Recruitment of NF-κB into Mitochondria Is Involved in Adenine Nucleotide Translocase 1 (ANT1)-induced Apoptosis*

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Overexpression of adenine nucleotide translocase-1 (ANT1) is known to induce apoptosis (Bauer, M. K., Schubert, A., Rocks, O., and Grimm, S. (1999) J. Cell Biol. 147, 1493–1501), but the mechanisms involved remain unclear. In this study we show that ANT1 overexpression results in a recruitment of the IκBα-NF-κB complex into mitochondria, with a coincident decrease in nuclear NF-κB DNA binding activity. In this situation, NF-κB transcriptionally regulated genes with antiapoptotic activity, such as Bcl-2, MnSOD, and c-IAP2, are down-regulated, and consequently, cells are sensitized to apoptosis. Accordingly, co-expression of p65 partially interferes with the proapoptotic effect of ANT1 overexpression. Despite the high identity of the two isoforms, overexpression of ANT2 does not exert an apoptotic effect; this lack of apoptotic activity is correlated with the absence of mitochondrial IκBα-NF-κB recruitment or changes in NF-κB activity. Thus, we propose that the mitochondrial recruitment of NF-κB observed following ANT1 overexpression has an important role in ANT1 proapoptotic activity.

Apoptosis is a form of cell death that plays a role in development, tissue homeostasis, and disease. The induction of apoptosis is governed by an elaborate array of checks and balances in the cell. Studies of apoptosis induction in “in vitro” systems have demonstrated that mitochondria are required for apoptosis stimulated by a variety of different factors. The ANT1 protein is localized in the inner mitochondrial membrane and exchanges cytosolic ADP for mitochondrial ATP. Three isoforms (ANT1, ANT2, and ANT3) with tissue-specific expression patterns have been described in humans. ANT interacts with several proteins of the outer mitochondrial membrane (peripheral benzodiazepine receptor, porin/VDAC, and Bax) as well as the matrix (cyclophilin D) to form the permeability transition pore (PTP). The PTP appears to be an important regulator of the apoptotic process. Opening of the pore leads to a loss of mitochondrial transmembrane potential, ΔΨm, which 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 (6, 7). Proteins of the bcl-2 family essentially control the release of cytochrome c. Antiapoptotic members of the family (Bcl-2 and Bcl-XL) prevent cytochrome release, whereas the proapoptotic members Bax and Bak exert the opposite effect. Bax has been shown to interact with ANT to induce PTP opening and cytochrome c release (9). Several pharmacological compounds interfere with PTP. For instance, cyclosporin A, through its binding to cyclophilin D, prevents PTP opening, and bongkrekic acid and atracyloside are, respectively, a blocker and an inducer of apoptosis via binding of two different conformational states of ANT (10). In addition, alongside their modulation of pore formation by ANT, Bcl-2 and Bax also have been reported to influence ANT ADP/ATP antiporter activity (11). Although ANT is a pore component, recently it has been shown, using mitochondria lacking ANT, that this protein is not essential for MPTP activity (12).

In addition, it has been demonstrated that overexpression of ANT1 (but not ANT2) can induce apoptosis (13); these results demonstrate the specificity of ANT in this pathway. The lack of apoptosis induction by ANT2 contrasts with the high 80% amino acid sequence identity of ANT2 with ANT1 (14). Moreover, the apoptotic activity of ANT1 appears not to depend upon its known role in ADP/ATP exchange because cell death still occurs in several transport-inactive mutants. A critical region of ANT1 (amino acids 102–141) required for apoptosis has been identified (13), and intriguingly, this region overlaps with the Bax binding site of ANT (9) and contains a Vpr-binding peptide motif (15). Moreover, these sites also overlap the region of ANT in which the three isoforms exhibit the highest degree of divergence, suggesting an isoform-specific regulation of ANT. Interestingly, hearts of ANT1-deficient mice exhibit a striking hypertrophic cardiomyopathy (16) that could be related to inhibition of apoptosis because of the lack of ANT1 expression in these animals.

