Insulin-like growth factor binding protein-3 increases intracellular calcium concentrations in MCF-7 breast carcinoma cells

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Abstract Insulin-like growth factor binding protein-3, IGFBP-3, specifically binds to IGFs with high affinity, but it is also capable of modulating the IGF-I signalling pathway or inducing apoptosis independently of its binding to IGFs. The molecular mechanisms underlying the action of IGFBP-3 have not been elucidated. In this study, we have demonstrated that binding of IGFBP-3 to a cell surface receptor in MCF-7 breast carcinoma cells induces a rapid and transient increase in intracellular free calcium. This increase was mediated via a pertussis toxin-sensitive pathway, indicating that the IGFBP-3 receptor may be specifically coupled to a Gi protein. The effect of IGFBP-3 on calcium concentrations was dose-dependent and also occurred when IGFBP-3 was complexed with either IGF-I or heparin, suggesting that the receptor binding site is probably located in the least conserved central domain of IGFBP-3. Neither IGFBP-1, nor IGFBP-5 (structurally the closest to IGFBP-3) altered intracellular calcium concentrations. These results provide evidence that a specific intracellular signal is triggered by IGFBP-3 binding to a cell surface receptor.

Key words: Insulin-like growth factor binding protein-3; Intracellular calcium; Breast carcinoma cell; Gi protein-coupled receptor

1. Introduction

The insulin-like growth factors, IGF-I and -II, participate in regulating proliferation and/or differentiation in diverse cell types via the type I IGF receptor (IGF-IR) (reviews in [1,2]). In all biological fluids, they are associated with high-affinity binding proteins, the IGFBPs, which act as carriers and modulate the availability of IGFs to their target cells [1,3,4]. One of these six IGFBPs, IGFBP-3, is present in most tissues [5] and is known to possess intrinsic activities that are unrelated to its IGF binding ability (review in [6]). These activities require binding to a specific cell surface receptor [7], but the nature of the latter and the signalling pathways that it activates remain unknown. These 'IGF-independent' effects of IGFBP-3 play a direct role in cell growth [8,9], they induce apoptosis [10] and they modulate IGF signalling [11]. Identification of the molecular mechanisms underlying the action of IGFBP-3 is therefore of primary importance.

One route would be to identify other events required for these effects and investigate a link with the influence of IGFBP-3-induced signalling. An increase in free cytosolic calcium is a key signal in the induction of apoptosis and IGFBP-3 has been shown to induce apoptosis directly [10] and/or to potentiate the effects of apoptotic agents [12,13]. We therefore investigated the possibility that IGFBP-3 may alter calcium levels in MCF-7 breast carcinoma cells and provide some clue as to the signalling pathways activated by IGFBP-3.

2. Materials and methods

2.1. Radioiodination of IGFBP-3

(N109D)-rhIGFBP-3 (Upstate Biotechnologies) was labelled by the chloramine-T method using Na ^{125}I (Amersham) and purified by gel filtration in a 0.1 M acetic acid, 0.15 M NaCl solution. Specific activity was approximately 100 μCi/μg IGFBP-3.

2.2. Binding of IGFBP-3 to the cell surface

The method used was similar to that described by Yamanaka et al. [14]. Confluent monolayers of MCF-7 cells ($\sim 1.7\times10^6$ cells/well) were incubated in serum-free medium for 16 h. The cells were washed once with cold HBSS (Hanks' balanced salt solution without $CaCl_2$ and $MgCl_2$, containing 25 mM HEPES, pH 7.4 and 25 mM $NaHCO_3$). Cell surface-bound endogenous IGFBPs were then removed by rinsing the cells once in cold HBSS containing 1 mM EDTA. The cells were then washed once in cold HBSS and incubated in 600 μl binding buffer (HBSS containing 1 mM $CaCl_2$ and 0.5% bovine serum albumin (BSA)) for 4 h at 4°C with [^{125}I]IGFBP-3 ($\sim$50 000 cpm) in the absence (triplicate) or in the presence (duplicate) of varying concentrations of unlabelled IGFBP-3 (and 400 nM in triplicate for determination of non-specific binding). They were then washed three times in cold phosphate-buffered saline (PBS) and solubilized with 0.5 M NaOH. Radioactivity of the cell lysates was measured in a γ-counter and specific binding was determined by subtracting non-specific binding from total counts.

