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Zinc Alters the Kinetics of IGF-II Binding to Cell Surface Receptors and Binding Proteins

Robert H. McCusker, Rebecca L. Mateski, and Jan Novakofski

Laboratory for Developmental Endocrinology, The Department of Animal Sciences, The University of Illinois, Urbana, IL 61801

The growth of most tissues is markedly depressed as a result of zinc deficiency by uncharacterized mechanisms that clearly involve the insulin-like growth factor (IGF) system. Herein, we describe the mechanism by which zinc (Zn^{2+}) maintains IGF-II in an active form by directly regulating IGF-II binding to IGF-binding proteins (IGFBPs) and the type 1 IGF receptor (IGF-1R). The specificity of Zn^{2+} effects was confirmed by using other cations that can (Cd^{2+} and Au^{3+}) or cannot (La^{3+}) mimic Zn^{2+} actions. Human fibroblasts, glioblastoma cells, and murine myoblasts were used to determine the kinetics of IGF-II binding to cell surface IGFBP-3, IGFBP-5, and the IGF-1R, respectively. Zn^{2+}, Cd^{2+}, and Au^{3+}, but not La^{3+}, decreased total binding and the affinity for [^{125}I]IGF-II association with IGFBP-3 and IGFBP-5. These effects were a result of lowered rate of ligand association without affecting rate of dissociation. In contrast, Zn^{2+} enhanced [^{125}I]IGF-II binding to the IGF-1R by enhancing the rate of ligand association and decreasing the rate of dissociation. Our previous work had shown that Zn^{2+} acts at physiological levels to alter IGF binding. Together with the current work, these findings imply that Zn^{2+} acts in vivo to prevent secreted IGF-II from binding to IGFBP-3 and IGFBP-5, thus maintaining IGF-II in an "active state," i.e., readily available for IGF-1R association.

Key Words: IGF; IGF-1R; IGFBP; zinc; affinity; kinetics; association rate.

Introduction

IGF-I and/or IGF-II mediate many of the growth-promoting actions of growth hormone and are required for both normal fetal development and postnatal growth *(1–4)*. They are produced by and act on a variety of cell types, making them autocrine and paracrine growth factors as well as hormones *(5)*. Six IGFBPs modulate the actions of these two growth factors *(6)*. Understanding IGFBPs and the mechanisms through which they inhibit or potentiate IGF activity is very important in designing methods to optimize IGF activity.

Extensive evidence demonstrates the importance of zinc in the growth and function of many cell types. Young, growing individuals are particularly susceptible to zinc deficiency, which results in depressed muscle growth, immune function, sexual maturation, and mental development beyond that associated with the depression in food intake associated with zinc deficiency *(7,8)*. Human zinc deficiency is widespread in underdeveloped countries and in populations with cereal-based diets. Animals respond to dietary zinc supplements with increased growth *(9,10)*. There is also extensive evidence on the crucial role for the insulin-like growth factors (IGFs) in regulating growth of most tissues. IGF-I is crucial for body growth, for muscle growth, for immune function, for sexual maturation, and for mental development *(1)*. Because of the central importance of IGF for growth and development, we know a great deal about the mechanism of IGF action *(11)* and we know a great deal about the biochemical/metabolic functions of zinc within metalloenzymes *(12,13)*. Given the similar consequences of zinc and IGF deficiency, it is surprising that the interaction between the two is so poorly understood.

When zinc deficiency is created experimentally, growth depression precedes changes in total body zinc content or changes in intracellular zinc levels *(14,15)*. In contrast, extracellular (serum) zinc levels closely parallel the physiological changes associated with experimental zinc deficiency *(16)* and parallel changes in growth rate when zinc is added as a dietary growth promoter for domestic animals *(9,10,17–19)*. Combined with the lack of clear evidence that the effects of zinc deficiency are mediated by changes in metalloenzymes within cells, it has been postulated for 20 yr that changes associated with zinc deficiency are related to changes in extracellular functions of zinc *(15)*. However, the extracellular mechanisms for zinc activity have not been defined.

Recently, a key to the mechanism of zinc function on the immune system was described. Depressed immune function associated with zinc deficiency most closely paralleled the amount of zinc bound to thymulin *(20–22)*. This suggests that zinc affects immune function by regulating the activity of a circulating hormone. No similar mechanism describ-

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Author to whom all correspondence and reprint requests should be addressed: Robert H. McCusker Jr., Ph.D., 207 Edward R. Madigan Laboratory, 1201 W. Gregory Dr., Urbana, IL 61801. E-mail: rmccuske@uiuc.edu

