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# Differential binding and internalization of insulin-like growth factor (IGF)-I in cultured human trophoblast and JEG-3 cells: possible modulatory effect of IGF binding proteins (BP)

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**The present study was undertaken to characterize and identify the insulin-like growth factor binding proteins (IGF BPs) secreted by placental cells and their possible modulatory effect on IGF-I binding to cell surface receptors. The experimental approach taken was comparative characterization of binding and internalization of IGF-I and its analog, [Gln$^3$, Ala$^4$, Tyr$^{15}$, Leu$^{16}$ (QAYL)]IGF-I, with reduced affinity for IGF BP, in two different placental cell culture models. One was human placental trophoblast in primary culture and the other, JEG-3 cells, a human choriocarcinoma cell line, representing placental trophoblasts. Binding of [$^{125}$I]IGF-I in both trophoblast and JEG-3 cells was time and temperature dependent. At 37°C, the plateau of [$^{125}$I]IGF-I binding to both the cells (1-2% specific binding per $10^5$ cells) was reached by 40-60 min. At 4°C, the time required to reach the plateau in both cells was increased to ~4 h. The maximum binding of [$^{125}$I]IGF-I to trophoblasts, however, was ~2 times higher than at 37°C, whereas in JEG-3 cells binding remained the same. Internalization of [$^{125}$I]IGF-I in trophoblast cells was low and temperature independent. At both 37 and 4°C, ≤ 30% of the total cell-associated [$^{125}$I]IGF-I was internalized. In contrast, internalization of [$^{125}$I]IGF-I in JEG-3 cells was rapid and temperature dependent. At 37°C, ≥ 60% of the total cell-associated [$^{125}$I]IGF-I was internalized by 40-60 min. At 4°C, internalization was slow and did not exceed 10% of the total cell-associated radioactivity. Binding of [$^{125}$I-QAYL]IGF-I to trophoblasts, in comparison to [$^{125}$I]IGF-I, was significantly different. The binding was undetectable at 37°C and it was low at 4°C. In JEG-3 cells, however, the binding and internalization of [$^{125}$I]-QAYL]IGF-I at both the temperatures were comparable to that of [$^{125}$I]IGF-I. Further characterization of the two [$^{125}$I]IGF-I bindings to the different placental cells was achieved by binding competition studies using unlabelled IGF-I, [QAYL]IGF-I and [Leu]IGF-I, another analog of IGF-I, [Leu$^{24}$, 1-62]IGF-I with reduced affinity for the IGF-I receptor, and α-IR3, a monoclonal antibody to the IGF-I receptor. The different potencies of IGF-I and its analogs, and α-IR3 in competing binding of two [$^{125}$I]IGF-Is in the different cells suggested that binding of IGF-I to JEG-3 cells was predominantly to IGF-I receptor, whereas to trophoblast cells it was to IGF BP. This was confirmed by affinity cross-linking studies. The major affinity cross-linked [$^{125}$I]IGF-I complex in trophoblast cells was shown to be a protein of Mr. ~43 kDa, corresponding to IGF BP-3. In JEG-3 cells, the major cross-linked [$^{125}$I]IGF-I and -[QAYL]IGF-I complexes were proteins of Mr ~130 kDa and >260 kDa, corresponding to the monomeric and multimeric forms of IGF-I receptor. The ~43 kDa complex in trophoblast was confirmed to be IGF BP-3 by identification of the characteristics of the IGF BP secreted by trophoblast by Western ligand and immunoblots of the conditioned media. JEG-3 cells did not secrete IGF BP. In conclusion, the membrane associated IGF BP-3 in trophoblast cells, shown here, imply an *in vivo* modulatory effect of membrane bound IGF BP-3 on IGF-I action in placenta. JEG-3 cells, not secreting IGF-BP, offer an attractive model to study the interactive mechanism of IGF-I and IGF BP-3 actions on the placenta.**

**Keywords:** Binding; internalization; IGF-I; JEG-3 cell; trophoblasts

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## Introduction

The placenta contains receptors for various growth factors (GFs), i.e., insulin-like growth factor (IGF)-I, insulin, epidermal growth factor and others (Armstrong *et al.*, 1983; Bala *et al.*, 1982; Hock & Hollenberg, 1980). The regulatory control of these GFs on the placenta may influence fetal growth. Recently, we have shown that IGF-I (one of two major IGFs) stimulates cellular differentiation (Bhaumick *et al.*, 1992) and placental lactogen secretion (Bhaumick and Bala, 1991) by human placental trophoblast cells in culture. This suggested that IGF-I has a regulatory role in placenta acting via its receptor.

In many IGF-I responsive cells, IGF-I also binds membrane-associated IGF binding proteins (BPs) secreted by these cells. These IGF BPs, by regulating IGF availability to these receptors, may in turn modulate IGF action. There are multiple forms of tissue and cell specific IGF BPs, identified as IGF BP-1 to -6 (Reviewed by Baxter, 1991; Drop *et al.*, 1991; Shimasaki *et al.*, 1991), that exist in soluble and membrane-associated forms (Clemmons *et al.*, 1986; Conover and Powell, 1991; McCusker *et al.*, 1990, 1991). Placental specific IGF BPs and their possible modulatory effects on IGF action have not yet been studied. The pregnancy specific protein pp 12, identified as IGF BP-1, was suggested to be a placental specific protein. Later, however, it was shown that pp 12/IGF BP-1 is synthesized by secretory/decidualized endometrium (reviewed by Drop *et al.*, 1991). More recently, Fant (1990), using the placental explant system, showed that the BP produced from placental tissue comes from fibroblast rather than trophoblasts. Ritvos *et al.* (1988), using JEG-3 cells, a human choriocarcinoma cell line, representing differentiated placental trophoblasts, also reported that these cells do not produce IGF BP. In context of these studies, it could be suggested that placental trophoblasts do not produce IGF BPs and IGF-I action on the placenta may not be directly affected by the placental cell produced IGF BP. However, preliminary studies in our laboratory, investigating the binding and internalization of IGF-I in primary cultured trophoblast cells, obtained from human term placenta, suggested that the binding of IGF-I to its receptor in these cells may be confounded by the presence of cell associated IGF BP. The present study was undertaken to investigate the possible presence of IGF BP on trophoblast membrane and its secretion by the cells. The experimental design planned was a detailed comparative characterization of binding and internalization of IGF-I and its analog [Gln$^3$, Ala$^4$, Tyr$^{15}$, Leu$^{16}$ (QAYL)]IGF-I, with reduced affinity for IGF BP (Bayne *et al.*, 1988), in primary trophoblast culture and JEG-3 cells. The results, as discussed below, led to the identification and characterization of IGF BP secretion by cultured trophoblasts and confirmed the absence of its production by JEG-3 cells.

