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CONTRACTING ORGANIZATION: University of Washington
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13. ABSTRACT ***(Maximum 200 Words)***

Mac25 is the first member of the IGFBP-rP family (IGFBP-rP1). IGFBP-rP1 is expressed in normal human mammary epithelial cells (HMECs) and upregulated in senescent HMECs. IGFBP-rP1 is not expressed in twelve/sixteen breast cancer cell lines and its expression is absent in estrogen receptor positive (ER^+) breast cancer cell lines.

IGFBP-rP1 may regulate HMEC growth. We tested the hypothesis that it suppresses growth in ER^+ breast cancer cells by transducing the cDNA into normal and human breast tumor cultures. We are testing the function of IGFBP-rP1 in normal HMECs by transducing AG11132 normal cells. We will determine if IGFBP-rP1 is regulated at the transcriptional level by characterization of the promoter. We are determining the binding properties of IGFBP-rP1 by growth assays in presence of IGF-I, IGF-II, R^3-IGF-I, and R^6-IGF-II and immunoprecipitation/phosphatase assays. We have determined IGFBP-rP1 tissue specificity by mRNA *in situ* hybridization.

We have ascertained that IGFBP-rP1 negatively regulates growth in ER^+ breast cancer cells. This growth inhibition is independent of apoptotic regulatory pathways. IGFBP-rP1 may inhibit growth through senescence-associated pathways. IGFBP-rP1 generated in ER^+ breast cancer cells exhibit two bands of higher molecular weight compared to protein found in ER^- breast cancer cells. We have potentially isolated two clones from a genomic library screen that may contain the 5'end of IGFBP-rP1 and may contain a portion of the promoter sequence.

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Karen Swisshelm 9/16/98

PI - Signature Date

U.S. Army Medical Research & Material Command
Fort Detrick, Maryland
Presentation of Annual Report
September 14, 1998

Mac25 (IGFBP-rP1), a new insulin-like growth factor binding protein and its role in breast cancer

Mentor: Karen Swisshelm, Ph.D.
Trainee: Heather-Marie P. Wilson

Attached cover letter, 1 copy, unnumbered

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INTRODUCTION

The IGF system has been implicated to play an active role in breast cancer development and progression (*1-4*). IGFBP-rP1 (mac25) was isolated by differential display while searching for genes that are overexpressed in senescent human mammary epithelial cells (HMECs) (*5*). Its sequence shares homology with the IGFBP family, but has been placed in the IGFBP-related protein family due to its low-affinity binding to IGFs. IGFBP-rP1 mRNA accumulates in senescent HMECs and is upregulated by all-trans-retinoic acid and synthetic retinoid fenretinide in normal, growing HMECs (*5*). There is an inverse correlation between IGFBP-rP1 mRNA expression and estrogen receptor positive breast cancer cells (*5, 6*). IGFBP-rP1 is upregulated by retinoic acid in normal human mammary epithelial cells and therefore may act as a downstream effector of retinoid chemoprevention by playing a role in growth inhibition. We hypothesize that IGFBP-rP1 functions are associated with senescence in normal human mammary epithelial cells and therefore may also act as a tumor suppressor in breast cancer cells. To determine the functional role of IGFBP-rP1 in human breast cancer, we have stably transfected three breast tumor cell lines, Hs578T (ER-), MCF-7 (ER+) and BT-474 (ER+), and one normal cell line, AG11132, with LXSN or L*IGFBP-rP1*SN retrovirus. In this report, I will describe experiments used to analyze IGFBP-rP1 effects on cell growth, cell cycle, expression levels of senescent markers, apoptosis, and changes in response to various growth factors. I will also show efforts undertaken to characterize the protein.

BODY

Aim 1: Experiments to address the potential of IGFBP-rP1 for modulating cell growth of ER+ breast cancer cells.

Previous Work/Background (August 1996-August 1998)
The full-length IGFBP-rP1 cDNA was cloned from a normal HMEC lambda Zap cDNA library into the BamHI site of an expression vector containing a cytomegalovirus (CMV) promoter and the gene for the selectable marker neomycin (vector from B.Vogelstein, unpublished). The IGFBP-rP1 cDNA was then cloned into the pBluescriptIIKS at the BamHI site. This allowed for easier manipulation in cDNA amplification and sequence analysis of the insert. The verified IGFBP-rP1 insert was ligated into the BamHI site in the mammalian expression vector conferring neomycin resistance, LXSN. Two packaging cell lines were used to generate virus for the transduction of selected breast cancer cell lines.

August 1998-August 1999
The focus of this year's research has been to elucidate the mechanism by which IGFBP-rP1 inhibits cell growth in tumor cells. We studied two mass transduced cultures and two clones each for LXSN-transduced (control) and IGFBP-rP1-transduced MCF-7 breast cancer cells. MCF-7 breast cancer cells do not have endogenous IGFBP-rP1 expression. We tested these cell lines for RNA and protein expression, percent of cells undergoing apoptosis, senescence-associated β-galactosidase activity, cell cycle activity, and growth kinetics. We also transduced BT474 breast cancer cells with LXSN and IGFBP-rP1 to determine if growth was inhibited by IGFBP-rP1 in another breast cancer cell line also lacking endogenous IGFPB-rP1 expression. We have recently transduced a normal breast cell line, AG11132, with IGFBP-rP1 and LXSN. We are beginning to test the resulting cell lines in the same manner as we did previously for MCF-7. The goal of theses experiments are to determine if increased IGFBP-rP1 expression is an effector or product of senescence. In addition to growth curves, apoptosis, and senescence-associated β-galactosidase activity analyses, we will be looking for changes in Rb phosphorylation and p16 protein expression.

Results and Discussion

The focus of our studies in Aim 1 is directed at elucidating the functional role played by IGFBP-rP1 in breast epithelial cell proliferation. To test our hypothesis that IGFBP-rP1 plays an inhibitory role, we transduced breast cancer lines lacking endogenous IGFBP-rP1 expression with IGFBP-rP1.

We originally transduced MCF-7 breast cancer cells with LXSN (control) and IGFBP-rP1, generating clonal cell lines and heterogeneous cell lines (composed of thousands of clones and more representative of an *in vivo* model). Growth curve analysis revealed 38% decline in growth potential over a seven day period in the heterogeneous cultures. In the clonal cell lines, there was a 74% reduction in growth **(Figure 1)**. These results suggested that IGFBP-rP1 functions as a growth inhibitory protein.

We wished to identify the potential pathway responsible for the observed growth inhibition and determine if IGFBP-rP1 was affecting the growth potential by increasing the rate of apoptotic cell death or by other mechanisms, such as senescence or terminal differentiation. The nuclear fragmentation assay was performed to determine the percent of apoptotic cells in a given population. This is accomplished by staining cell nuclei with fluorescent DAPI and scoring the number of cells exhibiting fragmented nuclei, a hallmark of apoptosis. Our data indicates that there are no significant differences in percentage of cells exhibiting apoptosis **(Table 1)**. Therefore, it is unlikely that apoptotic or regulated cell death mechanisms are responsible for the diminution of breast cancer cells in culture.

