Camptothecin Induces Nuclear Export of Prohibitin Preferentially in Transformed Cells through a CRM-1-dependent Mechanism*

Shipra Rastogi, Bharat Joshi1, Gina Fusaro2, and Srikumar Chellappan3

From the Drug Discovery Program, Department of Interdisciplinary Oncology, H. Lee Moffitt Cancer Center and Research Institute, University of South Florida, Tampa, Florida 33612

Prohibitin is a growth-suppressive protein that has multiple functions in the nucleus and the mitochondria. Our earlier studies had shown that prohibitin represses the activity of E2F transcription factors while enhancing p53-mediated transcription. At the same time, prohibitin has been implicated in mediating the proper folding of mitochondrial proteins. We had found that treatment of cells with camptothecin, a topoisomerase 1 inhibitor, led to the export of prohibitin and p53 from the nucleus to the mitochondria. Here we show that the camptothecin-induced export of prohibitin occurs preferentially in transformed cell lines, but not in untransformed or primary cells. Cells that did not display the translocation of prohibitin were refractive to the apoptotic effects of camptothecin. The translocation was mediated by a putative nuclear export signal at the C-terminal region of prohibitin; fusion of the nuclear export signal (NES) of prohibitin to green fluorescence protein led to its export from the nucleus. Leptomycin B could inhibit the nuclear export of prohibitin showing that it was a CRM-1-dependent event driven by Ran GTPase. Confirming this, prohibitin was found to physically interact with CRM-1, and this interaction was significantly higher in transformed cells. Delivery of a peptide corresponding to the NES of prohibitin prevented the export of prohibitin to cytoplasm and protected cells from apoptosis. These results suggest that the regulated translocation of prohibitin from the nucleus to the mitochondria facilitates its pleiotropic functions and might contribute to its anti-proliferative and tumor suppressive properties.

Mammalian cells are exposed to a variety of extracellular signals that induce proliferation, differentiation, and apoptosis (1–3). The E2F transcription factors play a major role in regulating the above processes in a signal-dependent fashion (4) and are known to regulate the expression of a wide variety of genes involved in cell-cycle progression as well as apoptosis. The transcriptional activity of E2F1 is mainly regulated by the retinoblastoma tumor suppressor protein, Rb (5–7), which recruits a variety of transcriptional co-repressors, including HDAC1,4 DNMT, polycomb proteins as well as chromatin-remodeling complexes like Brg and Brm (8–12) to mediate transcriptional repression. In addition, our laboratory found that prohibitin, a potential tumor suppressor protein, could bind to and repress the activity of E2F transcription factors (13, 14). The inhibition of E2F1 activity by prohibitin was reversed by specific extracellular signals like IgM stimulation and involved the recruitment of HDACs, N-CoR as well as chromatin-remodeling proteins like Brg and Brm (15, 16). Prohibitin was found to bind to E2F1 through a putative coiled-coil domain spanning residues 185–215 and this region alone could repress the transcriptional activity of E2F1 (17). Interestingly, the growth-suppressive properties of prohibitin strongly correlated with its ability to repress E2F1-mediated transcription. Prohibitin and a related protein, prohibitin 2 (also known as REA and BAP37), have also been shown to regulate the transcription of other factors like the estrogen receptor (18, 19) and myoD (20). These studies clearly demonstrate a nuclear function for prohibitin.

In addition to its proliferative properties, E2F1 can also induce cellular apoptosis in many systems (5, 21). Like its proliferative functions, the apoptotic activity of E2F1 can also be negated by Rb (22, 23). In a similar fashion, prohibitin has been shown to inhibit apoptosis induced by the topoisomerase I inhibitor camptothecin (24) as well as growth factor withdrawal (25). Thus like Rb, prohibitin also possesses the ability to regulate cell proliferation as well as apoptosis. An additional insight into the modulation of apoptosis by prohibitin was provided by the finding that it could physically interact with the p53 tumor suppressor protein and enhance its transcriptional activity (26). This transcriptional induction was mediated by enhanced recruitment of p53 to its target DNA binding sites, as seen by chromatin immunoprecipitation assays (26).

