Adenine nucleotide translocase 3 (ANT3) overexpression induces apoptosis in cultured cells

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Abstract Mitochondrial adenine nucleotide translocase 1 (ANT1), but not ANT2, can dominantly induce apoptosis [Bauer et al. (1999) J. Cell Biol. 439, 258–262]. Nothing is known, however, about the apoptotic activity of ANT3. We have transfected HeLa cells with the three human ANT isoforms to compare their potential as inducers of apoptosis. Transient overexpression of ANT3 resulted, like ANT1, in apoptosis as shown by an increase in the sub-G1 fraction, annexin V staining, low $\Delta\Psi_m$, and activation of caspases 9 and 3. Moreover, the apoptosis produced by ANT3 was inhibited by bongkrekic acid and by cyclosporin A. The pro-apoptotic activities of the ANT1 and ANT3 isoforms contrast with the lack of apoptotic activity of ANT2. This finding may help to identify the specific factors associated with the pro-apoptotic activities of ANT isoforms.

Key words: ADP/ATP carrier; Adenine nucleotide translocase; Mitochondrion; Apoptosis; Cell death

1. Introduction

Adenine nucleotide translocase (ANT) plays a role in the exchange of ATP for ADP through the inner mitochondrial membrane, thus supplying the cytoplasm with newly synthesized ATP in oxidative phosphorylation. ANT is a nuclear-encoded gene with an M_r of 32 000 and is thought to function as a homodimer [1,2]. It is an integral membrane protein comprising six transmembrane segments with both the N-terminal and C-terminal ends facing the intermembrane space [3–5]. Its crystallographic 2.2 Å structure in complex with carboxyatractyloside has recently been reported [6]. During the transport of ADP and ATP it is assumed that ANT changes from the c-conformation to the m-conformation in which the substrate binding site is oriented towards the cytoplasm or the matrix respectively [7]. Atractyloside and carboxyatractyloside are specific inhibitors of ANT that act by fixing the c-conformation, whereas bongkrekic acid (BKA) inhibits ANT by fixing the m-conformation [7]. In humans there are three closely related isoforms that are expressed in a tissue-specific manner. ANT1 is mainly expressed in the heart, skeletal muscle and brain, ANT2 is expressed in liver and in cells capable of proliferation and ANT3 is ubiquitously expressed [8–10]. Even though the amino acid sequence identity among these isoforms is very high (70–80%) it is thought that some functional differences might exist to explain their expression patterns. In support of this, there is some evidence that ANT2 could favor the mitochondrial uptake of cytosolic ATP in situations where oxidative phosphorylation is inhibited [9].

Apart from its role in nucleotide translocation, ANT is a core component of the so-called mitochondrial permeability transition pore (MPTP). This is a protein complex found in the mitochondrial contact sites that, when it is open, acts as a non-specific channel, allowing the free passage of molecules under 1500 Da through the inner mitochondrial membrane [11,12]. As a consequence, mitochondria lose their mitochondrial membrane potential ($\Delta\Psi_m$) and swell [13,14]. Several pharmacological and physiological regulators of MPTP exist, among them calcium has a significant role [11,15,16]. Inhibitors of ANT affect MPTP; thus, atractyloside activates MPTP whereas BKA inhibits it [17]. The involvement of MPTP in apoptosis has been reported by a number of groups [18–20]. In a search for pro-apoptotic proteins, Bauer et al. [21] identified ANT1 as a protein that can dominantly induce apoptosis. The overexpression of ANT1 produced a rapid cell death, with a concomitant decrease in $\Delta\Psi_m$ and an increase in nucleosomal DNA degradation. Cell death was sensitive to a caspase inhibitor and to inhibitors of MPTP, indicating altogether that apoptosis took place and that MPTP was involved [21]. Interestingly, the overexpression of ANT2 had no effect on cell death [21]. This contrasts with the ability of both isoforms to interact with proteins of the Bcl2 family, such as Bax and Bcl2 [22,23]. The isoform specificity of the apoptotic activity of ANT prompted us to investigate the activity of the third ANT isoform (ANT3), whose physiological role has not been studied to date. We observed that overexpression of ANT3 in HeLa cells induces apoptosis mainly through the mitochondrial pathway. This result will help in identifying the determinants of the pro-apoptotic activity of ANT1 and ANT3 that are absent in ANT2.

