Journal of Cellular Biochemistry 66:256–267 (1997)

Use of Lanthanum to Accurately Quantify Insulin-Like Growth Factor Binding to Proteins on Cell Surfaces

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Abstract Insulin-like growth factor binding proteins (IGFBPs) are found both associated with cells and in extracellular fluids. Cell-associated IGFBPs increase [^{125}I]-IGF binding to cell monolayers, whereas extracellular (soluble, released) IGFBPs decrease binding. In the current study, we show that either IGFBP-3 or IGFBP-5 are the major forms of IGFBP released from monolayers of human GM10 fibroblasts, T98G glioblastoma cells and forskolin-treated bovine MDBK cells. IGFBPs represent the most abundant [^{125}I]-IGF-I binding site on GM10 and T98G cell monolayers, but 4–17% of the total cell-associated IGFBPs are released from the cell monolayer at 8°C during their quantification. Most of the IGFBPs ($>$ 70%) are released from MDBK cells. Quantitative estimates of [^{125}I]-IGF binding to the cell monolayers are altered because of the ability of the released IGFBPs to reduce the amount of radiolabeled ligand that is available to bind to the cell surface. Lanthanum (La^{3+}) depresses IGFBP release from all three cell types ($>$ 80% for GM10 and T98G cells and $>$ 65% for MDBK cells). The effect was cation specific, noted with La^{3+} or Zn^{2+} but not with either Mn^{2+}, Sr^{2+} or Se^{3+}. The effect was also IGFBP specific; La^{3+} markedly depressed the release of IGFBP-3 and IGFBP-5, but had less of an effect on IGFBP-2 and IGFBP-4. Concomitant with a decrease in IGFBP-3 and IGFBP-5 release, La^{3+} caused an increase in [^{125}I]-IGF-I binding to cell-associated IGFBPs and type I IGF receptors. The released soluble IGFBPs have a three- to 20-fold greater affinity (K_a) for [^{125}I]-IGF-I compared to cell-associated IGFBPs. La^{3+} did not alter the affinity constants of cell-associated IGFBPs. In summary, we have identified a means to prevent loss of IGFBPs from cell monolayers during binding assays. This procedure will be useful in accurately quantifying the levels of IGFBPs on cell monolayers and in determining the role of cell-associated IGFBPs in controlling IGF activity. Retention of cell-associated low affinity IGFBPs may be important in controlling the size of the pericellular IGF pool and in regulating IGF-I access to the type I IGF receptor. J. Cell. Biochem. 66:256–267.

Key words: zinc; IGFBP; IGF; des-(1-3)-IGF-I; receptor; fibroblasts; glioblastoma; kidney epithelial cells; affinity; T98G; GM10; MDBK

The insulin-like growth factor binding proteins (IGFBPs) modulate IGF actions because of their ability to bind IGF-I and IGF-II [Jones and Clemmons, 1995; McCusker and Clemmons, 1992]. The various forms of extracellular soluble IGFBP have been shown to control IGF actions by blocking IGF association with the type I IGF receptor [Gopinath et al., 1989; Han et al., 1988; Knauer and Smith, 1980; Ritvos et al., 1988; McCusker et al., 1991]. This inhibitory action was recognized early in the study of IGFBP action when the IGFBPs were considered solely as carrier proteins. Work on the carrier protein role of the IGFBPs proceeded without rival until they were shown to potentiate IGF activity [Elgin et al., 1987]. With this finding it was apparent that soluble IGFBPs could not act to potentiate IGF activity. In addition to their presence in extracellular fluids, IGFBP-1, IGFBP-2, IGFBP-3 and IGFBP-5 have been shown to bind to cultured cells [Conover, 1991; Clemmons et al., 1987; Martin et al., 1992; McCusker et al., 1990; Orlowski et al., 1989; Holly et al., 1989; DeVroede et al., 1986]. IGFBP cell-association may control cellular activity by direct interactions with integral membrane proteins, the extracellular matrix or cell-associated glycosaminoglycans [Hodgkinson et al., 1994; Jones et al., 1993a, 1993b; Arai et al.,

Contract grant sponsor: National Institutes of Health, contract grant number AG-02331.

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Received 20 February 1997; accepted 17 April 1997

1996]. Indeed IGFBP-1 has IGF-independent actions mediated through integrin receptors [Jones et al., 1993b]. The mechanism by which the IGFBPs enhance IGF activity however is still undefined. The existence of cell-associated IGFBPs implies that they may control IGF activity by localizing the IGFs onto cell surfaces. This effect does not appear to involve direct interaction of IGFBPs with the type I IGF receptor, but provides a reservoir of IGFs in the pericellular milieu.

We have previously shown that IGFBPs that are associated with monolayers of human fibroblasts and glioblastoma cells are released even at 8°C and they act as partitioning agents in controlling the localization of [^{125}I]-IGF-I between the extracellular fluid and cell surface [McCusker et al., 1990]. Partitioning is a function of the distribution of IGFBPs between extracellular fluids and the cell surface. There are several means to quantify extracellular soluble IGFBP content: IGF-binding capacity, ligand blot analysis and radioimmunoassay. Each of these assays has distinct advantages and problems especially relevant to quantifying cell-associated IGFBPs. Ligand blot and radioimmunoassay of cell extracts do not differentiate between intracellular and cell-associated IGFBPs since, at present, there are no means to extract only cell-surface associated IGFBPs. Thus, the only means to quantify cell surface-associated IGFBP content is by direct [^{125}I]-IGF-I binding assays or affinity crosslinking.

In the current studies, we have investigated the factors controlling the release of IGFBPs from cell monolayers. In our previous work, it was difficult to accurately determine the amount of IGFBP on cell monolayers due to their release during direct [^{125}I]-IGF-I binding assays. IGFBP release is responsible for unusual binding characteristics including the 'paradoxical increase' in [^{125}I]-IGF-I binding that occurs following the addition of unlabelled IGF-I. This phenomenon limits the usefulness of Scatchard analysis for monolayer binding data [McCusker et al., 1990]. To obtain a more complete and reliable estimate of monolayer binding sites, it would be beneficial to maintain the IGFBP cell surface association during the binding assay. In this report, we demonstrate that IGFBP release from cell monolayers can be suppressed by the addition of either La^{3+} or Zn^{2+}. La^{3+} is particularly useful in quantifying IGFBP abundance on cell monolayers because it does not affect the affinity of cell-associated IGFBPs.

MATERIALS AND METHODS

Materials

GM10 and T98G (1690) cells are a human fibroblast cell line derived from a 12-week-old fetus and a human glioblastoma cell line, respectively. Both cell lines were purchased from the NIGMS Human Genetic Mutant Cell Repository (Camden, NJ). MDBK cells are bovine kidney epithelial cells and were purchased from the American Type Culture Collection (Rockville, MD). Culture medium was purchased from Hazelton Systems (Denver, PA). Calf serum (CS) was purchased from Colorado Serum (Denver, CO). Unlabeled recombinant human IGF-I was purchased from Bachem (Torrence, CA) and sodium-insulin was purchased from E.R. Squibb (Princeton, NJ). Des-IGF-I was purchased from GroPep (Adelaide, Australia). The four-point mutated QAYL-IGF-I (Gln^3,Ala^4,Tyr^{15},Leu^{16}-IGF-I) was a generous gift from M.A. Cascieri at Merck Sharp and Dohme (Rahway, NJ). IGF-I was iodinated as previously described [D'Ercole et al., 1976]. All other chemicals were purchased from Sigma (St. Louis, MO) unless noted.