Prohibitin has been shown to contribute to the folding and transport of mitochondrial proteins, especially in yeast systems (27–29). Further, fractionation studies on rat liver had shown the presence of prohibitin on the mitochondria, suggesting that it has a mitochondrial function in mammalian systems as well (30–32). Indeed, our earlier studies have shown that, although prohibitin is predominantly nuclear in human breast cancer cell lines like MCF-7 and T47D, it translocates to the cytoplasmic compartment upon camptothecin treatment and shows mitochondrial localization (26). The translocation of prohibitin to the mitochondria occurred with a simultaneous translocation of the p53 protein; indeed, p53 has been shown to translocate to the mitochondria in response to apoptotic signals (33–36). These studies show that prohibitin has both nuclear and mitochondrial functions, and the net effect of prohibitin on mitochondrial localization (33–36). These studies show that prohibitin has both nuclear and mitochondrial functions, and the net effect of prohibitin on mitochondrial localization is probably a vital element in the regulation of cellular functions.

The appropriate distribution of proteins between the nucleus and cytoplasm is maintained by a dynamic balance between nuclear import and nuclear export, mediated through nuclear pore complexes (37). Active nuclear export of proteins is mediated by short amino acid...
stretches termed nuclear export signals (NESs) (38, 39). The NESs identified to date are rich in hydrophobic amino acids such as leucine or isoleucine, and the leucine residues are critical for their activity (38, 40). Leucine-rich NES containing proteins form a ternary complex with CRM-1/exportin 1 in conjunction with the GTP-bound form of Ran in the nucleus and exported as trimeric complex to the cytoplasm (39, 41–43). In the present study, we demonstrate that prohibitin undergoes nuclear export upon camptothecin treatment mainly in cancer cells; normal cell lines appear to be resistant to this effect of camptothecin. Although the translocation of prohibitin occurred simultaneously with that of p53, prohibitin could translocate to the cytoplasmic compartment independent of p53. Furthermore, the export of prohibitin was inhibited by leptomycin B, which is an inhibitor of CRM-1-mediated export suggesting involvement of this member of importin β family. This observation led to the identification of a putative NES in prohibitin, and deletion of this hydrophobic amino acid stretch caused inhibition of camptothecin-mediated export. Interestingly, cell lines that did not show the translocation of prohibitin were refractory to the apoptotic effects of camptothecin. Inhibition of the binding of prohibitin to CRM-1 by a peptide prevented the export of prohibitin and negatively impacted apoptosis. These results suggest that the nuclear export of prohibitin contributes to its multiple cellular functions.

**MATERIALS AND METHODS**

**Cell Lines, Plasmids, and Transfections**—Breast carcinoma cell lines MCF-7, T47D, and MDA-MB231, human primary fibroblast cell line HSF8, human embryonic lung fibroblast cell line WI-38, p53 negative osteosarcoma cell line Saos-2, and human non-small cell lung cancer cell line H1299 were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Immortalized but non-tumorigenic human breast epithelial cell line MCF-10A was cultured in Dulbecco’s modified Eagle’s medium/F12K containing 5% horse serum, insulin, EGF, cholera toxin, and hydrocortisone. SV40-transformed human lung fibroblast WI-38-VA13 was obtained from ATCC and cultured in Minimal essential medium eagle with Earle’s salt and l-glutamine containing 10% fetal bovine serum, sodium pyruvate, and non-essential amino acids. Human aortic endothelial cells were grown in EGM bullet kit medium containing 5% fetal bovine serum from Clonetics.

