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13. ABSTRACT *(Maximum 200 Words)*

CC3 was identified as a metastasis suppressor protein in vivo. This laboratory demonstrated that CC3 impairs apoptotic resistance of cells derived from aggressive tumors and inhibits production of angiogenic factors by these cells. CC3 expression was introduced into two breast carcinoma cell lines derived from metastatic tumors and expressing very low levels of this protein. Expression of exogenous CC3 lead to enhancement of their apoptotic responses to growth factors withdrawal and treatment with cytotoxic drugs. However, there was no effect of CC3 expression on angiogenic activity of breast cancer cells which was very low even prior to introduction of CC3.

Because CC3 protein has no significant homologies to other known proteins, we have conducted analysis of cellular proteins that interact with CC3. Mass-spectrometric analysis of isolated protein revealed an amazing consistency among types of proteins that form complexes with CC3. Out of seven proteins identified, five belong to the family of importins of beta class and one is exportin, i.e. all these are proteins that serve as nuclear transport receptors. This amazing specificity in CC3 interactions pointed to a possibility that CC3 might be a factor involved in regulation of nuclear transport. Indeed, CC3 associates with nuclear envelope. Future work will focus on the mechanism through which CC3, via its possible role in nuclear transport, influences apoptosis.

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Table of Contents

INTRODUCTION

Development of aggressive and metastatic phenotypes in tumors largely depends on acquisition by tumor cells of ability to support angiogenesis and resist apoptosis. Human gene CC3 is able to negatively affect both of these features of metastatic cells. CC3 is a metastasis suppressor of variant small cell lung carcinoma (vSCLC) and a mouse melanoma in vivo (14, 9). We have identified two likely mechanisms whereby CC3 acts as a metastasis suppressor. First, forced expression of CC3 restores apoptotic responses of tumor cells to a wide variety of signals (14,16). Since the ability to resist apoptosis is an essential part of metastatic phenotype, the pro-apoptotic function of CC3 is likely to contribute to suppression of metastasis. Second, we have found that expression of CC3 in tumor cells results in suppression of their angiogenic activity in vitro (12). Enforced expression of CC3 in tumor cells leads to reduced expression of angiogenic stimulatory factors and increase in antiangiogenic factor production. Since angiogenesis is necessary for the development of metastasis, ability of CC3 to suppress angiogenesis is crucial in its metastasis suppressing ability. We proposed to examine how enforced expression of CC3 in breast carcinoma cell lines with low levels of CC3 might affect their ability to undergo apoptosis and inhibit angiogenesis.

BODY

Task 1. To examine effects of CC3 on angiogenic properties of breast cancer cells.

We have examined angiogenic properties in vitro of three breast carcinoma cell lines: two lines with almost undetectable levels of CC3 (MDA-MB-361 and 468) and one with moderate levels of CC3 protein (DU4475). The assays were conducted with conditioned medium collected from these cell lines and cultures of primary human umbilical vein endothelial cells (HUVEC). Proliferation of HUVEC cultures was examined in a tritiated thymidine incorporation assay; conditioned medium from small cell lung carcinoma and glioblastoma cell lines was used a positive control for angiogenic properties as described (12). However, we have found that the angiogenic activity in vitro of breast carcinoma cell lines is not significant (data not shown). Conditioned media from MDA-MB361, 468 and DU4475 did not induce proliferation of HUVEC cells over background values. Therefore we could not proceed with analysis of a potential inhibitory effect of CC3 on angiogenic properties of breast carcinomas in vitro.

Task 2. To identify angiogenic modulators whose expression levels are affected by CC3 (Months 15 - 24).

This task could not be accomplished because, as explained above, breast carcinoma cell lines examined under Task 1have no appreciable angiogenic activity in vitro.

Task 3. Examine the effect of exogenous CC3 expression on apoptosis of the breast cancer cells (Months 9 – 24).

Expression of CC3 in breast carcinoma cell lines increases their sensitivity to apoptotic signals.

Expression vector for CC3 was introduced into two breast carcinoma cell lines, MDA-MB-468 and 361, both derived from metastatic lesions and containing very low levels of endogenous CC3. About

40 clones were isolated from each line and only two clones in each line were found to express moderate levels of exogenous CC3. These clones were subjected to treatment with several inducers of apoptosis; in all cases the level of death was found to be higher in CC3-expressing clones compared to neo controls (Table 1).