2.3. Determination of intracellular free Ca^{2+} concentration

Changes in intracellular Ca^{2+} concentration in response to IGFBP-3 were measured using a QuantiCell 700 dynamic imaging microscopy system (Visitech Int. Ltd., UK), with 30–40 MCF-7 cells per field as previously described [15]. The cells were cultured on glass coverslips, washed, and incubated at 37°C for 60 min in PBS–calcium-free HEPES medium (20 mM HEPES/Tris, pH 7.4, 5.4 mM KCl, 2 mM Na_2PO_4, 0.8 mM $MgCl_2$, and 5 mM glucose) containing 5 μM Fura-2/AM. Before analysis, the cells were washed twice with the same buffer. After background recording for 40 s (20 images), the experiment was initiated by adding different concentrations of IGFBP-3, IGFBP-1 or IGFBP-5. Fluorescence images were obtained at intervals of 2 s and intracellular Ca^{2+} concentrations were calculated over 200 s from the ratio of the fluorescence intensities at 340 and 380 nm on a pixel basis. The dose–response curve for intracellular calcium concentrations was obtained by integrating the area under the curve (measuring Ca^{2+} transient peaks plotted as a function of time for each field from the addition of IGFBP-3 until the end of image recording, 200 s) and averaging the fluorescence from the whole field of cells chosen.

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Ca^{2+} stimulation curves were determined for each concentration of IGFBP-3 tested.

3. Results

Addition of 20 nM IGFBP-3 to MCF-7 cells increased intracellular free calcium concentrations within 2–4 s, a return to basal levels occurring 20–30 s later (Fig. 1). The response to IGFBP-3 was therefore transient, where calcium was released into the cytoplasm from intracellular stores and then re-absorbed. The viability of the cells in terms of calcium response was confirmed by their reaction to thapsigargin (1 μM) which induced total and irreversible calcium release from intracellular stores. As shown in Fig. 2, the response was dose-dependent: it was evident using 1–2 nM IGFBP-3 and maximal using 20–50 nM IGFBP-3; $ED_{50} = 3.02$ nM.

In view of the rapid response to IGFBP-3, internalization seemed improbable and the existence of a membrane receptor for IGFBP-3 most likely. We therefore set out to characterize the binding between IGFBP-3 and the cell surface in MCF-7 cells. Fig. 3 shows the competition curve and Scatchard analysis for a typical experiment. The mean association constant calculated from four experiments was $0.4 \pm 0.03 \times 10^8$ M^{-1} and the mean number of binding sites was 4.9×10^5 sites/cell. Non-specific binding was 7–9%. Binding to the cell surface (Fig. 3) and the ability to increase calcium concentrations (Fig. 1) appeared to be specific to IGFBP-3 in MCF-7 cells, since two other IGFBPs, IGFBP-1 and IGFBP-5 (the latter being structurally the most similar to IGFBP-3) had no effect (Fig. 4).

Table 1
Effects of IGF-I/IGFBP-3 and heparin/IGFBP-3 complexes on intracellular calcium concentrations in MCF-7 cells

Treatment	Cytosolic Ca^{2+} (percent of maximum)
IGFBP-3 (20 nM)	100
Heparin (200 μg/ml)	0
IGF-I (1 μM)	0
Heparin/IGFBP-3	94.7
IGF-I/IGFBP-3	170

MCF-7 cells were incubated with IGF-I (1 μM), heparin (200 μg/ml), or IGFBP-3 (20 nM) either alone or previously incubated for 50 min at room temperature with either IGF-I (1 μM) or heparin (200 μg/ml). Intracellular calcium concentrations were measured as described in Section 2. Results of a typical experiment are presented (mean for three independent points).