2. Materials and methods

2.1. Cell culture and transfection

HeLa cells (ATCC-CC-1) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 100 U/ml penicillin/streptomycin and supplemented with 10% fetal bovine serum (FBS) (Gibco BRL). The cells were incubated at 37°C in a 95% air, 5% CO_2 atmosphere. Cells were transfected by standard calcium phosphate precipitation. A co-transfected green fluorescent protein (GFP) expression plasmid was used to assess the transfection efficiency (around 80% in all cases). When indicated, cells were incubated with a combination of

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cyclosporin A (CsA) (5 μM, Sigma) plus trifluoropyrasine (TFP) (2 μM, Sigma) or with BKA (50 μM, Sigma).

2.2. Plasmid constructs and cDNAs

Reverse transcription polymerase chain reaction was used to obtain the cDNAs of the human ANT isoforms from HeLa cell RNA. cDNAs encoding full-length ANT isoforms were cloned into the pcDNA3.1-Zeo(+) vector (Invitrogen). The plasmid pGFP-N3 (Clontech) was used to assess the transfection efficiency by co-transfection.

2.3. Cell fractionation

HeLa cells were collected, washed with phosphate-buffered saline (PBS) and centrifuged at $500\times g$ for 2 min. The pellets were re-suspended in 1 ml of homogenization buffer (250 mM sucrose, 1 mM EGTA, 10 mM HEPES pH 7.4, 1 mM phenylmethylsulfonyl fluoride, Complete-Mini Protease Inhibitor cocktail tablets (Roche)). Cells were disrupted by 50 strokes in a glass homogenizer and then centrifuged at $1500\times g$ for 10 min at 4°C to remove unbroken cells, plasma membranes and nuclei. Supernatants were further centrifuged at $10\,000\times g$ for 10 min and the resultant pellet corresponded to the mitochondria-enriched fraction, and the supernatants were used as crude cytosolic extracts. Protein levels were quantified using the Bradford method (Bio-Rad).

2.4. Immunoblotting

Homogenates and mitochondrial extracts of transiently transfected cells were prepared as described above. The samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on 12.8% acrylamide gels and transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore). Membranes were blocked overnight with 5% skimmed milk containing 0.5% Tween

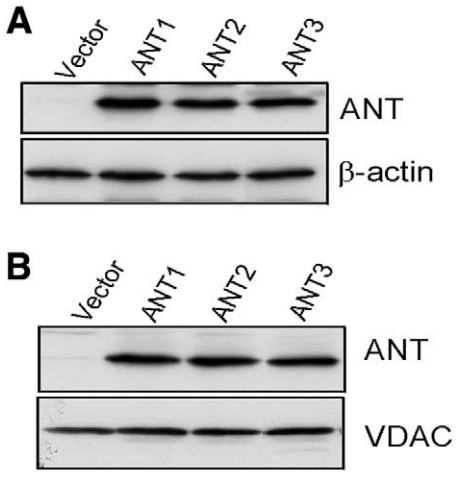

Fig. 1. Overexpression of ANT1, ANT2 and ANT3 proteins in HeLa cells 24 h after transfection. Proteins from cell homogenates (35 μg) (A) and purified mitochondrial fractions (35 μg) (B) of transfected cells were separated by SDS–PAGE, transferred to membranes and analyzed for the expression of ANT, β-actin and VDAC with specific antibodies as indicated.