A forward primer carrying a MluI site and a reverse primer carrying EcoRI were used for preparing full-length Myc-tagged Phb by PCR; the fragment was subcloned into pBS-Myc-Mum1. N-terminal Myc-tagged prohibitin was released by EcoRI digestion and subcloned into the pCDNA3 mammalian expression vector. This plasmid was called Myc-Phb and was used for transient transfection experiments. To prepare NES-deleted Myc-tagged prohibitin (1–243), a reverse primer carrying termination codon was designed, and PCR was performed using T7 primer with Myc-phb as a template. PCR product was subcloned into pCDNA3 and named Myc-Phb-DNES. GFP-NES was prepared by a PCR-based overlap extension protocol. Appropriate primer sets were designed, and full-length GFP-NES was prepared, TA-cloned, and subcloned back into pCMS-EGFP, a commercially available vector from Clontech Laboratories. Fusion between the NES of prohibitin and GFP as well as the integrity of GFP sequence was verified by sequencing prior to use in transient transfection experiments. Transient transfections were performed with Lipofectamine reagent (Invitrogen) using 1 μg of DNA for each construct on MCF-7 cells cultured in 8-well chamber slides. Primer sequences are as follows: Phb-MluI-F (5′-TCA GTA ACG CTG GCC AAA GTG TT-3′), Phb-243-R (5′-TTA AGC TTC CAG CTT TCG CAG-3′), Phb-NES-F (5′-TAC AAG GCT GCT GAG GAC ATT GCT-3′), Phb-NES-R (5′-TGA TCA TTA CTG GGG GAG CTG GAG GAC CAC-3′), GFP-NES-F (5′-CTT GCC ACA ACC CGG GAT CCA C-3′), and GFP-NES-R (5′-CTC AGG AGC TTT TCA GCT CAG CTC GTC CAT-3′).

**Drug Treatment, Antibodies, and Immunostaining**—The prohibitin monoclonal and polyclonal antibodies were purchased from NeoMarkers, Inc. (Fremont, CA). The p53 polyclonal, CRM-1 polyclonal, Myc tag polyclonal, and hemagglutinin tag monoclonal antibodies were purchased from Santa Cruz Biotechnology. Cells were plated onto poly-d-lysine (Sigma) coated 8-well glass chamber slides (10,000 cells per well) for immunostaining. Cells were either untreated or treated with either 30 μM camptothecin or the dose mentioned for 4 h (Sigma) in the presence or absence of 10 ng/ml leptomycin B. Cells were fixed in 3.5% paraformaldehyde for 25 min, permeabilized in 0.2% Triton X-100/ phosphate-buffered saline for 5 min, and blocked in 5% normal goat serum in phosphate-buffered saline at room temperature for 1 h. Primary antibody incubations were performed overnight at 4° C. After washing, secondary antibody incubation was performed with goat anti-mouse IgG Alexa Fluor-488 (green) and goat anti-rabbit IgG Alexa Fluor-546 (red) for 30 min at room temperature. DNA was labeled with Hoechst staining at a final concentration of 0.0025 mg/ml. Cells were visualized with a Zeiss LSM 510 (Zeiss, Thornwood, NY) confocal microscope, and areas of co-localization were determined using LSM 510 software (Zeiss).

**TUNEL Staining**—MCF-7, MCF-10A, WI-38, and WI-38-VA13 cells were plated onto poly-d-lysine (Sigma) coated 8-well glass chamber slides (10,000 cells per well). The cells were either treated with varying concentrations of camptothecin, namely 15, 30, 60, and 120 μM, for 4 h or the cells were treated with 30 μM camptothecin for varying durations (2, 4, 8, or 16 h). The cells were fixed and stained according to manufacturer’s instructions using Promega’s DeadEnd Colorimetric TUNEL system.

**Immunoprecipitation and Immunoblotting**—Cell lysates (50–200 μg) were treated with 1 μg of the appropriate primary antibody in a volume of 100 μl at 4° C for 1 h. A 3-mg aliquot of protein G-Sepharose in a 100-μl volume was added to each sample and incubated for an additional hour. The binding was performed in a buffer containing 20 mM HEPES, pH 7.9, 40 mM KCl, 1 mM MgCl₂, 0.1 mM EGTA, 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.1 mM NaF, 0.1 mM Na₃VO₄, 0.5% Nonidet P-40, and 3 mg of bovine serum albumin per ml. The beads were washed six times with 600 μl of the same buffer, boiled in 20 μl of SDS sample buffer, and separated on 8 or 10% polyacrylamide gels. After semi-dry transfer to supported nitrocellulose membranes, the blots were probed with the appropriate antibody. The proteins were detected by using an enhanced chemiluminescence assay system from Amer sham Biosciences.