## Results

### *Binding and Internalization of [$^{125}$I]IGF-I and -[QAYL]IGF-I*

The comparison of [$^{125}$I]IGF-I binding and internalization in trophoblast and JEG-3 cells is shown in Figure 1. [$^{125}$I]IGF-I

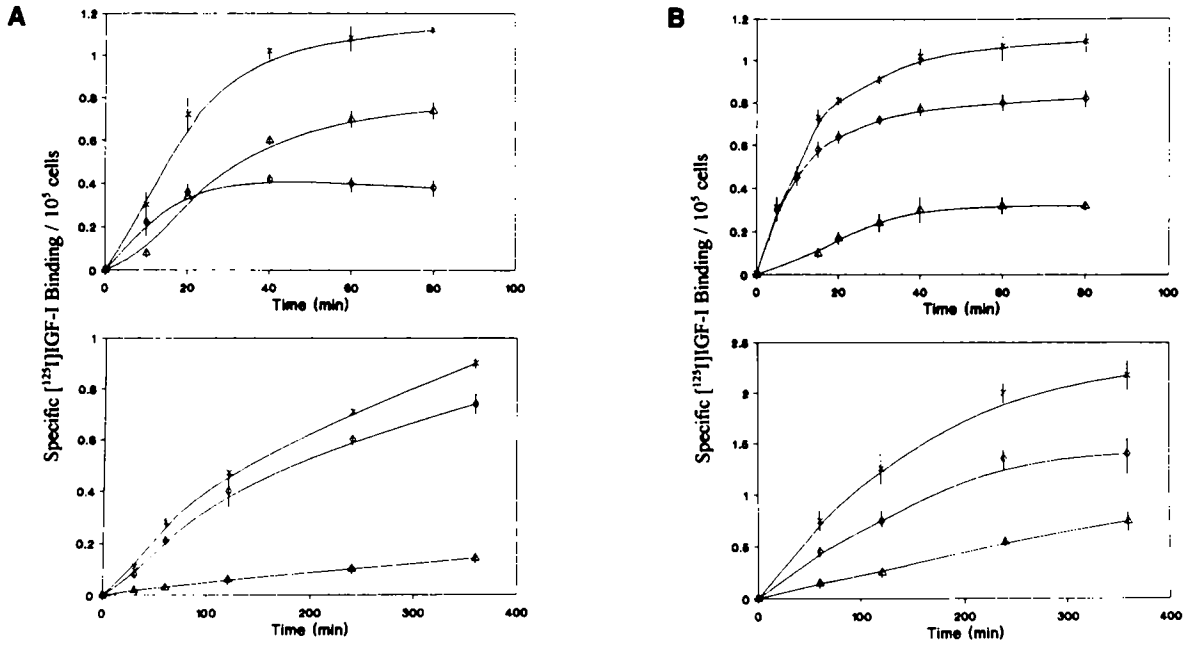


**Figure 1** Binding and internalization of [$^{125}$I]IGF-I in JEG-3 (A) and trophoblast (B) cells. The cells were incubated with [$^{125}$I]IGF-I at 37°C (upper panels) or 4°C (lower panels), as described in the Materials and methods. The total cell-associated (total binding, X), acid extractable (surface bound, ◇) and non-extractable (internalized, △) radioactivity was determined at different time points. The data, corrected for non-specific cell-associated radioactivity, are expressed as a percent of the total radioactivity added to each binding reaction. The points represent the mean ± SD of three experiments done in triplicate.

binding to trophoblast and JEG-3 cells at 37°C was comparable. Binding reached saturation in both cell types by 40 min, with a maximum total binding of ~1% per $10^5$ cells. At 4°C, the time required to reach saturation of [$^{125}$I]IGF-I binding in both cells increased to ~4 h. The total maximum binding to JEG-3 cells (1.1 ± 0.08% per $10^5$ cells) remained same as at 37°C (1.5 ± 0.5% per $10^5$ cells), whereas the maximum total binding to trophoblasts (2.2 ± 0.5% per $10^5$ cells) was ~2 times ($p < 0.05$) higher than that at 37°C (1.1 ± 0.3% per $10^5$ cells). The two cell types revealed that [$^{125}$I]IGF-I internalization in trophoblasts was low, ≤ 30% of the total cell-associated radioactivity, and not temperature dependent. Internalization of [$^{125}$I]IGF-I in JEG-3 cells, however, was temperature-dependent. A rapid internalization was seen at 37°C, reaching a plateau of 60% of the total cell-associated radioactivity by 60 min. At 4°C, the internalization was significantly ($p < 0.05$) lower and did not exceed 10% of the total cell-associated radioactivity.

The binding of [$^{125}$I-QAYL]IGF-I to trophoblast, in contrast to [$^{125}$I]IGF-I, was undetectable at 37°C and was significantly ($p < 0.01$) lower at 4°C. The maximum binding of [$^{125}$I-QAYL]IGF-I to trophoblasts was 0.84 ± 0.2% per $10^5$ cells, as shown in Figure 2. This was ~62% lower than the binding of [$^{125}$I]IGF-I to trophoblast at the same temperature. In JEG-3 cells, however, as shown in Figure 3, binding and internalization of [$^{125}$I-QAYL]IGF-I were comparable to that of [$^{125}$I]IGF-I in these cells. The binding of [$^{125}$IQAYL]IGF-I to JEG-3 cells at 37°C reached a plateau, 0.7 ± 0.2% per $10^5$ cells, by 40 min and approximately 60% of this was rapidly internalized, reaching a plateau by 80 min. At 4°C, similar to [$^{125}$I]IGF-I, the time to reach the plateau of [$^{125}$I-QAYL]IGF-I binding was increased to 4 h, but unlike [$^{125}$I]IGF-I, the maximum binding remained the same (0.9 ± 0.1% per $10^5$ cells). Also, similar to [$^{125}$I]IGF-I, internalization at 4°C was lower and did not exceed 10% of the total cell-associated radioactivity.

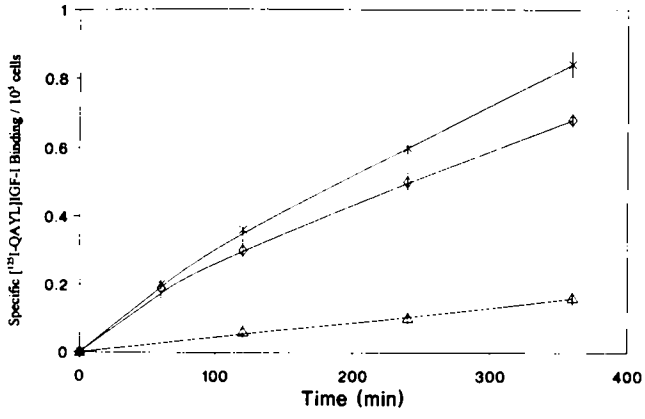


**Figure 2** Binding of [$^{125}$I-QAYL]IGF-I to trophoblasts. The cells were incubated with [$^{125}$I-QAYL]IGF-I at 4°C and the total cell-associated (total binding, X), acid extractable (surface bound, ◇) and non-extractable (internalized, △) radioactivity was determined at different time points. The data are expressed as described in Figure 1. The points represent mean ± SD of three experiments done in triplicate.