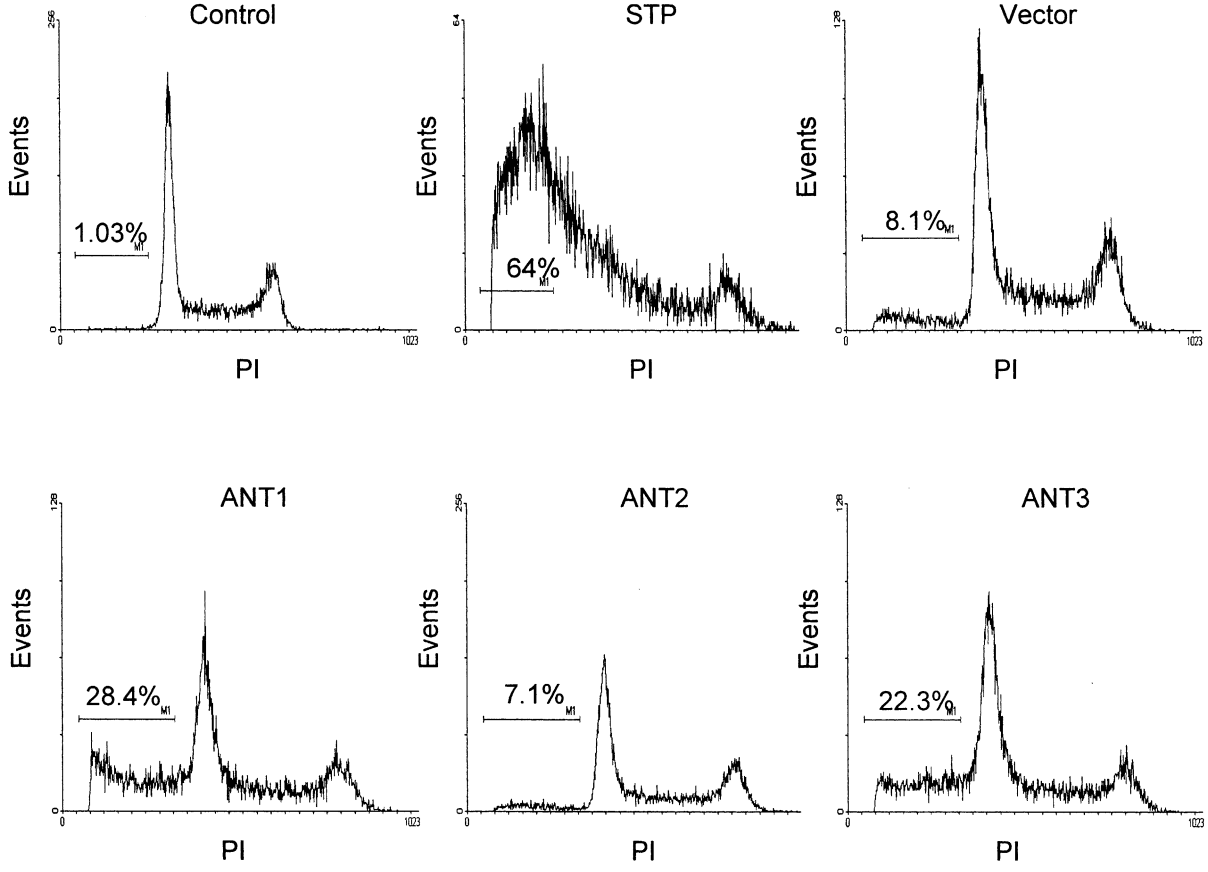

Fig. 2. Nucleosomal DNA degradation produced by overexpression of ANT1, ANT2 and ANT3 proteins. Twenty-four hours after transfection, HeLa cells overexpressing ANT1, ANT2 and ANT3 proteins, untransfected cells (control) and cells transfected with the empty vector (vector) were stained with PI and analyzed for sub-G1 DNA fraction by flow cytometry. As a positive control, HeLa cells were treated with 0.1 μM staurosporine (STP) for 16 h, and analyzed in the same way. The percentage of the sub-G1 fraction is indicated.

and then incubated with different primary antibodies: ANT (1:100; Q-18, Santa Cruz Biotechnology), β-actin (1:10 000; AC-15, Sigma) or VDAC (1:1000; 31HL, Calbiochem Biosciences). For caspase 9 analysis, whole homogenate membranes were stripped and reprobed with a polyclonal antibody against cleaved caspase 9 (Asp 315) (1:1000, Cell Signaling). Antibody binding was detected with horseradish peroxidase-coupled anti-mouse, anti-rabbit or anti-goat (Santa Cruz) secondary antibodies and an enhanced chemiluminescence (ECL) detection kit (Amersham Biosciences).