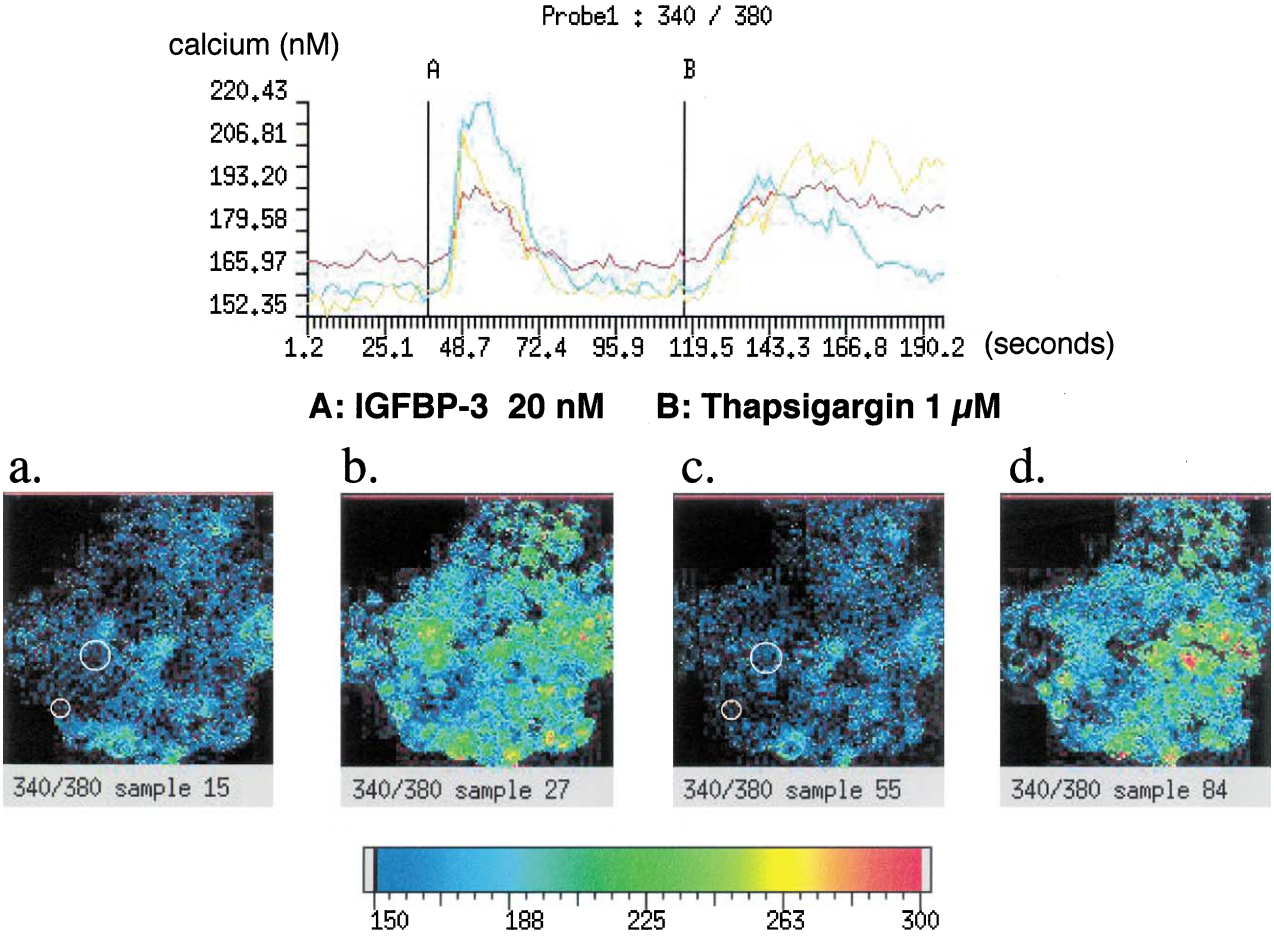

Fig. 1. IGFBP-3 increases intracellular calcium concentrations in MCF-7 cells. MCF-7 cells cultured on glass coverslips were incubated with Fura-2/AM. After background recording for 40 s to determine basal intracellular calcium concentrations as described in Section 2, cells were incubated (A) with IGFBP-3 (20 nM), then (B) with thapsigargin (1 μM). The results of a typical experiment are shown, in which two representative cells (blue and yellow lines) or the whole field (red line) were analyzed and intracellular calcium quantified. The slides from left to right show representative views of the cells (a) before addition of IGFBP-3, (b) after addition of IGFBP-3, (c) after return to basal levels and before addition of thapsigargin, and (d) after addition of thapsigargin.

