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THE INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN BP-25 IS EXPRESSED BY HUMAN BREAST CANCER CELLS

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Specific binding proteins are thought to modulate the effects of IGF-I. Previous work has demonstrated that media conditioned by human breast cancer cells contains IGF-I binding activity. Radiolabelled IGF-I incubated with serum-free conditioned media from the breast cancer cell line MDA-MB 231 eluted with an apparent M.W. of 35-40 kDa when analyzed by gel filtration chromatography at pH 7.4. The M.W. of this binding activity corresponded to that of BP-25, a binding protein cloned from the hepatocellular carcinoma cell line HepG2. Two breast cancer cell lines, MDA-MB 231 and Hs578T, were found to express BP-25 RNA. Specific BP-25 radioimmunoassay detected BP-25 production in the conditioned media of these two cell lines. Immunoprecipitation confirmed that metabolically labelled MDA-MB 231 released 30 kDa BP-25 into its medium. This study demonstrates that some breast cancer cells express the IGF-I binding protein, BP-25.

IGF-I is a mediator of the effects of growth hormone and a mitogen for both normal and malignant cell lines. These mitogenic effects are thought to be a result of the interaction between IGF-I and the type I IGF receptor, however IGF-I is also associated with specific serum and extracellular binding proteins that may also modulate the biological actions of IGF-I(1).

We have previously shown that IGF-I is a potent mitogen for human breast cancer cell lines and these cells produce an IGF-I related protein that is associated with a binding

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Abbreviations used: Insulin-like growth factor I-IGF-I, Molecular weight-M.W., Radioimmunoassay-RIA, Sodium dodecyl sulfate-SDS, Polyacrylamide gel electrophoresis-PAGE, Improved minimal essential media-IMEM, Phosphate buffered saline-PBS

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activity (2). Using gel filtration chromatography, we have found that the binding activity produced by the breast cancer cell line MDA-MB 231 has an apparent M.W. of 28-33 kDa. Since a cDNA for a low molecular weight IGF-I binding protein (BP-25) has been recently isolated from both placenta and the human hepatocellular carcinoma cell line HepG2 (3, 4, 5), we have examined breast cancer cell line RNA for BP-25 expression. Additionally, we have examined serum-free media conditioned by breast cancer cells for BP-25 protein production by RIA and immunoprecipitation with a specific BP-25 antiserum.

Materials and Methods

Cell lines

The cell line MCF-7 was obtained from the Michigan Cancer Foundation, Detroit MI. 172 is a normal mammary epithelial cell line derived from reduction mammoplasty tissue and was a gift from Martha Stampfer, Lawrence Berkeley Laboratories, Berkeley, CA. All other cell lines were obtained from American Type Culture Collection, Rockville, MD. Cancer cell lines were maintained in IMEM (Biofluids, Rockville, MD) with 2mM glutamine, 0.05 mg/ml gentamicin and 10% fetal calf serum (Gibco, Detroit, MI). 172 was maintained as previously described (6).

RNA was obtained using the guanadinium thiocyanate method (7).

Binding studies

Serum-free conditioned media was obtained as previously described (8). The conditioned media was concentrated 100 fold by ultrafiltration using an Amicon filter with a 5 kDa M.W. cutoff.

The conditioned medium was filtered on a 1.5cm x 85cm Sephadex G-100 column, equilibrated with 1x PBS pH 7.4, at a flow rate of 15 ml/hr. 2ml fractions were collected and 1 ml aliquots were measured on an LKB 1275 gamma counter.

IGF-I binding activity was assayed by the charcoal separation method as previously described (9).

Northern analysis and RNAse protection assay

A 970 bp BamHI fragment of the BP-25 cDNA (3) was subcloned into a pGem vector (Promega, Madison, WI). This fragment was random primed labelled and used as a probe for Northern analysis. For RNAse protection assays antisense RNA probes were transcribed and labelled with ^{32}P UTP according to the instructions of the manufacturer. Northern blots utilizing 10μg of total RNA were performed as previously described (10). Final washing was done in .1X SSC, 0.1% SDS at 65°C for 15 minutes.

RNAse protection assays were performed as previously described(11). Briefly, 30μg of total RNA was hybridized with probe in an 80% formamide (vol/vol) buffer. Samples were hybridized for 12-16 hours at 50°C followed by digestion with RNAse A. The samples were precipitated with 1 μg tRNA and 2 volumes of absolute ethanol. The pellets were lyophilized then resuspended in an 80% formamide loading buffer. The samples were run on a 6% polyacrylamide gel with 8M urea. pBR322 was digested and endlabelled with ^{32}P dCTP and used as size standards. The dried gels were exposed to X-ray film in the presence of a Quanta III (Dupont, Wilmington, DE) intensifying screen.