Nuclear factor κB (NF-κB) is a dimeric transcription factor involved in the expression of proteins necessary for innate immunity (17), apoptosis, and cell proliferation (18). NF-κB typically forms a heterodimer with the p50 and p65 (RelA) subunits and is mainly regulated by intracellular compartmentalization. The inactive form of NF-κB is retained in the cytoplasm upon association with the inhibitory IκBα protein (19). Exposure of cells to a variety of stimuli, including tumor necrosis factor-α (TNFα), induces phosphorylation of IκBα, which allows subsequent ubiquitination and degradation of the inhibitor, thus leading to nuclear entry and DNA binding of NF-κB...
Mitochondrial NF-κB in ANT1-induced Apoptosis

The aim of this study is to determine the molecular mechanisms involved in the induction of apoptosis by ANT1 overexpression in transiently transfected cells. We report here that the overexpression of ANT1 (but not ANT2) potentiates the recruitment of p65(NF-κB) into the mitochondria of overexpressing cells. Subsequently, nuclear NF-κB activity diminishes the transcription of antiapoptotic genes such as Bcl-XL and the caspase inhibitors c-IAP2 and c-IAP1, is associated with nuclear NF-κB activity (23). The scavenger enzyme superoxide dismutase MnSOD2 is also regulated by nuclear NF-κB and participates in the redox regulation that is essential for protecting cells from apoptosis (24). Inhibition of NF-κB activity by E2F1 and c-Myc has been associated with the suppression of MnSOD2 expression; this mechanism is responsible for apoptosis induction (25). Recently, more complex aspects of NF-κB regulation have been proposed. For example, studies indicate that NF-κB shuttling into and out of the nucleus could play an important role in apoptosis induction (26).

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EXPERIMENTAL PROCEDURES

Biological Reagents and Antibodies—Antibodies against p65(NF-κB) (C-20) and against ANT (Q-18) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anticaspase-9 antibody (Asp-315) was from Cell Signaling, anti-p65(NF-κB) (p50) from Sigma. Human TNFα (Sigma) was cultured in Dulbecco’s modified Eagle’s medium containing 100 units/ml penicillin/streptomycin and supplemented with 10% fetal bovine serum (Invitrogen). The cells were incubated under an atmosphere of 95% air and 5% CO2 at 37 °C. A co-transfected GFP expression plasmid was used in a 1:10 ratio to the vectors used. Apoptotic cells were detected by flow cytometry after staining with fluorescein isothiocyanate-conjugated annexin V and propidium iodide (PI) using a commercially available kit (Annexin V-FLUOS kit, Roche Applied Science). Cells were considered apoptotic when they were annexin V-positive and PI-negative. Staining of cells by PI was an indicator of the loss of plasma membrane integrity. Flow cytometry was performed using an EPICS-XL-MCL (Beckman Coulter, Inc., Fullerton, CA)cytometer.

Mitochondrial Membrane Potential—The mitochondrial membrane potential (ΔΨm) was measured by flow cytometry using hexamethylene bisacetoxymethine (Molecular Probes). Transiently transfected cells were loaded with 250 ng/ml DiIC1, 5 (50) in 20 min in Dulbecco’s modified Eagle’s medium without fetal bovine serum. At the end of the incubation the cells were washed twice in PBS and resuspended in a total volume of 0.5 ml of PBS, and the ΔΨm was analyzed by flow cytometry in a Coulter EPICS-XL-MCL (Beckman Coulter, Inc.).

Nuclear Extract Preparation—Extracts were prepared using a modified method from Digman et al. (27). For the isolation of nuclear protein extracts HeLa cells were washed with PBS, scrapped off the plates in PBS, and briefly centrifuged (16,000 × g, 4 °C, 30 s). Cells were washed again with ice-cold PBS, pelleted, and resuspended at 4 °C in 400 μl of buffer A (10 mM Hepes, pH 7.4, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM PMSF, and protease inhibitors (5 μg each of aprotinin, leupeptin, and pepstatin/ml)). Cells were allowed to swell on ice for 15 min; then 25 μl of Nonidet P-40 (0.5%) was added, and the suspension was thoroughly mixed for 10 s. The homogenate was centrifuged (16,000 × g, 4 °C, 60 s), and the nuclear pellet was resuspended in 50 μl of ice-cold buffer A. A 10 μl aliquot was transferred to a fresh tube and 2 μl of 10 mM proteinase K (Sigma) was added. Nuclear lysates were maintained on ice for 15 min with occasional mixing. The nuclear extract was cleared (16,000 × g, 4 °C, 5 min), and the supernatant containing the proteins from the nuclear extract was transferred to a fresh tube, and the protein concentrations were determined by the Bradford assay (Bio-Rad).