Methods

Both GM10 and T98G cells were grown in Eagle's minimal essential medium (EMEM) containing 10% CS and 10 U/ml penicillin and 10 µg/ml streptomycin [McCusker et al., 1990]. MDBK cells were grown in Dulbecco's minimal essential medium (DMEM) containing 10% heat-inactivated CS and antibiotics. For binding assays, cells were cultured on 2-cm^2 multiwell plates (Falcon 3047, Oxnard, CA) and initially plated at 1×10^4 cells/cm^2. Confluent, 7–8-day cultures were used for the binding assays. Binding of IGF-I to cell monolayers was performed as previously described [McCusker et al., 1991]. The cultures were washed twice with phosphate buffered saline (PBS) and once with Hank's balanced salt solution then incubated (at 8°C) with 250 µl of assay buffer (EMEM, 20 mM HEPES and 1 mg/ml bovine serum albumin, pH 7.4). Unlabeled IGF-I or insulin were added at the indicated concentrations in the presence or absence of cations. Between 70,000 and 90,000 cpm (≈1 ng/ml) of

[^{125}I]-IGF was added/well. In all but two experiments (Table III and Fig. 8), 10 µg/ml of sodium-insulin was included in all assay wells to block [^{125}I]-IGF binding to the type I IGF receptor [McCusker et al., 1990]. At the end of the incubation period (~3 h) the assay buffer was collected and transferred into 12 × 75 mm tubes. The cells were then rinsed, solubilized with 0.3 M NaOH and transferred to tubes. Cell bound [^{125}I]-IGF-I was determined by counting with a gamma-spectrometer (Beckman, Fullerton, CA).

The assay buffer (250 µl) was incubated for 20 min at 8°C with 250 µl of 1% human immunoglobulin and 500 µl of 25% polyethylene glycol 8000. The mixture was vortexed and centrifuged as previously described [McCusker et al., 1991]. [^{125}I]-IGF-I bound to IGFBPs in the pellet was quantified by gamma spectrometry and is an estimate of the soluble IGFBP activity that has been released from the cell surface during the binding assay.

The results are expressed either as counts per minute of [^{125}I]-IGF-I bound or as changes in [^{125}I]-IGF-I bound as a percentage of the control. Binding affinity (K_a) and number of binding sites (R_o) of the cell-associated IGFBPs or soluble 'released' IGFBPs were calculated by Scatchard analysis. The computer program that was used corrects the amount free at each point to be the [total added] − [amount bound to cell-associated IGFBPs + amount bound to soluble 'released' IGFBPs].

Ligand blot analysis was performed as previously described [McCusker et al., 1989]. In brief, confluent cell monolayers (which had been solubilized with 1 × Laemmli buffer; 50 µl/cm^2) or assay buffer (250 µl collected/well plus 83 µl 4 × Laemmli buffer) were electrophoresed (30 µl samples) through 12.5% polyacrylamide gels. After electrophoresis, proteins were transferred to Immobilon-P. IGFBPs were then visualized by incubating the membrane with [^{125}I]-IGF-I followed by washing and autoradiography. IGFBP sizes were estimated by comparing their relative mobility to that of prestained molecular weight standards run in parallel lanes (Rainbow markers, Amersham, Arlington Heights, IL).

RESULTS

IGFBPs from cell monolayers were visualized by ligand blot analysis (Fig. 1). GM10 cell extracts contain primarily the 46,000 M_r IGFBP-3 with a lesser amount of the 32,000 M_r IGFBP-5 (left). In contrast, T98G cell extracts contain primarily the 32,000 M_r IGFBP-5 and lesser amounts of the 27,000 M_r IGFBP-4 (middle). Extracts of forskolin-treated MDBK cells contain primarily the 43,000 M_r IGFBP-3 followed by lesser amounts of the 27,000 M_r IGFBP-4 and the 34,000 M_r IGFBP-2 (right). Untreated MDBK cell extracts contain primarily IGFBP-2.

IGFBP release from cell monolayers occurs at 8°C as determined by ligand blot activity (Fig. 2). GM10 cells release only the 46,000 M_r IGFBP-3. IGFBP-3 release is depressed if either La^{3+} or Zn^{2+} are present during the binding assay. T98G cells release the 32,000 M_r IGFBP-5 and 27,000 M_r IGFBP-4. IGFBP-5 release is markedly depressed by La^{3+}, whereas IGFBP-4 release is only slightly affected. MDBK

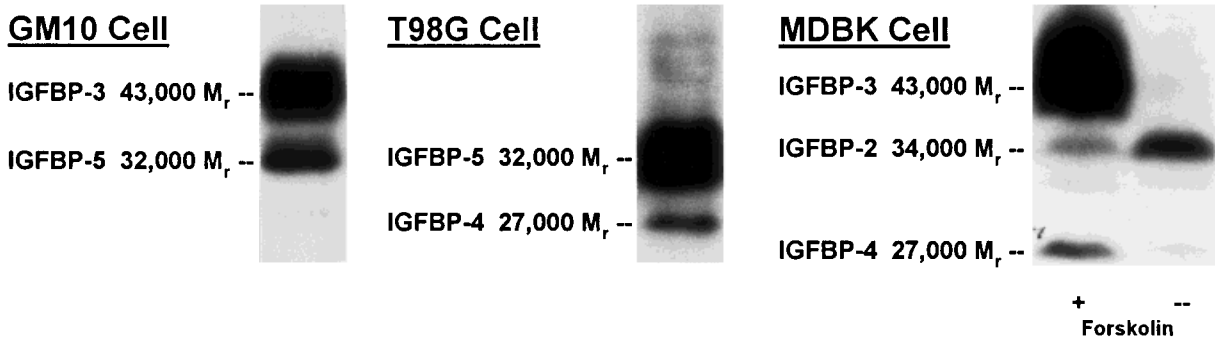

Fig. 1. IGFBP content of cell monolayers. Monolayer cultures were extracted with Laemmli sample buffer for SDS-PAGE and ligand blot analysis was performed as described in Materials and Methods. All cultures were incubated overnight (~18 h) in serum-free media before extraction. MDBK cells were either treated with (+) or without (--) 5 µM forskolin during the overnight incubation. The size of each IGFBP band was estimated by comparison to the migration of molecular weight standards run in parallel lanes. The identities of the IGFBPs have previously been published [Cohick and Clemmons, 1991; Camacho-Hubner et al., 1992].

cells, pre-treated overnight with forskolin, release IGFBP-3, IGFBP-2 and IGFBP-4 during the binding assay. The release of IGFBP-2 and IGFBP-3 is depressed by La^{3+} with IGFBP-3 being the most significantly suppressed. In contrast, in the absence of forskolin treatment, MDBK cells release IGFBP-2 and its release is slightly depressed by the presence of La^{3+}.

[^{125}I]-IGF-I binding to GM10 cell-associated IGFBPs and soluble released IGFBPs was performed to quantify the distribution of IGF binding activity and to determine the effect of multivalent cations on IGFBP release (Fig. 3). [^{125}I]-IGF-I binds to both cell-associated (top) and IGFBPs released into the assay buffer (bottom). La^{3+}, but not Zn^{2+} or Mn^{2+}, increases [^{125}I]-IGF-I binding to GM10 cell-associated IGFBPs. In contrast, both Zn^{2+} and La^{3+} depress IGFBP release from GM10 cells and Mn^{2+} is ineffective. Similar to Mn^{2+}, Sr^{2+} and Se^{3+} also do not affect binding of [^{125}I]-IGF-I to released or cell-associated IGFBPs (not shown). Thus La^{3+} and Zn^{2+} depress release of cell-associated IGFBPs, but Zn^{2+} apparently decreases IGF-I binding to cell-associated IGFBPs since an increase in [^{125}I]-IGF-I binding to cell-associated IGFBPs cannot be detected.

The addition of increasing concentrations of La^{3+}, not Zn^{2+}, is associated with an increase in cell surface [^{125}I]-IGF-I binding. Both La^{3+} and Zn^{2+} decrease binding activity in the assay buffer (Fig. 4). The effect of La^{3+} is evident at 10 μM, whereas between 10 and 50 μM Zn^{2+} is needed to depress IGFBP release from cell monolayers. La^{3+} significantly increases binding of [^{125}I]-IGF-I to GM10, T98G and forskolin-treated MDBK cells (Table I). Binding to cell-associated IGFBPs is increased by 51% for GM10 cells and 3.4- and 4.2-fold for T98G and MDBK cells, respectively. Concurrent with this effect, La^{3+} depresses the release of IGFBPs from the surface of all three cell types. IGFBP release is depressed by 84% for GM10 cells, 87% for T98G cells and 66% for MDBK cells.

