

Mitochondrial Chaperone Trap1 and the Calcium Binding Protein Sorcin Interact and Protect Cells against Apoptosis Induced by Antiblastic Agents

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Abstract

TRAP1, a mitochondrial chaperone (Hsp75) with antioxidant and antiapoptotic functions, is involved in multidrug resistance in human colorectal carcinoma cells. Through a proteomic analysis of TRAP1 coimmunoprecipitation complexes, the Ca²⁺-binding protein Sorcin was identified as a new TRAP1 interactor. This result prompted us to investigate the presence and role of Sorcin in mitochondria from human colon carcinoma cells. Using fluorescence microscopy and Western blot analysis of purified mitochondria and submitochondrial fractions, we showed the mitochondrial localization of an isoform of Sorcin with an electrophoretic motility lower than 20 kDa that specifically interacts with TRAP1. Furthermore, the effects of overexpressing or downregulating Sorcin and/or TRAP1 allowed us to demonstrate a reciprocal regulation between these two proteins and to show that their interaction is required for Sorcin mitochondrial localization and TRAP1 stability. Indeed, the depletion of TRAP1 by short hairpin RNA in colorectal carcinoma cells lowered Sorcin levels in mitochondria, whereas the depletion of Sorcin by small interfering RNA increased TRAP1 degradation. We also report several lines of evidence suggesting that intramitochondrial Sorcin plays a role in TRAP1 cytoprotection. Finally, preliminary evidence that TRAP1 and Sorcin are both implicated in multidrug resistance and are coregulated in human colorectal carcinomas is provided. These novel findings highlight a new role for Sorcin, suggesting that some of its previously reported cytoprotective functions may be explained by involvement in mitochondrial metabolism through the TRAP1 pathway. *Cancer Res*; 70(16); 6577–86. ©2010 AACR.

Introduction

The intrinsic or acquired resistance to anticancer drugs remains one of the most serious problems responsible for the failure of cancer chemotherapy (1). The cellular basis underlying multidrug resistance is not fully understood, and several mechanisms have been proposed. Our group recently showed that TRAP1, a mitochondrial chaperone (Hsp75) with antioxidant and antiapoptotic functions (2), is involved in the multidrug resistance of human colorectal carcinoma cells (3). In fact, TRAP1 protein levels are increased in

HT-29 colorectal carcinoma cells resistant to 5-fluorouracil (FU), oxaliplatin (l-OHP), and irinotecan (IRI), and in the majority of human colorectal cancers. Furthermore, HT-29 colorectal carcinoma and Saos-2 osteosarcoma cells transfected with TRAP1 exhibited a phenotype resistant to FU-, l-OHP-, and IRI-induced apoptosis, whereas a TRAP1 dominant-negative deletion mutant sensitized tumor cells to apoptotic cell death (3). Recent studies showed that TRAP1 can be proposed as a novel molecular target in localized and metastatic prostate cancer (4), and some other tumors (5), whereas other authors have pointed out that TRAP1 protects against oxidative stress and against focal ischemia in rat brain mitochondria (6).

Little is known about TRAP1 signal transduction. Kang and colleagues (7) identified TRAP1 as a member of a cytoprotective network selectively active in mitochondria of tumor tissues. Other studies suggested an involvement of this chaperone in cell adhesion and synaptic morphology through the modulation of N-cadherin expression (8). Starting from this limited information, a proteomic analysis of TRAP1 coimmunoprecipitation complexes was performed in our laboratory to further characterize the TRAP1 network and evaluate protein interactors relevant for TRAP1 role in multidrug resistance. Among several proteins, we identified Sorcin as a new TRAP1 interactor. Sorcin is a 21.6-kDa Ca²⁺-binding protein that is a member of the penta EF-hand protein family

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and is widely distributed among mammalian tissues such as skeletal muscle, kidney, and brain, but most abundantly in cardiac muscle (9). After Ca^{2+} binding, the overall hydrophobicity of the protein increases, and this leads to the translocation from the cytoplasm to cell membranes, resulting in Ca^{2+} -dependent interaction with other proteins (e.g., the ryanodine receptor; ref. 10). Although several biochemical studies are available on the direct binding of Sorcin to both cardiac ryanodine receptor RyRs (9) and voltage-dependent L-type calcium channel $\text{CaV}1.2$ (11, 12), little is known about the function of this soluble Ca^{2+} -binding protein in chemoresistance. Indeed, intracellular Ca^{2+} concentration may play a role in the development of chemoresistance, as suggested by clinical trials with verapamil, a Ca^{2+} channel blocker which selectively enhances drug cytotoxicity in MDR cell lines (13). Thus, some studies, aimed at investigating the regulatory role of Ca^{2+} in Taxol resistance, have suggested that chemoresistant cells express higher levels of the Ca^{2+} -binding protein Sorcin and the antiapoptotic Bcl2, compared with the parent cell line (14). Both these proteins have previously been implicated in chemoresistance, in part due to their ability to modulate Ca^{2+} levels. Other reports suggest a correlation between Sorcin and the multidrug-resistant MDR1/P-glycoprotein: it has been shown that the knockdown of Sorcin induces the upregulation of MDR1 in HeLa cells (15); by contrast, a direct correlation between Sorcin and multidrug resistance overexpression has also been reported in human gastric cancer cell lines and vincristine-resistant gastric cancer cells (16–18). Finally, recent studies aimed at evaluating changes in gene expression profile in oral squamous cell carcinoma and non-small cell lung cancer identified a cDNA homologous to Sorcin expressed only in tumors, apparently responsible for drug resistance and poor prognosis (19, 20).

This study shows for the first time that TRAP1 and a Sorcin isoform with an electrophoretic motility of 18 kDa interact in mitochondria, and that the result of this interaction may serve as an additional or alternative indirect route by which both proteins can regulate apoptosis and chemoresistance in colorectal carcinoma cells.

Materials and Methods

Chemicals, cell cultures, constructs, short hairpin RNAs and small interfering RNAs

I-OHP, FU, and IRI and all reagents were purchased by Sigma-Aldrich unless otherwise specified. MG-132 was purchased by Merck Chemicals Ltd-Calbiochem; shepherdin and control scrambled peptides were synthesized by PRIMM as reported in ref. (7).

Human Saos-2 osteosarcoma and HCT-116 colon carcinoma cells were purchased from the American Type Culture Collection (ATCC) and cultured in DMEM containing 10% fetal bovine serum, 1.5 mmol/L glutamine, and 100 U/mL penicillin and streptomycin. Cell lines are routinely monitored in our laboratory by microscopic morphology check. Authentication of cell lines has been checked before starting this study, <1 year ago, through standardized techniques by evaluating, respectively, the haplotype in Saos-2 cells (HLA A2,

A3, Bw16, and Bw47) and the presence of a mutation in codon 13 of the *ras* proto-oncogene in HCT-116 cells, according to ATCC product description.