**Delivery of Prohibitin NES to Cells**—A peptide of the sequence ITYLPAGQSVLQL corresponding to the NES of prohibitin was synthesized with a cysteine residue added at the C terminus for coupling to the carrier molecule, penetratin (44). 10 μM Phb-NES peptide was conjugated to 1 μM penetratin (QBiogene) in the presence of 10 mM tris-(2-carboxyethyl)phosphine as described previously (44). The final conjugate was diluted to 150 μM and aliquoted and stored in −80° C until further use. For competition assay, MCF-7 cells were plated on chamber slides as described earlier. The cells were treated with camptothecin (30 μM) for 4 h either in the presence of 10 μM Phb-NES peptide or a control peptide of the sequence FSGKNGFIC. A double immunofluorescence experiment was performed for prohibitin and p53 using monoclonal antibody for prohibitin and polyclonal antibody for p53. Secondary antibody incubation was performed with goat anti-mouse IgG Alexa Fluor-
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488 (green) and goat anti-rabbit IgG Alexa Fluor-546 (red) and after mounting cells, slide was observed by fluorescence microscopy using Leica DM LB2 microscope (40×/0.75 numerical aperture) with a Qimaging Retiga1300 camera.

RESULTS

Subcellular Localization of Prohibitin and p53 Changes Preferentially upon Camptothecin Treatment—We had observed earlier that the subcellular localization of prohibitin and p53 changes in breast cancer cells upon treatment with camptothecin (26). A double immunofluorescence experiment was conducted to examine whether this event was specific to camptothecin, or the translocation occurred when the cells were treated with other apoptotic agents as well. Human breast carcinoma cell lines MCF-7 and T47D were immunostained with a monoclonal antibody against prohibitin, and its presence was detected by secondary antibody labeled with Alexafluor-488, a green fluorochrome. At the same time, p53 was detected using a polyclonal antibody and a secondary antibody labeled with Alexafluor-546, a red fluorochrome. Cells were visualized by confocal microscopy. As shown in Fig. 1A, the majority of prohibitin (left panel) and p53 (middle panel) were found to be localized in the nucleus of MCF-7 cells; both the proteins were found to co-localize (right panel), indicating a physical interaction. As seen earlier, treatment of the cells with 30 μM camptothecin for 4 h led to the translocation of both proteins to the cytoplasmic compartment. Similar results were also obtained in T47D cells (Fig. 1B).

Both the cell lines were also treated for 4 h with different apoptotic agents like tamoxifen (10 μM), which is a competitive antagonist for estrogen receptor; taxol (10 nm), which is a microtubule inhibitor; 5-fluorouracil (10 nm), an analog of uracil and thymine; doxorubicin (2 nm), which is a DNA-intercalating drug; etoposide (30 μM), a topoisomerase II inhibitor; and cisplatin (20 μM), which is a DNA cross-linker. As shown in Fig. 1, the apoptotic agents other than camptothecin were unable to induce the translocation of prohibitin and p53 to cytoplasm in either cell line. We conclude that the observed nuclear export of these proteins is a phenomenon associated with apoptosis induced by the topoisomerase 1 inhibitor, camptothecin.

Nuclear Export of Prohibitin and p53 Does Not Occur in Untransformed Cells—The observation that prohibitin and p53 translocates to the cytoplasm in response to camptothecin was made in breast cancer cell lines. Experiments were designed to examine whether such an event took place in untransformed, immortalized cells as well as cultured primary human cells. Untransformed human diploid fibroblast cell lines, WI-38 and HSFe (Fig. 2, A and B), untransformed mammary epithelial cell line MCF-10A (Fig. 2C), or cultured primary human aortic endothelial cells (HAECs, Fig. 2D) were treated with 30 μM camptothecin as described earlier, and the subcellular localization of prohibitin and p53 was ascertained by double immunofluorescence. As seen in Fig. 2, prohibitin and p53 were found to be co-localized in the nucleus in all the four primary cell lines. Interestingly, they did not translocate to the cytoplasm upon camptothecin treatment, as in several transformed cell lines (Figs. 1 and 4 and data not shown). This experiment suggests that the nuclear exit of prohibitin and p53 is an event that takes place preferentially in transformed cell lines derived from human cancer.

