NF-κB and IκBα Are Found in the Mitochondria

EVIDENCE FOR REGULATION OF MITOCHONDRIAL GENE EXPRESSION BY NF-κB*

**Patricia C. Cogswell‡, David F. Kashatus‡§, Jayne A. Keifer‡§, Denis C. Guttridge‡,
Julie Y. Reuther‡, Cindy Bristow‡, Sophie Roy¶, Donald W. Nicholson¶,
and Albert S. Baldwin, Jr.‡§‖**

*From the ‡Lineberger Comprehensive Cancer Center, ‖Department of Biology, and §Curriculum in Genetics and Molecular
Biology, University of North Carolina, Chapel Hill, North Carolina 27599-7295 and the ¶Department of Pharmacology,
Biochemistry, and Molecular Biology, Merck Frosst Centre for Therapeutic Research,
Point Claire-Dorval, Quebec H9R 4P8, Canada*

The transcription factor NF-κB has been shown to be predominantly cytoplasmically localized in the absence of an inductive signal. Stimulation of cells with inflammatory cytokines such as tumor necrosis factor α or interleukin-1 induces the degradation of IκB, the inhibitor of NF-κB, allowing nuclear accumulation of NF-κB and regulation of specific gene expression. The degradation of IκB is controlled initially by phosphorylation induced by the IκB kinase, which leads to ubiquitination and subsequent proteolysis of the inhibitor by the proteasome. We report here that NF-κB and IκBα (but not IκBβ) are also localized in the mitochondria. Stimulation of cells with tumor necrosis factor α leads to the phosphorylation of mitochondrial IκBα and its subsequent degradation by a nonproteasome-dependent pathway. Interestingly, expression of the mitochondrially encoded cytochrome *c* oxidase III and cytochrome *b* mRNAs were reduced by cytokine treatment of cells. Inhibition of activation of mitochondrial NF-κB by expression of the superrepressor form of IκBα inhibited the loss of expression of both cytochrome *c* oxidase III and cytochrome *b* mRNA. These data indicate that the NF-κB regulatory pathway exists in mitochondria and that NF-κB can negatively regulate mitochondrial mRNA expression.

The transcription factor NF-κB has been studied extensively due to its interesting regulation and to the range of biological processes that it controls. Five members of the immediate NF-κB/Rel family have been identified: p50/NF-κB1, p65/RelA, p52/NF-κB2, RelB, and c-Rel. The classic form of NF-κB is the heterodimer of the p50 and p65 subunits (reviewed in Ref. 1). In most cells, NF-κB is complexed with members of the IκB family, IκBα, IκBβ, and IκBε, which typically function to inhibit the action of this group of transcription factors. Stimulation of cells with stimuli such as TNFα[1] leads to activation of the IκB kinase (IKK), which phosphorylates IκBα or IκBβ on

N-terminal serines (1, 2). Phosphorylated IκB then is targeted for ubiquitination and subsequent degradation by the proteasome, allowing NF-κB to accumulate in the nucleus (3). In the nucleus, NF-κB is a positive regulator of gene expression through its ability to bind to target sequences in the regulatory regions of genes encoding cytokines, cytokine receptors, anti-apoptotic proteins, and cell cycle regulators (1, 4). Additionally, NF-κB has been shown to negatively regulate MyoD mRNA through a post-transcriptional mechanism that appears to require the transcriptional activity of NF-κB (5). Presumably through its ability to regulate gene expression, NF-κB dysregulation contributes to a variety of diseases, including oncogenesis, arthritis, and cancer cachexia (4, 6).

Recently, more complex aspects of NF-κB regulation have been proposed. For example, studies indicate that NF-κB shuttles into and out of the nucleus in unstimulated cells (7–9). Thus, it was found that leptomycin B, an inhibitor of nuclear export, leads to the accumulation of NF-κB and IκB in the nucleus without an external stimulus. Whether NF-κB and IκB shuttle in a complex has been questioned (7). Additionally, it has been proposed that IκB can be degraded by a nonproteasomal mechanism following stimulation of cells with cytokines (10). In that study, the use of inhibitors with distinct specificities indicated that phosphorylated IκBα can be degraded by calpain in addition to the more characterized proteasome pathway.

The vertebrate mitochondrial genome is circular with ∼17 kb of DNA (11) (for a review, see Ref. 12). The mitochondrial genome encodes two ribosomal RNAs, 22 tRNAs, and at least 13 peptides, which contribute to complex I, complex III, complex IV, and complex V of the electron transport system (11, 12). A regulatory region associated with the origin of replication also serves as a promoter region for two large mitochondrial RNA transcripts that are processed into individual RNAs for the structural RNAs and mRNAs (see Ref. 13). For example, the mRNAs encoding cytochrome *c* oxidase I, II, and III (CoxI, -II, and -III, respectively) are derived from a common precursor RNA (12).

We report here that the NF-κB subunits p50 and p65 along with IκBα, but not IκBβ, are found in the mitochondria as well as in the cytoplasm of proliferating cells. Quiescent liver cells exhibit largely p50 and IκBα in the mitochondria with little detectable p65. Electron microscopy and biochemical approaches confirm the localization of these proteins to mitochondria. Recently, others have found IκBα and p65 in the mitochondria of Jurkat T cells (14). Interestingly, our data indicate that mitochondrial IκBα is phosphorylated on N-terminal serines in response to cellular TNFα stimulation, followed by

** To whom correspondence should be addressed: Lineberger Comprehensive Cancer Center, CB #7295, University of North Carolina, Chapel Hill, NC 27599. Tel.: 919-966-3652; Fax: 919-966-0444; E-mail: jhall@med.unc.edu.

