of transcriptionally competent NF-κB dimers is achieved after phosphorylation-induced degradation or dissociation of IκB-α molecules. At least two kinases (IKKa and IKKβ) that are specific for the conserved tandem serines in IκB-α molecules have been identified. They show an extensive homology and form homo- as well as heterodimers within the cell. IKKs are localized in the signalsome, a multiprotein complex (700–900 kDa). This complex also contains NEMO/IKKγ, a structural component that is crucial for the correct assembly and functionality of the signalsome (for a review, see Refs. 7 and 8). An alternative mechanism that links tyrosine kinases to NF-κB activation has been described that uses phosphorylation of tyrosine 42 of IκB-α to dissociate IκB-α/NF-κB complexes (9).

Phosphorylation of IκB-α on serines 32 and 36 allows its specific recognition by the E3RS ubiquitin protein ligase, which then transfers ubiquitin to lysines 20 and 21. The polyubiquitinated IκB-α molecule is thereafter recognized and degraded in situ by the proteasome 26 S complex (10). The N-terminal domain of IκB-α thus appears to integrate NF-κB activation signals. We used this regulatory domain as a bait in a yeast two-hybrid screening of a Jurkat cDNA library to search for protein interactors and therefore potential regulators of NF-κB activation. In this report, we describe and characterize the interaction between IκB-α and ANT, the mitochondrial ATP/ADP translocator (10). ANT is a central component of the mitochondria permeability transition pore (11). The opening of this pore, during induction of apoptosis allows the release of molecules, such as caspases or apoptosis-inducing factor, which are important to amplify the cell suicide response. The possible functional importance of the IκB-α/ANT interaction in the context of regulation of apoptosis is discussed.

MATERIALS AND METHODS

Biological Reagents and Cell Culture—Anti-IκB-α antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit anti-serum anti-IκB-α, anti-Myc epitope, and anti-F1 ATPase monoclonal antibody were kindly provided by Jean Imbert (U119, Marseille, France), Jean-François Tanti (INSERM, EPI E99–11, Nice, France), and Joel Lunardi (Commissariat à l’Energie Atomique, Grenoble, France), respectively.

Human embryonic kidney 293 cells were maintained in Dulbecco’s modified Eagle’s medium containing 2 mM glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, 1 mM pyruvate, and 10% fetal calf serum (Life Technologies, Inc.). Cells were transfected by the calcium phosphate method as previously described (12).

HTLV-I* infected MT4 cells (13) and cells of the human leukemic T-cell line Jurkat were maintained in RPMI 1640 medium containing 50 units/ml penicillin, 50 μg/ml streptomycin, 1 mM pyruvate, and 5% fetal calf serum.

Yeast Two-hybrid and cDNA Cloning—IκB-α cDNA encoding amino acids 2–72 was subcloned into the GAL4 DNA-binding vector pAS2.1 (CLONTECH, Palo Alto, CA). The resulting plasmid, pAS-IκB2–72, was used as the bait in a two-hybrid screening of a human Jurkat cDNA library (CLONTECH) in the S. cerevisiae HF7c strain according to the Matchmaker Two-Hybrid System II Protocol (CLONTECH). Positive yeast clones were selected for prototrophy for histidine and expression of β-galactosidase. Yeast DNA was recovered. Sequencing of posi-

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‡ To the Matchmaker Two-Hybrid System II Protocol (CLONTECH). Positive yeast clones were selected for prototrophy for histidine and expression of β-galactosidase. Yeast DNA was recovered. Sequencing of posi-

1 The abbreviations used are: HTLV, human T-cell lymphotropic virus; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PTP, permeability transition pore.
Yeast HF7c cells were cotransfected with expression vectors encoding the indicated GALA DNA-binding domain (DB)-fused and GALA transcription activation domain (AD)-fused proteins. Colonies were tested for their ability to grow on medium lacking histidine, tryptophan, and leucine, and galactosidase activity was monitored by a standard filter assay. Growth on selective medium and β-galactosidase activity are scored as a range from no growth and no β-galactosidase activity (−) to activity generated by the positive control (+).

| Yeast two-hybrids (DB/AD) | Growth on selective medium | β-Galactosidase assay |
|---------------------------|---------------------------|---------------------|
| IκB-α-(2–72)/vector       | −                        | −                   |
| IκB-α-(2–72)/α-t-Antigen   | −                        | −                   |
| p53/T-Antigen             | +                        | +                   |
| IκB-α-(2–72)/ANT1(171–297)| +                        | +                   |
| IκB-α-(2–72)/ANT2(152–298)| +                        | +                   |
| IκB-α-(2–72)/ANT1(171–297)| +                        | +                   |
| p53/ANT1(171–297)         | −                        | −                   |

IκB-α Interacts with ANT

Yeast two-hybrid system

Yeast HF7c cells were cotransfected with expression vectors encoding the indicated GALA DNA-binding domain (DB)-fused and GALA transcription activation domain (AD)-fused proteins. Colonies were tested for their ability to grow on medium lacking histidine, tryptophan, and leucine, and β-galactosidase activity was monitored by a standard filter assay. Growth on selective medium and β-galactosidase activity are scored as a range from no growth and no β-galactosidase activity (−) to activity generated by the positive control (+).

