MOLECULAR AND CELLULAR BIOLOGY, Aug. 2004, p. 7091–7101 Vol. 24, No. 16
0270-7306/04/$08.00+0 DOI: 10.1128/MCB.24.16.7091–7101.2004

Inhibition of Nuclear Import by the Proapoptotic Protein CC3

Frank W. King and Emma Shtivelman*

Cancer Research Institute, University of California San Francisco, San Francisco, California 94115

Received 25 January 2004/Returned for modification 29 April 2004/Accepted 26 May 2004

We report here that the normal cellular protein CC3/TIP30, when in excess, inhibits nuclear import in vitro and in vivo. CC3 binds directly to the karyopherins of the importin β family in a RanGTP-insensitive manner and associates with nucleoporins in vivo. CC3 inhibits the nuclear import of proteins possessing either the classical nuclear localization signal or the M9 signal recognized by transportin. CC3 also inhibits nuclear translocation of transportin itself. Cells modified to express higher levels of CC3 have a slower rate of nuclear import and, as described earlier, show an increased sensitivity to death signals. A mutant CC3 protein lacking proapoptotic activity has a lower affinity for transportin, is displaced from it by RanGTP, and fails to inhibit nuclear import in vitro and in vivo. Together, our results support a correlation between the ability of CC3 to form a RanGTP-resistant complex with importins, inhibit nuclear import, and induce apoptosis. Significantly, a dominant-negative form of importin β1 shown previously to inhibit multiple transport pathways induces rapid cell death, strongly indicating that inhibition of nuclear transport serves as a potent apoptotic signal.

The bidirectional transport of macromolecules between the nucleus and cytoplasm through the nuclear pore complex (NPC) is an active and regulated process. It depends on carrier proteins known as importins and exportins (or karyopherins) that compose the importin β family with 22 members in humans (reviewed in reference 47). Karyopherins can interact with their cargoes directly or use adapter proteins. Importin β1 mediates nuclear import of proteins containing a basic nuclear localization signal (NLS) by using importin α (1, 16, 50). Using snurportin as an adapter, importin β imports small nuclear riboproteins (37). Importin β2, also known as transportin, recognizes the M9-type nuclear localization sequences and imports M9-containing RNA-binding proteins without requiring an adapter molecule (13, 34, 39).

Importin β family members mediate interactions of transport complexes with the NPC. They also bind to the small GTPase Ran that controls the directionality of the transport (reviewed in references 14 and 24). Importins of the β family bind specifically to the GTP loaded form of Ran found in the nucleus. The importin-cargo complex docks at the cytoplasmic face of the NPC, followed by the translocation across the NPC via interactions with nucleoporins (5). At the terminal nucleoporin, the cargo is released from importin into the nucleus by RanGTP. Whereas RanGTP initiates the release of cargo from importins in the nucleus, exportins require RanGTP for binding to their various cargoes and subsequent nuclear export (reviewed in reference 49).

Although the sequence similarity among proteins of importin β family is low, they share conserved functional domains. They contain a N-terminal binding site for RanGTP and a NPC interacting domain. The latter mediates binding to the GLFG or FxFG repeats present in some nucleoporins (reviewed in reference 43). The cargo-binding domain of importins is located in the C terminus (reviewed in reference 47). The common structural feature of importins is that they consist entirely of tandem HEAT (huntingtin-elongation A subunit-TOR) repeats. HEAT repeats form extended superhelical structures that are predicted to be involved in creation of protein recognition interfaces (17). After RanGTP binding, the interconnected hairpin turns formed by HEAT repeats are thought to facilitate conformational changes in the structure of importin, which induces cargo release (9).

Nucleocytoplasmic transport could be inhibited by interfering with the activity of proteins that mediate transport. The inactivation of RanGTP (21) or the addition of excess of nucleoporin-binding domain of importin β (25) block nuclear transport. Nuclear transport is also inhibited during infection by some lytic viruses (8, 12, 18, 19). We report here that a cellular protein CC3, also known as TIP30, can inhibit nuclear import of substrates with different types of import signals. CC3 was first identified as a metastasis suppressor of the variant small cell lung carcinoma (SCLC) in vivo (45). Recently, a deficiency of CC3/TIP30 in germ line cells was shown to increase susceptibility to tumorigenesis (20). Expression of CC3 in tumor cells was shown to predispose them to apoptosis induced by a wide variety of death signals; acute overexpression of CC3 in cells induces cell death directly (51). CC3-induced apoptosis does not appear to depend on p53 or involve classical downstream death responses, such as mitochondrial release of cytochrome *c* (51), and its mechanism remains unclear. We demonstrate here that the proapoptotic properties of CC3 might stem from its ability to inhibit nuclear transport.

MATERIALS AND METHODS

Identification of CC3-binding proteins. Cells were metabolically labeled with [^{35}S]methionine and lysed in buffer A containing 25 mM Tris (pH 7.5), 0.5% Triton X-100, 150 mM NaCl, and Complete protease inhibitors (Roche). Glutathione *S*-transferase (GST) or GST-CC3 was added to the clarified lysate, and the samples were diluted fivefold with buffer B (25 mM Tris [pH 7.5], 100 mM NaCl, and protease inhibitors), followed by mixing at 4°C for 1 h. Proteins bound to GST-CC3 or GST were recovered with glutathione-Sepharose, resolved by sodium dodecyl sulfate–5% polyacrylamide gel electrophoresis (SDS–5% PAGE), and detected by autoradiography. For protein identification, bands were excised from Coomassie blue stained gels and subjected to matrix-assisted laser desorption ionization–time of flight mass spectrometry analysis at the Protein

* Corresponding author. Mailing address: Cancer Research Institute, 2340 Sutter St., San Francisco, CA 94115. Phone: (415) 502-1985. Fax: (415) 502-3179. E-mail: eshtivel@cc.ucsf.edu.

Chemistry Core Facility, Howard Hughes Medical Institute, Columbia University, New York, N.Y.

Protein expression and purification. GST and His tag fusion proteins were expressed in bacteria under standard conditions. GST fusion proteins were purified by affinity chromatography on glutathione-Sepharose beads (Amersham Biotechnology) and eluted from the beads with 20 mM reduced glutathione or by cleavage with thrombin. His-tagged proteins were first purified on Ni-nitrilotriacetic acid (NTA) agarose (Qiagen), followed by passage through a Superose-12 gel exclusion column (Amersham Biotechnology) in column buffer (25 mM HEPES [pH 7.6], 1 mM dithiothreitol [DTT], 5% glycerol, 150 mM NaCl) and concentrated with a Centricon-10 (Millipore). Coupled in vitro transcription-translation of proteins was done with wheat germ extracts in the presence of [^{35}S]methionine according to the manufacturer's protocol (Promega).

Antibodies to CC3. Polyclonal antisera to CC3 was raised in rabbits by using purified recombinant GST-CC3 as an antigen. Immune serum was purified first on column of immobilized GST protein to remove the GST-reactive antibodies, followed by affinity purification on a column of immobilized CC3 protein.

Protein labeling with fluorescein isothiocyanate (FITC). Recombinant proteins at 5 to 10 mg/ml were dialyzed in labeling buffer (25 mM HEPES [pH 7.6], 5% glycerol, 150 mM NaCl). Fluorescein-5-maleimide (Molecular Probes) was added to achieve a 2:1 molar ratio of fluorescein to protein, and samples were incubated for 1 h on ice. Nonbound fluorescein was removed by using a NAP-5 column (Amersham Biotechnology), followed by concentration with a Centricon-10 spin column.

Coimmunoprecipitation. HeLa cell lysates for immunoprecipitation were prepared by sonication as described previously (54) and subjected to immunoprecipitation with MAb414 (Covance) or anti-transportin antibodies (Transduction Labs). The immune complexes were collected on protein G-agarose (Invitrogen) and analyzed by Western blotting with relevant antibodies.

In vitro nuclear import assays. The in vitro nuclear import assays were performed according to the published protocol (2). HeLa cells grown on coverslips were washed with phosphate-buffered saline and permeabilized with 30 μg of digitonin/ml in buffer E (25 mM HEPES [pH 7.6], 1 mM DTT, 5% sucrose, 110 mM KH_3CO_2, 5 mM MgH_3CO_2, 2.5 mg of bovine serum albumin [BSA]/ml) for 5 min at room temperature. Permeabilized cells were washed four times with buffer E and inverted onto 15 μl of import mixture containing the indicated proteins added with or without cytosol from HeLa cells in transport buffer (buffer E plus 0.5 mM ATP, 0.5 mM GTP, 10 mM phosphocreatine, and 20 U of creatine kinase/ml). Coverslips were incubated 20 min at room temperature. Reactions were stopped with 2% paraformaldehyde. When cells were further processed for immunofluorescence, they were permeabilized with 0.2% Triton X-100 in phosphate-buffered saline for 10 min at room temperature prior to antibody staining.