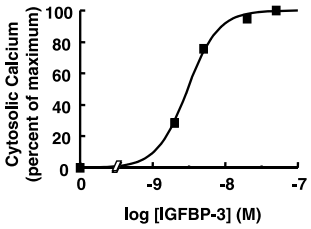

Fig. 2. The effect of IGFBP-3 on intracellular calcium concentrations in MCF-7 cells is dose-dependent. MCF-7 cells cultured on glass coverslips were incubated with Fura-2/AM. After background recording for 40 s to determine basal intracellular calcium concentrations as described in Section 2, cells were incubated with IGFBP-3. Separate and independent experiments were performed for each IGFBP-3 concentration. Dose–response curves for intracellular calcium concentrations were established as described in Section 2.

In many cases, transient increases of intracellular calcium depend on heterotrimeric G protein activation, so we tested a G protein inhibitor for its ability to block the IGFBP-3-induced increase in cytosolic calcium. This proved true for pertussis toxin, indicating that the specific receptor activated by IGFBP-3 in MCF-7 cells was probably coupled to a G protein (Fig. 5).

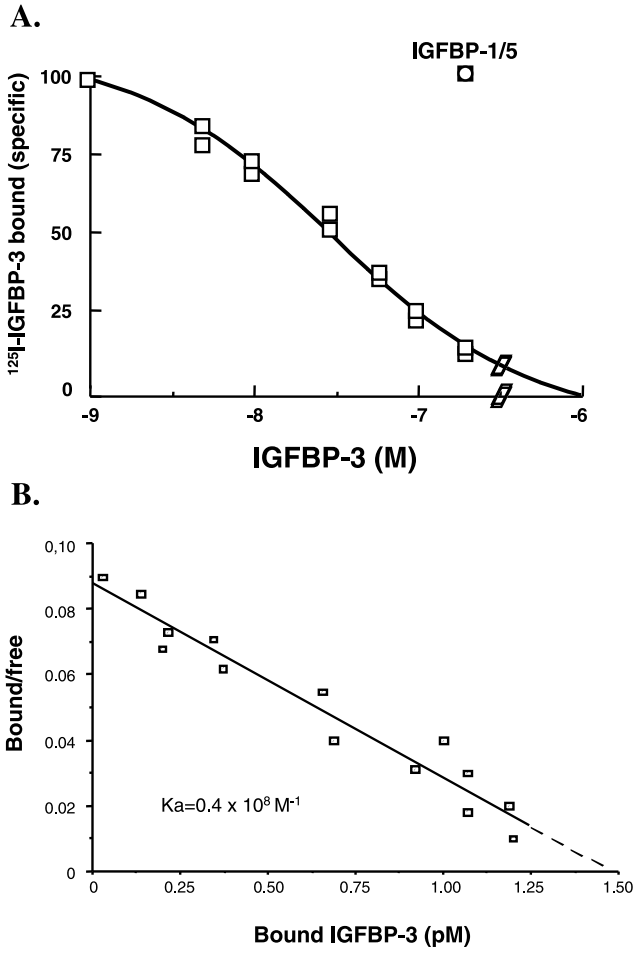

Fig. 3. rhIGFBP-3 binds to MCF-7 cells in monolayer culture. A: Cells were incubated with [^{125}I]IGFBP-3 in the presence of either various concentrations of IGFBP-3 (0–5 μM) or 5 μM of IGFBP-1 or -5. The displacement curve obtained after subtraction of non-specific binding determined in the presence of 400 nM IGFBP-3 is presented. Specific binding at 0 and 1 nM IGFBP-3 was 8.2% of total [^{125}I]IGFBP-3. B: Scatchard plot of the data. Results of a typical experiment.