Apoptotic Response to Camptothecin Is Distinctive in Transformed and Untransformed Cell Lines—The apoptotic response to camptothecin treatment at different concentrations as well as for different durations was evaluated by TUNEL staining in the untransformed human diploid fibroblast cell line WI-38 and a WI-38 cell line transformed with SV40 large T-antigen (WI-38-VA13). Similarly, we compared the TUNEL staining in the breast epithelial MCF-10A cells and MCF-7 cells derived from breast cancer. The untransformed cell lines showed significant resistance to apoptosis when the duration of treatment with 30 μM camptothecin was extended to 16 h, as determined by TUNEL staining (Fig. 3A). Similarly, WI-38 and MCF-10A cell lines showed remarkably less TUNEL-positive cells in comparison to the transformed cell lines WI-38-VA13 and MCF-7 at all the treatment doses of camptothecin (Fig. 3B). These results suggest the inherent differences in the transformed and untransformed cell lines to camptothecin induced apoptosis. Because a dose of 30 μM camptothecin and treatment duration of 4 h led to 40–60% TUNEL-positive cells, we chose these conditions for our further experiments.

Nuclear Export of Prohibitin and p53 in Transformed Cell Lines Correlates with Increased Apoptosis—The finding that prohibitin and p53 translocation occurs preferentially in transformed cells was further verified by using two pairs of closely related cell lines. MCF-10A is derived from normal mammary epithelial cells, whereas MCF-7 is derived from breast cancer. Similarly, we obtained a version of the WI-38 cell line that was transformed with SV40 large T-antigen (WI-38-VA13). Prohibitin and p53 were predominantly found in the nucleus of all the four cell lines. As shown in Fig. 4A, camptothecin treatment led to the nuclear export of prohibitin and p53 in the transformed MCF-7 cells as well as the WI-38-VA13 cells but not the normal MCF-10A or WI-38 cells. It thus appears that cellular transformation correlates with the nuclear export of prohibitin and p53.

Attempts were then made to examine whether the changes in the localization of prohibitin and p53 correlated with induction of apoptosis by camptothecin. Toward this purpose, PARP cleavage, a measure of...
caspase activity, was assessed by Western blotting (45) of lysates from MCF-7, MCF-10A, WI-38, and WI-38-VA13 cells treated with camptothecin for different periods (Fig. 4B). MCF-7 and WI-38-VA13 cells, which showed the nuclear export of prohibitin and p53, show significant amounts of PARP cleavage at 4 and 8 h after camptothecin treatment, as judged by the appearance of a lower 86-kDa band. At the same time, untransformed cell lines MCF-10A and WI-38 did not show cleavage of PARP suggesting that the translocation of p53 and prohibitin to the cytoplasm contributes to apoptosis induced by camptothecin.

Nuclear Export of Prohibitin Does Not Require Functional p53—Our previous studies have shown that prohibitin co-localizes with p53 in the nucleus of breast cancer cell lines and treatment with camptothecin caused both the proteins to translocate to the cytoplasmic compart-
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Camptothecin-induced nuclear export of prohibitin does not require p53.

Sao2, MDA-MB231, and H1299 cells, which carry deletions or mutations of the p53 gene, were treated with camptothecin (30 μM) for 4 h, immunostained with an anti-prohibitin monoclonal antibody, and visualized by confocal microscopy. Prohibitin was found to exit the nucleus in these cells as well.

FIGURE 5. Camptothecin-induced nuclear export of prohibitin does not require p53.

Non-small cell lung carcinoma cell line H1299, breast cancer cell line MDA-MB231, and osteosarcoma cell line Saos-2 were plated on polylysine-coated glass chamber slides, treated with camptothecin, and stained for prohibitin. Prohibitin appears predominantly in the nucleus of control cells, whereas treatment with camptothecin induced translocation of prohibitin to the cytoplasm. Addition of LMB inhibits the camptothecin-induced translocation, suggesting that the export of prohibitin from nucleus is a CRM-1-dependent event.

Loss of a Putative NES Prevents Camptothecin-induced Translocation of Prohibitin to the Cytoplasm—To examine whether a potential NES localized mainly to the cytoplasm in all the three cell lines. There was not a complete ablation of prohibitin in the nucleus as we had seen in MCF-7 and T47D cells. At the same time, the observation that prohibitin can migrate to the cytoplasmic compartment suggests that the nuclear export of prohibitin is not dependent on the presence of wild-type p53 in the cell.