Full-length TRAP1 cDNA was cloned in pRc-CMV vector (Invitrogen; ref. 21). cDNAs for 22- and 18-kDa sorcin isoforms were cloned in pRc-CMV or p-cDNA 3.1 vectors each carrying HA or Myc epitopes at the COOH terminus, respectively. Small interfering RNAs (siRNA) of TRAP1 and Sorcin were purchased from Qiagen. For knockdown experiments, siRNAs were diluted to a final concentration of 20 nmol/L and transfected according to the manufacturer's protocol. TRAP1-stable interference was achieved by transfecting HCT-116 cells with TRAP1 (*TGCTGTTGACAGTGAGCGACCCGGTCCCTGTACTCAGAAATAGTGAAGCCACAGATGTATTTCTGAGTACAGGGACCGGGTGCCTACTGCCTCGGA*) or scrambled (sequence containing no homology to known mammalian genes) short hairpin RNAs (shRNA) (Open Biosystem).

Fluorescence microscopy

The analysis of immunofluorescence was performed with a confocal laser scanner microscopy Zeiss 510 LSM (Carl Zeiss Microimaging GmbH), equipped with Argon ionic laser (Carl Zeiss Microimaging GmbH) whose λ was set up to 488 nm, a HeNe laser whose λ was set up to 546 nm, and an immersion oil objective, 63 \times /1.4 f.

Cotransfection experiment in cultured HCT116 cells were with 18-kDa Sorcin and mitochondria-targeted red fluorescence protein (p-mitoRFP) expression vectors.

Proteomic analysis of TRAP1 coimmunoprecipitation complexes

Lysis and immunoprecipitation procedures. TRAP1 complexes were isolated from Saos-2 osteosarcoma cell total extracts by affinity purification. Stable cells expressing TRAP1-HA were lysed and then incubated onto anti-HA agarose-conjugated antibody overnight under gentle stirring. Beads were collected by centrifugation (3,000 rpm for 5 minutes*), extensively washed with lysis buffer supplemented with 150 mmol/L NaCl to eliminate nonspecific bound proteins. Elution of the desired protein complexes was performed by competition with HA peptide in elution buffer. The eluted proteins were precipitated in methanol/chloroform and then loaded onto a 10% SDS-PAGE. The gel was stained with colloidal Coomassie blue (Thermo Scientific). Protein bands were excised from the gel, reduced, alkylated, and digested with trypsin (22).

Mass spectrometry analysis and protein identification

Peptide mixtures extracted from the gel were analyzed by nanochromatography tandem mass spectrometry (MS/MS) on a CHIP MS Ion Trap XCT Ultra equipped with a capillary 1100 high-performance liquid chromatography system and a chip cube (Agilent Technologies). Peptide analysis was performed using data-dependent acquisition of one MS scan (mass range from 400–2,000 m/z) followed by MS/MS scans of the three most abundant ions in each MS scan. Raw data from nanochromatography

MS/MS analyses were used to query a nonredundant protein database using the in-house MASCOT software (Matrix Science).

Cytotoxicity assays

Apoptosis was evaluated by cytofluorimetric analysis of Annexin V and 7-amino-actinomycin D-positive cells by using the FITC-Annexin V/7-amino-actinomycin D kit (Beckman Coulter). Stained cells were analyzed by "EPICS XL" Flow Cytometer (Beckman Coulter). Ten thousand events were collected per sample. Positive staining for Annexin V as well as double staining for Annexin V and 7-amino-actinomycin D were interpreted as signs of, respectively, early and late phases of apoptosis (23). In specific experiments, HCT-116 wild-type (WT) or HCT sh-TRAP1 cells were transiently cotransfected with pRc-CMV (mock) or 18-kDa Sorcin constructs and pEGFP-F (Clontech) and, 24 hours later, treated with 10 μ mol/L, FU, IRI or l-OHP. Drug-induced apoptosis was evaluated by propidium iodide labeling. The transfection vector pEGFR-F, which encodes for a farnesylated-enhanced green fluorescent protein (EGFP-F), was used as a reporter vector both to monitor transfection efficiency and as a cotransfection marker. Experiments were performed at least thrice using three replicates for each drug concentration.

Immunoblot analysis

Total cell lysates were obtained by homogenization of cell pellets in cold lysis buffer [20 mmol/L Tris (pH 7.5) containing 300 mmol/L sucrose, 60 mmol/L KCl, 15 mmol/L NaCl, 5% (v/v) glycerol, 2 mmol/L EDTA, 1% (v/v) Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride, 2 mg/mL aprotinin, 2 mg/mL leupeptin, and 0.2% (w/v) deoxycholate] for 1 minute at 4°C and further sonication for an additional 30 seconds at 4°C. Mitochondria were purified using the Qproteome Mitochondria Isolation kit (Qiagen) according to the manufacturer's protocol. Mitochondrial subfractionation was performed according to Tang and colleagues (24). Briefly, 150 μ g of mitochondria prepared from HCT-116 cells were swollen with hypotonic medium to break the outer mitochondrial membrane, keeping intact the inner mitochondrial membrane. The swollen mitochondria were pelleted by centrifugation; the supernatant contained the soluble proteins released from the inner mitochondrial membrane; and the pellet was the mitoplast fraction. Immunoblot analysis was performed as previously reported (2). Specific proteins were detected by using the following mouse monoclonal antibodies from Santa Cruz Biotechnology: anti-TRAP1 (sc-13557), anti-Sorcin (sc-100859), anti-cMyc (sc-40), anti-COX4 (sc-58348), anti-cytochrome *c* (sc-13156), anti-tubulin (sc-8035), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; sc-69778). The rabbit polyclonal anti-Mn-SOD was purchased from Upstate Biotechnology, and anti-F1-ATPase (sc-16690) was from Santa Cruz Biotechnology. Anti-Sorcin polyclonal antibody was a kind gift from Prof. E. Chiancone, University of Rome "La Sapienza", Rome, Italy.

Semiquantitative reverse transcription-PCR analysis

Total RNA was extracted using the Trizol Reagent (Invitrogen). The following primers used were as follows: TRAP1, forward 5'-GACGCACCGCTCAACAT-3', reverse 5'-CACATCAAACATGGACGGTTT-3'; Sorcin 22 kDa (transcript b) forward 5'-CGCAGTCTGCAGCATGGCG-3', reverse 5'-AAGCCGGCAAGTCTCCAGGT-3'; Sorcin 18 kDa (transcript a) forward 5'-GGCCACTCTGCAAGAAGGCA-3', reverse 5'-TCCGGGAGCCCCCTCCATACT-3'; GAPDH, forward 5'-CAAGGCTGAGAACGGGAA-3', reverse 5'-GCATCGCCCACTTGATTTT-3'. Reaction conditions were 95°C for 10 minutes, followed by 35 cycles of 30 seconds at 95°C, 30 seconds at 60°C, 5 minutes at 72°C, and 7 minutes at 72°C.