*Binding competition and affinity cross-linking studies*

Next we compared the binding characteristics of the two IGF-Is in the different cell types by competitive binding and affinity cross-linking studies. Competitive binding studies were performed using [$^{125}$I]IGF-I or -[QAYL]IGF-I as radioligands and unlabelled IGF-I, [QAYL]IGF-I, [Leu]IGF-I, an other analog of IGF-I with reduced affinity for IGF-I receptor (Caseieri *et al.*, 1988) and α-IR3, a monoclonal antibody to IGF-I receptor, as inhibitors. The binding of [$^{125}$I]IGF-I to trophoblasts at 37°C was inhibited by IGF-I and its analogs in the order IGF-I > [Leu]IGF-I > [QAYL] IGF-I (Figure 4, lower panel). α-IR3 did not inhibit binding. At 4°C (Figure 4, upper panel), the potency of IGF-I to inhibit [$^{125}$I]IGF-I binding was significantly ($p < 0.01$) higher than at 37°C, as reflected by the differences in $IC_{50}$ (the 50% inhibition of binding at 4°C (0.3 ± 0.1 ng/ml) and at 37°C (3 ± 1 ng/ml). Additionally, the order of potency of inhibition by [Leu]IGF-I and [QAYL]IGF-I was reversed ([Leu]-IGF-I < [QAYL]IGF-I) and α-IR3 inhibited the binding by

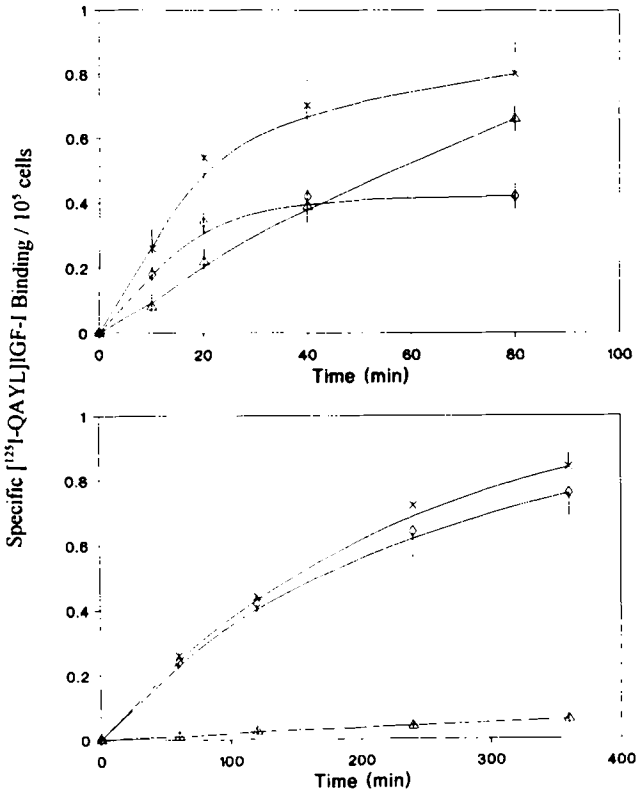


**Figure 3** Binding and internalization of [QAYL]IGF-I in JEG-3 cells. JEG-3 cells were incubated with radiolabelled [QAYL]IGF-I at 37°C (upper panel) or 4°C (lower panel) as described in the Materials and methods. The total cell-associated (total binding, X), acid extractable (surface bound, ◇) and non-extractable (internalized, Δ) radioactivity was determined at different time points. The data are expressed as described in Figures 1 and 2. The points represent mean ± 0.5 range of two experiments done in triplicate.

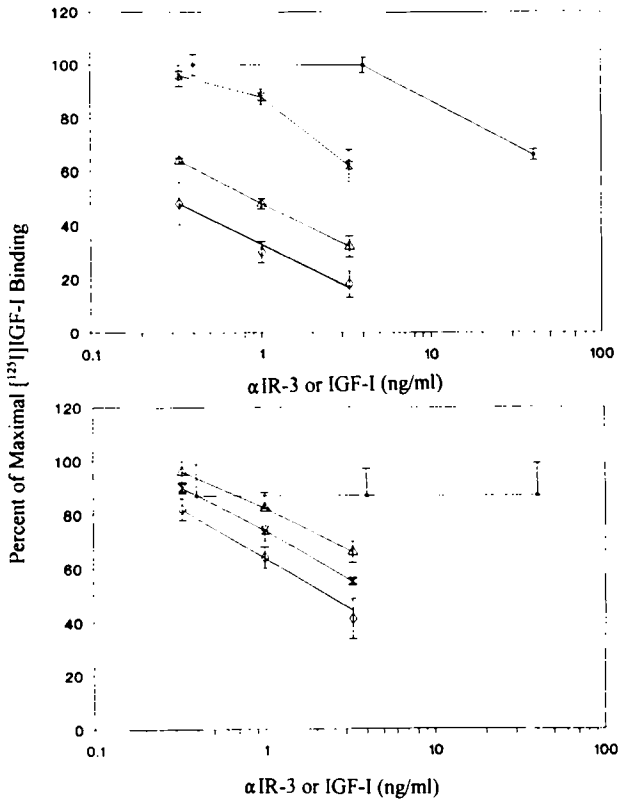


**Figure 4** Competition of [$^{125}$I]IGF-I binding to trophoblast cells. Trophoblasts were incubated with [$^{125}$I]IGF-I in the presence and absence of unlabelled IGF-I (◇), [QAYL]IGF-I (Δ), [Leu]IGF-I (X), or α-IR3 (■) at 37°C (lower panel) and 4°C (upper panel) in binding buffer as described in the Materials and methods. Results are expressed as a percentage of the radiolabelled IGF-I specifically (total binding minus non-specific binding) bound to the cells in the absence of unlabelled hormone (maximal specific binding). The points representing all the IGFs are mean ± SD of three experiments done in triplicate. The points representing α-1R3 are mean ± 0.5 range of two experiments done in duplicate.

~30% at the highest (30 ng/ml) of the three concentrations tested. Binding of [$^{125}$I-QAYL]IGF-I (Figure 5), performed only at 4°C, since its binding at 37°C was undetectable, was competed by IGF-I and [QAYL]IGF-I with equal potency. [Leu]IGF-I did not compete and α-IR3 completely inhibited the binding (equalled to nonspecific binding) at all three concentrations tested.

