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TITLE: Role of IGFBP-3/IGFBP-3 Receptor Interacton in Normal and Malignant Mammary Growth

PRINCIPAL INVESTIGATOR: Youngman Oh, Ph.D.

CONTRACTING ORGANIZATION: Oregon Health Sciences University
                         Portland, Oregon 97201-3098

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PREPARED FOR: U.S. Army Medical Research and Materiel Command
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Youngman Oh, Ph.D.

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11. SUPPLEMENTARY NOTES

13. ABSTRACT *(Maximum 200 Words)*
This report will detail the investigations of my laboratory into the biological actions of insulin-like growth factor binding protein 3 (IGFBP-3), which has been shown to exhibit activity in an IGF-independent manner. Data indicate that IGFBP-3 alone can bind to breast cancer cell surfaces, and subsequently exert inhibitory effects on cell growth. We have isolated, cloned, expressed, and purified a novel IGFBP-3 interacting protein, clone 4-33, and demonstrated a specific interaction with IGFBP-3 in a human breast cancer cell system. Further, transient overexpression of clone 4-33 in breast cancer cells results in an increase in the specific binding of IGFBP-3 to the cell surface. To facilitate more detailed investigation into the interaction and functions of these two proteins, we have generated a polyclonal α4-33 antibody which is specific and functional in a variety of immunoassays. Also to this end, we have generated IGFBP-3 stably transfected human breast cancer cell lines, in which expression of IGFBP-3 is inducible. Additionally, we have localized the IGFBP-3 receptor binding domain within the midregion of the IGFBP-3 molecule, residues 88-148. Further, we have examined naturally occurring and generated synthetic IGFBP-3 proteolytic fragments, and characterized IGF and insulin binding affinity compared to intact IGFBP-3.

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FOREWORD

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N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

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PI - Signature   Date

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INTRODUCTION:

In this project, I proposed investigation of the biological significance and mechanism of the IGF-independent action of IGFBP-3, as well as identification and characterization of an IGFBP-3 receptor in the human breast cancer system. My laboratory employed the yeast two-hybrid system to identify a potential IGFBP-3 receptor from human breast cancer cells (1). In the past year, we have characterized an IGFBP-3 interacting protein in the breast cancer system, utilizing several new tools and reagents generated within the laboratory. Additionally, we identified and initially characterized new lower affinity members of the IGFBP superfamily, the IGFBP-related proteins (rPs) (2). With respect to one of these related proteins, IGFBP-rP2, and IGFBP-3 we have demonstrated an involvement of the IGF axis in cell growth inhibition induced by sodium butyrate, a dietary micronutrient (see below).

BODY:

I. IGFBP-3 Interacting Protein (Tasks 1- 5).

In our continuing investigation of the biological importance of IGFBP-3, we are characterizing a novel protein which we isolated based upon its ability to specifically interact with IGFBP-3. An internal fragment of the IGFBP-3 cDNA, coding for 61 amino acids, was used as bait in a screen of an Hs578T human breast cancer cell cDNA library to identify potentially interacting clones. This screen yielded three independent positive clones. Two of these cDNAs matched sequences in the GenBank database: (1) Eps8 - epidermal growth factor receptor kinase substrate, and (2) GRP78 / BiP - glucose regulated stress protein, or human immunoglobulin heavy chain binding protein. The third cDNA, clone 4-33, was not identified in the database and represents a novel gene / protein.

Clone 4-33 protein is comprised of 240 amino acids, and is leucine rich (18%) with a predicted molecular weight of 26 kilodaltons (Appendix 1, panel A). There is a region of basic amino acids at the N-terminus of the sequence, three N-glycosylation sites withing the midregion, and a leucine zipper motif and putative transmembrane domain at the C-terminus. There are also potential phosphorylation sites.

As reported previously, Northern analysis of clone 4-33 mRNA indicates wide distribution in human tissues and breast and prostate cancer cell lines. Transiently expressed, EGFP-fused clone 4-33 protein displays a strong and specific interaction with IGFBP-3 in a coimmunoprecipitation assay. Immunofluorescent detection of transiently expressed clone 4-33 protein reveals a perinuclear and sometimes diffusely cytoplasmic or cell surface localization pattern.

We have cloned, expressed and purified clone 4-33 as a Glutathione S-Transferase (GST) fusion protein. The purified protein was used to inject rabbits for the production of clone 4-33-specific antibodies (Appendix 1, panel B). Rabbits were subsequently injected twice more at 6 week intervals to boost the antibody titre. Antisera from rabbit #6 gave the best results and was processed for further experiments. The antisera reacts specifically with purified GST:: 4-33 fusion protein (intact and Thrombin digested), and Hs578T conditioned media (CM) and cell lysate (CL) as compared to preimmune serum (Appendix 2, panel a). The antisera can also specifically immunoprecipitate clone 4-33 protein as demonstrated using transiently transfected EGFP::4-33 fusion protein in COS-7 cells. Immunoprecipitation of EGFP::4-33 is readily demonstrated using an antibody against the EGFP protein on an immunoblot of immunoprecipitated cell lysates. No immunoprecipitation is detected using preimmune sera, or from cell lysates transfected with unfused EGFP (Appendix 2, panel b). On an immunoblot of cell lysates from normal and cancerous human breast and prostate cell lines, the clone 4-33 antisera recognizes a species which migrates at roughly 32 kilodaltons (Appendix 2, panel c). We have also demonstrated

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specificity of the clone 4-33 antisera in immunocytochemical staining of transiently transfected human breast cancer cell lines (Appendix 3).

The estrogen responsive human breast cancer cell line MCF-7 does not produce detectable levels of IGFBP-3 protein under standard culture conditions, as compared to its counterpart, the estrogen non-responsive cell line Hs578T which produces very high levels of IGFBP-3. Interestingly, a differential immunocytochemical pattern appears between these two cell lines with respect to clone 4-33, both endogenous and transiently transfected. In MCF-7 cells endogenous levels of clone 4-33 protein appear to be low and difficult to differentiate from background immunostaining. Highly overexpressed levels of transiently transfected C-terminally Flag-tagged clone 4-33 is readily detectable at 24 – 48 hours post-transfection. In Hs578T cells, where endogenous IGFBP-3 is produced at high levels, endogenous clone 4-33 protein is easily detectable in a perinuclear and cytoplasmic / membrane associated pattern. Transiently transfected clone 4-33 is also detectable at 24 – 48 hours post-transfection, but overall levels of transfected protein appear to be lower than in the MCF-7 cell line, suggesting the possible existence of a regulatory pathway in the IGFBP-3 producing Hs578T cells.

When clone 4-33 is transiently transfected into Hs578T or MCF-7 human breast cancer cell lines, specific binding of exogenously added IGFBP-3 to the cell surface is reproducibly increased by 20-60% as compared to transfected vector alone (Appendix 4), suggesting an increase of the direct interaction between IGFBP-3 and clone 4-33 protein at the cell surface. This binding can be readily competed using unlabelled IGFBP-3, demonstrating specificity.

We are currently in the process of expressing clone 4-33, Flag-tagged at the C-terminus, using the baculovirus protein expression system (Appendix 5). This system will generate a protein product that more closely approximates the natural protein produced in humans than the GST system in bacteria. The protein purified from this system will be used for N-terminal sequencing to identify the natural N-terminus of the mature protein, as well as *in vivo / in vitro* studies with endogenous and laboratory purified IGFBP-3 to further elucidate the biological significance of the interaction of these two proteins.

II. Generation of inducible IGFBP-3 stably transfected human breast cancer cell lines.

The estrogen responsive human breast cancer cell line MCF-7 does not produce detectable levels of IGFBP-3 protein under standard culture conditions . We therefore chose this cell line to generate an inducible IGFBP-3 stably transfected cell line. The Ecdysone-Inducible expression system from Invitrogen (Carlsbad, CA) is a thoroughly tested and much used method providing for the generation of stably transfected cell lines in which the gene of interest is under the control of an inducible promoter, activated by the exogenous addition of ecdysone, or an ecdysone analog (Appendix 6). We have utilized this system to generate inducible IGFBP-3 expressing MCF-7 cell lines. These lines express IGFBP-3 at

varying levels depending upon the amount of inducer added to the culture medium, and the overall length of induction (Appendix 7) These cell lines will be extremely useful in studies of IGFBP-3 function, as negative and positive controls for experiments are all within a single cell line.

III. Involvement of IGFBP-3 and IGFBP-rP2 in growth inhibition induced by sodium butyrate.

Dietary factors play a vital role in both the development and prevention of human cancers, including breast and colon cancer. One dietary micronutrient, sodium butyrate (NaB), is a major nontoxic short-chain fatty acid in the large intestine, produced naturally during the bacterial fermentation of dietary fibers. NaB is a potent growth inhibitor and initiates cell differentiation for many cell types, including breast cancer cells (3).

As reported previously, my laboratory has identified connective tissue growth factor (CTGF) as a member of the IGFBP superfamily (4, 5). This protein is now designated IGFBP-related protein 2 (IGFBP-rP2). We have performed preliminary investigations into the effect of NaB on IGFBP-3 and IGFBP-rP2, two IGFBPs that are known to be involved in the regulation of growth of estrogen-nonresponsive breast cancer (Hs578T) cells, estrogen-responsive breast cancer (MCF-7) cells, and normal human mammary epithelial cells (HMEC).

NaB inhibited cell proliferation in both cancerous and noncancerous mammary cells to a similar degree (up to 90%) as measured by incorporation of [3H]thymidine (Appendix 8). Northern blot analysis showed that NaB treatment at physiological concentrations resulted in a stimulation of IGFBP-3 and IGFBP-rP2 mRNA expression in a dose dependent manner in the cancerous mammary cells. It also showed that NaB treatment resulted in a stimulation of IGFBP-rP2 but not of IGFBP-3 mRNA expression in noncancerous (HMEC) mammary cells (Appendix 9). Western ligand blot and immunoblot analyses demonstrated a concurrent increase in the IGFBP-rP2 protein levels in conditioned media from all these cell lines and an increase in IGFBP-3 protein levels in only the cancerous cells, following NaB treatment as well. There seemed to be no significant NaB effect on the levels of IGFBP-2, -4, and -5 in these cell lines (Appendix 10).

IV. Generation of proteolytic fragments of IGFBP-3 (Task 11).

Our studies of the IGFBP-3 molecule have included the investigation of proteolytic fragments, which have been demonstrated to occur *in vivo*. We looked at proteolytic fragments derived from plasmin-digested recombinant human IGFBP-3, synthetic fragments generated using the baculovirus expression system, and IGFBP-3 fragments in normal human urine. With each of these reagents we demonstrated retention of IGF binding of an N-terminal IGFBP-3 fragment, albeit with significantly reduced affinity

as compared to the intact molecule. In addition, we demonstrated that these N-terminal fragments can bind specifically to insulin, and inhibit insulin receptor autophosphorylation. (6, Appendix 11).

V. Identification of IGFBP-3 receptor binding sites within the IGFBP-3 protein (Task 12).

My laboratory has evaluated IGFBP-3 binding sites on breast cancer cell membranes by competitive binding studies with IGFBPs 1-6 and various forms of IGFBP-3, including synthetic fragments. These data demonstrate that specific, high affinity IGFBP-3 receptors are present on these cell surfaces. Studies with the synthetic fragments of IGFBP-3 have localized the binding domain within the IGFBP-3 molecule, within the region between residues 88-148. (7, Appendix 12).

KEY RESEARCH ACCOMPLISHMENTS

- Cloning, expression, and purification of a novel IGFBP-3 interacting protein, clone 4-33.
- Generation of polyclonal a4-33 antibody.
- Demonstration of specific interaction between IGFBP-3 and clone 4-33.
- Generation of inducible IGFBP-3 stably transfected human breast cancer cell lines.
- Generation of IGFBP-3 proteolytic fragments and subsequent characterization of IGF and insulin binding affinity.
- Identification of IGFBP-3 receptor binding sites within the IGFBP-3 protein molecule.

REPORTABLE OUTCOMES

1. Vorwerk, et al., 1998. Journal of Clinical Endocrinology and Metabolism, 83(4):1392-1395.
2. Yamanaka, et al., 1999. Endocrinology, 140(3):1319-1328.
3. Ingermann, A.R. and Oh, Y. 1999. 81st Annual Meeting of The Endocrine Society, abstract P2-585.

CONCLUSIONS:

In summary, my laboratory has demonstrated IGFBP-3 / IGFBP-3 receptor interactions in the human breast cancer cell system, identified and initially characterized an IGFBP-3 interacting protein from breast cancer cells, and generated a polyclonal antibody against this interacting protein, and generated inducible IGFBP-3 stably transfected cell lines. In addition, we have identified IGFBP-3 and IGFBP-rP2 as potential links between the IGF axis and sodium butyrate induced cell growth inhibition in breast cancer. As laid out in the Statement of Work, we have accomplished Technical Objective 1, Tasks 1-5. Tasks 6 (months 13-19) and 7 (months 16-26) are currently underway. In preparation for Technical Objective 2, we have generated inducible IGFBP-3 stably transfected MCF-7 lines. These and other valuable reagents generated during the course of Technical Objective 1 will be employed for identification and characterization of the IGFBP-3 mediated signal transduction pathway in human breast cancer cells. Technical Objective 3, Task 10 is underway. Tasks 11 and 12 are finished and published work., and Task 13 is in preparation.

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2. Baxter RC; Clemmons DR; Conover C; Drop SLS; Holly J; Mohan S; Oh Y; Rosenfeld RG. Recommendations for nomenclature of insulin-like growth factor binding protein (IGFBP) superfamily. Endocrinology, 1998, 139:4036.

3. Mandal M; Kumar R: Bcl-2 expression regulates sodium butyrate-induced apoptosis in human MCF-7 breast cancer cells. Cell Growth Different, 1996, 7:311.

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6. Vorwerk P; Yamanaka Y; Apagnoli A; **Oh Y**; Rosenfeld RG. Insulin and IGF binding by IGFBP-3 fragments derived from proteolysis, baculovirus expression and normal human urine. J. Clin. Endocrinol. Metab., 1998, 83:1392-1395.

7. Yamanaka Y; Fowlkes JL; Rosenfeld RG; **Oh Y**. Characterization of insulin-like growth factor binding protein-3 (IGFBP-3) binding to human breast cancer cells. Endocrinology, 1999, 140:1319-1328.

APPENDICES:

1. Clone 4-33 protein structure and antibody production.

2. Characterization of α4-33 polyclonal Ab.

3. Specificity of α4-33 polyclonal antibody in immunofluorescent staining of transiently transfected human breast cancer cells.

4. Binding of IGFBP-3 to clone 4-33 transfected cell surfaces.

5. Baculovirus expression system.

6. Ecdysone-inducible stable transfection system.

7. Inducible IGFBP-3 stably transfected MCF-7 cell lines.

8. Inhibition of DNA synthesis by NaB in cancerous and normal human mammary cells.

9. Regulation of IGFBP-3 and IGFBP-rP2 mRNA by NaB.

10. Regulation of IGFBP-3 and IGFBP-rP2 protein by NaB.

11. Yamanaka, et al., 1999. Endocrinology, 140(3):1319-1328.

12. Vorwerk, et al., 1998. Journal of Clinical Endocrinology and Metabolism, 83(4):1392-1395.

Appendix 1. Clone 4-33 protein structure and antibody production.