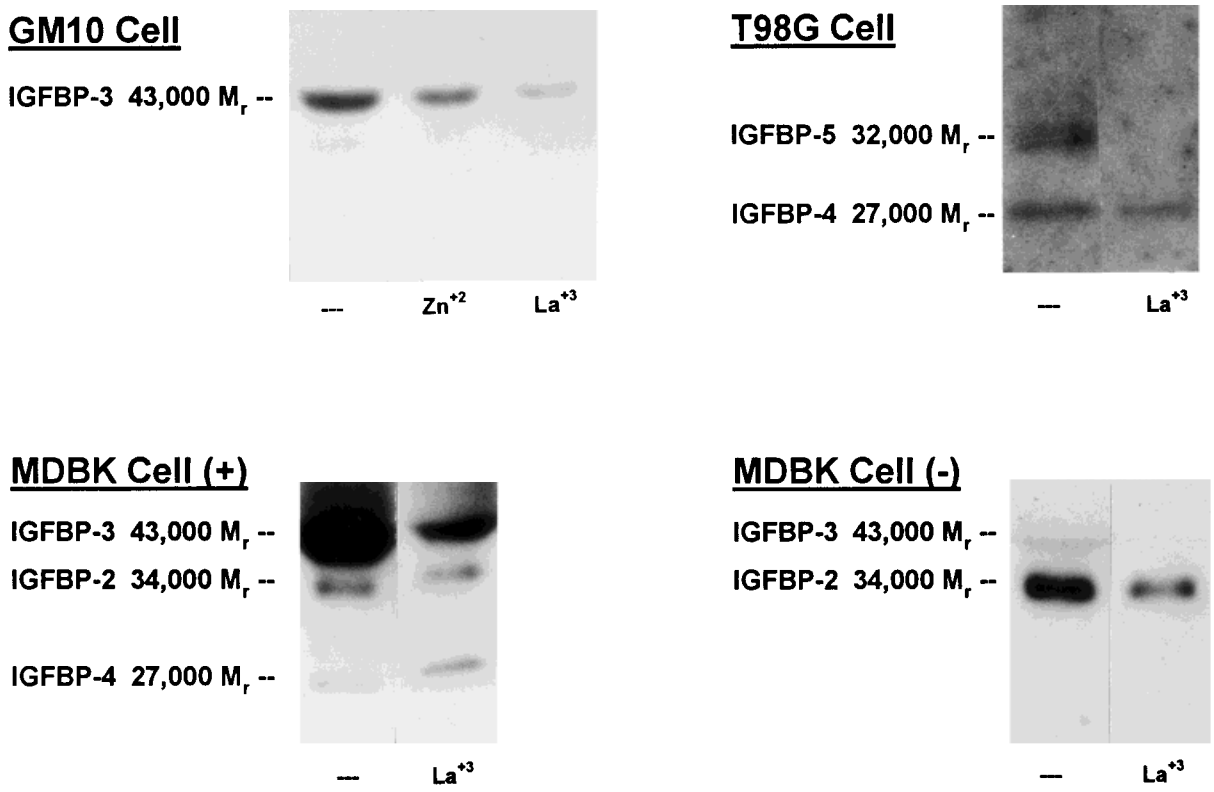

Fig. 2. Release of IGFBPs from cell monolayers. Assay buffer was collected at the end of a typical binding assay, except that no [^{125}I]-IGF-I was added. The cultures were incubated with or without La^{3+} (100 μM) or Zn^{2+} (200 μM). Samples were analyzed by ligand blot analysis as described in Materials and Methods. The size of each band was estimated by comparison to the migration of molecular weight standards run in parallel lanes. The identities of the IGFBPs have been confirmed previously [Cohick and Clemmons, 1991; Camacho-Hubner et al., 1992].

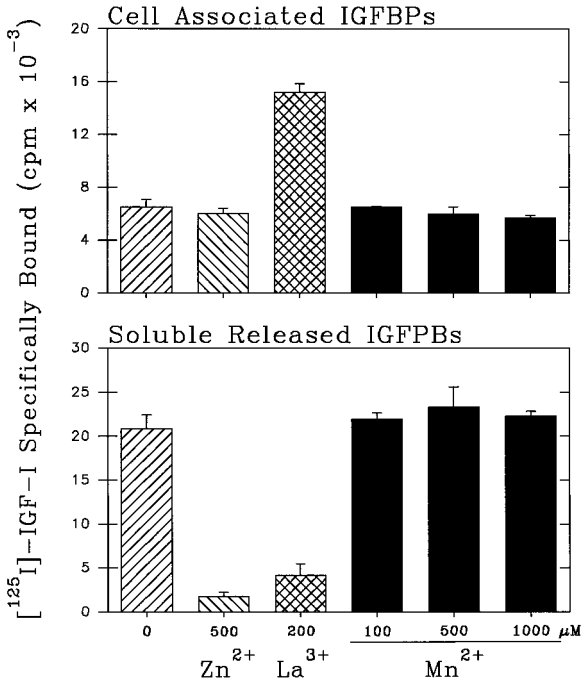

Fig. 3. Cation specific effects on IGFBP release from GM10 cell monolayers. Binding was determined with and without the addition of La^{3+}, Zn^{2+} or Mn^{2+} at the indicated concentrations. All wells contained 10 μg/ml of insulin to prevent binding to the type I IGF receptor. Thus, [^{125}I]-IGF-I binding to the cell-associated IGFBPs (top) and released IGFBPs (bottom) was quantified as described in Materials and Methods. Other cations that were tested include Se^{3+} and Sr^{3+}. They were without effects on [^{125}I]-IGF-I binding to either cell-associated or released IGFBP-3. Data represent the means ± SD of duplicates.

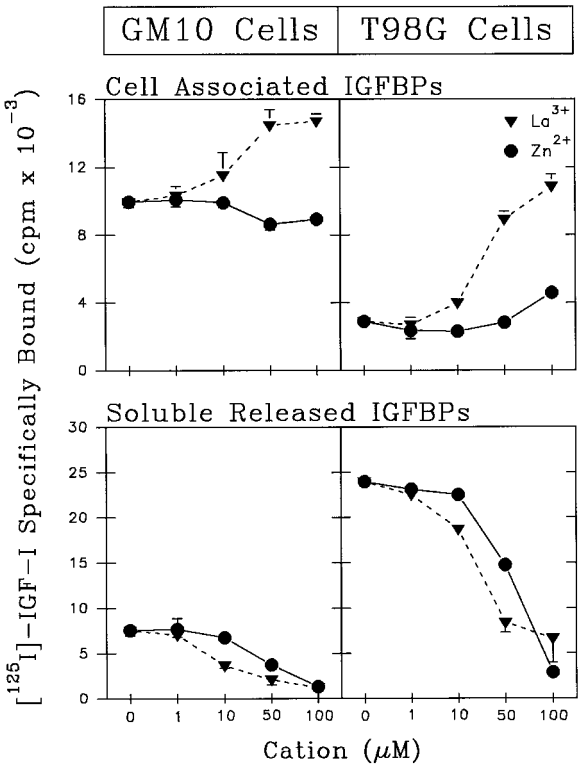

Fig. 4. Dose dependent effects of La^{3+} and Zn^{2+} on [^{125}I]-IGF-I binding to GM10 and T98G IGFBPs. Binding was analyzed with and without the addition of La^{3+} or Zn^{2+} at the indicated concentrations. All wells contained 10 μg/ml of insulin to prevent binding to the type I IGF receptor. Thus, [^{125}I]-IGF-I binding to the cell-associated IGFBPs (top) and released IGFBPs (bottom) was quantified using GM10 (left) and T98G (right) cells as described in Materials and Methods. Data represent the means ± SD of duplicates. Error bars are shown only if they are larger than the symbols.

T98G cells release the most IGFBP activity followed by MDBK cells and finally GM10 cells.