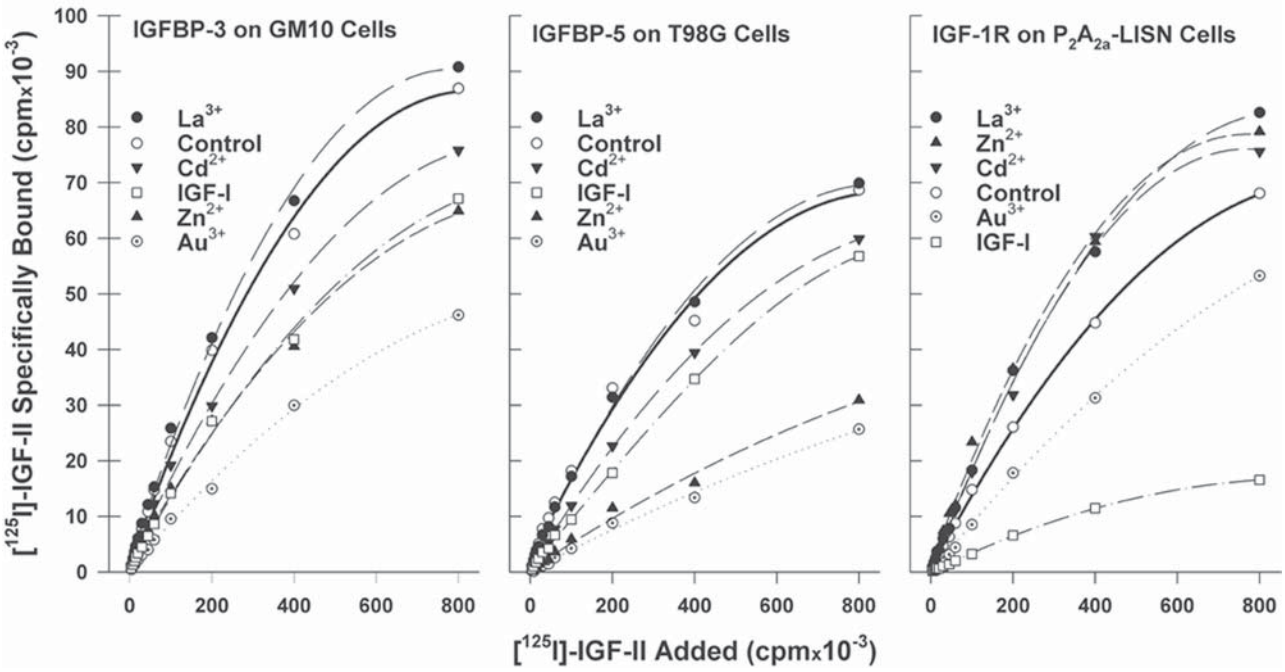

Fig. 1. Effect of various cations or IGF-I on [^{125}I]IGF-II binding to IGFBP-3, IGFBP-5, and the IGF-1R on GM10, T98G and P_2A_{2a}-LISN cells, respectively. [^{125}I]IGF-II was added at varying doses. Cations were added at 200 μ*M* and IGF-I at 900 ng/mL. Legends are arranged to correspond to treatment effects noted within each panel. Values were taken from a single representative experiment.

ing the extracellular requirements of zinc for body growth, sexual maturation, or neural development has been described. Herein, we define a mechanism that explains many of the growth abnormalities associated with inadequate zinc availability. Similar to the requirement of zinc for thymulin activity, we will demonstrate that zinc is required for normal growth because it is needed to aintain IGF-II in an active state, i.e., not bound to inhibitory IGFBPs but "free" and available for IGF-1R binding (Fig. 1).

The IGFs bind to soluble IGFBPs, to IGFBPs on the cell surface, and to at least three receptors on the cell surface (the IGF-1R, the type 2 IGF receptor, and the insulin receptor). IGF-I and IGF-II association with the IGF-1R is required for cellular signaling *(23)*. The presence of IGFBPs in extracellular fluids *(24,25)* and, on the cell surface *(25)* modulate, and in most cases, markedly inhibit IGF activity. Several cations modulate IGFBP binding activity. Specifically, Zn^{2+} lowers the affinity (K_a) by which IGF-I and IGF-II bind to cell-associated IGFBPs but Zn^{2+} does not depress the affinity of the IGF-1R *(26–28)*. An effect of Zn^{2+} on IGF interaction, either [ligand]–[receptor] or [ligand]–[binding protein], can be the result of either altered "on-rate" (rate of association) or "off-rate" (rate of dissociation). In order to define which mechanism occurs, the rate of association and dissociation of IGF-II was examined through various techniques using cell types with a single dominant cell surface binding site with the goal to determine how Zn^{2+} affects [^{125}I]IGF-II binding to cell-associated IGFBP-3, IGFBP-5, and the IGF-1R.

Results

Binding Assay: [^{125}I]IGF-II Binding

[^{125}I]IGF-II binding was assessed by adding increasing amounts of ligand to GM10, T98G, and P_2A_{2a}-LISN cultures. From our previous work, the primary binding sites on these cell surfaces differ: GM10 human fibroblast surfaces contain both IGFBP-3 and IGFBP-5 (but primarily IGFBP-3 and very little binding to the IGF-1R or IGF-2R), T98G surfaces contain primarily IGFBP-5 (very little binding to the IGF-1R or IGF-2R), and P_2A_{2a}-LISN cells contain the IGF-1R (with no evidence of IGF binding to cell-associated IGFBPs or the IGF-2R) *(28,29)*. Over the 20-fold concentration range of added [^{125}I]IGF-II, ligand binding was measurable at all doses but was not saturated even at the highest dose (approx 27 ng/mL [^{125}I]IGF-II).

La^{3+} did not affect [^{125}I]IGF-II binding to either GM10 IGFBP-3 (Fig. 1, left) or T98G IGFBP-5 (Fig. 1, middle). IGF-1, Zn^{2+}, Cd^{2+}, and Au^{3+} depressed binding to both IGFBP-3 and IGFBP-5. In contrast, [^{125}I]IGF-II binding to the IGF-1R on P_2A_{2a}-LISN cells was depressed by IGF-I and Au^{3+} and increased by La^{3+}, Zn^{2+}, and Cd^{2+} (Fig. 1, right).

Our previous work had shown that ligand affinity determined with a constant amount of labeled peptide and increasing amounts of unlabeled peptide was dependent on the unlabeled protein and largely independent of the labeled peptide, e.g., affinities were similar with [^{125}I]IGF-II/IGF-II and [^{125}I]IGF-I/IGF-II but dissimilar with [^{125}I]IGF-II/

Table 1
Equilibrium Binding Kinetics for [^{125}I]IGF-II
Binding to Cell-Associated IGFBP-3, IGFBP-5, and IGF-1R[a]

Binding Moiety Treatment	Capacity % control (μL/well)		Affinity; K_a % control (pM^{-9})		Hill coefficient	Effect
IGFBP-3						
Control	100	(29. 3)	100	(627)	1.01	
Zn^{2+}	60		75		0.96	K_a ⌋
La^{3+}	118		119		1.00	K_a ″
Cd^{2+}	75		83		0.96	K_a ⌋
Au^{3+}	29		34		0.98	K_a ⌋⌋
IGFBP–*5*						
Control	100	(26.9)	100	(867)	1.01	
Zn^{2+}	20		31		0.99	K_a ⌋⌋
La^{3+}	101		96		1.00	K_a —
Cd^{2+}	50		38		0.99	K_a ⌋⌋
Au^{3+}	13		11		0.99	K_a ⌋⌋
IGF-1R						
Control	100	(14.8)	100	(296)	0.99	
Zn^{2+}	170		173		1.01	K_a ″″
La^{3+}	144		127		0.98	K_a ″
Cd^{2+}	125		122		1.02	K_a ″
Au^{3+}	52		33		1.01	K_a ⌋⌋

[a]Data from Fig. 1 (and additional data from the same assays) were subjected to Scatchard analysis using a Lotus 123 (Cambridge, MA) spreadsheet program written by the author. Data for binding capacity, affinity, and number of binding sites are expressed as a percentage of control. Raw data for controls are shown in parentheses. Indicated comparisons (Effect) are relative to control for each cation. The Hill coefficient is an indication of linearity of the Scatchard lines, $n = 1$.