Dounce homogenization, cell extracts were laid over the top of a Percoll/supernatants and pellets were designated as cytosolic and mitochondrial. Mitochondria were highly purified as described.

Mitochondria were lysed in total buffer (20 mM Heps, pH 7.4, 50 mM NaCl, 20% glycerol, 1% Nonidet P-40, 1 mM MgCl₂, 1 mM CaCl₂, and 0.5 mM EDTA, 0.1 mM EGTA, 0.5 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, and 0.5 µg/ml of protein from total mitochondria extract or 50 µg of protein from digitonin supernatant were subjected to an electrophoretic mobility shift assay analysis. IκB-α and NF-κB complexes were dissociated with a 0.6% deoxycholate treatment during 10 min at room temperature.

The NF-κB probe was used in a synthetic double-stranded oligonucleotide containing the NF-κB binding site of the interleukin-2 gene promoter (5′-GATCCAAGGGACTTTCCATG-3′). The end-labeled probe was incubated with extract samples for 20 min at 37°C. Complexes were separated by electrophoresis on a 5% nondenaturing polyacrylamide gel in 0.5× TBE. The dried gels were subjected to autoradiography (X-Omat; Eastman Kodak Co.).

RESULTS

Isolation of ANT as a Potential IκB-α-binding Protein—Activation of the transcription factor NF-κB is regulated by phosphorylation of the N-terminal domain of the inhibitory subunit IκB-α. We used this N-terminal regulatory domain (IκB-α amino acids 2–72) as the bait in a yeast two-hybrid-based approach to identify new IκB-α interactors and therefore potential NF-κB regulators. By the screening of a commercial human Jurkat cell library, −6 × 10⁵ double yeast transformants were obtained, among which 232 grew on selective medium and only 88 turned blue when tested in a filter lift β-galactosidase assay. Fifty-six clones were further characterized by sequencing.

Primary nucleotide sequencing revealed one cDNA clone encoding a protein not previously described in GenBank and two clones corresponding to two isoforms of the adenine nucleotide translocator: ANT1(171–297) and ANT2(152–298).

The interaction between ANT and IκB-α was further characterized in the yeast two-hybrid system. Full-length IκB-α could not be used in the two-hybrid system because of a constitutive activity of reporter genes (not shown; see Ref. 16). As shown in Table I, the DB-IκB-α-(2–72) fusion protein cannot act as activator of transcription when coexpressed in yeast with GAL4 AD nor in combination with AD-T Ag (large T antigen) fusion protein (lanes 1 and 2). The AD-ANT fused protein cannot act as activator of transcription when coexpressed in yeast with GAL4 DB or in combination with the GAL4 DB fusion protein (lanes 7 and 8). On the contrary, co-expression of IκB-α-(2–72) with the portion of ANT1 or ANT2 isolated during the screening (lanes 4 and 5) or with the full-length ANT1 cDNA (lane 6) induced expression of the two reporter genes, thus demonstrating a direct and specific interaction between the two partners. We performed in vitro binding assay to confirm, in an independent experimental setting, the physical interaction between ANT and IκB-α proteins. The C-terminal...
part of ANT isolated during the screening as well as the full-length ANT protein sequences were fused to GST and used in pull-down experiments with recombinant IxB-α. The size of the recombinant proteins was checked by SDS-PAGE and Coomassie Blue staining (Fig. 1A, upper panel). The GST protein or a non-relevant fusion protein could not interact with IxB-α (Fig. 1A, lower panel, lanes 2–5). By contrast, the C-terminal domain of ANT1 (lanes 6 and 7) as well as the full-length protein (lanes 8 and 9) interact specifically and in a dose-dependent manner with IxB-α.

These in vitro results confirm the specificity of the ANT and IxB-α interaction.