Electrophoretic Mobility Shift Assays—HeLa cells transiently transfected with ANT1pcDNA3, ANT2pcDNA3, or empty pcDNA3 vector were used at various time points following transfection. In separate

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Proteinase K Treatment—Mitochondrial extracts were incubated in homogenization buffer without protease inhibitors in the presence of 15 ng/ml proteinase K (Sigma) for 10 min on ice. Protease inhibitors were added to stop the reaction, and proteins were analyzed by Western blotting as described below.

Immunoblotting—Homogenates and cytosolic and mitochondrial extracts of transiently transfected cells were prepared as described above. The samples were separated by SDS-PAGE on 12.5% acrylamide gels and transferred to a polyvinylidene difluoride membrane (Millipore). The membrane was blocked with 5% skimmed milk over night and then incubated with various primary antibodies (ANT (1:100), p65 (1:4000), β-actin (1:10,000), and VDAC (1:1000)). For caspase-9 analysis, cytosolic extracts were separated by SDS-PAGE on 12.5% acrylamide gels, transferred to polyvinylidene difluoride membranes, and incubated with a polyclonal antibody against cleaved caspase-9 (1:1000). The binding of antibodies was detected with a horseradish peroxidase-conjugated anti-mouse (Bio-Rad 170-6516 (1:3000)), anti-rabbit (Santa Cruz sc-2004 (1:3000)), or anti-goat (Santa Cruz sc-2354 (1:3000)) secondary antibody using an enhanced chemiluminescence (ECL) detection kit (Amersham Biosciences).

Assessment of Apoptosis by Flow Cytometry—The viability of transiently transfected cells was analyzed at different times after transfection of the vectors used. Apoptotic cells were detected by flow cytometry after staining with fluorescein isothiocyanate-conjugated annexin V and propidium iodide (PI) using a commercially available kit (Annexin V-FLUOS kit, Roche Applied Science). Cells were considered apoptotic when they were annexin V-positive and PI-negative. Staining of cells by PI was an indicator of the loss of plasma membrane integrity. Flow cytometry was performed using an EPICS-XL-MCL (Beckman Coulter, Inc., Fullerton, CA) cytometer.

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experiments HeLa cells were stimulated with 20 ng/ml TNF-α for 60 min before the cells were collected. For gel retardation assays, a double-stranded oligonucleotide containing a consensus NF-κB DNA binding site (5′-TCT AGA GTT GAG GGG ACT TTC CCA G-3′, obtained from Roche Applied Science) was end-labeled using [α-32P]dCTP and Klenow enzyme. Nuclear protein extracts (10 μg) were incubated for 10 min on ice with binding buffer (25 mM Hepes, pH 7.6, 0.5 mM dithiothreitol, 12.5 mM NaSO4, 50 mM KCl, 1 mg/ml bovine serum albumin, 5% glycerol, 0.1% Nonidet P-40, and 2.5 μg of poly(dI-dC) (deoxyinosinic-deoxyric acid)). The DNA probe (30,000 cpm) was added and incubated for 20 min at room temperature in a final volume of 25 μl. Samples were run on 5% non-denaturing polyacrylamide gels in 0.5× Tris-borate-EDTA at 350 V and 4 °C for 60 min. Supershift experiments were performed by adding anti-p65 antibody (Santa Cruz Biotechnology) to the sample after the initial incubation and keeping the complex on ice for 10 min, followed by addition of radiolabeled oligonucleotide and incubation for 20 min at room temperature. In the competition experiments, 100-fold molar excess of unlabeled double-stranded oligonucleotides was included.

RT-PCR—Total RNA was isolated from transiently transfected HeLa cells using the Qiagen RNA isolation kit. 5 μg of RNA was reverse-transcribed into cDNA with Moloney murine leukemia virus reverse transcriptase (Invitrogen). A total of 5 μl of cDNA was amplified in a 50-μl reaction mix using TaqDNA-polymerase (Roche Applied Science), and specific primer pairs were used. The primers used were the following: Bel-xL (5′-CAT GCC CCA TCC TTA CAT TCA AA), e-LAP2 (567-bp product) (5′-primer, 5′-GTC TAG TAT TAG CCA TGG TCA CACC C; 3′-primer, 5′-CGT CAG CCC CCC CAC AAG GAT CCT), PCR reactions were performed after 5 min of denaturation at 94 °C, repeating the cycle 1 min at 96 °C, 1 min at 55 °C, and 1 min at 72 °C for 30 cycles. PCR products (10 μl) were analyzed in an ethidium bromide-stained 1.2% agarose gel and quantified by densitometry. Cyclophilin A was used as an internal standard, and the results were corrected to this value.

