Membrane-associated Insulin-like Growth Factor-binding Protein-3 Inhibits Insulin-like Growth Factor-I-induced Insulin-like Growth Factor-I Receptor Signaling in Ishikawa Endometrial Cancer Cells*

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The function of cell surface-associated insulin-like growth factor-binding proteins (IGFBPs) is controversial. Both inhibition and facilitation of IGF action as well as IGF-independent effects have been reported. We examined the influence of endogenous cell surface-associated IGFBPs on IGF-I receptor (IGF-IR) function in Ishikawa endometrial cancer cells by comparing the effects of IGF-I and its truncated analog des-(1–3)-IGF-I on several components of the IGF-IR signal transduction pathway in the absence of significant amounts of soluble IGFBPs. IGF-I and des-(1–3)-IGF-I are known to have similar affinities for IGF-IR, although the affinity of des-(1–3)-IGF-I for IGFBPs is greatly reduced. Here we show that the two ligands were equipotent not only in IGF-IR binding but also in receptor activation in NIH 3T3 cells overexpressing IGF-IR and possessing a relatively small number of cell surface-associated IGFBPs. In contrast, des-(1–3)-IGF-I manifested a remarkably higher potency as compared with IGF-I in inducing short and middle term cellular responses in IGF-IR-transfected Ishikawa endometrial cancer cells possessing a high number of both the receptor and the cell membrane-bound IGFBP-3. Thus, this difference in the effects of IGF-I and des-(1–3)-IGF-I can be attributed to the attenuation of IGF-I-mediated IGF-IR signaling by membrane-bound IGFBP-3.

Insulin-like growth factors (IGFs)1 and II are closely related polypeptides that have significant homology to insulin. IGFs appear to be important regulators of normal and malignant cell growth (1, 2). Most of the cellular effects of IGF-I and IGF-II are mediated by the IGF-I receptor (IGF-IR). The receptor is a heterotetrameric complex composed of two α-subunits that bind the ligand and two β-subunits possessing tyrosine kinase activity. Binding of the ligand to IGF-IR leads to auto-phosphorylation of the β-subunit and tyrosine phosphorylation of the major receptor substrate (IRS-1) followed by activation of certain downstream signaling cascades (2). This results in expression of immediate early gene products such as c-Fos (3–5). c-Fos as well as other Fos family proteins can dimerize with Jun family proteins forming the AP-1 transcription regulatory complex. AP-1 modulates gene expression by binding to the 12-O-tetradecanoylphorbol-13-acetate-response element (TRE) present in promoter regions of numerous target genes, which ultimately brings about various cellular effects.

The biological actions of IGFs are modulated by a family of IGF-binding proteins (IGFBPs). At least six IGFBPs have been cloned and characterized. These proteins are found both in extracellular fluid and on the cell surface. The function of soluble IGFBPs is well defined. Their affinity for IGFs is higher than that of IGF receptors. Therefore, IGFBPs can inhibit the action of IGFs by sequestering these growth factors in the extracellular space (6). The function of membrane-associated IGFBPs is less well understood. Both inhibition and activation of cellular functions by these proteins have been demonstrated depending on cell type (6, 7). The situation is even more complex since the same IGFBP species being associated with the cell surface can display either an IGF receptor-dependent or -independent mode of action. It has been well documented that the inhibitory effects of IGFBP-3 often appear to be IGF-independent (8–10), whereas its stimulatory effects are associated with facilitation of the IGF action (11–17).

In addition, we have recently shown that in Ishikawa endometrial cancer cells tamoxifen treatment results in a marked decrease in the number of cell surface-associated IGFBP-3 that was accompanied by stimulation of cell proliferation (18). Most interestingly, this effect was associated with stimulation of IGF-I-induced IGF-IR tyrosine phosphorylation, one of the earliest events in the receptor activation. This correlation allows us to hypothesize that the cell surface-associated IGFBP-3 may attenuate the ligand-stimulated IGF-IR activity. The inhibition of IGF-I-induced functions by cell surface-associated IGFBP-3 was also suggested in recent publications (19, 20); however, the possibility of inhibition of IGF-IR signaling by these binding proteins has not been directly studied.