IGF-I combinations *(26–28)*. Thus, before the determination of association and dissociation rates for [^{125}I]IGF-II, using assays in which unlabeled peptides are not used, it was critical to quantify the binding of [^{125}I]IGF-II with the current design. The data in Fig. 1 confirm our earlier cation findings.

Binding Assay: Affinity Constants

Affinity constants (Table 1) were determined by quantifying [^{125}I]IGF-II binding with varying levels of tracer using both data in Fig. 1 and data from additional assays. "Capacity" estimates the total amount of IGF-II that can be bound to the binding site at saturation. K_a is directly proportional to the strength of the ligand-binding site interaction and R_o indicates the number of binding sites. The Hill coefficient is an indication of linearity of Scatchard plots (1.0 perfect linearity). The range of [^{125}I]IGF-II added to the wells was approx 0.13–27 ng/mL IGF-II. The Scatchard plots from these experiments were all linear, as evidenced by only one binding constant (K_a) and by Hill coefficients that were all near 1.0 (Table 1). Linearity of the Scatchard plots indicates that only a single affinity binding site is being quantified and in most cases linearity indicates that a single type of binding site is present. The linearity found herein indicates that a single binding moiety is being quantified on each cell type: IGFBP-3 on GM-10 fibroblasts cells, IGFBP-5 on T98G glioblastoma cells, and the IGF-1R on P_2A_{2a}-LISN muscle cells.

The affinity constant K_a of cell-associated IGFBP-3 or cell-associated IGFBP-5 was at least twofold greater than that of the IGF-1R in the absence of added cations, i.e., control cultures (bold faced in Table 1). Thus, in the absence of added cations, [^{125}I]IGF-II will preferentially bind to cell-associated IGFBPs.

The IGF-1R and cell-associated IGFBPs do not respond the same to the presence of multivalent cations. Zn^{2+} decreased binding capacity of both IGFBP-3 and IGFBP-5 by decreasing the binding affinity and the number of binding sites. IGFBP-5 was affected more than IGFBP-3 (Table 1). In contrast, Zn^{2+} increased binding capacity of the IGF-1R via an increase in affinity without a change in the number of binding sites compared to controls. La^{3+} did not markedly affect the binding capacity or affinity of cell-associated IGFBP-3 or IGFBP-5 but La^{3+} increased both parameters for [^{125}I]IGF-II binding to the IGF-1R. Cd^{2+} (which can mimic Zn^{2+}) decreased the binding capacity and affinity of cell-associated IGFBPs (IGFBP-5 affected more than IGFBP-3) but increased binding capacity and the affinity

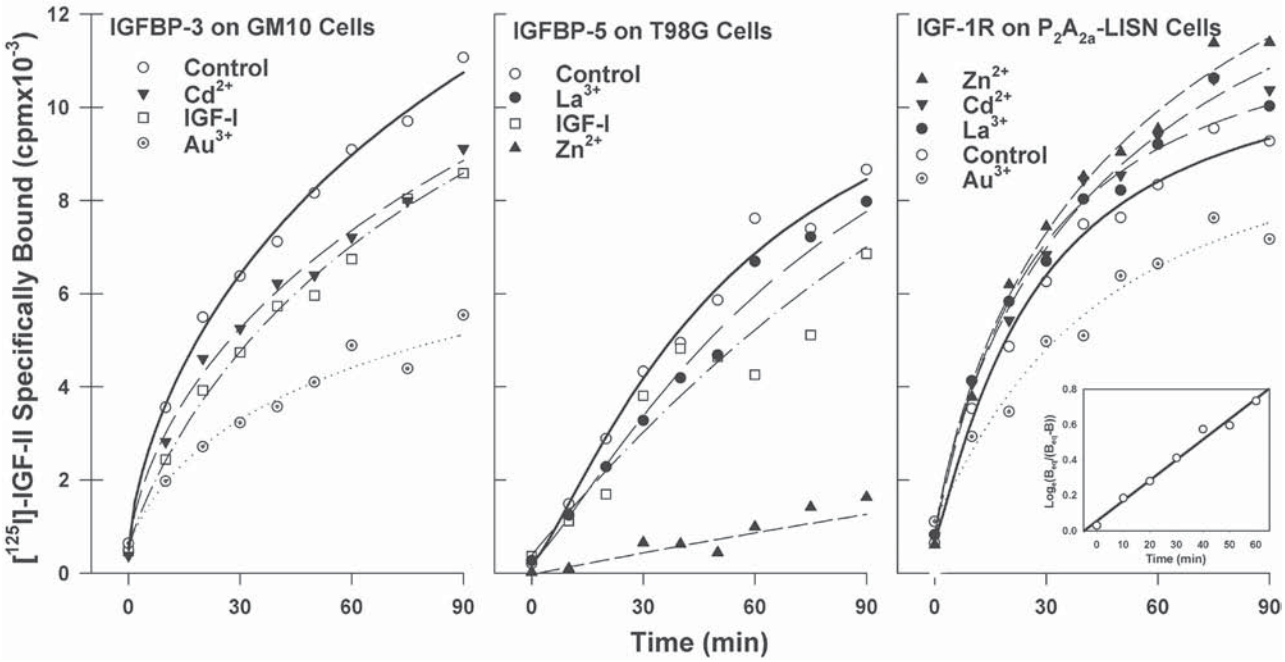

Fig. 2. Effect of cations or IGF-I on [^{125}I]IGF-II association to IGFBP-3, IGFBP-5, and the IGF-1R. Cations were added at 200 μ*M* and IGF-I at 100 ng/mL. Binding was stopped at the indicated times. Rate of association was calculated after the data was linearized as shown in the inset for P_2A_{2a}-LISN cell control data [ln (B_{eq}/(B_{eq}–B) is unitless; B_{eq} = cpm bound at equilibrium, i.e., 4 h; B = cpm bound at appropriate time point]. Legends are arranged to correspond to treatment effects noted within each panel. Data were taken from a single representative experiment.

of the IGF-1R. Au^{3+} (which can replace Zn^{2+} at its binding site) decreased [^{125}I]IGF-II binding capacity of both cell-associated IGFBPs (IGFBP-5 affected more than IGFBP-3) and the IGF-1R owing to changes in ligand affinity. These findings clearly indicate that cations markedly alter IGF-II binding. From a physiological standpoint the data indicate that Zn^{2+} depresses IGF-II binding to IGFBPs while enhancing IGF-II binding to the receptor. The mechanism of action for Zn^{2+} or the other cations was explored next.