RESULTS

In an effort to understand more fully the mechanism of ANT apoptosis induction in mammalian cells, we transiently transfected cells with individual ANT1- and ANT2-encoding plasmids. First of all, we characterized the apoptotic model induced specifically by ANT1 overexpression.

Comparison of the effects of overexpression of ANT1 and ANT2 in HeLa cells revealed an increase in the percent of detached and floating cells in the culture medium 24 h after transfection with an ANT1 expression construct (Fig. 1A). Overall transfection efficiency, measured by co-transfection with GFP, was about 70–80% in all transfection assays analyzed, and Western analysis, using a non-isoform-specific antibody that detects both ANT isoforms, demonstrated ANT increases in homogenate and also in mitochondria of HeLa transfected cells, indicating a correct localization of overexpressed protein in the mitochondrial membrane (Fig. 1B).

To characterize the floating and attached cells we analyzed apoptotic parameters such as procaspase-9 cleavage and annexin V-FITC staining at different time points following ANT1 or ANT2 transfection. Analysis was performed in total ANT1-transfected cells or in floating cells separated from attached cells. Lysates of both populations were prepared separately as described under “Experimental Procedures.” The number of floating cells in the medium after ANT2 or vector transfection was low, and it was not possible to collect sufficient amounts to process for subsequent analysis.

A significant increase in cleaved procaspase-9 was observed in total ANT1-transfected cells and also in floating and attached cells obtained after ANT1 overexpression, whereas the cleavage was negligible after ANT2 or empty vector transfection in HeLa cells (Fig. 1C). These data demonstrate that caspase-9 is involved in apoptosis induced by ANT1.

Fig. 2 shows changes in annexin V-FITC staining in transiently ANT1- or ANT2-transfected cells after 4 or 24 h of protein expression. Cells were considered apoptotic when they were annexin V-positive and PI-negative. As shown in Fig. 2A, the percentage of annexin V-FITC staining was similar in ANT2-transfected cells when compared with vector-transfected cells at 4 or 24 h of expression. In contrast, ANT1 overexpression resulted in increased numbers of annexin-positive cells at 4 h as well as at 24 h of overexpression. In parallel we analyzed separately floating and attached cells obtained after 4 and 24 h of ANT1 overexpression. The results indicate that almost all floating cells were annexin-positive at 4 h as well as at 24 h of expression. 24% of attached cells were annexin V-positive at 4 h, and this value decreased to 13.5% at 24 h of overexpression. The number of attached ANT1-transfected cells with annexin labeling observed at 24 h is very similar to values obtained after ANT2 or empty vector transfection. Furthermore, to assess whether caspase activation is required for induction of apoptosis by ANT1, ANT1- and vector-transfected cells were treated with the general caspase inhibitor Z-VAD-Fmk. As shown in Fig. 2B the presence of the caspase inhibitor significantly decreased the number of annexin-positive or apoptotic cells.
Flow cytometric determinations of DilC1(5) were used to analyze changes in mitochondrial membrane potential ($\Delta \Psi_{m}$) in ANT1- and ANT2-transfected cells. Untransfected control cells treated with the chemical uncoupler CCCP (carbonyl cyanide $m$-chlorophenylhydrazone) were used to quantify the maximum decrease of $\Delta \Psi_{m}$ in HeLa control cells. We co-transfected ANT1 or ANT2 together with an expression vector for GFP into HeLa cells and analyzed changes in the number of cells with low $\Delta \Psi_{m}$ after 4 hours of co-expression. Fig. 3A shows that ANT1-transfected, GFP-positive cells had an increased number of cells with a decreased $\Delta \Psi_{m}$ compared with vector-transfected, GFP-positive cells or ANT2 GFP-positive cells (Fig. 3B). Interestingly, the increase in the number of cells with a decreased $\Delta \Psi_{m}$ was observed in attached ANT1 cells, as well as in ANT1 floating cells. The increase in the number of cells with annexin V labeling and a low $\Delta \Psi_{m}$, especially in attached ANT1-transfected cells after 4 h of transfection, suggests that this increase is an early and isoform-specific effect of ANT1 overexpression.