To confirm that measurements of cell surface binding were quantifying binding only to IGFBPs and not receptors, binding specificity studies were performed using GM10 cells (Fig. 5). There was minimal binding of [^{125}I]-QAYL-IGF-I and low binding of [^{125}I]-des-IGF-I relative to [^{125}I]-IGF-I to the cell monolayer (top) in the presence of insulin excess. [^{125}I]-QAYL-IGF-I binding to the cell-associated (top) and released IGFBPs (bottom) was not affected by the addition of La^{3+}. In contrast, La^{3+} increased specific binding of both [^{125}I]-IGF-I and [^{125}I]-des-IGF-I to the cell-associated IGFBPs (twofold and 50%, respectively) and decreased specific binding to the soluble 'released' IGFBPs. Thus, [^{125}I]-IGF-I and [^{125}I]-des-IGF-I specifically bind to cell-associated IGFBPs and released IGFBPs (primarily IGFBP-3). Binding of [^{125}I]-IGF-I was three- to fivefold greater than that of [^{125}I]-des-IGF-I. In control cultures, the competitive binding curve for [^{125}I]-IGF-I shows a paradoxical increase in binding with the addition of low levels of unlabelled IGF-I; an effect that was not present with La^{3+}.

Analysis of binding specificity using T98G cells gave similar results (Fig. 6). [^{125}I]-QAYL-IGF-I and [^{125}I]-des-IGF-I had relatively low binding compared to [^{125}I]-IGF-I to the cell monolayers (top) and to released IGFBPs (bottom). Specific binding of [^{125}I]-IGF-I to the cell monolayer was increased more than twofold and specific binding to the soluble 'released' IGFBPs was decreased by >80% with the addition of La^{3+}. Binding of [^{125}I]-des-IGF-I to released IGFBPs was depressed by La^{3+} but binding to cell-associated IGFBPs was not affected by La^{3+}. Thus, [^{125}I]-IGF-I and [^{125}I]-des-IGF-I specifically bind to IGFBPs (primarily IGFBP-5)

TABLE I. Effect of La^{3+} on [^{125}I]-IGF-I Binding to GM10, T98G and MDBK Cell-surface and Released IGFBPs

Cell	Binding site	Control	La^{3+}
GM10	Surface IGFBPs (7,207)	100 ± 9	151 ± 13*
	Released IGFBPs (6,972)	100 ± 16	16 ± 5*
T98G	Surface IGFBPs (4,179)	100 ± 15	340 ± 24*
	Released IGFBPs (18,870)	100 ± 11	13 ± 3*
MDBK	Surface IGFBPs (1,343)	100 ± 24	419 ± 58*
	Released IGFBPs (10,519)	100 ± 9	34 ± 4*

Values represent the mean ± SE for the change in specific binding of [^{125}I]-IGF-I in the presence of 200 μM La^{3+}; data are expressed as percent of same site control. The number of determinations are 10, 9 and 8 for GM10, T98G and MDBK cells, respectively. Average control values (cpm bound) are shown in parentheses.
*Indicates significant differences ($P < 0.05$) between control and La^{3+} values within rows by paired *t*-tests.

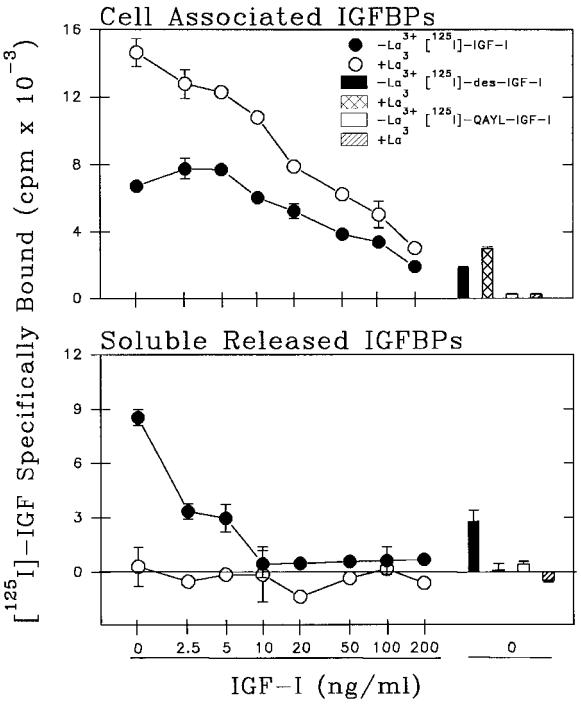

Fig. 5. [^{125}I]-IGF binding to GM10 cell-associated IGFBP and soluble 'released' IGFBP-3. Binding was analyzed with and without the addition of 200 μM La^{3+}. All wells contained 10 μg/ml of sodium-insulin to prevent binding to the type I IGF receptor. Binding was assessed with [^{125}I]-IGF-I (line plots), [^{125}I]-des-IGF-I (left two bars) and [^{125}I]-QAYL-IGF-I (right two bars). Competitive binding curves were also generated for [^{125}I]-des-IGF-I and [^{125}I]-QAYL-IGF-I but only data for the basal binding are shown for simplicity. [^{125}I]-IGF binding to cell-associated IGFBPs (top) and soluble released IGFBPs (bottom) was quantified as described in Materials and Methods. Data represent the means ± SD of duplicates. Error bars are shown if they are larger than the symbols.

both on the cell monolayer and in the assay buffer; although the amount of [^{125}I]-IGF-I bound was 10-fold greater. In control cultures, the competitive binding curve for [^{125}I]-IGF-I shows a paradoxical increase in binding with the addition of low levels of unlabelled IGF-I and this effect was not present with La^{3+}.

Binding specificity of both cell-associated and released IGFBPs was also determined for forskolin-treated MDBK cells (Fig. 7). Insulin prevented binding to the type I IGF receptor, confirmed by the extremely low binding of [^{125}I]-QAYL-IGF-I to the cell monolayer (top). [^{125}I]-QAYL-IGF-I binding to the cell-associated and released IGFBPs was low and not affected by the addition of La^{3+}. In contrast, La^{3+} greatly increased specific binding of [^{125}I]-IGF-I and [^{125}I]-des-IGF-I to cell-associated IGFBPs and it decreased specific binding to the soluble released IGFBPs (primarily IGFBP-3) by >60%. Thus, [^{125}I]-IGF-I and [^{125}I]-des-IGF-I specifically bind to IGFBPs both on the cell monolayer and in the assay buffer; the amount bound was similar for the two radiolabeled ligands.

Scatchard analysis was performed using data generated with the [^{125}I]-IGF-I and [^{125}I]-des-IGF-I competition data for the three cell lines (Table II). Non-linear Scatchard plots yielded affinity constants, K_a, and the number of binding sites, R_o for high and low affinity sites.[1]

The affinity of the high and low sites of GM10 cell-associated IGFBP-3 was similar for both [^{125}I]-IGF-I and [^{125}I]-des-IGF-I and was not affected by the addition of La^{3+}. The K_a of the high affinity site was approximately fivefold that of the low but there were about twofold more (R_o) low than high affinity sites. The increase in specific binding to the cell monolayer

[1]It should be noted that the [^{125}I]-des-IGF-I competition was performed with unlabelled IGF-I. Although Scatchard analysis is designed for use with homologous ligand combinations, this analysis was used as a means to estimate the K_a of the high affinity cell surface binding site in the absence of La^{3+}, since this cannot be done when [^{125}I]-IGF-I is used. Although interpretations based on this analysis are restricted, our data clearly indicate that similar affinity constants are obtained for cell-associated IGFBPs when [^{125}I]-IGF-I and [^{125}I]-des-IGF-I binding are compared.

