Published in final edited form as:

Biochem J. ; 420(1): 37–47. doi:10.1042/BJ20090082.

Biochemical evidence of the interactions of membrane type-1 matrix metalloproteinase (MT1-MMP) with adenine nucleotide translocator (ANT): potential implications linking proteolysis with energy metabolism in cancer cells

Ilian A. Radichev, Albert G. Remacle, Nor Eddine Sounni, Sergey A. Shiryaev, Dmitri V. Rozanov, Wenhong Zhu, Natalya V. Golubkova, Tatiana I. Postnova, Vladislav S. Golubkov, and Alex Y. Strongin¹

Burnham Institute for Medical Research, La Jolla, CA 92037, U.S.A.

Abstract

Invasion-promoting MT1-MMP (membrane type-1 matrix metalloproteinase) is a key element in cell migration processes. To identify the proteins that interact and therefore co-precipitate with this proteinase from cancer cells, we used the proteolytically active WT (wild-type), the catalytically inert E240A and the C-end truncated (tailless; Δ CT) MT1-MMP-FLAG constructs as baits. The identity of the pulled-down proteins was determined by LC-MS/MS (liquid chromatography tandem MS) and then confirmed by Western blotting using specific antibodies. We determined that, in breast carcinoma MCF cells (MCF-7 cells), ANT (adenine nucleotide translocator) efficiently interacted with the WT, E240A and Δ CT constructs. The WT and E240A constructs also interacted with α -tubulin, an essential component of clathrin-mediated endocytosis. In turn, tubulin did not co-precipitate with the Δ CT construct because of the inefficient endocytosis of the latter, thus suggesting a high level of selectivity of our test system. To corroborate these results, we then successfully used the ANT2-FLAG construct as a bait to pull-down MT1-MMP, which was naturally produced by fibrosarcoma HT1080 cells. We determined that the presence of the functionally inert catalytic domain alone was sufficient to cause the proteinase to interact with ANT2, thus indicating that there is a non-proteolytic mode of these interactions. Overall, it is tempting to hypothesize that by interacting with pro-invasive MT1-MMP, ANT plays a yet to be identified role in a coupling mechanism between energy metabolism and pericellular proteolysis in migrating cancer cells.

Keywords

adenine nucleotide translocator (ANT); energy metabolism; mitochondrion; membrane type-1 matrix metallo-proteinase (MT1-MMP); pericellular proteolysis; protein-protein interaction

INTRODUCTION

Malignant cells widely employ pericellular proteolysis to penetrate the extracellular matrix and basement membrane and to invade distant tissues [1–3]. Proteolysis of the extracellular matrix components, cell surface adhesion signalling receptors, growth factors and cytokines is essential for the efficient migration and invasion of cancer cells [4]. In neoplasms, the

elevated expression of MMPs (matrix metalloproteinases) is frequently associated with the invasion of malignant cells as well as with the metastasis and neovascularization of tumours [5–7].

MT1-MMP (membrane type-1 MMP) is a multifunctional invasion-promoting enzyme that is expressed in multiple, distinct cell/tissue origin, cancer types [8]. It is becoming increasingly clear that MT1-MMP functionality is very important for cell locomotion when compared with the soluble MMP species [3,9]. Our observations together with the results of other laboratories suggest that MT1-MMP is a key enzyme in tumour cell invasion and metastasis. Transfection of only one MT1-MMP gene causes a pro-migratory phenotype change and increases the tumorigenicity of cancer cells [10]. These changes suggest that MT1-MMP regulates multiple cell functions and affects, both directly and indirectly, a number of cell regulation pathways. The induction of these pathways and the associated changes in cell metabolism suggest that they jointly contribute to stimulate cell migration and invasion [11]. The coupling mechanisms and the adaptor proteins that link MT1-MMP functionality with these diversified downstream pathways including energy metabolism, however, are not precisely understood.

To identify these putative adaptor proteins, which directly interact with cell surface-associated MT1-MMP, we successfully performed multiple pull-down experiments using cells of distinct tumour types. To our surprise, we found that MT1-MMP directly interacts with ANT (adenine nucleotide translocator) and that ANT, specifically the ANT2 isoform, in addition to its expression in mitochondria, is also present at the plasma membrane of cancer cells. We determined that the presence of either the active or functionally inert MT-CAT (catalytic domain of MT1-MMP) is sufficient to cause the interactions of the proteinase with ANT2, thus suggesting that a non-proteolytic parameter of these interactions exists. Our results directly correlate with the observations that in both promastigotes and amastigotes of *Leishmania* the mitochondrial ANT is also present at the plasma membrane [12,13]. In *Leishmania*, the plasma membrane ANT plays an important and specific role as part of a negative chemotaxis response to the host ATP in order to prevent phagocytosis of the parasite by host neutrophils.

In summary, it is tempting to hypothesize that by interacting with MT1-MMP, ANT plays a role in a coupling mechanism between the energy metabolism and pericellular proteolysis. We believe that additional studies should be conducted to determine precisely the role that is played by an unconventional ANT–MT1-MMP axis in regulating either energy metabolism or chemotaxis or both in cancer cells.

EXPERIMENTAL

General reagents and antibodies

All reagents were purchased from Sigma–Aldrich unless indicated otherwise. EZ-link sulfo-NHS-SS-biotin [sulfosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate] and EZ-link sulfo-NHS-LC-biotin [sulfosuccinimidyl-6-(biotinamido)hexanoate] were from Pierce. GM6001 (a potent, wide-range hydroxamate inhibitor of MMPs, including MT1-MMP) was from Chemicon.

Soluble MT1-MMP constructs

The individual proteolytically active MT-CAT and MT-CAT-PEX (soluble catalytically inert E240A MT1-MMP construct that includes the catalytic and haemopexin domains) were expressed in *Escherichia coli* BL-21 (DE3) Codon Plus cells (Stratagene) [14,15]. In addition to the inert (E240A) catalytic domain, the MT-CAT-PEX construct included the hinge region, the PEX domain (haemopexin domain) and the C-terminal V5 and His $\times$ 6 tags (Figure 1). The MT-CAT and MT-CAT-PEX recombinant constructs were purified from the inclusion bodies

and the soluble fraction of *E. coli* cells respectively. MT-CAT was then refolded to restore its native conformation.

Recombinant ANT2 constructs

The cDNA clone 3867331 (Invitrogen) was used as a template for PCR using the AccuPrime Pfx DNA polymerase (Invitrogen) and 5'-CACCATGACAGATGCCGCTGTGTCC-3' and 5'-TGTGTACTTCTTGATTTCATCATAACAAGAC-3' as the forward and the reverse primers respectively to generate the full-length human ANT2 cDNA (SLC25A5; GenBank® accession number BC056160). The PCR product was re-cloned in the pLenti6/V5-D-TOPO vector. Carrying out PCR with 5'-CACCATGACAGATGCCGCTGTGTCC-3' (the forward primer) and 5'-

TTACTTGTTCATCGTCGTCCTTGTAGTC*ctctctccatcgagTGTGTACTTCTTGATTTCATCATAACAAGACAAGCAC-3'* (the reverse primer; the FLAG sequence is underlined; the stop codon and the C-terminal peptide linker are in boldface and in lower-case respectively), the ANT2 construct was C-terminally tagged with an LDGGG peptide linker followed by a FLAG tag. The additional construct we designed was ANT2 C-terminally tagged with RFP (red fluorescent protein). The recombinant RFP sequence with the XhoI and BstBI restriction sites was generated by PCR using the 5'-CCTACT**CGAG**TATGGCCTCCTCCGAGGACGTC-3' and 5'-CCTAT**TCGA**ATTAGGCGCCGGTGGAGTGGCGGCCCT-3' oligonucleotides as the forward and reverse primers respectively (the XhoI and BstBI restriction site are shown in boldface). The resulting PCR product was inserted in-frame downstream of the C-terminus of the ANT2 sequence in the pLenti6/V5-D-TOPO vector, thus resulting in the ANT2-RFP fusion construct. The authenticity of the recombinant constructs was confirmed by DNA sequencing.