In initial observations of the IGFBP-rP1-transduced MCF-7 breast cancer cells, we reported a change in morphology reminiscent of senescent cells (DAMD17-96-1-6247 Annual Report, September 1998). These cells were larger, flattened, having a larger cytoplasmic nuclear ratio than the LXSN (control) and parental (non-transduced) MCF-7 cells. These observed morphological changes have been reported in senescent cells (*7, 8*). Therefore, we tested the transduced cells for senescence-associated β-galactosidase staining using a protocol developed by Judith Campisi and collegues who determined that at pH 6, β-galactosidase activity was detectable in senescent cells, both *in vivo* and *in vitro* (*9*). Senescence-associated β-galactosidase activity is increased in IGFBP-rP1-transduced MCF-7 breast cancer cells **(Figure 2)**. We observed a 33% higher activity in the heterogeneous cell lines and a 41% increase in the clonal cell lines. From this data and the change in morphology to a senescent phenotype, we hypothesize that IGFBP-rP1 functions to inhibit growth by inducing changes associated with cellular senescence.

We transduced another breast cancer cell line, BT474, lacking endogenous IGFBP-rP1 expression. The purpose of this experiment was to determine if IGFBP-rP1 exhibited growth suppressing capabilities in another breast cancer cell line. In three growth curves using heterogeneous cell lines, IGFBP-rP1 inhibited growth between 15% and 54% in the transduced BT474 breast cancer cells **(Figure 3)**.

The next step is to distinquish if IGFBP-rP1 is acting as an inducer of senescence or is upregulated as a result of senescence in normal human mammary epithelial cells. To test for this, we have transduced AG11132, normal HMECs, at an early passage with LXSN (control) and IGFBP-rP1. We are currently in the process of testing these cells for changes in senescence-associated β-galactosidase activity, tumor suppressor gene $p16^{INK4A}$ (p16) and retinoblastoma protein (Rb) protein expression, and growth. Preliminary western immunoblot analysis has shown phosphorylated Rb protein, responsible for allowing cells to progress into S phase, to be decreased in IGFBP-rP1-transduced MCF-7 breast cancer cells **(Figure 7)**.

Aim 2: Experiments for promoter studies: library screening and clone analysis.

Previous Work/Background (August 1996-August 1998)

A human genomic library, enriched for chromosome 4, was screened and ten clones were isolated. Clones were analyzed by restriction digest, DNA-DNA hybridization of dot blots, PCR, and sequence analysis. PCR analysis using various

primers within the IGFBP-rP1 sequence and two primer surrounding the HindIII insert site suggested the presence of 5' region to the IGFBP-rP1 cDNA. Four clones showed promise in containing the promoter region.

August 1998-August 1999

We have continued our studies of trying to isolate and characterize the promoter region of IGFBP-rP1. To do this, we decided to clone the inserts isolated from our library screen into pBluescriptIIKS. This would allow easier manipulation of the cDNA for amplification and sequencing purposes. We have isolated two clones that contain the 5' end of IGFBP-rP1 and 3 putative clones that may also contain this region.

Results and Discussion

IGFBP-rP1 mRNA is upregulated in the presence of retinoic acid in normal human mammary epithelial cells (HMECs) (*5*). In a literature review of other members of the IGFBP superfamily (IGFBPs 1-6) as potential examples of how IGFBP-rP1 may be regulated, IGFBP-4 is upregulated and IGFBP-5 is downregulated in the presence of retinoic acid in human breast carcinoma cells (*10-13*). Location of the 5' transcription start site varies between 34 and 772 nucleotides upstream from the ATG translation initiation start codon. Four of the six IGFBPs lack the TATA consensus sequence, and four out of six IGFBPs contain G/C rich regions.

IGFBP-rP1 is homologous to prostacyclin-stimulating factor (PSF). The promoter for PSF has been isolated. The PSF promoter does not contain a TATA box and has many G/C rich regions, as well as a consensus sequence for Sp1 binding. We analyzed the reported sequence and found what may be a novel retinoic acid response element (RARE). Even though the PSF promoter has been cloned, we are continuing our search to isolate the IGFBP-rP1 promoter for the following reasons. First, the reported PSF promoter may not be complete. Second, we would like to verify the RARE to determine if it is indeed a novel response element. In general, RARE contain two specific sequences with 1-5 miscellaneous nucleotides in between them. The RARE found in the PSF promoter contains 6 miscellaneous nucleotides which are located between the two specific sequences. Third, we want to locate potential enhancer sites.

The cDNA inserts from the four clones determined by PCR analysis to potentially contain the promoter region were ligated into the pBluescriptIIKS plasmid vector and used to transform XL1-Blue *E.coli*. The resulting colonies were screened for successful ligation. Additional PCR screening was completed using primers specific for regions within the IGFBP-rP1 cDNA sequence. Automated sequencing was performed using primers flanking the cDNA insert site and a primer at the 5' end of IGFBP-rP1 in the direction of the promoter region. The results indicated that we had *E.coli.* sequence, suggesting contamination.

We decided to revisit the original ten λ phage clones obtained by the secondary screening of the genomic library. Southern Blot analysis was performed with λ phage from the ten clones. A 42 base pair probe from the 5' end of IGFBP-rP1 was used to detect inserts containing a portion of the IGFBP-rP1 cDNA **(Figure 4A)**. Signals indicative of successful hybridization were seen in clones 4 and 7. Faint signals were observed in clones 5, 6, and 8 **(Figure 4B)**.

The next steps will be to purify clones 4 and 7, ligate into pBluescriptIIKS, sequence analyzed, and search for transcription start sites and motifs for different binding sites. Once a promoter region has been successfully isolated, we will clone the sequence into a promoterless Luciferase reporter vector. We will test whole versus truncated versions to determine the minimal amount of promoter region necessary for activity. We will also test for potential response to regulators such as retinoic acid and estrogen.

Aim 3: Experiments to determine binding characteristics of IGFBP-rP1 using purified IGFBP-rP1 protein and a specific antibody.

Previous Work/Background (August 1996-August 1998)

We previously demonstrated 33 and 34 kDa IGFBP-rP1 proteins expressed by IGFBP-rP1 transduced MCF-7 cells. Hs578T breast cancer cells have endogenous IGFBP-rP1 (positive control) that is 31 kDa. Computer sequence analysis revealed the following motifs for potential modification: 4 casein kinase phosphorylation sites, 4 protein kinase C phosphorylation sites, 9 myristoylation sites, and one N-glycosylation sites.

August 1998-August 1999

This year's focus was directed at determining the potential post-translational modification suggest by the higher molecular weight proteins in the IGFBP-rP1 transduced MCF-7 cells **(Figure 5)**.

Results and Discussion

We transduced another ER+ breast cancer cell line, BT474, with IGFBP-rP1. Western analysis revealed the 33 and 34 kDa IGFBP-rP1 proteins expressed and not the 31 kDa protein.