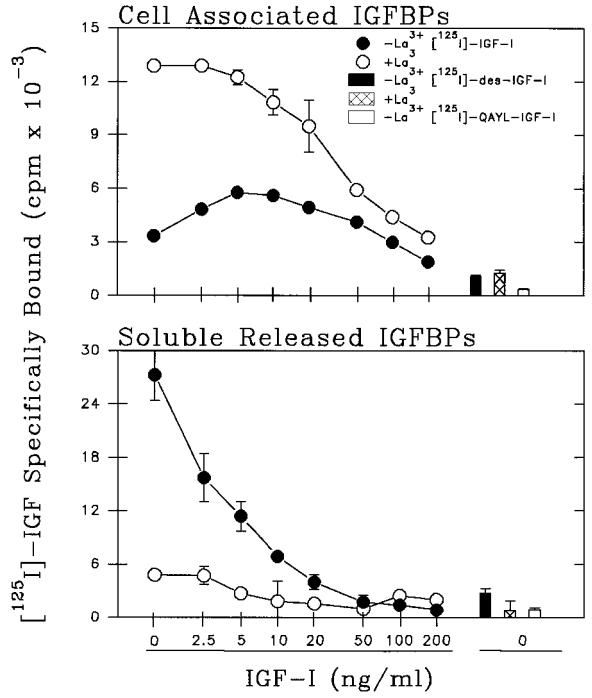

Fig. 6. [125I]-IGF binding to T98G cell-associated IGFBPs and soluble 'released' IGFBPs. Binding was determined with and without the addition of 200 μM La^{3+}. All wells contained 10 μg/ml of sodium-insulin to prevent binding to the type I IGF receptor. Binding was assessed with [125I]-IGF-I (line plots), [125I]-des-IGF-I (left two bars) and [125I]-QAYL-IGF-I (right bar). Competitive binding curves were also generated for [125I]-des-IGF-I and [125I]-QAYL-IGF-I but only data for the basal binding are shown for simplicity. [125I]-IGF binding to cell-associated IGFBPs (top) and released IGFBPs (bottom) was quantified as described in Materials and Methods. [125I]-QAYL-IGF-I binding was not performed with the addition of La^{3+}. Data represent the means ± SD of duplicates. Error bars are shown if they are larger than the symbols.

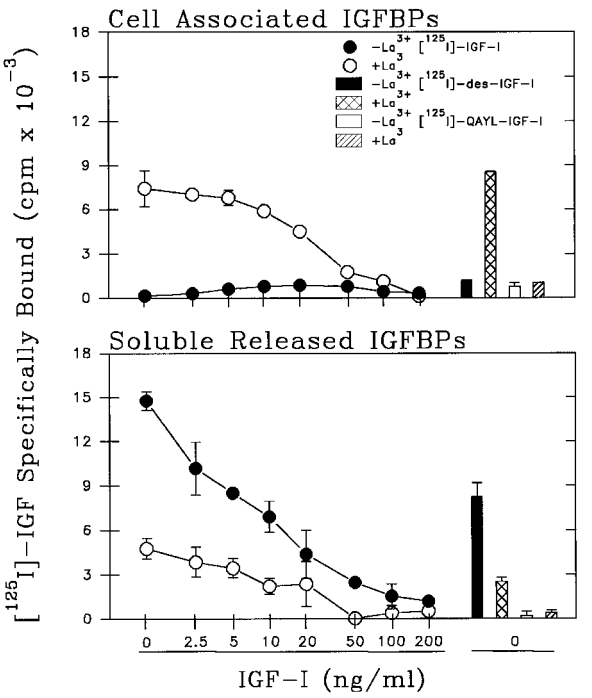

Fig. 7. [125I]-IGF binding to forskolin-treated MDBK cell-associated IGFBPs and soluble 'released' IGFBPs. Binding was analyzed with and without the addition of 200 μM La^{3+}. All wells contained 10 μg/ml of sodium-insulin to prevent binding to the type I IGF receptor. Cells were treated with 5 μM forskolin overnight prior to the assay. Binding was assessed with [125I]-IGF-I (line plots), [125I]-des-IGF-I (left two bars) and [125I]-QAYL-IGF-I (right two bars). Competitive binding curves were also generated for [125I]-des-IGF-I and [125I]-QAYL-IGF-I but only data for the basal binding are shown for simplicity. [125I]-IGF binding to cell-associated IGFBPs (top) and released IGFBPs (bottom) was quantified as described in Materials and Methods. Data represent the means ± SD of duplicates. Error bars are shown if they are larger than the symbols.

with La^{3+} was due to an increase in the number of high affinity sites. The K_a of the soluble 'released' IGFBP-3 was 10-fold that of the cell-associated IGFBP-3 with [125I]-IGF-I and 2.3-fold for [125I]-des-IGF-I. The number of binding sites released in the absence of La^{3+} was low (≈4%) relative to the total number of binding sites. [125I]-IGF-I binding to released IGFBP-3 was undetectable with La^{3+}. Therefore, La^{3+} prevented the loss of cell-associated IGFBP-3. The prevention of the loss of this small number of IGF binding sites with La^{3+} was associated with twice as much detectable binding to cell-associated IGFBP-3 (Table I and Fig. 5).

For T98G cell-associated IGFBP-5, the K_a of the high and low affinity sites were similar for both [125I]-IGF-I and [125I]-des-IGF-I in the presence and absence of La^{3+}. K_a of the high affinity site was approximately fourfold that of the low affinity site and there were similar number (R_o) of high and low affinity sites. The increase in specific binding of [125I]-IGF-I to the cell monolayer with the addition of La^{3+} was due to an increase in the number of high affinity sites. The K_a (high) of the soluble 'released' IGFBPs was 16-fold that of the cell-associated IGFBPs for [125I]-IGF-I. The K_a of the low affinity released sites were 10-fold greater than that of the cell-associated low affinity IGFBPs. The number of binding sites released in the absence of La^{3+} was approximately one-sixth of the total number of binding sites and was depressed by the addition of La^{3+}. Hence, La^{3+} depressed the loss of cell-associated IGFBPs (primarily IGFBP-5). The prevention of the loss of this small number of IGFBP binding sites with La^{3+} was associated with twice as much detectable binding to cell-associated IGFBP-5 (Table I and Fig. 6).

TABLE II. Effect of La^{3+} on the Affinity and Number of Cell Surface and Released IGFBPs*

			Binding to cell surface IGFBPs				Binding to released soluble IGFBPs			
			Affinity: K_a (nM^{-1})		Sites: R_o ($N \times 10^{-9}$)		Affinity: K_a (nM^{-1})		Sites: R_o ($N \times 10^{-9}$)	
Cell	[^{125}I]	Treatment	High	Low	High	Low	High	Low	High	Low
GM10	IGF-I	Control	—	0.13	—	130.5	5.26	—	5.6	—
		La^{3+}	0.41	0.08	62.2	111.6	—	—	—	—
	des-IGF-I	Control	0.58	0.07	9.2	17.5	1.32	—	4.1	—
		La^{3+}	0.56	0.11	12.7	17.9	—	—	—	—
T98G	IGF-I	Control	—	0.15	—	102.1	6.36	—	20.7	—
		La^{3+}	0.39	0.12	59.6	48.4	—	1.40	—	7.4
	des-IGF-I	Control	0.42	0.11	7.9	8.8	—	1.30	—	4.4
		La^{3+}	0.42	0.12	7.7	7.4	—	—	—	—
MDBK	IGF-I	Control	—	0.09	—	25.2	1.32	0.17	29.2	39.4
		La^{3+}	0.35	0.11	47.0	31.4	0.91	—	12.5	—
	des-IGF-I	Control	—	0.12	—	21.7	1.67	0.14	13.6	42.0
		La^{3+}	0.27	0.10	44.7	29.7	0.81	—	7.2	—

*Data represent analysis from competitive binding studies shown in Figures 5, 6 and 7. The labelled peptide varied (either [^{125}I]-IGF-I or [^{125}I]-des(1-3)-IGF-I), the unlabelled peptide was consistently IGF-I. Insulin was present in all wells to prevent binding to the type I IGF receptor.
— indicates that K_a or N of sites either not calculable or not detectable.

For forskolin-treated MDBK cell-associated IGFBPs (primarily IGFBP-3), the K_a of the high affinity site could not be calculated for [^{125}I]-IGF-I or [^{125}I]-des-IGF-I without La^{3+}. In the presence of La^{3+}, the K_a of the high affinity site was similar for both [^{125}I]-IGF-I and [^{125}I]-des-IGF-I. K_a of the high affinity site was threefold higher than the low affinity site and there were slightly greater number (R_o) of high affinity sites. The increase in specific binding of [^{125}I]-IGF-I and [^{125}I]-des-IGF-I to the cell monolayer with the addition of La^{3+} was primarily due to an increase in the number of high affinity sites. The K_a (high) of the soluble 'released' IGFBPs was three- to fourfold higher than that of the cell-associated IGFBPs. The K_a of the low affinity site was similar to that of the cell-associated IGFBPs. The number of binding sites released in the absence of La^{3+} was approximately three quarters of the total number of binding sites. Release was depressed by the addition of La^{3+}. Hence, La^{3+} depressed the loss of cell-associated IGFBPs. In this case, loss of most of the IGFBPs from the cell monolayer with enhanced binding ability decreased binding to the remaining cell-associated IGFBPs by >75% (Table I and Fig. 7).