Stably transfected cells

Human breast carcinoma MCF cells (MCF-7 cells) and human fibrosarcoma HT cells (HT1080 cells) were obtained from the A.T.C.C. (Manassas, VA, U.S.A.). Cells were cultured routinely in high-glucose DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% (v/v) FBS (fetal bovine serum), 100 units/ml penicillin and 100 µg/ml streptomycin. MCF cells stably transfected with the WT (wild-type) MT1-MMP (MCF-MT-WT), the catalytically inert MT1-MMP-E240A mutant (MCF-MT-E240A) and the C-end-truncated, tailless, MT1-MMP lacking amino acids 563–582 of the cytoplasmic tail (MCF-MT-ΔCT) were constructed and characterized previously [16,17]. The FLAG-tagged MT1-MMP construct in the pcDNA3-zeo vector (MT-WT-FLAG) and the stably transfected breast carcinoma MCF cells (MCF-MT-WT-FLAG) were obtained and characterized in our previous work [18]. In the present study we generated, in addition, the MT-E240A-FLAG and MT-ΔCT-FLAG constructs in the pcDNA3-zeo plasmid and the stably transfected breast carcinoma MCF cells (MCF-MT-E240A-FLAG and MCF-MT-ΔCT-FLAG cells respectively). In these constructs, the FLAG tag sequence was inserted between Asp³⁰⁷ and Lys³⁰⁸ of the peptide sequence of the hinge region of MT1-MMP.

To co-express recombinant ANT2 with MT1-MMP, we used the original HT cells, which express MT1-MMP naturally, and MCF cells which have already been transfected with the full-length WT MT1-MMP construct in the pcDNA3-zeo plasmid (MCF-MT-WT cells). As a control, we used the parental MCF cells which do not express MT1-MMP [17]. These three cell lines were transfected with the pLenti6/V5-D-TOPO vector (Invitrogen), which included the ANT2-FLAG or the ANT2-RFP constructs, using Lipofectamine™ 2000. Cells transfected with the control pLenti6/V5-GW/lacZ construct were used as controls. The stable transfectants were selected with 10 µg/ml blasticidin. ANT2-expressing clones were identified among the blasticidin-resistant clones by Western blotting and also by immunocytochemistry with the FLAG M2 antibody. To avoid clonal effects, five to six blasticidin-resistant ANT2-producing individual clones were combined and used in our further studies. As a result, the

following stably transfected cell lines were isolated and used in the present study: MCF-mock (MCF cells transfected with the control pLenti6/V5-GW/Lac Z vector), MCF-MT-WT/ANT2-FLAG, MCF-ANT2-FLAG, HT-mock (HT cells transfected with the control pLenti6/V5-GW/Lac Z vector) and HT-ANT2-FLAG.

Transient transfection

MCF-MT-WT cells (1×10^5 per well of a 24-well dish) were transfected with the ANT2-RFP pLenti6/V5-D-TOPO construct (0.8 μ g of total DNA) using Lipofectamine™ 2000. In 6 h, the medium was replenished and the cells were incubated for an additional 18 h. To determine the transfection efficiency, cells were also transfected with the pLenti6-V5-D-TOPO-RFP control construct. The presence of RFP was determined by microscopy of the transfected cells. In most cases, transfection efficiency was over 60%. Transiently transfected cells were immediately used in our immunostaining experiments.

Immunoblotting

Cells (1×10^5) were lysed in 50 μ l of SDS sample loading buffer (0.125 M Tris/HCl, pH 6.8, 20% glycerol, 2% SDS, 0.005% Bromophenol Blue and 5% 2-mercaptoethanol). The lysates (4–6 μ g of total protein each) were analysed by Western blotting with the following antibodies: murine monoclonal FLAG M2 antibody (dilution 1:4000), murine monoclonal antibody (clone 3G4) to the MT-CAT (Chemicon; dilution 1:6000); rabbit polyclonal antibody AB815 to the hinge region of MT1-MMP (Chemicon; dilution 1:2000); murine monoclonal ANT antibody (Mitoscience; clone 5F51BB5AG7, dilution 1:1000; this antibody does not discriminate between ANT1, ANT2 and ANT3), rabbit polyclonal α -tubulin antibody (Cell Signaling Technology; dilution 1:1000), murine monoclonal ATP synthase antibody (BD Transduction Laboratories, clone 10; 1:2000 dilution) and rabbit polyclonal Bax and Bcl-2 antibodies (a gift from Dr Stan Krajewski, Burnham Institute for Medical Research; dilution 1:4000). The species-specific peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch; dilution 1:3000) and a SuperSignal West Dura Extended Duration Substrate kit (Pierce) were used for detection of the immuno-positive protein bands.

Immunoprecipitation of the MT1-MMP-FLAG and ANT2-FLAG constructs

All operations were performed either directly on ice or at 4 °C. Cells (1×10^8) were lysed in 10 ml of buffer A [20 mM Tris/HCl, pH 7.9, 150 mM KCl, 5 mM MgCl₂, 10% glycerol, 0.5% NP40 (Nonidet P40), 1 mM EGTA, 10 μ g/ml nocodazole, 10 μ g/ml cytochalasin D, 1 mM PMSF and a protease inhibitor cocktail and phosphatase inhibitor cocktails 1 and 2]. The lysate was centrifuged (40 min at 20000 g). The supernatant fraction (20–40 mg of total protein) was incubated for 4 h with 40 μ l of 50% anti-FLAG M2-agarose bead slurry. The beads were collected and washed six times with 0.5 ml of buffer A to remove the impurities. The beads were then incubated for 1 h with 20 μ l of 0.2 mg/ml FLAG peptide to elute the FLAG constructs. The samples were centrifuged and the supernatant fraction was analysed further by Western blotting and silver staining.

In the experiments employing the soluble MT-CAT and MT-CAT-PEX constructs, the aliquots (20 mg of total protein each) of the MCF-ANT2-FLAG cell lysate were incubated with MT-CAT and MT-CAT-PEX (10 μ g each) for 1 h at 4 °C. The samples were then precipitated using anti-FLAG beads as described above.

Immunoprecipitation of the untagged cellular MT1-MMP constructs

Cells (1×10^8) were lysed in buffer A. The lysates (10 ml each) were precleared using 50 μ l of 50% slurry of Protein G-Sepharose beads (GE Healthcare). MT1-MMP was precipitated for 4 h from the precleared extracts using a 3G4 monoclonal antibody and 40 μ l of 50% slurry

of Protein G–Sepharose beads. MT1-MMP was eluted from the beads with 100 mM glycine (pH 2.5). The eluted fractions were analysed by silver staining of the gels and Western blotting.