The are several possible explanations behind the observed increase in secreted IGFBP-rP1 protein size in the ER+ breast cancer cell lines. The first explanation is that the protein is post-translationally modified in the ER+ cell. We ruled out N-glycosylation as the reason due to the fact that there is only one possible N-glycosylation site, and it has been previously demonstrated that the endogeneous IGFBP-rP1 expressed by Hs478T breast cancer cells is composed of a 27 kDa core protein and 4 kDa of N-linked sugars (*14*). Therefore, the higher molecular weight bands we are seeing are most likely have some other modification in addition to glycosylation. Myristoylation was also eliminated as a possibility because all known myristoylated proteins are modified at a site immediately adjacent to the amino terminal (*15-17*). There are no such myristoylation sites present in IGFBP-rP1. Because of the small difference in molecular weights (31 kDa vs 33/34 kDa), we focused our efforts on phosphorylation studies. The growth potentiating activities of IGFBP-1 and IGFBP-3 are regulated by the phosphorylation status of the protein (*18*). We hypothesized that the same regulation could be occurring with the IGFBP-rP1 protein. To test this hypothesis, we decided to phosphatase treat conditioned medium containing the secreted recombinant IGFBP-rP1 protein from MCF-7 breast cancer cells. If the protein were phosphorylated, we would

see a shift to a lower molecular weight protein by Western immunoblot. To date, two Westerns have shown the signal from higher molecular weight proteins to be diminished when treated with phosphatase **(Figure 6)**. However, we have not observed the emergence of a lower molecular weight protein. This may be a result of the dephosphorylated protein being sensitized to proteases secreted by the MCF-7 cells. We are currently working to determine if this is the case by phosphatase treating in the presence of various protease inhibitors. We have also successfully immunoprecipitated the IGFBP-rP1 protein and will be able to phosphatase treat the protein in a controlled buffer rather than the conditioned medium which contains many unknown factors.

Another possibility for the increase in molecular weight of the IGFBP-rP1 protein when secreted by ER+ cells may be that it is partially processed. There are several examples of proteins (all peptide hormone precursors) that are only partially processed by tumor cells. Several of these proteins are ACTH, calcitonin, and somatostatin (*19, 20*). Our collaborator, Roger Birnbaum, has also shown significant levels of these prohormones or processing intermediates when using tumor cell cultures (unpublished data).

Conclusion

We have demonstrated that IGFBP-rP1 inhibits growth of two breast cancer cell lines, MCF-7 and BT474. We have shown that this is independent of apoptotic mechanisms. We are currently revising a manuscript showing our findings, adding growth experiments that support the IGF-independent inhibitory function of IGFBP-rP1. This will be done by growing IGFBP-rP1-transduced MCF-7 cells in the presence of αIR3 antibody. αIR3 antibody neutralizes the IGF type-I receptor. Therefore, any increased growth suppression observed in the IGFBP-rP1-transduced MCF-7 compared with empty-vector transduced MCF-7 cells will unlikely be due to IGF inhibition.

Our data of the senescence-associated β-galactosidase assays and observed morphological changes in cell structure suggest that IGFBP-rP1 may inhibit function through senescent or senescent-like mechanisms in MCF-7 breast cancer cells. I am now focused on determining if IGFBP-rP1 is an effector of senescence or if its expression is increased as a result of senescence. We have added to our studies of breast cancer cells to normal human mammary epithelial cells (HMECs), AG11132 (Coriell). We have transduced early passage AG11132 with IGFBP-rP1 and will be testing the resulting cell strains for increase of levels of the following senescent markers: Rb, p16, and SA-β-galactosidase activity. AG11132 normal breast cells are well-defined in our laboratory. Examination of normal cells, in addition to our previous observations from tumor cells, will enable us to make more accurate predictions of the true function of IGFBP-rP1. Hopefully, we will be able to definitively answer if senescence is indeed the growth inhibitory mechanism used by IGFBP-rP1.

REFERENCES

1. Toropainen, E., Lipponen, P., and Syrjanen, K. (1995) Expression of insulin-like growth factor I (IGF-I) in female breast cancer as related to established prognostic factors and long-term prognosis. *Eur J Cancer* **9,** 1443-8.
2. Yu, H., Levesque, M. A., Khosravi, M. J., Papanastasiou-Diamandi, A., Clark, G. M., and Diamandis, E. P. (1996) Associations between insulin-like growth factors and their binding proteins and other prognostic indicators in breast cancer. *Br J Cancer* **74,** 1242-7.
3. Del Giudice, M. E., Fantus, I. G., Ezzat, S., McKeown-Eyssen, G., Page, D., and Goodwin, P. J. (1998) Insulin and related factors in premenopausal breast cancer risk. *Breast Cancer Res Treat* **47,** 111-20.
4. Hankinson, S. E., Willett, W. C., Colditz, G. A., Hunter, D. J., Michaud, D. S., Deroo, B., Rosner, B., Speizer, F. E., and Pollak, M. (1998) Circulating concentrations of insulin-like growth factor-I and risk of breast cancer [see comments]. *Lancet* **351,** 1393-6.
5. Swisshelm, K., Ryan, K., Tsuchiya, K., and Sager, R. (1995) Enhanced expression of an insulin growth factor-like binding protein (mac25) in senescent human mammary epithelial cells and induced expression with retinoic acid. *Proc Natl Acad Sci U S A* **92,** 4472-6.
6. Murphy, M., Pykett, M. J., Harnish, P., Zang, K. D., and George, D. L. (1993) Identification and characterization of genes differentially expressed in meningiomas. *Cell Growth Differ* **4,** 715-22.
7. Peacocke, M., and Campisi, J. (1991) Cellular senescence: a reflection of normal growth control, differentiation, or aging? *J Cell Biochem* **45,** 147-55.
8. Barrett, J. C., Annab, L. A., and Futreal, P. A. (1993) Genetic and molecular basis for cellular senescence. *Adv Exp Med Biol* **330,** 27-43.
9. Dimri, G. P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E. E., Linskens, M., Rubelj, I., Pereira-Smith, O., and et al. (1995) A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc Natl Acad Sci U S A* **92,** 9363-7.
10. Sheikh, M. S., Shao, Z. M., Hussain, A., Chen, J. C., Roberts, C. T., Jr., LeRoith, D., and Fontana, J. A. (1993) Retinoic acid and estrogen modulation of insulin-like growth factor binding protein-4 gene expression and the estrogen receptor status of human breast carcinoma cells. *Biochem Biophys Res Commun* **193,** 1232-8.
11. Sheikh, M. S., Shao, Z. M., Hussain, A., Clemmons, D. R., Chen, J. C., Roberts, C. T., Jr., LeRoith, D., and Fontana, J. A. (1993) Regulation of insulin-like growth factor-binding-protein-1, 2, 3, 4, 5, and 6: synthesis, secretion, and gene expression in estrogen receptor- negative human breast carcinoma cells. *J Cell Physiol* **155,** 556-67.
12. Adamo, M. L., Shao, Z. M., Lanau, F., Chen, J. C., Clemmons, D. R., Roberts, C. T., Jr., LeRoith, D., and Fontana, J. A. (1992) Insulin-like growth factor-I (IGF-I) and retinoic acid modulation of IGF-binding proteins (IGFBPs): IGFBP-2, -3, and -4 gene expression and protein secretion in a breast cancer cell line. *Endocrinology* **131,** 1858-66.

