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Award Number: DAMD17-01-1-0619

TITLE: Phosphorylation of intracellular IGF binding protein-3 by the IGF signaling cascade is essential for its growth-enhancing effect in mammary epithelial cells

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REPORT DATE: July 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2002	Annual (1 Jul 01 - 30 Jun 02)

4. TITLE AND SUBTITLE
Phosphorylation of intracellular IGF binding protein-3 by the IGF signaling cascade is essential for its growth-enhancing effect in mammary epithelial cells

5. FUNDING NUMBERS
DAMD17-01-1-0619

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

The insulin-like growth factors (IGF) are involved in processes leading to tumorigenesis and metastasis. The IGFs stimulate growth of mammary epithelial cells, the site of origin of ductal breast carcinomas. Their ability to stimulate growth is modulated by IGF binding protein-3. The goal of these studies is to determine how IGFBP-3 enhances IGF action. Two established mammary epithelial cell lines genetically engineered to express IGFBP-3 will be the experimental models. We have found that the ability of IGF-I to activate chemical signals within the cell that lead to gene activation is enhanced in cells expressing IGFBP-3. Studies in progress will determine if IGFBP-3 enhances IGF-I stimulated cell cycle progression in these cells and if IGF-I phosphorylates IGFBP-3 via IGF receptor activation. Establishing whether or not the IGF signaling cascade results in phosphorylation of IGFBP-3 is central to the overall hypothesis that intracellular IGFBP-3 plays a role in IGF-I stimulation of cell cycle progression. Further work in this area using breast tumor specimens will determine whether this pathway is disrupted in breast cancer. Potential therapies for breast cancer may include treatments that alter phosphorylation or dephosphorylation of the IGFBP-3 protein.

14. SUBJECT TERMS
breast cancer, mammary, insulin-like growth factor, phosphorylation, binding protein

15. NUMBER OF PAGES
6

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION:

The insulin-like growth factors (IGF) are involved in processes underlying tumorigenesis and metastasis, including cell cycle progression, inhibition of apoptosis, and cell migration. High circulating levels of IGF-I in pre-menopausal women have been shown to be associated with an increased risk of breast cancer, supporting a role for IGFs in tumor progression (1). The IGFs stimulate growth of both normal and transformed mammary epithelial cells, the site of origin of ductal breast carcinomas. Their ability to stimulate growth is modulated by IGF binding protein-3. Clinical data suggest that IGFBP-3 may promote tumor growth e.g. highly malignant breast tumors make more IGFBP-3 compared to tumors with a more positive prognosis and high serum levels of IGFBP-3 are associated with poor prognosis and a decrease in disease free survival (2). This is supported by in vitro data showing that breast tumor cells (3) and nontransformed mammary epithelial cells (4) that have been genetically engineered to constitutively express IGFBP-3 exhibit an enhanced responsiveness to IGF-I in terms of DNA synthesis. IGFBP-3 is a secreted protein and most paradigms have focused on events occurring in the extracellular environment or at the cell surface. However, we hypothesize that IGFBP-3 may function within the cell to influence IGF-I-stimulated cell cycle progression. Therefore the purpose of this project is to determine if intracellular modification of IGFBP-3 by IGF-I represents an important component of IGFBP-3 action. The specific aims of this proposal are to determine if IGFBP-3 enhances IGF-I stimulated cell cycle progression in mammary epithelial cells and if phosphorylation of IGFBP-3 is required for this effect.

BODY:

The experimental models that are being used for these studies are two established cell lines that have been transfected to overexpress IGFBP-3: (1) the estrogen receptor-positive human breast tumor cell line MCF-7 and (2) the MAC-T non-tumorigenic bovine mammary epithelial cell line that differentiates and produces milk proteins under appropriate stimuli. The constitutive expression of IGFBP-3 by both of these cell lines is associated with enhanced IGF-I responsiveness. To begin to determine if IGFBP-3 alters IGF-stimulated DNA synthesis through intracellular mechanisms, we investigated whether the activation of signaling cascades was enhanced in response to IGF-I in MAC-T +BP3 cells. We found that the phosphatidyl inositol 3-kinase (PI3K) pathway was augmented in +BP3 cells in that phosphorylation of Akt was stimulated to a greater degree by IGF-I, as well as for an extended time period compared to mock transfected control cells (mock). Several additional findings support an intracellular mechanism of action for IGFBP-3 in mediating these effects. Firstly, the enhanced activation of IGF-I by endogenous IGFBP-3 could not be mimicked by the addition of exogenous IGFBP-3 to control cells. Secondly, IGF analogues that bind to IGFBP-3 but not the IGF receptor failed to elicit this response, suggesting binding of extracellular IGFBP-3 to IGF-I is not required, and lastly +BP3 cells also exhibited enhanced activation of Akt in response to TGF-α. The later response suggests that IGFBP-3 acts downstream of the IGF receptor itself. The details of these studies are reported in reference (5). We are in the process of repeating these studies with MCF-7 +BP-3 breast tumor cells to see if similar results are observed. This will be particularly interesting because it has recently been reported that addition of exogenous IGFBP-3 to MCF-7 cells inhibits the ability of IGF-I to activate signal transduction cascades (6).

