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14. ABSTRACT Prohibitin, a potential tumor suppressor protein, was originally identified by its ability to induce G1/S arrest in human fibroblasts. Mutations in the prohibitin gene were subsequently found in sporadic breast tumors. Our experiments in B cells and breast cancer cells suggest that prohibitin protects against apoptosis induced by camptothecin, a topoisomerase I inhibitor. A human B cell line (Ramos) stably over-expressing prohibitin and treated with camptothecin exhibits 50% less apoptosis compared to the parental cell line. BT 549 breast cancer cells, which express high levels of endogenous prohibitin, exhibit 20% less death from camptothecin than ZR 751 cells, which have low levels. E2F transcriptional activity increases in response to camptothecin, but this increase is attenuated in cells overexpressing prohibitin. Moreover, we find that prohibitin and p53 associate in vitro and co-localize in the breast cancer cell lines MCF7 and T47D. Functionally, prohibitin may activate p53 mediated transcription and augment p53 binding to a target promoter. Further, prohibitin was specifically exported from the nucleus of breast cancer cells, but not normal cells.. The role of this in cellular apoptosis is being evaluated. Our studies are elucidating the mechanisms whereby prohibitin affects the chemotherapeutic response and may help in directing therapeutic strategies for breast cancer treatment.					
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Introduction

Prohibitin, a potential tumor suppressor protein, was originally identified by its ability to induce G1/S arrest in human diploid fibroblasts. The

prohibitin (Phb) gene was subsequently shown to be mutated in several sporadic breast tumors. We have shown that prohibitin binds Rb and represses all the five transcriptionally active E2Fs (Wang *et al.*, 1999a). Prohibitin co-immunoprecipitates with both Rb and E2F1, and contacts each protein using different domains. Certain signaling cascades such as IgM stimulation of B cells reverses prohibitin-mediated repression of E2F1; Rb remains inert to this stimulus (Wang *et al.*, 1999b). It had been shown earlier that microinjection of antisense oligonucleotides against prohibitin promotes entry into S phase (Nuell *et al.*, 1991); (Jupe *et al.*, 1995). Supporting this observation, colony formation of various breast cancer cell lines is repressed by prohibitin. Repression by prohibitin requires the same domains that are used to bind to Rb and E2F; deletion of either of these domains abrogates prohibitin mediated growth arrest. Immunocytochemical studies indicate that prohibitin is highly expressed in neoplastic foci of various tumor types (Coates *et al.*, 2001). It has been suggested that prohibitin associates with the IgM receptor in murine B lymphocytes (Terashima *et al.*, 1994). In yeast, however, it has been found that prohibitin might associate with the mitochondrial inner membrane (Coates *et al.*, 2001); (Berger & Yaffe, 1998); (Steglich, 1999); (Nijtmans *et al.*, 2000). Most recently, prohibitin-2 was found to associate with and repress the nuclear estrogen receptor. Also known as REA for repressor of estrogen activity, this protein is unique in its ability to selectively bind the unliganded estrogen receptor and to maintain it in a repressed state (Delage-Mourroux *et al.*, 2000); (Montano *et al.*, 1999).

Given the growth suppressive function of prohibitin and its ability to repress E2F-mediated transcription, it was examined whether prohibitin also affects the apoptotic process. It was found that the expression of prohibitin protected cells from death induced by the chemotherapeutic drug camptothecin, but not other agents. While Rb family members were functionally inactivated during drug treatment, prohibitin levels were elevated. In breast cancer cell lines, prohibitin was mainly a nuclear protein. Camptothecin treatment induced the migration of prohibitin out of the nucleus and into the cytoplasm, to peri-nuclear regions. In addition, it was found that prohibitin could functionally interact and activate the p53 tumor suppressor protein. Further studies funded by the project and carried out by Gina Fusaro, the former graduate student working on this project, showed that prohibitin enhances the transcriptional activity of p53. Further, it was found that the translocation of prohibitin from the nucleus to the cytoplasm occurs only in cancer cells, not normal cells. Becky Kinkade, in collaboration with a post-doctoral scientist in the lab, has been characterizing the nuclear export of prohibitin in breast cancer cells and its role in cellular apoptosis.

Body

We made significant advances on studies dealing with the nuclear export of prohibitin during the project period. These studies originated from the first specific aim of the grant, which deals with prohibitin and cellular apoptosis. Based on our findings on prohibitin-mediated regulation of p53 and translocation of these proteins in response to camptothecin, efforts were made to understand the molecular mechanisms involved. It was found that prohibitin has a potential nuclear export signal (NES) close to its C-

terminal end, spanning residues 257 to 270. This is a leucine-rich region that has all the features of a NES. This is a novel observation, since the presence of a NES on prohibitin has not been reported before.

Attempts were made to assess whether the putative NES was necessary for the nuclear export of prohibitin in response to camptothecin treatment. A deletion mutant was constructed, where the residues 242-273 of prohibitin was deleted. This was cloned with a myc tag. MCF-7 cells were transiently transfected with this construct or a myc-tagged wild-type prohibitin construct and the localization of prohibitin in the presence or absence of camptothecin was assessed by immunofluorescence using an anti-myc antibody which recognizes the transfected myc-tagged prohibitin, but not the endogenous prohibitin. It was found that the myc-tagged wild-type prohibitin translocated out of the nucleus upon camptothecin treatment, but the mutant protein with residues 242-273 deleted remained in the nucleus. This suggests that the C-terminal hydrophobic region is necessary for prohibitin translocation.