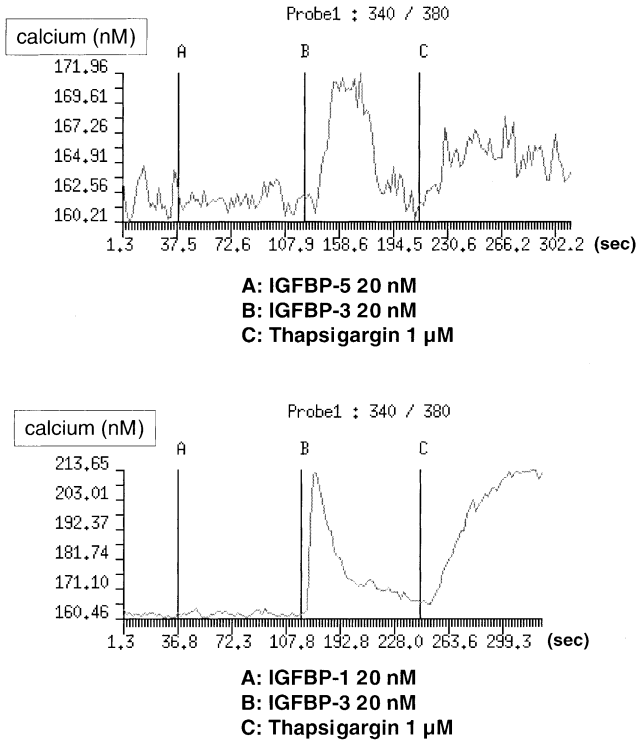

Fig. 4. Neither IGFBP-1 nor IGFBP-5 affect intracellular calcium concentrations in MCF-7 cells. MCF-7 cells were incubated (A) with either 20 nM IGFBP-1 (lower panel) or 20 nM IGFBP-5 (upper panel), then (B) with IGFBP-3 (20 nM) and (C) treated with thapsigargin (1 μM). Intracellular calcium concentrations were measured as described in Section 2. The results of a typical experiment are shown in which the whole field was analyzed and intracellular calcium quantified.

With a view to locating the position of the putative receptor binding site, we investigated the difference between the effects of IGFBP-3 alone and complexed with either heparin or IGF-I on the IGFBP-3-induced cytosolic calcium increase. Neither IGF-I (1 μM) nor heparin (200 μg/ml) alone changed intracellular calcium concentrations (Table 1). The IGFBP-3–heparin complex had the same effect on calcium levels as IGFBP-3 alone and the IGFBP-3–IGF-I complex induced a larger calcium increase than IGFBP-3 alone.

4. Discussion

This study provides the first evidence that an IGF binding protein, IGFBP-3, is capable of specifically transmitting an intracellular signal, giving rise to an increase in cytosolic free calcium in MCF-7 breast carcinoma cells. The kinetics of this transient increase were extremely rapid, suggesting that internalization of IGFBP-3 was highly unlikely and therefore that a membrane IGFBP-3 receptor capable of mediating intracellular signalling must exist. The results of the specific binding experiments were reproducible, with high non-specific binding (7–9%) owing to the stickiness of radiolabelled IGFBP-3. Consequently, at 200 nM IGFBP-3, displacement of [^{125}I]IGFBP-3 remained incomplete and the true association constant was probably underestimated and the calculated