- 240 amino acids
- predicted MW = 26 kDa
- PI = 8.4
- leucine-rich (18%)
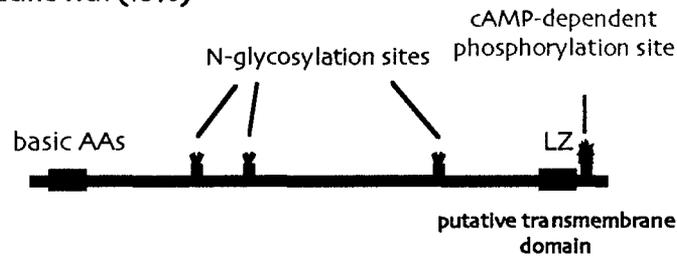

A. Schematic representation of clone 4-33 protein.
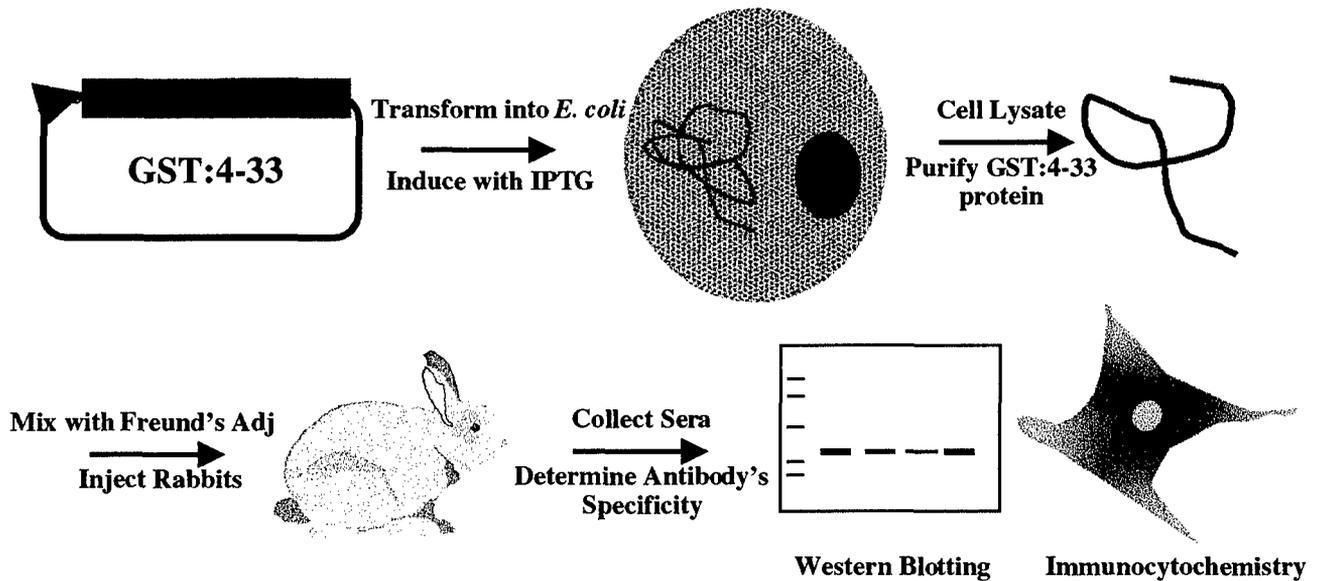

B. Production of α4-33 polyclonal antibody.

Appendix 2. Characterization of α4-33 polyclonal Ab.
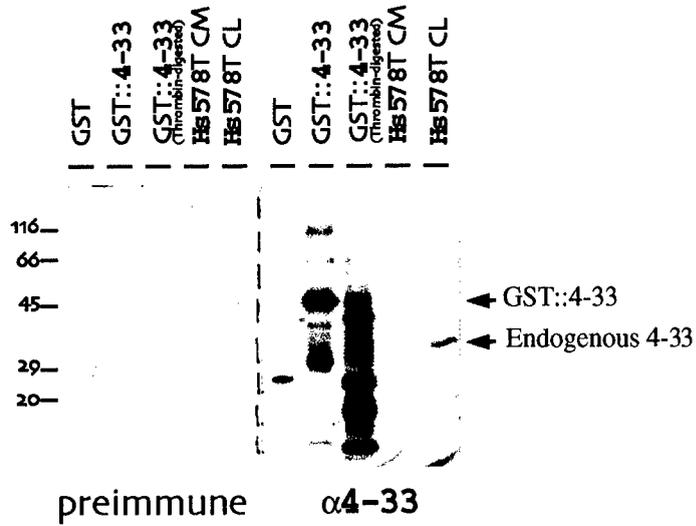
**A:** Preimmune serum vs. α4-33 polyclonal antibody.
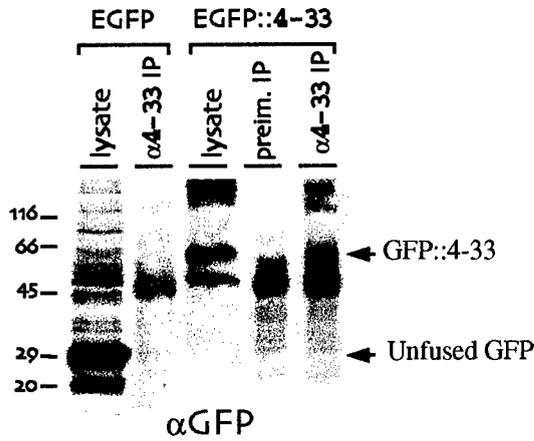

B: Immunoprecipitation of clone 4-33.
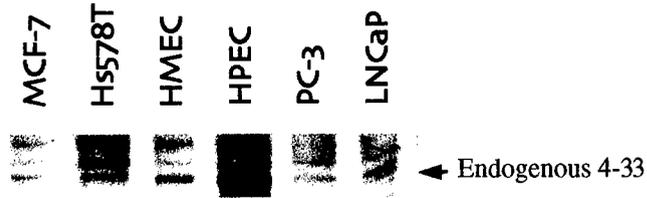
C. Immunoblot of clone 4-33 in cell lysates of human breast cancer cell lines (MCF-7, Hs578T) and normal mammary cells (HMEC), normal prostate cells (HPEC) and prostate cancer cell lines (PC-3, LNCaP).

Specificity of α4-33 polyclonal antibody in immunofluorescent staining of transiently transfected human breast cancer cells.
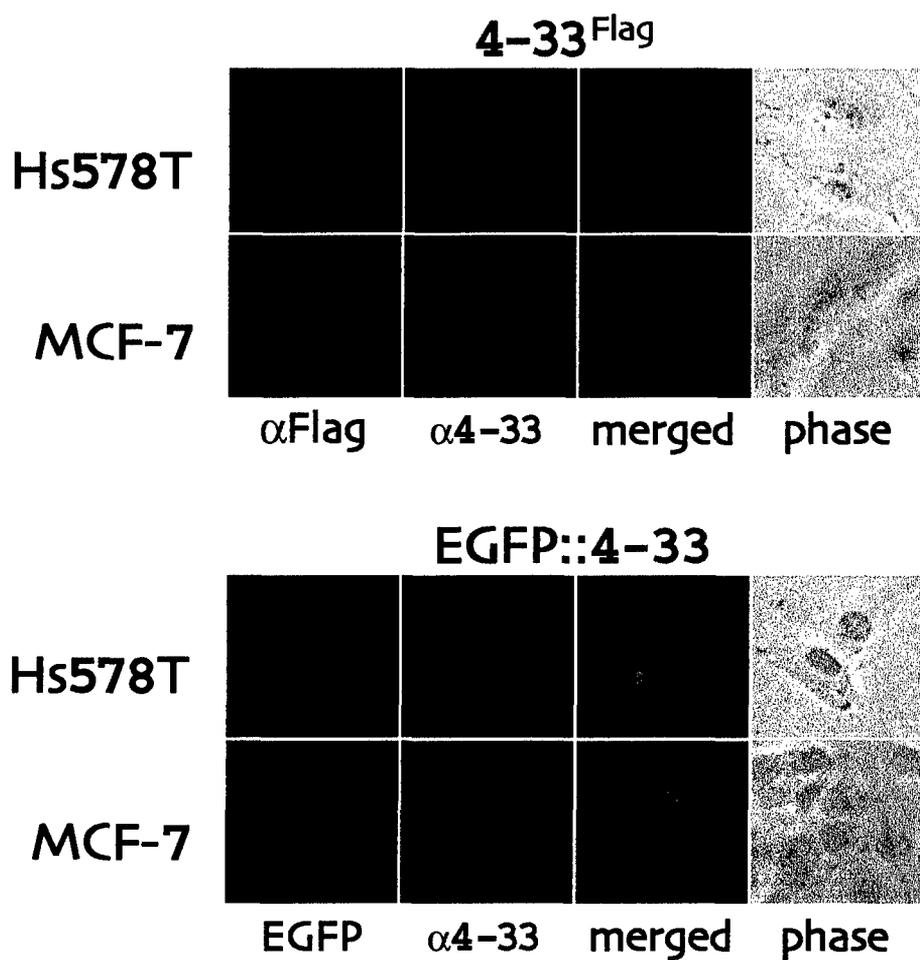

α4-33 polyclonal antibody recognizes transiently expressed Flag-tagged or EGFP-fused clone 4-33.

## Appendix 4. Binding of IGFBP-3 to clone 4-33 transfected cell surfaces.
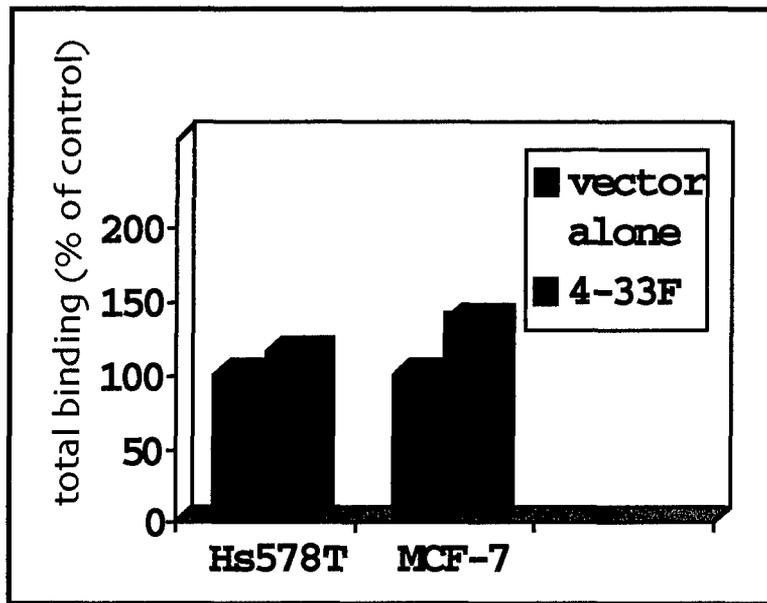
A: Increased binding of labelled IGFBP-3 to cells overexpressing clone 4-33 protein. Binding to Hs578T cells was increased by 20%, MCF-7 cells by 40%.
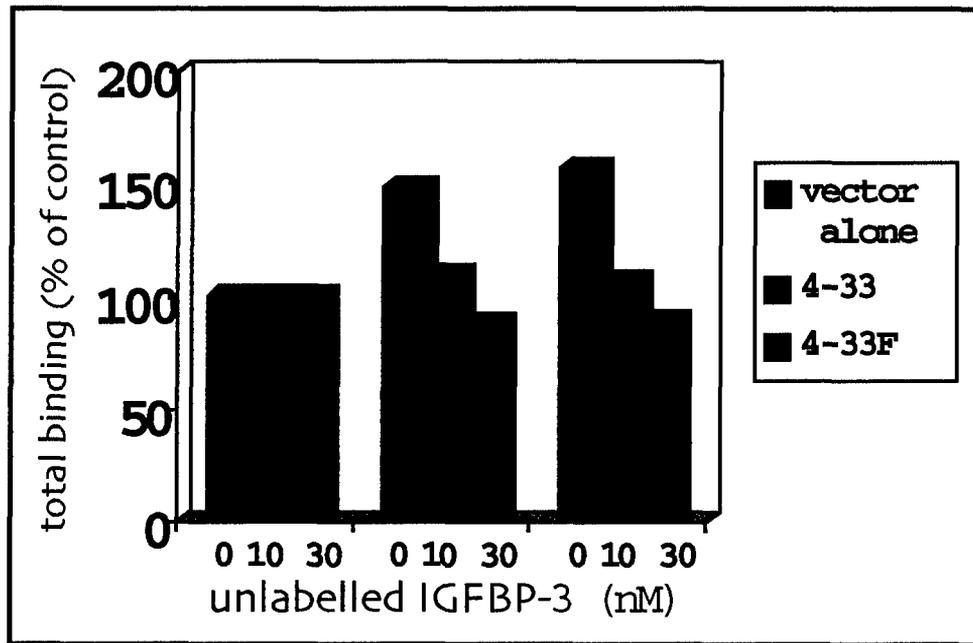

B: Transfection of untagged or Flag-tagged clone 4-33 resulted in 50-60% increased binding of labelled IGFBP-3 to Hs578T human breast cancer cell surfaces. Specificity was demonstrated using unlabelled IGFBP-3 in a competitive binding assay.

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Appendix 5. Baculovirus expression system.
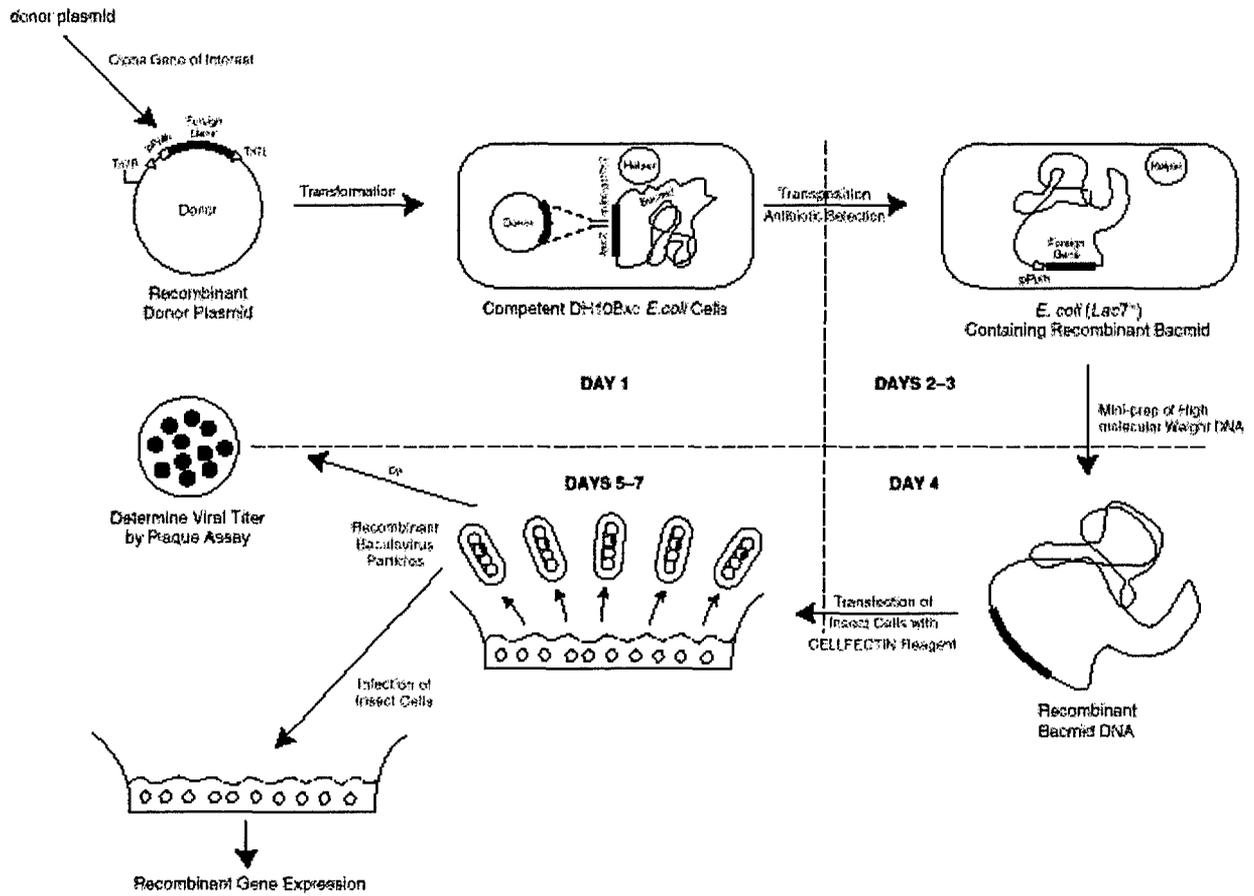
Appendix 6. Ecdysone-inducible stable transfection system.