Association Assay: [^{125}I]IGF-II Binding

[^{125}I]IGF-II binding affinity could be affected by cations as a result of changes in either the rate of association or dissociation. The mechanism will determine whether cations, such as the micronutrient Zn^{2+}, blocks IGF inactivation (depresses association rate to IGFBPs) or if Zn^{2+} can activate IGF-II (increase the rate of dissociation from inhibitory IGFBPs). Timed association experiments were conducted to test for the former.

Assays from a typical experiment with cell-associated IGFBP-3 show that [^{125}I]IGF-II binding increased over the 3-h incubation (Fig. 2, left). At 180 min, [^{125}I]IGF-II binding was decreased with the addition of Cd^{2+}, Au^{3+}, or IGF-I when compared to control.

Assays from a typical experiment with cell-associated IGFBP-5 show that [^{125}I]IGF-II binding also increased over the 3-h incubation (Fig. 2, middle). At 180 min, [^{125}I]IGF-II binding was decreased by the addition of Zn^{2+}, but not by La^{3+} or IGF-I.

Assays from a typical experiment with IGF-1Rs show that [^{125}I]IGF-II binding increased over the 3-h incubation (Fig. 2, right). At 180 min, [^{125}I]IGF-II binding was decreased by the addition of Au^{3+}, but increased by the addition of other cations; Zn^{2+}> Cd^{2+} > La^{3+}.

Association Assay: Association Constants

Association constants were calculated from the data in Fig. 2 and additional assays. An example of a linear plot generated from the data to calculate rate of association for the IGF-1R is shown in the inset (Fig. 2, right). All data were linear for the first 60 min and only the 0–60 min time points were used to calculate association constants.

With cell-associated IGFBP-3 and [^{125}I]IGF-II (Table 2, top), Zn^{2+} depressed binding compared to control and depressed the rate of association. La^{3+} did not significantly affect binding or the rate of association. Both Au^{3+} and Cd^{2+} depressed [^{125}I]IGF-II binding and rate of association compared to control.

With cell-associated IGFBP-5 (Table 2, middle), Zn^{2+}, Cd^{2+}, and Au^{3+} depressed binding and the rate of association compared to control. La^{3+} did not affect binding, although it lowered the rate of association.

For the IGF-1R (Table 2, bottom), Zn^{2+}, Cd^{2+}, and La^{3+} increased [^{125}I]IGF-II binding and Au^{3+} decreased binding

Table 2
Association Rates for [^{125}I]IGF-II Binding to Cell-Associated IGFBP-3, IGFBP-5, and IGF-1R[a]

Binging Moiety Treatment	Bound peptide % control		(cpm)	Rate of association % control		(min^{-1})
IGFBP-3						
Control	100		(7,271)	100		(6.6)
Zn^{2+}	64 ± 7	*		30 ± 5	*	
La^{3+}	115 ± 7			85 ± 12		
Cd^{2+}	62 ± 8	*		42 ± 8	*	
Au^{3+}	24 ± 1	*		10 ± 2	*	
IGFBP-5						
Control	100		(6,391)	100		(4.0)
Zn^{2+}	27 ± 5	*		18 ± 5	*	
La^{3+}	96 ± 4			71 ± 4	*	
Cd^{2+}	49 ± 5	*		19 ± 2	*	
Au^{3+}	16 ± 2	*		17 ± 5	*	
IGF-1R						
Control	100		(8,081)	100		(7.9)
Zn^{2+}	132 ± 5	*		126 ± 8	¶	
La^{3+}	113 ± 1	*		116 ± 15		
Cd^{2+}	124 ± 2	*		111 ± 20		
Au^{3+}	42 ± 2	*		13 ± 5	*	

[a] Values represent mean ± SEM for the slope ($\leftrightarrow 10^3$) of linearized timed association experiments (rate of association) or amount of [^{125}I]IGF-II bound (Bound, cpm) at equilibrium, i.e., 4 h time point. Data are expressed as a percentage of control with raw data in parentheses for control only. All cations added at 200 μ*M*. For IGFBP-3; $n = 12$ for Zn^{2+}, $n = 8$ for La^{3+}, and $n = 4$ for Cd^{2+} and Au^{3+}. For IGFBP-5; $n = 2$ for all cations. For the IGF-1R; $n = 3$ for all comparisons except Au^{3+} ($n = 2$). *$p < 0.05$ or ¶ $p = 0.06$ for the effect of cation alone versus control.

when compared to controls. Only Zn^{2+} enhanced the rate of ligand association, whereas Au^{3+} markedly depressed the rate of ligand association.

Together these data indicate that cation effects on IGF-II binding can all be explained based simply on their ability to alter the rate of ligand association. This does not preclude effects on rate of dissociation, which was tested next.

Dissociation Assay: [^{125}I]IGF-II Binding

[^{125}I]IGF-II binding was assessed to determine if either cations or IGF-I affect the rate of dissociation of bound [^{125}I]IGF-II. There was no release of prebound [^{125}I]IGF-II from cell-associated IGFBP-3 controls prior to 3 h and only approx 20% of the ligand was released after 4 h (Fig. 3, left). Only Au^{3+} enhanced the rate of ligand dissociation. In contrast, all other treatments slightly depressed the release of [^{125}I]IGF-II at 4 h compared to the control. There was essentially no loss of bound ligand in wells treated with Zn^{2+}, Cd^{2+}, or La^{3+}. There was essentially no release of prebound [^{125}I]IGF-II from cell-associated IGFBP-5 for any treatment even at the 4 h time point (Fig. 3, middle). Approximately 20% of prebound ligand was released from the IGF-1R in control wells with most of the loss occurring after 90 min (Fig. 3, right). Au^{3+} and Zn^{2+} depressed ligand dissociation while Cd^{2+} or La^{3+} had no effect. In contrast, IGF-I markedly increased the rate of [^{125}I]IGF-II dissociation from the IGF-1R with the loss of >40% after 1 h and 90% after 4 h.

These data indicate that cations, except Au^{3+}, do not increase IGF-II dissociation from IGFBP-3 or IGFBP-5.