13. Shemer, J., Yaron, A., Werner, H., Shao, Z. M., Sheikh, M. S., Fontana, J. A., LeRoith, D., and Roberts, C. T., Jr. (1993) Regulation of insulin-like growth factor (IGF) binding protein-5 in the T47D human breast carcinoma cell line by IGF-I and retinoic acid. *J Clin Endocrinol Metab* **77,** 1246-50.
14. Oh, Y., Nagalla, S. R., Yamanaka, Y., Kim, H. S., Wilson, E., and Rosenfeld, R. G. (1996) Synthesis and characterization of insulin-like growth factor-binding protein (IGFBP)-7. Recombinant human mac25 protein specifically binds IGF-I and -II. *J Biol Chem* **271,** 30322-5.
15. Aitken, A., and Cohen, P. (1984) Identification of N-terminal myristyl blocking groups in proteins. *Methods Enzymol* **106,** 205-10.
16. Boutin, J. A. (1997) Myristoylation. *Cell Signal* **9,** 15-35.
17. Resh, M. D. (1996) Regulation of cellular signalling by fatty acid acylation and prenylation of signal transduction proteins. *Cell Signal* **8,** 403-12.
18. Clemmons, D. R. (1997) Insulin-like growth factor binding proteins and their role in controlling IGF actions. *Cytokine Growth Factor Rev* **8,** 45-62.
19. Cate, C. C., Pettengill, O. S., and Sorenson, G. D. (1986) Biosynthesis of procalcitonin in small cell carcinoma of the lung. *Cancer Res* **46,** 812-8.
20. Stewart, M. F., Crosby, S. R., Gibson, S., Twentyman, P. R., and White, A. (1989) Small cell lung cancer cell lines secrete predominantly ACTH precursor peptides not ACTH. *Br J Cancer* **60,** 20-4.
21. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular cloning, a laboratory manual.*, Vol. 1, 2 ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
22. Birnbaum, R. S., and Wiren, K. M. (1994) Changes in insulin-like growth factor-binding protein expression and secretion during the proliferation, differentiation, and mineralization of primary cultures of rat osteoblasts. *Endocrinology* **135,** 223-30.
23. Wilson, E. M., Oh, Y., and Rosenfeld, R. G. (1997) Generation and characterization of an IGFBP-7 antibody: identification of 31kD IGFBP-7 in human biological fluids and Hs578T human breast cancer conditioned media. *J Clin Endocrinol Metab* **82,** 1301-3.
24. FitzGerald, M. J., Webber, E. M., Donovan, J. R., and Fausto, N. (1995) Rapid DNA binding by nuclear factor kappa B in hepatocytes at the start of liver regeneration. *Cell Growth Differ* **6,** 417-27.

RESEARCH ACCOMPLISHMENTS (August 1998-August 1999):

Aim 1. IGFBP-rP1 inhibits growth in breast epithelial cells.

- MCF-7 breast cancer cells transduced with IGFBP-rP1 were analyzed for cell growth curves, beta-galactosidase activity, flow cytometry, and nuclear fragmentation analyses. BT474 cells were subjected to cell growth curves.
- Growth curves show that growth is suppressed in the presence of IGFBP-rP1 in both MCF-7 and BT474 breast cancer cells.
- Senescence-associated β-galactosidase activity is increased in the rP1-transduced MCF-7 cells and there is no change in rate of apoptosis according to nuclear fragmentation assay results. It is likely that IGFBP-rP1 is using a senescent or senescent-like pathway to inhibit growth. This is further supported by the alteration in morphology of the rP1-transduced MCF-7 which have a large, flattened appearance with a large cytoplasmic to nuclear ratio, characteristic of senescent cells.
- Flow cytometry results have not yielded conclusive results.
- Response to various growth factors have not yielded conclusive results.
- Transduced AG11132 (normal HMECs) with empty-vector and IGFBP-rP1.

Aim 2. Experiments for promoter studies: library screening and clone analysis.

- Two clones isolated from genomic library screen enriched for chromosome 4 contain 42 base pairs located at the 5' end of IGFBP-rP1. Another three clones may also contain this region.

Aim 3. IGFBP-rP1 is post-translationally modified in ER+ breast cancer cells.

- Transduced rP1 protein was detected using Western immunoblot analysis and has been successfully immunoprecipitated with anti-rP1 Ab.
- Higher MW bands are observed in MCF-7 and BT474 IGFBP-rP1 transduced breast cancer cells.
- Glycosylation and phosphorylation studies have been undertaken to determine type of post-translational modification. It is unlikely that the upper bands are due to glycosylation, because computer analysis reveals one putative glycosylation site. The 31kDa IGFBP-rP1 secreted by Hs578T breast cancer cells is formed of a 27 kDa core protein and 4 kDa of N-linked sugars (*14*).
- Treatment with calf intestinal phosphatase results in decreased signal of the upper molecular weight proteins, but no presence of a lower 31 kDa protein. My hypothesis is that dephosphorylation of the rP1 protein makes it susceptible to proteolytic degradation by proteases secreted by the MCF-7 cells. Our next step will be to phosphatase treat the immunoprecipitated IGFBP-rP1 protein.

REPORTABLE OUTCOMES

Oral Presentation

Introduction of IGFBP-rP1/mac25 into MCF-7 breast cancer cells induces growth inhibition and senescent morphology. H-M.P.Wilson, R.S.Birnbaum, M.Poot, and K.Swisshelm. 81st Annual Meeting of The Endocrine Society, San Diego USA, June 12-15, 1999. *(HW was awarded a travel grant to present this work orally)*

Papers

K.L.Haugk, H-M.P.Wilson, K.Swisshelm, and L.S.Quinn. IGF Binding Protein-Related Protein 1: An Autocrine/Paracrine Factor Which Inhibits Skeletal Myoblast Differentiation In Response to IGF. *(in press, Endocrinology)*

H-M.P.Wilson, R.S.Birnbaum, M.Poot, and K.Swisshelm. Retroviral introduction of the insulin-like growth factor binding protein-related protein 1 (IGFBP-rP1) in MCF-7 breast cancer cells inhibits proliferation. *(in preparation for resubmission)*

MATERIAL AND METHODS

Cell Growth Curves.

MCF-7 cells containing LXSN and L*IGFBP-rP1*SN were plated on 60-mm tissue culture plates at 50,000cells/plates and grown in complete αMEM with 0.75 mg/ml G418. Duplicate plates were trypsinized and cells counted on days 1, 2, 3, 5, and 7 by a hemocytometer.

Nuclear fragmentation Assay as a parameter for apoptosis.

Transduced and parental MCF-7 cells (50,000) were innoculated in 60-mm tissue culture dishes in complete αMEM. After seven days, cells were harvested by trypsinization. To avoid loss of cells, the conditioned medium, PBS wash, cells and trypsin, and the complete αMEM used to rinse the plates were combined and pelleted by centrifugation. The cells were resuspended and fixed in freshly prepared methanol:acetic acid (3:1) and placed at -20°C for a minimum of 24 hours. Fixed cells were repelleted, resuspended in freshly prepared methanol:acetic acid (3:1), applied to ethanol-cleaned glass slides and stained with 20 μM Hoechst 33258 (in PBS) for 30 minutes. Nuclei were analyzed on a Zeiss fluorescent microscope using standard DAPI excitation and emission filters.

Senescence-associated β-galactosidase Activity

Transduced MCF-7 cells were plated at 25,000 cells/p35 tissue culture dishes in duplicates and grown for 3 days in complete αMEM. On day 3, cells were washed twice with PBS and fixed with 2% formaldehyde, 0.2% glutaradehyde in PBS. Cells were rinsed twice with PBS. Stain solution containing X-gal was overlaid onto cells and incubated at 37°C overnight. The cells were rinsed twice with H_2O the following day. Approximately 500 cells were counted from each plate and percent of cells exhibiting blue stain, indicative of senescence-associated β-galactosidase activity, were scored.