Type I IGF receptor binding was quantified for the three cell types. As published [McCusker et al., 1990], 10 μg/ml sodium-insulin effectively blocks binding to the type I IGF receptor. Thus type I IGF receptor binding was directly assessed for each radiolabeled ligand as the difference in binding between the absence and presence of insulin (Table III). GM10 cells have very few type I IGF receptors. La^{3+} slightly increases the amount of insulin competable binding. Similarly, T98G cells have few receptors and La^{3+} slightly increases the amount of insulin competable binding. However, MDBK cells have numerous receptors with similar insulin competable binding of [^{125}I]-IGF-I and [^{125}I]-des-IGF-I but lower binding of [^{125}I]-QAYL-IGF-I. La^{3+} increased the amount of insulin competable binding for all three ligands. Thus, insulin competable binding can be used to quantify binding to the type I IGF receptor. The few type I IGF receptors on GM10 and T98G cells indicate that the IGFBPs are the major binding site for IGFs on these cells. In the absence of La^{3+}, the receptor is the major binding site on MDBK cells. When quantified in the presence of La^{3+}, IGFBPs represent approximately 45% of the MDBK cell surface binding sites.

The usefulness of these findings is demonstrated by a practical application (Fig. 8). [^{125}I]-IGF-I binding to MDBK cells was performed without insulin (Fig. 8, top left). Specific [^{125}I]-IGF-I binding to the cell monolayer was lowered in a dose dependent manner by an over-

TABLE III. Insulin Displacement of IGF Binding to GM10, T98G and MDBK Cell-Surface Type I IGF Receptors

Cell	[^{125}I]	Treatment	Insulin competable cpm
GM10	IGF-I	Control	0 (−511)
		La^{3+}	0 (−267)
	des-IGF-I	Control	375 ± 170
		La^{3+}	689 ± 206
	QAYL-IGF-I	Control	183 ± 95
		La^{3+}	272 ± 6
T98G	IGF-I	Control	0 (−103)
		La^{3+}	702 ± 1444
	des-IGF-I	Control	696 ± 56
		La^{3+}	727 ± 415
	QAYL-IGF-I	Control	193 ± 59
		La^{3+}	ND
MDBK	IGF-I	Control	5,313 ± 157
		La^{3+}	6,867 ± 259
	des-IGF-I	Control	4,953 ± 491
		La^{3+}	5,763 ± 156
	QAYL-IGF-I	Control	2,276 ± 525
		La^{3+}	2,371 ± 618

Values represent the mean ± SD of duplicate determinations for the decrease in specific binding of the labelled ligand with the addition of 10 µg/ml sodium insulin.
ND, not determined.

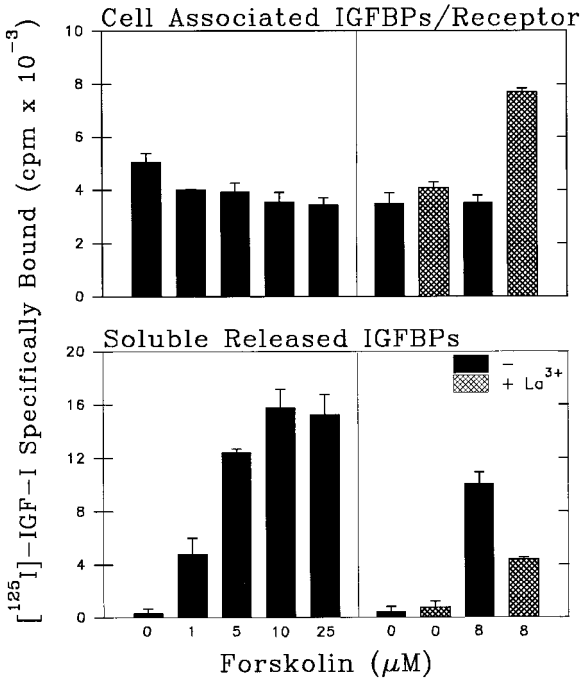

Fig. 8. [^{125}I]-IGF-I binding to MDBK cells. Left panel: Binding assays were performed with and without an overnight pre-treatment with the indicated dose of forskolin. Insulin was not added. Therefore ligand binding to both the type I IGF receptor as well as IGFBPs was quantified. Binding to the cell surface (top, left) and released IGFBPs (bottom, left) was assessed with [^{125}I]-IGF-I. Right panel: Binding was determined with and without an overnight pre-treatment with 8 µM forskolin. The wells did not contain insulin to allow binding to the type I IGF receptor as well as IGFBPs. [^{125}I]-IGF-I binding to the cell surface (top, right) and released IGFBPs (bottom, right) was assessed with and without the addition of La^{3+} (200 µM). Data represent the means ± SD of duplicates.

night pre-treatment with forskolin. Such an effect could be interpreted as a decrease in cell surface binding sites, either IGFBPs or receptors. However forskolin increases IGFBP release during the binding assay in a dose dependent manner (Fig. 8, bottom left). The released IGFBPs bind the [^{125}I]-IGF-I and thus lower association with the cell surface. In the experiment in the right panel, La^{3+} does not affect [^{125}I]-IGF-I binding to the cell monolayer of non-forskolin-treated cultures but markedly increases binding to forskolin-treated cells (top right). Very little IGFBP is released in non-forskolin-treated cultures and La^{3+} does not affect release (bottom right). However IGFBPs are released from forskolin-treated cultures and the release is depressed by La^{3+}.

DISCUSSION

The 43,000 M_r IGFBP on GM10 cells was previously shown to be IGFBP-3 [Camacho-Hubner et al., 1992]. IGFBP-3 was released from the cells during binding assays at 8°C. Release was almost completely blocked by La^{3+}. T98G cells released a 31,000–32,000 M_r IGFBP that we have identified as IGFBP-5 [Camacho-Hubner et al., 1992]. IGFBP-5 release was similarly prevented by the addition of La^{3+}. At least 80% of cell surface [^{125}I]-IGF-I binding to these cells is non-insulin displaceable due to the abundance of IGFBP-3 and IGFBP-5 on GM10 and T98G cell surfaces, respectively, which are the major cell surface IGF-binding sites. We had previously shown that [^{125}I]-IGF-I binding to the surface of these two cell types is affected by the release of IGFBPs during binding assay; i.e., there was a decrease in IGF binding to cell surfaces with increased release of IGFBP [McCusker et al., 1990]. This study extends this finding by showing that the loss of only 4 to 17% of cell surface IGFBPs can depress cell surface binding by 50%. This effect is possible because of the increase in affinity of the released IGFBPs as compared to their affinity when they are cell-associated.

The main goal of this study was to find a means to prevent loss of cell-associated IGFBPs without affecting binding affinity. This would make it possible to accurately quantify the

IGFBP content of cell monolayers. After screening several divalent and trivalent cations, we discovered that La^{3+} blocked the release of hIGFBP-3, bIGFBP-3 and hIGFBP-5 from GM10, MDBK and T98G cells, respectively. In parallel with the decrease in IGFBP release from cell surfaces, there was an increase in [^{125}I]-IGF-I binding to the cell surface. This was the only cation causing both of these effects. Zn^{2+} also blocked IGFBP release from cell surfaces, as directly demonstrated by ligand blot analysis and decreased IGFBP activity in the assay buffer, but Zn^{2+} did not increase binding to cell surfaces. This suggests that zinc may interfere with IGF binding affinity and is not useful for the sole purpose of quantifying IGFBPs.