GST pull-down assays. GST or GST fusion proteins at 10 μM were mixed for 1 h at 4°C with 7.5 μl (bed volume) of glutathione-Sepharose in a final volume of 20 μl in buffer F (25 mM HEPES [pH 7.6], 1 mM DTT, 5% glycerol, 100 mM KCl, 0.1% Triton X-100). Glutathione-Sepharose beads with bound GST fusion proteins were washed with reaction buffer (buffer F plus 5 mM $MgCl_2$, 1 mg of BSA/ml, protease inhibitors), mixed with 10 μl of the in vitro-translated proteins in a final volume of 100 μl, and incubated for 1 h at 4°C. When His-RanQ69L was added, reactions also contained 1 mM GTP, 10 mM phosphocreatine, and 20 U of creatine kinase/ml. Glutathione-Sepharose beads were washed with buffer F and resuspended in the SDS sample buffer for gel analysis. Pull-down assays with Ni-NTA agarose (Qiagen) were performed in a similar manner, except that the buffers contained 0.5% Triton X-100, 10% glycerol, and 5 mg of BSA/ml.

Microinjections. 3T3 cells grown in Dulbecco modified Eagle medium plus 10% fetal bovine serum on 4-cm glass bottom plates (MatTek Corp.) were microinjected into the cytoplasm with the indicated recombinant proteins by using Eppendorf Transjector 5246, followed by incubation at 37°C. Protein localization of live or fixed cells was determined by fluorescence microscopy.

Transfection assays. Rat1 cells were plated onto 3.5-cm dishes and transfected immediately after plating with 0.5 μg of DNA and Fugene (Roche). Cells were harvested 24 h later, fixed, stained with propidium iodide, and analyzed on FACS Calibure for enhanced green fluorescent protein (EGFP) expression and DNA content.

RESULTS

Identification of proteins associated with CC3. To identify proteins that associate with CC3, purified GST-CC3, and GST were incubated with extracts prepared from MCF7 or HeLa cells metabolically labeled with [^{35}S]methionine. GST or GST-CC3, together with the bound cellular proteins, were collected on glutathione-Sepharose, and bound proteins were resolved by SDS-PAGE. Nine distinct protein bands were detected in a complex with GST-CC3 but not GST in both MCF7 and HeLa (Fig. 1). When the experiment was performed with larger amounts of cell extracts, bands were visible on Coomassie blue-stained gels (data not shown). These protein bands were excised from the gel and subjected to analysis by mass spectrometry.

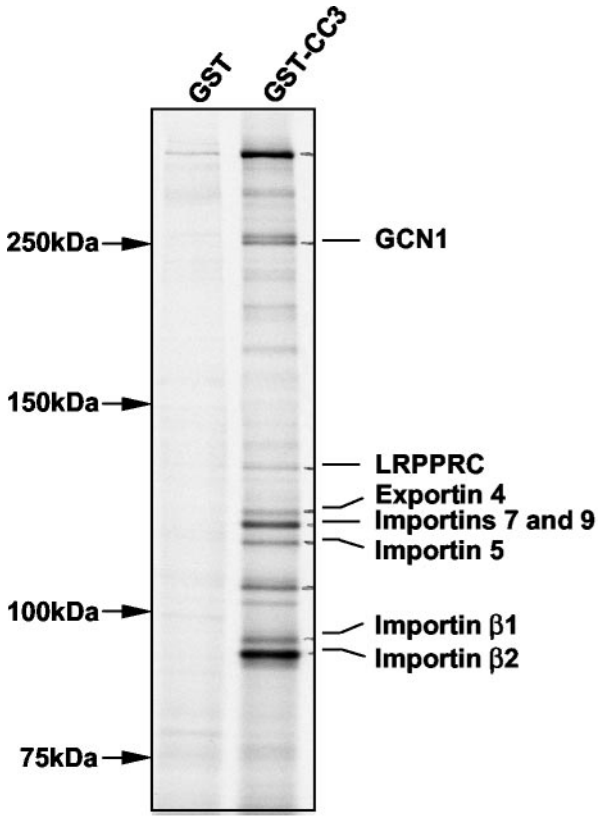

FIG. 1. Identification of proteins associated with CC3. HeLa cells were metabolically labeled with [^{35}S]methionine and lysed. Cytosolic protein extracts were incubated with GST or GST-CC3. Bound proteins were recovered on glutathione-Sepharose, resolved by SDS-PAGE, and detected by autoradiography.

Positive identification was possible for proteins in seven of nine bands, and a total of eight proteins were identified (one of the bands contained two individual proteins). Six of these were nuclear transport receptors of the importin β family (Fig. 1)—importins β1, β2 (transportin), and β3 (importins 5, 7, and 9) and exportin 4, which mediates nuclear export of the translation initiation factor eIF-5A (26). Two other proteins were LRPPRC, also known as leucine-rich protein-130 (11), implicated in the nucleocytoplasmic shuttling of RNA (27, 32), and human GCN, a homologue of yeast GCN1 that is necessary for activation of GCN2 kinase (31). All eight proteins found to bind to CC3 in vitro shared one common structural feature: they contain HEAT repeats. Importin β proteins are entirely composed of 18 to 19 HEAT repeats (reviewed in reference 47); HEAT repeats are present in the LRPPRC sequence (27) and in the sequence of GCN1 (data not shown).

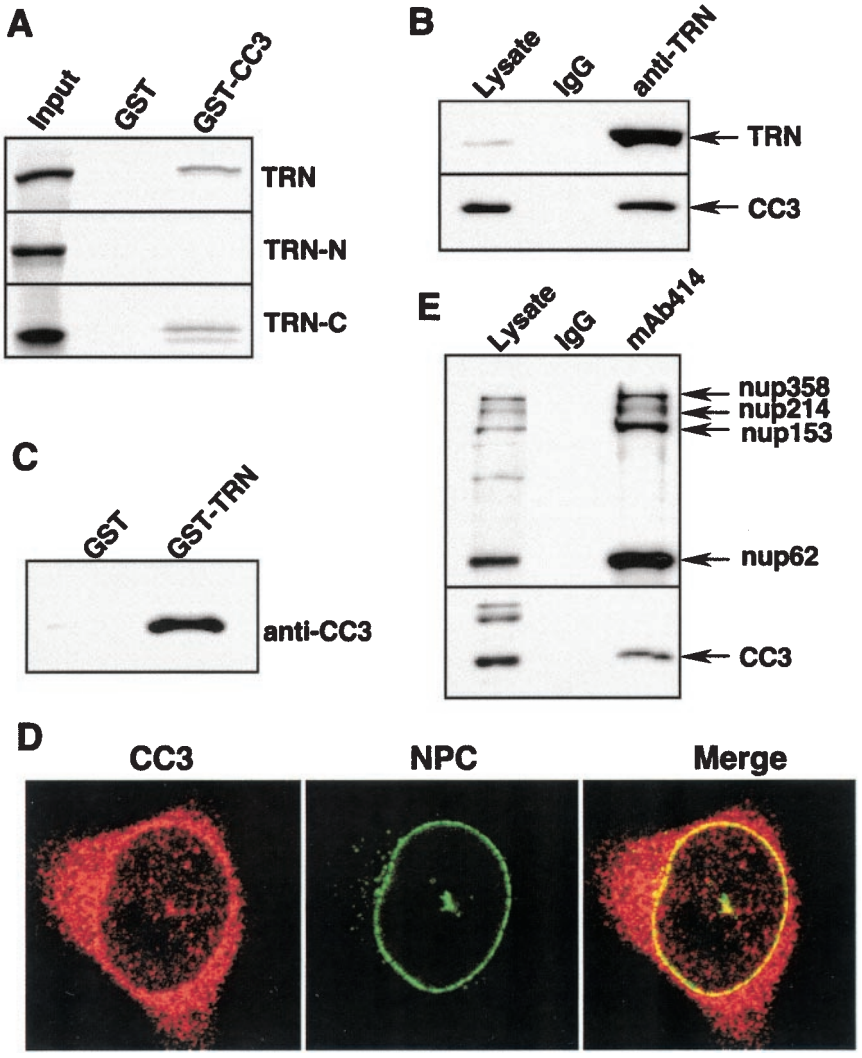

FIG. 2. CC3 associates with transportin and the NPC in vitro and in vivo. (A) In vitro-translated transportin polypeptides: full-length (TRN), N-terminal amino acids 1 to 581 (TRN-N), and C-terminal amino acids 581 to 890 (TRN-C) were incubated with GST or GST-CC3. Complexes were recovered with glutathione-Sepharose, washed, resolved by SDS-PAGE, and detected by autoradiography. (B) HeLa cell extracts were subjected to immunoprecipitation with a control immunoglobulin G (IgG) or anti-transportin antibody. The immune complexes were resolved by electrophoresis and analyzed by Western blotting with antibodies to transportin (TRN) and CC3. (C) Purified CC3 at 1 μM was mixed with GST or GST-transportin for 1 h at 4°C. Complexes were recovered with glutathione-Sepharose, resolved by SDS-PAGE, and detected by Western blotting with anti-CC3 antibody. (D) HeLa cells were processed for immunofluorescence with the anti-CC3 rabbit polyclonal antibody and anti-NPC mouse monoclonal MAb414 (Covance), followed by detection with anti-rabbit IgG-CY3 (red) and anti-mouse IgG-FITC (green), and analyzed by confocal microscopy. (E) MAb414 was used to immunoprecipitate components of the NPC from HeLa cells extracts as described in Materials and Methods. The immune complexes were analyzed by Western blotting with MAb414 and antibodies to CC3.