Radioimmunoassay of conditioned media

Cells (2 x 10^7) were placed in T-75 flasks (Costar, Cambridge, MA) and grown in IMEM with 10% fetal calf serum. When the cells approached confluence, 10 ml of serum-free conditioned media was collected per flask and lyophillized as described (8).

BP-25 levels in the conditioned media samples were analyzed by RIA using standards and tracer prepared from purified HepG2 cell-derived BP-25 (9) and a rabbit polyclonal antiserum raised against the same protein (12). The specificity of this RIA for BP-25 has been validated in separate experiments (P.D.K. Lee, manuscript in preparation). Briefly, lyophilized samples were reconstituted to 0.5x original volume in RIA buffer (PBS, pH 7.4, 0.1% BSA). 100μl of standard or sample was incubated with 100μl of

1:1000 dilutions of antiserum for 1 hour at room temperature, followed by a 16 hour incubation with 5-10K cpm/tube ^{125}I-BP-25 at 4°C. Bound counts were separated using agarose-immobilized goat anti-rabbit immunoglobulin (Biorad, Richmond, CA). Samples were assayed in duplicate and after serial dilution. Data were analyzed using the IBM-PC Data Reduction Program (M.L. Jaffee and Assoc., Silver Spring, MD).

Metabolic Labelling and Immunoprecipitation of BP-25 protein

In vitro labeling of proteins was performed using ^{35}S cysteine and ^{35}S methionine as previously described (13). ^{35}S labelled proteins released in the conditioned medium from MDA-MB 231, HepG2, and MCF-7 were immunoprecipitated with 10µg of BP-25 antiserum as previously described(14). Precipitates were separated by electrophoresis on 10% SDS/PAGE gels, and subsequently autoradiographed. Pre-labelled M.W. markers (Biorad) were run in parallel lanes.

Results and Discussion: Radioiodinated IGF-I (1ng - specific activity 2000 Ci/mmol) was incubated with 1ml of 100x serum-free concentrated media conditioned by the breast cancer cell line MDA-MB 231. Gel filtration chromatography on the Sephadex G-100 column at pH 7.4 shows that labelled IGF-I incubated with conditioned media eluted with an apparent M.W. of 35-40 kDa, while labelled IGF-I alone eluted at the expected M.W of 7.5 kDa(Fig 1). Addition of excess unlabelled IGF-I (10 µg) could partially displace

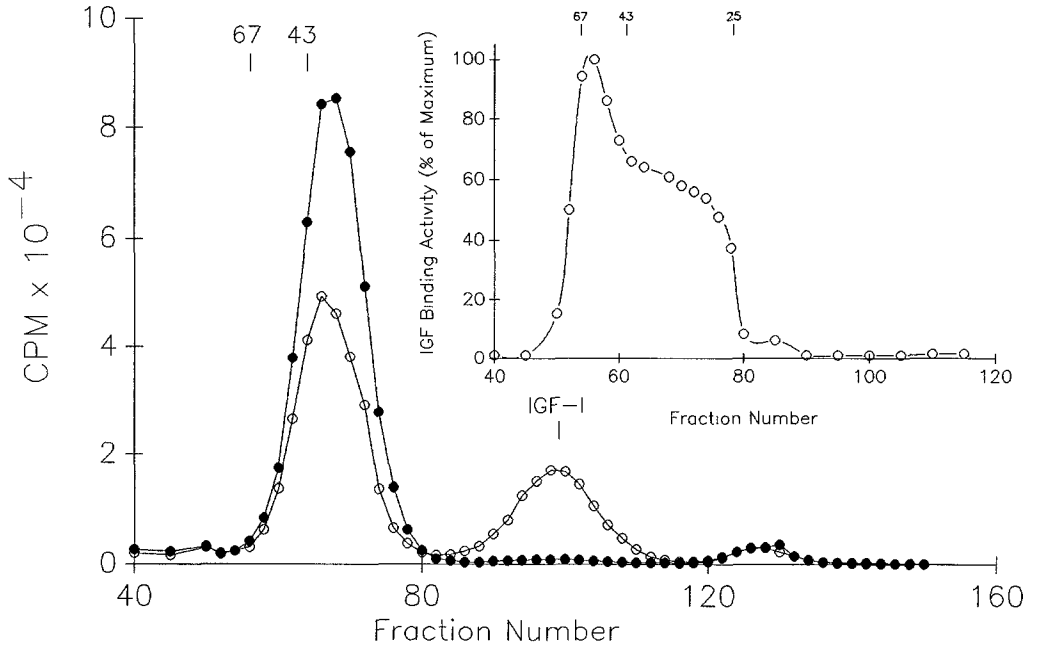

Figure 1-Gel filtration of binding protein-radiolabelled IGF-I complex. 1 ml of 100x concentrated conditioned media from the cell line MDA-MB 231 was incubated with labelled IGF-I at 4°C overnight and chromatographed at pH 7.4 with Sephadex G-100. Closed circles represent labelled IGF-I and conditioned media. Open circles represent labelled IGF-I, conditioned media plus 10µg unlabelled IGF-I. The positions of protein standards 67 kDa bovine serum albumin, 43 kDa ovalbumin and labelled IGF-I alone are marked. Inset: 100x concentrated conditioned media from cell line MDA-MB 231 was run on the same column, with eluted fractions tested for IGF binding protein activity by charcoal binding assay. The positions of the protein standards are noted.