Figure 6 shows the binding competition of [$^{125}$I]labelled-IGF-I and -[QAYL]IGF-I to JEG-3 cells at 4°C. Similar to [$^{125}$I-QAYL]IGF-I in trophoblast cells, bindings of both the [$^{125}$I]IGF-Is to JEG-3 cells were equipotently competed by IGF-I and [QAYL]IGF-I. [Leu]IGF-I did not compete and α-IR3 inhibited the binding effectively. Similar observations were made at 37°C (results not shown).

In the affinity cross-linking studies, [$^{125}$I]IGF-I binding to both trophoblasts and JEG-3 cells was cross-linked, whereas due to low [$^{125}$I-QAYL]IGF-I binding to trophoblasts, cross-linking was only performed in JEG-3 cells. As shown in Figure 7A, the predominant labelling of both [$^{125}$I]IGF-I or [$^{125}$I-QAYL]IGF-I to JEG-3 cells was at ~130 kDa and to higher $M_r$ species, >220 kDa. In contrast, the predominant labelling of [$^{125}$I]IGF-I in trophoblasts (Figure 7B) was to a 45 kDa species and no labelling around 130 kDa or high $M_r$ species was observed. This suggested that the binding of [$^{125}$I]IGF-I to JEG-3 cells is mainly to α-subunit and the possible non-reduced α/β dimer of the IGF-I receptor as previously shown by us in the placenta and the kidney (Bhaumick & Bala, 1987; 1985) and by others in JEG-3 cells (Ritvos *et al.*, 1988). In trophoblast, however, the binding of IGF-I was predominantly to a low molecular weight non-receptor protein with an apparent $M_r$ of 45 kDa.

*Identification and characterization of IGF BP secreted by trophoblasts and JEG-3 cells*

Next we investigated the IGF BP secretion by the cells. Conditioned media from both cells were separated by SDS-PAGE electrophoresis and were subjected to Western ligand and immunoblotting. The ligand blot shown in Figure 8 (lane a) revealed the presence of [$^{125}$I]IGF-I binding activity in the trophoblast conditioned medium with apparent $M_r$ of 37 and 40 kDa. This suggested that the trophoblast secrete essentially one IGF BP corresponding to the $M_r$ of doublet IGF BP-3, 38/40 kDa. In contrast, JEG-3 cells, revealed no [$^{125}$I]IGF-I binding activity (Figure 8, lane b) in conditioned medium indicating that these cells do not secrete IGF BP. Identification of the trophoblast secreted IGF BP to IGF BP-3 was confirmed by immunoblot analysis using α-IGF BP-1, -2a-3 antisera. As shown in Figure 8, lane c, the trophoblast secreted protein immunoreacted with α-IGF BP-3 and migrated to a $M_r$ of ~38–40 kDa doublet. The antisera, α-IGF BP-1 or -2 did not show any immunoreactivity (results not shown).

## Discussion

The present study was undertaken to investigate whether IGF-I signalling in placental trophoblasts is influenced by IGF BP(s) secreted by the cells. This was done by comparative characterization of the binding and internalization

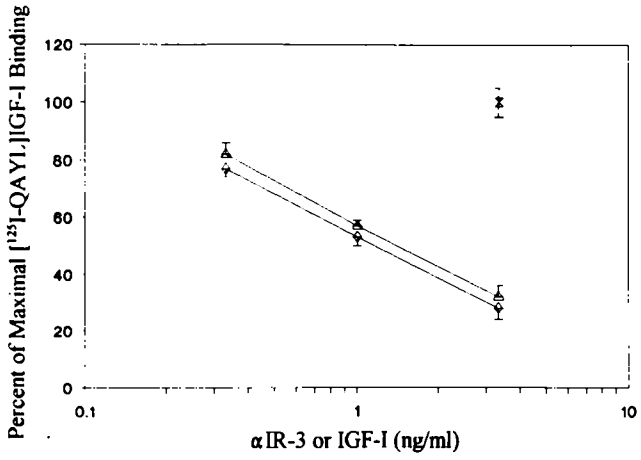


**Figure 5** Competition of [$^{125}$I-QAYL]IGF-I binding to trophoblast cells. The cells were incubated at 4°C in binding buffer, as described in Figure 4, with [$^{125}$I-QAYL]IGF-I in the presence and absence of unlabelled IGF-I (◇) [QAYL]IGF-I (△), [Leu]IGF-I (X), or α-IR3 (all data points equalled non-specific binding, see text). The expression of results is as described in Figure 4. Each point represents the mean ± SD of three experiments done in triplicate.

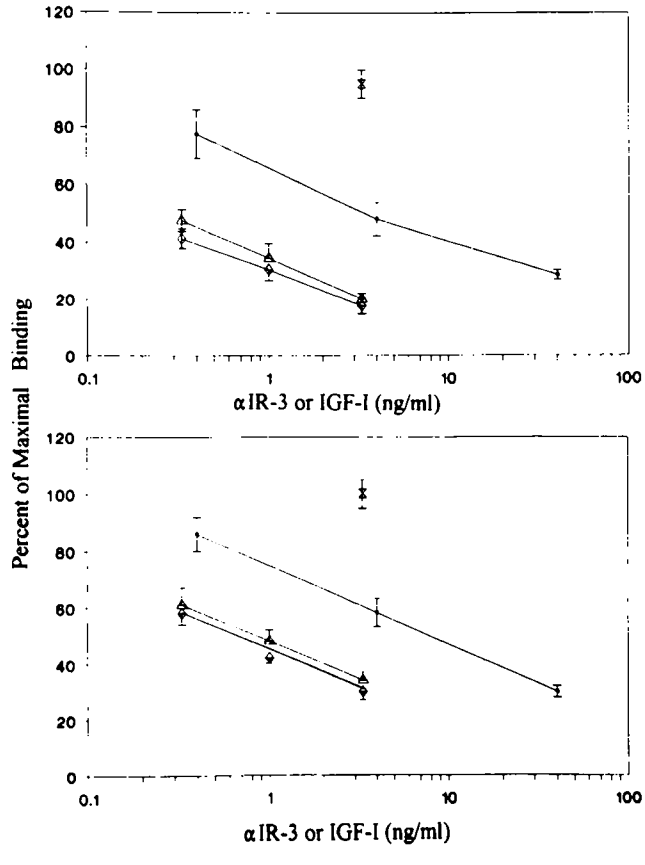


**Figure 6** Competition of [$^{125}$I]IGF-I and [$^{125}$I-QAYL]IGF-I binding to JEG-3 cells. Cells were incubated with [$^{125}$I]IGF-I or [$^{125}$I-QAYL]IGF-I in the presence or absence of unlabelled IGF-I (◇), [QAYL]IGF-I (△), [Leu]IGF-I (X) or α-IR3 (■) at 4°C as described in the materials and methods. Results are expressed as described in Figures 4 and 5. The points represent the mean ± 0.5 range of two experiments performed in triplicate.

of IGF-I and its analogue, [QAYL]IGF-I, with reduced affinity for IGF BP (Bayne *et al.*, 1988), in two different placental cell culture models. One was a primary culture of human placental trophoblasts established in our laboratory (Bhaumick & Bala 1991a and Bhaumick *et al.*, 1992) and the other, JEG-3 cells, a human choriocarcinoma cell line, representing placental trophoblasts.