1. Ligate gene
of interest
into pIND.

pIND
&
pIND(SP1)

Zeocin™
Resistance

pVgRXR

Neomycin
Resistance
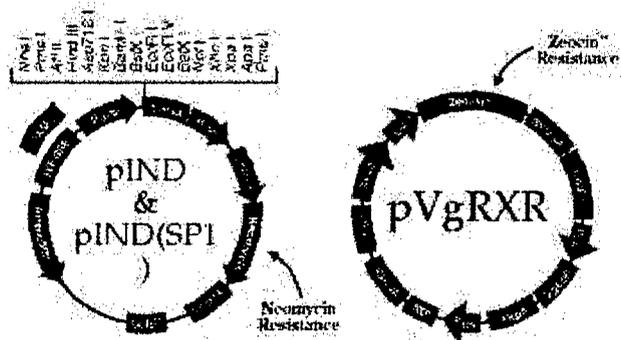
2. Cotransfect
recombinant
pIND and
pVgRXR into
mammalian cells.

3. Induce expression   ←── inducing agent

5xE/GRE   P$_{\mathrm{HSP}}$ gene of interest
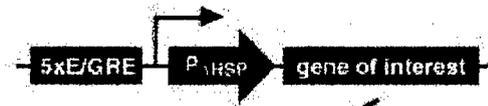
4. Assay for
expressed protein.
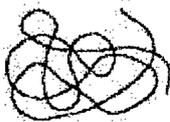
Appendix 7. Inducible IGFBP-3 stably transfected MCF-7 cell lines.
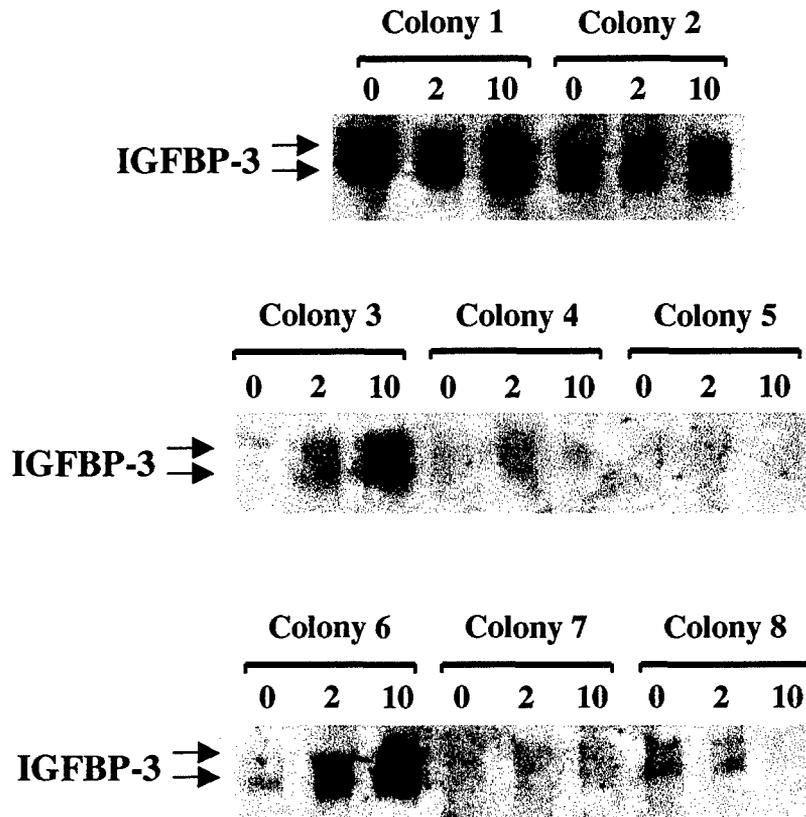
MCF-7:BP-3 inducible cell lines 1 - 8, showing induction of IGFBP-3 protein levels with the addition of 0, 2, and 10 µM Ponasterone A to the culture medium. In colony 1, BP-3 is constitutively active, colonies 2, 3, and 6 are inducible for IGFBP-3 expression.

Appendix 8. Inhibition of DNA synthesis by NaB in cancerous and normal human mammary cells.
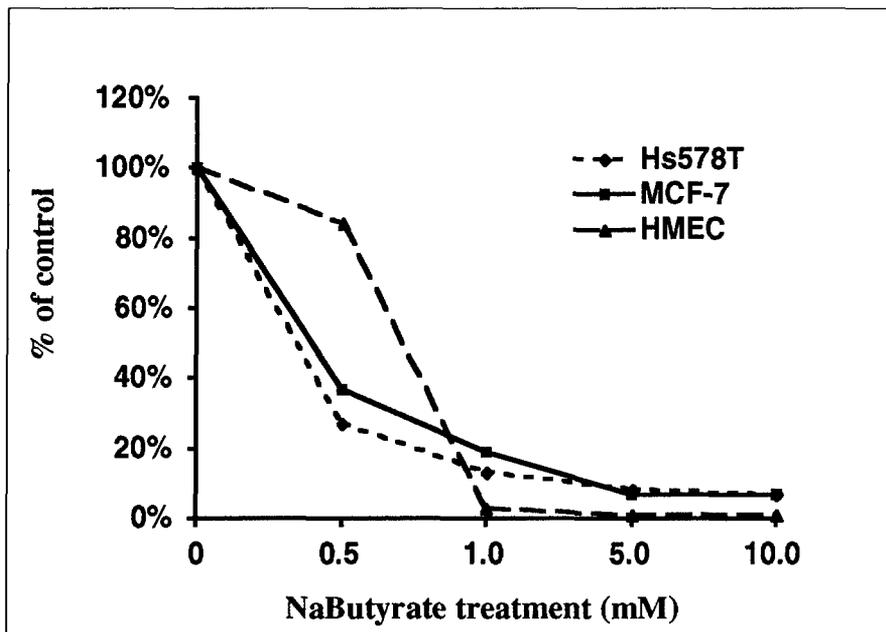
Effect of sodium butyrate on DNA synthesis in normal Human Mammary Epithelial Cells, and Hs578T and MCF-7 human breast cancer cells.

Appendix 9. Regulation of IGFBP-3 and IGFBP-rP2 mRNA by NaB.

NaB treatment

	control	0.1 mM	0.5 mM	1 mM	5 mM	10 mM

Hs578T

HMEC

IGFBP-3 mRNA
(2.4 kb)

NaB treatment

	control	0.1 mM	0.5 mM	1 mM	5 mM	10 mM

Hs578T

HMEC
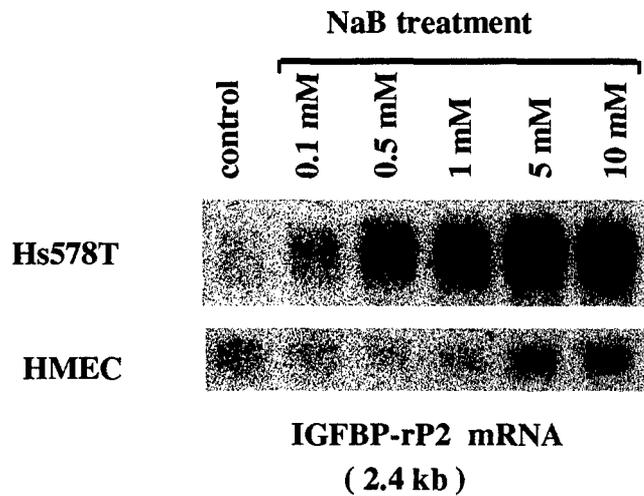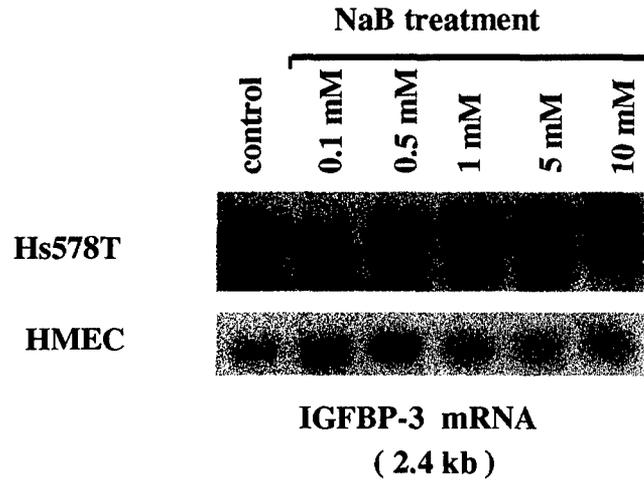
IGFBP-rP2 mRNA
(2.4 kb)

Effect of NaB on IGFBP-3 and IGFBP-rP2 mRNA levels in cancerous Hs578T and non-cancerous HMEC human breast cell lines. Both IGFBP-3 and IGFBP-rP2 mRNAs were induced by NaB in the cancerous Hs578T, but only IGFBP-rP2 mRNA was induced in the non-cancerous HMEC cells. Neither IGFBP-3 nor IGFBP-rP2 mRNA was detectable from the cancerous MCF-7 cell line.

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Appendix 10. Regulation of IGFBP-3 and IGFBP-rP2 protein by NaB.

IGFBP-3 (protein)
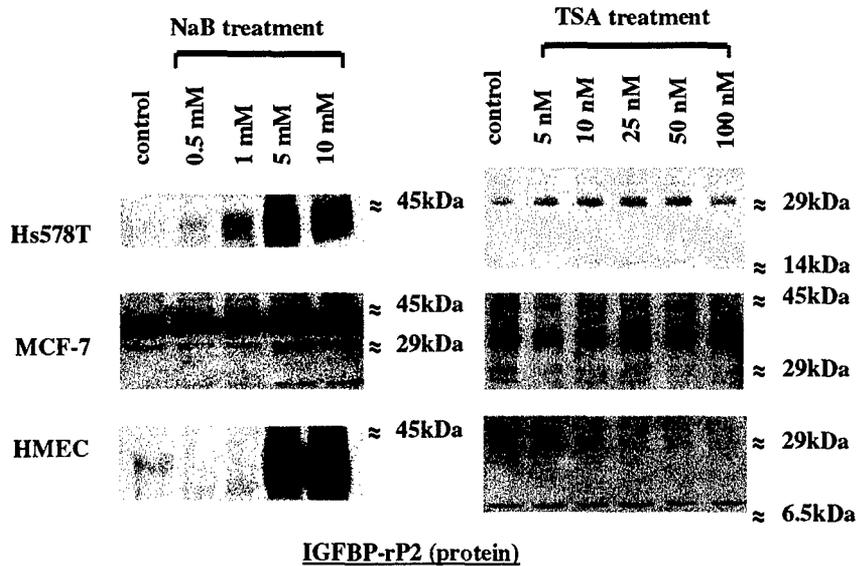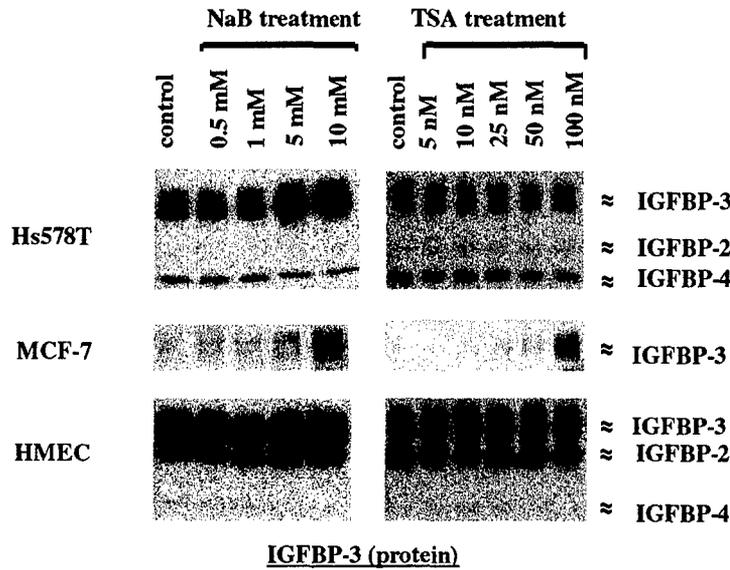
IGFBP-rP2 (protein)

Effect of NaB on IGFBP-3 and IGFBP-rP2 protein levels in cancerous Hs578T and MCF-7 and non-cancerous HME human breast cell lines. IGFBP-rP2 protein levels were increased by NaB in all cell lines. IGFBP-3 levels, however, were only increased in the cancerous Hs578T and MCF-7 cells, not the non-cancerous HMEC cells.