In the present study we directly examined the above hypothesis by comparing the effects of IGF-I and its truncated analog des-(1–3)-IGF-I on several components of the IGF-IR signal transduction pathway in cells possessing a high amount of membrane-associated IGFBPs under conditions where the amount of soluble IGFBPs is insignificant. IGF-I and des-(1–3)-IGF-I are known to have a similar affinity for IGF-IR (21), but in contrast to IGF-I, the affinity of des-(1–3)-IGF-I for IGFBPs is greatly reduced (22, 23). Thus, differences in the effects of IGF-I and des-(1–3)-IGF-I (both of which bind to IGF-IR, but only IGF-I also interacts with the cell surface-
associated IGFBPs would be attributed to the IGF-dependent function of these binding proteins. One of the advantages of such an experimental approach is that in this system the number of cell surface-associated IGFBPs remains constant during the assay; therefore, possible IGF-independent effects of the cell surface-associated IGFBP-3 do not interfere in the determination of its IGF-dependent modulation of IGF-IR function.

Here we demonstrate that in IGF-IR-transfected Ishikawa cells possessing a high number of both the receptor and the cell membrane-bound IGFBP-3, des-(1–3)-IGF-I manifested a remarkably higher potency as compared with IGF-I in inducing short and middle term cellular responses. These data indicate that in Ishikawa cells the membrane-bound IGFBP-3 negatively controls IGF-IR function in an IGF-IR-dependent manner.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human recombinant IGF-I and des-(1–3)-IGF-I were purchased from Groep Pty. Ltd. (Adelaide, Australia). Human recombinant IGF-I and des-(1–3)-IGF-I were generously supplied by Dr. C. Maack (Cetrix Pharmaceutical Inc., Santa Clara, CA). Sodium chloride was purchased from Sigma (St. Louis, MO). Hepes (1, Hepsarin), bicine (bicine, bicine, and phosphatidylcholine) from Sigma. Phosphate-buffered saline (PBS) and calcium chloride solutions were from Sigma. Lipofectin reagent and G418 (Geneticin) were from Life Technologies, Inc. Dulbecco’s modified Eagle’s medium (DMEM) and CaCl2/MgCl2-free PBS were from Biological Industries (Beth Haemek, Israel).

**Cell Culture**—Ishikawa endometrial cancer cells and NIH 3T3 mouse fibroblasts were grown in DMEM containing penicillin (100 units/ml), streptomycin (0.1 mg/ml), nystatin (12.5 μg/ml), and 15% dialyzed fetal calf serum, respectively.

**Transfections**—Transfections of NIH 3T3 and Ishikawa cells were performed essentially as described previously (3, 14). Briefly, cells were co-transfected with pSVPl-IGF-I and pSVneo using Lipofectin reagent. Primary selection was done by growing transfected cells in the presence of 100 μg/ml G418 (Geneticin). Screening of clones for IGF-I expression was performed by flow cytometry (FACStar, Becton Dickinson, Mountain View, CA) using anti-IGF-IR antibody (IGF-IR-3B7, Santa Cruz Biotechnology, Santa Cruz, CA). The IGF-IR number was then determined as described below.

**Iodination Procedures**—IGF-I, IGF-II, des-(1–3)-IGF-I, and IGFBP-3 were iodinated by a mild chloramine-T procedure and were separated from the free iodine by gel filtration on a Sephadex G-25M PD-10 column (Pharmacia Biotech Inc.). Specific radioactivity was about 9000 cpm/nmol for IGF-I and des-(1–3)-IGF-I and 1100 cpm/nmol for IGFBP-3.