Immunofluorescence staining

Cells were cultured to 50% confluence on coverslips in DMEM/10% FBS medium. Cells were fixed for 10 min in 4% (w/v) paraformaldehyde, permeabilized using 0.1% Triton X-100, washed with PBS containing 0.1% Tween 20 and blocked for 1 h in 1% casein. Cells were then stained for 1 h at ambient temperature using the MT1-MMP antibody 3G4 (dilution 1:1000) followed by an Alexa Fluor® 488-conjugated goat anti-mouse secondary antibody (Molecular Probes; dilution 1:200). The slides were then mounted in mounting medium (Vector) that contained DAPI (4',6-diamidino-2-phenylindole) for the nuclear staining. Images were acquired at a $\times 400$ original magnification on an Olympus BX51 fluorescence microscope equipped with an Olympus MagnaFire digital camera and MagnaFire 2.1C software.

Biotinylation and pull-down of cell surface-associated proteins

For the isolation of the plasma membrane proteins, HT-mock and HT-ANT2–FLAG cells were surface biotinylated with membrane-impermeable NHS-LC-biotin [succinimidyl-6-(biotinamido)hexanoate]. The biotin-labelled proteins were pulled-down using streptavidin–Sepharose beads as previously described [19]. The precipitated samples were analysed by Western blotting with the FLAG, ANT and ATP synthase antibodies.

MS

The sample precipitated from the MCF-MT-E240A–FLAG cell lysate using FLAG beads was used to determine the identity of the ANT2 and tubulin bands that co-precipitated with the MT-E240A–FLAG construct. Similarly, the sample precipitated from the HT-ANT2–FLAG cell lysate using FLAG beads was used to identify the MT1-MMP bands that co-precipitated with the ANT2–FLAG construct. The samples were separated by gel electrophoresis and stained with Coomassie Simply Blue (Invitrogen). The individual protein bands of ANT (33 kDa), tubulin (50 kDa) and MT1-MMP (55–60 kDa) were excised from the gel and subjected to an in-gel tryptic digest. The digest peptides were extracted and then identified by LC-MS/MS (liquid chromatography tandem MS) using an LTQ XL Linear Ion Trap mass spectrometer (Thermo Scientific). MS/MS spectra were searched against the Swiss-Prot database using the SEQUEST Sorcerer software. Only the peptides with a probability score that exceeded 0.95 and a cross-correlation (X_{corr}) value that exceeded 2.0 were considered further and annotated.

RESULTS

Proteomic analysis shows that ANT interacts with MT1-MMP in breast carcinoma MCF cells

To identify the cellular proteins that interact with cellular MT1-MMP and that are pulled-down with this proteinase, we used the FLAG-tagged MT1-MMP constructs. To avoid interference of the FLAG tag with the trafficking processes, the tag sequence was inserted into the hinge region rather than directly linked to the C-terminus of MT1-MMP. Breast carcinoma MCF cells were stably transfected with the WT, E240A catalytically inert and C-end truncated, tailless, FLAG-tagged MT1-MMP constructs (MT-WT–FLAG, MT-E240A–FLAG and MT- Δ CT–FLAG respectively). We specifically selected MCF cells for our studies because the parental cell line is deficient in both MT1-MMP and MMP-2 [20]. As controls, we used MCF cells stably transfected with the respective untagged MT1-MMP constructs. The MT1-MMP constructs we used in the present study are shown in Figure 1.

Cells were lysed and cellular MT1-MMP was precipitated from the lysates using anti-FLAG beads (Figure 2). The FLAG-tagged material was specifically eluted from the beads using the

FLAG peptide and separated by gel electrophoresis. The selected protein bands of the MCF-MT-E240A sample were excised from the gel and subjected to in-gel tryptic proteolysis. The tryptic peptides were analysed by LC-MS/MS to determine the identity of the material. The untagged MT-E240A construct was used as a control in these experiments to identify the non-specific bands, which were not analysed further. This method allowed us to readily confirm the identity of the MT1-MMP bands we observed in the samples. Thus MCF-MT-WT-FLAG and MCF-MT- Δ CT cells that expressed the proteolytically active MT1-MMP constructs exhibited both the full-length enzyme and the proteolytically degraded MT1-MMP species. In contrast, the full-length MT1-MMP was primarily observed in MCF-MT-E240A cells which expressed the inert proteinase that was incapable of self-proteolysis. These results correlate well with our earlier observations and the findings of other laboratories [17].

Earlier studies revealed that MT1-MMP is internalized rapidly in clathrin-coated vesicles, whereas the tailless MT- Δ CT remains predominantly on the cell surface [21–23]. In agreement, our LC-MS/MS analysis of the precipitated samples determined that both the MT-WT-FLAG and MT-E240A-FLAG constructs pulled-down tubulins, including α - and β -tubulins, which are the essential components of the microtubulin cytoskeleton and clathrin-dependent endocytosis. In turn, the tailless MT- Δ CT-FLAG construct is not efficiently internalized through the clathrin-dependent internalization pathway [16,19,23]. In agreement, the MT- Δ CT-FLAG samples did not contain tubulins. The identity of tubulins, specifically α -tubulin, was confirmed by an in-gel trypsin digestion followed by LC-MS/MS analysis of the tryptic peptides (Supplementary Table S1 at <http://www.BiochemJ.org/bj/420/bj4200037add.htm>).

In contrast with α -tubulin, all three of the samples (MT-WT-FLAG, MT-E240A-FLAG and MT- Δ CT-FLAG) included a prominent 33 kDa band (Figure 2A). LC-MS/MS analysis of the tryptic peptides of the 33 kDa band identified, with high confidence, multiple unique tryptic peptides from the sequences of the highly homologous mitochondrial ANT2 and ANT3 (Supplementary Table S2 at <http://www.BiochemJ.org/bj/420/bj4200037add.htm>).

Pull-down analysis of MT1-MMP and ANT in breast carcinoma MCF cells

To support the specific interactions of MT1-MMP with ANT, we co-immunoprecipitated the ANT-MT1-MMP complex from MCF-MT-E240A cells using the monoclonal 3G4 antibody against the MT-CAT (Figure 2B). The precipitated sample was separated by gel electrophoresis and stained using a silver stain. In addition to the heavy- and light-chain IgG antibody bands, the MT1-MMP-E240A and the 33 kDa ANT bands were readily detected in the stained gels. An aliquot of the precipitated material was analysed by Western blotting using the murine monoclonal ANT antibody (clone 5F51BB5AG7). This immunoblotting analysis confirmed the presence of high levels of ANT in the sample. In contrast, a control MCF-MT-E240A cell sample, which was immunoprecipitated using the FLAG antibody, did not contain ANT.

To corroborate these observations, we analysed the samples using Western blotting with the MT1-MMP, α -tubulin and ANT antibodies. The samples were immunoprecipitated from MT-WT-FLAG, MT-E240A-FLAG and MT- Δ CT-FLAG MCF cells using anti-FLAG beads. In agreement with the results shown in Figure 2(A), both the full-length enzyme and the proteolytically degraded MT1-MMP form were detected in MT-WT-FLAG and MT- Δ CT-FLAG MCF cells, whereas MT1-MMP was predominantly represented by a full-length mature enzyme in the MT-E240A-FLAG cells. As controls, we used the samples that we immunoprecipitated with anti-FLAG beads from MT-WT, MT-E240A and MT- Δ CT MCF cells, which express the untagged MT1-MMP constructs. The presence of ANT was readily detected in all of the three experimental samples but not in the controls. α -Tubulin was absent in the sample from MT- Δ CT-FLAG cells (Figure 2C).