Discussion

The goal of the current study was to test mechanism(s) by which multivalent cations affect IGF-II binding to cell surfaces. Cell types differ markedly in the type of IGF binding moiety expressed at the plasma membrane. The human fibroblasts used in this study do have IGF-1Rs but the major binding site on the surface of these cells is IGFBP-3 *(26)*. Similarly, human glioblastoma cells have very few IGF-1Rs relative to the abundance of IGFBP-5 *(27)*. In contrast myoblasts, in particular the P_2A_{2a}-LISN transfected cells used in the current work, have little if any cell-associated IGFBPs but have the IGF-1R as their major cell surface IGF binding site *(28)*. Clearly different cell types within tissues present various binding moieties for the IGFs and partitioning among the various binding sites will ultimately control the tissue response to extracellular IGFs.

The current data clearly indicate complex interactions between cations, IGFBPs and IGF-1R. This work shows that Zn^{2+} enhances [^{125}I]IGF-II binding to the IGF-1R while depressing [^{125}I]IGF-II binding to IGFBP-3 and IGFBP-5. The inhibitory effect is more pronounced for IGFBP-5 than for IGFBP-3. Two other cations, which bind to proteins at the same site as Zn^{2+} (Cd^{2+} and Au^{3+}), had similar depressing effects on the IGFBPs. La^{3+}, which can replace Ca^{2+} at its binding site on proteins and was used in these studies as a negative control, is largely without effect on IGFBPs affinity *(29)*, although it tends to enhance IGF-1R binding. In contrast, Au^{3+} had a depressing effect on IGF-II binding to the IGF-1R versus an enhancing effect for Zn^{2+} and Cd^{2+}. These effects are the result of affinity changes, which can be explained by changes in the rates of ligand association.

Whether Zn^{2+} affects the rate of association or dissociation is not a trivial concern. These results have far-reaching implications regarding the application of the results. An effect on rate of dissociation would indicate that Zn^{2+} would act to free IGFs within extracellular fluids long after secretion, chelation by IGFBPs, and export into the circulation. Our findings imply that Zn^{2+} does not have such a potential. In contrast, our results clearly show that Zn^{2+}, and possibly other micronutrients, depress the rate of association. This implies that Zn^{2+} will prevent IGF chelation by IGFBPs and maintain them in the free "active" state within the tissue of production. Thus, Zn^{2+} should enhance the autocrine/paracrine effects of the IGFs more so than their endocrine effects. We now have exciting new data that clearly indicate

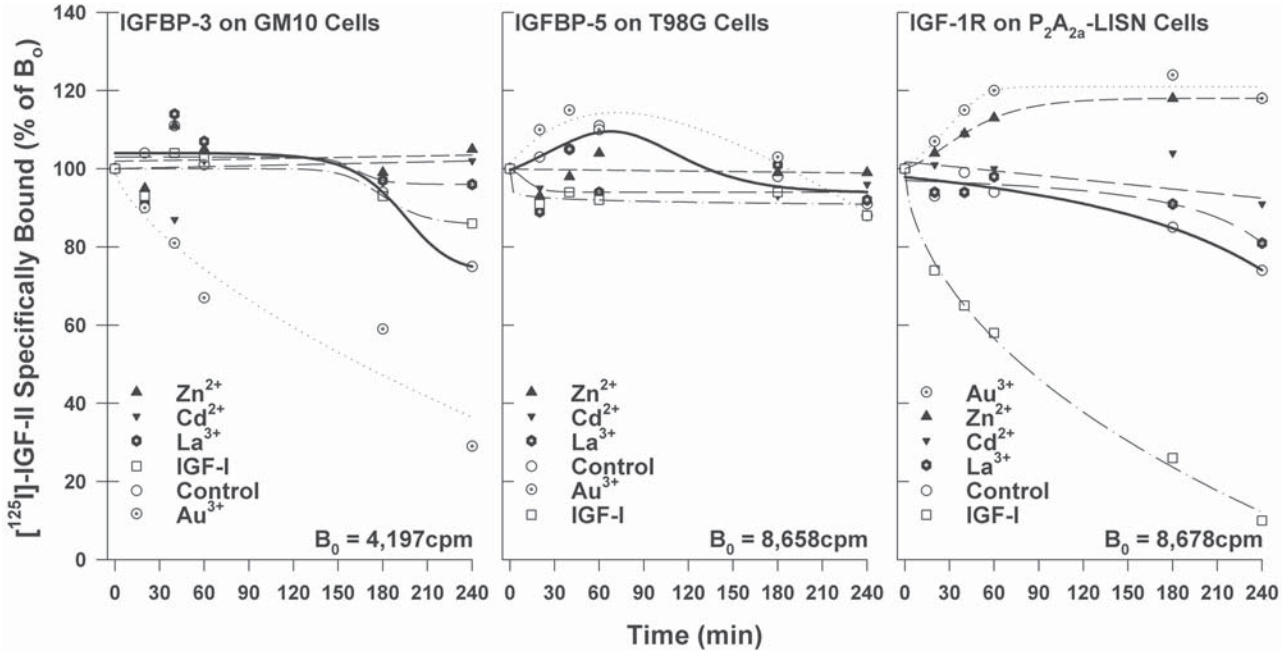

Fig. 3. Effect of various cations or IGF-I on [^{125}I]IGF-II dissociation from IGFBP-3, IGFBP-5, and the IGF-1R. [^{125}I]IGF-II was added and allowed to bind for 3 h. The cultures were extensively rinsed and fresh buffer was added containing buffer alone or buffer plus either cations at 200 μ*M* or IGF-I at 100 ng/mL. The amount of [^{125}I]IGF-II still bound at each time was quantified. The cpm bound at time 0 (B_0) are indicated (lower right corner of each graph). Legends are arranged to correspond to treatment effects noted within each panel. Values were taken from a single representative experiment.

this potential. Zn^{2+} depresses both IGF-I and IGF-II binding to secreted/soluble IGFBP-5 *(30)*. Depressed binding to the soluble IGFBP enhanced binding to cell surface IGF-1R receptors on BC_3H-1 muscle cells. These results clearly indicate that Zn^{2+} partitions both IGF-I and IGF-II away from the inhibitory soluble IGFBP-5 but toward the cell surface receptor involved in signal transduction within cells. Thus, Zn^{2+} should enhance IGF activity by this mechanism.