2.5. Fluorometric determination of caspase 3 activity

Caspase 3 activity was determined by fluorometry. Cytosolic extracts were incubated with the fluorogenic substrate Ac-DEVD-AMC (Promega).

2.6. Assessment of apoptosis by flow cytometry

The viability of transiently transfected cells was analyzed 24 h after transfection. 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 Molecular Biochemicals). Cells were considered apoptotic when they were annexin V-positive and PI-negative. Staining of cells by PI was used as an indicator of the loss of plasma membrane integrity. PI staining was also used to measure nucleoso-

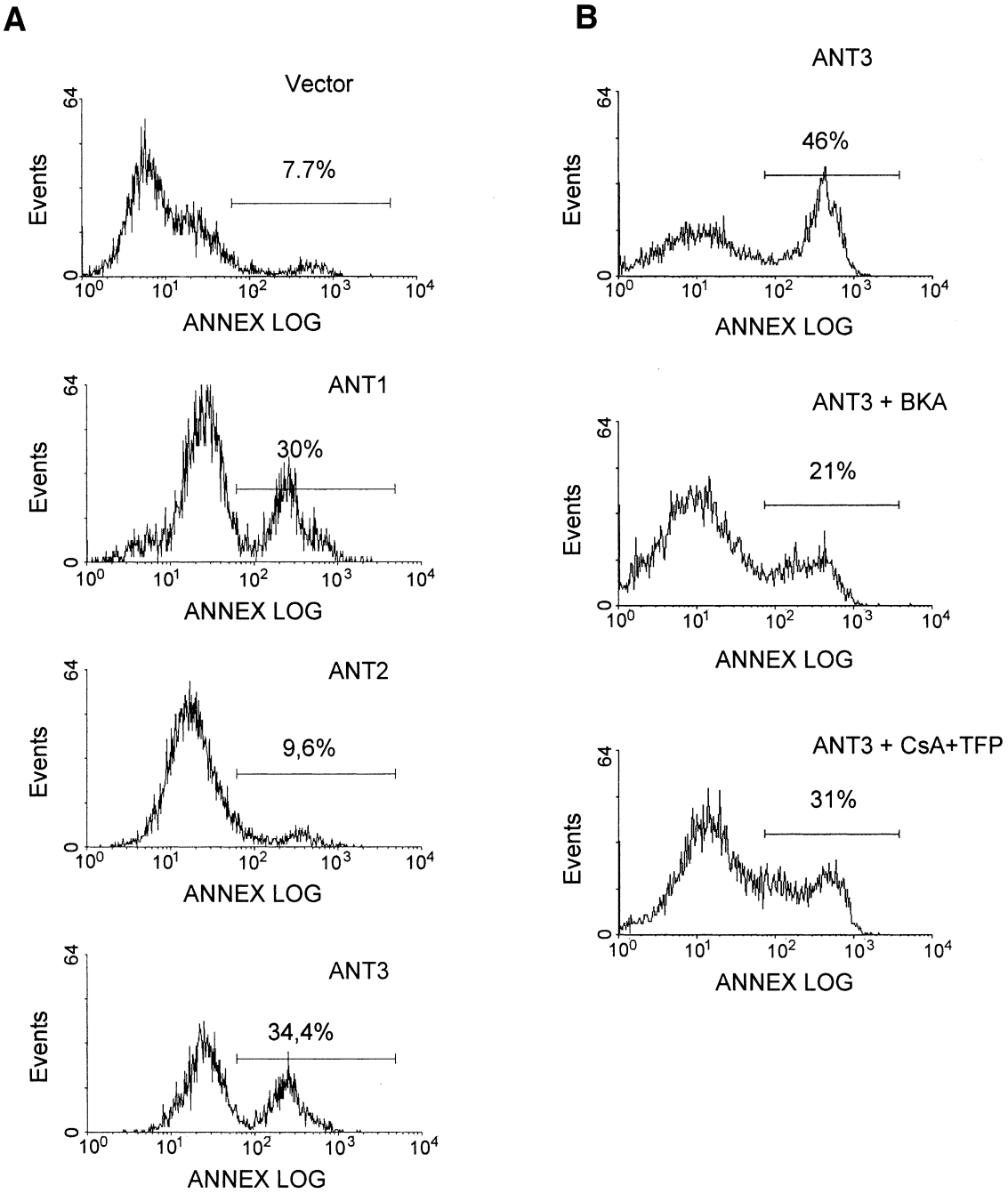

Fig. 3. Apoptosis induced by overexpression of ANT1, ANT2 and ANT3 proteins. A: Twenty-four hours after transfection, HeLa cells overexpressing ANT proteins and cells transfected with the empty vector (vector) were analyzed for apoptosis by annexin V and PI staining by flow cytometry. B: Cells transfected with ANT3 plasmid were treated with BKA (50 μM) and with CsA (5 μM) plus TFP (2 μM) during the whole procedure. Apoptosis was measured as in A. The percentage of annexin V-positive, PI-negative cells is indicated.

mal DNA degradation. Flow cytometry was carried out using an EPICS XL MCL (Beckman) cytometer.

2.7. Mitochondrial membrane potential

$\Delta\Psi_m$ was measured by flow cytometry using hexamethylindodicarbocyanine iodide ($DiIC_1(5)$) (Molecular Probes). Transiently transfected cells were loaded with 40 nM $DiIC_1(5)$ for 20 min in DMEM without FBS. At the end of the incubation period the cells were washed twice in PBS, re-suspended in a total volume of 0.5 ml with PBS and the $\Delta\Psi_m$ was analyzed by flow cytometry in a Coulter EPICS-XL-MCL (Beckman) cytometer.

3. Results

HeLa cells were transfected with the expression vectors containing the coding sequences for human ANT1, ANT2 and ANT3, resulting in the overexpression of these proteins (Fig. 1) detected by means of a non-isoform-specific antibody against ANT. The mitochondria of transfected cells showed a marked increase in the amount of ANT (Fig. 1B), compared to the control, mock-transfected cells, indicating that ANT