Pull-down analysis of MT1-MMP and ANT2 in fibrosarcoma HT cells

To confirm that the specific interactions of ANT with MT1-MMP that we observed in MCF cells took place in the distinct cell system, we used human fibrosarcoma HT cells that naturally express the full-length enzyme of MT1-MMP [24,25]. HT cells were stably transfected with the ANT2-FLAG (FLAG-tagged ANT2 construct) (Figure 1). We specifically selected ANT2 for our subsequent studies because the overexpression of ANT3 normally leads to rapid apoptosis of the transfected cells [26,27] and also because ANT2 was shown to be specifically associated with cancer [28–30].

As a control, we used HT cells stably transfected with the lacZ-expressing lentiviral vector (HT-mock cells). Immunoprecipitation was performed with the lysates from ANT2-FLAG and mock cell lysates using anti-FLAG beads. The FLAG-tagged construct was eluted from the beads using the FLAG peptide. As an additional control, the HT-mock cell lysates were immunoprecipitated using the murine monoclonal 3G4 antibody against MT-CAT. The samples were then analysed by gel electrophoresis using the silver-stained gels (Figure 3A, left panel). In ANT2-FLAG cells (lane ANT2-FLAG) and mock cells (lane MOCK) the positions of ANT2-FLAG and naturally produced ANT respectively were slightly different because of the presence of the additional FLAG sequence in the tagged recombinant construct. The 33 kDa ANT2 band was markedly increased in the ANT2-FLAG sample compared with the controls. The 3G4 antibody to the MT-CAT precipitated MT1-MMP and ANT, both of which are naturally produced by mock cells (Figure 3A, lane MOCK/MT-CAT). The additional Western-blot analysis using the MT1-MMP antibodies verified that MT1-MMP naturally produced by HT cells was pulled down with the ANT2-FLAG construct, thus supporting the selectivity and specificity of our assay system (Figure 3A, right panel). The LC-MS/MS analysis of the 55–60 kDa MT1-MMP bands that were pulled-down by FLAG beads from the HT-ANT2-FLAG cells identified multiple unique peptides from the MT1-MMP sequence, thus confirming their MT1-MMP origin (Supplementary Table S3 at <http://www.BiochemJ.org/bj/420/bj4200037add.htm>).

To demonstrate that cellular ANT2 specifically interacts with MT1-MMP, we fractionated HT-ANT2-FLAG and control HT-mock cell lysates using anti-FLAG beads. As an additional control for these experiments, we analysed the levels of Bcl-2, Bax and ATP synthase (a mitochondrial marker) in the samples. The absence of Bcl-2, Bax and ATP synthase and, in turn, the presence of MT1-MMP in the fractionated samples would indicate the specific pull-down of the latter. Furthermore, because Bcl-2 and Bax were shown to dynamically interact with mitochondrial ANT [31,32], the absence of both of these proteins in the pull-down samples would indicate the absence of mitochondrial contamination. The cell lysate, the unbound proteins and the sample, which was specifically retained by the beads, were each analysed by Western blotting with the MT1-MMP, ANT, ATP synthase, Bax and Bcl-2 antibodies. As an additional control, we used HT-mock cells, which were surface biotinylated, lysed and then processed in parallel with the HT-ANT2-FLAG cell samples. ATP synthase and MT1-MMP were absent in the mock cell sample eluted from anti-FLAG beads. In contrast, both ANT2-FLAG and MT1-MMP, but not ATP synthase, Bax or Bcl-2, were detected in the HT-ANT2-FLAG samples that were specifically precipitated by anti-FLAG beads (Figure 3B). These results again suggested that the ANT2-FLAG construct specifically pulled-down MT1-MMP from the HT ANT2-FLAG cells.

To confirm that MT1-MMP interacts with non-mitochondrial ANT, we extracted HT-ANT2-FLAG cells using 0.1 and 0.5% NP40 detergent. The total cell lysate and the extracted samples were analysed for the presence of both the solubilized ANT and the mitochondrial marker p110 (see Supplementary Figure S1, left panel, at <http://www.BiochemJ.org/bj/420/bj4200037add.htm>). A fraction of both ANT2-FLAG and naturally synthesized ANT was readily released by 0.1% NP40. In turn, p110 was not detected

in the 0.1% NP40-solubilized fraction. The use of 0.5% NP40 led to the disruption of the mitochondria and to the release of p110 and the residual ANT. An additional immunoprecipitation of the 0.1 and 0.5% NP40-solubilized samples using anti-FLAG M2-agarose beads followed by Western blotting with MT1-MMP 3G4 antibody confirmed that the non-mitochondrial ANT2-FLAG fraction co-precipitated with MT1-MMP (Supplementary Figure S1, right panel).

To demonstrate that the ANT2-FLAG construct was expressed on the cell surface in addition to the intracellular milieu, we used surface biotinylation of HT-ANT2-FLAG cells with membrane-impermeable biotin. Cell surface biotinylation was followed by lysis of the cells. The lysate was fractionated using streptavidin beads. The unfractionated lysate, the unbound material and the biotin-labelled proteins, which bound the beads, were analysed by Western blotting with the FLAG antibody. This experiment (Figure 3C) clearly demonstrated that a noticeable fraction of the total cell ANT2 was expressed at the cell surface and therefore this fraction was accessible to membrane-impermeable biotin.

Pull-down analysis of cell surface-associated ANT2

To determine if ANT2 was also associated with the plasma membrane in MCF cells, we surface biotinylated MCF-MT-WT-FLAG, MCF-MT-E240A-FLAG, MCF-MT-WT and MCF-MT-E240A cells (the latter two were controls). The cells were then lysed and the lysates were immunoprecipitated with anti-FLAG beads. The samples were then evaluated by Western blotting with horseradish peroxidase-conjugated Extravidin. Figure 4(A) shows that, in a manner consistent with our other results, both MT-WT-FLAG and MT-E240A-FLAG constructs pulled-down the 33 kDa band that corresponded to ANT2. An additional Western blot confirmed that the band that co-precipitated with MT-E240A-FLAG in MCF cells was recognized by the ANT antibody, thus confirming that this band is ANT. As expected, the ANT antibody did not detect any positive material in the control MT-E240A sample.

To elucidate whether the interactions of MT1-MMP with ANT2 are initiated during the secretion process in the intracellular milieu or whether these interactions take place only at the cell membrane, cell surface-biotinylated MCF-MT-E240A-FLAG cells were lysed and the biotin-labelled, cell surface proteins were precipitated from the lysates using streptavidin beads. The unbound, biotin label-depleted fraction was immunoprecipitated with anti-FLAG beads. The cell lysate, the streptavidin-depleted (unbound), the streptavidin-precipitated and the anti-FLAG-precipitated fractions were evaluated by Western blotting with the FLAG, ANT and ATP synthase antibodies. Figure 4(B) demonstrates that the MT1-E240A-FLAG construct specifically and efficiently pulled-down ANT2. These results agree with our findings in HT cells and suggest that the MT1-MMP-ANT2 interactions occur largely inside the cell, rather than exclusively at the cell surface, post-secretion.

Consistent with our results using HT cells (Figure 3) and in contrast with ANT2, mitochondrial ATP synthase did not efficiently co-precipitate with the MT-E240A-FLAG construct and therefore was absent from the FLAG fraction but was present in the biotin-labelled protein fraction. The presence of ATP synthase on the cell surface in our cell system is not entirely surprising. In addition to its localization in the mitochondrial inner membrane, ATP synthase β -chain has been shown to be ectopically localized on the surface of the vascular endothelial cells and of hepatocytes and to be regulated by a principal plasma apolipoprotein [33,34].