**Determination of IGF-IR and Cell Surface-associated IGFBPs**—IGF-IR assay was performed using 125I-labeled IGF-I essentially as we described previously (24). Cell surface-associated IGFBPs were determined by a procedure using unlabeled IGF-3 in the presence of 100 nM unlabeled des-(1–3)-IGF-I in every assay point to exclude 125I-IGF-I binding to IGF receptors (25). Radioligand binding was measured in monolayers of wild-type or transfected Ishikawa cells (150,000 cells/well), wild-type NIH 3T3 cells (100,000 cells/well), and IGF-IR-transfected NIH 3T3 cells (25,000 cells/well) in 24-well plates. Cells were washed twice with PBS and incubated for 2.5 h at 4 °C with 150,000 cpm of either 125I-IGF-I or 125I-labeled des-(1–3)-IGF-I in 0.2 ml of PBS containing 1 mg/ml essentially globulin-free bovine serum albumin (Sigma). After incubation, cells were washed three times with ice-cold PBS and dissolved in 0.5 M NaOH. Radioactivity was then measured in a γ-counter. The Kd values and the number of binding sites were analyzed as described by Munson and Rodbard (26) using the LIGAND program for the final Scatchard analysis.

**IGFBP-3 Binding Assay**—Ishikawa cells were trypsinized and seeded in 24-well plates (100,000 cells/well) in DMEM containing 0.5% fetal calf serum. The binding assay was carried out 8 h after plating to minimize the number of the naturally occurring cell surface-associated IGFBPs. Cells were washed with PBS followed by a 2.5-h incubation at room temperature with 100,000 cpm/well 125I-IGFBP-3 in the presence or absence of increasing concentrations of unlabeled IGFBP-3 in 200 μl of PBS containing 1 mg/ml essentially globulin-free BSA. The cells were then washed, and radioactivity was measured as above.

**Release of IGFBPs from the Cell Surface**—Ishikawa cells were seeded in 6-well plates at 300,000 cells/well. Two days later, cells were washed twice with PBS and incubated with 1 ml of PBS containing 0.5 mM AEBSF, 10 μg/ml leupeptin, 10 μg/ml aprotonin, 10 μg/ml trypsin inhibitor, and different test agents for 45 min at room temperature. Washouts from 3 wells were combined, desalted, and concentrated to a final volume of 40–50 μl in Centricon microconcentrators (10-kDa molecular mass cut-off).

**Chloride Treatment**—Cells were treated with sodium chloride according to published procedures (27, 28) and incubated in 7.5–7.75 mM sodium chloride solutions. DMEM supplemented with 15% dialyzed fetal calf serum and containing either 30 mM sodium chloride and 80 mM sodium chloride or 108 mM sodium chloride and 1 mM sodium sulfate for 48 h with medium replacement after 24 h. Cells were then plated in the same media into 24-well plates at a density of 80,000 cells/well. Two days later, the number of the cell surface-associated IGFBPs was determined as above.

**IGFBP Ligand Blots and Polyethylene Glycol-IGG Precipitation Assay**—Ligand blotting was performed essentially as described earlier (18). Samples were subjected to SDS-PAGE under non-reducing conditions followed by electrophoretic transfer to a nitrocellulose membrane. Filters were incubated overnight with 1.5 × 106 cpm 125I-IGF-II, washed, dried, and then analyzed by a radioactive image analyzer (BAS 1000, Fuji Photo Film Co., Tokyo, Japan). The rainbow 125I-methylated protein molecular mass markers (Amersham Corp., Buckinghamshire, United Kingdom) were used. Polyethylene glycol-IGG precipitation assay was performed as described (24).