Since IGF binding to cell surfaces is affected by IGFBP release, it was impossible to accurately quantify binding to IGFBPs on the cell surface unless La^{3+} was used to prevent release. This effect was most easily demonstrated with MDBK cells which were shown to bind fourfold more IGF-I when La^{3+} was added. These studies also showed that the effect of forskolin on [^{125}I]-IGF-I binding to cell surfaces could not be properly assessed unless La^{3+} was used to prevent the loss of IGFBPs. Without La^{3+}, forskolin appeared to depress [^{125}I]-IGF-I binding to the cell surface binding sites. However, forskolin actually increases [^{125}I]-IGF-I binding to the cell surface when IGFBP release is inhibited. Using typical binding assay conditions, the stimulatory effect of forskolin on MDBK cell proliferation [Cohick and Clemmons, 1991] would be difficult to explain. Using La^{3+} to quantify IGFBPs would lead to the conclusion that the effect of forskolin might be due to enhanced levels of cell-associated IGFBP-3 which has been shown to enhance the effect of IGF-I on proliferation of these cells [Cohick and Clemmons, 1993].

Release of IGFBPs into binding assay buffer also alters the shape of [^{125}I]-IGF-I competitive binding curves. A paradoxical increase in cell surface binding occurs with the addition of low levels of unlabelled ligand. This phenomenon has been reported by several labs [Orlowski et al., 1989; Van Obberghen-Schilling et al., 1981; DeVroede et al., 1986; Clemmons et al., 1986; Ballard et al., 1988; Menuelle and Plas, 1993; McCusker et al., 1990]. A complete explanation for such binding characteristics has been difficult to provide but is associated with the presence of high affinity IGFBPs in the assay buffer and low affinity binding sites on the cell surface [McCusker et al., 1990]. This binding phenomenon is corrected if IGFBP release from the cell surface is blocked by the addition of La^{3+}. Our previous work using human GM10 cells and subsequent work of others showed that the affinity of the released IGFBPs (principally IGFBP-3) was higher than those on the cell surface [Conover, 1991, 1992; McCusker et al., 1990]. This study extends these findings to T98G cells (principally human IGFBP-5) and bovine MDBK cells (primarily IGFBP-3). This study also shows that when IGFBP release is prevented by La^{3+} the affinities of the IGFBPs that remain on the cell surface are unchanged.

Although IGF-I that is bound to either IGFBP-3 or IGFBP-5 in extracellular fluids is very tightly bound, when these IGFBPs attach cell surfaces, the affinity for IGF-I decreases. This effect will result in the release of some of the IGF-I which could then bind to either IGFBPs in solution or type I IGF receptors. One of the key parts of this mechanism, cell-association, is modified by multivalent cations. Therefore it is possible that cations could target IGF-IGFBP complexes to the cell surface. This would result in IGF-I release thus increasing the potential for receptor activation.

The mechanism by which the IGFBPs are released from cell surfaces is currently under investigation. La^{3+} can antagonize Ca^{2+} activated proteases and other enzymes. Thus, one possible mechanism of release could be proteolysis, since IGFBP-3 and IGFBP-5 are cleaved by proteases that are secreted by cell in culture [Tonner et al., 1995; Fowlkes et al., 1994; Kanzaki et al., 1994; Camacho-Hubner et al., 1992; Conover and Kiefer, 1993]. However, there was no apparent change in the size of either IGFBP-3 or IGFBP-5 after release from cell surfaces compared to their size in solubilized cell extracts. Further evidence against this possibility was the increase in ligand affinity of released IGFBPs compared to those remaining on the cell surface. Proteolysis of IGFBP-3 decreases ligand affinity [Blat et al., 1994; Lamson et al., 1993]. It is possible, if IGFBP-binding sites exist, that they could be enzymatically cleaved and thus release IGFBPs; however this possibility awaits further characterization of the cell surface components that bind to IGFBP-3 and IGFBP-5.

IGFBPs have been reported to enhance and to inhibit IGF actions. Generally the soluble high affinity IGFBPs are inhibitory and the cell-associated lower affinity forms enhance ac-

tivity. Several studies support the idea that there is a delicate balance between the IGF inhibiting activity and IGF potentiating activity. Slight changes in culture conditions can result in a change in this equilibrium [Coleman and Etherton, 1994; Bourner et al., 1992]. These changes in some cases probably reflect differences in levels of IGFBP at the cell surface. Therefore it is important, when measuring IGF activity, to quantify the amount of IGFBPs present in extracellular fluids and on the cell surface. This is not possible if a considerable amount of cell-associated IGF binding activity is released during the binding assay. With the use of La^{3+}, IGFBP release from cell surfaces was limited and the number and affinity of binding sites on the cell surface can be accurately quantified.

Type I IGF receptor binding can be assessed with insulin displacement and IGFBP binding with IGF-I displacement beyond that of insulin. Insulin at the dose used does compete for most if not all binding to the type I IGF receptor [Kull et al., 1983; Baxter and Williams, 1983; Tollefsen et al., 1987; McCusker et al., 1990]. There are numerous other reports of insulin competing for all of the [^{125}I]-IGF-I binding sites as well as numerous papers citing incomplete and poor displacement by insulin. We believe that the latter findings are due to the frequently unrecognized presence of IGFBPs on the cell surface. It should also be emphasized that the affinity of the cell-associated IGFBPs is similar to that of the type I IGF receptor [McCusker et al., 1990]. Thus, binding affinity cannot be used to distinguish binding between the receptor and IGFBPs. In the current work, La^{3+} increased [^{125}I]-IGF-I binding to the type I IGF receptor. This is not a direct effect on either receptor affinity or number (unpublished data). Instead, by preventing the release of IGFBPs and their strong IGF binding activity, more [^{125}I]-IGF-I is available for receptor binding. Thus similar to cell-associated IGFBPs, receptor number and affinity cannot be accurately quantified if IGFBPs are released during the binding assay.

In summary, our data clearly show that multivalent cations effect cell-association of IGFBPs and the affinity of the [IGF]-[IGFBP] complex is lowered. Hence evolution has developed a system by which the type I IGF receptor is activated due to changes in the affinity of the IGFBPs for their ligand. It is becoming apparent that the [IGFBP]-[IGF] complex is not only a transport mechanism for the IGFs but the complex is necessary for maximal IGF activity. Hence, IGF activity appears to be balanced by soluble-extracellular IGFBP inhibitory activity and cell-associated [IGFBP]-[IGF] stimulatory complexes. Whether the stimulatory effects of the IGFBPs can be explained solely by an increase in the pericellular concentration of IGFs with IGFBP cell-association requires further investigation.

ACKNOWLEDGMENTS

The authors thank Dr. Louis E. Underwood for providing the iodinated IGF peptides. The authors are grateful to Margaret Cascieri for supplying the [Gln^3,Ala^4,Tyr^{15},Leu^{16}]-IGF-I.

REFERENCES

Arai T, Clarke J, Parker A, Busby W, Nam T, Clemmons DR (1996): Substitution of specific amino acids in insulin-like growth factor (IGF) binding protein 5 alters heparin binding and its change in affinity for IGF-I in response to heparin. J Biol Chem 271:6099–6106.

Ballard FJ, Ross M, Upton FM, Francis GL (1988): Specific binding of insulin-like growth factors 1 and 2 to the type 1 and type 2 receptors respectively. Biochem J 249:721–726.

Baxter RC, Williams PF (1983): Reciprocal modulation of insulin and insulin-like growth factor-I receptor affinity by calcium. Biochem Biophys Res Commun 116:62–67.

Blat C, Villaudy J, Binoux M (1994): In vivo proteolysis of serum insulin-like growth factor (IGF) binding protein-3 results in increased availability of IGF to target cells. J Clin Invest 93:2286–2290.

Bourner MJ, Busby WH, Siegel NR, Krivi GG, McCusker RH, Clemmons DR (1992): Cloning and sequence determination of bovine insulin-like growth factor binding protein-2 (IGFBP-2): Comparison of its structural and functional properties with IGFBP-1. J Cell Biochem 48:215–226.

Camacho-Hubner C, Busby WH, McCusker RH, Wright G, Clemmons DR (1992): Identification of the forms of insulin-like growth factor-binding proteins produced by human fibroblasts and the mechanisms that regulate their secretion. J Biol Chem 267:11949–11956.