Co-localization of MT1-MMP and ANT2 on the surface of MCF cells

For the co-localization experiments we used MCF-MT-WT cells transiently transfected with the pLenti6/V5-D-TOPO construct that expressed the ANT2-RFP chimera. At 24 h after transfection, the cells were fixed, stained with an MT1-MMP 3G4 monoclonal antibody and

the co-localization of the MT1-MMP immunoreactivity with the RFP fluorescence was analysed using a fluorescence microscope. As a control, we used MCF-MT-WT cells transiently transfected with the pLenti6/V5-GW/LacZ vector without the ANT2-RFP insert. The bulk of ANT2-RFP was observed in the intracellular mitochondrial fraction, whereas a portion of ANT2-RFP was also detected in the plasma membrane. In the plasma membrane compartment, ANT2-RFP was consistently co-localized with the MT1-MMP immunoreactivity, thus supporting the results of our pull-down experiments (Figure 5).

ANT2 interacts with MT-CAT

To identify the individual domain of MT1-MMP that interacts with ANT2, we used MCF-MT-WT/ANT2-FLAG, MCF-ANT2-FLAG, MCF-MT-WT and MCF-mock cells. The cell lysates were immunoprecipitated with anti-FLAG beads to pull-down the cellular ANT2-FLAG-MT1-MMP complex. The presence of MT1-MMP in the precipitated samples was detected using the Ab815 antibody against the hinge region of MT1-MMP that recognizes both the full-length proenzyme-enzyme and the 44 kDa degraded, membrane-associated, species of the proteinase [17]. As expected, MT1-MMP was not detected in the samples precipitated with the anti-FLAG antibody from the control MCF-ANT2-FLAG, MCF-MT-WT and MCF-mock cells because these cells do not express either MT1-MMP or ANT2-FLAG or both. MT1-MMP was efficiently co-precipitated with the ANT2-FLAG construct only in MCF-MT-WT/ANT2-FLAG cells. The full-length forms rather than the degraded species of MT1-MMP were predominantly observed in these samples (Figure 6A).

To confirm these results, we incubated the MCF-ANT2-FLAG cell extracts with the purified soluble MT-CAT and the MT-CAT-PEX constructs. ANT2-FLAG was then immunoprecipitated from the samples with anti-FLAG beads. The levels of the co-immunoprecipitated MT1-MMP species were measured by Western blotting with the MT1-MMP 3G4 antibody (Figure 6B). Both MT-CAT-PEX and MT-CAT were efficiently co-precipitated with ANT2-FLAG. Based on these observations, we conclude that the MT-CAT alone is sufficient to cause the interactions of the proteinase with ANT2.

The expression of ANT2 does not affect the levels of cellular MT1-MMP

To determine whether the ANT2 expression affects the levels of MT1-MMP, we used Western blotting with the MT1-MMP 3G4 antibody to measure both the levels of cellular MT1-MMP and cell surface-associated, biotin-labelled MT1-MMP in HT-mock and HT-ANT2-FLAG cells. Figure 7 shows that both the total cell and the cell surface-associated levels of MT1-MMP were similar in HT-mock cells compared with those in HT-ANT2-FLAG cells.

In agreement, gelatin zymography of the medium aliquots did not demonstrate any difference in the level of activation of MMP-2 in HT-mock cells compared with HT-ANT2-FLAG cells and also in MCF-MT-WT and MCF-mock cells relative to MCF-MT-WT/ANT2-FLAG cells (results not shown). Because MCF cells do not produce MMP-2 naturally, MCF-MT-WT, MCF-mock and MCF-MT-WT/ANT2-FLAG cells were each incubated with the external purified MMP-2 proenzyme and the gelatin zymography of medium aliquots then followed. Our multiple additional experiments demonstrated that ANT2 did not affect the level of the presentation of MT1-MMP on cell surfaces and the rate of internalization of cell surface-associated MT1-MMP (results not shown).

DISCUSSION

Pro-migratory type-1 transmembrane proteinase MT1-MMP is a key regulator of cell migration in both normal and malignant cells. Evidence is emerging that MT1-MMP promotes cell migration and invasion by both proteolytic and non-proteolytic mechanisms and that the

localization of the proteolytic activity of MT1-MMP at the cell surface, as opposed to the extracellular soluble proteinases, is especially important to the cell migration and invasion processes [3,7,11,35,36]. Stimulation of a complex downstream network of molecular, biochemical and cellular events is required for the initiation and efficient continuation of cell migration processes. This essential network emerges in migrating cells to function in concert with MT1-MMP and to control signalling, transport, transcription, degradation, cell division and other critical cell functions including energy metabolism [11]. From a biochemical perspective, high energy metabolism, in addition to the efficient pericellular proteolysis of the extracellular matrix, appears to be mandatory for migratory cancer cells [37]. The biochemical links that couple energy metabolism, cell migration and pericellular proteolysis, however, are not precisely understood.

In eukaryotes, ATP production and energy metabolism greatly vary in different tissues and cells. These differences result from the differential expression of genes involved in oxidative phosphorylation and also from the kinetics of ATP and ADP exchange in the cell [38–40]. Neoplastic cells sustain a high rate of glycolysis even under aerobic conditions and therefore tumour cell growth depends to a large extent on glycolysis for ATP synthesis, compared with normal cells. ANT catalyses ADP/ATP exchanges across the mitochondrial inner membrane and links the mitochondrial ATP production with cellular ATP consumption. In humans, ANT has four isoforms, ANT1, ANT2, ANT3 and ANT4, each of which is encoded by a distinct gene [41–43]. The four ANTs are nuclear-encoded proteins located in the inner mitochondrial membrane [44]. There are six transmembrane domains in the ANT sequence. ANT1 is primarily expressed in heart and skeletal muscles, ANT2 is either weakly or not expressed at all in human tissues, whereas ANT3 is ubiquitously expressed and ANT4 transcripts are exclusively present in the liver, testis and brain. Among the four ANTs, ANT2 levels are significantly increased in proliferating migratory neoplastic cancer cells with a high glycolytic rate [28,29]. ANT2 is required for cancer cell glycolysis, which is up-regulated in cancer cells in order to generate more energy from glycolysis compared with normal cells [45]. In agreement with the apparent functional role of ANT2 in cancer, transcriptional silencing of ANT2 induces apoptosis in human breast cancer cells and inhibits tumour growth [46].

In our pull-down studies, we have been looking for the proteins that interact with recombinant MT1-MMP in breast carcinoma cells. One of the proteins we identified by LC-MS/MS analysis of the samples was ANT. The presence of ANT was then confirmed by Western blotting using specific antibodies. We then confirmed the interactions of ANT with MT1-MMP, which is naturally expressed in fibrosarcoma cells. For this purpose, we used the recombinant ANT2 construct as a bait in our pull-down reactions. According to our results, a noticeable fraction of ANT2 was present in the plasma membrane fraction in cancer cells, whereas the bulk of this enzyme was localized to the mitochondria (results not shown).

ANTs, however, are not exclusively mitochondrial proteins. For example, ANT is localized in the plasma membrane compartment in *Leishmania* and is a component of a negative chemotactic response that allows the parasite to evade phagocytosis by host neutrophils [12, 13]. Furthermore, the mitochondrial ATP-binding cassette transporter isoform was demonstrated to switch the sorting mode from co-translational endoplasmic reticulum targeting to post-translational mitochondrial import in COS7 cells [47], suggesting that a similar mechanism may exist for mitochondrial ANT. From this perspective, it is important that the C-terminal sequence of ANT2 (KKYT) represents a consensus endoplasmic reticulum retrieval/retention signal (KKXX), suggesting the potential role of the COPI (coatamer protein I) pathway in the trafficking of cellular ANT [48]. Accordingly, it is likely that the endoplasmic reticulum is the cell compartment where the interactions of ANT with MT1-MMP are initiated.