Binding of [$^{125}$I]IGF-I to both trophoblast and JEG-3 cells was time- and temperature-dependent and comparable at 37°C. At 4°C, the binding of [$^{125}$I]IGF-I to trophoblasts was increased by ~2 times compared to that at 37°C, whereas binding to JEG-3 cells remained the same. This differential pattern of [$^{125}$I]IGF-I binding to the two cells was accentuated by hormone internalization patterns in the cells. In trophoblasts, the internalization of [$^{125}$I]IGF-I was low and temperature-independent, while in JEG-3 cells, internalization was high, rapid and temperature-dependent. The lower rate of [$^{125}$I]IGF-I internalization in trophoblasts could be explained by differences in the metabolic states of a primary culture and a cell line. However, the temperature independent internalization implied that the classical hormone-receptor internalization by endocytosis may not be the mechanism of IGF-I internalization in trophoblasts. Alternatively, the binding of IGF-I to its receptor in trophoblast may be confounded by the presence of membrane-bound IGF BP(s) secreted by the cells.

The latter hypothesis was initially investigated by studying the binding and internalization of [$^{125}$I-QAYL]IGF-I in the two cells and then analyzing detailed comparative binding characteristics of the two IGF-Is by competitive binding and affinity cross-linking studies. The binding and internalization of [$^{125}$I-QAYL]IGF-I to trophoblast and JEG-3 cells revealed that [QAYL]IGF-I did not bind to trophoblasts at 37°C and its binding was significantly ($p<0.01$) lower at 4°C. The binding and internalization of [$^{125}$I-QAYL]IGF-I in JEG-3 cells, however, were similar to that of [$^{125}$I]IGF-I. These differences were further expanded in the competitive binding studies. [$^{125}$I]IGF-I binding to trophoblasts was most effectively inhibited by IGF-I and then by [QAYL]IGF-I and [Leu]IGF-I, the other IGF-I analog with reduced affinity for the IGF-I receptor. α-IR3, an antibody to the IGF-I receptor, was only inhibitory at 4°C at a high concentration and had no effect at 37°C. Moreover, there was a temperature-dependent difference in the potencies of IGF-I and its analogs in their ability to inhibit [$^{125}$I]IGF-I binding to the cells. The potency of IGF-I to inhibit [$^{125}$I]IGF-I at 4°C was significantly ($p<0.05$) higher than at 37°C and the potencies of [Leu]IGF-I and [QAYL]IGF-I were reversed for the two temperatures. At 37°C, potency of [Leu]IGF-I > [QAYL] IGF-I in inhibiting the binding, whereas at 4°C, the order was reversed. The characteristics of [$^{125}$I]IGF-I binding to trophoblast were significantly different from the characteristics of its binding to JEG-3 cells and that of [$^{125}$I-QAYL]IGF-I binding to both JEG-3 and the trophoblast cells. Binding of [$^{125}$I]IGF-I in JEG-3 cells and [$^{125}$I-QAYL]IGF-I in JEG-3 and trophoblast cells were equipotently inhibited by IGF-I and [QAYL]IGF-I. [Leu]IGF-I had no effect and α-IR3 inhibited the bindings at both temperatures. These differential inhibition of [$^{125}$I]IGF-I and -[QAYL]IGF-I binding to trophoblast by IGF-I, its analogs and α-IR3 strongly supported the suggestion that IGF-I binding to its trophoblast receptors may be confounded by IGF BP(s). This will then explain the temperature-dependent differential inhibitory potencies of IGF-Is and α-IR3. The increased inhibitory potencies of IGF-I, [QAYL]IGF-I and α-IR3 seen at 4°C could be explained due to the decrease in recycling and/or degradation of membrane receptors at lower temperature resulting in an increase in surface receptors available for IGF-I binding.

Affinity cross-linking studies revealing the binding of IGF-I to a low molecular weight non-receptor protein confirmed that IGF-I binding to trophoblast was mainly to IGF BP. The electrophoretic mobility of the affinity cross-linked complex, ~45 kDa, suggested the protein to be IGF BP-3 of $M_r$ ~37 kDa bound to IGF-I of $M_r$~7.5 kDa. IGF BP-3, a glycosylated protein, is shown to migrate as a single unit protein with an apparent Mr of 40–45 kDa on SDS-gel under reducing condition and as a doublet under non-