Prohibitin Contains a Leucine-rich Nuclear Export Signal at Its C-terminal End—Because the nuclear export of prohibitin did not require the presence of functional p53, attempts were made to understand the mechanisms underlying the translocation process. Several studies have demonstrated that a leucine-rich amino acid sequence in the correct configuration can serve as a signal to direct protein transport out of the nucleus (39, 42, 43, 47). An examination of prohibitin protein sequence revealed a putative leucine/isoleucine-rich motif between amino acids 257 and 270 that had the features of a nuclear export signal. CRM-1, a member of the importin family, has been implicated in the export of NES-containing proteins (41, 47–49). The nuclear export mediated by CRM-1 has been shown to be inhibited by leptomycin B (LMB) (48, 50–52). Based on the identification of a putative NES in prohibitin, we next determined whether LMB would inhibit the cytoplasmic transport of prohibitin. MCF-7, T47D, and WI-38 cells were cultured on chamber slides and treated with camptothecin in the presence or absence of LMB for 4 h. As shown in Fig. 6, prohibitin remains localized predominantly in the nucleus of control cells, whereas treatment with camptothecin induces translocation of prohibitin to the cytoplasm. Addition of LMB inhibits the camptothecin-induced translocation, suggesting that the export of prohibitin from nucleus is a CRM-1-dependent event.

Reduced Prohibitin-CRM-1 Interaction in MCF-10A Cells Correlates with Less Nuclear Export—To further examine the functionality of the prohibitin NES, amino acids 243–272 deleted fused to a Myc tag at the C-terminus of prohibitin. Myc-tagged prohibitin constructs were transfected into MCF-7 cells, and the expression and the localization of the proteins was assessed by immunostaining using a monoclonal antibody to the Myc tag. As shown in Fig. 7A, the cells transfected with full-length Myc-tagged prohibitin show predominant nuclear staining, whereas treatment with camptothecin shows a significant reduction in nuclear staining. Further, the treatment of transfected cells with camptothecin in the presence of LMB shows nuclear accumulation of Myc-tagged prohibitin. The cells transfected with Myc-tagged NES deletion mutant of prohibitin (Myc-NESPhb) also showed nuclear localization; but interestingly, camptothecin treatment did not lead to the translocation of the protein to the cytoplasm. The amount of the NES protein remained comparable in transfected cells treated with camptothecin in the presence or absence of LMB. This result suggests that the C-terminal NES is essential for the translocation of prohibitin upon camptothecin treatment.

NES of Prohibitin Can Facilitate the Cytoplasmic Translocation of GFP—To further examine the functionality of the prohibitin NES, amino acids 243–272 of prohibitin were fused to the C terminus of pCMS-EGFP. This construct was then transfected into the MCF-7 cells. Although wild-type GFP localized predominantly to the nucleus, GFP-NES localized mainly to the cytoplasm (Fig. 7C). This result strongly suggests that the prohibitin NES is functional and directs the export of proteins out of the nucleus.

Reduced Prohibitin-CRM-1 Interaction in MCF-10A Cells Correlates with Less Nuclear Export—Results from the above immunofluorescence experiments raised the possibility that prohibitin physically interacts with CRM-1 in the cell. This was examined by an immunoprecipitation-
Western blot experiment. Cell lysates from control or camptothecin-treated MCF-7 and MCF-10A cells were immunoprecipitated with antibodies to prohibitin and the presence of CRM-1 in the immunoprecipitates was examined by Western blotting. As shown in Fig. 8, a significant amount of CRM-1 could be detected in prohibitin immunoprecipitations from control as well as camptothecin-treated MCF-7 cells. In contrast, there was very little CRM-1 associated with prohibitin in MCF-10A cells. Western blots of the lysates also showed that MCF-7 cells had more CRM-1 than MCF-10A cells, whereas MCF-10A cells had significantly higher amounts of prohibitin than MCF-7 cells; levels of actin were comparable in both the cell lines. This result seems to suggest that increased association of CRM-1 with prohibitin in MCF-7 cells facilitates the nuclear export of prohibitin in response to camptothecin, whereas the lack of CRM-1-prohibitin interaction in MCF-10A cells correlates with nuclear retention of prohibitin upon camptothecin treatment. It thus appears that there could be alterations in the nuclear export machinery as well, in transformed cells compared with normal cells.