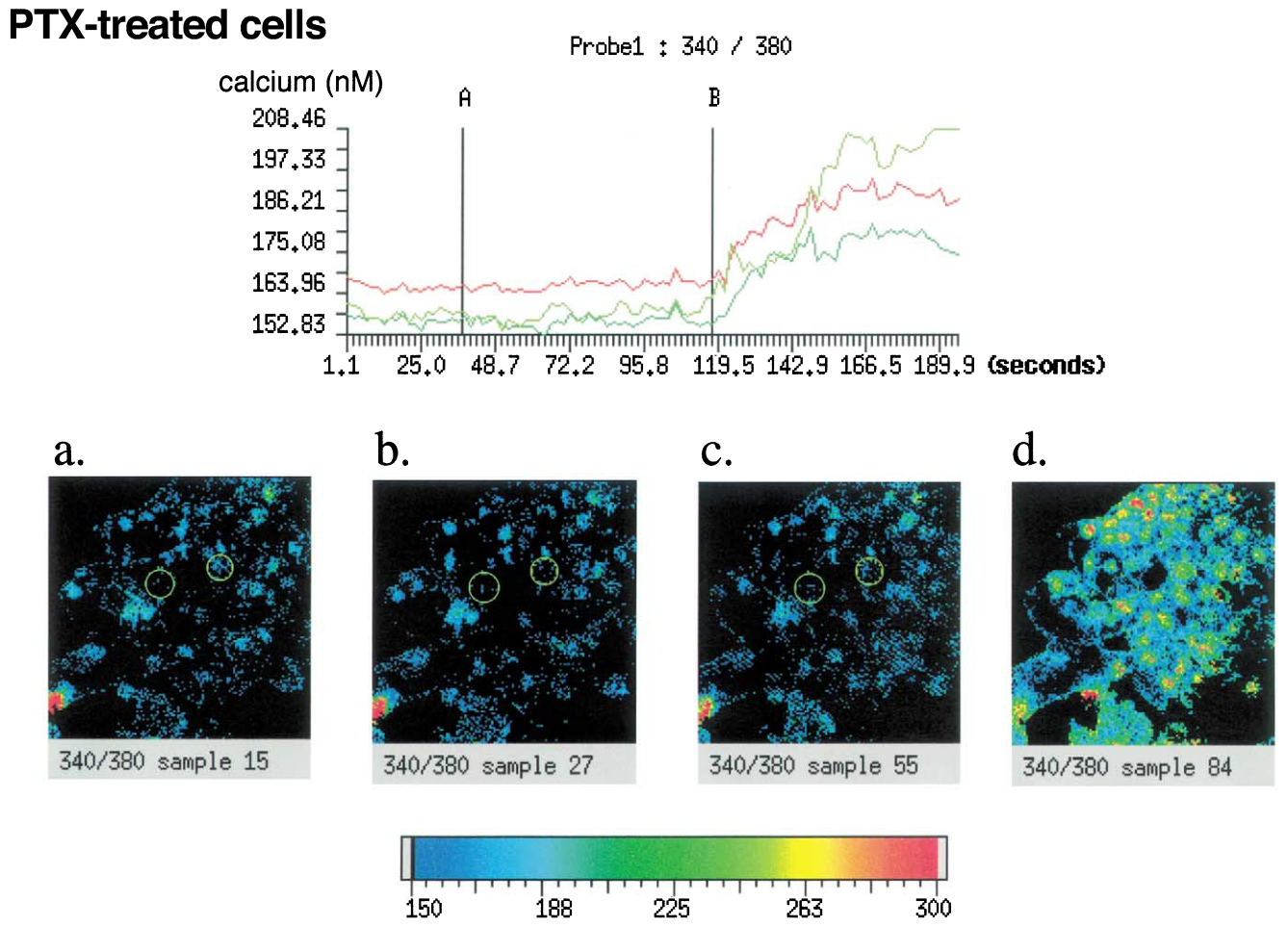

Fig. 5. IGFBP-3 increases intracellular calcium concentrations via a pertussis toxin-sensitive pathway in MCF-7 cells. MCF-7 cells cultured on glass coverslips were treated with or without 200 ng/ml pertussis toxin (PTX) for 16 h at 37°C and incubated with Fura-2/AM. After background recording for 40 s to determine basal intracellular calcium concentrations as described in Section 2, cells were incubated (A) with IGFBP-3 (20 nM), then (B) with thapsigargin (1 μM). The results of a typical experiment are shown, in which two representative cells (green and yellow lines) or the whole field (red line) were analyzed and intracellular calcium quantified. The slides from left to right show representative views of the cells (a) before addition of IGFBP-3, (b) after addition of IGFBP-3, (c) after return to basal levels and before addition of thapsigargin, and (d) after addition of thapsigargin.

number of binding sites, overestimated. In their work on breast cancer cell lines, Yamanaka et al. [14] found a similar number of binding sites to ours in Hs578T cells, but for MCF-7 cells they found 20–30 times fewer sites with three times stronger affinity. However, these values were the highest ones calculated from curvilinear plots, suggesting a two-site binding model. In our experiments, the Scatchard plots were apparently linear, which probably means a smaller proportion of high-affinity binding sites in our cells than in those of Yamanaka et al. [14].