The ability of the putative hydrophobic region to act as a genuine NES was next examined. Towards this purpose, the corresponding region was fused to GFP. Control GFP vector or the GFP-NES fusion was transfected into MCF-7 cells. It was found that while GFP remained predominantly in the nucleus, the GFPNES construct was distributed in the nucleus and the cytoplasm, suggesting that this region could act as a genuine NES.

The hydrophobic nuclear export signals of proteins carry out their function in association with proteins of the importin family. The major exportin protein is crm-1, whose function can be inhibited by leptomycin-B. It was next examined whether the nuclear export of prohibitin is leptomycin-B sensitive. MCF-7 cells were treated with camptothecin for 4 hours in the presence or absence of leptomycin B. It was found that the nuclear export of prohibitin was inhibited by leptomycin B, suggesting that it is a crm-1 dependent event. It was also found that prohibitin could be found in association with crm-1 in MCF-7 cells, as seen by immunofluorescence.

The effect of preventing prohibitin translocation on cellular apoptosis is being assessed. We are examining whether preventing the binding of prohibitin to crm-1, or preventing its translocation by other means will affect the response of cells to camptothecin as well as other chemotherapeutic agents. We predict that preventing the nuclear export of prohibitin will reduce the apoptosis induced by camptothecin, but not other agents.

These results were published in J. Biol.Chem last year (Rastogi et al., 2006 Feb 3;281(5):2951-9); this grant is acknowledged in the paper.

Thus this pre-doctoral fellowship has resulted in two publications in reputed journals. We expect to continue these studies in the future. Work on prohibitin and breast cancer is being actively pursued in the lab and we hope to make more headway in understanding this protein.

Key Research Accomplishments

The following key research accomplishments in breast cancer cell lines have been supported by this award:

- Prohibitin is protective against apoptosis induced by the chemotherapeutic drug camptothecin.
- Prohibitin attenuates E2F activity during camptothecin induced apoptosis, when Rb family members are inactive.
- Prohibitin is a nuclear protein in untreated breast cancer cells, but migrates to the cytoplasm after induction of apoptosis.
- Prohibitin associates with p53 and activates p53 transcriptional activity.
- Prohibitin promotes p53 binding to an endogenous promoter but represses E2F1 binding.

Reportable Outcomes

The following reportable outcomes have been supported by this award:

- Manuscripts

1. **Fusaro, G.**, Dasgupta, P., Rastogi, S., Joshi, B., and Chellappan, S. Prohibitin induces the transcriptional activity of p53 and is exported from the nucleus upon apoptotic signaling. J Biol Chem. 2003 Nov 28;278(48):47853-61..
2. **Fusaro, G.**, Wang, S., and Chellappan, S. Differential regulation of Rb family proteins and prohibitin during camptothecin induced apoptosis. (2002). Oncogene, 21: 4539 - 4548.
3. Wang, S., **Fusaro, G.**, Padmanabhan, J., and Chellappan, S. (2002). Prohibitin co-localizes with Rb in the nucleus and recruits N-CoR and HDAC1 for transcriptional repression. Oncogene, 21: 8388 - 8396.
4. Rastogi S, Joshi B, **Fusaro G**, Chellappan S. (2006) Camptothecin induces nuclear export of prohibitin preferentially in transformed cells through a CRM-1-dependent mechanism. J Biol Chem. 2006 Feb 3;281(5):2951-9.

- Presentations

1. "The Role of the Prohibitin Gene in Apoptosis of Breast Cancer Cells." Poster presented at the Department of Defense Era of Hope Breast Cancer Meeting, Orlando, Florida. September 25 - 28, 2002.

2. Camptothecin induces nuclear export of prohibitin preferentially in transformed cells through a CRM-1-dependent mechanism. AACR Annual Conference, Anaheim California April 2005.

- Degrees Obtained

Doctor of Philosophy degree from Columbia University, Department of Pathology, awarded to Gina Fusaro (PI) on May 21. 2003.

- List of Personnel Supported by this Award

Gina Fusaro, Ph.D. (PI)

Rebecca Kinkade (February 2004- April 2005).

Conclusions

Our data suggests several important functions for prohibitin in breast cancer cells. First, in the course of receiving genotoxic insult from camptothecin, cells degrade Rb while simultaneously increasing levels of prohibitin protein. In the presence of hyper-proliferatory signals from transcription factors such as E2F, cells induce apoptosis to prevent uncontrolled growth. Prohibitin may protect cells from death by providing a means to rein in E2F activity and thus attenuate hyper-proliferatory signals. Such an activity for prohibitin might have vital implications for the selection of drugs to treat breast cancer, because tumors that express high protein levels of prohibitin may be resistant to certain apoptosis inducing drugs.

Second, prohibitin interacts with the p53 tumor suppressor protein physically and functionally. These proteins associate *in vivo* and this association is altered in response to apoptotic signals from camptothecin. By activating p53 activity, prohibitin may aid in promoting p53 mediated cell cycle arrest or apoptosis in tumors that express both of these proteins. Furthermore, prohibitin can affect the binding of at least two different transcription factors, E2F1 and p53, to target promoters: prohibitin activates p53 and promotes its binding to target promoters, while repressing E2F1 activity and preventing its recruitment to promoter regions. Prohibitin may thus potentially be an important modulator of gene activity.

Third, we find that the translocation of prohibitin from the nucleus to the cytoplasm is a regulated event, that is mediated by a specific nuclear export signal. Attempts are being made to understand the role of this translocation in modulating cellular apoptosis.

We have evidence that prohibitin may intersect both the Rb/E2F pathway and the p53 pathway, providing a link between proliferation and growth control. Our studies are thus elucidating the mechanisms whereby prohibitin affects the chemotherapeutic response and may help in directing therapeutic strategies for patients with breast cancer.

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