Statistical analysis

The paired Student's *t* test was used to establish the statistical significance between different levels of apoptotic cell death in cell lines overexpressing 18-kDa Sorcin or TRAP1, or in which Sorcin and/or TRAP1 expression had been knocked down compared with the respective mock-transfected control. Statistically significant values ($P < 0.05$) are reported in the Tables.

Results

Sorcin is a mitochondrial TRAP1 interactor

A "fishing for partners" strategy combined with mass spectrometric procedures was carried out to identify TRAP1 protein partners specifically interacting with the bait. Previous studies from our group showed that TRAP1 gene expression increased upon adaptation of human osteosarcoma cells to chronic mild oxidizing conditions and a phenotype resistant to H₂O₂- or cisplatin-induced DNA damage and apoptosis was generated upon transfection with TRAP1 (21). Therefore, Saos-2 osteosarcoma cells were stably transfected with the HA-TRAP1 construct or the empty vector as control. Total protein lysates were incubated with anti-HA-conjugated agarose beads and the retained samples were eluted by competition with HA peptide. The sample and the control were fractionated by 10% SDS-PAGE. The entire lanes from the gel were cut into slices, and each gel slice was submitted to the identification procedure (25). The resulting peptide mixtures were directly analyzed by mass spectrometry (liquid chromatography/tandem mass spectrometry, LC-MS/MS) and identified by the MASCOT protein database search. Proteins identified in the control and the sample lanes were discarded, whereas those proteins solely identified in the sample and absent in the control were selected as putative TRAP1 interactors. Among the putative TRAP1 binding partners, several mitochondrial proteins, mainly components of the membrane carrier family, were identified. Other interactors include cytoskeleton proteins as tubulin and some actin isoforms, as well as proteins involved in cell remodeling, as previously suggested by Kubota and colleagues (8), protein biosynthesis, posttranslational modifications and cell cycle regulation, as previously described (26). We focused our attention on a protein of ~18 kDa identified as Sorcin by LC-MS/MS analysis. No trace of this protein could be observed in

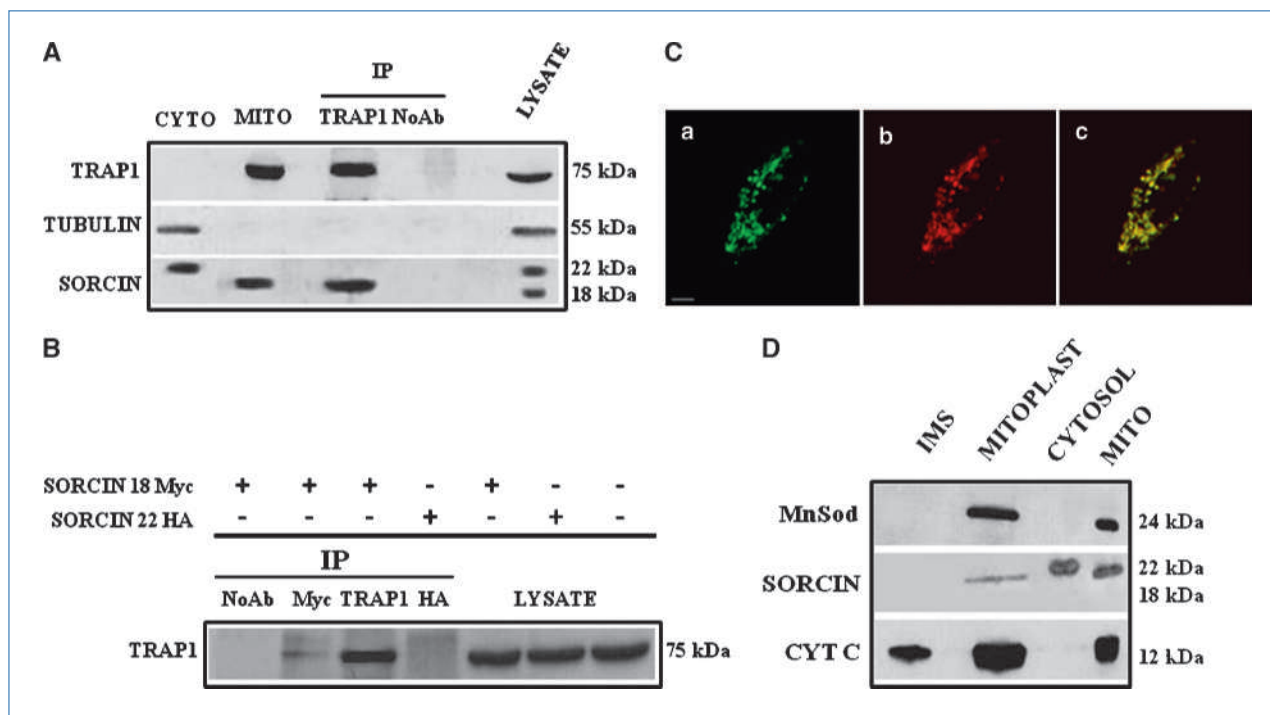


Figure 1. TRAP1 interacts with Sorcin within the mitochondria. **A**, total cell lysates from HCT-116 cells were fractionated in the cytosolic (CYTO) and mitochondrial (MITO) fractions, separated by SDS-PAGE and immunoblotted with mouse monoclonal anti-TRAP1, anti-Sorcin, and anti-tubulin antibodies. No Ab, total cellular extracts incubated with protein A/G-Sepharose without antibodies; IP, immunoprecipitation with the corresponding antibodies. **B**, HCT-116 were transfected with expression vectors specific for the 22- or 18-kDa Sorcin isoforms carrying, respectively, HA or Myc epitopes at the COOH terminus. Total cell lysates, and TRAP1 and Sorcin immunoprecipitates were analyzed by SDS-PAGE and immunoblotted with anti-TRAP1 antibodies. **C**, cotransfection experiment in HCT116 cells with pcDNA 3.1 Myc plasmid expressing 18-kDa sorcin cDNA (a) or mitochondria-targeted red fluorescence protein (mitoRFP) plasmid (p-mitoRFP); b). Fluorescence microscopy shows that the chimeric sorcin protein (a) colocalizes with the fluorescent protein tag of mitochondria (b). c, merge. Scale bar, 2 μ m. **D**, mitochondria prepared from HCT-116 cells were swollen with hypotonic medium to break the outer mitochondrial membrane, keeping the inner mitochondrial membrane intact as described in Materials and Methods. The swollen mitochondria were pelleted by centrifugation; the supernatant contained the soluble proteins released from the IMS and the pellet the mitoplast fraction. The purity of fractions was assessed by using the listed antibodies specific for the submitochondrial compartments.

any gel slice from the control lane. Interestingly, the Sorcin identified showed an electrophoretic mobility corresponding to a molecular mass slightly lower (18 kDa) than that reported in the Swiss Prot databank (22 kDa).