Table 1. Expression of CC3 predisposes breast cancer cells to apoptosis in response to a variety of treatments

	MDA-MB-468 NEO	MDA-MB-468 CC3	MDA-MB-361 NEO	MDA-MB-361 CC3
Serum withdrawal*	9.0	19.2	1.1	3.9
Etoposide 50 μg/ml	34.5	62.1	5.8	18.6
Taxol 50 nM	36.1	55.1	5.5	20.1
Worthmannin 10 nM	8.1	19.1		

* All treatments were for 24 hours. Data are shown as % of cells with subdiploid DNA content and are average of at least two experiments.

These data show that CC3 acts as a pro-apoptotic protein in breast carcinoma cell lines in a manner that is not signal-specific.

We have therefore conducted experiments aimed at identification of the mechanisms through which CC3 exerts its pro-apoptotic action.

Identification of proteins associated with CC3

In order to begin to elucidate how CC3 exerts its pro-apoptotic function on various cell types, we first identified proteins that associate with CC3. This was accomplished by exposing the GST-CC3 fusion protein to protein extracts prepared from breast carcinoma MCF7 and cervical carcinoma Hela cells metabolically labeled with ^{35}S-methionine. GST protein was used as a control for the specificity of interactions. GST or GST-CC3 together with the bound cellular proteins were isolated on glutathione-sepharose, and bound ^{35}S-labeled proteins were resolved by SDS-PAGE. As shown in Figure 1, 8 distinct protein bands of relatively high molecular weight were detected specifically in a complex with GST-CC3 but not GST (Figure 1). GST-CC3-bound protein bands were identical between HeLa and MCF7 cells. (data not shown). Same experiments were performed in which bound proteins were detected with silver stain or Coomassie Blue staining of the gels and they produced same set of proteins bound to GST-CC3 as detected by autoradiography of ^{35}S-methionine labeled proteins.

Each of the eight protein bands associated with GST-CC3 was excised from the Coomassie Blue stained gel and after tryptic digestion were subjected to analysis by mass spectrometry. Six of the eight protein bands were positively identified (Table 1). Because one of the bands actually contained two proteins of the same molecular weight, a total of seven proteins were identified. Five of them are members of the importing β family (reviewed in ref 15), which function as nuclear import receptors for various cellular proteins and ribonucleoproteins. One of the bands was actually a mixture of two importins, 7 and 9 (Table 2). Importin β_1, importin β_2 (transportin), importin β_3 (importins 5) and importins 7 and 9 are all involved in the nuclear import of substrates possessing the necessary nuclear localization sequence (NLS) (reviewed in 17, 10, 6). "Band 3" was identified as exportin-4 that has recently been shown to be a critical factor in the nuclear export of some proteins (8).

Table 2. CC3 binding proteins identified by mass spectrometry.

Band#	MW (kD)	Protein Identity
1	250	ND*
2	130	103 kD Leucine-rich protein
3	120	Exportin 4
4	115	Importin 7 and Importin 9
5	115	Importin 5 (importin β_3)
6	100	ND
7	90	Importin β_1
8	90	Importin β_2 (transportin, MIP)

*Positive identification was not possible.

Interactions between CC3 and importin β_2 (transportin)

Since CC3 can associate with several different members of the importin β family and an exportin, it is likely that CC3 may interact with a common domain of these nuclear transport receptors. Importins and exportins contain two domains in common: one is the binding domain for the Ran protein, which plays a key role in regulating the binding and release of the cargo substrates to and from transport proteins (reviewed in 15). The other common feature is the presence of so called HEAT repeats which are not necessarily conserved on the level of sequence homology but share a common structure. We have chosen transportin (5, 13), also known as MIP-1 and importin β2 to roughly locate the CC3 - interacting domain. Full-length cDNA of transportin was transcribed and translated in vitro in the presence of ^{35}S-Methionine. GST or GST-CC3 was added to this *in vitro* translated mix, followed by the recovery of complexes on glutathione-sepharose. GST-CC3 was able to form a stable complex with full-length transportin and its C-terminal domain (amino acids 581-890) but not with the transportin N-terminal amino acids 1-581 (Figure 2). The C-terminal portion of transportin as well as other importins contains its substrate-binding domain (5), whereas the N-terminus contains the Ran-binding site (11). These results suggest that transportin and other transport proteins interact with CC3 through a domain different from the Ran-binding domain. The interacting domain on transportin contains the HEAT repeats.