CC3 associates with transportin and the NPC in vitro and in vivo. We have examined interactions of CC3 protein with transportin (importin β_2) in vitro. GST-CC3, when incubated with transportin polypeptides translated in vitro, bound to the full-length transportin and its C-terminal domain (amino acids 581 to 890) but not to the N-terminal amino acids 1 to 581 (Fig. 2A). The N terminus of importin β contains domains responsible for binding to RanGTP, as well as to components of NPC (25). The in vitro-translated N-terminal domain of transportin was functional because it could still bind to the Ran protein (unpublished data).

Importantly, the association of CC3 and TRN was also observed between endogenous proteins in vivo. Transportin was immunoprecipitated from HeLa cells by using specific antibodies, and CC3 was easily detected in complex with transportin (Fig. 2B). CC3 could also be detected in a complex with immunoprecipitated importin β1 (data not shown).

To determine whether CC3 interactions with transportin are direct, GST-transportin was immobilized on beads and incubated with purified recombinant CC3. Under these conditions, CC3 bound to GST-transportin (Fig. 2C), demonstrating that the interaction of CC3 and transportin is a direct one.

The subcellular localization of endogenous CC3 was analyzed by immunofluorescent staining. CC3 protein was found to localize in the perinuclear area and form a distinct ring at the perimeter of the nucleus (Fig. 2D). The cytoplasmic struc-

ture to which CC3 is largely localized appears to be the endoplasmic reticulum, as evidenced from its staining with antibodies to calreticulin (data not shown). To verify that some of CC3 is found at the nuclear envelope, double staining was performed with antibodies to CC3 and monoclonal antibody MAb414, which reacts with several FxFG-containing nucleoporins. This revealed a partial overlap of CC3 localization with the NPC (Fig. 2D), indicating that some of cellular CC3 might be associated with the NPC.

To confirm the association of endogenous CC3 with the NPC by independent means, we examined complex formation between CC3 and NPC in vivo. As shown in Fig. 2E, CC3 was detected in a complex with nucleoporins that were immunoprecipitated with MAb414. Only a fraction of cellular CC3 present in the extracts was immunoprecipitated by MAb414, as expected from the results of cell staining that shows the bulk of CC3 in the perinuclear structures (Fig. 2D). It should be noted that complex formation between NPC and CC3 was significantly reduced when the salt concentration was increased from 100 to 150 mM NaCl (not shown). This finding strongly indicates that CC3 is not a component of NPC per se but is merely associated with it. We conclude that a fraction of cellular CC3 is found at the NPC, although it remains to be determined whether CC3 binds to NPC directly or through other proteins.

CC3 interactions with the NPC are independent of RanGTP. Interactions between CC3 and various karyopherins could indicate that CC3 is a "promiscuous cargo" or that it plays a role in nuclear transport. To distinguish between these possibilities, we investigated whether CC3 could be imported into nuclei in vitro and whether the localization of CC3 is affected by RanGTP. We used the in vitro nuclear import assay (2) with digitonin-permeabilized HeLa cells in the presence or absence of exogenously added RanQ69L. RanQ69L is a Ran mutant that binds but does not hydrolyze GTP and remains in the GTP-bound form (6). RanGTP inhibits nuclear transport of multiple proteins by preventing their association with importin β proteins (reviewed in references 24, 30, and 49). Transportin cargo GST-M9 (34) was used as a positive control, and it was efficiently imported into nuclei when cytosol (that contains nuclear transport factors) was added. As expected, import of GST-M9 was entirely inhibited by RanQ69L (Fig. 3A). However, CC3 localized at the nuclear envelope and in the perinuclear area, and neither the addition of cytosol nor the addition of RanQ69L had an effect on the localization of CC3 (Fig. 3A). We conclude that CC3 is not imported into the nucleus and binds to the nuclear envelope in a Ran-independent manner.

CC3 interactions with transportin and exportin 4 are independent of RanGTP. Having established that RanGTP has no effect on the interaction of CC3 with the nuclear envelope, we then examined how RanGTP might affect interactions of CC3 with karyopherins that normally release cargo proteins upon binding of RanGTP (15, 21, 41). GST-CC3 was incubated with in vitro-translated transportin in the absence or presence of increasing amounts of RanGTP. RanGTP had little effect on the binding between transportin and GST-CC3 (Fig. 3B), whereas it completely inhibited complex formation between transportin and its cargo GST-M9 (data not shown). RanGTP also had no effect on the direct binding of purified recombinant CC3 to GST-transportin when added in excess to the reaction mixture (data not shown).

Unlike importins, exportins require RanGTP for binding to their nuclear substrates, which are subsequently exported to the cytoplasm (reviewed in reference 14). However, GST-CC3 efficiently bound to His-exportin 4 in the absence of RanGTP (Fig. 3C), and the addition of RanGTP had no effect on this binding. These results show that CC3 interactions with transportin and exportin 4 are independent of the Ran GTPase; therefore, CC3 is unlikely to be a cargo of either of these karyopherins.

CC3 competes with M9 substrate but not with RanQ69L for binding to transportin. We sought to determine whether CC3 interferes with the binding of substrates or RanGTP to transportin. Figure 3D shows that RanGTP efficiently binds to transportin even in presence of a large excess of CC3. However, the binding of GST-M9, a transportin cargo, was inhibited when CC3 was present in excess (Fig. 3E). It is unclear whether M9 and CC3 compete for the same binding site on transportin or whether the binding of CC3 has an allosteric effect on transportin structure that inhibits subsequent binding of GST-M9.

CC3 inhibits nuclear import in vitro. We attempted to determine whether CC3 might play a role in regulating nuclear transport when associated with importins and/or components of the NPC. The in vitro nuclear import assay was used to monitor effects of CC3 on the nuclear import of GST-M9 (a transportin cargo) and GST-GFP-NLS (importin α:β1 cargo). Both substrates were imported into the nucleus in the presence of cytosol (Fig. 4A). However, the addition of purified CC3 to the transport mixture resulted in a dramatic decrease of the nuclear import for both GST-M9 and GST-GFP-NLS. Partial inhibition was observed with CC3 concentrations as low as 1 μM (data not shown); the addition of 8 μM CC3 resulted in complete inhibition (Fig. 4A). Inhibition of nuclear import by CC3 was not due to a nonspecific blocking of the NPC channel. The free diffusion of GST protein (most likely forming a dimer with a molecular mass of 50 kDa) through the NPC was unimpeded in the presence of 8 μM CC3 (unpublished data).

Using a different approach, the in vitro nuclear import assay was performed with cytosol prepared from either CC3-negative cells (N417neo) or from the same cells stably transfected to express high levels of exogenous CC3 (N417cc3) (45). As described previously (45, 51), the N417cc3 cells have a relatively high rate of spontaneous apoptosis under normal conditions and, unlike N417neo cells, are highly sensitive to a variety of death signals. Using quantitative analysis, we have estimated that the intracellular concentration of exogenously expressed CC3 in N417cc3 cells is ~9 μM. This is a rather high concentration, especially compared to a variety of other cell lines in which the concentration of endogenous CC3 varies from undetectable to 3 μM.

A significant proportion of CC3 protein from N417cc3 cells was found in the cytosol fraction (data not shown), which was used for the in vitro import assay. GST-M9 or GST-GFP-NLS was added to permeabilized HeLa cells in the presence of cytosol from N417neo or N417cc3 cells. The cytosol from N417neo cells fully supported the nuclear import of both GST-M9 and GST-GFP-NLS proteins (Fig. 4B). The same concentration of cytosol from N417cc3 cells failed to significantly support nuclear import of either substrate. We conclude