[1] The abbreviations used are: TNF, tumor necrosis factor; IKK, IκB kinase; CoxI, -II, and -III, cytochrome *c* oxidase I, II, and III, respectively; EMSA, electrophoretic mobility shift assay.

FIG. 1. **Visualization of p50/NF-κB1 and p65 in the mitochondria by electron microscopy.** Sections of U937 cells were stained with a p50 or p65 antibody and visualized with electron microscopy (see "Materials and Methods"). *Bar*, 0.25 μm. Pictures were taken at ×60,000 magnification.
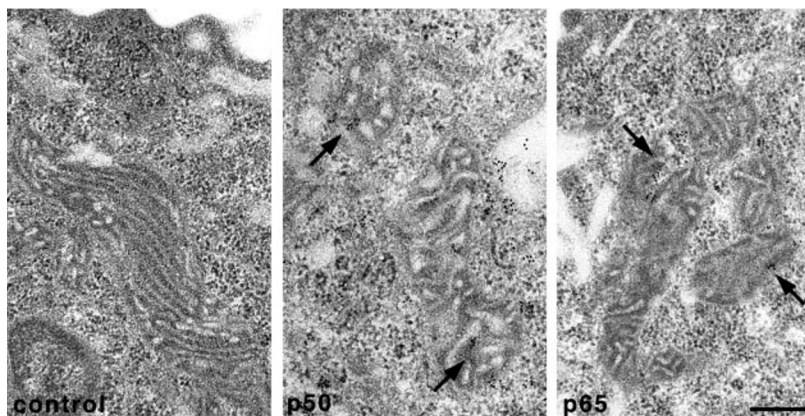
nonproteasomal degradation. TNFα treatment leads to a significant reduction in CoxIII and cytochrome *b* mRNA. Inhibition of NF-κB by mitochondrially localized superrepressor IκBα blocked the loss of both CoxIII and Cyt B mRNA. These data show that NF-κB is found in mitochondria, where it regulates mitochondrial specific gene expression.

MATERIALS AND METHODS

Cell Culture and Treatment with Cytokines and Protease Inhibitors—Cell lines were obtained from the Lineberger Comprehensive Cancer Center Tissue Culture Core Facility at the University of North Carolina (Chapel Hill, NC). U937 cells were grown in Dulbecco's modified Eagle's medium H supplemented with 10% fetal bovine serum and antibiotics. HT1080 V and HT1080 I lines were previously described (15). Cells were treated with 10 ng/ml human recombinant TNFα diluted in phosphate-buffered saline. Calpeptin, lactacystin, and MG132 (Calbiochem) were all resuspended in Me₂SO and used at a final concentration of 40 ng/ml.

Electron Microscopy and Immunocytochemical Labeling—U937 cells were suspended in serum-free medium and were mixed with a fixative containing 0.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. The cells were cooled to 10 °C on ice, followed by microwave irradiation using a 750-watt microwave oven until of a final temperature of 40 °C was obtained (16). The fixed cells were pelleted, rinsed in 0.1 M sodium cacodylate buffer, and postfixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 30 min. Following postfixation, the cell pellet was dehydrated through a graded series of ethanol washes and embedded in L. R. White resin (available from Ted Pella, Inc., Redding, CA). 70-nm ultrathin sections were taken of the embedded pellet, mounted on nickel grids, and stained using indirect immunocytochemical methods.

Immunocytochemical labeling, incubations, and washing steps were carried out at ambient temperature. The incubation and wash buffer for the primary antibody consisted of 0.05 M Tris-buffered saline with 0.1% fish skin gelatin and 0.01% Tween 20 (TBS/FGT) at pH 7.6. Grid-mounted sections were blocked with 0.2 M glycine in Hanks' balanced salts for 10 min, followed by a secondary blocking step with 5% goat serum in TBS/FGT for 10 min. The grids were incubated for 1 h with primary antibody (p50/NF-κB1; Upstate Biotechnology, Inc., Lake Placid, NY). After rinsing with TBS/FGT, the grids were incubated for 1 h in secondary antibody (goat anti-rabbit IgG 10 nm colloidal gold (BBI International, Ted Pella, Redding, CA), diluted 1:50 in 0.1 M TBS/FGT, pH 8.2. Negative controls were performed concurrently, consisting of incubation in normal rabbit IgG at the same repetitive dilutions and conditions. The immunogold-stained sections were post-stained with uranyl acetate followed by lead citrate, and the grids were observed and photographed using a LEO EM-910 transmission electron microscope (LEO Electron Microscopy, Inc., Thornwood, NY) at 80 kV.

Preparation of Mitochondria—Mitochondria were isolated from cell lines as described previously (17). Digitonin was added to the mitochondrial buffer A at 0.05% to aid in the disruption of the cells. Further purification was performed by ultracentrifugation at 30,000 rpm in gradient buffer (250 mM mannitol, 1 mM EGTA, 25 mM Hepes, 0.1% bovine serum albumin), pH 7.4, supplemented with 30% Percol (Sigma). The uppermost band was removed and washed two or three times with mitochondrial buffer A. Mitochondria that were analyzed by Western blot analysis were boiled in SDS sample buffer and loaded directly onto SDS-polyacrylamide gels. When electrophoretic mobility shift assays

(EMSAs) were performed, purified mitochondria were incubated in nuclear extract buffer for 10 min in ice. The sample was then centrifuged for 10 min at 12,000 rpm at 4 °C. The supernatants were used directly in the EMSA reaction (see below). Isolation of mitochondria from rat liver was as previously described (18).