Statistics

Significance for the following assays was determined using the Student's T-test non-paired, two-tailed analysis: growth curve analysis, apoptotic assays, and SA-β-galactosidase activity.

Purification of lambda DNA

Lambda DNA clones were amplified by large scale phage lysates following the protocol described (*21*). The amplified λ DNA was isolated using a Lambda DNA purification kit (Qiagen).

Southern analysis of λ DNA clones

Lambda DNA was run on a 0.8% agarose gel. The gel was denatured in 0.5N NaOH, 1M NaCl, rinsed with distilled H_2O, and neutralized with 1.5M Tris, pH 7.4, 3M NaCl. The λ DNA fragments were transferred to Zeta Blot membrane (Bio-Rad, Ca) by

capillary transfer. The blot was hybridized with ^{32}P-labeled probe made from 42bp at the 5' end of IGFBP-rP1 cDNA.

Immunodetection of secreted IGFBP-rP1 protein.

Cells were plated at 500,000 cells per 35-mm tissue culture dish in complete αMEM. On day 5, cells were washed once with PBS and fed 2 ml SFM. Twenty-four hours later (day 6), 750μl of conditioned medium was collected and immediately concentrated on nitrocellulose (*22*). The concentrated protein was eluted in 25ul of 1x sample buffer (0.5M Tris, 10% glycerol, 8M urea, and 2% SDS). Samples were boiled for 10 minutes and 20 μl was separated on a 15% polyacrylamide gel with a 4% polyacrylamide stacker. The gel was run at 100 volts for 30 minutes and 185 volts for 2 hours. Proteins were transferred onto Immuno-Blot PVDF membrane (Bio-rad) at 100 volts for 1 hour in transfer buffer (39mM glycine, 48mM Tris, 0.04% (w/v) SDS, 20% MeOH). The membrane was incubated with 10% hydrogen peroxide for 10 minutes, blocked 1 hour in 1%BSA/0.5% blotting-grade nonfat dry milk in 0.05% Tween-20/Tris Buffered Saline (TTBS), and incubated overnight at 4°C with anti-hRIGFBP-rP1 (*23*) at 1:2500 in 1%BSA/0.5% milk in TTBS. Blots were washed in TTBS and incubated for 3 hours with anti-rabbit Ig horseradish peroxidase linked whole antibody from donkey at 1:3000 in 1%BSA/0.5% nonfat dry milk in TTBS (Amersham Life Science). The IGFBP-rP1 protein was detected with Supersignal Chemiluminescent Substrate (Pierce, Rockford, IL) and CL-Xposure Film (Pierce).

Phosphorylation Studies

750μl of conditioned medium was collected, as described above. 20μM ethanolamine and 4μl calf intestinal phosphatase (Roche Molecular Biochemicals) was added. The conditioned medium was then incubated at 37°C for 1 hour, concentrated to nitrocellulose, and western immunoblotted as described above.

Nuclear Extracts

Cells were grown on two p150 tissue culture plates in completed αMEM medium and harvested at 80% confluency. Nuclear extracts were isolated using a protocol previously described (*24*).

Immunodetection of total Rb protein

10 μg of total protein from nuclear extracts was separated on a 15% polyacrylamide gel, transferred to PVDF immunoblot overnight at 4°C at 30 volts, and blocked with 1% BSA/4% blotting grade non-fat milk in TTBS for 1 hour at RT. The blot was incubated with 1μg Rb antibody/ml blocker for 3 hours at RT, washed with TTBS three times, and incubated with 1:2000 dilution of goat anti-mouse IgG horseradish peroxidase for 1 hour at RT. The Rb protein was detected with Supersignal Chemiluminescent Substrate (Pierce, IL) and CL-Xposure Film (Pierce).

FIGURE LEGENDS
Figures on pages 21-28

Figure 1. *IGFBP-rP1-transduced MCF-7 cells have diminished proliferation.* MCF-7 breast cancer cells were plated in duplicates at 50,000 cells/p60 in completed αMEM medium and counted on days 1, 3, 5, and 7. The histogram represents the mean and the bars represent the deviation from the mean of day 7 cumulative cell number.

Figure 2. *Senescence-associated β-galactosidase activity is higher in IGFBP-rP1-transduced MCF-7 breast cancer cells.* MCF-7 breast cancer cells were plated in duplicates at 25,000 cells/p35 in completed αMEM medium. Cells were fixed and stained on Day 2, as described in Material and Methods. Approximately 500 cells were counted from each plate to determine the percentage of cells stained blue.

Figure 3. *IGFBP-rP1-transduced BT474 breast cancer cells demonstrate inhibited growth.* BT474 breast cancer cells were plated in duplicates at 50,000 cells/p60 in completed αMEM and counted on days 1, 3, 5, and 7. The histogram represents the mean and the bars represent the deviation from the mean of day 7 cumulative cell number.

Figure 4. *Two clones demonstrate hybridization with ^{32}P-labeled 5' IGFBP-rP1 probe.* A. Probe diagram. A 42 base pair fragment, located at the 5' end of IGFBP-rP1, was used to generate ^{32}P-labeled probe. B. Southern blot. Southern blot analysis reveals clones 4 and 7 to show strong hybridization with the 42 base pair fragment. Three addition clones (clones 5, 6, and 8) show weaker hybridization.

Figure 5. *Western analysis reveals higher molecular weight IGFBP-rP1 proteins are secreted in the ER+ breast cancer cell lines MCF-7 and BT474.* Hs578T, transduced-MCF-7, and transduced- BT474 cells were grown to 90% confluency and incubated with serum-free medium overnight. 750μl of conditioned medium was collected, concentrated, separated by PAGE, and transferred to PVDF membrane. IGFBP-rP1 protein was detected by Western immunoblot using anti-rP1 antibody. ER- Hs578T breast cancer cells secrete endogenous IGFBP-rP1 with a molecular weight of 31 kDa. The IGFBP-rP1-transduced MCF-7 and BT474 breast cancer cell lines secrete IGFBP-rP1 proteins with molecular weights of approximately 33 and 34 kDa.

Figure 6. *33/34 kDa IGFBP-rP1 signal is decreased when treated with calf intestinal phosphatase.* Hs578T and transduced MCF-7 cells were grown to 90% confluency and incubated with serum-free medium overnight. 750μl of conditioned medium was collected and incubated with calf intestinal phosphatase for 1 hour at 37°C. The conditioned medium was concentrated, separated by PAGE, and transferred to PVDF membrane, and detected using anti-rP1 antibody. Hs578T and LXSN-transduced MCF-7 cells were used as controls. IGFBP-rP1 signal was decreased in transduced-MCF-7 breast cancer cells in various conditions in the presence of phosphatase (denoted by *).

Figure 7. *Phosphorylated Rb protein is decreased in IGFBP-rP1-transduced MCF-7 breast cancer cells.* Cells were grown to 80% confluency in completed αMEM medium and nuclear extracts collected. 10μg total protein was separated on a 15% polyacrylamide gel and transferred to PVDF immunoblot. Total Rb protein was detected using anti-Rb antibody (PharMingen).