Our group is involved in the study of molecular mechanisms involved in chemoresistance of colorectal cancers (3). Therefore, TRAP1/Sorcin interaction was confirmed in HCT-116 (Figs. 1A and 2A) and HT-29 (data not shown) colon carcinoma cells by coimmunoprecipitation experiments using either anti-TRAP1 (Fig. 1A) or anti-Sorcin (Fig. 2A) antibodies. As shown in Fig. 1A, Western blot analysis using anti-Sorcin antibodies revealed two specific bands in total lysates from HCT-116 cells. Because TRAP1 is almost completely localized in mitochondria, a subfractionation of total lysates was performed and revealed that the 22-kDa Sorcin band is present only in the cytosolic fraction, whereas the lower molecular weight (LMW) protein with an electrophoretic motility of \sim 18 kDa is localized in the mitochondria (Fig. 1A). Interestingly, we observed that TRAP1 interacts specifically with the 18-kDa Sorcin isoform (Fig. 1A). Accordingly, coimmunoprecipitation analyses using mitochondrial lysates confirmed the specific interaction between TRAP1

and the LMW Sorcin (Supplementary Fig. S1). The identification of the 18-kDa Sorcin as specific to the mitochondrial compartment and the finding that it interacts with TRAP1 in mitochondria were both unexpected because Sorcin has been previously described as a cytosolic protein, with no details on its specific organelle localization. The finding of two Sorcin isoforms with different electrophoretic mobilities and the specific interaction of TRAP1 with the LMW isoform was further confirmed by immunoblot analysis of immunoprecipitates from HCT-116 cells transfected with either 22- or 18-kDa Sorcin expression vectors (Fig. 1B). These results agree with quantitative reverse transcription-PCR (RT-PCR) analyses on RNA purified from HCT-116 cells showing the presence of two independent transcripts encoding for two Sorcin isoforms, namely variants a and b (data not shown), as also reported in the BLAST databases (Supplementary Fig. S2). Finally, fluorescence microscopy analysis shows that the LMW sorcin isoform (a) colocalizes with mitoRFP (b) in mitochondria (Fig. 1C). To further characterize the submitochondrial compartments involved in the interaction, we purified mitochondrial subfractions and showed that TRAP1/Sorcin binding occurs in the mitoplast fraction,

which was separated upon hypotonic shock of purified mitochondria (Fig. 1D; ref. 24). These results agree with the sub-mitochondrial localization of TRAP1.

The interaction between Sorcin and TRAP1 is specific and requires the NH₂-terminal domain of TRAP1

The binding specificity between the two proteins was confirmed using protein lysates from HCT-116 cells in which TRAP1 expression was notably decreased by shRNAs (sh-TRAP1). Indeed, in these cells, coimmunoprecipitation analysis failed to detect the interaction between TRAP1 and LMW Sorcin (compare coimmunoprecipitation analyses in Fig. 2A and B) due to the very low intracellular TRAP1 levels after RNA interference (compare TRAP1 protein levels in the lanes of total lysates in Fig. 2A and B). As previously shown, TRAP1 has a mitochondrial targeting sequence located at the NH₂ terminus of the protein (26). To define which TRAP1 domain was involved in TRAP1/Sorcin interaction, we performed the same coimmunoprecipitation experiments with protein lysates obtained from HCT-116 cells transfected either with a mutant TRAP1 in which the first 100 amino acids at the NH₂-terminal end were fused to the myc tag (Fig. 2C), or with the Δ N mutant lacking the NH₂-terminal domain of TRAP1 and carrying an HA epitope at the COOH terminus (Fig. 2D). The results indicate that Sorcin binding requires the NH₂ terminus of TRAP1 protein (Fig. 2C) and that the interaction between these two proteins is lacking when the first 100

amino acids are deleted (Fig. 2D). This also agrees with the cytosolic localization of this TRAP1 deletion mutant (data not shown).

Reciprocal regulation of TRAP1 and Sorcin

We then sought to characterize the regulation that occurs between the two proteins upon their binding. To this aim, we measured the levels of each protein by overexpressing or downregulating either Sorcin and/or TRAP1, respectively. Figure 3A shows that a decreased expression of the LMW Sorcin isoform is detected in mitochondria of sh-TRAP1 HCT-116 colon carcinoma cells compared with WT cells. Because TRAP1 has chaperone activity and is the only member of the HSP90 family with mitochondrial localization (26), and considering that Sorcin is devoid of a canonical mitochondrial localization sequence, we hypothesized that TRAP1 might be involved in Sorcin transport to mitochondria. With this aim, we showed that the transfection of TRAP1 expression vectors in cells with very low amount of TRAP1 (sh-TRAP1) restores mitochondrial levels of LMW Sorcin to those observed in WT cells (Fig. 3A). The fact that the "low Sorcin" phenotype in sh-TRAP1 cells is rescued upon TRAP1 transfection suggests an involvement of TRAP1 in the mitochondrial transport of the LMW Sorcin isoform. As a control, interference of either HSP90 or HSP70 by specific siRNAs did not affect the levels of Sorcin (data not shown). Conversely, TRAP1 protein levels decrease in colorectal carcinoma cells upon