CC3 associates with the nuclear envelope

The finding of CC3 interaction with a number of different nuclear transport proteins indicated that these interactions are not due to CC3 being a cargo substrate of all these proteins. Rather, it suggested that CC3 itself might play a certain role in nuclear transport or its regulation. All receptors of importin β family and exportins have two binding specificities in common: they can bind Ran-GDP (reviewed in 15) and they all interact with the components of nuclear pore, nucleoporins (4). A possibility therefore was considered that CC3 is a component of the nuclear pore. To start addressing this possibility we have first examined subcellular localization of endogenous and exogenously added CC3.

The subcellular localization of endogenous CC3 was examined by immunofluorescent staining (Figure 3A,B). CC3 was found to be enriched in perinuclear area and form a punctate ring around nuclei consistent with association with nuclear envelope. A weak nuclear signal was detected as well.

Next we examined if exogenous CC3 added to permealized HeLa cells will also localize to the nuclear envelope and if this ability could be affected by addition of cytosolic factors or transportin or if CC3 could be imported into nuclei. We have performed in vivo nuclear import assays (1). Recombinant CC3 protein in transport buffer was added to HeLa cells permealized with digitonin.

Localization of the protein was detected by immunostaining of fixed cells. We have noticed that permealization with digitonin removes bulk of endogenous CC3 (Figure 3C) indicating that CC3 is not a true component of the nuclear pore complex or an authentic nuclear envelope protein. (Digitonin treatment does not disrupt nuclear envelope or pore structure). Permealized cells that were exposed to recombinant CC3 displayed intense staining around the nuclear envelope (Figure 3D) that was very similar to the localization of endogenous CC3 except a relative reduction in intranuclear staining. This indicates that exogenous CC3 assumes correct localization in permealized cells accumulating at the nuclear envelope. Addition of the cytosol fraction or purified transportin did not change the distribution of recombinant CC3 (data not shown) indicating that CC3 is not imported into nuclei.

These results suggest that CC3 could be a peripheral protein associated with nuclear envelope or nuclear pores and involved in some regulatory aspect of the trafficking of nuclear import/export receptors.

CC3 interactions with nuclei are independent of Ran-GTP

Interactions of importins of β family with their cargo proteins and with nucleoporins are both regulated by the small GTPase Ran. We examined if interactions of CC3 with the nuclei are affected by Ran (experiments examining the potential effect of Ran on interaction of CC3 with transportin are in progress).

An *in vitro* assay was developed to monitor interactions between the whole nuclei and CC3 in the presence or absence of transport factors. GST-CC3, GST-Transportin (as a positive control) or GST (negative control) were incubated with purified nuclei. Binding of proteins to nuclei was detected after pelleting nuclei through a sucrose cushion. Significant amounts of both GST-CC3 or GST-transportin were found associated with the nuclei pellets whereas only a trace of the GST control was detected in the nuclei pellet (Figure 4). This shows that CC3, similar to transportin, easily associates with the nuclei.

Ran-GTP has been shown to modulate the binding of importins and exportins not only to their cargo in but also to nucleoporins, structural components of the nuclear pores itself (4). RanQ69L is a non-hydrolysable Ran mutant, which remains in the GTP-bound form (7). To examine the effects of Ran-GTP, either GST-CC3 or GST-transportin was allowed to interact with purified nuclei in the presence of increasing amounts of RanQ69L. Similar to what was shown in previously published studies involving the binding of importin to nucleoporins (2, 4), lower concentrations of Ran-GTP increased transportin binding to nuclei, while higher Ran-GTP concentrations inhibited transportin-nuclei complex formation (Figure 5A). On the other hand, the amount of GST-CC3 detected in complex with purified nuclei was not affected by the presence of Ran-GTP (figure 6A, lanes 2-4).