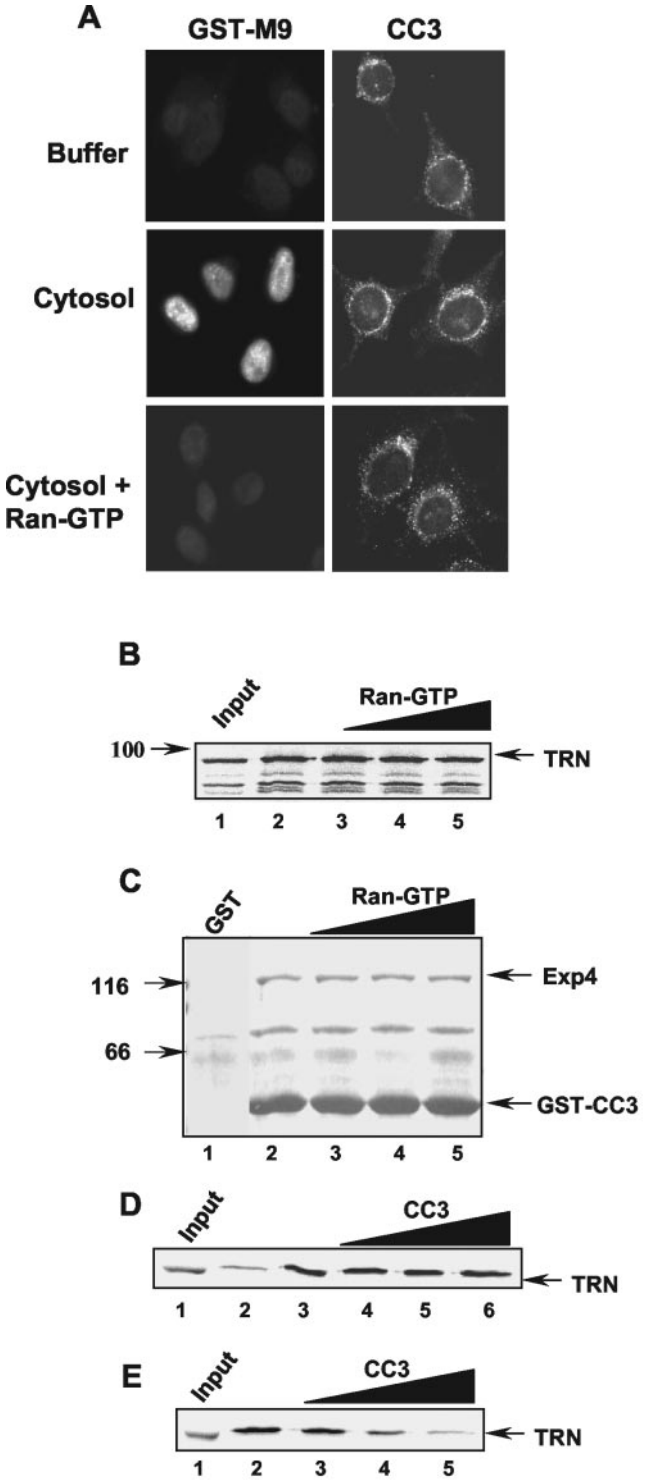

FIG. 3. Interactions of CC3 with NPC, transportin, and exportin 4 are not sensitive to RanGTP. (A) FITC-labeled GST-M9 or GST-CC3 at 0.5 μM were added to permeabilized HeLa cells in transport buffer with or without 50 μg of HeLa cytosolic extract and 8 μM RanQ69L. After incubation for 20 min at room temperature, the cells were fixed and examined by fluorescence microscopy. (B) In vitro-translated transportin (lane 1) was incubated with GST-CC3 in the absence (lane 2) or in the presence of 1, 4, or 8 μM RanQ69L (lanes 3 to 5). Complexes were recovered with glutathione-Sepharose, resolved by SDS-PAGE, and detected by autoradiography. (C) His-exportin 4 at 5 μM was incubated with GST (lane 1) or GST-CC3 without RanQ69L (lane 2) or in the presence of 2.5, 5, or 10 μM RanQ69L (lanes 3 to 5) for 1 h at 4°C. Complexes were recovered with glutathione-Sepharose, resolved by SDS-PAGE, and detected by staining with Coomassie blue. (D) In vitro-translated transportin was incubated with His-RanQ69L in the absence (lane 3) or presence of 1, 4, and 8 μM GST-CC3 (lanes 4 to 6). Complexes were recovered with Ni-NTA agarose and analyzed by SDS-PAGE, followed by autoradiography. Lane 2 shows the amount of transportin that was incubated without RanQ69L and bound to the Ni-NTA agarose in a nonspecific manner. (E) In vitro-translated transportin was incubated with GST-M9 in absence (lane 2) or in presence of 1, 4, or 8 μM CC3 (lanes 3 to 5). Complexes were analyzed as described for panel B.

that high levels of CC3 protein, either recombinant or cellular, inhibit nuclear import.

High levels of cellular CC3 diminish the rate of nuclear import in vitro. The results described above suggest that the rate of nuclear import could be slower in cells where CC3 is expressed at higher levels versus cells without CC3 expression or with very low CC3 levels. We have therefore performed in vitro import assays with isogenic cell lines differing in expression of CC3. We have first examined the effect of extraction with digitonin on cellular CC3 and found that most of the CC3 protein remains in the extracted cells (not shown). N417 clones described above were used in nuclear import assays. Because these cells grow in suspension, the assays were conducted in suspension and analyzed by fluorescence-activated cell sorting (FACS) as described previously (38). We found that whereas the import of GST-M9 into N417cc3 cells was insignificant for the first 20 min, in N417neo cells it has reached its maximum level by this time (Fig. 4C and D).

We also compared the rates of nuclear import in clones derived from the melanoma cell line C32r. The expression level of endogenous CC3 in C32r cells is extremely low, with an intracellular concentration well below 0.1 μM (data not shown). A high level of expression of exogenous CC3 was introduced into these cells (36). In vitro import assays showed practically no nuclear fluorescence after a 5-min incubation in C32cc3 cells, whereas C32neo cells showed a robust nuclear import at this time (Fig. 4E). Only after 20 to 30 min of incubation, the amount of imported substrate in C32cc3 cells reached levels seen in C32neo cells (Fig. 4E). Apparently, CC3 remaining in cells after mild extraction is capable of slowing down the rate of nuclear import, which strongly indicates that cells with different concentrations of cellular CC3 might have different rates of nuclear import in vivo.

CC3 inhibits nuclear translocation of transportin. The effect of CC3 on nuclear translocation of transportin was examined in permeabilized cells. Transportin alone, as shown previously (33), translocates into the nuclei and is not retained in remaining cytosolic structures (Fig. 4F1). However, in the presence of even relatively low concentrations of CC3, the nuclear translocation of transportin was diminished and much of it was "docked" at the nuclear envelope and in the extranuclear structures (Fig. 4F2). Accumulation of transportin in cytoplasmic structures was time dependent (unpublished data) and more pronounced at higher concentrations of CC3 (Fig. 4F), resembling the distribution of CC3 protein itself in permeabilized cells (Fig. 3A). These results suggest that CC3-mediated "docking" of importins at the NPC and in the cytoplasm could

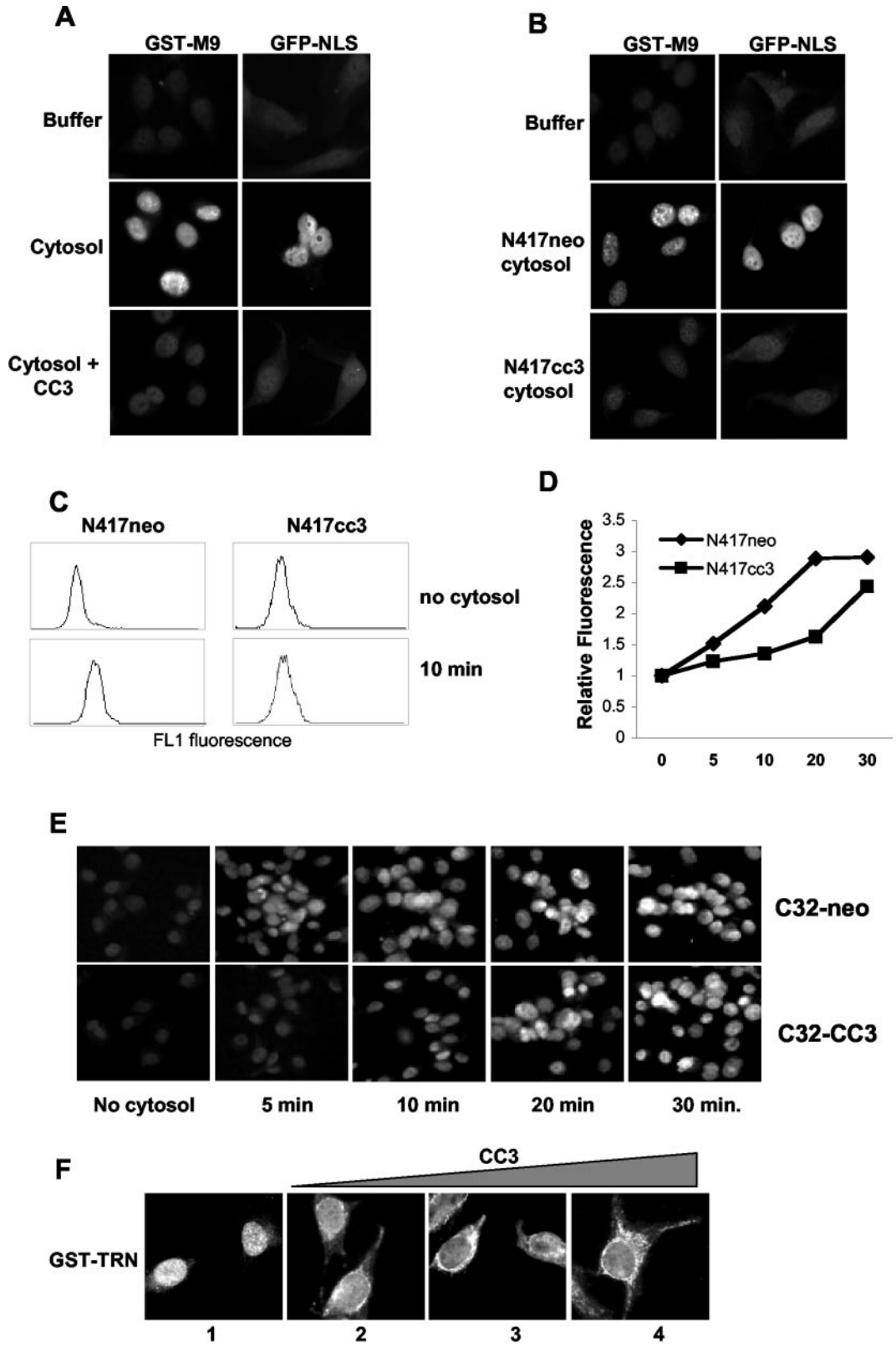

FIG. 4. CC3 inhibits nuclear import of substrates containing NLS or M9 import signals and nuclear translocation of transportin. (A) FITC-labeled GST-M9 at 0.5 μM or GST-GFP-NLS (1 μM) was added to permeabilized HeLa cells in transport buffer with or without 50 μg of HeLa cytosolic extract and 8 μM CC3. After incubation for 20 min at room temperature, cells were fixed, and the localization of import cargoes was examined by fluorescence microscopy. (B) FITC-GST-M9 or GST-GFP-NLS was added to permeabilized HeLa cells in transport buffer or with 150 μg of cytosolic extract prepared from either CC3-negative cells (N417neo) or from the same cells stably transfected to express high levels of