Recently it has been described that ANT binds to the IκBα-NF-κB complex in mitochondria (21). We hypothesized that as a result of ANT overexpression an increase of mitochondrial IκBα-NF-κB complex could be induced. We studied the mitochondrial localization of p65(NF-κB) in ANT1 or ANT2 transiently transfected cells (Fig. 4). Mitochondria were first isolated from cells and subjected to a proteinase K treatment, whose function was to hydrolyze proteins, hence allowing only NF-κB protected by mitochondrial membranes to be detected. Western blot analysis of ANT revealed an increase in expressed protein in homogenates and also in mitochondria from floating and attached HeLa cells. Expression of VDAC and $\beta$-actin was examined as a control for protein loading. Fig. 4A shows changes in p65(NF-κB) protein in homogenate, cytosol, and mitochondrial fractions from floating and attached ANT1- and empty vector-transfected cells after 24 h. Results indicated no changes in the amount of p65(NF-κB) in homogenates. Interestingly, however, a decrease in p65(NF-κB) was observed in the cytosol alongside an increase in the mitochondria from floating cells. Attached cells obtained after 24 h of overexpression did not show changes either in mitochondrial p65(NF-κB) or in cytosolic p65(NF-κB) levels despite ANT 1 overexpression in mitochondria. These results, indicating mitochondrial NF-κB recruitment, are in agreement with the annexin labeling obtained after 24 h of overexpression in that the results are high in floating cells and low in attached cells. In experiments using ANT2 or empty vector transfection no floating cells were obtained (Fig. 1B). As shown in Fig. 4B, ANT2 transient transfection did not grossly alter the p65(NF-κB) levels in mitochon-
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Fig. 3. Mitochondrial membrane potential (ΔΨm) decreases specifically in ANT1-overexpressing cells. Flow cytometric determinations of DilC₁(5) were used to determine changes in cells transiently co-transfected with the indicated plasmid and GFP after 4 h of overexpression. The percentage of GFP-positive cells with low ΔΨm levels is indicated. A, untransfected control cells treated or not with 5 μM chemical uncoupler CCCP and empty vector- or ANT1-transfected cells. T, total cells; A, attached cells; F, floating cells. B, ANT2- and empty vector-transfected cells.

dria, homogenate, or cytosol of HeLa transfected cells. The increase of NF-κB localization in mitochondria from HeLa ANT1-transfected cells thus seems to be specific to ANT1 overexpression and could lead to a decrease in nuclear NF-κB activity.

To verify the NF-κB nuclear binding activity following transient transfection of ANT1 or ANT2, we analyzed nuclear extracts obtained from HeLa cells 24 h after transfection by EMSA (electrophoretic mobility shift assay). Two bands corresponding to NF-κB DNA binding were observed in nuclear extracts from cells transfected with empty vector (Fig. 5A).

Specificity of the two DNA binding complexes was assessed in competition experiments by adding an excess of unlabeled NF-κB oligonucleotide. The use of a p65-specific antibody led to a reduction in DNA binding without the appearance of a supershifted band. Nuclear extracts from ANT1- and ANT2-overexpressing cells revealed differences in NF-κB DNA binding activity; these differences were an important decrease in ANT1- and no changes in ANT2-transfected cells (Fig. 5A).

These data support a recruitment of mitochondrial NF-κB because of increased ANT1 protein in transient transfection assays, which then affects nuclear NF-κB activity. Moreover,
NF-κB antiapoptotic phenotype. Attached cells also showed a decrease in floating cells according to their previously described apo-
tivation of NF-κB has been described in cells expressing ANT in vitro (13). Furthermore, our results demonstrate that only the ANT1 isoform is capable of increasing the nuclear availability of NF-κB, specifically by ANT1 and not by ANT2 overexpression, plays an important role in this apoptosis pathway. Thus, the diminished nuclear availability of NF-κB observed in ANT1-overexpressing cells results in a down-regulation of antiapoptotic genes, causing cells to become sensitized to induction of apoptosis. Importantly, the overexpression of NF-κB reduces the ANT1 apoptotic effect.

Interestingly, after ANT1 transfection cells become detached from the plate, and the number of floating cells increases. Floating cells have typical features of apoptotic cells, such as annexin labeling and cleavage of procaspase-9. Thus, the increase in floating cells could be considered an apoptotic index, as it occurs in response to other apoptotic stimuli (28). When we analyzed attached cells, apoptotic parameters were observed in those that overexpressed ANT1 at 4 h, almost disappearing later by 24 h.