Another protein that we identified and that specifically interacted with both the WT and the catalytically inert E240A mutant MT1-MMP but not with the tailless MT1-MMP- Δ CT construct was α -tubulin, an essential component of both the cell cytoskeleton and the clathrin-dependent internalization pathway. It is well established that in contrast with both the WT and the catalytically inert proteinase, the tailless MT1-MMP- Δ CT construct accumulates predominantly in the caveolin-enriched lipid rafts and therefore it is inefficiently internalized via the clathrin-microtubulin pathway [16,19,22,23]. Accordingly, the tailless mutant should not efficiently interact with tubulin. In agreement, tubulin did not co-precipitate with the tailless MT1-MMP, thus confirming the high level of precision and selectivity of our experimental methodology and serving as a control for our MT1-MMP-ANT study. We suspect that MT1-MMP interacts with tubulin indirectly rather than directly and that these interactions involve the components of the dynamin-dependent clathrin-coated pits. The analysis of the interactions of MT1-MMP with α -tubulin, however, is clearly beyond the scope of the present study.

Overall, our findings suggest the existence of a novel, unconventional and unforeseen, role of ANT in cancer cells. The ANT-MT1-MMP axis likely links energy metabolism, especially its glycolysis portion, to invasion-promoting MT1-MMP-mediated pericellular proteolysis. We believe our observations warrant additional detailed investigations focused on gaining an understanding of the mechanisms that co-ordinate the in-unison performance of the proteinases, the ATP/ADP exchange enzymes and the purinergic receptors in the cell migration and chemotaxis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations used

ANT, adenine nucleotide translocator
 DAPI, 4',6-diamidino-2-phenylindole
 DMEM, Dulbecco's modified Eagle's medium
 FBS, fetal bovine serum
 HT cell, HT1080 cell
 LC-MS/MS, liquid chromatography tandem MS
 MCF cell, MCF-7 cell
 MMP, matrix metalloproteinase
 MT1-MMP, membrane type-1 MMP
 MT-CAT, catalytic domain of MT1-MMP
 MT-CAT-PEX, soluble catalytically inert E240A MT1-MMP construct that includes the catalytic and haemopexin domains
 NP40, Nonidet P40
 PEX, haemopexin domain
 RFP, red fluorescent protein
 WT, wild-type

Acknowledgments

FUNDING

This work was supported by the National Institutes of Health [grant numbers CA83017, CA77470 and RR020843; to A.Y.S.].

REFERENCES

1. Lopez-Otin C, Bond JS. Proteases: multifunctional enzymes in life and disease. *J. Biol. Chem* 2008;283:30433–30437. [PubMed: 18650443]
2. Seiki M, Yana I. Roles of pericellular proteolysis by membrane type-1 matrix metalloproteinase in cancer invasion and angiogenesis. *Cancer Sci* 2003;94:569–574. [PubMed: 12841863]
3. Wolf K, Wu YI, Liu Y, Geiger J, Tam E, Overall C, Stack MS, Friedl P. Multi-step pericellular proteolysis controls the transition from individual to collective cancer cell invasion. *Nat. Cell Biol* 2007;9:893–904. [PubMed: 17618273]
4. Strongin AY. Mislocalization and unconventional functions of cellular MMPs in cancer. *Cancer Metastasis Rev* 2006;25:87–98. [PubMed: 16680575]
5. Hotary K, Li XY, Allen E, Stevens SL, Weiss SJ. A cancer cell metalloprotease triad regulates the basement membrane transmigration program. *Genes Dev* 2006;20:2673–2686. [PubMed: 16983145]
6. Itoh Y. MT1-MMP: a key regulator of cell migration in tissue. *IUBMB Life* 2006;58:589–596. [PubMed: 17050376]
7. Li XY, Ota I, Yana I, Sabeh F, Weiss SJ. Molecular dissection of the structural machinery underlying the tissue-invasive activity of membrane type-1 matrix metalloproteinase. *Mol. Biol. Cell* 2008;19:3221–3233. [PubMed: 18495869]
8. Egeblad M, Werb Z. New functions for the matrix metalloproteinases in cancer progression. *Nat. Rev. Cancer* 2002;2:161–174. [PubMed: 11990853]
9. Wolf K, Friedl P. Mapping proteolytic cancer cell-extracellular matrix interfaces. *Clin. Exp. Metastasis* 2009;26:289–298. [PubMed: 18600304]
10. Hotary KB, Allen ED, Brooks PC, Datta NS, Long MW, Weiss SJ. Membrane type I matrix metalloproteinase usurps tumor growth control imposed by the three-dimensional extracellular matrix. *Cell* 2003;114:33–45. [PubMed: 12859896]
11. Rozanov DV, Savinov AY, Williams R, Liu K, Golubkov VS, Krajewski S, Strongin AY. Molecular signature of MT1-MMP: transactivation of the downstream universal gene network in cancer. *Cancer Res* 2008;68:4086–4096. [PubMed: 18519667]
12. Detke S, Elsabrouty R. Identification of a mitochondrial ATP synthase-adenine nucleotide translocator complex in *Leishmania*. *Acta Trop* 2008;105:16–20. [PubMed: 17920025]
13. Detke S, Elsabrouty R. *Leishmania mexicana amazonensis*: plasma membrane adenine nucleotide translocator and chemotaxis. *Exp. Parasitol* 2008;118:408–419. [PubMed: 18031742]
14. Golubkov VS, Chekanov AV, Shiryaev SA, Aleshin AE, Ratnikov BI, Gawlik K, Radichev I, Motamedchaboki K, Smith JW, Strongin AY. Proteolysis of the membrane type-1 matrix metalloproteinase prodomain: implications for a two-step proteolytic processing and activation. *J. Biol. Chem* 2007;282:36283–36291. [PubMed: 17938169]
15. Ratnikov B, Deryugina E, Leng J, Marchenko G, Dembrow D, Strongin A. Determination of matrix metalloproteinase activity using biotinylated gelatin. *Anal. Biochem* 2000;286:149–155. [PubMed: 11038285]
16. Rozanov DV, Deryugina EI, Monosov EZ, Marchenko ND, Strongin AY. Aberrant, persistent inclusion into lipid rafts limits the tumorigenic function of membrane type-1 matrix metalloproteinase in malignant cells. *Exp. Cell Res* 2004;293:81–95. [PubMed: 14729059]
17. Rozanov DV, Deryugina EI, Ratnikov BI, Monosov EZ, Marchenko GN, Quigley JP, Strongin AY. Mutation analysis of membrane type-1 matrix metalloproteinase (MT1-MMP). The role of the cytoplasmic tail Cys(574), the active site Glu(240), and furin cleavage motifs in oligomerization, processing, and self-proteolysis of MT1-MMP expressed in breast carcinoma cells. *J. Biol. Chem* 2001;276:25705–25714. [PubMed: 11335709]
18. Golubkov VS, Boyd S, Savinov AY, Chekanov AV, Osterman AL, Remacle A, Rozanov DV, Doxsey SJ, Strongin AY. Membrane type-1 matrix metalloproteinase (MT1-MMP) exhibits an important intracellular cleavage function and causes chromosome instability. *J. Biol. Chem* 2005;280:25079–25086. [PubMed: 15878869]
19. Remacle AG, Rozanov DV, Baciuc PC, Chekanov AV, Golubkov VS, Strongin AY. The transmembrane domain is essential for the microtubular trafficking of membrane type-1 matrix metalloproteinase (MT1-MMP). *J. Cell Sci* 2005;118:4975–4984. [PubMed: 16219679]