**Intact Cell Tyrosine Phosphorylation**—Cells were seeded in 6-well plates and grown for 2 days. Confluent cell monolayers were serum-starved overnight before the experiment. Cells were washed twice with PBS followed by incubation with 0.3, 3, or 30 mM IGF-I or 125I-labeled IGF-I in 2 ml of PBS containing 1 mg/ml BSA (essentially globulin-free) at 37 °C for 3 min. Following stimulation, plates were placed on ice, and cells were washed rapidly with ice-cold PBS and frozen in liquid N2. Cells were then thawed on ice in 0.4 ml of lysis buffer (1% Triton X-100, 0.1% SDS, 50 mM HEPES, 150 mM sucrose, 80 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 2 mM EDTA, 2 mM EGTA, 2 mM sodium orthovanadate, 50 mM sodium fluoride, 0.5 mM AEBSF, 10 μg/ml leupeptin, 10 μg/ml aprotonin, and 10 μg/ml trypsin inhibitor), and the lysates were centrifuged at 12,000 × g for 20 min at 4 °C. The protein content was determined using BCA protein assay reagent (Pierce). 100-μg protein samples were fractionated by 7.5% SDS-PAGE under reducing conditions. The rainbow protein molecular mass markers (Amersham) were used. Proteins were then transferred to a polyvinylidene difluoride membrane (Bio-Rad), and the tyrosine-phosphorylated substrates were detected using rabbit polyclonal antiphosphotyrosine antibodies (Upstate Biotechnology Inc., Lake Placid, NY) at 1 μg/ml. Bands were detected by the Enhanced Chemiluminescence Kit (Amersham Corp.) according to the manufacturer’s suggested procedures. Quantitation was done by the Image Analysis System (GDS 5000, UVP Inc.). IRS-1 and the β-subunit of IGF-IR were identified by protein stripping and reblotting of the polyvinylidene difluoride membranes with anti-IRS-1 (IRS-1-CT, Upstate Biotechnology, Inc.) and anti-IGF-IR β-subunit (IGF-IRβ, Santa Cruz Biotechnology, Inc.) antibodies, respectively.

**Determination of c-Fos**—Confluent cells in 6-well plates were starved overnight in the serum-free medium, rinsed twice with PBS, and incubated with either IGF-I or des-(1–3)-IGF-I. Total cell lysates were then prepared according to the protocol recommended by Transduction Laboratories (Lexington, KY). Cells were washed with PBS, followed by addition of boiled buffer (1% SDS, 10 mM Tris-HCl, pH 7.4). After a 5-min boiling, the lysates were homogenized by repetitive passing through a 26-gauge needle and clarified by centrifugation at 12,000 × g at 4 °C. The nuclear pellet was resuspended in buffer containing 20 mM HEPES, pH 7.9, 1.5 mM MgCl2, 0.5 mM dithiothreitol, 0.5 mM AEBSF, 10 mM mg aprotonin, and 10 μg/ml leupeptin. The lysates were lysed by passing through the 26-gauge needle, and nuclei were collected by centrifugation at 12,000 × g at 4 °C. The nuclear pellet was resuspended in buffer containing 20 mM HEPES, pH 7.9, 1.5 mM MgCl2, 0.5 mM dithiothreitol, 25% (v/v) glycerol, 0.2 mM EDTA, 0.42 mM NaCl, 0.5 mM AEBSF, 10 μg/ml aprotonin, and 10 μg/ml leupeptin and was lysed by passing through the 21-gauge needle. Lysates were clarified by centrifugation at 12,000 × g at 4 °C and dialyzed against 50

Washouts from 3 wells were combined, desalted, and concentrated to a final volume of 40–50 μl in Centricon microconcentrators (10-kDa molecular mass cut-off).
TABLE I

| Cell types    | IGFRs | IGFBPs |
|---------------|-------|--------|
|               | number/cell \( \times 10^{-3} \) | number/cell \( \times 10^{-3} \) |
| Ishikawa      | 16.8 \( \pm \) 4.3 | 295.9 \( \pm \) 42.3 |
| IGR14         | 299.6 \( \pm \) 34.5 | 299.2 \( \pm \) 37.4 |
| IGR21         | 305.2 \( \pm \) 32.9 | 302.1 \( \pm \) 29.9 |
| MCF-7         | 28.0 \( \pm \) 5.6 | 92.4 \( \pm \) 8.8 |
| NIH-3T3       | 15.6 \( \pm \) 5.7 | 6.2 \( \pm \) 2.1 |
| NR9           | 971.9 \( \pm \) 54.3 | 6.7 \( \pm \) 2.9 |
| NR15          | 968.1 \( \pm \) 69.6 | 7.1 \( \pm \) 3.4 |

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In NIH 3T3 and Ishikawa cells were grown in DMEM supplemented with 10 or 15% fetal calf serum, respectively, in 24-well plates. The number of IGFRs and cell surface-associated IGFBPs was determined and calculated as described under “Experimental Procedures.” Data are means \( \pm \) S.E. of three independent experiments, each done in triplicate. Results on MCF-7 cells were calculated from our previously published data (25).