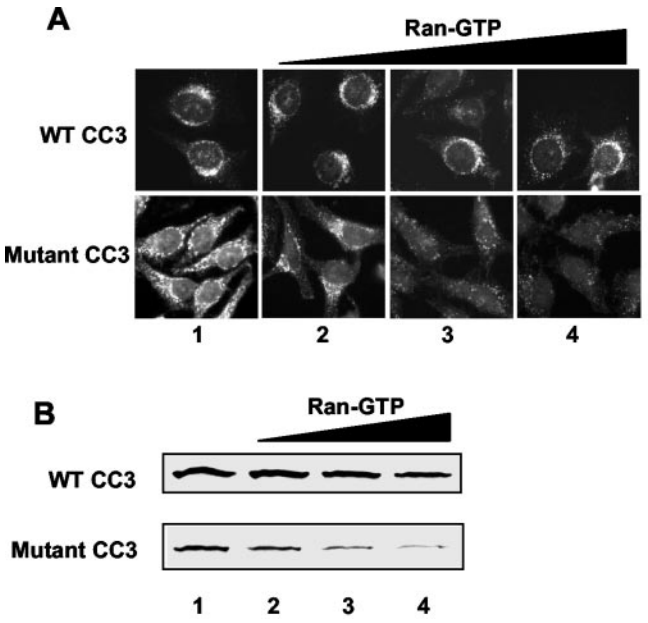

FIG. 5. Interactions of mutant CC3 with transportin and the NPC are vulnerable to dissociation by RanGTP. (A) GST-CC3 or GST-mutant CC3 at 0.5 μM was added to permeabilized HeLa cells in transport buffer without (panels 1) or with 1, 4, or 8 μM RanQ69L (panels 2 to 4). After incubation for 20 min at room temperature, cells were examined by immunofluorescence microscopy with anti-GST polyclonal antibodies. (B) In vitro-translated transportin was incubated with GST-CC3 or GST-mutant CC3 alone or in the presence of 1, 4, or 8 μM RanQ69L. The presence of transportin in complexes was detected by autoradiography.

be involved in the negative regulation of nuclear transport by CC3.

A mutant CC3 protein lacking proapoptotic properties shows weakened interactions with both transportin and NPC. We have examined how mutations that abolish the proapoptotic activity of CC3 affect its binding to importins and NPC (Fig. 5A). The design of mutant CC3, where glycines 28 and 31 were substituted with valine and alanine, respectively, was based on a previously published report (52). These mutations have been shown to inhibit the proapoptotic properties of CC3 (52).

We found that recombinant mutant CC3, when added in transport buffer to permeabilized HeLa cells, showed some association with the nuclear envelope but was strongly retained in remaining extranuclear structures (Fig. 5A1). Importantly, recombinant mutant CC3 was also found within the nuclei (Fig. 5A), as was transfected mutant CC3 (data not shown). The addition of increasing amounts of RanGTP to permeabilized cells inhibited the association of mutant, but not wild-type CC3, with the nuclear envelope. At higher concentrations of RanGTP, mutant CC3 failed to associate with the NPC or to be imported into nuclei (Fig. 5A). We conclude that the mutant CC3 has a weakened ability to interact with the NPC and is vulnerable to dissociation by excess RanGTP.

Differences in the affinities of wild-type and mutant CC3 for the NPC could be secondary to their different affinities for importins. To address this possibility, equal amounts of wild-type and mutant GST-CC3 proteins were incubated with the in vitro-translated transportin in the presence of increasing amounts of RanGTP. Mutant CC3 indeed displayed a weaker affinity for transportin and, more importantly, RanGTP inhibited binding of transportin to the mutant CC3 (Fig. 5B). The vulnerability of the mutant CC3-transportin complex to RanGTP, together with the observed partial nuclear import of mutant CC3, strongly suggest that, unlike the wild-type protein, mutant CC3 might behave more like an import cargo protein.

CC3 mutant lacking proapoptotic properties does not inhibit nuclear import in vitro or in vivo. We examined whether the mutant CC3 protein lacking proapoptotic activity can inhibit nuclear import. In vitro assays with GST-M9 or GST-GFP-NLS were performed in the presence of increasing amounts of mutant CC3. Unlike wild-type CC3 (Fig. 4A), mutant CC3 had no effect on the nuclear import of either the GST-M9 or the GST-GFP-NLS cargo proteins (Fig. 6A). Together, these results support a correlation between the ability of CC3 to form a RanGTP-resistant complex with importins and its ability to inhibit nuclear import. The proapoptotic activity of CC3 proteins appears to be related to their ability to inhibit nuclear import.

To address this possibility directly, the wild-type and mutant CC3 proteins were injected into the cytoplasm of live NIH 3T3 cells, together with the GST-GFP-NLS. As expected, GST-GFP-NLS alone (or coinjected with GST) assumed nuclear localization within 15 min after injection. Mutant CC3 had no effect on the localization of GST-GFP-NLS (Fig. 6B). However, the coinjection of wild-type CC3 led to a dramatic retention of substrate in the cytoplasm (Fig. 6B). At 30 min after coinjection of wild-type CC3 and GST-GFP-NLS, the majority of cells had very little nuclear GFP, and many cells assumed a rounded morphology (not shown). Within 2 h of injection, 70 to 80% of the GFP-positive cells injected with wild-type CC3 had floated up from the surface of the dish. At this time, at least 80 to 90% of cells injected with GST-GFP-NLS alone or together with mutant CC3 remained alive, as judged by their

CC3 (N417cc3). Cells were processed as for panel A. (C) In vitro import of FITC-GST-M9 (0.5 μM) was performed in suspension with N417neo and N417cc3 cells, and the nuclear fluorescence was analyzed by FACS. FL1 fluorescence histograms of live cells are shown for cells incubated for 30 min with GST-M9 but without cytosol (no cytosol) and for cells incubated with both GST-M9 and cytosol for 10 min. (D) Graphic representation of the results of the in vitro import assays with N417 clones performed as described for panel C. The data are shown as the relative fluorescence, where the fluorescence of cells incubated with substrate in the absence of cytosol is assigned an arbitrary value of 1. The experiment was performed twice, with nearly identical results each time. (E) FITC-labeled GST-M9 (0.5 μM) was added to permeabilized C32neo and C32cc3 cells in transport buffer with 50 μg of cytosolic extract prepared from N417neo cells. Cells were processed as for panel A after different incubation times. (F) GST-transportin at 0.5 μM was added to permeabilized HeLa cells in transport buffer alone (panel 1) or in the presence of 2, 4, or 8 μM CC3 (panels 2 to 4). After incubation for 20 min at room temperature cells were fixed and permeabilized with Triton X-100. The cellular localization of transportin was examined by immunofluorescence microscopy with polyclonal anti-GST antibodies and anti-rabbit IgG-FITC.