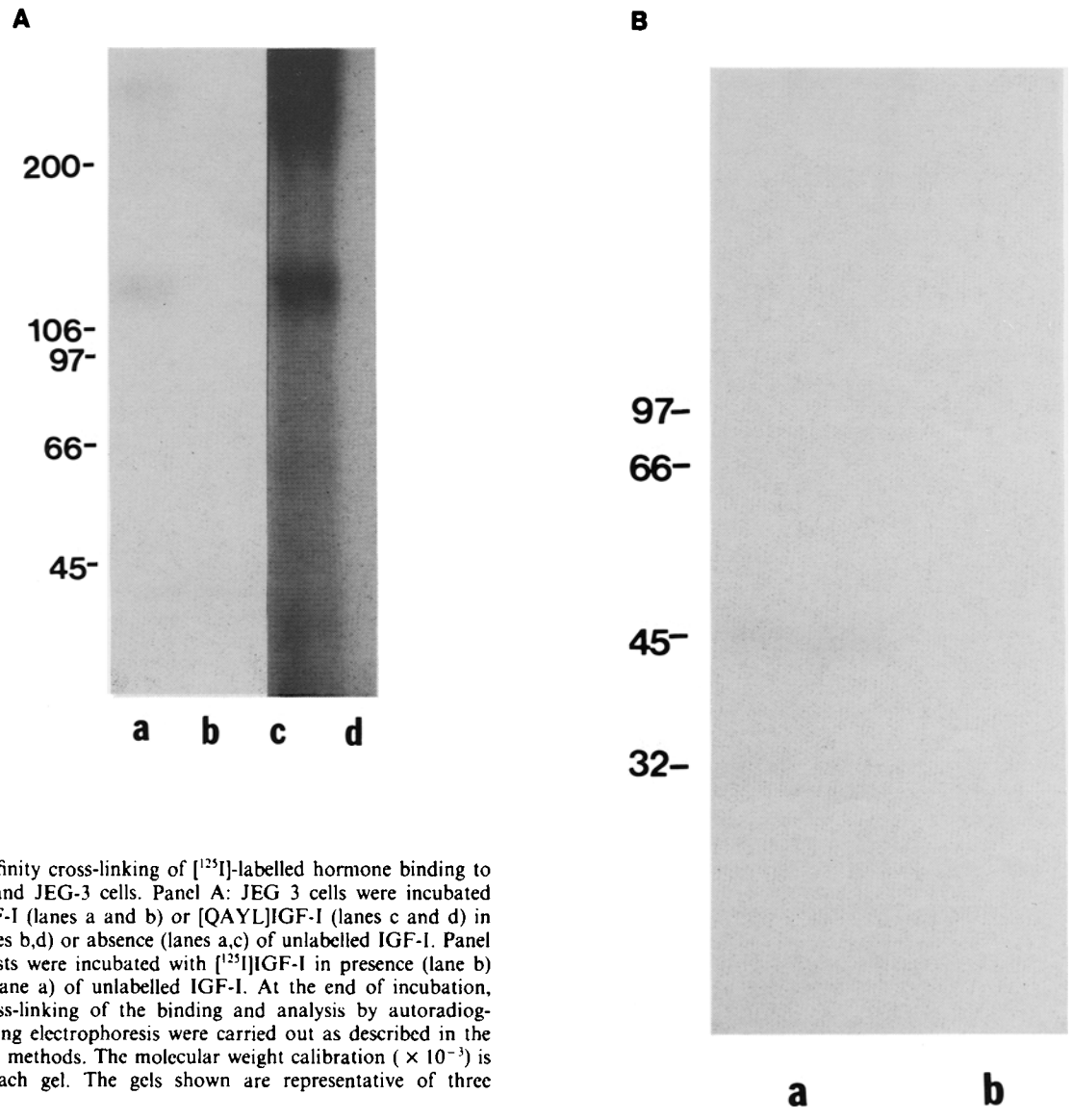


**Figure 7** Affinity cross-linking of [$^{125}$I]-labelled hormone binding to trophoblast and JEG-3 cells. Panel A: JEG 3 cells were incubated with [$^{125}$I]IGF-I (lanes a and b) or [QAYL]IGF-I (lanes c and d) in presence (lanes b,d) or absence (lanes a,c) of unlabelled IGF-I. Panel B: trophoblasts were incubated with [$^{125}$I]IGF-I in presence (lane b) or absence (lane a) of unlabelled IGF-I. At the end of incubation, chemical cross-linking of the binding and analysis by autoradiography following electrophoresis were carried out as described in the materials and methods. The molecular weight calibration ( $\times 10^{-3}$) is shown for each gel. The gels shown are representative of three experiments.

reducing conditions. Migration of the protein as a doublet under non-reducing condition is suggested to reflect its alternative glycosylation (Martin & Baxter, 1986).

Our failure to visualize [$^{125}$I]IGF-I binding in trophoblasts to the proteins corresponding to IGF-I receptor is possible due to the combination of low receptor binding and a low efficiency of affinity cross linking technique. In contrast to trophoblasts, the affinity cross-linked of [$^{125}$I]IGF-I and [$^{125}$IQAYL]IGF-I complexes in JEG-3 cells migrated to Mrs $\sim$130 kDa and $>$260 kDa, corresponding to $\alpha$-subunit and the non-reduced $\alpha/\beta$ dimers of the IGF-I receptor, as reported previously (Bhaumick & Bala, 1985; 1987). This and the results of competitive binding studies, strongly supported that IGF-I binding to JEG-3 cells is predominantly to the IGF-I receptor.

The confirmation that the trophoblast membrane protein that bound IGF-I to be IGF BP-3 was obtained by Western ligand and immunoblot analyses. Ligand blot analysis revealed the secretion of IGF BP by trophoblasts with an apparent $M_r$ of $\sim$37 and 40 kDa. This coincided with the migration of IGF BP-3 doublet, which is typically shown to have an electrophoretic mobility of 38/45 kDa under non-reducing condition. Immunoblot analysis of the protein confirmed its identity to IGF BP-3 by revealing its immunoreactivity to $\alpha$-IGF BP-3 antiserum. The absence of IGF binding activity in JEG-3 cell conditioned medium revealed by ligand blot analysis, confirmed the previous suggestion by Ritvos *et al.* (1988a & b) that JEG-3 cells do not produce any IGF BP. This also supported the conclusion of binding studies presented here, suggesting that the binding of IGF-I to JEG-3 cells is mainly to IGF-I receptors and not modulated by the membrane-associated IGF BP.

In summary, the findings of this study identified the secretion and membrane association of IGF BP by placental trophoblasts and confirmed that JEG-3 cells do not secrete IGF BP. The findings of IGF BP secretion by trophoblast, shown here, may appear to be contradictory to the previous studies, especially, the study reported by Fant (1990). His study suggested that the placental specific IGF BP, viz., IGF BP-3, is predominantly produced by pre-term placental cells (fibroblast) of mesenchymal origin. Trophoblasts of matured placenta secrete very little IGF BP. The success of the present study in identifying the placental trophoblast specific IGF BP-3 is possibly due to the quality of the cultured trophoblast used. As previously reported from our laboratory (Bhaumick *et al.*, 1992), evaluation of these cells by immunohistochemical staining followed by placental lactogen production revealed that over 80% of the cultured cells were differentiated trophoblast and only $<$2% of the cells were fibroblast and/or other endothelial cells. Therefore, use of this system had an advantage in detecting the IGF BP secreted by trophoblasts, a cell type in placenta which may produce IGF BP in smaller quantity in comparison to many other cells, especially fibroblast, in the tissue. To support

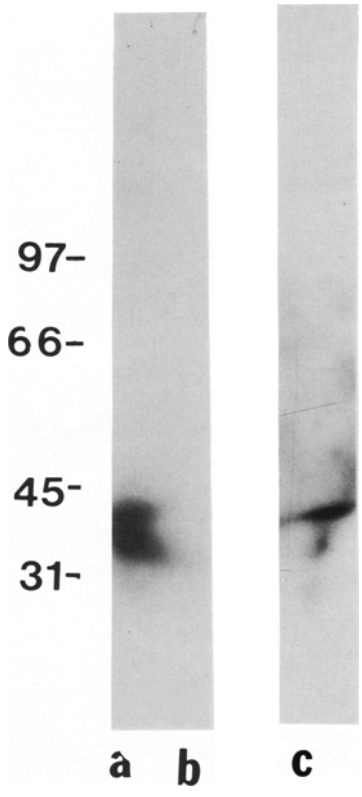


**Figure 8** Identification of IGF BP secreted by trophoblast cells. Conditioned media of trophoblast and JEG-3 cells were concentrated and ligand blotted with [$^{125}$I]IGF-I or immunoblotted with α-BP antiserum following gel electrophoresis as described in the materials and methods. Lanes a,b: ligand blots of trophoblast and JEG-3 conditioned medium; lane c: immunoblot of trophoblast conditioned medium. The molecular weight calibration on the left represents both the blots. The autoradiograms of the blots shown are representative of three experiments for ligand blots and two experiments for immunoblots.

this, when using a fibroblast reference control in the present study (data not shown), it was observed that IGF BP production by fibroblasts is 3–4 times higher than by trophoblasts. In this context, Fant's (1990) study with placental explants may not have been able to detect the IGF BP-3 production by trophoblasts due to fibroblast producing this protein in greater amounts. Thus, fibroblast IGF BP-3 would mask trophoblast IGF BP-3 production.