Table 1. *IGFBP-rP1 does not increase apoptosis in MCF-7 breast cancer cells.* MCF-7 breast cancer cells were plated in duplicates at 50,000 cells/p60 in completed αMEM medium. Conditioned medium, PBS wash, and trypsinized cells were harvested on day 7, fixed overnight, plated on glass slides, and stained with Hoechst. 500 cells were counted to determine the percentage of cells having fragmented nuclei, a hallmark of apoptosis.

STATEMENT OF WORK

Technical Objective 1
Test the potential of IGFBP-rP1 function.

Task 1: (Months 1-4) Prepare plasmids for transfection. Ligate full-length IGFBP-rP1 cDNA into retroviral vector. *Completed*

Task 2: (Months 5-12) Transfection of packaging cell lines and transduction of breast cancer cell lines, select clones and begin to test growth parameters. *Completed*

Task 3: (Months 15-24) Complete growth studies, demonstrating that IGFBP-rP1 inhibits growth in breast cancer cells. *Completed – paper for resubmission, oral presentation given*

Task 4: (Months 24-30) Test for mechanism behind growth suppression in transduced-breast cancer cell lines (apoptosis vs. senescence). *Completed*

Task 5: (Months 36-40) Test the function of IGFBP-rP1 in normal human mammary epithelial cells and analyze transduced-AG11132 normal cells for alterations in growth and markers of senescence. *In Progress*

Technical Objective 2
Characterize the mac25 promoter.

Task 1: (Months 4-7) Finish screening genomic libraries for IGFBP-rP1 promoter. *Completed*

Task 2: (Months 12-15, 24-32) Restriction and sequence analysis of promoter clones. *In Progress*

Technical Objective 3
Determine protein binding characteristics of mac25.

Task 1: (Months 1-12) Test antiserum generated mac25 peptide by Elisa. Have access to an antibody generated in another lab that has been successfully used in our lab. *Completed*

Task 2: (Months 12-28) Perform immunoblots for normal HMECs and breast tumor cell lines. *Completed*

Task 3: (Months 15-24) Determine ligand interaction with IGFBP-rP1. *Completed*

Task 4: (Months 28-36) Determine modification of higher molecular weight IGFBP-rP1 proteins secreted by transduced-ER+ breast cancer cells. *In Progress*

Technical Objective 4 (Additional)
Determine IGFBP-rP1 tissue specificity of RNA.

Task 1: (Months 5-12) Test mRNA expression localization in various mouse tissue. *Completed – abstract presented*

INTRODUCTION OF IGFBP-RP1/MAC25 INTO MCF-7 BREAST CANCER CELLS INDUCES GROWTH INHIBITION AND SENESCENT MORPHOLOGY. Heather-Marie P. Wilson, Roger S. Birnbaum, Martin Poot, and Karen Swisshelm. Department of Pathology, University of Washington, Seattle, WA 98195.

Insulin-like growth factor binding proteins (IGFBPs) may modulate growth of cultured human breast cancer cells by regulating the availability of insulin-like growth factors (IGFs). IGF binding proteins with lower affinity for IGFs are members of a related family, the IGFBP-rPs. Our studies of IGFBP-rP1 gene (mac25/TAF/PSF) suggest an additional role for the protein as IGFBP-rP1 expression is upregulated in senescent human mammary epithelial cells (HMECs). By retroviral transduction, we introduced the IGFBP-rP1 cDNA into MCF-7 breast cancer cells, which lack detectable endogenous IGFBP-rP1 expression. We observed a reduction of growth of 40% and 74% in pooled and clonal cell lines transduced with IGFBP-rP1, respectively, compared to cells transduced with the LXSN vector alone or the parental MCF-7 cell line. IGFBP-rP1-positive MCF-7 cells exhibited an altered morphology, from a pleomorphic, epitheliod cobblestone cell shape to an enlarged and flattened shape similar to senescent HMECs. Consistent with the morphologic change, we detected senescence associated-dependent β-galactosidase activity in the range of 60-85% of the cells. Our experiments suggest that IGFBP-rPs may facilitate potent replicative inhibitory function in mammary epithelial cells.

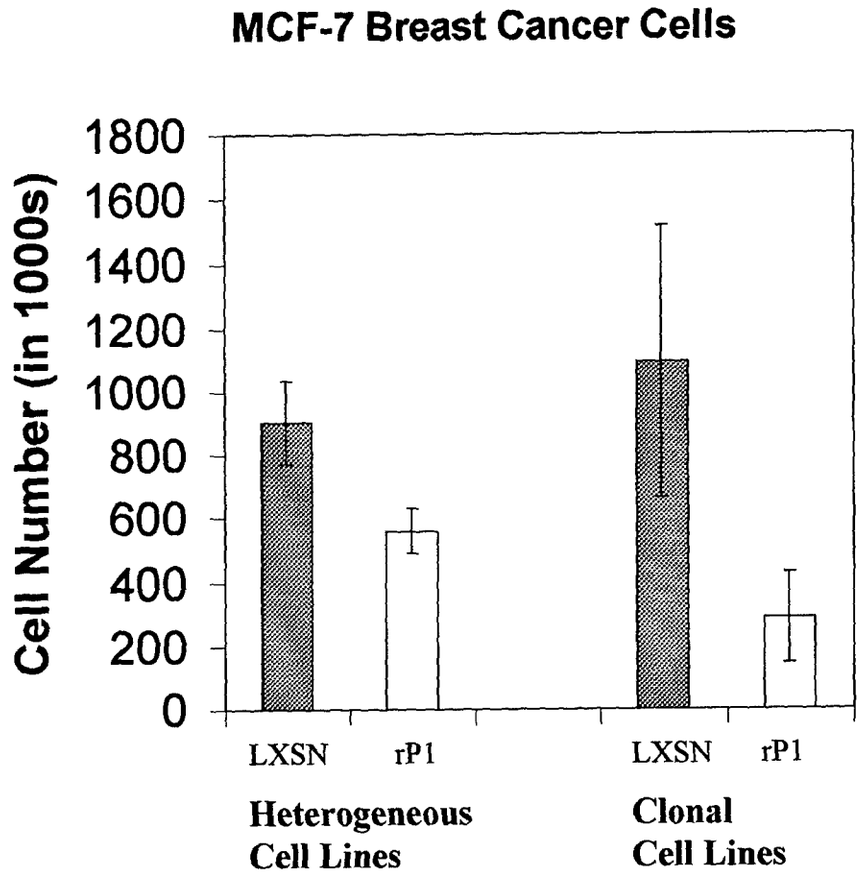

MCF-7 Breast Cancer Cells
Cell Number (in 1000s)
1800
1600
1400
1200
1000
800
600
400
200
0
LXSN
rP1
LXSN
rP1
Heterogeneous Cell Lines
Clonal Cell Lines

Figure 1.