Western Blot Analysis—For Western blotting analysis of cultured cells, equal amounts of protein were separated on a 12% SDS-polyacrylamide gel and transferred to nitrocellulose. Blots were blocked in 5% milk in 1× TBST (Tris-buffered saline, 0.5% Tween 20) and probed with either p65 (Rockland, Gilbertsville, PA), p50, IKKα, IKKβ, IKKγ (Upstate Biotechnology), IκBα (c-21), IκBβ, Skp-1 (Santa Cruz Biotechnology, Santa Cruz, CA), Lmp-2, (Affiniti Research Products, Exeter, UK), cytochrome *c* (PharMingen, San Jose, CA), or anti-phosphoserine-32 IκBα (New England Biolabs, Beverly, MA). Blots were probed with a secondary antibody conjugated to horseradish peroxidase (Promega Corp.) at a 1:15,000 dilution in 1× TBST. Protein bands were visualized with an enhanced chemiluminescence detection system (Amersham Life Sciences). Western blotting of rat liver mitochondria was performed as described (19).

EMSAs—EMSAs were performed as described previously (20). An oligonucleotide corresponding to an NF-κB site in the *H-2Kᵇ* gene was radiolabeled using [α-³²P]dCTP and the Klenow fragment of DNA polymerase I (Roche Molecular Biochemicals). For antibody supershift analysis, extracts were preincubated 15 min at room temperature with 1 μg of antiserum before the addition of the radiolabeled gel shift probe. Antibodies used in supershift analysis were identical to those utilized for Western blotting analysis.

IκB Kinase Assay—Cells were either treated or not treated with TNFα for specified times and harvested, and mitochondrial and cytoplasmic extracts were isolated in phosphate-buffered saline containing phosphatase inhibitors. Equal amounts of protein (500 μg) were immunoprecipitated using antibodies against the β subunit of IKK (a gift of Dr. F. Mercurio, Signal Pharmaceuticals, San Diego, CA). Kinase activity was determined by incubating the immunoprecipitates with 4 μg of glutathione *S*-transferase-IκBα (amino acids 1–54) wild type substrate or a mutated form of IκBα (S32T,S36T) in the presence of [γ-³²P]ATP, as described (21). The immunoprecipitates were subjected to SDS-PAGE, dried, and visualized by autoradiography.

Northern Blot Analysis—RNAs were isolated using Trizol as recommended by the manufacturer (Invitrogen). Northern blot analysis utilized 10–20 μg of total cellular RNA separated on 1.5% formaldehyde-agarose gels according to standard procedures. RNA samples were transferred overnight to nylon filter, UV cross-linked (Stratagene, La Jolla, CA), and probed with randomly labeled probe (Amersham Biosciences) corresponding to cytochrome *c* oxidase II or III or cytochrome B. Hybridization and wash conditions were obtained using Expresshyb (Stratagene) solution as described by the manufacturer. Blots were normalized for equal loading using a cDNA probe for glyceraldehyde-3-phosphate dehydrogenase.

RESULTS

Mitochondrial Localization of NF-κB—Studies were initiated in order to analyze the subcellular localization of NF-κB and IκB subunits. Electron microscopy of sections of U937 cells in association with immunocytochemistry indicated the presence of both the p50/NF-κB1 and p65 subunits localized in the inner matrix of the mitochondria as well as in the cytoplasm (see Fig. 1). The control (see "Materials and Methods") showed
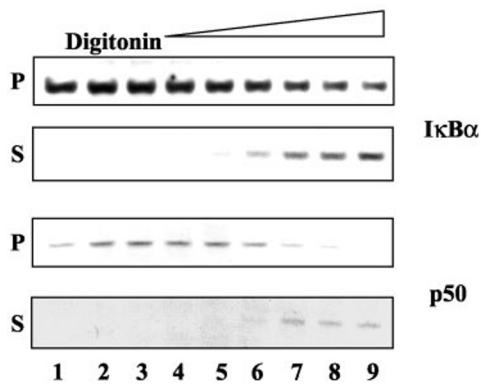
FIG. 2. **Localization of p50 and IκBα in the mitochondria of rat liver cells.** Mitochondria were isolated from rat liver and incubated with increasing amounts of digitonin. Pellet (*P*) and supernatant (*S*) from either untreated sample (*lane 1*) or samples treated with 0.1–0.8% digitonin (*lanes 2–9*) were separated on 10% SDS-PAGE and transferred to nitrocellulose. The membrane was probed with either an anti-p50 or anti-IκBα antibody.

no detectable labeling. Additionally, IκBα was detectable in the inner matrix of mitochondria.[2] After observing the presence of the NF-κB p50 and p65 subunits in the mitochondria using electron microscopy, we were interested in confirming the results biochemically. Mitochondria were first isolated from rat liver and subjected to increasing concentrations of digitonin, which functions to elute proteins in a manner dependent on the localization within the mitochondria. Supernatants and mitochondrial pellets at each concentration were analyzed by Western blotting using antibodies to IκBα, p50, or p65. The results show that both IκBα and p50 are present in the rat mitochondria (Fig. 2). With the addition of low concentrations of digitonin, IκBα protein was not detected in the supernatant. The addition of 0.4% digitonin (*lane 5*) leads to release of IκBα from mitochondria so that it is detected in the supernatant. However, the p50 protein was not visible in the supernatant until 0.5–0.6% digitonin was added (*lanes 6* and *7*). When these results are plotted against known mitochondrial components, release of p50 from the mitochondria following digitonin treatment corresponds to the release of fumarase,[2] a marker for the inner matrix of the mitochondria, whereas IκBα release from the mitochondria occurs earlier, indicating that IκBα localization is closer to the mitochondrial surface and presumably also in the inner matrix. p65 was not detected in the mitochondria of quiescent rat liver (data not shown, but see below regarding cells in culture).