Surface-association of IGF BP-3 to trophoblast membrane may imply that *in vivo* actions of IGF-I in placenta are modulated by membrane-bound IGF-BP. The suggestion that surface-associated IGF BP modulate IGF biological responses has been postulated to be via their acting as reservoirs of IGF binding sites and competing for the binding of IGF to their receptors (Clemmons *et al.*, 1991). In this context, membrane bound IGF BP-3 modulated action of IGF-I in placenta will be influenced by factors regulating the availability of both IGF-I and IGF BP-3. It is shown by our laboratory that binding and phosphorylating activities of IGF-I and insulin receptors are increased in the placentas of diabetic mothers (Bhaumick & Bala, 1988; 1989). Whether, this observation, according to the present suggestion, is related to the availability of membrane bound IGF BP-3 in trophoblast is not yet known. JEG-3 cells, not producing IGF BP, provides an attractive model to continue our study in exploring the mechanism of IGF-I action interactively with IGF BP-3 in the placentas of normal and diabetic patients. The advantage of JEG-3 cells is the possibility of manipulating the cells for the presence and absence of IGF BP-3. The wild type cells will be advantageous in studying the IGF-I action in the absence of IGF BP-3. An environment with the presence of IGF BP-3, for its modulatory effect, could be achieved either by the exogenous addition of IGF BP-3 or by transfecting the cells with IGF BP-3 gene to produce the protein endogenously.

## Materials and methods

### *Reagents*

Media and supplies for tissue culture were obtained from GIBCO (Burlington, Ontario) and most chemicals were obtained from Sigma Chemical Co. (St. Louis, MI). Disuccinimidyl suberate (DSS) was obtained from Pierce Chemicals (Rockford, IL). α-IR3, a monoclonal antibody to the IGF-I receptor, was obtained from Oncogene Science, Inc. (Uniondale, NY). α-BP antisera were gifts from Dr Clemmons (University of North Carolina, Chapel Hill, NC).

### *Hormones*

IGF-I was a gift from Lilly Research Laboratories (Indianapolis, Indiana). IGF-I analogs, [QAYL]IGF-I and [Leu]-IGF-I were gifts from Dr Cascieri at Merk Sharp and Dohme Research Laboratories. [$^{125}$I]IGF-I and -[QAYL]IGF-I (~150 μCi/μg) were prepared by a modification of the Chloramine-T method as previously described (Bayne *et al.*, 1988; Bala & Bhaumick, 1979) and according to instructions from Dr Cascieri, respectively.

### *Cell culture*

Normal term placentas, obtained from cesarean section deliveries, were processed for cell culturing as previously described (Bhaumick *et al.*, 1992; Bhaumick & Bala, 1991a). The cells ($1-2 \times 10^6$) were plated in 24 multiwell dishes (Linbro, Flow Laboratories, Mississauga, Ontario) with 1 ml of Dulbecco's modified medium (DMEM) containing 20 mM HEPES, 2 mM glutamine, 10% fetal bovine serum (FBS) and antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin, P/S). The cells were maintained in a humidified incubator at 37°C in a 5% $CO_2$ and 95% air atmosphere. The medium was changed daily for the first 5 days and every other day after that. Cells from 7–9 days in culture (~$1 \times 10^5$ cells/well) were used in the experiments.

Human choriocarcinoma, JEG-3 cells (American Type Culture Collection, Rockville, Maryland), were cultured as a monolayer in Eagle's Minimal Essential Medium (MEM) containing 1 mM pyruvate, nonessential amino acids, P/S and 10% FBS, following the supplier's instructions. The cells were either plated in 24 multiwell dishes (~$5 \times 10^4$ cells/plate) or in 60 mm petri dishes ($5 \times 10^5$ cells/plate) and grown to near confluency before using for the experiments.

### *Binding and internalization of IGF-I*

Trophoblasts ($1 \times 10^5$ trophoblast/well, after 7 days in culture) or JEG-3 cells ($5 \times 10^5$/well in 24 multiwell dishes at near confluency) grown in serum containing media were placed in serum-free medium (SFM) containing 0.25% bovine serum albumin (BSA) and P/S 24 h prior to binding to reduce the possible competition of IGF binding by the secreted IGF BP in the medium (McCuscker *et al.*, 1990). Cells were washed 3 times with $NaHCO_3$-free medium (MEM for JEG-3 and DMEM for trophoblasts, no differences were observed in the binding of the two cell types when they were grown in each others media) containing 1% BSA and 15 mM HEPES, (pH 7.4). They were then incubated in 300 μl of this medium containing [$^{125}$I]IGF-I or -[QAYL]IGF-I (50 000 c.p.m.) with or without 1 μg of unlabelled IGF-I under the conditions specified in text. After the incubation, cells were washed 3 times wih ice cold 1% BSA in phosphate buffered

saline [(PBS) (binding buffer)]. Surface-bound radioactivity was removed by incubating the cells in 500 µl of 0.2 M acetic acid containing 0.5 M NaCl (pH 2.8) for 10 min on ice. The optimum pH and time for the removal of surface bound radioactivity were determined previously (Bhaumick & Bala, 1991b). The cells were then washed once with PBS and solubilized with 1 N NaOH. Radioactivity associated with both the acid-extracted supernatant and solubilized cells, was determined using a γ-counter. Specific binding was calculated by subtracting the [$^{125}$I]labelled IGF-Is bound in the presence of unlabelled IGF-I from the total amount of labelled IGF-I bound. Total binding was taken as the sum of the surface bound (acid extractable) and internalized (acid non-extractable) radioactivity.

*Competitive binding studies*

Similar to binding and internalization studies, cells were grown or cultured in multiwell plates and were placed in SFM 24 h prior to binding. They were then washed 3 times with binding buffer and incubated in the same buffer containing 50 000 c.p.m. [$^{125}$I]labelled hormone with or without various concentrations of unlabelled IGF-I, [QAYL]IGF-I, [Leu] IGF-I (another IGF-I analogue, [Leu$^{24}$, 1-62]IGF-I has reduced affinity for IGF-I receptor, (Caseieri *et al.*, 1988), and α-IR3, a monoclonal antibody to IGF-I receptor, at 37°C for 1 h or 4°C, overnight. At the end of incubation, the cells were washed 3 times with binding buffer and the bound radioactivity was determined by solubilizing the cells in 1 N NaOH. Specific binding was calculated as described in the binding and internalization studies.