Percentage of MCF-7 Cells with β-galactosidase Activity

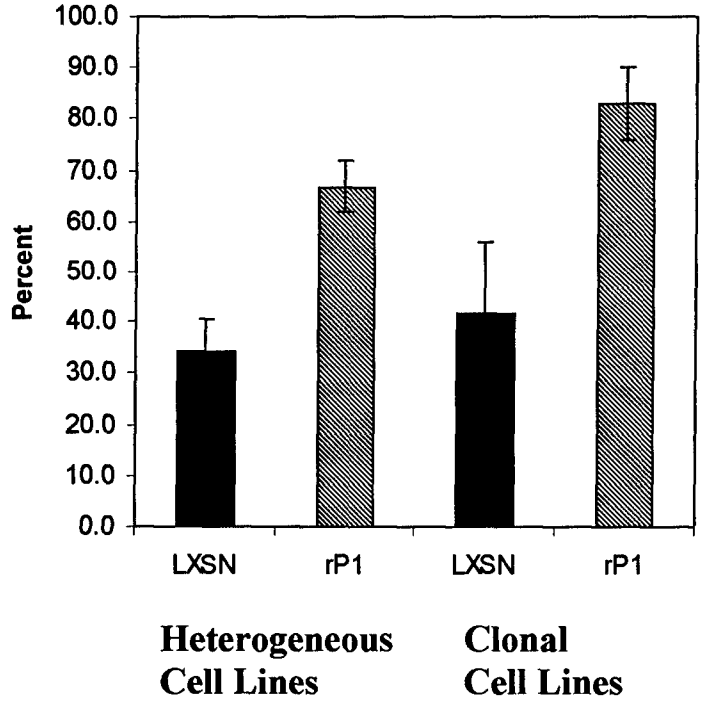


Figure 2.

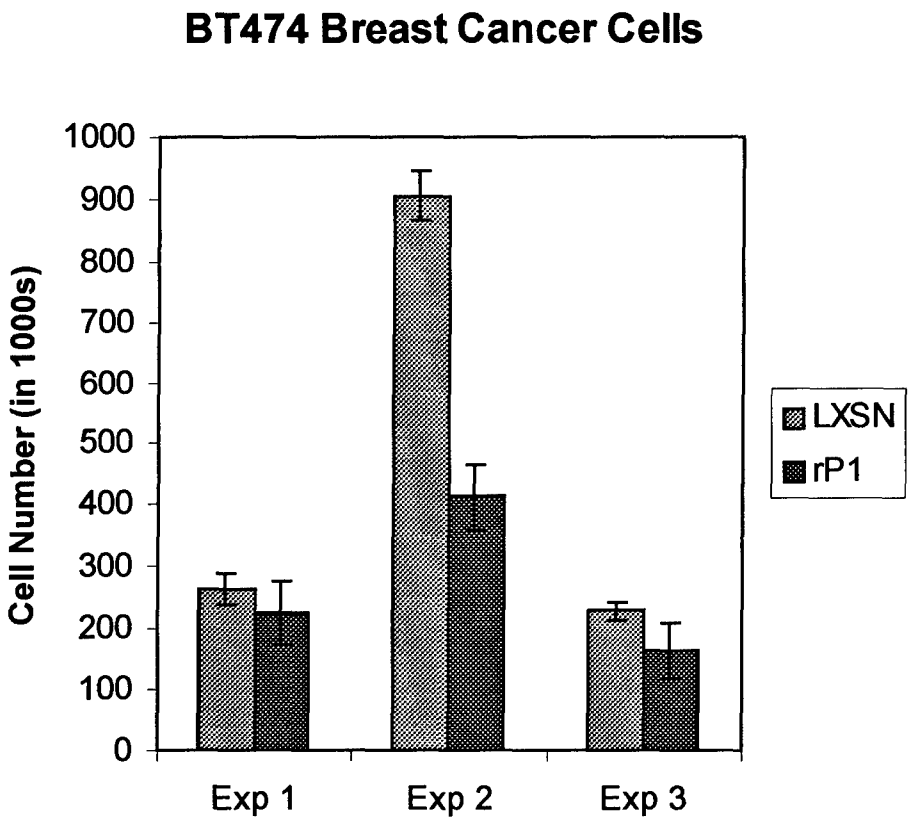
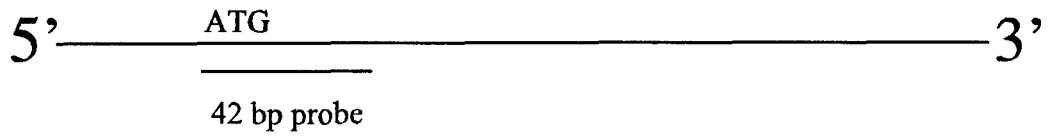

BT474 Breast Cancer Cells
Cell Number (in 1000s)
1000
900
800
700
600
500
400
300
200
100
0
Exp 1
Exp 2
Exp 3
LXSN
rP1

Figure 3.

Figure 4.

A. IGFBP-rP1 fragment used as probe.



B.

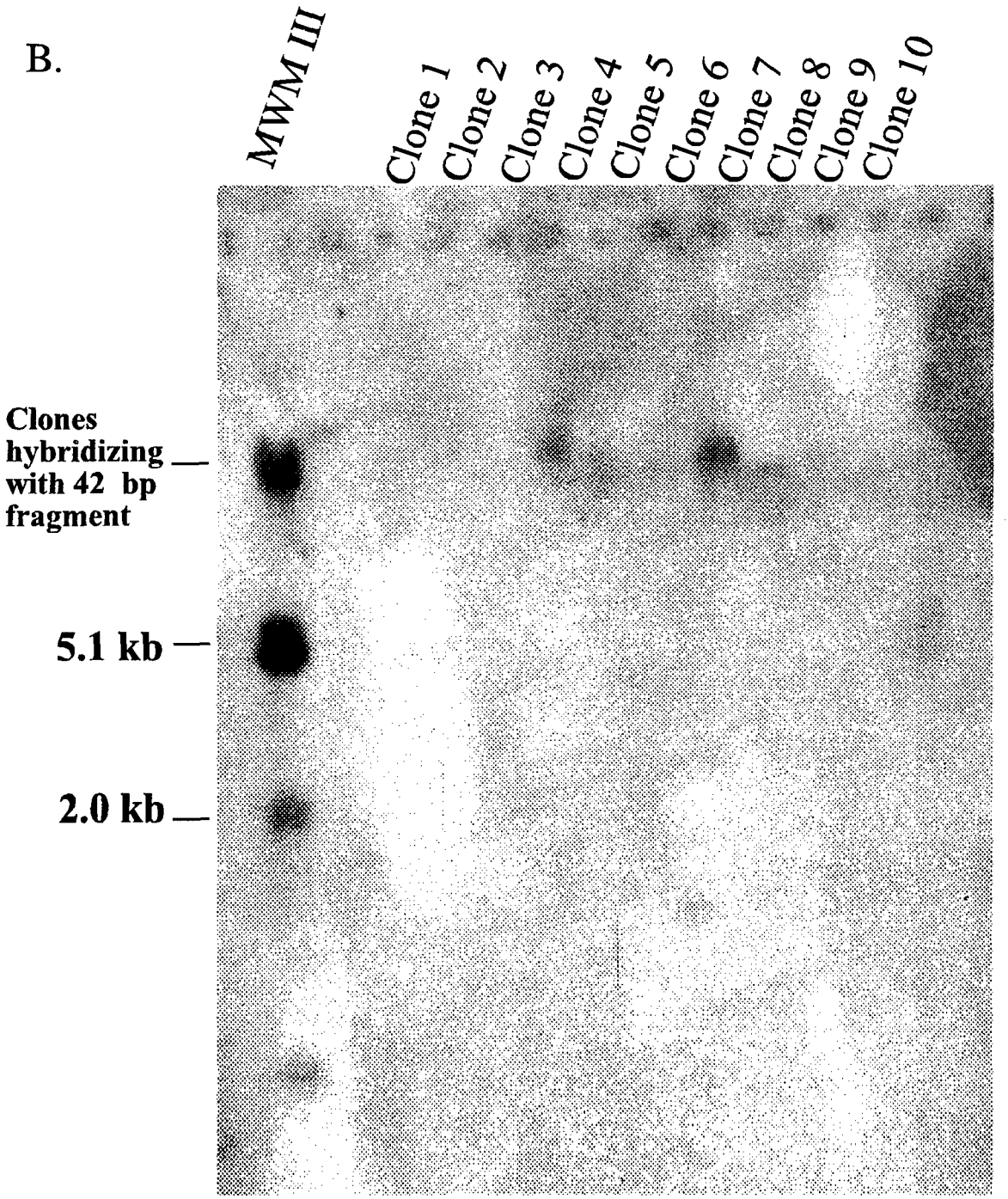


Figure 5.