Our previous work had shown that Zn^{2+} acts at physiological levels to modulate IGFBP-3, IGFBP-5, and IGF-1R binding *(26–28)*. Cell-associated IGFBP-3 and IGFBP-5 bound both IGF-I and IGF-II with a significantly greater affinity than did the IGF-1R unless Zn^{2+} was present. Those studies involved competitive binding assays (IGF-I competition for [^{125}I]IGF-I and IGF-II competition for [^{125}I]IGF-II) to assess affinity. The calculation of binding affinity based on varied levels of [^{125}I]IGF-II in the current work avoids complications associated with possible differences in binding avidity of unlabeled and radiolabeled ligand. However, one problem with the current assay is the large amount of radioactive ligand required. To decrease user exposure and the cost of the experiments, affinity measurements in Table 1 represent single determinations. Even with this limitation, the current affinity data confirm our previous findings with almost identical high affinity constants for all three binding moieties. This indicates that the changes noted with the single determinations are real and that both types of binding assays confirm cation-induced affinity changes.

A major difference was found using the two assay types. IGF-I and IGF-II binding to all three moieties resulted in nonlinear concave down Scatchard plots in our previous work using competitive binding assays. This phenomenon is a result of negative-cooperativity for IGF binding to the IGF-1R *(31)* and unknown causes for the two IGFBPs. Nonlinear Scatchard plots require the determination of affinity constants for both high- and low-affinity binding sites. Scatchard plots for all three binding moieties were linear when assessed with varying levels of [^{125}I]IGF-II in the current experiments. However, using the earlier competitive assay, the high-affinity binding constant is quantified when unlabeled IGF is present from 0 to 20 ng/mL and the low-affinity site is quantified when unlabeled IGF is added between 20 and 500 ng/mL *(26–28)*. Thus, the data presented herein represent only the high-affinity binding sites of the IGF-1R and cell-associated IGFBPs, because the amount of labeled [^{125}I]IGF-II peaked at 27 ng/mL. Indeed, the affinity constants herein are nearly identical to $K_{a\,hi}$ presented in the earlier studies. Further support that the low-affinity binding sites of the receptor and IGFBPs were not saturated by even the highest dose of [^{125}I]IGF-II is provided by the observation that specific binding was still increasing between the addition of 400,000 and 800,000 cpm of [^{125}I]IGF-II (Fig. 1). Our previous data clearly indicated that the high-affinity binding sites were those affected by cations, as evidenced by the identical responses found in the current work.

K_a is a function of the "on rate" and "off rate" of ligand binding, i.e., rate of association and rate of dissociation *(32)*. Each of these components can be altered independently. Changes in the rate of dissociation could not be accurately quantified because of the extremely slow rate of dissociation at both 4 and 25°C and because of the combined active secretion and release of IGFBPs from the cell surface at 37°C. However there was no evidence that Zn^{2+} increased the rate of IGF-II dissociation from cell-associated IGFBPs, a phenomenon that would depress K_a. We were able to show that Au^{3+} did increase the rate of dissociation of IGF-II from IGFBP-3. This latter effect was specific, as it did not occur in the presence of Zn^{2+}, Cd^{2+}, or La^{3+}. Although Zn^{2+}, Cd^{2+}, and Au^{3+} bind to the same site on proteins (reviewed in *33*), Au^{3+} is considerably larger than the other two cations. Its presence may have altered IGFBP-3 structure differently than the presence of the other cations, enough to affect dissociation rate. Although we have tested additional metals, no other physiologically relevant cation has been found that affects IGFBP binding to either IGF-I or IGF-II. At this point it is unknown if an unidentified micronutrient increases the rate of IGF dissociation from either IGFBP-3 or IGFBP-5 and thereby serves as a release mechanism for IGFs.

Our experiments show Zn^{2+} acts to partition IGF-II away from cell-associated IGFBP-3 and IGFBP-5 and onto the cell surface IGF-1R by modulating the rate of association. Zn^{2+} depressed the affinity of cell-associated IGFBP-5 more so than that of IGFBP-3, indicating IGFBP differences. The change in affinity paralleled the effects of Zn^{2+} on the rate of association; Zn^{2+} had a greater effect on IGFBP-5 association constants than on IGFBP-3. Rate of association was suppressed by more than 70% for IGFBP-5 and by more than 50% for IGFBP-3, whereas affinity was suppressed by 70% and 25%, respectively. Thus the changes in binding affinity could be a result of changes only in rate of association without requiring changes in the rate of dissociation for both IGFBP-3 and IGFBP-5.

Zn^{2+} did depress the rate of dissociation of [^{125}I]IGF-II from the IGF-1R. Thus, both enhanced rate of association and attenuated rate of dissociation play a role in Zn^{2+}'s effect on the IGF-1R. These effects would indicate that IGF-II is more likely to bind to the IGF-1R in the presence of Zn^{2+} and once there will remain bound longer. Thus, Zn^{2+} should markedly accentuate IGF-II actions via the IGF-1R. IGF-I increased the rate of dissociation of [^{125}I]IGF-II from the IGF-1R but had no such effect on either IGFBP-3 or IGFBP-5. Thus, at least by this assay, there was no evidence of IGF-I-induced negative cooperativity for IGFBP binding but there was for IGF-1R binding. This effect of IGF-I on [^{125}I]IGF-II binding to the IGF-1R was similar to the effect of IGF-I on [^{125}I]IGF-I binding to the IGF-1R *(34)*.

A very important remaining question is whether Zn^{2+} binding sites exist within IGFBP-3 and IGFBP-5, since there is none within either IGF-I or IGF-II *(35)*. The nature of the Zn^{2+} effect is related to the probable site of interaction with IGFBP-3 and IGFBP-5. Zn^{2+} has a variety of functions including a role as an enzyme cofactor, enhancement of DNA binding, and the stabilization of protein structure *(36)*. In the extracellular environment, Zn^{2+} often binds to proteins with a tetrahedral coordination to four cysteine residues, thus stabilizing tertiary structure *(13)* and preventing oxidation of cysteine residues in this oxidizing environment *(37)*. A structure-stabilizing motif for Zn^{2+} consists of four cysteine residues arranged in two pairs. Cysteines within each pair are usually separated by 1 to 20 or more amino acids, and the pairs are usually separated by 20 or more residues; this latter intervening sequence serves as a loop or spacer permitting the two pairs to be juxtaposed. In the absence of Zn^{2+}, the cysteines within each pair are either reduced, share a hydrogen ion, or form a disulfide bond but are not involved in disulfide bonds with other residues *(12)*. In the presence of Zn^{2+}, disulfide bonds are not present but instead Zn^{2+} binds to the cysteine residues with a tetrahedral coordination.