*Affinity cross-linking of IGF-I binding*

For affinity cross-linking studies, trophoblast in multiwell plates and JEG-3 cells in 60 mm plates were incubated in binding buffer containing [$^{125}$I]labelled IGF-Is with or without unlabelled IGF-I at 4°C, overnight. The cells were then washed 3 times with PBS and the cross-linking of the cell bound [$^{125}$I]labelled IGF-Is were performed by incubating the cells with 1 mM DSS, in PBS, at 4°C for 30 min. The cells were then solubilized in PBS containing 1% Triton X-100. The solubilized cells (trophoblasts pooled from ~10 wells and JEG-3 cells from 1-60 mm plate) were concentrated in Centricon 10 concentrators (Amicon, Mississauga, Ontario). The concentrated samples were boiled in SDS-polyacrylamide gel (PAGE) sample buffer (Laemmli, 1970) and electrophoresed on a 5–13% gradient SDS-PAGE under reducing conditions. The gel was then dried and autoradiographed as previously described (Bhaumick & Bala, 1985).

*Ligand blotting*

Ligand blotting was done by the method of Yang *et al.* (1989). In brief, medium (24 h in SFM) from day 9 trophoblasts or near confluent JEG-3 cells was concentrated 10 times in a Centricon 10 concentrator. These samples were electrophoresed on 5–13% SDS-PAGE under non-reducing condition and the proteins transferred to polyvinylidene fluoride (Immobilon PVDF; Millipore, Mississauga, Canada). Transferred membranes were incubated overnight at 4°C with ~1 × 10$^6$ c.p.m. of [$^{125}$I]IGF-I in 20 ml of 50 mM Tris-HCl (pH 7.4) containing 1% BSA and 0.1% Tween 20. At the end on this incubation, the membranes were then washed, air dried and autoradiographed as described above.

*Immunoblotting*

Samples were electrophoresed and transferred to Immobilin PVDF as described for ligand blotting. The membranes were blocked with 7% non-fat skim milk for 1 h at room temperature. The blot was then probed with a 1/1000 dilution of BP antisera diluted in PBS containing 1% Tween 20 and 1% BSA (PBS-T), for 1 h at room temperature. The blot was then washed 3 times for 10 min in PBS containing 1% Tween 20 (PBS-T). The blot was then incubated with a 1/5000 dilution of donkey anti-rabbit antisera (diluted in PBS-T) for 45 min at room temperature. The blot was then washed 3 times in PBS-T as described above. The amount of BP present was then visualized using CSPD (Chemiluminescent substrate, Bio/Can, Mississauga, Ontario) following the manufacturer's directions, and autoradiography.

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**References**

Armstrong, G.D., Hollenberg, M.D., Bhaumick, B. & Bala, R.M. (1983). Bradshaw, R.A., Gill, G.N. (eds). Alan R. Liss Inc.: New York. pp. 227–236.

Baxter, R.C. (1991). *Acta Endocrinologica*, **124**, 33–40.

Bala, R.M. & Bhaumick, B. (1979). *Can. J. Biochem.*, **57**, 1289 1298.

Bala, R.M., Bhaumick, B., Armstrong, G.H. & Hollenberg, M.D. (1982). Spencer, E.M. (ed). de Grayte-Berlin: New York. pp. 491–507.

Bayne, M.L., Applebaum, J., Chicchi, G.G., Hayes, N.S., Green, B.G. & Cascieri, M.A. (1988). *J. Biol. Chem.*, **263**, 6233–6239.

Bhaumick, B. & Bala, R.M. (1985). Ontogeny and characterization of basic somatomedin receptors in rat placenta. *Endocrinol.*, **116**, 492–498.

Bhaumick, B. & Bala, R.M. (1987). *Endocrinol.*, **120**, 1439–1448.

Bhaumick, B. & Bala, R.M. (1991a). *Trophoblast Res.*, **5**, 171 -182.

Bhaumick, B. & Bala, R.M. (1991b). *Placenta*, **12**, 373.

Bhaumick, B., George, D.& Bala, R.M. (1992). *J. Clin. Endocrinol. Metab.*, **74**, 1005–1011.

Cascieri, M.A., Chicchi, G.G., Applebaum, J., Hayes, N.S., Green, B.G. & Bayne, M.L. (1988). *Biochem*, **27**, 3229 -3233.

Clemmons, D.R., Elgin, R.G., Han, V.K.M., Casella, S.J., D'Ercole, A.J. & Van Wyk, J.J. (1986). *J. Clin. Invest.*, **77**, 1548–1556.

Clemmons, D.R., Camacho-Hubner, C., Jones, J.I., McCusker, R.H. & Busby Jr., W.H. (1991). Spencer, E.M. (eds). Elsevier: New York, Amsterdam, London, Tokyo, pp. 475–486.

Conover, C.A. & Powell, D.R. (1991). *Endocrinol*, **129**, 710–716.

Drop, S.L.S., Brinkma, A., Kortleve, D.J., Groffen, C.A.H., Schuller, A. & Zwarthoff, E.C. (1991). Spencer, E.M. (ed). Elsevier: New York, Amsterdam, London, Tokyo, pp. 311–328.

Fant, M. (1990). *Placenta*, **11**, 123–133.

Hock, R.A. & Hollenberg, M.D. (1980). *J. Biol. Chem.*, **255**, 10731 -10736.

Laemmli, U.K. (1970). *J. Biol. Chem.*, **261**, 8754 -8760.

McCusker, R.H., Camacho-Hubner, C., Bayne, M.L., Cascieri, M.A. & Clemmons, D.R. (1990). *J. Cell Physiol.*, **144**, 244–253.

McCusker, R.H., Busby, W.H., Dehoff, M.H., Camacho-Hubner, C. & Clemmons, D.R. (1991). *Endocrinol.*, **129**, 939–949.

Ritvos, O., Rutanen, E.-M., Pekonen, F., Jalkanen, J., Suikkari, A.-M. & Ranta, T. (1988a). *Endocrinol.*, **122**, 395–401.

Ritvos, O., Ranta, T., Jaklanen, J., Suikkari, A.-M., Voutilainen, V., Bohn, H., Rutanen, E.-M. (1988b). *Endocrinol.*, **122**, 2150–2157.

Shimasaki, S., Shimonaka, M., Zhang, H.-P. & Ling, N. (1991). Spencer, E.M. (ed). Elsevier: New York, Amsterdam, London, Tokyo, pp. 343 -358.

Yang, Y., Wang, J.F., Orlowski, C., Nissley, S. & Rechler, M. (1989). *Endocrinol.*, **125**, 1540–1544.