TNFα Treatment of U937 Cells Causes a Loss of IκBα in the Mitochondria—To further analyze the localization of IκBα and NF-κB in the mitochondria and to determine potential responses to cytokine treatment, U937 cells were studied by Western blotting of mitochondrial and cytoplasmic extracts (Fig. 3). Cells were stimulated with TNFα over a 1-h time course. After separation of cytoplasmic and mitochondrial components, the mitochondrial pellet was resuspended in a volume of buffer equal to one-tenth volume of the cytosolic fraction, consistent with published estimates that mitochondria represent one-tenth the total volume of a human cultured cell (22). Protein assays confirmed that concentrations of the two fractions were approximately equivalent. Assuming that overall protein concentrations are similar in the mitochondria and cytoplasm of living cells, we loaded equal amounts of protein from each fraction in order to observe physiologically comparable levels of each subunit. First, the data support the EM data indicating that p50 and IκBα were localized in the mito-
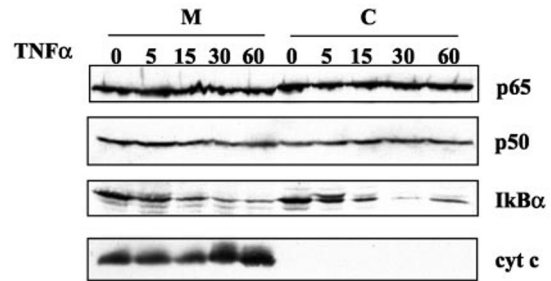

FIG. 3. **Changes in p50 and IκBα in the mitochondria of U937 cells upon TNFα stimulation.** U937 cells were treated with 10 ng/ml TNFα over a 1-h time course, and mitochondria (*M*) and cytoplasmic (*C*) fractions were prepared and analyzed by Western blot analysis. Proteins were fractioned on SDS-polyacrylamide gels, transferred to nitrocellulose, and incubated with antibodies specific for p65, p50, IκBα, and cytochrome *c*.

chondria. Second, the data indicate that the p65 subunit of NF-κB is located in the mitochondria of U937 cells. Additionally, the data indicate that the mitochondrial p65 exhibits slightly faster migration in SDS gels (*e.g.* compare the mobility of the mitochondrial p65 with cytoplasmic p65) (Fig. 3), possibly due to a protein processing event associated with mitochondrial import (see Ref. 12). The results also show that following TNFα treatment, there is degradation of IκBα by 30 min and resynthesis at 60 min in both the cytoplasm and mitochondria of U937 cells (Fig. 3). The overall kinetics of loss of mitochondrial IκBα is slightly different from what we observe in the cytoplasm. It is noted that a slower migrating form of mitochondrial IκBα is detected following TNFα treatment, suggestive of induced phosphorylation (see below). There appears to be very little change in p65 in either the mitochondria or cytoplasm of these cells following TNFα treatment; however, p50 shows a reduction in the mitochondria following TNFα treatment of cells. To confirm the absence of mitochondrial contamination of the cytoplasmic fractions, the Western blot was probed with a cytochrome *c* antibody. Other experiments show that there is little or no cytoplasmic contamination of mitochondria and that IκBβ is not found in the mitochondria (see Fig. 5, *B* and *C*). Additionally, c-Rel was not detected at appreciable levels in the mitochondria of U937 cells. Identical results were found when using the HT1080 cell line, showing that the localization is not cell type-specific.

TNFα Stimulation Results in Increased p50 DNA Binding Activity in the Mitochondria—To determine whether the NF-κB components in the mitochondria were able to bind DNA, mitochondrial extracts were prepared, and mitochondrial proteins were eluted in nuclear extract buffer. The results from an EMSA show two binding complexes (complexes I and II). The NF-κB DNA binding activity is low in the mitochondria in untreated cells and increases with TNFα treatment, with the strongest binding occurring at the 1-h time point (Fig. 4). To identify which components of NF-κB contribute to this binding activity, supershift analysis was performed on the mitochondrial extract that was treated with TNFα for 1 h. The use of a p50-specific antibody led to a supershifted band from complex II (Fig. 4). However, when a p65-specific antibody was added to the binding reaction, there was a reduction in the DNA binding in complex II without the appearance of a supershifted band. Complex I could not be supershifted with either antibody. These results indicate that p50 is the major NF-κB subunit found in binding complex II, possibly existing as a homodimer. It remains possible that additional NF-κB components may be part of the DNA binding activity. The data also suggest that the presence of p50 and p65 in the mitochondria is presumably not as a heterodimer, possibly suggesting distinct mitochondrial sublocalization for these two NF-κB subunits.
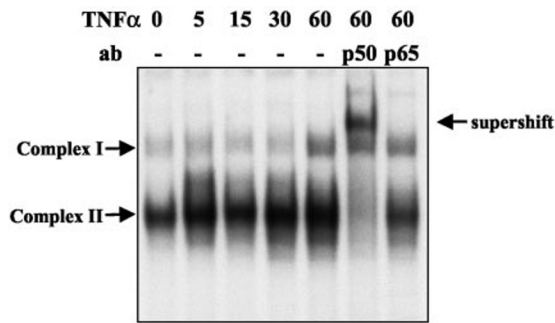
---

[2] P. Cogswell and A. Baldwin, unpublished results.