A model was produced illustrating the probable Zn^{2+} binding motif in IGFBP-1 through IGFBP-5 and the IGF-1R (Fig. 4); IGFBP-6 lacks cysteine residues analogous to numbers 6 and 7 and thus does not completely fit this model. Disulfide pairings for all but cysteines 5 and 6 have been confirmed *(38–42)*. Cysteine residues 1 and 2 are separated by two amino acids (proline–glycine) and form a disulfide bond in IGFBP-6 *(38)*. The conservation of a probable disulfide bond between cysteines 1 and 2 in IGFBP-3 and IGFBP-5 is based on the disulfide isomerase activity of IGFBPs *(43)*. Disulfide isomerase activity, or the ability of a protein to refold other proteins by disulfide bond formation and rearrangement, is associated with the presence of a reactive tetrapeptide -Cys-X-Y-Cys- sequence within proteins *(44)*. The two cysteines within this motif form an unstable disulfide bond, and this instability confers both disulfide isomerase activity *(45)* and the ability to react with Zn^{2+}. Cysteines 1 and 2 within IGFBP-1 through IGFBP-6 all contain a motif (–C—A/E/P–P/G–C–) indicative of disulfide isomerase activity, consistent with the presence of an unstable disulfide bond between these two residues. The existence of a disulfide bond between cysteines 5 and 6 is supported by indirect evidence. First, there are no free cysteines within the IGFBPs *(46,47)*; second, all other cysteines are involved in previously defined disulfide linkages, and third, the ability of disulfide bonds to form between cysteine residues separated by only one amino acid residue *(48)*. Alternatively, other pairs of cysteines may form a zinc-binding site. The 3-D structure of an IGFBP-5 peptide containing cysteines 9, 10, 11, and 12 indicates that the side chains of these four cysteine residues form a pocket *(41)* wherein zinc may reside (Fig. 4 left inset). The 3-D structure of the remaining portions of IGFBP-5 has not been determined. Until the 3-D structure of an IGFBP is described the exact proximity of these two cysteine pairs (1–2 and 5–6)

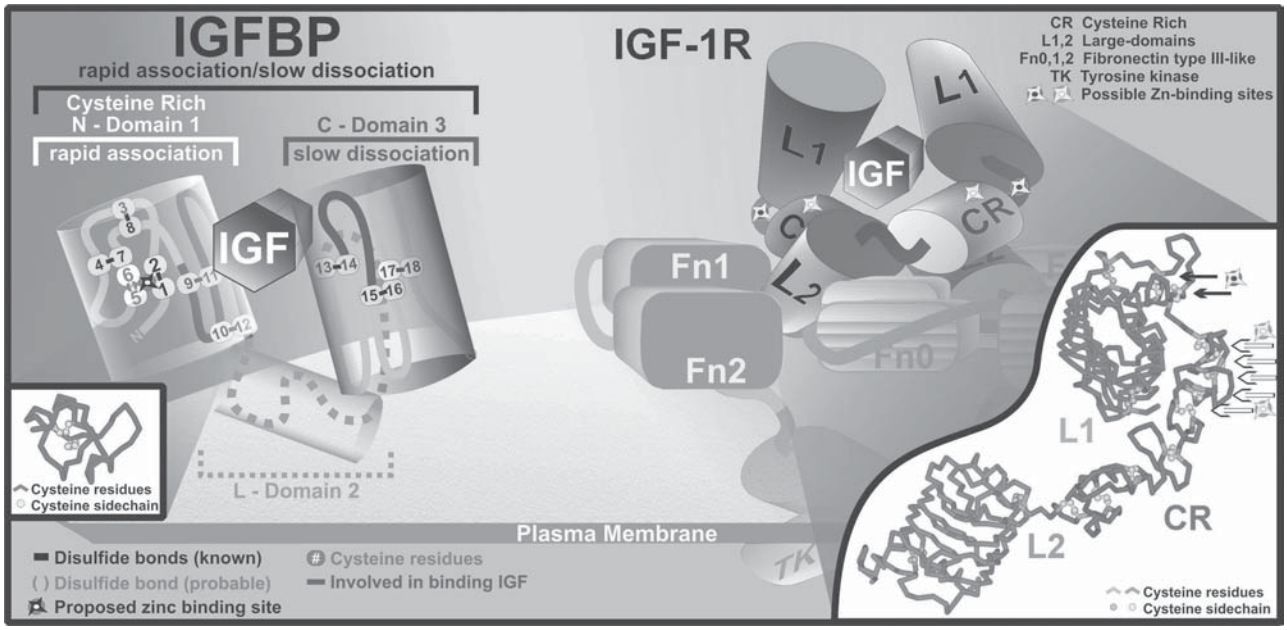

Fig. 4. Model for the mode of action of zinc on IGFBP-3, IGFBP-5, and IGF-1R ligand binding. The model is a cartoon of IGFBP structure with the proposed disulfide bond arrangements for IGFBP-1 through IGFBP-5 *(36–40)*. All of the cysteines conserved in IGFBP-1 through IGFBP-5 are purportedly involved in disulfide bonds [because IGFBPs do not contain free cysteines *(44,45)*], but the disulfide bond linking cysteines 5 and 6 has not been confirmed at this time. In IGFBP-1 through IGFBP-5, Cys1 and Cys2 are separated by two intervening amino acids (GluPro, AlaPro, or ProPro) and Cys5 and Cys6 by a single intervening glycine *(58)*. A classical Zn^{2+} binding motif *(13)* would form by the near-proximity of four cysteine residues: numbers 1, 2, 5, and 6. The tertiary structure of a truncated form of IGFBP-5 containing cysteines 9–11 has been determined *(39)* and coordinates (MMDB Id: 12156, PDB Id: 1BOE) rendered using CnD3 (left inset). The illustration for the structure of the IGF-1R heterodimer is based on the structure of the insulin receptor *(50)*. A set of two L1, CR, and L2 regions, one from each half of the heterodimer, form a binding pocket for IGF. The structure of the L1, CR, and L2 regions has been determined *(49)* and coordinates (MMDB Id: 11440; PDB Id: 1IGR) used to render a version using the NCBI 3-D viewer (right inset): CnD3 version 3. 0 (program and structure coordinates from http://www. ncbi. nlm. nih. gov/Structure).

will remain unknown, but they do form the most likely Zn^{2+} binding site and, most important, this proposed Zn-binding site is located within the N-terminus of the IGFBPs.