Translocation of Prohibitin in the Presence of Camptothecin Is Disrupted in the Presence of Phb-NES Peptide—To assess the importance of the NES in the translocation of prohibitin, a different strategy was employed. A peptide corresponding to NES of prohibitin was synthesized and tested for its ability to disrupt the nuclear export of prohibitin. As shown in Fig. 9, deletion of the NES abolishes the nuclear export of prohibitin. Addition of the NES leads to the export of GFP to the cytoplasm.

FIGURE 7. Prohibitin requires a putative leucine-rich nuclear export signal (NES) in the C-terminal region for nuclear export. A, a schematic showing the location and sequence of the putative NES of prohibitin. Structure of vectors expressing Myc-tagged full-length or ΔNES-Phb as well as a GFP fusion protein with the NES of prohibitin is also shown. B, loss of NES inhibits translocation of prohibitin to the cytoplasm. MCF-7 cells were transfected with Myc-tagged full-length prohibitin (top panel), Myc-tagged prohibitin lacking NES (middle panel), and vector pCDNA3 (bottom panel). Transfected cells were treated with camptothecin (30 μM) or camptothecin (30 μM) in the presence of inhibitor of CRM-1-mediated transport leptomycin B (10 ng/ml) for 4 h, immunostained with an anti-Myc monoclonal antibody, and visualized by confocal microscopy. Deletion of the NES abolishes the nuclear export of prohibitin. C, the NES of prohibitin can induce nuclear export of GFP. MCF-7 cells were transfected with EGFP (left) and EGFP-NES fusion (right) and visualized by confocal microscopy. Addition of the NES leads to the export of GFP to the cytoplasm.
sized and conjugated to a carrier peptide, penetratin, for delivery into the cells. The effect of delivering this peptide on the nuclear export of prohibitin was assessed by a double immunofluorescence experiment. As shown in Fig. 9A, prohibitin and p53 translocate from nucleus to cytoplasm upon camptothecin treatment in control MCF-7 cells that did not receive any peptide; similarly, the translocation was not affected in cells treated with a control peptide (Fig. 9B). In contrast, the Phb-NES peptide could inhibit the translocation of prohibitin to a great extent in MCF-7 cells while translocation of p53 remained unaffected (Fig. 9C). This result suggests that translocation of prohibitin is indeed being carried out through NES, which is effectively competed out in the presence of Phb-NES peptide. The effect of this peptide on camptothecin-mediated apoptosis was examined by assessing the cleavage of PARP by Western blotting (Fig. 10). MCF-7 cells treated with camptothecin in the presence of Phb-NES peptide did not show cleavage of PARP as judged by the appearance of a lower band. At the same time, untreated MCF-7 cells and cells exposed to control peptide showed marked PARP cleavage upon treatment with camptothecin (Fig. 10). This suggests that inhibition of the nuclear export of prohibitin can prevent camptothecin-mediated apoptosis.

DISCUSSION

The subcellular localization of prohibitin has been variously attributed to the mitochondria or the nucleus (13–16, 24, 26–32, 53–55). A mitochondrial function for prohibitin has been suggested in systems as varied as yeast or mammalian systems in earlier studies. Further, a role for prohibitin has been proposed in mitochondrial integrity in yeast, and its loss of function led to metabolic defects. In contrast, studies from many laboratories, including ours have attributed a predominantly nuclear function for prohibitin (13–16, 24, 26–55). It has been shown recently that prohibitin is localized to the nucleus and cytoplasm of LnCAP cells (55); similarly, studies on prohibitin in rat ovary showed that prohibitin translocated from the cytoplasm to the nucleus in atretic follicles (54). A protein very similar to prohibitin (prohibitin 2, REA, and BAP37) has been described to repress the transcriptional activity of the estrogen receptor (18, 19) as well as myoD (20). Studies from our laboratory have consistently shown predominantly nuclear localization of prohibitin in cultured human cells and its ability to repress E2F-mediated transcription by recruiting co-repressors like HDAC-1 and N-CoR (53) as well as chromatin remodeling proteins like Brg1/Brm (15, 16). In addition, our laboratory has demonstrated that prohibitin translocates from the nucleus to the cytoplasmic compartment, specifically to the mitochondria, in response to apoptotic signaling as shown by Western blots in nuclear and cytoplasmic fractions from camptothecin-treated cells (26). This raises the possibility that prohibitin carries out both nuclear and mitochondrial functions in the cell. Studies presented here demonstrate that this nuclear export of prohibitin is a signal-specific event that occurs preferentially in transformed cell lines, whereas it remains nuclear in untransformed cells.