Characterization of Insulin-Like Growth Factor Binding Protein-3 (IGFBP-3) Binding to Human Breast Cancer Cells: Kinetics of IGFBP-3 Binding and Identification of Receptor Binding Domain on the IGFBP-3 Molecule*

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ABSTRACT

Insulin-like growth factor binding protein-3 (IGFBP-3) binds to specific membrane proteins located on human breast cancer cells, which may be responsible for mediating the IGF-independent growth inhibitory effects of IGFBP-3. In this study, we evaluated IGFBP-3 binding sites on breast cancer cell membranes by competitive binding studies with IGFBP-1 through -6 and various forms of IGFBP-3, including synthetic IGFBP-3 fragments. Scatchard analysis revealed the existence of high-affinity sites for IGFBP-3 in estrogen receptor-negative Hs578T human breast cancer cells (dissociation constant (K_d) = 8.19 ± 0.97 × 10^{-9}.M and 4.92 ± 1.51 × 10^5 binding sites/cell) and 30-fold fewer receptors in estrogen receptor-positive MCF-7 cells $(K_d$ = 8.49 ± 0.78 × 10^{-9} M and 1.72 ± 0.31 × 10^4 binding sites/cell), using a one-site model. These data demonstrate binding characteristics of typical receptor-ligand interactions, strongly suggesting an IGFBP-3:IGFBP-3 receptor interaction. Among IGFBPs, only IGFBP-5 showed weak competition, indicating that IGFBP-3 binding to breast cancer cell surfaces is specific and cannot be attributed to

nonspecific interaction with glycosaminoglycans. This was confirmed by showing that synthetic IGFBP-3 peptides containing IGFBP-3 glycosaminoglycan-binding domains competed only weakly for IGFBP-3 binding to the cell surface. Rat IGFBP-3 was 20-fold less potent in its ability to compete with human IGFBP-3[Escherichia coli], as well as 10- to 20-fold less potent for cell growth inhibition than human IGFBP-3, suggesting the existence of species specificity in the interaction between IGFBP-3 and the IGFBP-3 receptor. When various IGFBP-3 fragments were evaluated for affinity for the IGFBP-3 receptor, only those fragments that contain the midregion of the IGFBP-3 molecule were able to inhibit ^{125}I-IGFBP-3[Escherichia coli] binding, indicating that the midregion of the IGFBP-3 molecule is responsible for binding to its receptor. These observations demonstrate that specific, high-affinity IGFBP-3 receptors are located on breast cancer cell membranes. These receptors have properties that support the notion that they may mediate the IGF-independent inhibitory actions of IGFBP-3 in breast cancer cells. (*Endocrinology* **140**: 1319–1328, 1999)

T HE INSULIN-LIKE GROWTH factors (IGFs), IGF-I and IGF-II, have been recognized as major regulators of mammary epithelial cell and breast cancer cell growth (1–4). Both IGF-I and IGF-II serve as potent mitogens for a number of breast cancer cell lines *in vitro* (1, 2, 4–6), and IGF-I or IGF-II messenger RNAs are detectable in the majority of human breast tumor specimens (7, 8). Six distinct IGF binding proteins (IGFBPs), which can bind IGFs with high affinity, have been identified and have been designated IGFBP-1 through IGFBP-6 (9–14). IGFBPs, which are produced by breast cancer cells, can either potentiate or inhibit IGF-induced DNA synthesis in cultured fibroblasts (15–17) and can interfere with IGF effects in transformed cells (18–20).

Delbe *et al.* (21) have demonstrated that purified mouse IGFBP-3 can bind to the chick embryo fibroblast cell surface and inhibit cell growth. Our laboratory and others have

demonstrated a significant inhibitory effect of exogenous IGFBP-3 on the growth of Hs578T estrogen receptor (ER)-negative human breast cancer cells (22), human IGFBP-3 transfected mouse Balb/c fibroblast cells (23), and human IGFBP-3 transfected fibroblast cells, which were derived from mouse embryos homozygous for a targeted disruption of the type I IGF receptor gene (24). Factors that are known to inhibit the growth of human breast cancer cells, such as transforming growth factor-β (TGF-β) (25), retinoic acid (26), and antiestrogens (27, 28), may do so through their effects on IGFBP-3. We have demonstrated that the antiproliferative effects of TGF-β and retinoic acid in human breast cancer cells are mediated, at least in part, through IGFBP-3 action (29, 30), whereas Huynh *et al.* have shown that antiestrogen-induced growth inhibition of MCF-7 human ER-positive breast cancer cells is mediated similarly through increased IGFBP-3 action (31). In addition, Hembree *et al.* (32) have reported that treatment of human ectocervical cells with a retinoic acid receptor-specific ligand increases IGFBP-3 levels and suppresses cell proliferation. Furthermore, Buckbinder *et al.* (33) have demonstrated that IGFBP-3 is induced by the p53 tumor suppressor gene and is probably a mediator in p53 signaling. Previous studies from our laboratory have demonstrated that IGFBP-3 binds to specific membrane pro-

teins (20, 26, and 50 kDa) located on Hs578T cells (34), which may be responsible for mediating these growth inhibitory effects of IGFBP-3 (22). Those were further confirmed by studies from Rajah *et al.* (35), showing that IGFBP-3 binds to specific membrane proteins and induces apoptosis through a p53- and IGF-independent mechanism in human prostate cancer cells and IGF receptor-negative mouse fibroblasts. Thus, recent studies have revealed that IGFBP-3 may have specific biological effects in various cell systems, including human breast cancer cells, which are not mediated through their interactions with IGFs (IGF-independent actions).

In this study, we characterize IGFBP-3 binding to breast cancer cells by employing competitive binding assays, and demonstrate a single class of specific, high-affinity membrane receptors for IGFBP-3, and identify a receptor binding domain on the IGFBP-3 molecule.

Materials and Methods

Materials

HPLC-purified hIGFBP-1 from human amniotic fluid was kindly provided by Dr. D. R. Powell (Baylor College of Medicine, Houston, TX) (36). Recombinant human IGFBP-3 (rhIGFBP-3), a nonglycosylated 29K core protein expressed in *Escherichia coli* cells, was the generous gift of Celtrix, Inc. (Santa Clara, CA) (37). rhIGFBP-2, -4, -5, and -6 were purchased from Austral Biologicals (San Ramon, CA). Intact rat IGFBP-3, NH$_2$-terminal rat IGFBP-3 (rat IGFBP-3$^{1-160}$), and COOH-terminal rat IGFBP-3 (rat IGFBP-3$^{161-265}$), which were purified from normal rat serum, were gifts from Dr. Nicholas Ling (Neurocrine Biosciences, Inc., San Diego, CA). Pooled human serum IGFBPs were prepared as previously described (36). Briefly, human sera from healthy male adult volunteers were collected and pooled. This pooled sera were chromatographed over a Sephadex G-50 column in formic acid to separate IGF peptides from the IGFBPs. IGF-I was purchased from Bachem California, Inc. (Torrance, CA). IGF-II was kindly provided by Eli Lilly & Co. (Indianapolis, IN). Iodination was performed by a modification of the chloramine-T technique, to specific activities of 350–500 μCi/μg for IGF-I and -II and to 100 μCi/μg for IGFBP-3$^{Escherichia\ coli}$ peptides. Reagents used for SDS-PAGE were purchased from Bio-Rad Laboratories, Inc. (Richmond, CA). Na^{125}I was obtained from Amersham Corp. (Arlington Heights, IL).

Cell Cultures

Hs578T (ER-negative) and MCF-7 (ER-positive) human breast cancer cell lines were obtained from the American Type Culture Collection (Rockville, MD). Hs578T and MCF-7 were maintained in DMEM supplemented with 4.5 g/liter glucose, 110 mg/liter sodium pyruvate, and 10% FBS. Stock cultures were subcultured every 3 days.

Preparation of human IGFBP-3 fragments produced by matrix metalloproteinase-3 (MMP-3)

IGFBP-3 fragments (fragments a–e) were produced by MMP-3, as previously described (38, 39). In brief, 60 μg rhIGFBP-3$^{Escherichia\ coli}$ were digested by 200 ng MMP-3 (kindly provided by Dr. Hideaki Nagase, University of Kansas Medical Center, KS) in a total vol of 60 μl of 50 mM Tris (pH 7.5), 0.15 M NaCl, 10 mM CaCl$_2$, 0.02% NaN$_3$, 0.05% Brij 35 for 8 h at 37 C. The digestion was stopped by the addition of EDTA (final concentration: 10 mM), and the digestion products were analyzed by SDS-PAGE under reducing conditions. Five rhIGFBP-3$^{Escherichia\ coli}$ fragments were described as either NH$_2$-terminal IGFBP-3 fragments: a) IGFBP-3$^{1-109}$ and b) IGFBP-3$^{1-99}$, or COOH-terminal IGFBP-3 fragments: c) IGFBP-3$^{100-264}$, d) IGFBP-3$^{110-264}$, and e) IGFBP-3$^{177-264}$ (38, 39). To separate rhIGFBP-3$^{Escherichia\ coli}$ fragments produced by MMP-3 digestion, the digestion mixture was passed through a heparin-Sepharose column (Sigma Chemical Co.). All fractions were analyzed by SDS-PAGE and stained with Coomassie Blue. The wash fractions contained only the smallest IGFBP-3 fragments (fragments a and b) produced by

MMP-3, which correspond to the first 100–110 NH$_2$-terminal amino acids of IGFBP-3. Fragments c–e bound to the heparin column, and all three fragments were eluted. The calculated protein concentrations were performed using a modified Bradford method obtained from Bio-Rad, and were read against a BSA standard.

Preparation of synthetic human IGFBP-3 peptides

Peptides containing heparin binding domains were produced by solid-phase peptide synthesis, using 9-fluorenylmethoxycarbonyl chemistry, as previously described (39). The sequences are as follows: ^{149}CKKGHAKDSQRYKVDYESQS^{167} (peptide IV) and ^{213}CDKKG-FYKKKQ[C-Acm]RPSKGR^{230} (peptide VI). Peptides were purified on a Vydac C-8 HPLC column, using a Gilson automated HPLC system, and were shown to be more than 98% pure. Sequence verification was performed by electrospray mass spectrometry. Peptides were synthesized with an additional NH$_2$-terminal cysteine for use in thiol-coupling reactions. The internal cysteine in peptide VI was acetylmethylated, because it is normally involved in disulfide bond formation.

Baculovirus expression of rhIGFBP-3 fragments representing NH$_2$-terminal domain or midregion of IGFBP-3 molecule

Plasmid constructs for IGFBP-3$^{1-87}$, IGFBP-3$^{88-148}$, and IGFBP-3$^{88-183}$ were prepared to express proteins in a baculovirus expression system (Life Technologies), as previously described (40). In brief, PCR was employed to make all constructs, using human IGFBP-3 complementary DNA (cDNA) as template, and further to add FLAG epitope sequences (DYKDDDDK) and a new stop codon immediately following COOH-termini of IGFBP-3 fragments. The signal peptide sequences of IGFBP-3 cDNA were ligated to NH$_2$-termini of IGFBP-3 fragments. After sequencing, the FLAG-tagged IGFBP-3 fragment cDNAs were subcloned into pFASTBAC1 baculovirus expression vector and transfected into Sf9 insect cells, and viral recombinants were identified by immunoblotting with the anti-FLAG M2 antibody (Eastman Kodak Co.). Recombinant proteins were purified with anti-FLAG M2 affinity column using the serum-free media of HIGH-5 cells after infecting for 3 days at 27 C. The purified proteins were subjected to SDS-PAGE in a 15% gel and were stained with Coomassie Blue or transferred to nitrocellulose for immunodetection. Protein fractions were pooled, concentrated, and quantified by comparison with known amounts of BSA, by silver-staining (Bio-Rad).

^{125}I-IGFBP-3 cell binding assay

Monolayer binding assays were performed as previously described (22). Confluent monolayers of human breast cancer cells (0.2 $\times$ 10^6 cells/well in 24-multiwell plates) were incubated in serum-free medium overnight. The cells were washed once with cold washing buffer (HBSS without CaCl$_2$ and MgCl$_2$, containing 25 mM HEPES and 25 mM NaHCO$_3$, pH 7.4). Cell surface-bound endogenous IGFBP-3 was then removed by rinsing the cells once with cold washing buffer containing 1 mM EDTA. The cells were washed once with cold washing buffer and incubated in 250 μl of binding buffer (HBSS without MgCl$_2$, containing 25 mM HEPES, 25 mM NaHCO$_3$, 1 mM CaCl$_2$, and 0.5% BSA, pH 7.4) for 3 h at 15 C with ^{125}I-IGFBP-3 (50,000 cpm) in the absence or presence of various concentrations of unlabeled IGFBP-3. The cells were washed with PBS and solubilized with 0.6 N NaOH. Radioactivity of the cell lysates was determined, and specific binding was calculated by subtracting nonspecific binding (cpm in the presence of unlabeled 10% human serum IGFBP fractions or 100 nM unlabeled IGFBP-3) from total binding. Binding parameters were determined by the curve-fitting program LIGAND (41). Cells from parallel wells were gently detached from plates by Trypsin/EDTA, and cell numbers were counted with a Coulter Counter (Coulter, Ltd., Beds, UK).

Specificity of IGFBP-3 binding to cell surfaces

Cell binding assays were performed as described above, by incubation of Hs578T cells with rh-^{125}I-IGFBP-3 in the absence or presence of various concentrations of: 1) HPLC-purified IGFBP-1 from human amniotic fluid; and 2) rhIGFBP-2, -4, -5, and -6, or 3) intact rat IGFBP-3. 100

nM unlabeled IGFBP-3 was used for determination of nonspecific binding. These materials were characterized by Western ligand blots, to verify that they were intact and biologically active.

Identification of domains of IGFBP-3 responsible for binding to the IGFBP-3 receptor and to heparin

Cell binding assays were performed, as described above, by incubation of Hs578T cells with ^{125}I-IGFBP-3 in the absence or presence of various concentrations of: 1) human IGFBP-3 fragments produced by MMP-3 digestion; 2) intact rat IGFBP-3, rat IGFBP-3$^{1-160}$, or rat IGFBP-3$^{161-265}$; 3) synthetic IGFBP-3 peptides IV and VI; and 4) baculovirus-expressed rhIGFBP-3 fragments IGFBP-3$^{1-87}$, IGFBP-3$^{88-148}$, and IGFBP-3$^{88-183}$.

Monolayer cell replication assay

Hs578T cells were grown in 24-multiwell dishes until 60% confluent (3×10^4 cells/well) and then changed to 1% FBS containing media with or without reagents for 94 h. Cells were then gently detached from plates by trypsin-EDTA, and cell number was counted using a Coulter counter (Coulter, Ltd.).

Statistical analysis

Data were analyzed with a two-tailed Student's t test, using the software program Instat 2.01 (Graphpad Software, Inc., San Diego, CA). Values are expressed as means ± SD.

Results

Characterization of IGFBP-3 binding to the breast cancer cells

Previous studies have demonstrated that IGFBP-3 has growth-inhibitory effects in ER-negative Hs578T and MDA-231 cells and ER-positive MCF-7 cells (31). In all three cell lines, the inhibitory effects of IGFBP-3 have been shown to be IGF-independent. Further studies have demonstrated the existence of IGFBP-3 interacting proteins on the cell surface of Hs578T cells (22); these cell surface proteins seem to have the characteristics of a specific IGFBP-3 receptor. To determine the characteristics of IGFBP-3 binding to its receptor on Hs578T and MCF-7 cells, competitive binding data regarding receptor affinity, number, and specificity were gathered. Because our previous studies have demonstrated that the binding of ^{125}I-IGFBP-3 to the cell surface of Hs578T cells was increased by divalent cations ($CaCl_2$ or $MnCl_2$) in a dose-dependent manner (22), characterization of specific IGFBP-3 binding to the cell surface was performed in the presence of 1 mM $CaCl_2$. Figure 1 shows the competitive binding curve and Scatchard analysis for the binding of ^{125}I-IGFBP-3 to Hs578T and MCF-7 monolayers. Calculated binding affinity and number of binding sites for IGFBP-3 in Hs578T and MCF-7 cells are shown in Table 1. In both Hs578T cells and MCF-7 cells, the specific binding of ^{125}I-IGFBP-3 was inhibited by unlabeled IGFBP-3 in a dose-dependent manner, with approximately 50% nonspecific binding (Fig. 1A). Scatchard analysis revealed a best fit for a one-site model in both Hs578T and MCF-7 cell lines ($K_d = 8.19 \pm 0.97 \times 10^{-9}$ and $8.49 \pm 0.78 \times 10^{-9}$ M, respectively) (Fig. 1B). The dissociation constants, number of binding sites/cell, and the percentage of specific binding for both cell lines are shown in Table 1, with data obtained from three independent experiments.