RESULTS

Comparison of IGF-I and des-(1–3)-IGF-I Binding and the Ligand-induced Tyrosine Phosphorylation in NIH 3T3 Cells—To elucidate the role of cell surface-associated IGFBPs in modulating the IGF-IR signaling, we compared the effects of IGF-I and des-(1–3)-IGF-I on several components of the IGF-IR signal transduction pathway under conditions that do not favor significant accumulation of soluble IGFBPs. Since des-(1–3)-IGF-I binds to IGFBPs with extremely low affinities, the difference in effects of IGF-I and des-(1–3)-IGF-I on IGF-IR signaling in cells possessing a sufficiently high number of membrane IGFBPs could be attributed to modulation of the receptor function by these proteins. To validate this approach it should be examined whether both ligands are equipotent not only in IGF-IR binding but also in the receptor activation in cells where IGFRs substantially outnumber cell surface-associated IGFBPs.

NIH 3T3 mouse fibroblasts were chosen for this purpose since we found these cells to have a very low number of membrane IGFBPs (6,200 molecules/cell) as compared with other cell lines tested in our laboratory (Table I). However, the IGFR number in the wild-type NIH 3T3 cells was also quite low (15,600 molecules/cell), thus reducing sensitivity of the receptor function assay. Therefore, the cells were stably transfected with hIGF-IR. The NR9 and NR15 transfected clones used in the experiments showed a dramatic increase in IGF-IR number (971,900 and 968,000 molecules/cell, respectively) without a significant change in number of the cell surface-bound IGFBPs (6,700 and 7,100 molecules/cell, respectively), i.e. 96% of the total IGF-I binding sites in these clones were represented by IGF-IR. Fig. 1 demonstrates Scatchard plots of \( ^{125}\text{I}-\text{IGF-I} \) and \( ^{125}\text{I}-\text{des-(1–3)-IGF-I} \) binding to NR9 cells. The data clearly indicate that both ligands have the same affinity to hIGF-IR (\( K_d = 2 \text{ nM} \)). As shown in Fig. 2, the IGF-I and des-(1–3)-IGF-I induced hIGF-IR tyrosine autophosphorylation in these cells with a similar dose response. Thus, the above results demonstrate equipotency of IGF-I and des-(1–3)-IGF-I in both IGF-IR binding and activation.

Effects of cell surface-associated IGFBPs on IGF-IR function were studied in Ishikawa endometrial cancer cells naturally expressing a large number of cell surface-associated IGFBPs (mostly IGFBP-3), which is about 20-fold higher than that of IGF-IR (24). IGFBP-3 is known to be capable of attaching to both the extracellular matrix and the cell membrane (6, 7). Its characterisation of Association of IGFBPs with Ishikawa Cells—To understand the nature of IGFBP association with the Ishikawa cell surface, we examined the ability of various agents to remove these proteins from cells. As shown in Fig. 3A, a 45-min incubation of cells with buffer alone or a divalent metal-ion chelator (EDTA (5 mM)) did not cause release of the IGFBPs into the incubation medium. Cell exposure to a high ionic strength solution (1 M NaCl) led to a dissociation of only a trace amount of IGFBPs. Essentially the same effect was ob-
Chartracterization of IGFBP-3 association with the Ishikawa cell surface. A, ligand blotting of IGFBPs removed from Ishikawa cells by different treatments. Subconfluent cells in 6-well plates were washed twice with PBS and incubated with buffer alone (lane 2), 5 mM EDTA (lane 3), 1 mM NaCl (lane 4), 5 units/ml heparinase and heparitinase (lane 5), 1 mM NaSCN (lane 6), and 1 unit/ml phosphatidylinositol-phospholipase C (lane 7) at room temperature for 45 min. Washouts from 3 wells were combined and concentrated as described under “Experimental Procedures,” followed by ligand blotting. Human serum (4 μl) was used as a control (lanes 1 and 8). B, immunoblot of the NaSCN-washout (see panel A, lane 6) with anti-IGFBP-3 antibody.