31 kDa
Hs578T
BT474 (non-transduced)
BT474 LXSN
BT474 IGFBP-rP1
34 kDa
33 kDa
MCF-7 (non-transduced)
MCF-7 LXSN, T1
MCF-7 LXSN, T2
MCF-7 LXSN, cl.2
MCF-7 LXSN, cl.6
MCF-7 IGFBP-rP1, T1
MCF-7 IGFBP-rP1, T2
MCF-7 IGFBP-rP1, cl.9
MCF-7 IGFBP-rP1, cl.10

Figure 6.

Hs578T
LXSN, cl.2 RT
LXSN, cl.2 37C
IGFBP-rP1, cl.9 RT
IGFBP-rP1, cl.9 37C
*
IGFBP-rP1, cl.9 37C
IGFBP-rP1, cl.9 37C + Ethanolamine
*
IGFBP-rP1, cl.9 37C + Ethanolamine
IGFBP-rP1, cl.9 RT
IGFBP-rP1, cl.9 37C
IGFBP-rP1, cl.9 37C, pH 9.5
*
IGFBP-rP1, cl.9 37C, pH 9.5
**
IGFBP-rP1, cl.9 37C, pH 9.5
*
IGFBP-rP1, cl.9 37C, pH 9.5 + Ethanolamine
**
IGFBP-rP1, cl.9 37C, pH 9.5 + Ethanolamine
LXSN, cl.2 RT
LXSN, cl.2 37C

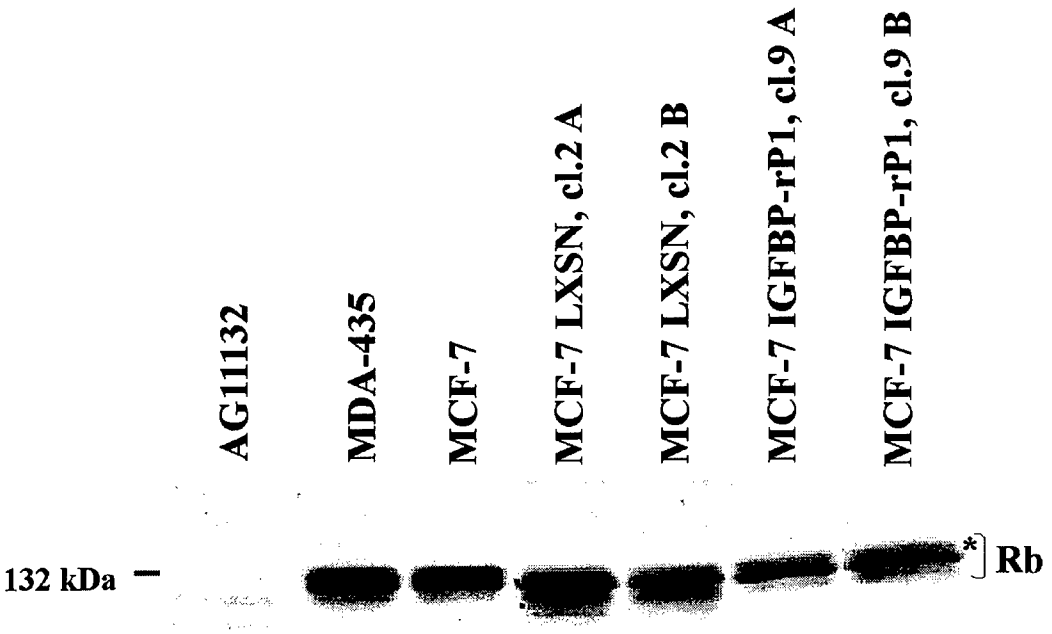

AG11132
MDA-435
MCF-7
MCF-7 LXSN, cl.2 A
MCF-7 LXSN, cl.2 B
MCF-7 IGFBP-rP1, cl.9 A
MCF-7 IGFBP-rP1, cl.9 B
132 kDa
*
Rb

Figure 7.

Table 1. Percentage of cells with nuclear fragmentation

Parental	**1.5%**
Heterogeneous Cell Lines	
LXSN	**1.2%**
IGFBP-rP1	**0.9%**
Clonal Cell Lines	
LXSN	**0.7%**
IGFBP-rP1	**0.9%**

Accepted for publication by Endocrinology

IGF Binding Protein-Related Protein 1: An Autocrine/Paracrine Factor Which Inhibits Skeletal Myoblast Differentiation But Permits Proliferation in Response to IGF.

Kathleen L. Haugk, Heather-Marie P. Wilson, Karen Swisshelm, and LeBris S. Quinn

Abstract

Skeletal myogenic cells respond to the insulin-like growth factors (IGF-I and IGF-II) by differentiating or proliferating, which are mutually exclusive pathways. What determines which of these responses to IGF skeletal myoblast undergo is unclear. IGF binding protein-related protein 1 (IGFBP-rP1) is a secreted protein with close homology to the IGF binding proteins (IGFBPs) in the N-terminal region. IGFBP-rP1, previously called mac25 and IGFBP-7, is highly expressed in C2 skeletal myoblasts during the proliferative phase, but downregulated during myoblast differentiation. To determine the role of IGFBP-rP1 in myogenesis, IGFBP-rP1 was overexpressed in C2 myoblasts using a retroviral vector. Western blots indicated the resulting C2-rP1 myblasts secreted approximately 27-fold higher levels of IGFBP-rP1 than control C2-LX myoblasts which were transduced with a control vector (LXSN). Compared to C2-LX myoblasts, the differentiation responses of C2-rP1 myoblasts to IGF-I, IGF-II, insulin and des(1-3)IGF-I were significantly reduced ($p<0.05$). However, proliferation responses of C2-rP1 and C2-LX myoblasts to these same factors were not significantly different. Exposure of control C2-LX myoblasts to factors secreted by C2-rP1 myoblasts using a transwell co-culture system reduced C2-LX myoblasts differentiation significantly ($p<0.05$). Experiments with the mitogen-activated protein kinase (MAPK) kinase (MEK) inhibitor PD098059 suggested that IGFBP-rP1 inhibits a MAPK-dependent differentiation pathway. In confirmation of this idea, levels of phosphorylated ERK-2 (extracellular signal-regulated kinase-2, a MAPK) were reduced in C2-rP1 myoblasts compared to C2-LX myoblasts. These findings indicate that IGFBP-rP1 may function as an autocrine/paracrine factor which specifies the proliferative response to the IGFs in myogenesis.

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 23 Aug 01

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2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management

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