Because we have previously demonstrated that competition for ^{125}I-IGFBP-3 binding to the cell surface is also ob-
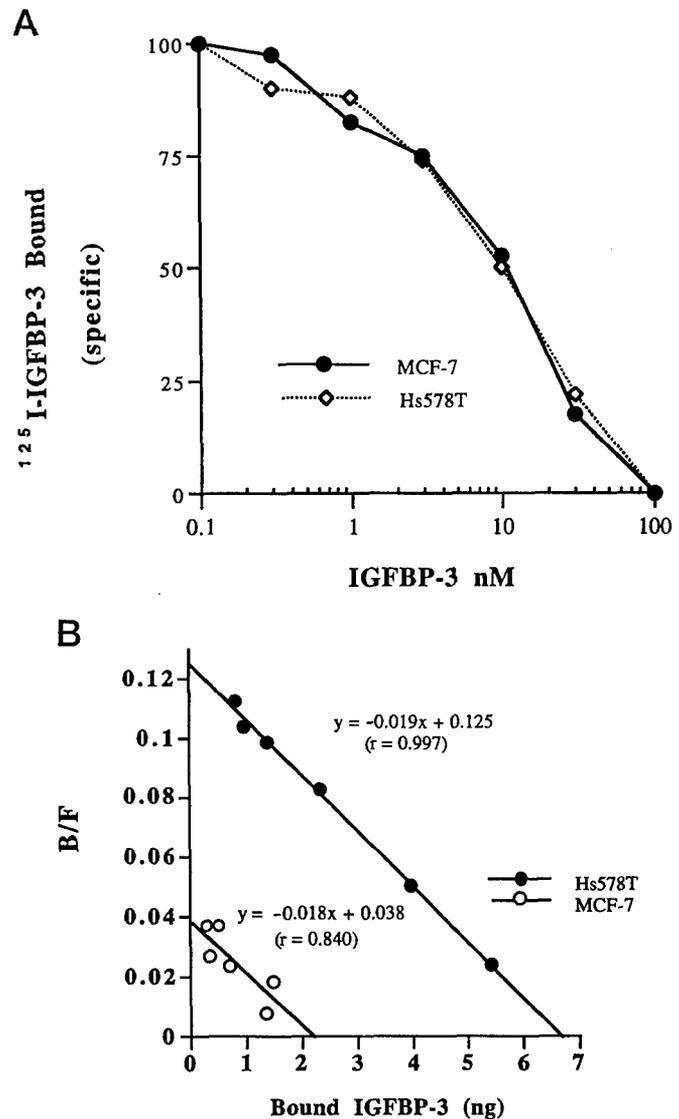

FIG. 1. A, Competitive binding of ^{125}I-IGFBP-3 to ER-negative Hs578T and ER-positive MCF-7 monolayers by unlabeled IGFBP-3. Cells were grown to confluence in 24-multiwell plates and maintained in serum-free media for 16 h. Cells were incubated for 3 h at 15 C with ^{125}I-IGFBP-3 (50,000 cpm/well) in the presence of the indicated amounts of unlabeled IGFBP-3, and then were washed and solubilized and the total cell-associated radioactivity determined. Each point of the curve represents the mean of three independent experiments carried out in triplicate. B, Scatchard plot of IGFBP-3 binding to Hs578T and MCF-7 cells, using 100 nM unlabeled IGFBP-3 for determination of nonspecific binding. The level of binding was determined and analyzed, using the LIGAND computer program to gain the best fit for one-site analysis, after subtraction of ^{125}I-IGFBP-3 binding in the presence of 100 nM unlabeled IGFBP-3. The *circles* indicate experimental data, and the *solid lines* indicate the resolved high-affinity and nonspecific components of the fitted curves. B/F indicates the ratio of bound-to-free ligand. Results are representative of three independent experiments.

served with 10% human serum IGFBP fractions derived from G-50 acid chromatography (22), the competitive binding assay was also performed after nonspecific binding was determined in the presence of unlabeled 10% human serum IGFBP fractions. Scatchard analysis of specific binding data

TABLE 1. Characteristics of IGFBP-3 binding to IGFBP-3 receptor in human breast cancer cells

	K_d (M)	Binding sites/cell	Specific binding (%)
Hs578T cells	$8.19 \pm 0.97 \times 10^{-9}$	$4.92 \pm 1.51 \times 10^5$	11.2 ± 3.0
MCF-7 cells	$8.49 \pm 0.78 \times 10^{-9}$	$1.72 \pm 0.31 \times 10^{4a}$	5.1 ± 0.6^b

Binding studies were carried out on Hs578T cells and MCF-7 cells as described in *Materials and Methods*. The K_d value and the number of IGFBP-3 receptors were determined using LIGAND computer program to gain the best fit for one-site analysis after subtraction of ^{125}I-IGFBP-3 binding in the presence of 100 nM unlabeled IGFBP-3. Each result is the mean $\pm$ SD for three independent experiments.
  $^a P < 0.01$; $^b P < 0.05$ (Hs578T cells *vs.* MCF-7 cells).

revealed curvilinear plots in both cell lines, and statistical analysis resulted in preference for a two-site binding model (data not shown). Analysis of the high-affinity binding site in Hs578T cells (K_d = 8.11 $\pm$ 0.88 $\times$ 10^{-9} M; 5.79 $\pm$ 1.32 $\times$ 10^5 binding sites/cell) and in MCF-7 cells (K_d = 9.92 $\pm$ 1.12 $\times$ 10^{-9} M; 3.52 $\pm$ 0.61 $\times$ 10^4 binding sites/cell) demonstrates similar binding affinities in the two cell lines but approximately 20-fold less binding sites/cell in MCF-7 cells. Low-affinity binding characteristics in Hs578T cells (K_d = 2.20 $\pm$ 0.34 $\times$ 10^{-5} M; 3.08 $\pm$ 0.58 $\times$ 10^7 binding sites/cell) and in MCF-7 cells (K_d = 2.10 $\pm$ 0.27 $\times$ 10^{-5} M; 2.26 $\pm$ 0.45 $\times$ 10^7 binding sites/cell) show a similar pattern in the two cell lines. In both cell lines, the dissociation constants for high-affinity binding and the number of binding sites/cell are similar to those observed when 100 nM unlabeled IGFBP-3 was employed for nonspecific binding. Under both conditions, the high-affinity dissociation constant in Hs578T cells is similar to that of MCF-7 cells. These findings suggest that a binding site with a K_d of 8–9 $\times$ 10^{-9} represents the high-affinity site for IGFBP-3 in both cell lines and that quantitative differences in specific binding between the two cell lines can be entirely explained by differences in the numbers of high-affinity binding sites per cell.

Specificity of IGFBP-3 binding to Hs578T cell surface

To establish the specificity of IGFBP-3 binding to Hs578T cells, competitive binding assays were performed, where the complete family of IGFBPs was used to compete for binding of ^{125}I-IGFBP-3 (Fig. 2, A and B). Neither IGFBP-1, 2, 4, nor 6 were able to compete for ^{125}I-IGFBP-3 binding. In contrast, IGFBP-5 was able to weakly compete, with approximately 10% of the potency of IGFBP-3 (24% displacement of ^{125}I-IGFBP-3 at IGFBP-5 concentrations of 30 nM).

To evaluate the specificity of human IGFBP-3 binding to Hs578T cells, compared with IGFBP-3 from other species, intact rat IGFBP-3 was used for competitive binding assays. Rat IGFBP-3 consists of 265 amino acids, one amino acid longer than human IGFBP-3, and shows 83% homology with human IGFBP-3 (42). Most of the amino acid sequence variations between the two IGFBP-3s occur in the midregion of the IGFBP-3 molecules. Although intact rat IGFBP-3 inhibited ^{125}I-IGFBP-3$^{Escherichia\ coli}$ binding in a dose-dependent manner, rat IGFBP-3 was 20-fold less potent than human IGFBP-3$^{Escherichia\ coli}$ in competing for ^{125}I-hIGFBP-3 binding, suggesting the existence of species specificity in the interaction between IGFBP-3 and the IGFBP-3 receptor (Fig. 3A). These differences are unlikely to be attributable to glycosylation of rat IGFBP-3, because data from our laboratory and others indicate no difference in the ability of glycosylated and nonglycosylated human IGFBP-3 to associate with cell
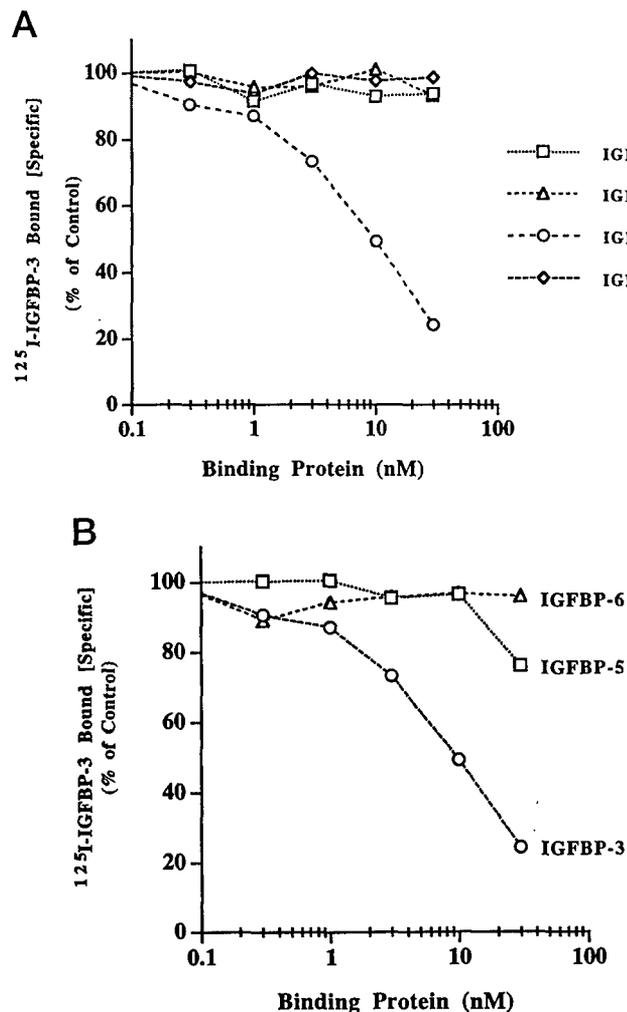
FIG. 2. Specificity of IGFBP-3 binding to the IGFBP-3 receptor in Hs578T cells. Binding studies were carried out on Hs578T monolayers, as described in *Materials and Methods*, in the absence or presence of indicated concentrations of unlabeled IGFBP-1, 2, 3, or 4 (A) or IGFBP-3, 5, or 6 (B). Cell-associated radioactivity was determined as described in Fig. 1.

surfaces (data not shown; Ref. 43). Assays for inhibition of Hs578T cell growth were consistent with the relative affinities of IGFBP-3 preparation for cell surface binding. When human IGFBP-3 was added, it showed a significant inhibitory effect on monolayer growth of Hs578T cells (10 nM, $P < 0.005$). This inhibitory effect of IGFBP-3 was dose dependent. On the other hand, rat IGFBP-3 showed a growth inhibitory effect with 10–20 times less potency, compared with human
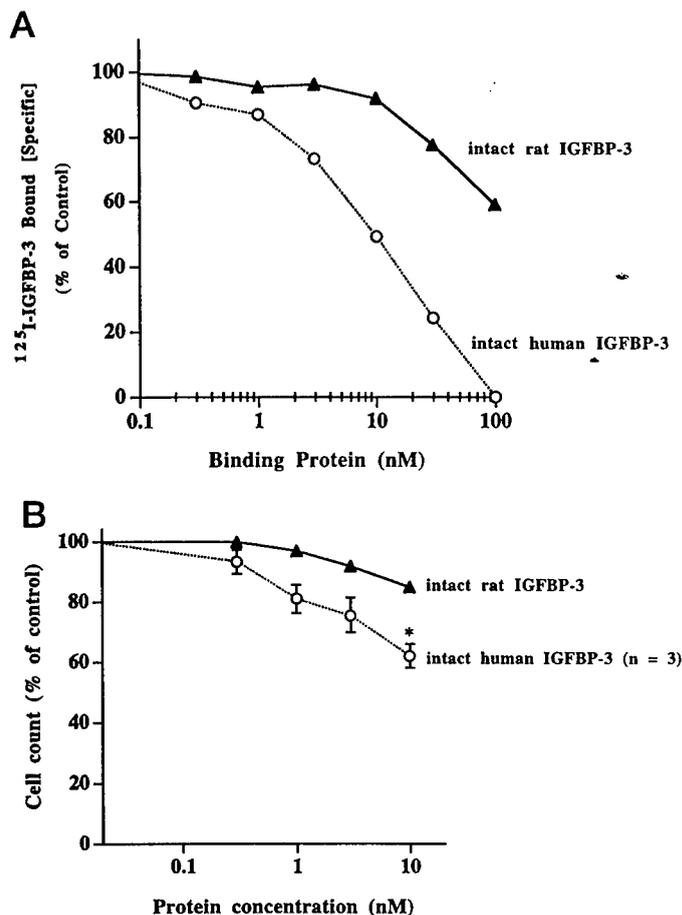
FIG. 3. A, Competitive binding of intact rat IGFBP-3 with ^{125}I-IGFBP-3 in Hs578T cells. Binding studies were carried out on Hs578T monolayers, as described in *Materials and Methods*, in the absence or presence of indicated concentrations of unlabeled intact rhIGFBP-3 and intact rat IGFBP-3. Cell-associated radioactivity was determined as described in Fig. 1. B, Effects of rhIGFBP-3 and rat IGFBP-3 on monolayer growth in Hs578T cells. Hs578T cells were grown in 24-multiwell dishes until 60% confluent (0.1×10^6 cells/well) and then changed to 1% FBS containing media with or without reagents for 94 h. Cells were then gently detached from plates by trypsin-EDTA, and cell number was counted using a Coulter counter. Statistical significance, in comparison with control values, is indicated by * ($P < 0.005$).

IGFBP-3 (Fig. 3B). These data suggest that the less-homologous midregion of the IGFBP-3 molecule contributes significantly to the binding site on IGFBP-3 for cell surface association, because the amino acid sequences from COOH-terminal residues 157–249 between human and rat IGFBP-3 are almost identical.