To investigate the molecular mechanism(s) by which ANT1 induces apoptosis, changes in cell number with low ΔΨm after ANT1 or ANT2 transfection were studied. Results demonstrated that only the ANT1 isoform is capable of increasing the number of cells with a low potential. This effect is observed within 4 h of ANT1 overexpression. Thus, the early increase in annexin labeling and in cell number with low ΔΨm observed in attached cells suggests that these cells acquired the phenotype of apoptotic cells early and then were progressively detached from the plate, becoming apoptotic, floating cells.

ANT is a protein that belongs to the mitochondrial mem-

**DISCUSSION**

The results presented here demonstrate that the ANT1 isoform specifically induces programmed cell death when overexpressed in HeLa cells. These results are essentially in agreement with those of Bauer et al. (13). Furthermore, our results demonstrate that the recruitment of NF-κB into mitochondria, specifically by ANT1 and not by ANT2 overexpression, plays an important role in this apoptosis pathway. Thus, the diminished nuclear availability of NF-κB observed in ANT1-overexpressing cells results in a down-regulation of antiapoptotic genes, causing cells to become sensitized to induction of apoptosis. Importantly, the overexpression of NF-κB reduces the ANT1 apoptotic effect.

![Fig. 4. p65(NF-κB) is increased in mitochondria from ANT1-overexpressing HeLa cells.](image)
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Intriguingly, the ANT2 isoform, which is over 90% identical to ANT1, was inactive for apoptosis and for changing mitochondrial membrane potential \( \Delta \Psi_m \) despite the fact that transfected cells overexpressed ANT2 and the protein was localized in mitochondria. Together these observations suggest functional differences between ANT isoforms. Moreover, it has been suggested that the ANT2 isoform, unlike ANT1, could import glycolytic ATP into mitochondria, and a differential regulation of ANT2 gene expression has also recently been demonstrated (30). However, it has also been reported that there are no important differences in the transport activity or kinetic parameters of ANT isoforms (31). In addition, deletion studies of ANT1 demonstrated that apoptosis induction by ANT1 does not depend upon its function as a mitochondrial carrier (13).

Some evidence has been reported indicating different affinities between certain proteins and ANT1 or ANT2 isoforms. For instance, ARL2 and its binding partner (BART) were found to be predominantly associated with ANT1 in mitochondria, and the structurally homologous ANT2 does not bind this complex (32). On the other hand, using specific antibodies it was found that ANT1 might have a higher affinity for cyclophilin D, an MPTP component, which suggests a greater involvement of ANT1 than ANT2 in MPTP activity (33). These observations predict the isoform specificity of ANT-protein interactions and could explain the different activity of specific isoforms in apoptosis induction.

It has been reported that the apoptotic effect of ANT1 is dependent upon MPTP activity because it is inhibited by cyclophilin D co-transfection or by treatment with bongkrekic acid, a known MPTP inhibitor (13). However, overexpression of ANT1 did not produce cell death in yeast, indicating that it is not ANT1 per se that has the ability to directly disturb the cell (13). Thus, the interaction between ANT1 and another protein must be responsible for inducing apoptosis. This putative protein probably inhibits MPTP; thus, ANT1 overexpression would be predicted to displace this protein, leading to MPTP opening. Recently, cyclophilin D has been described as being able to inhibit apoptosis (34). Thus, we can speculate that when ANT1 is overexpressed, cyclophilin D would be displaced from MPTP resulting in MPTP opening.

Recently an intramitochondrial pool of IκBα-NF-κB has been demonstrated to be associated with ANT. The interaction was originally detected in a yeast two-hybrid system and confirmed in an in vitro system, although differences in the affinities of ANT isoforms to the complex were not analyzed (21). However, little is known about the function of the mitochondrial NF-κB pathway.