To determine if cell cycle progression is enhanced in +BP3 cells in response to IGF-I, flow

cytometry experiments are in progress. At this point, flow cytometry conditions have been established using wild-type MAC-T cells. Exponentially growing cells treated with or without IGF-I were collected at multiple time points. Cellular DNA was labeled with bromodeoxyuridine prior to fixation. Our next step is to determine the proportion of cells in each phase of the cell cycle at a given time for both MAC-T and MCF-7 cells transfected with IGFBP-3 or vector alone. It is anticipated that +BP3 cells treated with IGF will have a greater proportion of cells in G2/M and S phase and a smaller number in G0/G1 compared to mock cells treated with IGF by the later time points. In addition, since our previous results (4) have shown that MAC-T +BP3 cells have a very low basal rate of DNA synthesis relative to mock cells in the absence of IGF, we also anticipate that +BP3 cells should have a greater number of cells in G0/G1 compared to mock cells in the absence of IGF. If the anticipated results are obtained, additional experiments will be conducted to determine which components of the cell cycle are involved. The comparison between MAC-T and MCF-7 cells will determine if IGFBP-3 functions similarly in non-transformed and transformed mammary epithelial cells.

The ability of IGF-I to phosphorylate IGFBP-3 will be determined by metabolic labeling with ^{32}P-orthophosphate. Cell lysates will be collected at time points over the course of 1 h and immunoprecipitated with IGFBP-3 antisera. Immunoprecipitates will be analyzed by electrophoresis, dried, autoradiographed, and quantitated by phosphorimager analysis, or immunoblotted for determination of total IGFBP-3. In addition to this approach, we have begun studies using standard immunoprecipitation approaches to determine if IGFBP-3 coprecipitates with proteins that are phosphorylated on either tyrosine or serine/threonine.

It should be noted that the postdoctoral fellow working on this project left Dr. Cohick's laboratory to accept a full-time position when the cell cycle progression work was ongoing. As a result, a one-year extension has been approved for this project. It is anticipated that in the coming year the cell cycle progression studies and IGFBP-3 phosphorylation studies will be completed.

KEY RESEARCH ACCOMPLISHMENTS:

Not applicable as a major portion of the work has not yet been completed.

REPORTABLE OUTCOMES:

- Grill CJ, Sivaprasad U, Cohick WS. 2002 Constitutive expression of IGF binding protein-3 by mammary epithelial cells alters signaling through akt and p70S6 kinase. J Mol Endocrinol 29:153-162 (a portion of Dr. Grill's salary was supported through this grant).

- Grill CJ, Cohick WS. 2001 Potentiation of IGF-I action in mammary epithelial cells expressing IGFBP-3 involves alterations in the PI3 kinase signaling cascade. 83rd Annual Meeting of the Endocrine Society, p 189 (a portion of Dr. Grill's salary was supported through this grant).

- The postdoctoral training offered to Dr. Constance Grill through support from this grant contributed to her success in obtaining employment by Schering-Plough in the Division of

Oncology.

- It is anticipated that the remaining work supported by this grant will result in one additional abstract to be presented at a national meeting and at least one additional publication.

- It is anticipated that the remaining work supported by this grant will form the basis of an Idea grant to be submitted to the DOD Breast Cancer Research Program in May, 2003

CONCLUSIONS:

Establishing whether or not the IGF signaling cascade results in phosphorylation of IGFBP-3 is central to the overall novel hypothesis that intracellular IGFBP-3 plays a role in IGF-I stimulation of cell cycle progression. Future studies will determine the actual phosphorylation sites involved, including mutational analysis and generation of stable cell lines expressing mutated IGFBP-3 for use in functional studies. In addition, studies will be designed to determine the phase(s) of the cell cycle that is influenced by IGFBP-3. Further work in this area using breast tumor specimens will determine whether this pathway is disrupted in breast cancer. In addition this work will help to explain why IGFBP-3 has been reported to have opposing effects on mammary cell growth. Potential therapies for breast cancer may include treatments that alter phosphorylation or dephosphorylation of the IGFBP-3 protein.

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