IGFBP-3 provoked a rise in intracellular calcium following activation of a pertussis-sensitive G-coupled receptor. In view of the specificity of pertussis toxin, it would seem that the IGFBP-3 receptor may be specifically coupled to a Gi protein in MCF-7 cells. The nature of both the Gi protein and the receptor are currently under investigation. It is noteworthy that we [11] and others [7] have shown that IGFBP-3 can bind to cell surface proteins with an apparent molecular mass of 30–40 kDa, which coincides with that of most G protein-coupled receptors. Furthermore, it has recently become evident that Gi proteins play an important role in the IGF signalling pathway: Gβγ proteins mediate MAP kinase activation by IGF-I [16,17] and activation of a Gi protein is a prerequisite for IGF-I-induced proliferation and differentiation in 3T3-L1 adipocytes and HIRcB cells [18]. We previously demonstrated that IGFBP-3 can down-regulate the IGF-I signalling pathway [11] and it is conceivable that this effect and the so-called IGF-independent effects of IGFBP-3 (for review [6]) may result from activation of this IGFBP-3 receptor coupled to a pertussis toxin-sensitive G protein.

In the experiments described above, we used commercial (N109D)-IGFBP-3 (recombinant *Escherichia coli* protein). The same results were obtained using either wild-type recombinant *E. coli* IGFBP-3 or a baculoviral glycosylated IGFBP-3 preparation (data not shown). This indicates that the N109D mutation caused no experimental artefacts and that glycosylation made no difference in the responses observed to IGFBP-3. Since neither IGFBP-1 nor IGFBP-5 (structurally the most closely related to IGFBP-3) competes for the binding of [^{125}I]IGFBP-3 to the cell surface receptor and had any effect on intracellular calcium concentrations in MCF-7 cells, it can be concluded that the mechanism of action of IGFBP-3 is specific. From this and a previous study suggesting that the mid-region of IGFBP-3 is probably re-

sponsible for IGFBP-3 binding to the cell surface [14], it can be hypothesized that the binding site for the IGFBP-3 receptor would be situated in the least conserved region among the IGFBPs, the central domain of IGFBP-3. An explanation for the observation that IGFBP-3 complexed with IGF-I was more effective in increasing calcium concentrations than IGFBP-3 alone may lie in greater conformational stability conferred by complex formation with IGF-I and hence more favorable interaction with the receptor. This would be consistent with the physiological potential of such a mechanism, particularly since, for the most part, IGFBP-3 circulates in the cellular environment as an IGF–IGFBP-3 complex. The greater efficacy of this complex also indicates that IGF-I does not mask the binding site on IGFBP-3 for its receptor. Although our binding sites appear to be similar to those described by Yamanaka et al. [14], it has been reported in other studies [19,20] that IGF-I binding to IGFBP-3 diminishes, but does not totally inhibit, its interaction with cell surface binding sites. It would seem that in the latter model sufficient binding sites could remain unblocked for IGFBP-3/IGF-I complexes to affect intracellular calcium. As regards the similar effects on calcium levels of IGFBP-3 complexed with heparin and IGFBP-3 alone, the implication would be that the IGFBP-3 binding site for its receptor is nowhere close to the heparin binding site which is situated in the C-terminal region. Heparin has been shown to inhibit IGFBP-3 binding to the membranes of GM-10 fibroblasts and C6 glioma cells [21], suggesting that it might compete for binding to structurally similar heparan sulfate proteoglycans on the cell surface. However, the inhibition of IGFBP-3 binding by heparin was not total [21] and it would seem that IGFBP-3 may bind to sites other than the cell surface heparan sulfate proteoglycans. In our cell model, heparin clearly failed to inhibit the IGFBP-3-induced increase in intracellular calcium and in this instance IGFBP-3 action could not involve heparan sulfate proteoglycans.

In conclusion, our results reveal an essential molecular mechanism of rapid action for IGFBP-3 in MCF-7 cells, providing direct evidence of signalling pathways that it activates and opening new doors in research on the so-called IGF-independent activities of IGFBP-3.

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