was properly directed to the mitochondria. Apoptosis results in the degradation of nucleosomal DNA that can be quantified by flow cytometry by analyzing the sub-G1 fraction of DNA content. The overexpression of ANT1 and ANT3, 24 h after transfection, produced an important increase in the sub-G1 fraction (Fig. 2), indicating that extensive DNA degradation occurred. In contrast, the empty vector or ANT2 overexpression did not generate this effect (Fig. 2). As a positive control, staurosporine treatment (0.1 μM, 16 h) resulted in extensive DNA degradation as indicated by a marked increase in the sub-G1 fraction (Fig. 2). Cells were analyzed for apoptosis by staining of annexin V (Fig. 3). Whereas the transfection of empty vector or the overexpression of ANT2 showed only slight staining for annexin V, the overexpression of ANT1 and ANT3 resulted in massive cell death with a high percentage of annexin V-positive cells (Fig. 3A). In order to determine whether MPTP is involved in the cellular death produced by ANT3 overexpression, we made use of two known MPTP inhibitors, BKA and CsA used in combination with TFP [24]. We observed that the pro-apoptotic effect of ANT3 was effectively inhibited by these compounds (Fig. 3B). To assess mitochondrial involvement in this apoptotic process, $\Delta\Psi_m$ was measured. The empty vector or the overexpression of ANT2 had no effect, resulting in an almost homogeneous population with normal $\Delta\Psi_m$ (Fig. 4). In contrast, following the overexpression of ANT1 or ANT3 the cell population became biphasic: one half of the cells showed normal $\Delta\Psi_m$ whereas the other half had very low $\Delta\Psi_m$ (Fig. 4). Finally, we measured caspase 9 and caspase 3 activities, normally used as indicators of the involvement of the intrinsic or mitochondrial pathway of apoptosis. Caspase 9 activity was determined by measuring the amount of the cleaved, active caspase 9 by Western blot (Fig. 5A). The overexpression of ANT1 and ANT3 resulted in the activation of caspase 9 whereas ANT2 overexpression had no effect. In line with the activation of caspase 9, caspase 3 was also specifically induced by ANT3 overexpression (Fig. 5B).