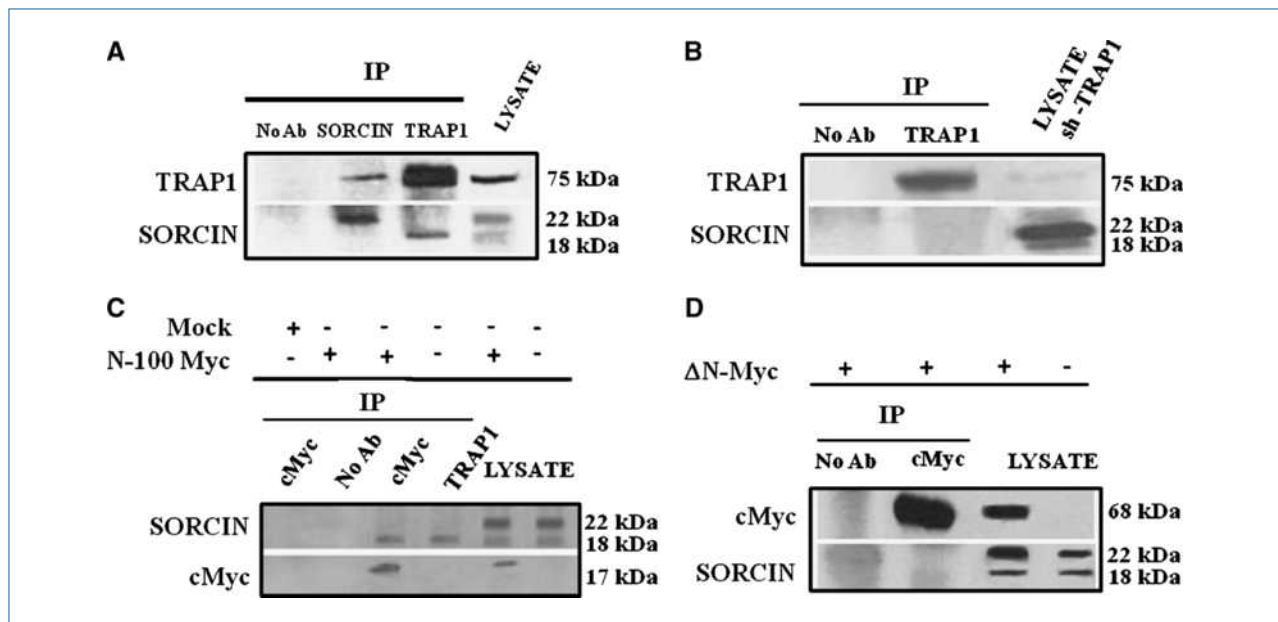


Figure 2. Characterization of TRAP1 domains involved in the interaction with Sorcin. Protein lysates from different cells (see below) were immunoprecipitated with the indicated antibodies, analyzed by SDS-PAGE, and immunoblotted with the listed antibodies. No Ab, total cellular extracts incubated with protein A/G-Sepharose without antibodies; IP, immunoprecipitation with the corresponding antibodies. A, WT HCT-116 cells. B, HCT-116 cells in which TRAP1 expression was stably decreased by shRNA (sh-TRAP1). C, HCT-116 cells were transfected with a mutant TRAP1 expression vector containing cDNA sequences coding for amino acids 1 to 100 at the NH₂ end, fused to cMyc epitope at the COOH terminus (N100-Myc). D, HCT-116 cells were transfected with a mutant TRAP1 construct lacking 100 amino acids at the NH₂ end and containing cDNA sequences coding for amino acids 101 to 604 fused to cMyc epitope at the COOH terminus (Δ N-Myc).