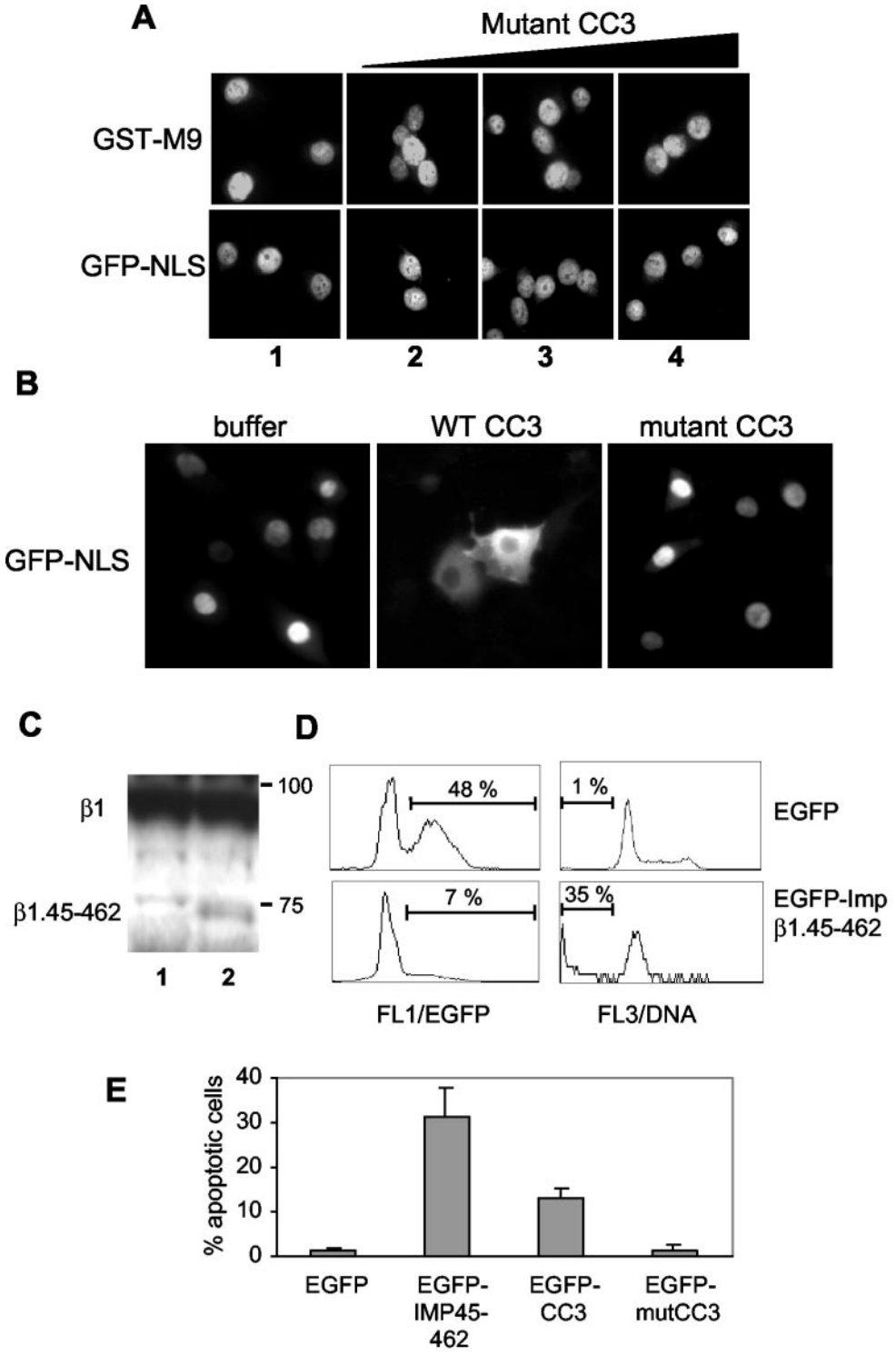

FIG. 6. Inhibition of nuclear import by CC3 and dominant-negative importin leads to apoptosis. (A) GST-M9-FITC (0.5 μM) or GST-GFP-NLS (1 μM) was added to permeabilized HeLa cells in transport buffer with 50 μg of HeLa cytosolic extracts without (panels 1) or with 2, 4, or 8 μM of mutant CC3 protein (panels 2 to 4). After incubation for 20 min at room temperature, cells were fixed, and the cellular localization of proteins was examined by fluorescence microscopy. (B) GST-GFP-NLS (0.5 mg/ml) was injected into cytoplasm of NIH 3T3 cells with GST-CC3 (5 mg/ml) or GST-mutant CC3 (5 mg/ml), followed by incubation at 37°C. Protein localization was examined by fluorescence microscopy. (C) Analysis of expression of transiently transfected EGFP–Impβ1.45-462. HEK293 cells were transfected with empty vector (lane 1) or EGFP–Impβ1.45-462 (lane 2), harvested after 20 h, and analyzed by Western blot with antibodies to importin β1 provided by K. Weis. The migration of molecular size standards is shown. The blot was exposed for a longer period to detect the protein band corresponding to EGFP–Impβ1.45-462 (β1.45-462), which is expressed at very low levels compared to endogenous importin β1 (β1). (D) Rat1 cells were transfected with indicated constructs and analyzed for EGFP expression (FL1/EGFP, right panels) and DNA content (FL3/DNA, right panels) by FACS. The numbers show the percentage of EGFP-positive transfected cells and apoptotic cells with subdiploid DNA content. (E) Analysis of apoptotic DNA fragmentation in EGFP-positive transfected cells. The results are averages of at least three independent experiments.

morphological appearance. We conclude that there is a direct relationship between the ability of CC3 to inhibit nuclear import and induce cell death.

Inhibition of nuclear transport by a dominant-negative importin leads to cell death. Because the findings described above indicate that inhibition of nuclear import by CC3 induces cell death, we investigated whether inhibition of nuclear transport could be a more general apoptosis-inducing pathway. To this end, we used a "dominant-negative" form of importin β1 that contains the NPC-binding domain but lacks the Ran GTP-binding domain (25). This protein, Impβ1.45-462, efficiently inhibits multiple nuclear transport pathways by occupying importin docking sites on NPC in a RanGTP-resistant manner (25). Mammalian expression construct for EGFP–Impβ1.45-462 fusion protein was prepared, and its expression was verified by Western blotting with antibodies to importin β1. As shown in Fig. 6C, the expression level of EGFP–Impβ1.45-462 was manyfold lower than that of endogenous importin β1, potentially indicating that expression of the dominant-negative importin is incompatible with cell growth or survival. The toxicity of Impβ1.45-462 in *Xenopus* oocytes was indeed noted by Kutay et al. (25). To monitor specifically transfected populations, we used EGFP fusion constructs throughout transfection experiments with Rat1 cells that are susceptible to killing by overexpressed CC3 (51). As soon as 18 to 20 h after transfection, more than 30% of cells expressing dominant-negative importin β1 succumbed to apoptosis, as measured by DNA fragmentation (Fig. 6D and E). Transient expression of CC3 but not the mutant CC3 induced apoptosis in ca. 13% of transfected cells. These numbers are most likely a gross underestimate of the actual extent of cell death in transfected populations. First, Rat1 cells undergo a rapid disintegration after the onset of apoptotic process that results in deceptively low numbers of cells with fragmented DNA (unpublished observations). Second, on average 40 to 50% of cells were positive for EGFP fluorescence in cultures transfected with EGFP or EGFP-mutCC3, but only 25 to 30% of transfected cells expressed EGFP-CC3 and only 3 to 7% of cells expressed EGFP–Impβ1.45-462 (Fig. 6D). Moreover, levels of fluorescence in cells positive for EGFP–Impβ1.45-462 expression were much lower than in cells expressing EGFP (Fig. 6D), a finding corresponding to the Western blot results (Fig. 6C). Third, the total cell numbers in cultures transfected with EGFP-CC3, and particularly with EGFP–Impβ1.45-462, were dramatically reduced compared to EGFP or EGFP-mutCC3 (not shown), indicating either lack of proliferation or massive death of transfected cells.

We have also tested a similarly truncated form of transportin (amino acid residues 54 to 484), but it failed to inhibit import of GST-GFP-NLS and did not induce cell death (results not shown). It is tempting to speculate that the lack of inhibitory activity of this transportin fragment is related to the fact that transportin-mediated import might involve docking sites on NPC that are different from those for importin α:β-mediated import (25, 44, 48). It is also possible that blocking the import of transportin cargo proteins might not have immediate lethal consequences for cells. In any case, the lack of nuclear import inhibitory activity of the NPC-binding domain of transportin is likely to be relevant to its lack of proapoptotic activity. We conclude that inhibition of nuclear import could be a general pathway triggering apoptosis.

DISCUSSION

The present study was initiated in order to elucidate the mechanism through which CC3, when overexpressed in tumor cells, induces apoptosis. CC3 was initially identified as a gene whose expression is absent in metastatic SCLC cells and whose ectopic expression suppressed metastasis of SCLC and other tumors (29, 45). CC3/TIP30-deficient mice spontaneously develop tumors in their second year of life (20), a finding that strongly indicates that CC3 acts as an authentic tumor suppressor. Studies in vitro showed that CC3 has a proapoptotic activity: SCLC cells forced to express CC3 had a high rate of spontaneous apoptosis under normal growth conditions and became highly sensitive to a variety of death signals that they would otherwise resist (45). Acute overexpression of CC3 induced cell death in transiently transfected cells (51). However, the mechanisms underlying the proapoptotic activity of CC3 remained a mystery. The sequence of CC3 did not provide any clues in this regard, even though significant sequence homology was reported between CC3 and the members of a large family of short-chain dehydrogenases-reductases or SDRs (3). CC3 was also identified as a human immunodeficiency virus Tat-binding protein, named TIP30, and shown to stimulate Tat-induced transcription (53). It was later reported that CC3/TIP30 has a kinase activity and that the residues 22 to 35 comprise the ATP-binding domain of CC3/TIP30 kinase (52). This same domain, well conserved between CC3 and SDR enzymes, was predicted to serve as an NADP(H) binding site (4). We were unable to detect a kinase activity of CC3 protein, either recombinant or immunoprecipitated from cells (unpublished results).

In order to understand the possible biochemical function of CC3, we have identified proteins interacting with CC3. Unexpectedly, we have found that CC3 interacts with six members of the importin β family, including five importins and exportin 4. One of the two nonkaryopherin proteins found associated with CC3, LRPPRC, was implicated in the nucleoplasmic shuttling of RNA (27, 32). The other, GCN has not been studied in higher eukaryotes, although its yeast homologue GCN1 is known to play a role in regulation of translation through activation of GCN2 kinase (31).