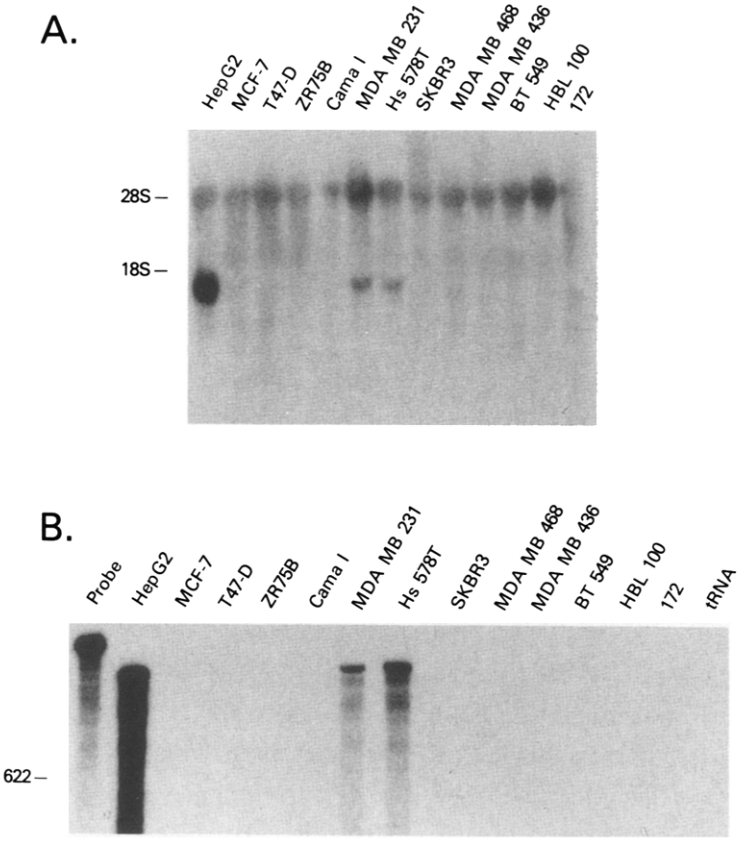

Figure 2-Northern analysis and RNAse protection assay of breast cancer cell lines using BP-25 probe. (A) Northern analysis of 10µg RNA per lane. Migration of 28S and 18S ribosomal RNA is noted. (B) RNAse protection with 30 µg of RNA per lane. Position of the 622bp pBR322 MspI fragment is noted.

the bound labelled IGF-I(Fig 1). When the M.W. of IGF-I was subtracted, the binding activity had a M.W. of 28-33 kDa. Furthermore, when the charcoal IGF-I binding protein assay was used to examine MDA-MB 231 conditioned media size fractionated by neutral gel filtration chromatography, the binding activity was found over a broad M.W. range from 67 to 25 kDa(Fig 1, inset). These data suggest that at least one 28-33 kDA species of binding protein is produced by the cell line MDA-MB 231, but larger molecular weight binding proteins may be produced as well.

Because the binding activity produced by MDA-MB 231 corresponds in size to that of the cloned IGF-I binding protein BP-25 (3), we next examined breast cancer cell lines for transcription of the BP-25 gene. Northern analysis (Fig 2A) demonstrated a 1.65 kb band in HepG2, MDA-MB 231, and Hs578T. In addition, a larger band that co-migrated with 28S ribosomal RNA was detected in all cell lines. A larger transcript has been described in HepG2 and it has been proposed that it could represent differential splicing of the BP-25 mRNA (3). However, this larger band could also represent cross hybridization between 28S ribosomal RNA and the BP-25 probe. To address these