4. Discussion

We have demonstrated that the overexpression of ANT3 results in cell death; transfected cells have a high sub-G1 fraction, are annexin V-positive, have decreased $\Delta\Psi_m$ and in-

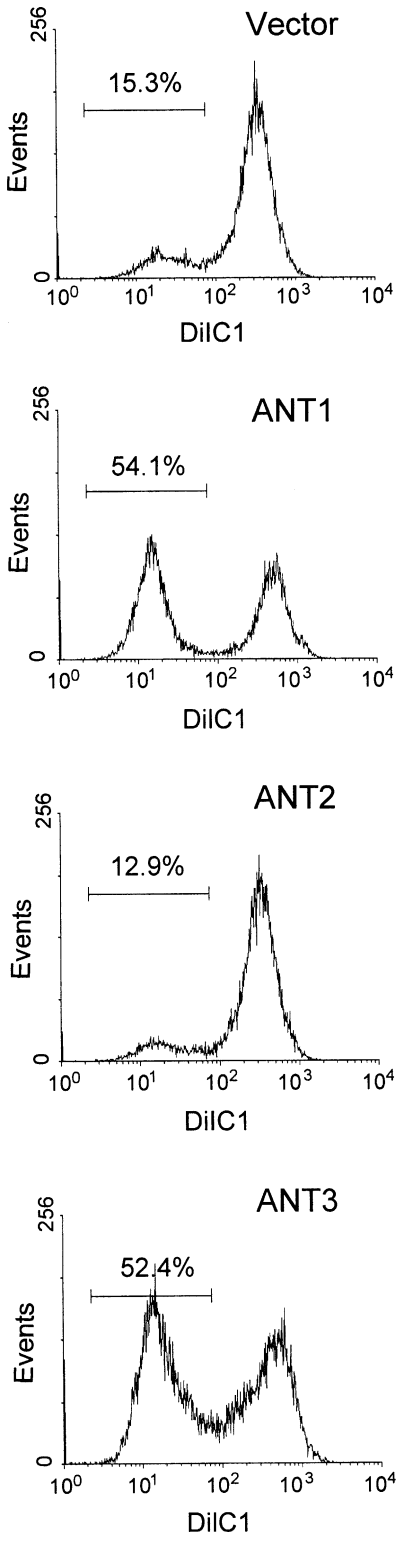

Fig. 4. Effects of ANT isoform overexpression on mitochondrial membrane potential ($\Delta\Psi_m$). Twenty-four hours after transfection, HeLa cells overexpressing ANT isoforms and cells transfected with the empty vector (vector) were incubated with the potential-sensitive probe $DiIC_1(5)$ and analyzed by flow cytometry. The percentage of cells with low $\Delta\Psi_m$ is indicated.