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FIG. 3. Characterization of IGFBP-3 association with the Ishikawa cell surface. A, ligand blotting of IGFBPs removed from Ishikawa cells by different treatments. Subconfluent cells in 6-well plates were washed twice with PBS and incubated with buffer alone (lane 2), 5 mM EDTA (lane 3), 1 mM NaCl (lane 4), 5 units/ml heparinase and heparitinase (lane 5), 1 mM NaSCN (lane 6), and 1 unit/ml phosphatidylinositol-phospholipase C (lane 7) at room temperature for 45 min. Washouts from 3 wells were combined and concentrated as described under “Experimental Procedures,” followed by ligand blotting. Human serum (4 μl) was used as a control (lanes 1 and 8). B, immunoblot of the NaSCN-washout (see panel A, lane 6) with anti-IGFBP-3 antibody.

FIG. 4. Scatchard analysis of 125I-IGFBP-3 binding to Ishikawa cells. Binding assays were performed as described under “Experimental Procedures” in the presence of increasing concentrations of unlabeled IGFBP-3. The results are adjusted for 100,000 cells. A representative of three similar experiments is shown.

Effects of Membrane IGFBPs on Short and Medium Term IGF-IR Signaling—Because of a low IGF-IR number in Ishikawa cells (Table I), sensitivity of the tyrosine phosphorylation assay in whole cells was not sufficient for performing a quantitative analysis of the IGF-1 and des-(1–3)-IGF-I-induced receptor autophosphorylation at low ligand concentrations (data not shown). To increase the assay sensitivity in this cell system, Ishikawa cells were transfected with hIGF-IR. From several transfectant sublines obtained, two clones (IGR14 and IGR21) with an approximately equal number of IGF-IRs and membrane-bound IGFBPs (Table I) were utilized. Comparison of the effects of these two ligands at three different concentrations (0.3, 3, and 30 nM) indicates that des-(1–3)-IGF-I is about one order of magnitude more potent than IGF-I, i.e. an at least 10 times higher concentration of IGF-I was required to produce the same effect on IGF-IR and IRS-1 tyrosine phosphorylation as that induced by des-(1–3)-IGF-I (Fig. 5).

To examine whether differences in the effects of IGF-I and des-(1–3)-IGF-I can also be observed downstream of IRS-1 tyrosine phosphorylation, medium term mitogenic signaling events (namely expression of the immediate early gene-encoded product c-Fos and the binding capacity of the AP-1 (Fos/Jun) transcriptional regulatory complex) were studied. The time course of IGF-I- and des-(1–3)-IGF-I-induced expression of c-Fos in the IGR21 clone is shown in Fig. 6A. Results indicate that the level of c-Fos protein, which was undetectable in non-stimulated cells, was appreciable after a 45-min incubation with each ligand. The maximal c-Fos level was observed after about 90 min followed by its down-regulation as seen by 135 min of stimulation. At all time points the effect of des-(1–3)-IGF-I on c-Fos induction was clearly stronger than that of IGF-I (Fig. 6C). Determination of a dose response of the IGF-I and des-(1–3)-IGF-I effects (Fig. 6B) at 90 min of incubation revealed that to obtain the same level of c-Fos expression, a 20 times higher concentration of IGF-I than that of des-(1–3)-IGF-I should be applied (Fig. 6D). During this period of time, no detectable accumulation of soluble IGFBPs was observed by either ligand blotting or IGF-I binding assay using polyethylene glycol-IgG precipitation (not shown).

To test whether differences observed in induction of the c-Fos by IGF-I and des-(1–3)-IGF-I may affect binding capacity of the AP-1 transcriptional regulatory complex, we examined its binding to the oligonucleotide representing the TRE consensus sequence in IGR21 cell nuclear extracts. The analysis was performed following a 90-min treatment with 30 ng IGF-I or des-(1–3)-IGF-I when the maximal c-Fos expression was observed (see Fig. 6, A and C). Using the electrophoretic mobility shift assay approach (see “Experimental Procedures”), we found that both IGF-I and des-(1–3)-IGF-I significantly enhanced AP-1 binding capacity (Fig. 7, compare lanes 2 and 3 to lane 1). The specificity of binding was confirmed by competition with a 100-fold excess of non-labeled TRE oligonucleotide. Results demonstrate that at the concentration applied, des-(1–3)-IGF-I is 2 times more effective than IGF-I in stimulating AP-1 binding (Fig. 7, compare lanes 2 and 3), which is consistent with the pattern of c-Fos induction by the two ligands (see Fig. 6).