Characterization of the receptor binding domain on the IGFBP-3 molecule

To determine the binding domain on the IGFBP-3 molecule responsible for binding to its receptor in Hs578T cells, human IGFBP-3 fragments, produced by MMP-3 digestion and rat IGFBP-3 fragments (rat IGFBP-3$^{1-160}$ and rat IGFBP-3$^{161-265}$), were compared for their binding affinities for Hs578T monolayers in competitive binding assays. Figure 4A demonstrates that COOH-terminal fragments of human
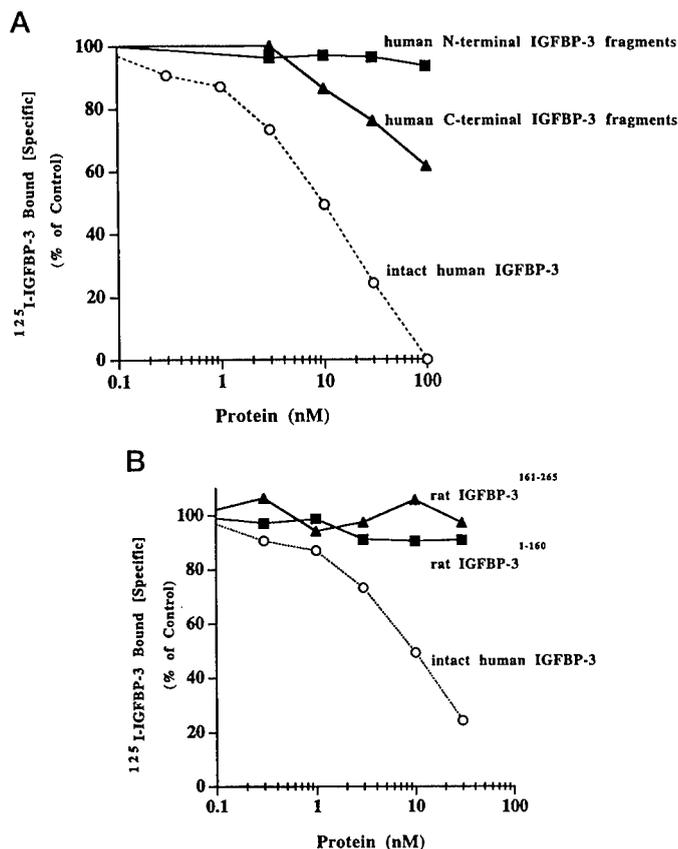

FIG. 4. Competitive binding of ^{125}I-IGFBP-3 to Hs578T monolayers by human IGFBP-3 fragments (A) or rat IGFBP-3 fragments (B). Binding studies were carried out on Hs578T monolayers, as described in *Materials and Methods*, in the absence or presence of indicated concentrations of unlabeled human IGFBP-3 fragments (A) or unlabeled rat IGFBP-3 fragments (B). Cell-associated radioactivity was determined as described in Fig. 1.

IGFBP-3 (a mixture of IGFBP-3$^{100-264}$, IGFBP-3$^{110-264}$, and IGFBP-3$^{177-264}$) inhibited ^{125}I-IGFBP-3 binding in a dose-dependent manner, with 10-fold less potency than intact human IGFBP-3. In contrast, NH$_2$-terminal fragments of human IGFBP-3 (a mixture of IGFBP-3$^{1-109}$ and IGFBP-3$^{1-99}$) did not inhibit ^{125}I-IGFBP-3 binding. In addition, neither rat NH$_2$- nor COOH-terminal IGFBP-3 fragments inhibited ^{125}I-IGFBP-3 binding, as shown in Fig. 4B.

To further narrow down the receptor binding site on the IGFBP-3 molecule, and, especially, to evaluate the significance of the midregion (44) and the heparin-binding domains near the COOH-terminus of the IGFBP-3 molecule (45) for receptor binding, we have generated two synthetic peptides containing the heparin-binding domain, as well as baculovirus-expressed rhIGFBP-3 fragments corresponding to the midregion of the IGFBP-3 molecule (Fig. 5). Peptide IV contains the sequence ^{149}KKGHA^{153}, which resembles a short heparin-binding domain (BBXBX; B = basic amino acid and X = nonbasic amino acid) and peptide VI contains the sequence ^{219}YKKKQCRP^{226}, which resembles a long heparin-binding motif (XBBBXXBX) (45, 46). IGFBP-3$^{1-87}$ represents the highly conserved NH$_2$-terminal region, whereas IGFBP-3$^{88-183}$ and IGFBP-3$^{88-148}$ correspond to the nonconserved midregion of IGFBP-3, with or without heparin binding do-
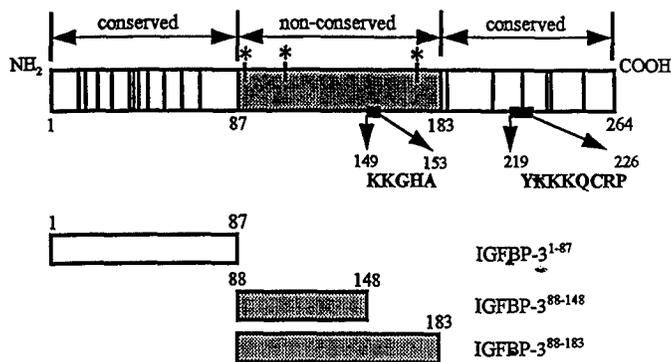
FIG. 5. Schematic diagram of the structure of human IGFBP-3 and baculovirus-expressed IGFBP-3 fragments. The conserved and non-conserved regions among the high-affinity IGFBPs are indicated. The *vertical lines* represent cysteine residues. The sequences of two putative heparin binding domains are indicated. Symbols (*) represent putative N-glycosylation sites. IGFBP-3^{1-87} represents the conserved NH$_2$-terminal region, whereas IGFBP-3^{88-148} and IGFBP-3^{88-183} correspond to the nonconserved midregion of IGFBP-3, with or without heparin binding domains, respectively.

mains, respectively. Competition binding assays revealed that the two peptides that contain the heparin binding domains (peptide IV and VI) weakly inhibited ^{125}I-IGFBP-3 binding (Fig. 6A); 15% and 29% of ^{125}I-IGFBP-3 were displaced by 10 μM of peptide IV or VI, respectively, confirming that IGFBP-3 binding to the Hs578T cell surface cannot be attributed to interaction with GAGs. Indeed, as shown in Fig. 6B, the K_d for peptide VI was significantly lower than that of the high-affinity component of IGFBP-3 binding in Hs578T cells (Fig. 1B). On the other hand, the low-affinity binding characteristics of IGFBP-3 are virtually identical to those of peptide VI ($K_d = 2.03 \times 10^{-5}$ M, 3.88×10^7 binding sites/cell). As would be predicted, when peptide VI was added, it showed no significant inhibitory effect on monolayer growth of Hs578T cells, at concentrations up to 300 nM (data not shown).

On the other hand, IGFBP-3 fragments, representing the midregion of IGFBP-3, inhibited ^{125}I-IGFBP-3 binding with equipotency to intact IGFBP-3 (40% and 60% inhibition at the concentrations of 10 and 30 nM, respectively), regardless of heparin binding domains. The NH$_2$-terminal fragment, IGFBP-3^{1-87}, showed no inhibition of IGFBP-3 binding at concentrations up to 30 nM (Fig. 7). These data indicate that the midregion of the IGFBP-3 molecule, even without a putative heparin binding motif, is probably responsible for the binding of IGFBP-3 to Hs578T cell surfaces and the accompanying growth-inhibitory effect.

Discussion

Our laboratory and others have demonstrated a significant IGF-independent inhibitory effect of: 1) exogenous IGFBP-3 on the growth of Hs578T ER-negative human breast cancer cells (22); 2) human IGFBP-3 transfected mouse Balb/c fibroblast cells (23); and 3) human IGFBP-3 transfected fibroblast cells, which were derived from mouse embryos homozygous for a targeted disruption of the type I IGF receptor gene (24). We have recently demonstrated that the antiproliferative effects of TGF-β and retinoic acid in human breast
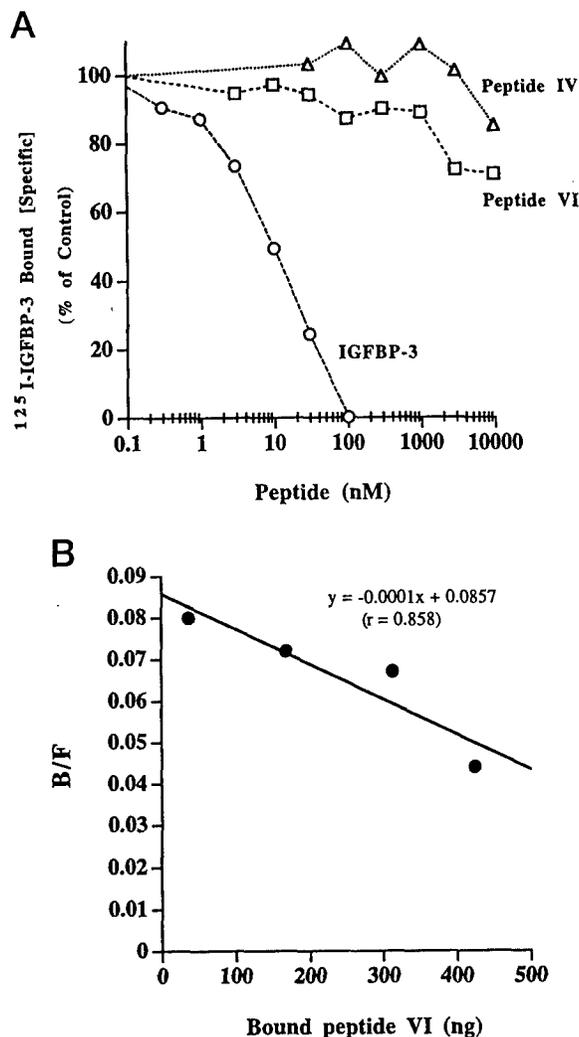

FIG. 6. A, Competitive binding of ^{125}I-IGFBP-3 to Hs578T monolayers by human IGFBP-3 peptides IV and VI. Binding studies were carried out on Hs578T monolayers, as described in *Materials and Methods*, in the absence or presence of indicated concentrations of unlabeled human IGFBP-3 peptides IV or VI. Cell-associated radioactivity was determined as described in Fig. 1. B, Scatchard plot of IGFBP-3 to Hs578T cells using peptide VI as a competitor. Binding studies were carried out on Hs578T monolayers, as described in *Materials and Methods*, in the absence or presence of indicated concentrations of unlabeled human IGFBP-3 peptides VI. Cell-associated radioactivity was determined as described in Fig. 1.

cancer cells are mediated, at least in part, through transcriptional regulation of IGFBP-3 (29, 30) and that the growth inhibitory effects of IGFBP-3 in these cells are not mediated through interaction with IGFs (IGF-independent actions). The significance of IGFBP-3 as a mediator of the growth inhibitory effects of other hormones has been studied by others, as with antiestrogens and the p53 tumor suppressor gene (31, 33). Furthermore, we have recently demonstrated that IGFBP-3 binds to specific membrane proteins (20, 26, and 50 kDa) located on Hs578T cells (22), which may be responsible for mediating the growth inhibitory effects of IGFBP-3 (34). In the same context, previous studies have demonstrated that IGFBP-3 binds to specific membrane proteins and induces apoptosis through a p53- and IGF-independent
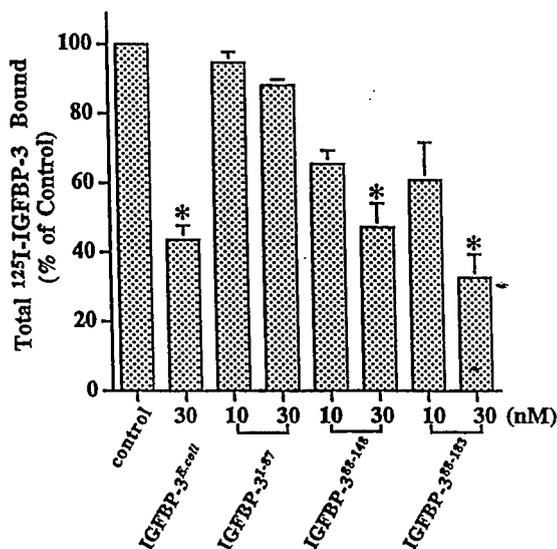
FIG. 7. Competitive binding of ^{125}I-IGFBP-3 to Hs578T monolayers by baculovirus-expressed rhIGFBP-3 fragments. Experiments were performed using Hs578T monolayers, as described in Fig. 6A. Statistical significance, in comparison with control values, is indicated by * ($P < 0.01$).

mechanism in human prostate cancer cells, supporting our previous results indicating IGF-independent action of IGFBP-3 and IGFBP-3 receptors (35).

IGFBP production by breast cancer cells is heterogeneous; the predominant secreted IGFBPs seem to correlate with the ER status of the cells (47). ER-negative cells predominantly secrete IGFBP-3 and IGFBP-4 as major species, whereas ER-positive cells secrete predominantly IGFBP-2 and IGFBP-4 as major species (48–50). The differential expression of IGFBPs in these two classes of breast cancer cells suggests that the biological significance of IGFBPs and, especially, of IGFBP-3 and its own receptor, may depend upon the ER status of the cells. In this study, accordingly, we used two human breast cancer cell lines: Hs578T cells (ER-negative) and MCF-7 cells (ER-positive). High-affinity ^{125}I-IGFBP-3 binding data in Hs578T cells and MCF-7 cells reveal similar binding affinities in the two cell lines, regardless of whether 10% human serum IGFBP fractions or 100 nM IGFBP-3 was employed for nonspecific binding. Receptor affinities were identical in the two cell lines, although 20- to 30-fold lower receptor number/cell was observed in MCF-7 cells, relative to Hs578T cells, entirely accounting for the differences in specific binding between the two cell lines. These data indicate that high-affinity binding can be attributed to a specific IGFBP-3 binding site, which seems to have all the characteristics of a peptide ligand-receptor interaction and which can be found, although at varying concentrations, in both Hs578T and MCF-7 cells. The presence of a single high-affinity binding site for IGFBP-3 on Hs578T cells is consistent with our previous identification of a single binding site (estimated at 23 kDa) by monolayer cross-linking (34). On the other hand, studies employing cross-linking to Hs578T cell lysates suggested the presence of as many as three specific IGFBP-3 interacting proteins. The additional two binding sites may reflect either: 1) intracellular proteins contained within the whole cell ly-

sates; 2) components of an IGFBP-3 receptor complex; or 3) precursor or degradation forms of the IGFBP-3 receptor.

While Hs578T cells seem to have the same putative IGFBP-3 receptors as MCF-7 cells, the former has about 30-fold more IGFBP-3 receptors than the latter. From these results, it would be predicted that IGFBP-3 would have more growth inhibitory effects on Hs578T cells than on MCF-7 cells, because of the higher IGFBP-3 receptor concentrations in Hs578T cells. Thus, the differences in the cellular response to IGFBP-3 between ER-negative cell lines and ER-positive cell lines may depend, at least in part, on the concentration of IGFBP-3 receptors, although further studies will be necessary in additional cell lines. Nevertheless, in our previous studies (22), the threshold for IGFBP-3 growth inhibitory effects was in the range of 10^{-9}–10^{-8} M, which is consistent with the affinity of IGFBP-3 for the IGFBP-3 receptor ($K_d = 8.19 \pm 0.97$ nM). These binding characteristics are also in the range of those estimated by Delbe et al. in chick embryo fibroblasts ($K_d = 10^{-8}$ M and 60,000 binding sites/cell) (21).