Clemmons DR, Elgin RG, Han VKM, Casella SJ, D'Ercole AJ, Van Wyk JJ (1986): Cultured fibroblast monolayers secrete a protein that alters the cellular binding of somatomedin-C/insulin-like growth factor I. J Clin Invest 77: 1548–1556.

Clemmons DR, Han VKM, Elgin RG, D'Ercole AJ (1987): Alterations in the synthesis of a fibroblast surface associated 35 K protein modulates the binding of somatomedin-C/insulin-like growth factor I. Mol Endocrinol 1:339–347.

Cohick WS, Clemmons DR (1991): Regulation of insulin-like growth factor binding protein synthesis and secretion in a bovine epithelial cell line. Endocrinology 129: 1347–1354.

Cohick WS, Clemmons DR (1993): Regulation of IGFBP secretion and modulation of cell growth in MDBK cells. Growth Regulat 3:20–23.

Coleman ME, Etherton TD (1994): Porcine insulin-like growth factor (IGF)-binding protein-3 elicits biphasic effects on IGF-I stimulated DNA synthesis in neonatal porcine skin fibroblasts. Domest Anim Endocrinol 11:299–305.

Conover CA (1991): Glycosylation of insulin-like growth factor binding protein-3 (IGFBP-3) is not required for potentiation of IGF-I action: Evidence for processing of cell-bound IGFBP-3. Endocrinology 129:3259–3268.

Conover CA (1992): Potentiation of insulin-like growth factor (IGF) action by IGF-binding protein-3—Studies of underlying mechanism. Endocrinology 130:3191–3199.

Conover CA, Kiefer MC (1993): Regulation and biological effect of endogenous insulin-like growth factor binding protein-5 in human osteoblastic cells. J Clin Endocrinol Metab 76:1153–1159.

D'Ercole AJ, Underwood LE, Van Wyk JJ, Decedue CJ, Foushee DB (1976): Specificity, topography and ontogeny of the somatomedin C receptor in mammalian tissues. In Pecile A, Muller R (eds): "Growth Hormone and Related Peptides." Amsterdam: Excepta Medica, pp 190–205.

DeVroede MA, Tseng LY-H, Katsoyannis PG, Nissley SP, Rechler MM (1986): Modulation of insulinlike growth factor I binding to human fibroblast monolayer cultures by insulinlike growth factor carrier proteins released to the incubation media. J Clin Invest 77:602–613.

Elgin RG, Busby WH, Clemmons DR (1987): An insulin-like growth factor binding protein enhances the biogic response to IGF-I. Proc Natl Acad Sci USA 84:3254–3258.

Fowlkes JL, Enghild JJ, Suzuki K, Nagase H (1994): Matrix metalloproteinases degrade insulin-like growth factor-binding protein-3 in dermal fibroblast cultures. J Biol Chem 269:25742–25746.

Gopinath R, Walton PE, Etherton TD (1989): An acid stable insulin-like growth factor (IGF)-binding protein from pig serum inhibits binding of IGF-I and IGF-II to vascular endothelial cells. J Endocrinol 120:231–236.

Han VKM, Lauder JM, D'Ercole AJ (1988): Rat astroglial somatomedin/insulin-like growth factor binding proteins: Characterization and evidence of biologic function. J Neurosci 8:3135–3143.

Hodgkinson SC, Napier JR, Spencer GSG, Bass JJ (1994): Glycosaminoglycan binding characteristics of the insulin-like growth factor-binding proteins. J Mol Endocrinol 113:105–112.

Holly JMP, Smith CP, Dunger DB, Edge JA, Biddlecombe RA, Williams AJK, Howell R, Chard T, Savage MO, Rees LH, Wass JAH (1989): Levels of the small insulin-like growth factor-binding protein are strongly related to those of insulin in prepubertal and pubertal children but only weakly so after puberty. J Endocrinol 121:383–387.

Jones JI, Clemmons DR (1995): Insulin-like growth factors and their binding proteins: Biological actions. Endocrine Rev 16:3–34.

Jones JI, Gockerman A, Busby WH, Camacho-Hubner C, Clemmons DR (1993a): Extracellular matrix contains insulin-like growth factor binding protein-5—Potentiation of the effects of IGF-I. J Cell Biol 121:679–687.

Jones JI, Gockerman A, Busby WH, Wright G, Clemmons DR (1993b): Insulin-like growth factor binding protein-1 stimulates cell migration and binds to the alpha 5 beta 1 integrin by means of its Arg-Gly-Asp sequence. Proc Natl Acad Sci USA 90:10553–10557.

Kanzaki S, Hilliker S, Baylink DJ, Mohan S (1994): Evidence that human bone cells in culture produce insulin-like growth factor-binding protein-4 and -5 proteases. Endocrinology 134:383–392.

Knauer DJ, Smith GL (1980): Inhibition of biologic activity of multiplication stimulating activity by binding to its carrier protein. Proc Natl Acad Sci USA 77:7252–7254.

Kull FC, Jacobs S, Su Y, Svoboda ME, Van Wyk JJ, Cuatrecasas P (1983): Monoclonal antibodies to receptors for insulin and somatomedin-C. J Biol Chem 258:6561–6566.

Lamson G, Giudice LC, Cohen P, Liu F, Gargosky S, Muller HL, Oh Y, Wilson KF, Hintz RL, Rosenfeld RG (1993): Proteolysis of IGFBP-3 may be a common regulatory mechanism of IGF action in vivo. Growth Regulat 3:91–95.

Martin JL, Ballesteros M, Baxter RC (1992): Insulin-like growth factor-I (IGF-I) and transforming growth factor-beta 1 release IGF-binding protein-3 from human fibroblasts by different mechanisms. Endocrinology 131:1703–1710.

McCusker RH, Clemmons DR (1992): The insulin-like growth factor binding proteins: Structure and biological functions. In Schofield PN (ed): "The Insulin-Like Growth Factors: Structure and Biological Functions." Oxford: Oxford University Press, pp 110–150.

McCusker RH, Camacho-Hubner C, Clemmons DR (1989b): Identification of the types of insulin like growth factor binding proteins that are secreted by muscle cells in vitro. J Biol Chem 264:7795–7800.

McCusker RH, Camacho-Hubner C, Bayne ML, Cascieri MA, Clemmons DR (1990): Insulin-like growth factor (IGF) binding to human fibroblast and glioblastoma cells: The modulating effect of cell released IGF binding proteins (IGFBPs). J Cell Physiol 144:244–253.

McCusker RH, Busby WH, Dehoff MH, Camacho-Hubner C, Clemmons DR (1991): Insulin-like growth factor (IGF) binding to cell monolayers is directly modulated by the addition of IGF-binding proteins. Endocrinology 129:939–949.

Menuelle P, Plas C (1993): Glycogenic effect of insulin, insulin-like growth factors II and I and association to their specific receptors in cultured rat hepatocytes. Endocrine J 1:527–533.

Orlowski CC, Chernausek SD, Akeson R (1989): Actions of insulin-like growth factor-I on the B104 neuronal cell line: Effects on cell replication, receptor characteristics, and influence of secreted binding protein on ligand binding. J Cell Physiol 139:469–476.

Ritvos O, Ranta T, Jalkanen J, Suikkari AM, Voutilainen R, Bohn H, Rutanen EM (1988): Insulin-like growth factor (IGF) binding protein from human decidua inhibits the binding and biological action of IGF-I in cultured choriocarcinoma cells. Endocrinology 122:2150–2157.

Tollefsen SE, Thompson K, Petersen DJ (1987): Separation of the high affinity insulin-like growth factor I receptor from low affinity binding sites by affinity chromatography. J Biol Chem 262:16461–16469.

Tonner E, Beattie J, Flint DJ (1995): Production of insulin-like growth factor binding protein (IGFBP), IGFBP-3 protease, and expression of IGF-I receptors by cells of the sheep immune system. Eur J Endocrinol 132:118–122.

Van Obberghen-Schilling EE, Rechler MM, Romanus JA, Knight AB, Nissley SP, Humbel RE (1981): Receptors for insulin like growth factor I are defective in fibroblast cultured from a patient with leprechaunism. J Clin Invest 68:1356–1365.