Although the nucleocytoplasmic shuttling of proteins that are smaller than 50 kDa can occur by passive diffusion through aqueous channels that exist around the perimeter of the nuclear pore complex (39, 43), prohibitin, which is only 30 kDa may require active transport, because it is largely bound to multiprotein complexes like E2F transcription factor family, Rb, HDACs, N-CoR, and Brg1/Brm to perform its nuclear function. The active nuclear import and export of proteins are mediated by short amino acid stretches termed as nuclear localization signals and nuclear export signals, respectively. Our results show that prohibitin has a well defined nuclear export signal. The most common type of export sequence resembles the one in the human immunodefi-
cytotoxic virus, type 1 Rev protein and typically comprises a core of large hydrophobic amino acids that specify recognition by the CRM-1 export receptor (56). An earlier study has demonstrated that in Rev-type NESs in different cellular functional proteins, the relative export activity varies considerably (57, 58). The weakest NESs were found in the p53 tumor suppressor and the p53-regulated proteins p21 and hdm2, which are all normally localized to the nucleus (59). The preferential repression of the export of prohibitin by the peptide could be explained by the differences in the NESs of prohibitin and p53. All of the Rev-type NESs were inactivated by mutation of key hydrophobic residues and by treatment with the CRM-1-specific export inhibitor, leptomycin B (57). Leucine-rich NES-containing substrates form ternary complex with CRM-1/exportin 1, in conjunction with the GTP-bound form of Ran in the nucleus, and are exported as a trimeric complex to the cytoplasm. We demonstrate that the translocation of prohibitin is mediated through its interaction with CRM-1, as in the case of other proteins (60–62). The removal of predicted nuclear export signal inhibited the translocation of prohibitin, and addition of this moiety to GFP facilitated its export suggesting that the Phb NES is functional and plays crucial role in translocation of prohibitin.

One interesting observation is the correlation between prohibitin translocation and the apoptotic response in various cell lines. The fact that, at the times points (4–8 h) of camptothecin treatment, non-transformed cell lines MCF-10A and WI 38 do not show translocation or high amount of PARP cleavage suggests that the translocation of prohibitin to the mitochondria might have an effect on the apoptotic process. Various studies have shown that p53 translocates to the mitochondria in response to apoptotic signaling (33–36). Our finding that prohibitin can affect p53 function and that p53 also translocates to the mitochondria supports the contention that these events contribute to the cellular apoptotic process (26). Prohibitin has been found to affect apoptosis by other groups as well; in one case, prohibitin levels in the mitochondria were found to be enhanced during apoptosis induced by the telomerase inhibitor, 3,3′-diethyloxadacarbocyanine iodide (63). Thus the results presented here support our contention that the subcellular localization of prohibitin might affect cellular apoptosis. Another interesting observation is that the endogenous prohibitin levels influence the camptothecin-induced apoptosis. Our earlier studies have shown that Ramos cells expressing prohibitin are resistant to camptothecin-induced apoptosis (24). As shown in Fig. 7, MCF10A cells appear to have more prohibitin in comparison to MCF-7; also MCF10A cells show reduced PARP cleavage suggesting that levels of prohibitin also influence the apoptotic response in various cell lines.

A role for prohibitin in the genesis of breast cancer has been proposed by many groups (64–66). Earlier studies had detected mutations in the prohibitin gene in sporadic breast cancers. Alterations in prohibitin function in cancer appear to be a reasonable hypothesis given its strong anti-proliferative properties. In addition to the anti-proliferative properties of the protein, elegant studies from the Jupe laboratory have shown that the 3′-untranslated region of the prohibitin messenger RNA could have anti-proliferative and tumor-suppressive effects (65, 67, 68). The studies described in this report raise the possibility that differences in the translocation of prohibitin could be another dimension that determines the growth and apoptosis of tumor cells.

In conclusion, our studies show a dynamic partitioning of prohibitin between nuclear and cytoplasmic compartments of tumor cells in a signal-dependent manner, which appear to affect the apoptotic properties of the cell. Further studies on the mechanisms underlying this regulated translocation of prohibitin will throw light on hitherto unknown differences between tumor cells and normal cells.