Taken together, the results demonstrate that in Ishikawa cells, membrane-bound IGFBP-3 can attenuate the IGF-I-induced IGF-IR signaling.
Cell surface-associated IGFBP-3 has been shown to produce either inhibition (8, 9, 19, 20) or potentiation (12–17) of cellular IGF effects. The direction of the effect may depend on the cell type as was particularly demonstrated in the study by Rogers et al. (20), which showed that preincubation with human recombinant IGFBP-3 potentiates the mitogenic action of IGF in bovine fibroblasts but inhibits this effect in human placental fibroblasts. However, it is unclear whether modulation of IGF-IR signaling is involved in these phenomena. To clarify this question we employed a well established difference between affinities of IGFBPs for IGF-I and its naturally occurring truncated analog des-(1–3)-IGF-I in examining short and middle term cell signaling responses to IGF-IR activation. This was performed under conditions where the amount of soluble IGFBPs was insignificant. We have recently shown that IGF-I and des-(1–3)-IGF-I display a similar affinity for IGF-IR (24) in Ishikawa endometrial cancer cells, which is consistent with the data obtained in L6 myoblasts (21) as well as rat cortical and medullar renal tissue (32). Here, in NIH 3T3 mouse fibroblasts overexpressing IGF-IR we demonstrated directly that radiolabeled IGF-I and des-(1–3)-IGF-I bind to IGF-IR with the same affinity (Fig. 1). It was reported previously that IGF-I and des-(1–3)-IGF-I display a similar affinity for IGF-IR (24) in Ishikawa cells (where more than 95% of 125I-labeled IGF-I is bound to IGF-IR) (23). Affinity of the cell surface-associated IGFBPs for IGF-I is sufficient to show that in these cells IGFBP-3 is specifically associated with the cell membrane. This is consistent with the data of Oh et al. (9) showing the presence of specific membrane proteins responsi-
FIG. 7. Effects of IGF-I and des-(1–3)-IGF-I on AP-1 binding capacity in IGR21 cells. Subconfluent cell monolayers were incubated in 75-cm² cell culture flasks in serum-free medium for 24 h. Cells were washed twice with PBS and treated for 30 min with buffer alone or with 30 nM IGF-I or des-(1–3)-IGF-I. Nuclear extracts were then prepared as described under “Experimental Procedures.” Samples (10 μg of protein) were incubated with [γ-32P]ATP end-labeled TRE oligonucleotide at room temperature for 30 min with or without 3-min preincubation in the presence of a 100-fold excess of non-labeled TRE oligonucleotide. DNA-protein complexes were separated on 5% Tris borate/EDTA polyacrylamide gel. Lane 1, buffer alone; lane 2, IGF-I, lane 3, des-(1–3)-IGF-I, lanes 4 and 5, IGF-I and des-(1–3)-IGF-I after preincubation with a cold probe; lane 6, [γ-32P]ATP end-labeled TRE oligonucleotide alone. Representative of four similar experiments is shown. Statistical analysis of four experiments is shown as mean ± S.E.

We have demonstrated here for the first time that in Ishikawa endometrial cancer cells, IGFBP-3 specifically associated with the cell membrane inhibits IGF-IR signaling in an IGF-dependent manner as revealed by measurement of short and middle term receptor-mediated responses. These results are consistent with our previous findings showing that treatment with different human cancer cell growth modulators (tamoxifen and estradiol) results in modulation in cell surface-associated IGFBPs (18, 25). These changes are negatively correlated with both cell growth and IGF-IR activity. Taken together our data strongly suggest that in certain cell types, biological action of IGFs is negatively controlled not only by their sequestering by soluble IGFBPs, but also due to attenuation of the IGF-IR signaling by the cell surface IGFBP-3. The latter activity adds a novel regulatory loop to the powerful IGF system. Its nature is unclear and will therefore be the subject of further studies.

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