In spite of its association with multiple karyopherins, CC3 is not a "promiscuous" cargo protein that uses different transport receptors, as has been shown for some ribosomal proteins and the signal recognition particle SRP19 (10, 22, 42). RanGTP regulates interactions of karyopherins with their cargoes but not with CC3. In addition, the observed association of CC3 with nucleoporins in vivo argues against the possibility that CC3 is simply a promiscuous cargo. Our results do not allow us to distinguish between a direct association of CC3 with NPC or an association that is mediated by karyopherins, but in any case it is not disrupted by RanGTP. Most importantly, we show that excess of CC3 inhibits nuclear import in vitro and in vivo.

A clue to the mechanism of this inhibition might be found in the ability of CC3 to associate with importins and inhibit the translocation of transportin (and possibly other importins) through the NPC. It is also possible that the ability of CC3 to

compete with cargo proteins for binding to transportin (Fig. 3E) contributes to the inhibition of nuclear import by CC3. We suggest, however, that the inhibitory effect of CC3 on nuclear import is best explained by the observations that its interactions with karyopherins and NPC are insensitive to RanGTP. This would have profound consequences, not so much due to the sequestration of importins but due to the occupancy of importin docking sites on the NPC. Importin-CC3 complexes would remain bound to the nucleoplasmic side of the NPC, insensitive to the effects of nuclear RanGTP. As a result, the docking sites on the NPC will be occupied unproductively, which would lead to the inhibition of nuclear transport. This mechanism is not dissimilar to the mode of action of the dominant-negative fragment of importin β1 that binds to Nup153 in a Ran-GTP resistant manner (25).

By showing that a mutant CC3, deficient in apoptotic activity, does not inhibit nuclear import, the present study supports a correlation between the proapoptotic potential of CC3 and its negative effect on the nuclear transport. Insensitivity to RanGTP could be a key feature in both the proapoptotic and import-inhibiting functions of CC3. This is emphasized by our findings that a mutant CC3 protein, deficient in both these functions, dissociates from transportin and the NPC in presence of RanGTP. This hypothesis is further strengthened by our demonstration that a RanGTP-resistant dominant-negative form of importin that blocks nuclear transport is a potent inducer of apoptosis. The mechanisms through which these two different proteins induce apoptosis might be similar, as mentioned above, and their ability to inhibit many transport pathways is probably relevant to their death-promoting activity. Impβ1.45-462 inhibits import and export mediated by a variety of karyopherins (25), apparently as a consequence of the nonproductive occupation of the docking sites on NPC shared by many karyopherins. CC3 inhibits at least two major import pathways, and it would be interesting to determine whether other transport pathways could be affected as well. Binding of CC3, at least in vitro, to a number of karyopherins, might indicate that it has a potential to inhibit multiple transport pathways.

Our findings that both CC3 and importin β1.45-462 induce apoptosis strongly indicate that inhibition of nuclear transport could serve as general apoptotic signal. This should not be surprising because nucleocytoplasmic traffic is a fundamental cellular process. However, importin β1.45-462 is an artificially created inhibitor, whereas CC3 is a normal cellular protein. The need for a cellular protein capable of inhibiting nuclear import is not immediately clear. CC3 is expressed in all normal tissues examined, but its levels are highly variable in different tumors and tumor cell lines. Concentrations of endogenous CC3 in tumor cell lines analyzed vary from undetectable to 3 μM (data not shown). Concentration of importins is considered to be ~10 μM. It would appear that most cells do not have enough CC3 protein to sequester importins. However, it would be unreasonable to expect that cells will express high amounts of a protein that inhibits nuclear transport, since it would present a danger of spontaneously shutting down cellular processes that depend on nuclear trafficking. Indeed, the highest concentration of exogenously expressed CC3 is found in N417cc3 cells (i.e., ~9 μM), and these cells are highly prone to undergo spontaneous apoptosis even under normal growth conditions. As is evident from our experiments, a relatively high concentration of CC3 is needed to induce apoptosis, whereas very low expression levels of importin β1.45-462 induce a massive apoptosis (Fig. 6C and D). We suggest that the mechanisms for inhibition of nuclear import by CC3 might involve nonproductive occupancy of importin docking sites at the NPC, similar to β1.45-462. However, CC3 will have to compete with multiple cargo proteins for binding to multiple members of importin β family at the cytoplasmic face of the NPC. Although dominant-negative importin inhibits many nuclear transport pathways by nonproductively occupying some limited sites on NPC, a similarly truncated transportin does not inhibit nuclear import (and does not induce apoptosis). Therefore, interactions of CC3 with transportin and likely with some of the other importins at the NPC might have no effect on nuclear import in general and not lead to apoptosis. This could explain the difference in the efficiency of killing by CC3 versus importin β1.45-462.

We believe that under certain conditions cells might have to inhibit nuclear transport, perhaps temporarily. There is some published evidence to support this. The classical nuclear import/export pathways of poly(A) RNA are inhibited by heat shock (28). In addition to heat shock, several other types of stress in *S. cerevisiae* inhibit protein import (46). Nuclear import has been shown to be downregulated by phosphorylation (23). Reduction in the rate of nuclear import occurs in aged fibroblasts (40), suggesting that cells indeed possess mechanisms to negatively regulate nuclear transport. We speculate that under conditions of stress, the levels of CC3 protein could be elevated or it could be recruited to the nuclear envelope, resulting in reduced efficiency of nuclear transport. Indeed, expression of CC3 was reported to be significantly increased after heat shock treatment (35) and gamma irradiation (7). Our preliminary results indicate that the association of CC3 with importins is increased in preapoptotic cells compared to cells cultured under normal conditions. Obviously, further study is needed to fully understand the physiological significance of CC3-induced inhibition of the nuclear import pathways.

ACKNOWLEDGMENTS

We thank Karsten Weis for RanQ96L and anti-importin β1 antibodies, Gideon Dreyfuss for GST-M9 and GST-transportin constructs, Brian Cullen for transportin cDNA, Dirk Gorlich for His-exportin 4 plasmid, Ian Macara for the GST-GFP-NLS plasmid, and Ulrike Kutay for importin β1.45-462. We are indebted to Andy Finch for his guidance in microinjections.

This study was supported by the grant DAMD17-01-1-0191 from USAMRAA to E.S.

REFERENCES

1. **Adam, S. A., and L. Gerace.** 1991. Cytosolic proteins that specifically bind nuclear location signals are receptors for nuclear import. Cell **66:**837–847.
2. **Adam, S. A., R. S. Marr, and L. Gerace.** 1990. Nuclear protein import in permeabilized mammalian cells requires soluble cytoplasmic factors. J. Cell Biol. **111:**807–816.
3. **Baker, M. E.** 1999. TIP30, a cofactor for HIV-1 Tat-activated transcription, is homologous to short-chain dehydrogenases/reductases. Curr. Biol. **9:**R471.
4. **Baker, M. E., L. Yan, and M. R. Pear.** 2000. Three-dimensional model of human TIP30, a coactivator for HIV-1 Tat-activated transcription, and CC3, a protein associated with metastasis suppression. Cell Mol. Life Sci. **57:**851–858.
5. **Ben-Efraim, I., and L. Gerace.** 2001. Gradient of increasing affinity of importin beta for nucleoporins along the pathway of nuclear import. J. Cell Biol. **152:**411–417.