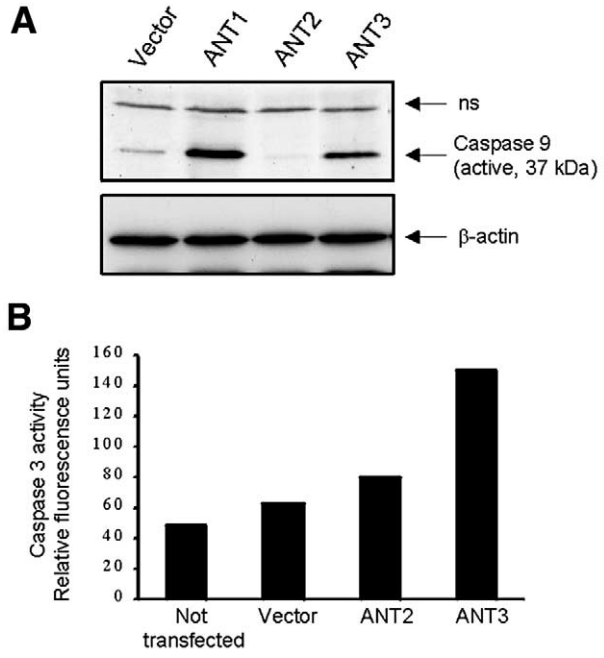

Fig. 5. Effects of ANT isoform overexpression on caspases 9 and 3 activation. A: Twenty-four hours after transfection, HeLa cells overexpressing ANT1, ANT2 or ANT3 proteins and cells transfected with the empty vector (vector) were analyzed for caspase 9 activity. Proteins from whole cell homogenates (35 μg) were separated by SDS–PAGE and incubated with a specific antibody against the activated caspase 9. ns, non-specific band. β-Actin was used as a loading marker. B: Caspase 3 activity was analyzed fluorometrically by incubating cytosolic fractions of ANT2- and ANT3-overexpressing cells with fluorogenic substrate.

creased caspase 9 and 3 activities. All these results are consistent with the induction of apoptosis as described previously for ANT1 [21] and confirmed in our own experiments. Our results also confirm the lack of effect of the overexpression of ANT2 in spite of its high amino acid identity with ANT1 and ANT3, demonstrating that the effects of ANT1 and ANT3 are not due to an artifact of overexpression. The different capacity for producing apoptosis of ANT isoforms can be related to the finding that ANT1 and ANT2 have distinct mitochondrial localizations and different abilities to interact with cyclophilin D [25]. In fact, there is evidence that the pro-apoptotic effect of ANT1 is dependent upon MPTP activity, since it is inhibited by the co-transfection of cyclophilin D or by treatment of cells with BKA [21], a known inhibitor of MPTP [17]. Similarly, we have shown that the pro-apoptotic effect of ANT3 overexpression can be inhibited by BKA and by CsA+TFP, indicating that, most likely, both ANT1 and ANT3 produce apoptosis through the same mechanism. It is interesting to note that in cultured cells the major ANT isoform expressed is ANT2, with very low, if any, levels of ANT1 or ANT3 ([8], and data not shown). This may suggest that ANT isoforms can induce apoptosis when expressed in the wrong place or at the wrong time. This assumption could explain why, despite its strong pro-apoptotic effect in HeLa cells, ANT1 is the major isoform expressed in skeletal muscle, heart or brain [8,9] where it does not induce apoptosis and has an important bioenergetic role. Interestingly, Bauer et al. [21] reported that overexpression of ANT1 in yeast did not induce cell death, indicating that it is not ANT1 per se that has the ability to directly disturb the cell, but an interaction between ANT1 and another protein that must be responsible for inducing apoptosis. This putative protein probably inhibits MPTP and apoptosis; thus, the overexpression of ANT1 would displace this protein from being attached to the MPTP or other relevant complexes, in this way opening the pore and inducing apoptosis [21]. A similar mechanism could explain the effects of ANT3. Of course, the interaction has to be isoform-specific, since ANT2 has no effect at all on apoptosis. ANT is a protein capable of multiple protein–protein interactions. It interacts with other MPTP constituents, like cyclophilin D and VDAC [11], and can also interact with members of the Bcl2 family of proteins like Bax and Bcl2 [22,23]. In addition, ANT can bind the IκBα/NF-κB complex, the inactive pool of NF-κB transcription factor, through its interaction with IκBα [26]. This may represent another putative mechanism involved that may require further attention because of the important role of NF-κB in apoptosis. Some of these interactions with other proteins have been demonstrated to be totally or partially isoform-specific. Thus, it seems that cyclophilin D has more affinity for ANT1 than for ANT2 [25], suggesting perhaps a major involvement of ANT1 in the MPTP activity. It has also been reported that ANT1 and ANT3 but not ANT2 bind ARL2 (ADP-ribosylation factor-like 2) and BART (binder of ARL2, with unknown function) [27]. Direct involvement of ARL2-BART in apoptosis induced by ANT has yet to be demonstrated, but it is interesting to note that commonly used cell lines, susceptible to the induction of apoptosis by overexpression of ANT1 or ANT3, have almost undetectable levels of BART-ARL2 proteins [27]. Our finding that overexpression of ANT3, like that of ANT1, induces apoptosis should be an important aid in the search for the putative ANT–protein interactions responsible for the induction of apoptosis.

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