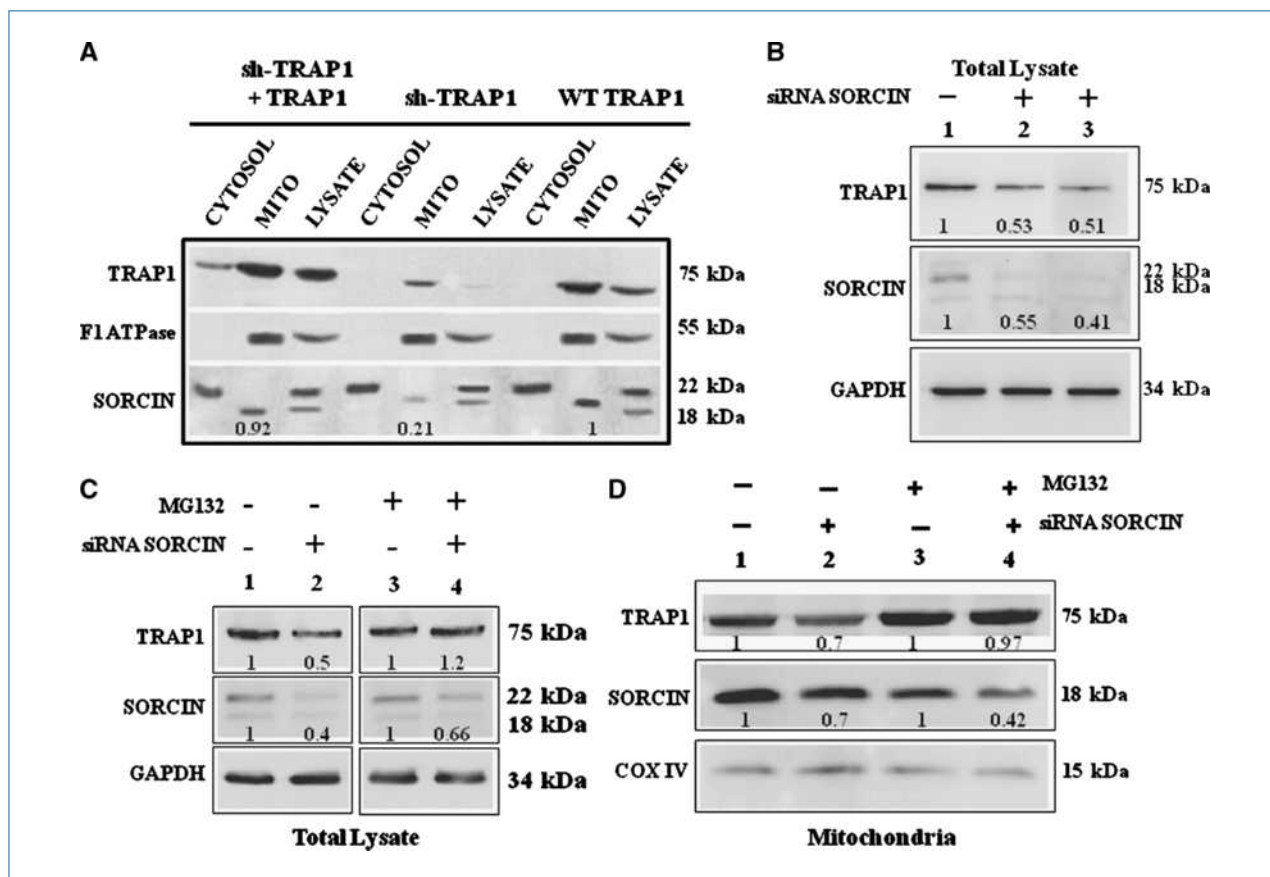


Figure 3. TRAP1/Sorcin reciprocal regulation. A, total cell lysates from WT HCT-116, sh-TRAP1, or sh-TRAP1 cells transfected with TRAP1 full-length construct were fractionated in the cytosolic (CYTOSOL) and mitochondrial (MITO) fractions as described in Materials and Methods, separated by SDS-PAGE, and immunoblotted with mouse monoclonal anti-TRAP1 and anti-SorcIn antibodies, and rabbit polyclonal anti-F1 ATPase antibodies. Note that the densitometric band intensities of LMW SorcIn in mitochondria of indicated cell populations are 1 (WT), 0.21 (sh-TRAP1 cells), and 0.92 (sh-TRAP1+TRAP1), respectively. Three independent experiments were performed with similar results. B, total cell lysates from HCT-116 cells transfected with nonspecific siRNA as a control (line 1) or two independent siRNAs of SorcIn (lines 2 and 3) were separated by SDS-PAGE and immunoblotted with mouse monoclonal anti-TRAP1, rabbit polyclonal anti-SorcIn, and mouse monoclonal anti-GAPDH antibodies. Note that densitometric band intensities for TRAP1 or 22-kDa SorcIn are indicated by numbers by assuming protein levels of the controls (WT cells) are equal to 1. Three independent experiments were performed with similar results. C and D, TRAP1 and SorcIn immunoblot analysis of total cell lysates (C) and mitochondrial fractions (D) from HCT-116 cells transfected with siRNA-negative control (lines 1 and 3) or siRNA of SorcIn (lines 2 and 4), and cultured in the presence of the proteasome inhibitor MG-132 (250 nmol/L) for 48 h (lines 3 and 4), or the inhibitor-free solvent (lines 1 and 2). Note that densitometric band intensities for TRAP1 or 18-kDa SorcIn are indicated by numbers by assuming protein levels of the respective control (mock-transfected cells) are equal to 1. Data represent the mean of three independent experiments.

transient downregulation of SorcIn expression by specific siRNAs (Fig. 3B). These results show a reciprocal regulation between TRAP1 and SorcIn. Previous data suggest that this calcium-binding protein could be involved in the regulation of the gene expression of MDR1 in HeLa cells (15). The subcellular fractionation analysis by Western blot shown in Fig. 3C and D indeed suggests that SorcIn's functional role could be to contribute to TRAP1 protein stability. In fact, the decreased TRAP1 expression observed upon SorcIn interference (Fig. 3C, lane 2) is prevented by the pretreatment of colon carcinoma cells with the proteasome inhibitor MG132 (Fig. 3C, lane 4). The same results were obtained using purified mitochondrial lysates (Fig. 3D). In agreement with posttranslational regulatory mechanisms, we confirmed that mRNA levels of TRAP1 were

not affected by SorcIn interference, thus excluding transcriptional regulation (Supplementary Fig. S3).

TRAP1/SorcIn interaction is prevented upon Ca^{2+} chelation

It is known that SorcIn may modulate the cytoplasmic release of Ca^{2+} , mainly from smooth endoplasmic reticulum in neurons (27). We therefore evaluated whether TRAP1/SorcIn interaction could be modulated by chelating free Ca^{2+} with ethylene glycol tetraacetic acid. As shown in Supplementary Fig. S4, binding between the two proteins is greatly decreased in the presence of 0.1 mmol/L ethylene glycol tetraacetic acid, being completely abolished at a higher concentration (0.5 mmol/L) of the Ca^{2+} chelator. This suggests a regulatory role of Ca^{2+} in TRAP1/SorcIn binding.

TRAP1 and Sorcin are both implicated in multidrug resistance and are coupled in human colorectal carcinomas

We previously showed the role of TRAP1 in the resistance of colon carcinoma cells to FU-, I-OHP-, and IRI-induced apoptosis (3), and data are available on Sorcin involvement in multidrug resistance in gastric cancer cell lines (16). Here, we confirm an analogous role of Sorcin in drug resistance in HCT-116 colon carcinoma cells exposed to the same three drugs. Indeed, HCT-116 were cultured for 48 hours in the presence of FU, IRI, or I-OHP, and evaluated for the rate of apoptotic cell death. Cell sensitivity to each drug is expressed as a ratio between drug-induced and vehicle-induced apoptosis (Table 1). Interestingly, protection by the 18-kDa Sorcin isoform toward the programmed cell death induced by FU, I-OHP, and IRI is observed (Table 1). To study the functional effects of TRAP1/Sorcin interaction on the protection from apoptosis, we evaluated the rates of apoptotic cell death in colorectal carcinoma HCT-116 cells treated with I-OHP upon transient (siRNA) or stable (shRNA) downregulation of TRAP1 and/or Sorcin gene expression. As shown in Table 2, the silencing of either TRAP1 or Sorcin by siRNA enhanced drug-induced apoptosis. However, TRAP1/Sorcin double knockdown did not induce any additional effect on cell death (Table 2), thus suggesting that the two proteins concomitantly contribute to cytoprotection by interacting and working together in a common pathway. Accordingly, in parallel experiments, drug-induced cell death was evaluated in sh-TRAP1 HCT-116 cells transfected with the 18-kDa Sorcin isoform (Table 1). Interestingly, although HCT-116 cells overexpressing the 18-kDa Sorcin isoform are characterized by a reduced sensitivity to FU,

I-OHP, and IRI, the downregulation of TRAP1 abolishes this apoptosis-resistant phenotype, further supporting the hypothesis that Sorcin and TRAP1 cooperate in protecting from cell death and that the cytoprotective activity of Sorcin requires TRAP1 to be functioning (Table 1).

It was recently shown that shepherdin, a mitochondria-directed peptidomimetic, induces programmed cell death by disrupting a cytoprotective pathway involving TRAP1, selectively active in tumor cell mitochondria (7). We have previously shown that, indeed, this network is activated in colon carcinoma cells (3). Here, we show that Sorcin interference by siRNAs does not increase sensitivity to shepherdin-induced apoptosis either in the presence or absence of TRAP1 (Supplementary Table S1). Again, these results suggest an interdependent activity on the part of both proteins, which interact to protect cells from mitochondrial apoptosis.

Based on the evidence that TRAP1 and Sorcin cooperate in inducing an apoptosis-resistant phenotype in colorectal carcinoma cells, we sought to evaluate TRAP1 and Sorcin expression in human colorectal carcinomas. Interestingly, we observed a concomitant upregulation of both Sorcin isoforms and TRAP1 protein levels in 44% of our series of 41 human colorectal carcinomas, and a parallel increase in TRAP1 and Sorcin mRNA expression levels evaluated by semiquantitative PCR analyses. Figure 4 shows the protein and mRNA levels of TRAP1 and Sorcin in three tumor specimens and in the respective normal noninfiltrated mucosas, chosen as representative analytic results of our series. A parallel upregulation of TRAP1 and Sorcin was observed in colorectal carcinoma cells resistant to FU, I-OHP, and IRI (data not shown).