Co-precipitation of ANT and IxB-α in Transfected Intact Cells—293 cells were transiently transfected with two expression vectors encoding Myc-tagged ANT and IxB-α. Cellular levels of IxB-α and ANT were visualized by direct Western blot analysis of total lysates with antibodies to IxB-α (Fig. 1B, lane 2 versus lane 1) or to Myc epitope (lane 10 versus lane 9). Cell soluble extracts were then subjected to immunoprecipitation with anti-IxB-α (lanes 3, 4, 11, and 12) or anti-Myc (lanes 5, 6, 13, and 14) rabbit polyclonal antibodies or with a non-immune serum (lanes 7, 8, 15, and 16). The precipitates were fractionated by SDS-PAGE and blotted with polyclonal IxB-α (lanes 1–8) or anti-Myc (lanes 9–16) monoclonal antibodies. Anti-IxB-α precipitates contained an anti-Myc reactive band (lane 12) that comigrates with MycANT (lane 10). Quantification of the bands lead to an estimate of 14% of total ANT associated with IxB-α. On the contrary, IxB-α could not be revealed after anti-Myc immunoprecipitation (lane 4). No anti-Myc or anti-IxB-α reactive proteins were precipitated by a non-immune serum (lanes 8 and 16). These results demonstrate that a specific interaction between ANT and IxB-α could occur in intact cells.

Localization of IxB-α and p65 NF-κB in Purified Mitochondria from Jurkat Cells—Since ANT is a mitochondrial protein, we looked for the presence of IxB-α in this organelle. Mitochondria preparation after subcellular fractionation exhibited minor contamination by cytosol, as revealed by assays for specific mitochondrial enzymes: lactate dehydrogenase (2.6%; data not shown). IxB-α was analyzed in total cell extracts (Fig. 2A, upper panel, lane 1) or in cytosolic (lane 2) or mitochondrial (lane 3) extracts. Equal amounts (250 μg) of proteins from cytosol or mitochondria were loaded. Although the majority of IxB-α was localized in the cytosolic fraction (lane 2), the protein could also be observed in the mitochondria (lane 3). The localization of p65 NF-κB was investigated. As shown in the lower panel, p65 was also present in the cytoplasm (lane 2) as well as in the mitochondria (lane 3).

In order to exclude a possible contamination of the mitochondrial fraction by cytosolic proteins or other cellular compartments such as the Golgi apparatus or the ER, mitochondria were further enriched and purified on a Percoll/metrizamide gradient. Lactate dehydrogenase activity was below 1% in the highly purified mitochondrial fraction (not shown). Western blot analysis with specific antibodies confirmed the presence of IxB-α and p65 in mitochondria (lane 5, upper and middle panels). Using densitometry scanning of the autoradiography, we calculated that approximately 7% of cellular total IxB-α is located in mitochondria. Cytochrome c was mainly detected in the mitochondrial fraction (lane 5, lower panel) and only faintly in the cytosol (lane 4, lower panel).

Immunoelectron microscopy analysis using anti-IxB-α anti-
bodies as primary antibodies was then performed on purified mitochondria (Fig. 2B). No signal could be detected when gold-conjugated secondary antibody was used alone or in combination with a nonrelevant primary antibody (see controls on Fig. 4, A and B). IκB-α staining was associated with the membrane or the internal crests, but not with the matrix.

We next assessed the sensitivity of IκB-α, p65, and Bcl2 in mitochondrial fractions to proteinase K (Fig. 2D, lanes 1–3, left panels). While Bcl2, which is associated with the external mitochondrial membrane, was completely degraded (lower panel, lanes 2 and 3), IκB-α and p65 remained unaffected (upper and middle panels, lanes 2 and 3), suggesting that IκB-α and p65
quire a higher digitonin concentration (0.4%; Fig. 2A) to dissociate IκBα. Specific binding was indicated (lanes 3 versus lane 1). Deoxycholate (Doc) treatment of cytosol (lane 2) or mitochondria preparation (lane 5) released NF-κB that was competent for specifically binding a 32P-labeled κB probe, as verified by competition assays with unlabeled probe (lanes 3 and 6). Taken together, these results confirm the existence of IκBα-NF-κB complexes in the mitochondria.

**Immunoelectron Microscopy Analysis of IκBα and p65 Subcellular Localization—**Immunoelectron microscopy analysis was then performed on intact Jurkat cells, using anti-IκBα or anti-p65 antibodies as primary antibodies. No signal could be detected when gold-conjugated secondary antibody was used in combination with a nonrelevant anti-cytokeratin antibody (Fig. 4A) or alone (Fig. 4B).