20. Deryugina EI, Bourdon MA, Jungwirth K, Smith JW, Strongin AY. Functional activation of integrin alpha V beta 3 in tumor cells expressing membrane-type 1 matrix metalloproteinase. *Int. J. Cancer* 2000;86:15–23. [PubMed: 10728589]
21. Jiang A, Lehti K, Wang X, Weiss SJ, Keski-Oja J, Pei D. Regulation of membrane-type matrix metalloproteinase 1 activity by dynamin-mediated endocytosis. *Proc. Natl. Acad. Sci. U.S.A* 2001;98:13693–13698. [PubMed: 11698655]
22. Lehti K, Valtanen H, Wickstrom SA, Lohi J, Keski-Oja J. Regulation of membrane-type-1 matrix metalloproteinase activity by its cytoplasmic domain. *J. Biol. Chem* 2000;275:15006–15013. [PubMed: 10748199]
23. Remacle A, Murphy G, Roghi C. Membrane type I-matrix metalloproteinase (MT1-MMP) is internalised by two different pathways and is recycled to the cell surface. *J. Cell Sci* 2003;116:3905–3916. [PubMed: 12915589]
24. Maquoi E, Franken F, Baramova E, Munaut C, Sounni NE, Remacle A, Noel A, Murphy G, Foidart JM. Membrane type 1 matrix metalloproteinase-associated degradation of tissue inhibitor of metalloproteinase 2 in human tumor cell lines. *J. Biol. Chem* 2000;275:11368–11378. [PubMed: 10753951]
25. Osenkowski P, Toth M, Fridman R. Processing, shedding, and endocytosis of membrane type 1-matrix metalloproteinase (MT1-MMP). *J. Cell Physiol* 2004;200:2–10. [PubMed: 15137052]
26. Zamora M, Granell M, Mampel T, Vinas O. Adenine nucleotide translocase 3 (ANT3) overexpression induces apoptosis in cultured cells. *FEBS Lett* 2004;563:155–160. [PubMed: 15063741]
27. Yang Z, Cheng W, Hong L, Chen W, Wang Y, Lin S, Han J, Zhou H, Gu J. Adenine nucleotide (ADP/ATP) translocase 3 participates in the tumor necrosis factor induced apoptosis of MCF-7 cells. *Mol. Biol. Cell* 2007;18:4681–4689. [PubMed: 17855512]
28. Chevrollier A, Loiseau D, Chabi B, Renier G, Douay O, Malthiery Y, Stepien G. ANT2 isoform required for cancer cell glycolysis. *J. Bioenerg. Biomembr* 2005;37:307–316. [PubMed: 16341775]
29. Giraud S, Bonod-Bidaud C, Wesolowski-Louvel M, Stepien G. Expression of human ANT2 gene in highly proliferative cells: GRBOX, a new transcriptional element, is involved in the regulation of glycolytic ATP import into mitochondria. *J. Mol. Biol* 1998;281:409–418. [PubMed: 9698557]
30. Jang JY, Choi Y, Jeon YK, Kim CW. Suppression of adenine nucleotide translocase-2 by vector-based siRNA in human breast cancer cells induces apoptosis and inhibits tumor growth *in vitro* and *in vivo*. *Breast Cancer Res* 2008;10:R11. [PubMed: 18267033]
31. Marzo I, Brenner C, Zamzami N, Jurgensmeier JM, Susin SA, Vieira HL, Prevost MC, Xie Z, Matsuyama S, Reed JC, Kroemer G. Bax and adenine nucleotide translocator cooperate in the mitochondrial control of apoptosis. *Science* 1998;281:2027–2031. [PubMed: 9748162]
32. Verrier F, Deniaud A, Lebras M, Metivier D, Kroemer G, Mignotte B, Jan G, Brenner C. Dynamic evolution of the adenine nucleotide translocase interactome during chemotherapy-induced apoptosis. *Oncogene* 2004;23:8049–8064. [PubMed: 15377997]
33. Martinez LO, Jacquet S, Esteve JP, Rolland C, Cabezón E, Champagne E, Pineau T, Georgeaud V, Walker JE, Terce F, et al. Ectopic beta-chain of ATP synthase is an apolipoprotein A-I receptor in hepatic HDL endocytosis. *Nature* 2003;421:75–79. [PubMed: 12511957]
34. Wang T, Chen Z, Wang X, Shyy JY, Zhu Y. Cholesterol loading increases the translocation of ATP synthase beta chain into membrane caveolae in vascular endothelial cells. *Biochim. Biophys. Acta* 2006;1761:1182–1190. [PubMed: 16996794]
35. D'Alessio S, Ferrari G, Cinnante K, Scheerer W, Galloway AC, Roses DF, Rozanov DV, Remacle AG, Oh ES, Shiryaev SA, et al. Tissue inhibitor of metalloproteinases-2 binding to membrane-type 1 matrix metalloproteinase induces MAPK activation and cell growth by a non-proteolytic mechanism. *J. Biol. Chem* 2008;283:87–99. [PubMed: 17991754]
36. Takino T, Watanabe Y, Matsui M, Miyamori H, Kudo T, Seiki M, Sato H. Membrane-type 1 matrix metalloproteinase modulates focal adhesion stability and cell migration. *Exp. Cell Res* 2006;312:1381–1389. [PubMed: 16473349]
37. Ridley AJ, Schwartz MA, Burridge K, Firtel RA, Ginsberg MH, Borisy G, Parsons JT, Horwitz AR. Cell migration: integrating signals from front to back. *Science* 2003;302:1704–1709. [PubMed: 14657486]

38. Faure Vigny H, Heddi A, Giraud S, Chautard D, Stepien G. Expression of oxidative phosphorylation genes in renal tumors and tumoral cell lines. *Mol. Carcinog* 1996;16:165–172. [PubMed: 8688152]
39. Lunardi J, Attardi G. Differential regulation of expression of the multiple ADP/ATP translocase genes in human cells. *J. Biol. Chem* 1991;266:16534–16540. [PubMed: 1885585]
40. Stepien G, Torroni A, Chung AB, Hodge JA, Wallace DC. Differential expression of adenine nucleotide translocator isoforms in mammalian tissues and during muscle cell differentiation. *J. Biol. Chem* 1992;267:14592–14597. [PubMed: 1378836]
41. Cozens AL, Runswick MJ, Walker JE. DNA sequences of two expressed nuclear genes for human mitochondrial ADP/ATP translocase. *J. Mol. Biol* 1989;206:261–280. [PubMed: 2541251]
42. Dolce V, Scarcia P, Iacopetta D, Palmieri F. A fourth ADP/ATP carrier isoform in man: identification, bacterial expression, functional characterization and tissue distribution. *FEBS Lett* 2005;579:633–637. [PubMed: 15670820]
43. Kuan J, Saier MH Jr. The mitochondrial carrier family of transport proteins: structural, functional, and evolutionary relationships. *Crit. Rev. Biochem. Mol. Biol* 1993;28:209–233. [PubMed: 8325039]
44. Pebay-Peyroula E, Dahout-Gonzalez C, Kahn R, Trezeguet V, Lauquin GJ, Brandolin G. Structure of mitochondrial ADP/ATP carrier in complex with carboxyatractyloside. *Nature* 2003;426:39–44. [PubMed: 14603310]
45. Belzacq AS, Brenner C. The adenine nucleotide translocator: a new potential chemotherapeutic target. *Curr. Drug Targets* 2003;4:517–524. [PubMed: 14535652]
46. Le Bras M, Borgne-Sanchez A, Touat Z, El Dein OS, Deniaud A, Maillier E, Lecellier G, Rebouillat D, Lemaire C, Kroemer G, et al. Chemosensitization by knockdown of adenine nucleotide translocase-2. *Cancer Res* 2006;66:9143–9152. [PubMed: 16982757]
47. Miyazaki E, Kida Y, Mihara K, Sakaguchi M. Switching the sorting mode of membrane proteins from cotranslational endoplasmic reticulum targeting to posttranslational mitochondrial import. *Mol. Biol. Cell* 2005;16:1788–1799. [PubMed: 15673615]
48. Stornaiuolo M, Lotti LV, Borgese N, Torrisi MR, Mottola G, Martire G, Bonatti S. KDEL and KKXX retrieval signals appended to the same reporter protein determine different trafficking between endoplasmic reticulum, intermediate compartment, and Golgi complex. *Mol. Biol. Cell* 2003;14:889–902. [PubMed: 12631711]