FIG. 4. **TNFα stimulation increases DNA binding activity of p50 in the mitochondria.** Mitochondria isolated from U937 cells that were treated with TNFα were incubated in nuclear extract buffer, and the resulting protein was analyzed by EMSA. Supershift analysis was performed on extract from the TNFα 1-h time point. Extracts were preincubated for 15 min with either a p65 or p50 antibody prior to the addition of the probe.

Phosphorylation of Mitochondrial IκBα in Response to TNFα Occurs on N-terminal Serines, and Degradation of IκBα in the Mitochondria following TNFα Stimulation Is Proteasome-independent—The fact that TNFα induced the degradation of IκBα in the mitochondria led us to explore whether IκBα is phosphorylated and degraded in a manner similar to that of cytoplasmic IκBα. First, we asked whether IκBα loss was dependent on a proteasome-mediated mechanism. Cells were pretreated with proteasome and calpain inhibitors and either left untreated or stimulated with TNFα. Mitochondria were purified, and proteins were eluted and analyzed by Western blotting. The results (Fig. 5A) show that upon treatment of cells with TNFα for 15 min, there is a significant loss of IκBα in both the mitochondrial and the cytoplasmic compartments. This loss in the cytoplasm can be blocked following pretreatment with either lactacystin, a proteasome-specific inhibitor, or with MG132, an inhibitor of both calpain and the proteasome (10) (Fig. 5A). When cells were pretreated with calpeptin, an inhibitor specific for calpain (10), there was a modest inhibition of the degradation of cytoplasmic IκBα but considerably less inhibition than with the other inhibitors. However, in the mitochondrial fraction, lactacystin had very little effect in blocking the degradation of IκBα. Additionally, cells pretreated with calpeptin showed more inhibition of mitochondrial IκBα degradation than lactacystin, unlike the cytoplasm, which showed the opposite effect. However, it is noted that calpeptin was able to inhibit the degradation of IκBα in the mitochondria only partially.

When an antibody specific for the phosphorylated form of IκBα (anti-phosphoserine 32) was used to probe the same blot, phosphorylated IκBα was visualized when cells were pretreated with all of the inhibitors in the cytoplasm but only in cells pretreated with calpeptin or MG132 in the mitochondria (Fig. 5A). Again, these results point to a lack of proteasome effect in the mitochondria. It is not clear whether the degradation of IκBα in the mitochondria is a calpain-specific pathway, since calpeptin does not completely block the degradation. This may be a function of the inhibitor, or there may be other factors involved in this process. Additionally, we cannot rule out that the lack of effect seen in the mitochondria when the cells are pretreated with lactacystin was due to the lack of permeability of the mitochondria to lactacystin.

To address whether the proteasome may not be relevant in mitochondrial IκBα degradation, cytoplasmic and mitochondrial extracts were prepared from U937 cells, and Western blotting was performed using antibodies specific for Lmp-2, a functional subunit of the proteasome (23) and Skp-1, a component of the ubiquitin ligase associated with IκBα ubiquitina-

tion (3). The results show that both proteins can only be found in the cytoplasm of U937 cells (Fig. 5B). Blots were also probed for cytochrome *c* as a mitochondrial marker. These results further support the possibility that the mitochondria lacks proteasome function. They also confirm that there is no cytoplasmic contamination of the mitochondria preparations.

NF-κB is regulated by multiple forms of IκB, including IκBα and IκBβ (1). Mitochondria blots from U937 cells were probed with IκBα and IκBβ antibodies (Fig. 5C). The results indicate that whereas IκBα is found in both the mitochondria and the cytoplasm, IκBβ is only found in the cytoplasm. Again, cytochrome *c* was used as a mitochondrial marker.

Evidence for Mitochondrial IKK Activity—In order to understand the mechanism of the induced phosphorylation of IκBα in the mitochondria in response to TNFα treatment, we explored whether IKK family members were present in or potentially associated with the mitochondria. Western blots of fractions from U937 cells enriched for mitochondria were probed with IKKα-, IKKβ-, and IKKγ-specific antibodies. The results show that all three IKK family members are present in the mitochondrial fraction (Fig. 6A). Blots were also probed with a cytochrome *c* antibody as a marker for mitochondria. Based on the phosphorylation on IκBα in the mitochondria and the presence of the IKK family members, we wanted to determine whether functional kinase activity could be detected in the mitochondria fraction. Large scale mitochondria and cytoplasmic extracts were prepared from U937 cells either left untreated or treated with TNFα. The results show that both in the cytoplasm and mitochondrial extracts, low levels of IKK kinase activity were seen in untreated cells (Fig. 6B). Following stimulation with TNFα, the IκB kinase activity in both the cytoplasm and mitochondria was greatly increased. These results indicate that the IKK family members are associated with the mitochondrial fraction and are potentially responsible for the phosphorylation of IκBα seen there.