Table 1. Rates of apoptotic cell death in colorectal carcinoma cells transiently transfected with pRc-CMV (mock) or 18-kDa Sorcin constructs and pEGFP-F vector treated with FU, I-OHP, or IRI

	<u>Apoptosis (% ± SD)</u>	<u>Ratio (±SD)</u>	<u>Apoptosis (% ± SD)</u>	<u>Ratio (±SD)</u>	<u>P</u>
	Mock		18-kDa Sorcin		
HCT-116 cells					
Vehicle	1.9 ± 0.2		2.4 ± 0.3		
10 µmol/L FU	9.6 ± 0.9	5.0 ± 1.2	3.3 ± 0.4	1.4 ± 0.4	0.008*
10 µmol/L I-OHP	10.5 ± 1.1	5.5 ± 1.3	1.9 ± 0.6	0.8 ± 0.4	0.008*
10 µmol/L IRI	12.7 ± 1.2	6.7 ± 1.5	3.4 ± 0.5	1.4 ± 0.5	0.004*
HCT-116 shRNA TRAP1					
Vehicle	2.2 ± 0.3		2.6 ± 0.2		
10 µmol/L FU	21.5 ± 0.9	9.7 ± 2.1	24.3 ± 0.5	9.3 ± 1.0	n.s.*
10 µmol/L I-OHP	24.9 ± 0.8	11.3 ± 2.2	25.1 ± 0.4	9.6 ± 1.0	n.s.*
10 µmol/L IRI	28.3 ± 0.9	12.9 ± 2.5	31.4 ± 0.7	12.1 ± 1.3	n.s.*

NOTE: Ratios are calculated between rates of apoptosis in drug- and vehicle-treated cells. *P* values indicate the statistical significance between the ratios of apoptosis in Sorcin- and mock-transfected cells. Apoptotic rates are calculated based only on sorted pEGFP-F-positive cells.

Abbreviation: n.s., not significant.

*Compared with mock-transfected cells.

Table 2. Rates of apoptotic cell death in colorectal carcinoma HCT-116 cells treated with I-OHP upon transient (siRNA) or stable (shRNA) downregulation of TRAP1 and/or Sorcin gene expression

	Apoptosis (% ± SD)	Ratio (± SD)	P
siRNA-negative control			
Control	2.6 ± 0.2		
10 µmol/L I-OHP	5.9 ± 0.4	2.3 ± 0.3	
30 µmol/L I-OHP	23.6 ± 1.2	9.1 ± 1.2	
siRNA Sorcin			
Control	1.1 ± 0.1		
10 µmol/L I-OHP	7.5 ± 0.5	6.8 ± 1.2	0.003
30 µmol/L I-OHP	19.0 ± 1.4	17.3 ± 3.1	0.013
siRNA TRAP1			
Control	1.6 ± 0.2		
10 µmol/L I-OHP	21.3 ± 1.4	13.3 ± 2.9	0.003
30 µmol/L I-OHP	28.0 ± 1.2	17.5 ± 3.3	0.014
siRNA TRAP1/Sorcin			
Control	1.7 ± 0.3		
10 µmol/L I-OHP	15.9 ± 1.1	9.3 ± 2.8	n.s.*
30 µmol/L I-OHP	28.6 ± 1.3	16.8 ± 4.5	n.s.*
shRNA TRAP1			
Control	2.4 ± 0.2		
10 µmol/L I-OHP	20.2 ± 1.2	8.4 ± 1.3	0.001
30 µmol/L I-OHP	27.3 ± 0.7	11.4 ± 1.3	0.007
shRNA TRAP1/siRNA Sorcin			
Control	2.6 ± 0.2		
10 µmol/L I-OHP	17.7 ± 1.0	6.8 ± 1.0	n.s.†
30 µmol/L I-OHP	32.4 ± 1.5	12.5 ± 1.6	n.s.†

NOTE: Ratios are calculated between rates of apoptosis in drug- and vehicle-treated cells. *P* values indicate the statistical significance between the ratios of apoptosis induced by I-OHP in transfected cells and the siRNA-negative control.

Abbreviation: n.s., not significant.

*Compared with either siRNA Sorcin or siRNA TRAP1-transfected cells.

†Compared with shRNA TRAP1 transfected cells.

Discussion

This article provides evidence of a new mitochondrial network involving TRAP1 and the Ca²⁺-binding protein Sorcin. Using mass spectrometry analysis of TRAP1 coimmunoprecipitating proteins, we identified Sorcin as a novel TRAP1 interactor. To our knowledge, this is the first time that Sorcin had been found to associate to a mitochondrial protein. This finding prompted us to investigate the subcellular localization of Sorcin because none of the previous data described the organelle compartmentalization of this protein. Cell fractionation experiments clearly indicate the occurrence of two main Sorcin isoforms: the 22-kDa isoform, quantitatively the major Sorcin band, is found in the cytosol, whereas the LMW 18-kDa isoform is specifically localized in the mitochondrial fraction. According to this finding, we show that TRAP1 interacts with the LMW Sorcin isoform within mitochondria, more specifically in mitoplasts (Fig. 1).

Sorcin is a Ca²⁺-sensitive protein, interacting with many important regulatory proteins involved in Ca²⁺ homeostasis. Consistent with these features, the existence of a functional role of Ca²⁺ in the interaction between Sorcin and TRAP1 is preliminarily shown by the interference of Sorcin mitochondrial localization upon Ca²⁺ depletion (see Supplementary Fig. S2). Although the role of Ca²⁺ in mitochondria is widely shown, little is known about the role of Ca²⁺ in TRAP1 signaling. Because Sorcin is highly sensitive to the modulation of Ca²⁺ levels, the interaction with TRAP1 and the consequent import of Sorcin into mitochondria, in which the homeostasis of calcium is strictly controlled (28), might contribute to defending the Ca²⁺-sensitive protein against perturbations of Ca²⁺ levels, most frequently observed in the cytosolic compartment. The selective binding of TRAP1 to LMW Sorcin could also be dependent on the same subcellular compartmentalization of both proteins. However, more in-depth analysis is required to characterize the molecular mechanisms involved in TRAP1/LMW Sorcin interaction (i.e., the effects of different protein structure/conformation of Sorcin isoforms on the interaction with TRAP1).

The interaction of Sorcin with a mitochondrial chaperone led us to investigate how the Ca²⁺-binding protein, which has no canonical mitochondria-targeting sequence, is imported into these organelles and to analyze whether Sorcin might be translocated into the mitochondrial compartment through a mechanism involving its interaction with TRAP1. Consistently with this assumption, we found that the localization of Sorcin in the mitochondria of human cells is dependent on the presence and abundance of TRAP1 (Fig. 3A). In fact, the levels of 18-kDa Sorcin decrease upon TRAP1 knockdown by shRNAs, whereas a rescue of this phenotype is achieved after transfection of colon carcinoma cells with TRAP1 expression vectors. Conversely, Sorcin interference might affect TRAP1 protein stability through posttranslational mechanisms, as suggested by preliminary experiments with the proteasome inhibitor MG132 (Fig. 3B–D). Although further investigation is necessary to clarify the mechanism involved in the regulation of TRAP1 expression by Sorcin in this regard,

it is important to underline that our findings are in line with previous studies indicating that the suppression of Sorcin induced a >3-fold increase in the expression of MDR1 (15), thus indicating a role by this protein in regulatory mechanisms of gene expression.