Analysis of anti-IκBα antibody-labeled cells revealed numerous beads distributed mainly in the cytosol with few in the nucleus (Fig. 4C). Beads were also found associated with mitochondria. No labeling could be detected within the endoplasmic reticulum or the plasma membrane, ruling out a nonspecific binding of the antibody to lipid membranes. A greater magnification of mitochondria showed that IκBα staining was localized mainly at the membrane level or in the internal crests (Fig. 4D).

Analysis of anti-p65 antibody labeled cells revealed a similar distribution of the molecule both in intact cells (Fig. 4E) and mitochondria (Fig. 4F).

Taken together, these results further confirm a mitochondrial localization of IκBα as well as of p65 NF-κB.

**IκBα-NF-κB Complexes Are Released from Mitochondria after Fas-induced Apoptosis—**The release of pro- or antiapoptotic molecules from the mitochondrial intermembrane space during apoptosis in several cellular systems is well documented (11). We analyzed the fate of IκBα, p65 NF-κB, and cytochrome c after induction of apoptosis upon engagement of the Fas surface molecule with the CH11 antibody. After cell treatment with increasing doses of CH11 antibody, mitochondria were isolated and analyzed for the presence of IκBα. As shown in Fig. 5A, engagement of the Fas receptor resulted in a dose-dependent release of IκBα (lanes 4–6, upper panel) as well as p65 (lanes 4–6, middle panel) and cytochrome c (lanes 4–6, lower panel) from mitochondria. In contrast, little to no degradation of these molecules could be visualized in the cytosol after CH11 treatment (lanes 1–3).

Electron microscopy analysis of CH11-treated Jurkat cells revealed cell shrinkage, perinuclear chromatIN condensation, and alterations of the mitochondrial structures, including swelling, disappearance of cristae membranes, and deestructurizing of the surrounding membranes (data not shown). In these cells, IκBα staining was highly reduced in the mitochondria (Fig. 5B, compare a with b). Similarly, CH11 treatment resulted in the disappearance of p65 reactivity (compare c with d).

These data suggest that IκBα and p65 complexes located in the intermembrane space of the mitochondria can be released in the cytosol following stimulation of Jurkat cells with apoptotic signals.

**Mitochondrial IκBα Is Not Degraded in MT4 Cells—**We
then performed experiments in order to determine if the mitochondrial IκB-α-NF-κB complexes were sensitive to activation signals. We used HTLV-I-infected MT4 cells that display a constitutive activation of NF-κB. The HTLV-I Tax protein is known to activate NF-κB by binding to the NEMO/IKKγ component of the signalsome, resulting in a constitutive activation of the IκB-α kinases (18). MT4 cells express low amounts of IκB-α (Fig. 6, lane 1) as visualized by Western blot analysis. Cell treatment (1 h, 50 μM) with the proteasome inhibitor ALLN, shown to prevent degradation of IκB-α, resulted in an increase in the cellular levels of IκB-α (lane 2). By contrast, the amount of IκB-α in the mitochondria was not affected by the ALLN treatment (compare lane 4 with lane 3). The nitrocellulose filter was then hybridized with a phosphospecific IκB-α antibody. Despite constitutive stimulation of IKK kinases, no phosphorylated IκB-α could be detected in cytosolic extracts (lane 6), suggesting that phosphorylated IκB-α is immediately degraded. Indeed, ALLN treatment allowed the visualization of phospho-IκB-α (lane 7). By contrast, no signal could be detected in the mitochondrial fraction (lane 8), even in the presence of ALLN (lane 9), suggesting that despite continuous firing of IκB kinases, mitochondrial IκB-α is protected from phosphorylation.

**DISCUSSION**

In this report, we show that IκB-α-NF-κB complexes are present in the mitochondrial intermembrane space via interaction of the N-terminal domain of IκB-α with the internal membrane protein ANT (adenine nucleotide translocator).

The interaction was originally detected in a yeast two-hybrid screening and checked by in vitro pull-down experiments with GST-ANT and recombinant IκB-α. A coprecipitation of the two molecules was observed in transfected 293 cells, demonstrating that the two proteins could interact in intact cells. Finally, immunoelectron microscopy allowed the unambiguous demonstration of IκB-α-NF-κB complexes in the mitochondrial intermembrane space. NF-κB and IκB-α were unaffected by a proteinase K treatment of intact mitochondria and could be extracted by digitonin lysis of the outer membrane.