NF-κB Negatively Regulates Expression of CoxIII and Cytochrome b mRNAs—In order to address a possible function of NF-κB components in the regulation of mitochondrial gene expression, we utilized HT1080 cells either containing an empty vector or expressing the modified form of IκBα known as IκBαSR (15). Interestingly, IκBαSR was shown to be in the mitochondria as well as the cytoplasm through its ability to be recognized by a FLAG tag antibody.[2] Importantly, the super-repressor form of IκBα was shown to be resistant to degradation in mitochondria of HT1080 cells in response to TNFα stimulation. HT1080 cells stably transfected with either a vector control or the IκBαSR were treated with TNFα, and RNA was isolated at several time points. The results show that HT1080 vector control cells exhibit a loss of CoxIII mRNA beginning at 1 h following TNFα treatment and continuing through 2 h (Fig. 7). The mRNA returns to near normal level following 4 h of TNFα stimulation. However, when HT1080 cells containing the IκBαSR were treated with TNFα, only a modest change in the CoxIII mRNA was observed. Since cytochrome *c* oxidase III plays an integral role in complex IV of the electron transport machinery (12), it is expected that the loss of CoxIII would impact changes in ATP and reactive oxygen species. Efforts to address such changes in response to TNF signaling have been hampered by significant differences in ATP and ROI levels between IκBα-expressing cells and vector control cells in the absence of TNF treatment (see "Discussion"). We have explored whether another mRNA, encoded in a region downstream of CoxIII, is also regulated differentially in the HT1080 V and HT1080 I cells. As with CoxIII, we find that CytB mRNA is down-regulated by TNFα in the HT1080 vector cells but not affected in the IκBα-expressing cells. Overall,
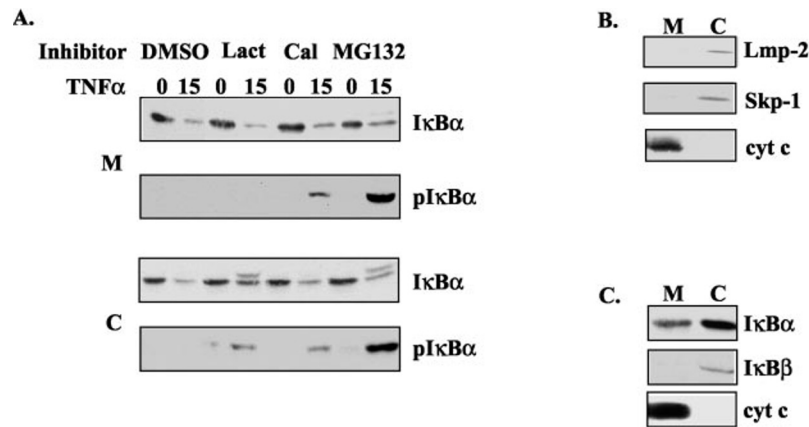
FIG. 5. **IκBα degradation upon TNFα stimulation is blocked by proteasome-specific inhibitors in the cytoplasm but not in the mitochondria.** *A*, U937 cells were pretreated with either Me₂SO (*DMSO*; *lanes 1* and *2*), lactacystin (*lanes 3* and *4*), calpeptin (*lanes 5* and *6*), or MG132 (*lanes 7* and *8*) for 15 min followed by stimulation with 10 ng/ml of TNFα for 15 min (*lanes 2*, *4*, *6*, and *8*). Mitochondrial (*M*) and cytoplasmic (*C*) proteins were isolated and analyzed on 12% SDS-polyacrylamide gels. Blots were first probed with a phospho-IκBα (*pIκBα*). Blots were stripped and reprobed with an IκBα antibody. *B*, proteins isolated from untreated U937 cells were analyzed on 12% SDS-polyacrylamide gels. Blots were probed with Lmp-1, Skp-1, and cytochrome *c* antibodies. *C*, mitochondrial (*M*) and cytoplasmic (*C*) extracts isolated from U937 cells were analyzed on 12% SDS-polyacrylamide gels. Blots were probed with either IκBα, IκBβ, or cytochrome *c* antibodies.

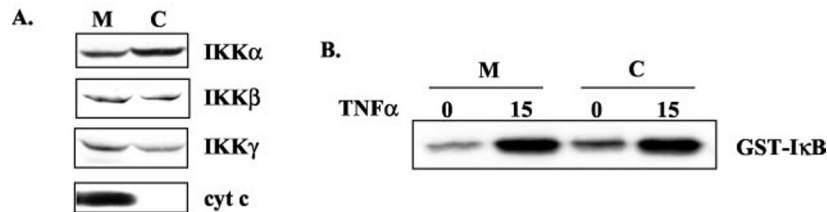FIG. 6. **IKKα, IKKβ, and IKKγ are all present and functional in the mitochondria.** *A*, mitochondria (*M*) and cytoplasmic (*C*) extracts isolated from U937 cells were run on 12% SDS-polyacrylamide gels and transferred to nitrocellulose. Blots were probed with either IKKα, IKKβ, IKKγ, or cytochrome *c*. *B*, U937 cells were either treated or not treated with TNFα, and mitochondrial and cytoplasmic extracts were prepared. 500 μg of protein was immunoprecipated with an anti-IKKβ antibody. Immunoprecipates were incubated with wild-type glutathione *S*-transferase-IκBα fusion protein in the presence of [γ-³²P]ATP. The immunoprecipitates were run on a 10% SDS-PAGE gel, dried, and exposed to film. The data represent three independent experiments.
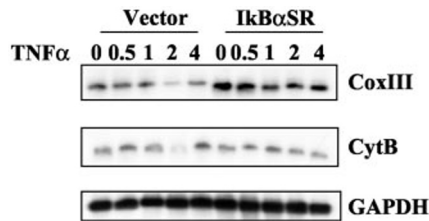