We hypothesized that the mitochondrial pool of IκBα-NF-κB would increase in response to ANT overexpression. Interestingly, our results demonstrate an increase in the reservoir of mitochondrial p65(NF-κB), especially in apoptotic, floating

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**Fig. 5.** Specific decrease in DNA binding activity of NF-κB in ANT1-overexpressing cells. A, nuclear extracts were isolated after 16 h of overexpression as described under “Experimental Procedures.” They were subjected to EMSA using an NF-κB consensus oligonucleotide. In competition assays, a 100-fold molar excess of unlabeled competitor oligonucleotide was added to the binding mixture. In supershift assays, nuclear extracts were preincubated with 1 μg of an antibody (Ab) recognizing the NF-κB subunit p65 prior to the binding reaction. B, nuclear extracts from vector- and ANT1-transfected cells after 8, 14, and 24 h of ANT overexpression and nuclear extracts from attached (A) and floating (F) cells previously isolated after 16 h of ANT1 overexpression were subjected to EMSA. n.s, nonspecific.
ANT1-overexpressing cells, whereas no such increase was observed when ANT2 was expressed. The increase in mitochondrial NF-κB results in a decrease of this complex in the cytosol, and that decrease, in turn, has an important repercussion for nuclear NF-κB activity. It has been assumed that inactivation of NF-κB increases the sensitivity of cells to apoptotic stimuli (25, 35, 36). In agreement with this suggestion, antiapoptotic genes such as the Bcl-XL, MnSOD2, and c-IAP genes are down-regulated in ANT1-overexpressing cells indicating sensitization to an apoptotic stimulus. Together, these

**Fig. 7.** Effects of ANT1 overexpression on mRNA levels of NF-κB-dependent genes Bcl-XL, MnSOD2, and c-IAP2. HeLa cells were transiently transfected with empty vector or ANT1 vector. 1 μg of total RNA was analyzed by RT-PCR after 8 h (A) or 14 h (B) of expression. Attached (A) and floating (F) cells were obtained separately. Data are shown as mean ± S.E. (n = 3). *, p ≤ 0.05 (ANT1 versus vector).

**Fig. 8.** p65(NF-κB) co-transfection reduces the apoptotic effect of ANT1 overexpression. A, Western blot of p65 and ANT from homogenates of transfected cells at 24 h with 4 μg of vector or ANT1 and co-transfected with p65(NF-κB) vector (2 μg) together with empty vector (2 μg) or ANT1 (2 μg). B, percentage of floating cells obtained following transfection with the indicated plasmids. C, percentage of annexin V-FITC-positive cells following transfection with the indicated plasmids. D, percentage of mortality measured by propidium iodide labeling. Data are shown as mean ± S.E. (n = 3), *, p ≤ 0.05 (vector versus ANT1 or versus cells co-transfected with ANT1 and p65). ●, p ≤ 0.05 (ANT1 versus cells co-transfected with p65 and ANT1).
results indicate that the affinity of the IκBα-NF-κB complex for ANT protein varies depending upon the ANT isoform.

In addition, the TNFα response of NF-κB activity is lower in ANT1-overexpressing cells than in vector-transfected cells, suggesting a diminished cytosolic NF-κB TNFα-sensitive pool. We demonstrated that the co-transfection of p65(NF-κB) together with ANT1 significantly reduces the apoptosis assessed as changes in the number of floating cells, cellular viability, or annexin V labeling, indicating that the cytosolic IκBα-NF-κB pool is limiting in ANT1-transfected cells.

The results presented here highlighting the importance of mitochondrial recruitment of IκBα-NF-κB by ANT1 overexpression could also explain the protective effect of cyclophilin D observed when it was co-expressed with ANT1 (34). In our model the overexpression of cyclophilin D would lead to IκBα-NF-κB removal from ANT1 binding and consequently a recovery of nuclear NF-κB. Thus, antia apoptotic genes up-regulated by NF-κB can control apoptosis induction.

On the other hand, ANT1 is highly expressed in skeletal muscle and heart (16), where this protein is one of the most abundant in mitochondria and makes up around 10% of all proteins in the inner mitochondrial membrane (37). In this biological context ANT does not induce apoptosis, and it has an important bioenergetic role. A similar phenomenon occurs in biological context ANT does not induce apoptosis, and it has an important bioenergetic role. A similar phenomenon occurs in biological situations.

Lastly, three ANT isoforms have been described in humans, ANT1, -2 and -3. We have demonstrated that ANT3, like ANT1, induces apoptosis when overexpressed in HeLa cells (38). In this situation, a decrease in NF-κB DNA binding and an increase in NF-κB in mitochondria were observed (data not shown). Thus, we propose that as for ANT1 overexpression, recruitment of NF-κB into mitochondria could be involved in the induction of apoptosis by ANT3 overexpression.

In summary, our results demonstrate that recruitment of mitochondrial IκBα-NF-κB associated with ANT1 overexpression plays a critical role in the induction of apoptosis by ANT1.

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