The ANT protein is localized in the inner mitochondrial membrane and exchanges cytosolic ADP for mitochondrial ATP (10). ANT interacts with several proteins of the outer membrane ( peripheral benzodiazepine receptor, porin/VDAC, Bax) as well as the matrix (cyclophilin) to form the permeability transition pore (PTP) or megachannel (19). The PTP appears as an important regulator of the apoptotic process. Opening of the pore leads to loss of the mitochondrial transmembrane potential, Δψmo, that can ultimately culminate in matrix swelling and outer membrane rupture, allowing the release of apoptogenic proteins such as cytochrome c, apoptosis-inducing factor, and procaspases (11, 20–22). The exit of cytochrome c is controlled by proteins of the Bcl-2 family that are anchored on the mitochondrial outer membrane. Antiapoptotic members of the family (Bcl-2, Bcl-xl) prevent cytochrome c release in contrast to the proapoptotic members Bax and Bak (23). Bax has been shown to interact with ANT to induce PTP opening and cytochrome c release (24).

Several pharmacological compounds interfere with PTP. For instance, bongkrekic acid and atractyloside are, respectively, blocker and inducer of apoptosis by binding two different conformational stages of ANT (20, 25). Cyclosporin A also blocks mitochondrial apoptotic signals by interacting with cyclophilin (26), while engagement of the benzodiazepine receptor facilitates apoptosis (27).
The observed interaction of IkB-α with ANT suggests that mitochondrial IkB-α-NF-κB complexes could be involved in the regulation of the apoptotic cascade. Apart from its well known functions in coordination of immune and inflammatory responses, NF-κB appears to promote cell survival (4, 5). For instance, inactivation of the p65 NF-κB (relA) gene resulted in embryonic death due to massive liver apoptosis (28). This property could be demonstrated in various cell lines by expression of a dominant negative form of the inhibitory subunit IkB-α. Replacement of serines 32 and 36 by alanines prevents phosphorylation/degradation of IkB-α and thus activation of NF-κB. Cells expressing the IkB-α superrepressor clearly become more susceptible to apoptosis induced by tumor necrosis factor, chemotherapeutic drugs, or γ-rays (29–31).

In order to determine if the mitochondrial pool of IkB-α-NF-κB complexes could be reached by activation signals, we used HTLV-1-infected MT4 cells that display a constitutive activation of NF-κB, due to an interaction of the viral Tax protein with IKKg to activate the IKK complex. Inhibition of the degradation pathway by ALLN increased cytosolic IkB-α levels and allowed detection of the serine phosphorylated form. In sharp contrast, mitochondrial IkB-α was not affected by ALLN treatment. These results show that mitochondrial IkB-α-NF-κB complexes are sequestered from NF-κB-activating signals.

Induction of apoptosis after engagement of the Fas death receptor leads to the exit of IkB-α-NF-κB from mitochondria. While cytochrome c can leave mitochondria through open VDAC (diameter 2.4–3 nm) (32), the liberation of procaspases and IkB-α-NF-κB complexes requires rupture of the outer membrane. We believe that this release in the cytosol could
increase the number of NF-κB complexes susceptible for activation.

Apoptosis should be considered as a multistep and integrative process. Before producing an appropriate outcome response, cells have to integrate pro- and anti-apoptotic influences. Moreover, cross-talks between apoptotic and survival pathways have already been demonstrated. NF-κB could be one of the important players at this cross-road. NF-κB is p65 NF-κB survival genes, is cleaved and inactivated during apoptosis (35). So is p55 NF-κB, whose cleavage produces a protein lacking its transactivation domain that has a dominant negative effect on NF-κB-induced survival genes (36). IκB-α could also be cleaved by caspase 3, to an N-terminal truncated and stable inhibitor of NF-κB activation (37).

Apoptotic pathways have characteristics of a self-amplifying process. Procaspases are released from the mitochondria (38), and activated caspases are able to induce the exit of cytochrome c to exacerbate the response (39). In contrast, NF-κB promotes, in many cells, survival effects via transcription of genes such as those for cIAP1 and cIAP2 that code for caspases inhibitors (40, 41). It is thus possible that the release of IκB-αNF-κB from the mitochondria is an additional control level. Reception of a survival signal at this point would limit or delay apoptosis, whereas continuous caspase activation will lead to NF-κB inactivation. Alternatively, IκB-αNF-κB could exert a transcriptionally independent regulatory function on apoptosis. This has been demonstrated for p53, which translocates to mitochondria during p53-mediated apoptosis. Translocation precedes mitochondrial dysfunctions changes and appears independent of p53 transcriptional activity (42). It is thus conceivable that IκB-αNF-κB could regulate opening of the PTP through its association with ANT.

The results presented here suggest that a reservoir of IκB-αNF-κB complexes in the intermembrane mitochondrial space could be involved in modulating apoptosis responses.

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