To examine the potential effect of transportin on CC3 binding to nuclei, GST-CC3 was allowed to interact with purified nuclei in the presence of increasing amounts of GST-Transportin. As shown in Figure 5B, the presence of a 10 fold molar excess of GST-Transportin to CC3 did not affect GST-CC3 interactions with the nuclei (Figure 5B). These results indicate that CC3 may interact directly with nuclear envelope or nuclear pore proteins in a Ran-independent and transportin-independent manner. Therefore, the binding mode of CC3 is different from this of importins and independent of CC3 binding to importins.

Gel filtration was used to examine if CC3 exist in cells as a component of protein complexes. The gel filtration profile of endogenous CC3 (Figure 6) indicates that CC3 can form very high molecular weight complexes. CC3 began to elute from the Superose 12 column in very high molecular weight complexes above 700 kD, and it finished eluting in the range of 60 kD, which may represent the dimer form of CC3 (Figure 6).

KEY RESEARCH ACCOMPLISHMENTS

- Breast carcinoma lines have undetectable angiogenic activity towards endothelial cells in vitro
- Expression of exogenous CC3 predisposes breast cancer cells with low endogenous CC3 levels to apoptosis induced by diverse signals
- CC3 protein interacts with at least six members of nuclear transport receptors family: five importins and one exportin
- Association of CC3 with transportin (importin β2) occurs independent of its Ran-binding domain
- CC3 is not imported into nucleus on its own or by its binding partner, transportin
- CC3 associates with the nuclear envelope as assessed by two independent assays
- Association of CC3 with nuclei is Ran-independent and is independent of CC3 binding to transportin.

CONCLUSIONS

We have demonstrated that the CC3 protein clearly associates with several nuclear transport receptors as well as purified nuclei in an *in vitro* system. Exactly how CC3 interacts with these transport factors will be examined through *in vitro* and *in vivo* techniques. The main thrust of the project will be to determine whether a link exists between CC3 association with the nuclear transport system and the pro-apoptotic properties of CC3.

The finding that CC3 is likely to be involved in the regulation of nuclear transport is very important. Nuclear transport is an integral part of how cells regulate their growth and proliferation. Change in subcellular localization of a number of cellular proteins that perform key roles in various cellular processes is one of the ways through which cells regulate metabolic, proliferative and apoptotic responses. At least one peripheral component of nuclear pore, CAN/Nup214 was shown to induce cell cycle arrest and apoptosis when overexpressed (3). The possibility that the proapoptotic protein CC3 is involved in regulation of nuclear transport is very exciting.

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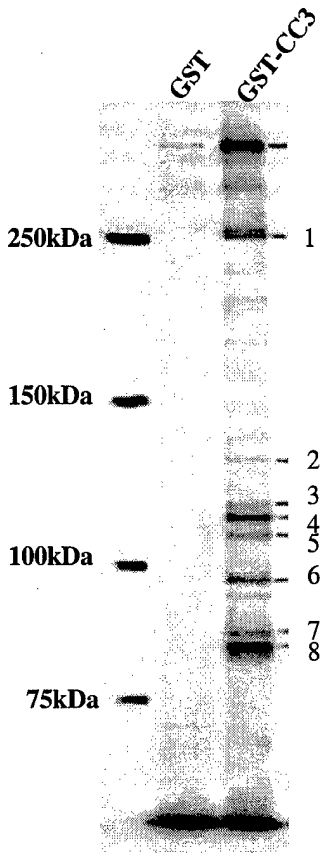

Figure 1. Proteins associated with CC3. Purified GST or GST-CC3 proteins were mixed with cell extract prepared from ^{35}S-methionine labeled HeLa cells and incubated for 1 hour on ice. The recombinant proteins and bound cellular proteins were recovered on glutathione-sepharose. After thorough washes proteins were resolved on SDS-PAGE and detected by autiradiography.