FIG. 7. **CoxIII mRNA is maintained following TNFα stimulation in the absence of NFκB.** HT1080 cells transfected with either a vector control or IκBα-SR were treated with 10 ng/ml TNFα for the specified times. RNA was isolated, and 10 μg was electrophoresed on 1.0% formaldehyde gels. RNAs were transferred to nylon membrane and probed with ³²P-labeled cDNAs corresponding to CoxIII and cytochrome *b*. The blots were stripped and reprobed with glyceraldehyde-3-phosphate dehydrogenase as a loading control.

these results indicate a role for NF-κB in the regulation of specific mitochondrial gene expression.

DISCUSSION

The results presented here demonstrate the localization of certain NF-κB subunits and IκBα to the mitochondria and are consistent with a recent publication showing that IκBα and p65 are found in the mitochondria (14). Interestingly, the major components of the NF-κB regulatory pathway are also apparently localized with the mitochondrial fraction, including the IκB kinase subunits IKKα and IKKβ as well as IKKγ. Consistent with this, TNFα treatment of U937 cells leads to the phosphorylation of degradation of the mitochondrially associated IκBα. In contrast with the degradation that occurs in the cyto-

plasm, the mitochondrial degradation of IκBα appears to be proteasome-independent, since a specific inhibitor of the proteasome (lactacystin) does not block degradation and because key components of the proteasome and ubiquitin ligase associated with IκBα ubiquitination are not found in the mitochondria. The use of inhibitors suggests that calpain may be partly involved with IκBα degradation in the mitochondria. The DNA binding assay indicates that an NF-κB complex containing p50, possibly as a homodimer, is released from IκBα and is capable of binding to DNA following TNFα stimulation. This response is correlated with the loss of CoxIII and CytB mRNAs following TNFα treatment. The involvement of NF-κB in this process is suggested by the use of the superrepressor form of IκBα, which inhibits the loss of CoxIII mRNA. The data raise many questions about the functions of NF-κB in the mitochondria.

Although great efforts were made to ensure the purity of the mitochondrial preparations, we cannot rule out the possibility of small amounts of contamination from other cellular organelles. We performed electron microscopy on our mitochondrial preparations and found that they were ~95% pure mitochondria. The other 5% of the preparation consisted of membranous debris, which may include Golgi, plasma membrane, and endoplasmic reticulum. Western blots were also performed on these preparations using various organelle markers, confirming that the mitochondrial preparations were highly purified. We did see some staining for endoplasmic reticulum in our mitochondrial fractions; however, the percentage of nonmitochondrial membrane in our preparations was low enough that we feel it did not contribute significantly to the protein content of the preparations. In addition, lack of endo-

Role of Mitochondrial NF-κB

plasmic reticulum staining of p65 or IκBα in the electron micrographs further supports the conclusion that NF-κB staining in our fractions is mitochondrial.

One obvious question is the potential mechanism whereby NF-κB regulates CoxIII and CytB mRNA levels. One can envision at least four mechanisms. One mechanism may be that NF-κB, possibly as a p50 homodimer, inhibits the processing of the RNA precursor at a level inhibiting CoxIII and CytB mRNA release. In this model, the release of mitochondrial p50 from IκBα may lead to an interaction with the precursor RNA and an inhibition of processing. Another mechanism could be that binding of NF-κB to mitochondrial DNA inhibits a transcriptional mechanism, possibly elongation, again somehow affecting CoxIII and CytB mRNA production. Another possibility is that p50 in association with IκBα serves as a positive mechanism in regulating CoxIII and CytB mRNA but that dissociation of the p50 and IκBα complex following TNF treatment leads to a loss of this control. Finally, we cannot rule out that a nuclear gene or genes regulated by NF-κB encode proteins that regulate specific mitochondrial mRNA accumulation. Obviously, more complex experimentation will be required to determine how NF-κB may regulate mitochondrial gene expression and whether other transcripts may be positively or negatively controlled by NF-κB. Along these lines, it is presently unclear whether p50 and IκBα are complexed in the mitochondria and whether the detection of p65 in the mitochondria is due to an interaction with p50 or IκBα. The recent paper from Bottero *et al.* (14) indicates that p65 and IκBα are associated in mitochondrial extracts. Western blotting did not reveal significant levels of other NF-κB subunits in the mitochondria of U937 cells (data not shown).

Another interesting question is how mitochondrial IκBα becomes phosphorylated and how mitochondrial or mitochondria-associated IKK becomes activated in response to TNF signaling (Fig. 6*B*). For cytoplasmic IKK, one current model is that IKK is recruited to factors associated with cytokine receptors, such as the TNF receptor (24), and becomes activated to phosphorylate IκBα or IκBβ. Presumably, rapid activation of mitochondrially associated IKK must occur by a distinct mechanism that is somehow dependent on TNF binding to its receptor on the membrane. The nature of the signal linking cell surface TNF binding to its receptor and the activation of the mitochondrial or mitochondria-associated IKK is unclear. We cannot rule out the possibility that the mitochondrial IκBα becomes phosphorylated by a kinase distinct from IKK or becomes phosphorylated in the cytoplasm and is transported into the mitochondria.