Apoptosis is the essential response induced in tumor cells by most chemotherapeutic agents. Several mechanisms are involved in resistance to chemotherapeutic agents in tumor cells (29). Among others, we recently studied, at a preclinical level, the role of TRAP1 in inducing a chemoresistant phenotype in human colorectal carcinoma (3), whereas a possible involvement of this mitochondrial chaperone in ovarian cancer has been suggested (30). Other studies have identified TRAP1 as a novel mitochondrial survival factor differentially expressed in localized and metastatic prostate cancer compared with normal prostate. Targeting this pathway could be explored as novel molecular therapy in patients with advanced prostate tumors (4). Sorcin has been implicated in chemoresistance and cancer as well. In fact, high levels of this protein have been reported in cancer cell lines and vincristine-resistant gastric cancer cell lines (16–18). Furthermore, Sorcin expression might be responsible for drug resistance and poor prognosis in non-small cell lung tumors (19, 20). The results reported here suggest that the previously described role of Sorcin in chemoresistance and cell survival may be dependent on its interaction with TRAP1 and involvement in TRAP1 mitochondrial antiapoptotic network.

To our knowledge, this study is the first evidence of a role played by Sorcin in resistance to FU, IRI, and I-OHP, three anti-blastic agents that represent the backbone of human colorectal carcinoma therapy (31). Interestingly, RNAi knockdown of

Sorcin and/or TRAP1 sensitizes human HCT-116 colon cancer cells to anticancer compounds that induce apoptosis, suggesting that targeting the TRAP1/Sorcin network directly in mitochondria may represent a novel therapeutic strategy for colorectal tumors. Indeed, it is very well established that regimens combining FU with IRI or I-OHP are equally effective as first-line treatment for advanced colorectal carcinoma (31), but achieve a response rate of 30% to 50% and an overall survival of 14 to 20 months (32). Thus, the development of novel strategies able to counteract multidrug resistance to improve the efficacy of systemic chemotherapy is a major issue in the clinical management of colorectal cancer patients. In such a perspective, our results suggest that the TRAP1/Sorcin pathway deserves to be further evaluated as a potential molecular target in human colorectal carcinoma for the clinical development of mitochondria-targeting agents. Indeed, a derivative of geldanamycin, 17AAG, is at present under clinical investigation in cancer patients due to its ability to block the HSP90/TRAP1 pathway, by binding the regulatory pocket in the NH₂-terminal domain of HSP90 (33), whereas other small molecules (e.g., shepherdin, gamitrinibs) have been designed to selectively accumulate in the mitochondria of human tumor cells targeting the HSP90/TRAP1 network and acting as ATPase antagonists (34). Finally, the clinical validation of novel biomarkers predictive of resistance/sensitivity to specific chemotherapy regimens is urgently required in human tumors. Thus, the novel preliminary finding of a parallel up-regulation of TRAP1 and Sorcin in human colorectal cancers highlights the existence of a functional link between the two

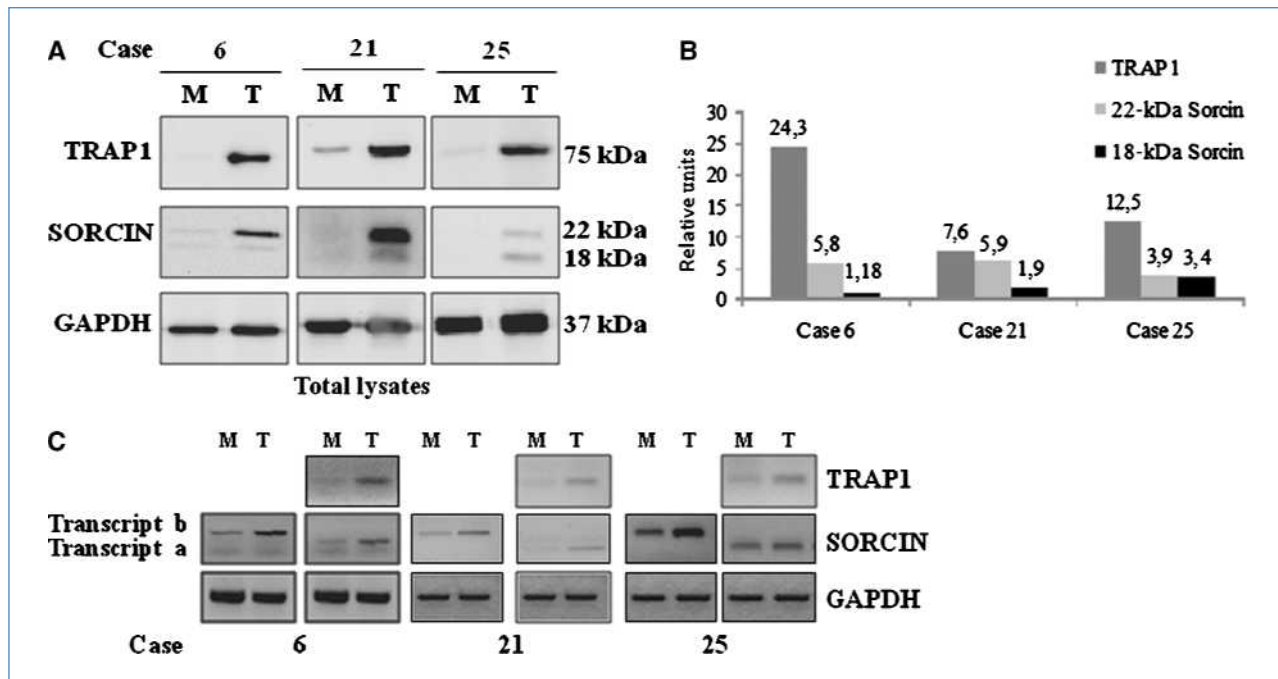


Figure 4. TRAP1 and Sorcin protein and mRNA levels in human colorectal carcinomas. A and C, protein (A) and mRNA (C) levels of TRAP1, and 18 and 22-kDa Sorcin in three colorectal tumors (T), and the corresponding noninfiltrated peritumoral mucosas (M) analyzed by immunoblot (A) and semiquantitative RT-PCR (C). B, densitometric analysis of TRAP1 and 18- and 22-kDa Sorcin isoforms in the immunoblot analysis reported in A. All the values are expressed as fold increases, by assuming protein levels of the controls (M) are equal to 1.

proteins, suggesting the opportunity to further characterize these two polypeptides as candidate biomarkers to predict the response of colon cancer patients to antitlastic treatments and personalize individual therapeutic strategies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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