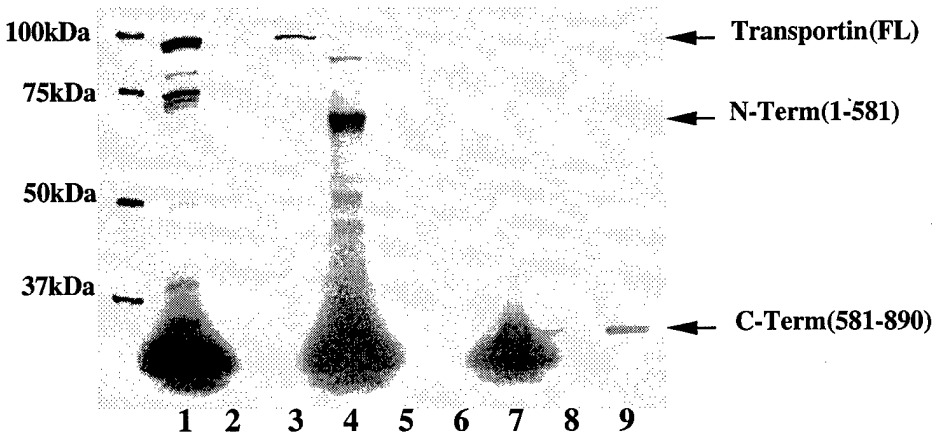

Figure 2. Interactions between CC3 and importin β_2 (transportin). Full-length (FL) transportin (lanes 1-3), transportin N-terminus residues 1-581 (lanes 4-6) or transportin C-terminus residues 581-890 (lanes 7-9) was translated in Wheat germ extract in the presence of ^{35}S-methionine as described in the Promega TNT® Transcription/Translation system. GST (lanes 2, 5 and 8) or GST-CC3 (lanes 3, 6 and 9) were mixed with each of the translated proteins and incubated on ice for 1 hour. The recombinant proteins were recovered by mixing in the presence of Glutathione-Sepharose for 30 min. at 4°C. Any ^{35}S-labeled proteins bound to GST-CC3 were resolved on SDS-PAGE, followed by detection on autoradiography film.

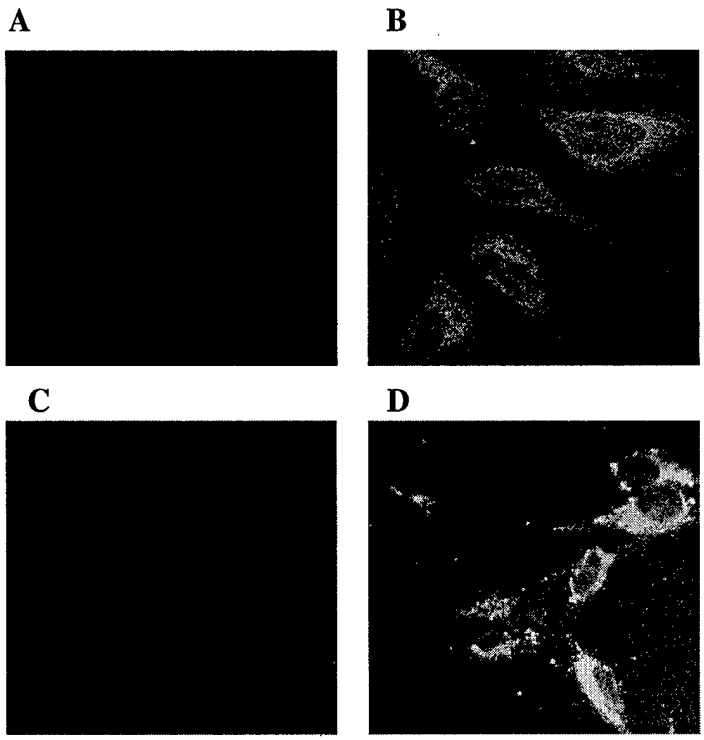

Figure 3. CC3 localizes to nuclear envelope and perinuclear area. HeLa cells were fixed with 2% paraformaldehyde, permealized with 0.2 % Triton X-100 and stained with control rabbit IgG (A) or immunoaffinity purified anti-CC3 antiserum (B). Bound antibodies were detected with anti-rabbit FITC conjugated antibodies. In C and D, HeLa cells were first permealized with digitonin as used for nuclear import assays (1) and incubated in transport buffer without (C) or with (D) added recombinant purified CC3 for 15 mins. Cells were then fixed and stained with anti-CC3 antiserum as in (B).