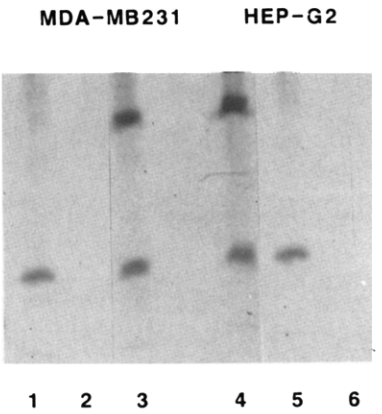

Figure 3-Immunoprecipitation of metabolically labelled conditioned media from HepG2 and MDA-MB 231 with normal rabbit serum (lanes 2 and 6), BP-25 antiserum (lanes 3 and 4), and BP-25 antiserum with 50µg of purified unlabelled BP-25 (lanes 1 and 5). M.W. was determined by comparison of protein migration with M.W. standards. The upper bands in lanes 3 and 4 are ~30kDa, the lower bands in lanes 1, 3, 4, and 5 are 10-15kDa.

possibilities, we used the RNAse protection assay which will only detect RNAs that are exactly complementary to the transcribed probe. When the cell line RNAs were examined with the 970bp probe, only HepG2, MDA-MB 231, and Hs578T were found to express BP-25 mRNA. No partially protected fragments were found in any of the other cell lines. Therefore it is unlikely that the larger band is a transcription product of the BP-25 gene, but rather represents cross hybridization between 28S ribosomal RNA and the BP-25 probe. The possibility that other closely related binding protein RNA species are transcribed by breast cancer cells has not been excluded, however.

Consistent with the RNA data, BP-25 was detected by RIA only in the conditioned media of confluent MDA-MB 231 (6 ng/ml) and in Hs578T (394 ng/ml). In comparison, adult human serum contained 15.6 ng/ml BP-25 while MCF-7 and T47D had insignificant levels (< 1ng/ml) detected. Although production rates were not assessed, it is clear that under similar *in vitro* conditions both Hs578T and MDA-MB 231 express BP-25 while other breast cancer cell lines do not.

Immunoprecipitation of metabolically labelled proteins released into the conditioned media by HepG2 and MDA-MB 231 cells with antiserum directed against BP-25 demonstrated a 30 kDa M.W. protein species (Fig 3, lanes 3 and 4, upper band). The antibody did not precipitate any specific proteins produced by MCF-7, consistent with the finding that this cell line does not express BP-25 mRNA (Fig 2A, 2B, and data not shown). No specific bands were immunoprecipitated with normal rabbit serum (Fig 3, lanes 2 and 6). When 50µg of purified unlabelled BP-25 was incubated with labelled HepG2 or MDA-MB 231 conditioned media, the 30kDa band was eliminated (Fig 3, lanes 1 and 5). Although the nucleotide sequence of BP-25 predicts a 25 kDa protein, expression of the BP-25 gene in *E. coli* or COS-1 cells has yielded proteins of 30-31 kDa M.W. suggesting that BP-25 is subject to post-translational modification (4,5). Therefore,

the size of the protein detected in breast cancer cells agrees with that of the expressed gene product. This suggests that authentic BP-25 is produced by these cells.

A 10-15 kDa protein was also identified by immunoprecitation of HepG2 and MDA-MB 231 conditioned media (Fig. 3, lanes 1,3,4, and 5, lower band) and unlabelled BP-25 did not compete with this smaller protein (Fig 3, lanes 1 and 5). BP-25 and other smaller M.W. IGF-I binding proteins have homologous NH_2 terminal amino acid sequences (14) and these smaller binding proteins could potentially cross react with the polyclonal BP-25 antiserum. Alternatively, the smaller M.W. protein could represent another unidentified protein that binds to BP-25.

This study demonstrates that some human breast cancer cell lines express BP-25. By cross linking conditioned media with labelled IGF-I, DeLeon *et al* have identified 45, 36, and 29 kDa IGF-I binding protein species in MDA-MB 231 (15). MCF-7 did not contain the 29 kDa binding protein. Since MDA-MB 231 expresses BP-25 mRNA and protein while MCF-7 does not, it appears that the 29 kDa protein identified by cross linking and by immunoprecipitation is BP-25. Immunoprecipitation of MDA-MB 231 conditioned media did not identify any of the higher M.W. species binding proteins. This suggests that the larger binding proteins represents either a different species of IGF-I binding protein or a multimeric form of BP-25. However, since MCF-7 produces the 45 and 36 kDa binding proteins (14) yet expresses no BP-25, it is unlikely that these higher molecular weight species are a result of post-translational modification of BP-25. The function and relative abundance of the higher M.W. binding proteins produced by breast cancer cells has not yet been fully defined. It is likely that an interaction between these different binding proteins, IGF-I, and the type I IGF receptor modulate the mitogenic effect of IGF-I.

The binding protein purified from amniotic fluid is identical to BP-25 and has been shown to enhance the mitogenic effects of IGF-I in human fibroblasts (16). Since IGF-I is a potent mitogen for breast cancer cells, expression of BP-25 could be important in regulating the growth of breast cancer.

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