Figure 1. Constructs of MT1-MMP and ANT2

The FLAG tag sequence was inserted in the hinge region and linked to the C-terminus of MT1-MMP and ANT2, respectively. In the ANT2-RFP chimaera, RFP was C-terminally linked to the ANT2 sequence. S, signal peptide; PRO, prodomain; CAT, catalytic domain; H, hinge region; PEX, PEX domain; ST, stalk region; TM, transmembrane domain; CT, cytoplasmic tail; L1–L5, short loop sequences that link the six transmembrane domains of ANT2.

Figure 2. MT1-MMP specifically interacts with ANT2 in breast carcinoma MCF cells
(A) MT1-MMP-FLAG was immunoprecipitated (IP) with anti-FLAG beads from MT-E240A-FLAG, MT-WT-FLAG, MT-ΔCT-FLAG and MT-E240A cells (control). The precipitated samples were separated by gel electrophoresis and stained with a silver stain.
(B) Left panel: MT1-MMP was immunoprecipitated from MT-E240A cells using the monoclonal antibody 3G4 against the catalytic domain. The precipitated sample was separated by gel electrophoresis and stained with a silver stain. Right panel: to show the presence of ANT2, an aliquot of the precipitated sample was analysed by Western blotting (WB) with the ANT antibody. ANT2 was absent in the control sample immunoprecipitated from MT-E240A cells with anti-FLAG beads.
(C) MT1-MMP-FLAG was immunoprecipitated with anti-FLAG

beads from MT-E240A-FLAG, MT-WT-FLAG and MT- Δ CT-FLAG cells and also from MT-E240A, MT-WT and MT- Δ CT cells (the latter three are controls). The immunoprecipitated samples were analysed by Western blotting with the ANT and α -tubulin antibodies. The presence of the MT1-MMP-FLAG constructs in the experimental samples was confirmed using Western blotting with the FLAG antibody (upper panels).

Figure 3. ANT2 specifically interacts with MT1-MMP in fibrosarcoma HT cells

(A) Left panel: ANT2-FLAG was immunoprecipitated (IP) with anti-FLAG-beads from ANT2-FLAG cells and mock-transfected control cells (two left lanes). As an additional control, naturally produced MT1-MMP was immunoprecipitated from mock cells using the 3G4 antibody against MT-CAT (right lane, MT-CAT). The precipitated samples were separated by gel electrophoresis and stained with a silver stain. Right panel: the ANT2-FLAG and mock samples precipitated with anti-FLAG beads were analysed by Western blotting (WB) with the ANT and MT1-MMP 3G4 antibodies. (B) The ANT2-FLAG and mock cell lysates (cell lysates) were fractionated using anti-FLAG-beads to separate the unbound and anti-FLAG-bound (IP) protein fractions. The fractions were analysed by Western blotting with the MT1-MMP Ab815 antibody to the hinge region and also with the ATP synthase, ANT, Bcl-2 and Bax antibodies. (C) The lysate of surface-biotinylated HT-ANT2-FLAG cells (cell lysate) was fractionated using streptavidin beads to separate the unbound and streptavidin-bound (IP) protein fractions. The fractions were analysed by Western blotting with the ANT antibody.

Figure 4. ANT2 is expressed on the surface of breast carcinoma MCF cells

(A) Left panel: MCF-MT-E240A-FLAG, MCF-MT-WT-FLAG, MCF-MT-E240A and MCF-MT-WT cells (the latter two are controls) were surface biotinylated with membrane-impermeable biotin and then lysed. The lysates were immunoprecipitated (IP) with anti-FLAG-beads. The precipitated samples were analysed by Western blotting (WB) with Extravidin conjugated with horseradish peroxidase (avidin-HRP). Right panel: Western blotting (WB) with the ANT antibody of MCF-MT-E240A-FLAG and MCF-MT-E240A samples confirms the presence of ANT in the FLAG-immunoprecipitated sample. (B) Surface-biotinylated MCF-MT-E240A-FLAG cells were lysed (cell lysate) and the biotin-labelled proteins were precipitated using streptavidin beads. The unbound fraction was immunoprecipitated with anti-FLAG beads (FLAG IP). The cell lysate, the unbound, the streptavidin-precipitated (streptavidin IP) and the FLAG IP fractions were analysed by Western blotting with the FLAG, ANT and ATP synthase antibodies.

Figure 5. Co-localization of MT1-MMP and ANT2 on the surface of MCF cells

MCF-MT-WT cells were transiently transfected with the ANT2-RFP chimera. The MCF-MT-WT/ANT2-RFP cells and MCF-MT-WT cells transiently transfected with the empty vector (control) were stained with the MT1-MMP antibody followed by an Alexa Fluor® 488-conjugated secondary antibody and the co-localization (merged) of the MT1-MMP immunoreactivity (green) with the RFP fluorescence (red) were analysed using fluorescence microscopy. Nuclei were stained with DAPI. The arrow points to the region with intense co-localization of the MT1-MMP immunoreactivity with ANT2-RFP.

BREAST CARCINOMA MCF-7 CELLS

Figure 6. The individual MT-CAT is sufficient for the ANT2 binding

Left: the MCF-MT-WT/ANT2-FLAG, MCF-ANT2-FLAG, MCF-MT-WT and MCF-mock cell lysates were immunoprecipitated (IP) with anti-FLAG beads to pull-down the cellular ANT2-FLAG-MT1-MMP complex. The presence of MT1-MMP in the precipitated samples was detected by Western blotting (WB) with the Ab815 antibody against the hinge region of MT1-MMP. Right: the soluble MT-CAT and MT-CAT-PEX constructs were each allowed to interact with ANT2-FLAG that was present in the MCF-ANT2-FLAG cell lysate. The samples were then immunoprecipitated using anti-FLAG-beads (IP). The precipitated material was analysed by Western blotting (WB) with the 3G4 antibody against MT-CAT.

Figure 7. ANT2 does not affect the expression of cellular MT1-MMP

The levels of total cell and cell surface-associated MT1-MMP in HT-mock and HT-ANT2-FLAG cells were determined by Western blotting (WB) with the 3G4 antibody against MT-CAT. The bands were scanned and the images were digitized. The ratio of the band density is shown at the bottom of the panel.