The localization of NF-κB to the mitochondria raises interesting questions concerning a potential role in regulating apoptosis. NF-κB activation in response to TNF signaling has been shown to inhibit apoptosis. Thus, it has been shown that NF-κB activates several gene products (namely Bcl-xL, A1/Bfl-2, IAP proteins, TRAF proteins, etc.) to inhibit the caspase cascade and to block cytochrome *c* release from mitochondria (4, 6). It is interesting to speculate that mitochondrial NF-κB may play a role in the suppression of apoptosis. As described above, it is predicted that modulation of CoxIII mRNA may ultimately impact ATP production, which is required for apoptosis induced by TNF. Along these lines, Bottero *et al.* (14) provide evidence that mitochondrial IκBα interacts with ANT, the adenine nucleotide transporter, which has been speculated to be involved with apoptosis through its ability to regulate the mitochondrial permeability transition (see Ref. 14). Additionally, we have observed that expression of superrepressor IκBα in cells alters basal levels of ATP (data not shown). Future studies will address mechanisms whereby mitochondrial NF-κB may control cell death mechanisms.

NF-κB is not the first nuclear encoded transcription factor to be localized to the mitochondria. Interestingly, the glucocorticoid receptor has been found in the mitochondria (25). Potentially important are observations that glucocorticoid receptor can physically interact with NF-κB subunits (26); thus, NF-κB/glucocorticoid receptor interactions in the mitochondria may function to regulate key processes involved in cell growth and apoptsosis. Experiments addressing these issues may reveal novel regulatory mechanisms associated with mitochondrial function.

REFERENCES

1. Ghosh, S., May, M., and Kopp, E. (1998) *Annu. Rev. Immunol.* **16,** 225–260
2. Zandi, E., and Karin, M. (1999) *Mol. Cell. Biol.* **19,** 4547–4551
3. Maniatis, T. (1999) *Genes Dev.* **13,** 505–510
4. Baldwin, A. (2001) *J. Clin. Inv.* **107,** 3–6
5. Guttridge, D., Mayo, M., Madrid, L., Wang, C.-Y., and Baldwin, A. (2000) *Science* **289,** 2363–2366
6. Baldwin, A. (2001) *J. Clin. Inv.* **107,** 241–246
7. Carlotti, F., Dower, S., and Qwarnstrom, E. (2000) *J. Biol. Chem.* **275,** 41028–41034
8. Huang, T., Kudo, N., Yoshida, M., and Miyamoto, S. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 1014–1019
9. Tam, W., Lee, L. Davis, L., and Sen, R. (2000) *Mol. Cell. Biol.* **20,** 2269–2284
10. Yan, Y., Weinman, S. Boldogh, I. Walker, R., and Brasier, A. (1999) *J. Biol. Chem.* **274,** 787–794
11. Anderson, S., Bankier, A., Barrell, B., de Bruijn, M., Coulson, A., Drouin, J., Eperon, I., Nierlich, D., Roe, B., Sanger, F., Schreier, P., Smith, A., Staden, R., and Young, I. (1981) *Nature* **290,** 457–465
12. Scheffler, I. (1999) in *Mitochondria*, pp. 367–384, Wiley-Liss, Inc., New York
13. Puranami, R., and Attardi, G. (2001) *Mol. Cell. Biol.* **21,** 548–561
14. Bottero, V., Rossi, F., Samson, M., Mari, M., Hofman, P., and Peyron, J.-F. (2001) *J. Biol. Chem.* **276,** 21317–21324
15. Wang, C.-Y., Mayo, M., and Baldwin, A. (1996) *Science* **274,** 784–787
16. Madden, V. J. (1998) *Microsc. Microanal.* **4,** Suppl. 2, 854–855
17. Yang, J., Liu, X. Bhalla. K. Kim, C. Ibrado, A. Cai, J., Peng, T.-I., Jones, D., and Wang, X. (1997) *Science* **275,** 1129–1132
18. Mancini, M., Nicholson, D., Roy, S., Thornberry, N., Peterson, E., Casciola-Rosen, L., and Rosen, A. (1998) *J. Cell Biol.* **140,** 1485–1495
19. Gervais, F., Thornberry, N., Ruffolo, S., Nicholson, D., and Roy, S. (1998) *J. Biol. Chem.* **273,** 17102–17108
20. Mayo, M., Wang, C.-Y., Cogswell, P., Rogers-Graham, K., Lowe, S., Der, C., and Baldwin, A. (1997) *Science* **278,** 1812–1815
21. Keifer, J. A., Guttridge, D., Ashburner, B., and Baldwin, A. (2001) *J. Biol. Chem.* **276,** 22382–22387
22. Posakony, J. W., England, J. M., and Attardi, G. (1977) *J. Cell Biol.* **74,** 468–491
23. Kingsbury, D. J., Griffin T., and Colbert, R. A. (2000) *J. Biol. Chem.* **275,** 24156–24162
24. Devin, A., Lin, Y., Yamaoka, S., Li, Z., Karin, M., and Liu, Z. (2000) *Mol. Cell. Biol.* **21,** 3986–3994
25. Scheller, K., Sekeris, C., Krohne, G. Hock, R., Hansen, I., and Scherer, U. (2000) *Eur. J. Cell Biol.* **79,** 299–307
26. Tao, Y., Williams-Skipp, C., and Scheinman, R. (2001) *J. Biol. Chem.* **276,** 2329–2332