To establish the specificity of IGFBP-3 binding to Hs578T cells, competitive binding assays were performed in which the family of IGFBPs was used to compete for binding of ^{125}I-IGFBP-3. IGFBP-1, 2, 4, and 6 were not able to compete for ^{125}I-IGFBP-3 binding, although IGFBP-5 weakly competed at a concentration of 30 nM, indicating that IGFBP-3 has highly specific binding sites on the cell surface. The mechanism of the cell surface binding for IGFBP-1 has been shown to result from $\alpha5\beta1$-integrin receptors, which would recognize the arginine-glycine-aspartic acid (RGD) tripeptide sequence in IGFBP-1 (51). The fact that IGFBP-3 lacks this RGD sequence and that no competition was seen with RGD sequence-containing IGFBPs, such as IGFBP-1 and IGFBP-2, indicate that the mechanism of IGFBP-3 cell surface binding is unlikely to be mediated through integrin receptors and is different from that of IGFBP-1. IGFBP-3, IGFBP-5, and IGFBP-6 have putative heparin binding amino acid sequences: residues ^{149}K-K-G-H-A^{153} and ^{219}Y-K-K-Q-C-R-P^{226} in IGFBP-3; residues ^{139}P-K-H-T-R-I^{144} and ^{225}Y-K-R-K-Q-C-K-P^{232} in IGFBP-5; residues ^{172}Y-R-K-R-Q-C-R^{178} in IGFBP-6. Heparin-like molecules are present on cell surfaces and in extracellular matrix (ECM), suggesting the possibility that IGFBP-3, 5, and 6 could bind to cell surfaces or to ECM through heparin-like molecules. Others have reported that heparin, which releases proteins attached to cell surface proteoglycans, could displace IGFBP-3 binding on human neonatal fibroblast (52) and rat sertoli cell surfaces (53), and that heparin modulates the binding of IGFBP-5 to osteoblastic cell membranes (54). Synthetic peptides, containing the C-terminal heparin binding motifs from IGFBP-3, -5, and -6, inhibit IGFBP-3 and IGFBP-5 binding to endothelial cell monolayers (55–57). Similarly, recent studies reported that the ability of IGFBP-3 to associate with the cell surface was lost in IGFBP-3 variants lacking residues 185–264 and in the ^{228}KGRKR^{232} → MDGEA mutant, suggesting that residues 228–232 of IGFBP-3 are essential for the cell-surface association in Chinese hamster ovary cells (58). However, it is of note that the ^{228}KGRKR^{232} → MDGEA (IGFBP-1 sequence) mutations are adjacent to the putative long binding domain (^{219}YKKKQCRP^{226}) affecting IGFBP-3 binding to heparin. It is possible that mutation of the basic residues 228–232 to

acidic residues results in disruption of heparin binding to the putative long binding domain of IGFBP-3, thereby affecting the ability of IGFBP-3 to associate with the cell surface.

On the other hand, when heparin and heparan sulfate linkages of glycosaminoglycans (GAGs) on the cell surface are enzymatically removed by pretreatment with heparinase or heparitinase, IGFBP-3 binding is only minimally affected in human breast cancer cells, despite exogenously added soluble heparin or heparan sulfate, which inhibits ^{125}I-IGFBP-3 binding to the cell surface in a dose-dependent manner (59). This suggests that soluble heparin or heparan sulfate forms a complex with IGFBP-3, thereby inhibiting binding of IGFBP-3 to cell-surface proteins specific for IGFBP-3, rather than competing with cell-surface GAGs for binding of IGFBP-3. In our study, IGFBP-5 and IGFBP-6 showed little or no ability to compete for ^{125}I-IGFBP-3 binding, suggesting that ^{125}I-IGFBP-3 binding to breast cancer cell surfaces through heparin-like molecules is not a major factor. This was further confirmed by experiments employing synthetic peptides IV and VI, which contain the two heparin binding domains, ^{149}K-K-G-H-A^{153} and ^{219}Y-K-K-K-Q-C-R-P^{226}, respectively, and have been shown to bind several different GAGs, such as heparin, heparan sulfate, and dermatan sulfate, but with different affinities (60). Even though peptides IV and VI competed with IGFBP-3 binding to the cell surface, the K_ds for peptides IV and VI were significantly lower (Fig. 6) than that of the high-affinity component of IGFBP-3 binding in Hs578T cells (Fig. 1B). Furthermore, in the presence of peptides IV or VI, the IGFBP-3 cell binding assay showed the single high-affinity binding site (data not shown). More evidently, the low-affinity binding characteristics of IGFBP-3, as shown in the two cell lines, when 10% human serum IGFBP fractions were used for nonspecific binding, are identical to those of peptide VI ($K_d = 2.03 \times 10^{-5}$ M, 3.88×10^7 binding sites/cell), suggesting that the low-affinity binding component may be the result of interactions between IGFBP-3 and GAG-like molecules on the cell surface or in the ECM. In that case, the curvilinear Scatchard plot observed in the presence of 10% human serum IGFBPs may be partially caused by competition by IGFBP-5 for the low-affinity site. We have shown that the C-terminal heparin binding motif from IGFBP-5 has an affinity for heparin similar to that of the homologous heparin binding motif from IGFBP-3, whereas the homologous IGFBP-6 consensus sequence binds heparin with much less affinity (60). These observations possibly explain why IGFBP-6 association with the cell surface has not been demonstrated.

Glycosylated rat IGFBP-3 inhibited rh-^{125}I-IGFBP-3$^{Escherichia\ coli}$ binding in a dose-dependent manner, although glycosylated rat IGFBP-3 shows 20-fold less potency than human IGFBP-3$^{Escherichia\ coli}$, differences which cannot be explained simply by glycosylation. These observations suggest that the differences in IGFBP-3 binding affinity to cell surfaces depend on the species of origin of IGFBP-3. Most of the amino acid sequence variations between human IGFBP-3 and rat IGFBP-3 occur in the midregion of the IGFBP-3 molecules (residues 86–184), suggesting that the binding site on IGFBP-3 for the IGFBP-3 receptor is located in the midregion of the IGFBP-3 molecule. This was supported by the different levels of competition for IGFBP-3 binding by human IGFBP-3

fragments or rat IGFBP-3 fragments. First, human N-terminal IGFBP-3 fragments, which contain residues 1–109, failed to inhibit ^{125}I-IGFBP-3 binding, indicating that the N-terminal domain of IGFBP-3 is not responsible for receptor binding. Secondly, a human mid-C-terminal fragment (residues 100–264) inhibited ^{125}I-IGFBP-3 binding, whereas a rat C-terminal fragment (161–265) did not compete for ^{125}I-IGFBP-3 binding, even though it contains a heparin binding motif that is identical to that of human IGFBP-3, indicating that the difference between the human C-terminal IGFBP-3 and rat C-terminal IGFBP-3 is attributed to the midregion, where human and rat IGFBP-3 contain different amino acid sequences. Whereas the C-terminal fragments of human IGFBP-3 contain the midregion of the IGFBP-3 molecule, the other peptides employed do not contain or only partially contain this region, consistent with the hypothesis that the midregion of the IGFBP-3 molecule is essential for IGFBP-3 binding to its receptor.

To test our hypothesis more directly, we have generated three rhIGFBP-3 fragments in a baculovirus expression system: 1) IGFBP-3$^{1-87}$, an NH$_2$-terminal fragment containing the highly conserved sequences among IGFBPs, including the 12-cysteine cluster; 2) IGFBP-3$^{88-148}$, a fragment containing the midregion of IGFBP-3, but without heparin binding domains; and 3) IGFBP-38$^{88-183}$, a fragment corresponding to the entire midregion of IGFBP-3, including heparin binding motif. Competitive binding assays revealed that only IGFBP-3 fragments, representing the midregion of IGFBP-3, bind to the putative IGFBP-3 receptor with equipotency, compared with intact IGFBP-3, indicating that the receptor binding site resides within the midregion of the IGFBP-3 molecule (possibly within amino acids 88–148).

These observations have shown that high-affinity IGFBP-3 receptors are located on breast cancer cell membranes. These receptors have properties that support the notion that they may mediate the IGF-independent inhibitory actions of IGFBP-3 in breast cancer cells. Recent studies postulated that the type V TGF-β receptor is the putative IGFBP-3 receptor in mink lung epithelial cells (61). It seems that IGFBP-3 binds to the type V TGF-β receptor specifically and competes with TGF-β for receptor binding. The growth inhibitory effect of IGFBP-3 is aborted by a TGF-$\beta1$ peptide antagonist in these cells, suggesting that IGFBP-3 is a functional ligand for the type V TGF-β receptor. However, at the present time, it is unclear whether the type V TGF-β receptor represents the primary IGFBP-3 receptor, whether it is identical to the putative receptor observed in breast and prostate cancer cell systems, and whether it plays a role in the growth-inhibitory actions of IGFBP-3 in those cell systems. Further characterization and sequencing of the IGFBP-3 receptor will be important in determining the mechanisms for the IGF-independent actions of IGFBP-3 in directly inhibiting cell growth in human breast cancer cells. Current studies are in progress to identify the IGFBP-3 receptor and to characterize the IGFBP-3 receptor-mediated signal transduction pathway(s) in human breast cancer cells.

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INSULIN AND IGF BINDING BY IGFBP-3 FRAGMENTS DERIVED FROM PROTEOLYSIS, BACULOVIRUS EXPRESSION AND NORMAL HUMAN URINE.

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ABSTRACT

Recombinant human IGFBP-3 was proteolysed with different concentrations of plasmin for various periods of time. The major IGFBP-3 fragment resulting from this digestion migrated at ca. 15 kDa in nonreducing SDS-PAGE. Following the identification of this fragment as an N-terminal IGFBP-3 fragment, by use of N-terminus-specific monoclonal antibody and amino acid sequence analysis, we constructed and expressed a similar fragment in a baculovirus expression system. The fragments resulting from plasmin digestion, as well as the baculovirus-expressed recombinant human IGFBP-3$^{1-97}$, retain weak IGF binding and show specific insulin binding on cross-linking and western ligand blot. RhIGFBP-3$^{1-97}$ can inhibit insulin receptor autophosphorylation in insulin receptor-overexpressing NIH 3T3 cells. Insulin and IGF binding to IGFBP-3 fragments could be further demonstrated in normal urine. These data indicate the physiological significance of IGFBP-3 fragments derived from proteolysis in vivo.

INTRODUCTION

IGFBPs are important regulators of IGF action, by modulating IGF binding to its receptors. IGFBP-3 is the major serum IGFBP and transports 70-90% of the circulating IGFs. In target cell systems, it sequesters IGFs and inhibits their hormonal action, but may, under specific conditions, also potentiate IGF action or exert IGF-independent effects (1,2). IGFBP-3 can be modified by proteolysis under various conditions. Initially described in human pregnancy serum, limited IGFBP-3 proteolysis and its significance in vivo have become intensive research subjects (3-6). Prostate specific antigen, matrix metalloproteinases, plasmin and thrombin have all been identified as IGFBP-3 proteases (7-12). Reports about limited proteolysis of IGFBP-3 by plasmin and the physiological relevance of the resulting fragments have provided different results with respect to size, sequence and IGF binding ability (11,12). Our laboratory has reported recently the ability of IGFBPs to specifically bind insulin (18). The N-terminal part of IGFBP-3 was shown to bind insulin and inhibit its interaction with the insulin receptor. In the present study, we have employed purified plasmin from human serum to proteolyse recombinant IGFBP-3$^{E.coli}$ in vitro, and have characterized the major resulting fragment and expressed it in a baculovirus expression system. Using baculovirus-expressed rhIGFBP-3$^{1-97}$, we describe the ability of this fragment to specifically bind IGFs and insulin and inhibit insulin receptor autophosphorylation.

MATERIAL AND METHODS

RhIGF-I, purchased from Bachem (Torrance, CA), was iodinated as described previously (13); IGFBP-3$^{E.coli}$ was a gift from Celtrix (Santa Clara, CA); plasmin was from Fluka (Ronkonkoma, NY), and ^{125}insulin was a gift from DSL (Webster, TX).

Proteolysis of IGFBP-3$^{E.coli}$ - 0.5 µg of IGFBP-3$^{E.coli.}$ were digested with different concentrations of plasmin (0.1 to 100 µg plasmin per 0.5 µg IGFBP-3) for 30 min at 37°C in Tris buffer (0,02 M tris, ph 7,4; 0,15 M NaCl). After digestion, the reaction was stopped with SDS sample buffer and loaded immediately on a 15% SDS-PAGE.

Expression and purification of human IGFBP-3$^{1-97}$ fragment- The cDNA for IGFBP-3$^{1-97}$ was generated by PCR amplification from the human IGFBP-3 cDNA and subcloned into pFASTBAC1 baculovirus expression vector (Gibco) using the unique EcoRI site at nt. position 917 in the IGFBP-3 cDNA (19). At the 3' end, a FLAG epitope (DYKDDDDK) followed by a stop codon and an unique XbaI site were introduced after aa position 97 of IGFBP-3. Primers (A) 5'-GTGAATTCTCGAGCTCGTCGA (915-935) and (B) 5'-GCTCTAGACTACTTGTCATCGTCGTCCTTGTAGTCG CGCAGGCGGCTGACGGCACTAG (1396-1418) were used, and the resulting PCR product was subcloned into the expression vector. After sequencing, the construct was introduced into viral DNA and transfected into SF-9 insect cells, according to the vendor's protocol. Western immunoblots (WIB) were performed with FLAG specific anti-M2 antibody (Kodak) and our polyclonal α-IGFBP-3 antibody (14). Large scale protein purification was done as described previously (13). Purity and concentration of IGFBP-3$^{1-97}$ was determined by staining with Coomassie Blue and comparison with known amounts of IGFBP-3$^{E.coli}$

Western ligand blot (WLB)- Samples of plasmin-digested IGFBP-3$^{E.coli}$ or IGFBP-3$^{1-97}$ were subjected to reducing SDS-PAGE (15% gel), electroblotted onto nitrocellulose filters, incubated overnight with 1.5x10^6 cpm of ^{125}IGF-I or ^{125}insulin, washed, dried, and exposed to Biomax MS film (Eastman Kodak) for 4 to 14 days .

Affinity cross-linking - Plasmin-digested IGFBP-3$^{E.coli}$ or IGFBP-3$^{1-97}$ was incubated with ^{125}IGF-I or ^{125}insulin (50,000 cpm) in the presence or absence of unlabeled ligand. After cross-linking with DSS, samples were subjected to SDS-PAGE (15% gel) and autoradiography on Biomax MS film.

Insulin receptor (IR) autophosphorylation assay - was performed as described previously (18). Briefly, confluent IR-overexpressing NIH3T3 cells were incubated in serum-free medium overnight and treated for 3 min with DMEM containing 75 ng/ml bovine insulin and different concentrations of IGFBP-3$^{1-97}$, which had been preincubated for 2h at 4°C. Cells were washed, solubilized and the lysate subjected to 8% SDS-PAGE. The β-subunit of the IR was detected by 4G10 anti-phosphotyrosine antibody.

Preparation of urine: Overnight urine from a healthy 4 year-old girl was filtered and concentrated with centriprep 10 (Amicon, Beverly, MA).