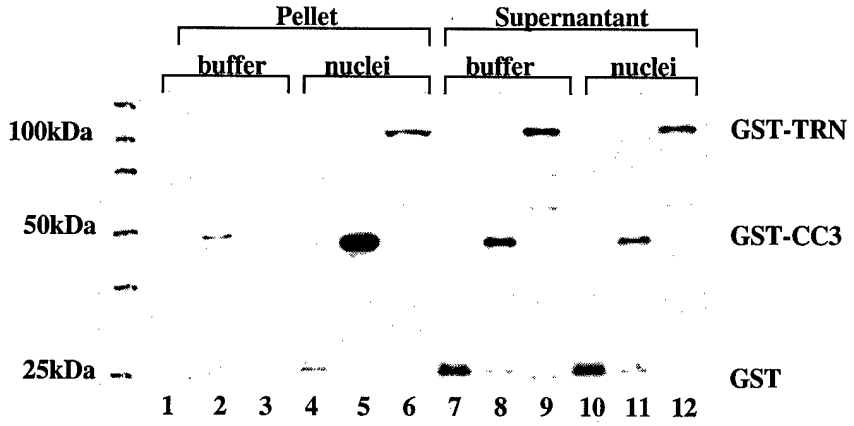

Figure 4. CC3 associates with isolated nuclei. Intact nuclei were isolated from HeLa cells by hypothnic lysis and homogenization. GST (lanes 1,4,7,10), GST-CC3 (lanes 2,5,8,11) or GST-Transportin (lanes 3,6,9,and 12) were incubated with reaction buffer alone (lanes 1-3 and 7-9) or with isolated nuclei (lanes 4-6 and 10-12) for 15 mins. The reactions were then layered on top of 25 % sucrose in reaction buffer and centrifuged at 20,000 g for 15 min. Recombinant proteins in the pellets and supernatants were analyzed by gel electrophoresis, blotting and detection with anti-GST antibodies.

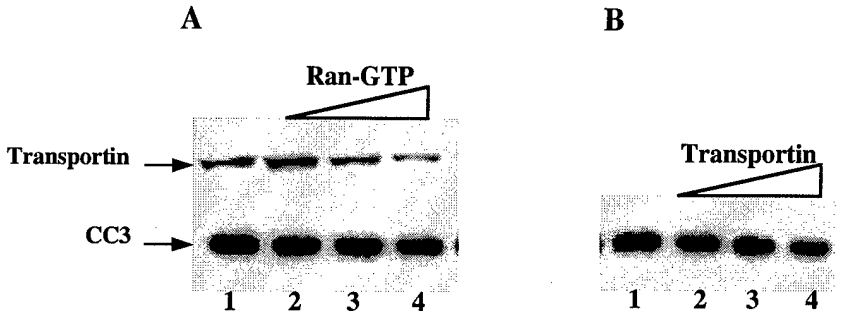

Figure 5. CC3 binding to nuclei is independent of Ran-GTP or transportin. (A). GST-CC3 (0.1 μM) or GST-Transportin (1 μM) were incubated with purified nuclei in the absence (lane 1) or presence of increasing amounts (0.7, 3.4 and 17 μM) of Ran-Q69L (lanes 2-4). Proteins bound to nuclei were analyzed as described in Figure4 (B). GST-CC3 (0.1 μM) was incubated with purified nuclei in the absence or presence of increasing amounts (0.04, 0.2 and 1 μM) of transportin. CC3 protein bound to nuclei was detected as in Figure 4.

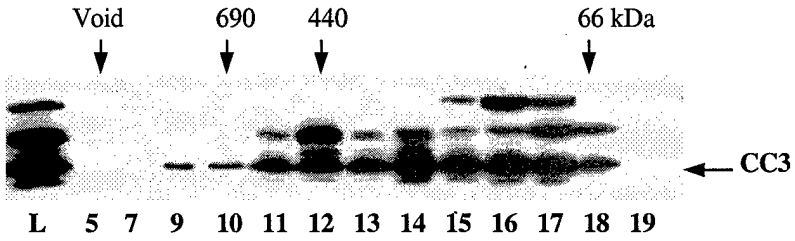

Figure 6. Endogenous CC3 is found in high molecular weight complexes. Solubilized HeLa cell extract (L) was applied to a Superose 12 gel exclusion column and eluted at 0.3 ml/min in 0.5 ml fractions. An aliquote of eluted fractions (5-19) was resolved on SDS-PAGE followed by Western blot analysis with anti-CC3 antibodies.