6. **Bischoff, F. R., C. Klebe, J. Kretschmer, A. Wittinghofer, and H. Ponstingl.** 1994. RanGAP1 induces GTPase activity of nuclear Ras-related Ran. Proc. Natl. Acad. Sci. USA **91:**2587–2591.
7. **Chaudhry, M. A., L. A. Chodosh, W. G. McKenna, and R. J. Muschel.** 2003. Gene expression profile of human cells irradiated in G_1 and G_2 phases of cell cycle. Cancer Lett. **195:**221–233.
8. **Chen, Z., Y. Li, and R. M. Krug.** 1999. Influenza A virus NS1 protein targets poly(A)-binding protein II of the cellular 3′-end processing machinery. EMBO J. **18:**2273–2283.
9. **Chook, Y. M., and G. Blobel.** 1999. Structure of the nuclear transport complex karyopherin-beta2-Ran x GppNHp. Nature **399:**230–237.
10. **Dean, K. A., O. von Ahsen, D. Gorlich, and H. M. Fried.** 2001. Signal recognition particle protein 19 is imported into the nucleus by importin 8 (RanBP8) and transportin. J. Cell Sci. **114:**3479–3485.
11. **DiSorbo, D., E. G. Shi, and W. L. McKeehan.** 1988. Purification form human hepatoma cells of a 130-kDa membrane glycoprotein with properties of the heparin-binding growth factor receptor. Biochem. Biophys. Res. Commun. **157:**1007–1014.
12. **Dobbelstein, M., J. Roth, W. T. Kimberly, A. J. Levine, and T. Shenk.** 1997. Nuclear export of the E1B 55-kDa and E4 34-kDa adenoviral oncoproteins mediated by a rev-like signal sequence. EMBO J. **16:**4276–4284.
13. **Fridell, R. A., R. Truant, L. Thorne, R. E. Benson, and B. R. Cullen.** 1997. Nuclear import of hnRNP A1 is mediated by a novel cellular cofactor related to karyopherin-beta. J. Cell Sci. **110**(Pt. 11)**:**1325–1331.
14. **Gorlich, D., and U. Kutay.** 1999. Transport between the cell nucleus and the cytoplasm. Annu. Rev. Cell Dev. Biol. **15:**607–660.
15. **Gorlich, D., N. Pante, U. Kutay, U. Aebi, and F. R. Bischoff.** 1996. Identification of different roles for RanGDP and RanGTP in nuclear protein import. EMBO J. **15:**5584–5594.
16. **Gorlich, D., S. Prehn, R. A. Laskey, and E. Hartmann.** 1994. Isolation of a protein that is essential for the first step of nuclear protein import. Cell **79:**767–778.
17. **Groves, M. R., and D. Barford.** 1999. Topological characteristics of helical repeat proteins. Curr. Opin. Struct. Biol. **9:**383–389.
18. **Gustin, K. E., and P. Sarnow.** 2001. Effects of poliovirus infection on nucleocytoplasmic trafficking and nuclear pore complex composition. EMBO J. **20:**240–249.
19. **Her, L. S., E. Lund, and J. E. Dahlberg.** 1997. Inhibition of Ran guanosine triphosphatase-dependent nuclear transport by the matrix protein of vesicular stomatitis virus. Science **276:**1845–1848.
20. **Ito, M., C. Jiang, K. Krumm, X. Zhang, J. Pecha, J. Zhao, Y. Guo, R. G. Roeder, and H. Xiao.** 2003. TIP30 deficiency increases susceptibility to tumorigenesis. Cancer Res. **63:**8763–8767.
21. **Izaurralde, E., U. Kutay, C. von Kobbe, I. W. Mattaj, and D. Gorlich.** 1997. The asymmetric distribution of the constituents of the Ran system is essential for transport into and out of the nucleus. EMBO J. **16:**6535–6547.
22. **Jakel, S., and D. Gorlich.** 1998. Importin beta, transportin, RanBP5 and RanBP7 mediate nuclear import of ribosomal proteins in mammalian cells. EMBO J. **17:**4491–4502**.**
23. **Kehlenbach, R. H., and L. Gerace.** 2000. Phosphorylation of the nuclear transport machinery downregulates nuclear protein import in vitro. J. Biol. Chem. **275:**17848–17856.
24. **Kuersten, S., M. Ohno, and I. W. Mattaj.** 2001. Nucleocytoplasmic transport: Ran, beta and beyond. Trends Cell Biol. **11:**497–503.
25. **Kutay, U., E. Izaurralde, F. R. Bischoff, I. W. Mattaj, and D. Gorlich.** 1997. Dominant-negative mutants of importin-beta block multiple pathways of import and export through the nuclear pore complex. EMBO J. **16:**1153–1163.
26. **Lipowsky, G., F. R. Bischoff, P. Schwarzmaier, R. Kraft, S. Kostka, E. Hartmann, U. Kutay, and D. Gorlich.** 2000. Exportin 4: a mediator of a novel nuclear export pathway in higher eukaryotes. EMBO J. **19:**4362–4371.
27. **Liu, L., and W. L. McKeehan.** 2002. Sequence analysis of LRPPRC and its SEC1 domain interaction partners suggests roles in cytoskeletal organization, vesicular trafficking, nucleocytosolic shuttling, and chromosome activity. Genomics **79:**124–136.
28. **Liu, Y., S. Liang, and A. M. Tartakoff.** 1996. Heat shock disassembles the nucleolus and inhibits nuclear protein import and poly(A)$^+$ RNA export. EMBO J. **15:**6750–6757.
29. **Liu, Y., A. Thor, E. Shtivelman, Y. Cao, G. Tu, T. D. Heath, and R. J. Debs.** 1999. Systemic gene delivery expands the repertoire of effective antiangiogenic agents. J. Biol. Chem. **274:**13338–13344.
30. **Macara, I. G.** 2001. Transport into and out of the nucleus. Microbiol. Mol. Biol. Rev. **65:**570–594.
31. **Marton, M. J., D. Crouch, and A. G. Hinnebusch.** 1993. GCN1, a translational activator of GCN4 in *Saccharomyces cerevisiae*, is required for phosphorylation of eukaryotic translation initiation factor 2 by protein kinase GCN2. Mol. Cell. Biol. **13:**3541–3556.
32. **Mili, S., H. J. Shu, Y. Zhao, and S. Pinol-Roma.** 2001. Distinct RNP complexes of shuttling hnRNP proteins with pre-mRNA and mRNA: candidate intermediates in formation and export of mRNA. Mol. Cell. Biol. **21:**7307–7319.
33. **Nakielny, S., and G. Dreyfuss.** 1998. Import and export of the nuclear protein import receptor transportin by a mechanism independent of GTP hydrolysis. Curr. Biol. **8:**89–95.
34. **Nakielny, S., M. C. Siomi, H. Siomi, W. M. Michael, V. Pollard, and G. Dreyfuss.** 1996. Transportin: nuclear transport receptor of a novel nuclear protein import pathway. Exp. Cell Res. **229:**261–266.
35. **Narita, N., I. Noda, T. Ohtsubo, S. Fujieda, M. Tokuriki, T. Saito, and H. Saito.** 2002. Analysis of heat-shock related gene expression in head-and-neck cancer using cDNA arrays. Int. J. Radiat. Oncol. Biol. Phys. **53:**190–196.
36. **NicAmhlaoibh, R., and E. Shtivelman.** 2001. Metastasis suppressor CC3 inhibits angiogenic properties of tumor cells in vitro. Oncogene **20:**270–275.
37. **Palacios, I., M. Hetzer, S. A. Adam, and I. W. Mattaj.** 1997. Nuclear import of U snRNPs requires importin beta. EMBO J. **16:**6783–6792.
38. **Paschal, B. M., C. Delphin, and L. Gerace.** 1996. Nucleotide-specific interaction of Ran/TC4 with nuclear transport factors NTF2 and p97. Proc. Natl. Acad. Sci. USA **93:**7679–7683.
39. **Pollard, V. W., W. M. Michael, S. Nakielny, M. C. Siomi, F. Wang, and G. Dreyfuss.** 1996. A novel receptor-mediated nuclear protein import pathway. Cell **86:**985–994.
40. **Pujol, G., H. Soderqvist, and A. Radu.** 2002. Age-associated reduction of nuclear protein import in human fibroblasts. Biochem. Biophys. Res. Commun. **294:**354–358.
41. **Rexach, M., and G. Blobel.** 1995. Protein import into nuclei: association and dissociation reactions involving transport substrate, transport factors, and nucleoporins. Cell **83:**683–692.
42. **Rout, M. P., G. Blobel, and J. D. Aitchison.** 1997. A distinct nuclear import pathway used by ribosomal proteins. Cell **89:**715–725.
43. **Ryan, K. J., and S. R. Wente.** 2000. The nuclear pore complex: a protein machine bridging the nucleus and cytoplasm. Curr. Opin. Cell Biol. **12:**361–371.
44. **Shah, S., and D. J. Forbes.** 1998. Separate nuclear import pathways converge on the nucleoporin Nup153 and can be dissected with dominant-negative inhibitors. Curr. Biol. **8:**1376–1386.
45. **Shtivelman, E.** 1997. A link between metastasis and resistance to apoptosis of variant small cell lung carcinoma. Oncogene **14:**2167–2173.
46. **Stochaj, U., R. Rassadi, and J. Chiu.** 2000. Stress-mediated inhibition of the classical nuclear protein import pathway and nuclear accumulation of the small GTPase Gsp1p. FASEB J. **14:**2130–2132.
47. **Strom, A. C., and K. Weis.** 5 June 2001, posting date. Importin-beta-like nuclear transport receptors. Genome Biol. **2:**REVIEWS3008. 1–3008.9. [Online.] http://genomebiology.com.
48. **Walther, T. C., M. Fornerod, H. Pickersgill, M. Goldberg, T. D. Allen, and I. W. Mattaj.** 2001. The nucleoporin Nup153 is required for nuclear pore basket formation, nuclear pore complex anchoring and import of a subset of nuclear proteins. EMBO J. **20:**5703–5714.
49. **Weis, K.** 2002. Nucleocytoplasmic transport: cargo trafficking across the border. Curr. Opin. Cell Biol. **14:**328–335.
50. **Weis, K., I. W. Mattaj, and A. I. Lamond.** 1995. Identification of hSRP1 alpha as a functional receptor for nuclear localization sequences. Science **268:**1049–1053.
51. **Whitman, S., X. Wang, R. Shalaby, and E. Shtivelman.** 2000. Alternatively spliced products CC3 and TC3 have opposing effects on apoptosis. Mol. Cell. Biol. **20:**583–593.
52. **Xiao, H., V. Palhan, Y. Yang, and R. G. Roeder.** 2000. TIP30 has an intrinsic kinase activity required for up-regulation of a subset of apoptotic genes. EMBO J. **19:**956–963.
53. **Xiao, H., Y. Tao, J. Greenblatt, and R. G. Roeder.** 1998. A cofactor, TIP30, specifically enhances HIV-1 Tat-activated transcription. Proc. Natl. Acad. Sci. USA **95:**2146–2151.
54. **Xu, L., Y. Kang, S. Col, and J. Massague.** 2002. Smad2 nucleocytoplasmic shuttling by nucleoporins CAN/Nup214 and Nup153 feeds TGFβ signaling complexes in the cytoplasm and nucleus. Mol. Cell **10:**271–282.