RESULTS

Proteolysis of IGFBP-3 by plasmin - Fig. 1A shows the immunoblot of IGFBP-3$^{E.coli}$ digested by different concentrations of plasmin, on a nonreducing 15% SDS-PAGE. At the lowest plasmin concentration, five different-sized IGFBP-3 fragments were detected (27-, 21-, 20-, 18-, and 15-kDa). On increasing the plasmin concentration 500-1000 fold, only the 15 kDa fragment remained stable. Similar results have been detected by digesting

0.5 µg IGFBP-3 with 15 µg plasmin for up to 4 hours (data not shown). Fig. 1B shows a WLB of plasmin-digested IGFBP-3 with [125]IGF-I (left panel) and [125]insulin (right panel). The predominant 15 kDa fragment is able to bind radiolabeld IGF-1, as well as insulin. Affinity cross-linking of IGFBP-3 proteolysed by plasmin with radiolabeled IGF-I or insulin, was also performed (Fig. 1C) The 15 kDa fragment could be crosslinked to radiolabeled IGF-I (left panel) and insulin (right panel).

plasmin 0 0.1 0.5 1 5 10 50 100
µg/100µl

29 kDa →

18 kDa →

14 kDa →
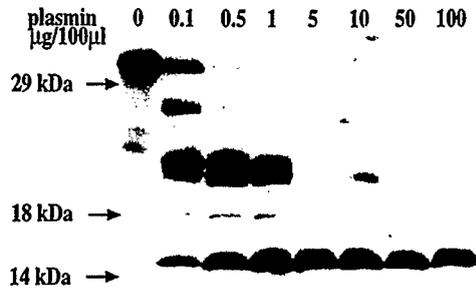
Figure 1A: WIB of plasmin-digested IGFBP-3. In each lane 0.5 µg nonglycosylated IGFBP-3 were digested with different amounts of plasmin as indicated above and separated on a 15% SDS-PAGE under nonreducing conditions.

WLB [125]IGF-I   WIB α BP-3   WLB [125]INS

28 kDa →

18 kDa →

14 kDa →
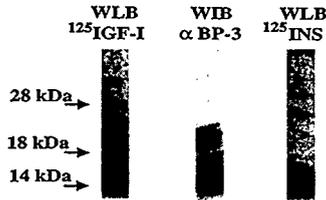
Figure1B: WLB of plasmin digested IGFBP-3. 5µg IGFBP-3[E.coli] were digested with 500µg plasmin and separated on a 15% SDS-PAGE under reducing conditions. WLB was done with radiolabled IGF-I (left lane) or insulin (right lane), and the control WIB is shown in the middle.

x-link [125]IGF-I   WIB α BP-3   x-link [125]INS

28 kDa →

18 kDa →

14 kDa →
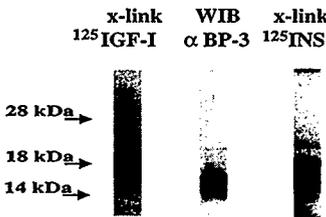
Figure1C: Cross-linking of plasmin digested IGFBP-3 (200 ng) to [125]IGF-I (left lane) or [125]insulin (right lane) and subsequent separation on a 15% nonreducing SDS-PAGE. WIB of the digestion is shown in the middle.

The NH$_2$-terminal sequence analysis of the 15 kDa IGFBP-3 fragment revealed that this fragment represents the NH$_2$ -terminal portion of IGFBP-3. Based on published cleavage sites for plasmin and other proteases, the size of the fragment, and the amino acid sequence of the 15 kDa fragment, we determined this fragment to be IGFBP-3[1-97].

Construction and expression of FLAG-tagged IGFBP-3[1-97] Fig. 2A is a WIB of the fractions collected during purification of FLAG-tagged IGFBP-3[1-97]. Analysis of the purified protein and subsequent Coomassie staining (data not shown) showed a protein of approximately 16,000 molecular weight and 99% purity.

Deglycosylation studies demonstrated a 15,000 Mw core protein with one N-linked glycosylation site. WLB of IGFBP-3[1-97] (Fig. 2 B) shows the ability of the synthetic fragment to bind [125]IGF-1 (left) or [125]insulin (right).

CM FT W VV E

14 kDA →
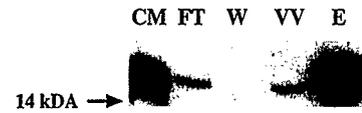
Figure 2A: WIB of baculovirus-expressed IGFBP-3[1-97] using anti-M2 antibody. CM-conditioned medium (30ml); FT-flow-through (30ml); W-wash (30ml); VV-void volume (1ml); E-elution (4ml). 25µl of each fraction were separated on a 15% SDS-PAGE under reduced conditions.

[125]IGF-I   [125]INS

← 14 kDA →

Figure 2B: WLB of baculovirus expressed IGFBP-3[1-97]. 12µg of IGFBP-3[1-97] were separated on a 15% gel under reduced conditions and incubated with ligand, as indicated above.

Cross-linking of IGFBP-3[1-97] to [125]IGF-I and [125]insulin was performed. For both [125]IGF-I and [125]insulin, a dose-dependent linear increase in binding of the ligand to the fragment, starting at < 30 nmol fragment concentration, could be detected (data not shown). Competitive affinity cross-linking was performed with 100 pmol IGFBP-3[1-97]. Both [125]IGF-I and [125]insulin bound specifically to the fragment, as shown by competition with unlabeled IGF-I or insulin, respectively (fig. 2C). Subsequent competition of labeled insulin with cold IGF-I or labeled IGF-I with cold insulin showed similar competition in both experiments (data not shown), indicating a similar, if not identical, binding site for IGF-I and insulin.

[125]IGF-I   [125]INS

cold peptide (pmol) 0 4 14 40 80   0 5 17 50 100

tracer trimer →

15kDa fragment →

tracer dimer →
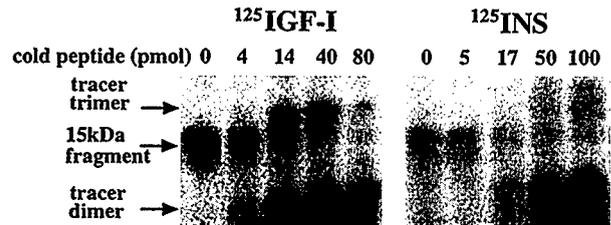
Figure 2C: Competitive affinity cross-linking of IGFBP-3[1-97] to [125]IGF-I (left) or [125]insulin (right). 100 pmol IGFBP-3[1-97] were crosslinked to [125]IGF-I or [125]insulin. Competition was done with the same unlabeled ligand at the concentrations indicated above.

Inhibition of Insulin receptor autophosphorylation by IGFBP-3[1-97]
Figure 3A shows the anti-phosphotyrosine WIB of total cell lysate from insulin receptor-overexpressing NIH 3T3 cells, after insulin stimulation in the absence or presence of different amounts of rhIGFBP-3[1-97]. The β-subunit of the insulin receptor was detected by anti-phosphotyrosine antibody. In contrast to full length IGFBP-3, the IGFBP-3[1-97] fragment was able to inhibit the autophosphorylation of the insulin receptor β-subunit in a dose-dependent manner. IGFBP-3[1-97] readily inhibited IR autophosphorylation more than 50% at a concentration of 10pmol and 90% at 100pmol (fig. 3B). It is of note that after preincubation of insulin and IGFBP-3[1-97], additional treatment with excess

insulin (100ng/ml) overcomes the inhibitory effect of IGFBP-3$^{1-97}$, suggesting that inhibition by IGFBP-3$^{1-97}$ is through insulin binding (data not shown).
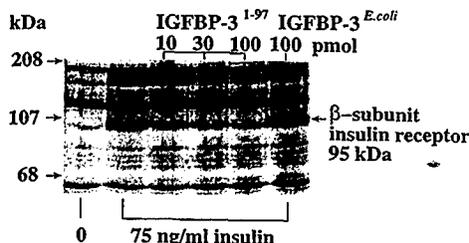

Figure 3A: Inhibition of IR autophosphorylation by IGFBP-3$^{1-97}$. Left lane: no insulin; lanes 2-6 (left to right): 75 ng/ml insulin; lanes 3-5: coincubation with 10, 30 or 100 pmol IGFBP-3$^{1-97}$; lane 6: coincubation with 100pmol IGFBP-3$^{E.coli}$.
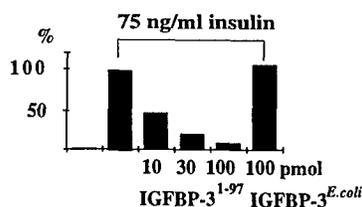

Figure 3B: Inhibition of IR autophosphorylation by IGFBP-3$^{1-97}$. Densitometric readings of 3 representative gels. Stimulation with 75 ng/ml insulin = 100%.

IGF-I and insulin binding of urinary IGFBP-3 fragments:

In concentrated urine, we detected IGFBP-3-related bands at 42/44 kDa, 29 kDa and 17.7 kDa by WIB with polyclonal αIGFBP-3 antibody under nonreducing conditions (data not shown). Under reducing conditions, the 17.7 kDa IGFBP-3 fragment migrates in 15% SDS-PAGE at approx. 14 kDa. Figure 4 shows a WLB of urine with radiolabeled IGF-I or insulin. The corresponding WIB identified two major IGFBP-3 fragments, at 29 and 14 kDa, both of which are detected by the radiolabeled ligands. The other bands (such as 18 kDa) could not be detected by IGFBP-3-immunoblot, and could result from other IGFBPs in urine which retain the ability to bind IGF-I and insulin.
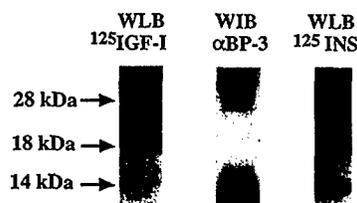

Figure 4: WLB of 30μl 500x concentrated urine under reducing conditions. The 29 kDa and the 14 kDa fragments are detected by IGFBP-3 antibody (middle). Both ^{125}IGF-I and ^{125}insulin can recognize these fragments in WLB. The 18 kDa band is not detected by WIB and presumably is a fragment of another IGFBP.

DISCUSSION

In vitro proteolysis of IGFBP-3 by plasmin is a convinient model for the study of the structure and action of IGFBP-3 fragments, since several laboratories have shown that plasmin is part of the *in vivo* proteolytic activity for IGFBP-3 under various conditions (6,10,12). In the present study, we describe an IGFBP-3 fragment derived from *in vitro* plasmin digestion. Previous studies identified this fragment as part of the N-terminal domain of IGFBP-3 (16). Because the N-terminus of IGFBP-3 is one of the highly conserved regions among the binding proteins and considered as a major IGF binding site, we expected the IGFBP$^{1-97}$ fragment to bind IGFs, although with reduced affinity. This assumption was supported by our previous report off a 17.7 kDa urinary IGFBP-3 fragment which binds IGF, although with reduced affinity compared to intact IGFBP-3. Similar to the IGFBP-3$^{1-97}$ peptide, this fragment is glycosylated at one site and the deglycosylated core protein migrates at ca. 15 kDa in a nonreducing SDS-PAGE (20). Western immunoblots performed with previously described monoclonal IGFBP-3 antibodies (16) confirmed this 17.7 kDa urinary fragment as an N-terminal fragment (data not shown).

Our laboratory has reported recently on the ability of IGFBPs, especially IGFBP-7, to bind insulin (18). It could be shown additionally, that under reduced conditions, IGFBP-3 and a synthetic fragment similar to the highly conserved N-terminal region of IGFBP-3 (aa 1-87) bind insulin in WLB. This N-terminal fragment was able to inhibit insulin-induced autophosphorylation of the insulin receptor and subsequent IRS-1 phosphorylation in insulin receptor-overexpressing NIH-3T3 cells. In the present study, we demonstrate the ability of a physiological IGFBP-3 fragment to bind IGF and insulin in WLB and solution cross-linking. We were able to express this fragment in a baculovirus system and to purify it. Due to oligomerisation of synthetic IGFBP-3$^{1-97}$ and possible conformational changes during immobilization of the fragment on the nitrocellulose membrane, binding to IGF-I and insulin in WLB could only be demonstrated under reducing conditions. Solution binding with subsequent cross-linking of the ligand to the fragment under more physiological conditions, however, could clearly demonstrate binding of both ligands to the fragment. The observation that this 15kDa IGFBP-3 fragment can bind IGF, albeit with decreased affinity, is consistent with our previous studies of IGFBP-3 fragments in urine (20), but in contrast with the report by Lalou et. al. (11); the failure of the latter group to detect specific IGF binding probably reflects technical aspects of the methods employed, although we cannot rule out the possibility that their plasmin-generated IGFBP-3 fragment differs from ours.

Insulin and IGF-I binding to a N-terminal urinary IGFBP-3 fragment described above was demonstrated by WLB with concentrated urine. Immunoprecipitation with a monoclonal antibody specific against the N-terminal part of IGFBP-3 and subsequent affinity cross-linking or WLB with ^{125}insulin or ^{125}IGF-I confirmed these findings (data not shown). For studying IGFBP-3 fragments from biological fluids, urine seems to be suitable, since the number and concentration of proteins, especially albumin and other IGFBPs, is limited and the concentration of the IGFBP-3 fragments is relatively high. Recent binding studies with biotinylated IGF-I in WLB demonstrated the IGF-I binding capacity of smaller IGFBP-3 fragments (17); this method was several times more sensitive than the usual WLB with radiolabeled ligand. By use of an extremely sensitive X-ray film, such as Biomax MS, however, we were able to detect binding with radioligands. The affinity of the described IGFBP-3 fragment for IGF-I, IGF-II (data not shown) and insulin is almost the same, in contrast to intact IGFBP-3, which has a much greater affinity for IGF than for insulin. IGFBP-3 proteolysis results,

therefore, in a dramatic decrease in IGF affinity, with a simultaneously increasing affinity for insulin. Insulin binding to proteolytic IGFBP-3 fragments and subsequent inhibition of insulin receptor action could play an important role in the microenvironment of the cell, where, in addition to IGFBP-3, a variety of other IGFBPs and IGF receptors compete for IGF. In contrast to IGFs, which are almost all trapped in complexes with IGFBPs, insulin is much more accessible for binding to the IGFBP-3 fragments. Whether the binding of insulin and subsequent inhibition of insulin action explain the IGF-independent actions of IGFBP-3 and its fragments remains to be seen (2, 11). This is unlikely to be the full explanation of IGFBP-3 action, however, in light of the ability of IGFBP-3 fragments to inhibit FGF (21).

Bereket and coworkers (15) reported the active involvement of insulin in the regulation of the IGFBP-3 protease activity in children with IDDM. They demonstrated that insulin administration during the first weeks of IDDM dramatically reduced serum proteolysis of IGFBP-3. It is also known that under conditions in which serum proteolysis of IGFBP-3 is increased (pregnancy, NIDDM, postsurgical catabolism or severe illness), insulin resistance is a common feature. How insulin itself is involved in the regulation of IGFBP-3 proteolysis, however, has to be further investigated.

ACKNOWLEDGMENTS

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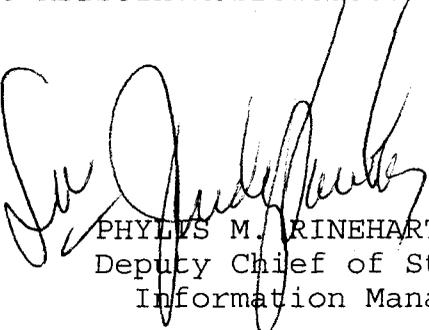
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