The importance of the N-terminal location of a Zn^{2+} binding site is relative to the interpretation of the data. Recently, both N-terminal and C-terminal fragments of IGFBP-2 and IGFBP-3 were shown to bind IGF-I using BIAcore instrumentation. N-termini were shown to be necessary for rapid ligand association and C-termini necessary for slow dissociation *(49,50)*. The current work indicates that Zn^{2+} depresses the rate of ligand association without evidence of an effect on ligand dissociation and so is presumably Zn^{2+} interacts with the N-terminal ends of the IGFBPs. A probable Zn^{2+}-induced structural change in the N-terminus of IGFBP-3 or IGFBP-5 would be consistent with the location of the proposed Zn^{2+} binding site(s) shown in Fig. 4.

The structure of the IGF-1R has not been fully elucidated. However, with partial structural determination of the IGF-1R *(51)* and more complete data on the insulin receptor *(52)* the probable binding site for zinc can at least be speculated. The α-subunits of the receptor form the central core "pocket" composed of two L1, two cysteine rich (CR), and two L2 domains. This pocket surrounds the IGF molecule during binding Fig. 4. The most likely zinc-binding site(s) for the IGF-1R is found in the CR region of the receptor. The CR domain contains 149 amino acids, 22 cysteine residues, and 11 disulfide bonds *(51)*. Upon more detailed examination of IGF-1R structure *(51)*, two of the disulfide bonds are juxtaposed at a bend in CR (Fig. 4 right inset, dark arrows) and five other disulfide bonds are clustered in a 41-amino-acid "backbone" region of the protein (open arrows). All of the cysteine residues that form disulfide bonds are separated by 7–12 amino acids. This type of structure has the capacity to bind four or five zinc molecules *(36)*. Upon ligand binding within the pocket formed by the two L1/CR/L2 units, the IGF-1R undergoes a conformational change thereby shifting the shape of the underlying structures and the intracellular tyrosine kinase domains *(53)*. It is easy to imagine an alteration in CR structure upon Zn^{2+} binding with the resultant increase in receptor affinity described herein. However, the consequences of these shifts on receptor activation remain to be studied. Unlike the smaller IGFBPs, regions of the receptor necessary for rapid ligand association and/or slow dissociation are not defined.

In summary, Zn^{2+} has been shown to accentuate IGF-I activity, although similar experiments have not been reported for IGF-II. Zinc potentiated the effect of IGF-I but not that of insulin *(54)*. Zinc exerts its major effect on DNA synthe-

sis during the IGF-I stimulatory phase of the cell cycle and chelation of extracellular zinc depresses cell proliferation without changes in intracellular Zn^{2+} levels *(55)* clearly implicating an extracellular mode of action. Furthermore, Zn^{2+} altered the effect of IGF-I but not insulin despite similar intracellular signaling mechanisms for these hormones/growth factors. The findings from this article describe an extracellular mechanism by which Zn^{2+} would affect IGF-I activity without affecting insulin activity (insulin does not bind to IGFBP-3 or IGFBP-5). Our data indicate that Zn^{2+} has the potential to enhance IGF-II activity by partitioning IGF-II away from IGFBPs onto the IGF-1R. This mechanism could explain many of the growth promoting actions of Zn^{2+} both in vitro and in vivo.

Materials and Methods

Cell Culture

GM10 (human fibroblasts) and T98G (human glioblastoma) cells were acquired from the NIGMS Human Mutant Genetic Cell Repository (Camden, NJ) and the American Type Culture Collection (Rockville, MD), respectively. P_2A_{2a}-LISN myoblasts *(56)* were a gift from Dr. Mike Kaleko from the Fred Hutchinson Cancer Research Center. Cells were passaged and plated once a week as previously described *(24,57)*. GM10 and T98G cells were grown in Eagle's minimum essential medium (EMEM, Gibco BRL, Grand Island, NY) plus 10% calf serum (Gibco). P_2A_{2a}-LISN cells were grown in DMEM (Dulbecco's modified Eagle's medium (Gibco BRL) plus 10% fetal bovine serum (JRH, Lenexa, KS). P_2A_{2a}-LISN cells were plated on gelatin-coated plates for all assays.

Iodine-125 Labeling

Recombinant human IGF-II (Bachem, Torrance, CA) was iodinated by incubating 5 µg aliquots with 2 mCi [iodine-125] (Amersham, Arlington Heights, IL) and 12 µg/mL chloramine-T in sodium phosphate buffer (500 m*M*, pH 7.4). The reaction was stopped by adding sodium-(meta)-bisulfite. Free ^{125}I was separated from the [^{125}I]labeled IGF-II with Millipore (UFC3 LGC 25) 10,000 kDa nominal molecular weight cut-off filter units *(28)*. The specific activity of the iodinated peptide averaged approx 250 µCi/µg.

Binding Assay, Varying Amounts of [^{125}I]IGF-II

Confluent 7–8 d cultures grown on 96-well plates were rinsed three times with PBS then incubated with Earle's balanced salt solution (EBSS) at 4°C for 3 h to allow release of any loosely associated IGFBPs from the cell surface and to minimize IGFBP release during the subsequent incubation. The cells were then rinsed twice with PBS and incubated 3 h at 4°C with assay buffer (EMEM, 20 m*M* HEPES, 1% BSA, pH 7.4; final volume 75 µL) plus or minus various cations (200 µ*M*), IGF-I (900 ng/mL), IGF-II (3,000 ng/mL for NSB), and varying doses of [^{125}I]IGF-II. [^{125}I]IGF-II was added over a 200-fold range of 4,000 to 800,000 cpm/well (i.e., approx 0.13–27 ng/mL or approx 17–3,500 p*M* IGF-II). Assay buffer for GM10 and T98G cultures also contained 10 µg/mL sodium insulin to minimize tracer binding to the IGF-1R *(24,29)*. At the end of 3 h, assay buffer was aspirated and cells were rinsed three times with PBS. Cells were solubilized at room temperature with 300 m*M* NaOH and transferred into 12 ↔ 75 mm tubes. Bound ligand was counted using a gamma counter. During all parts of the assay, plates were placed on a platform rotator to ensure adequate mixing. Affinity data, indicating the mode of action by which the specific binding is being affected, was calculated from the binding data *(32)*.

Association Assay, Varying Time

Confluent cultures grown on 24-well plates were rinsed three times with PBS and incubated at 4°C for 3 h with EBSS. Cells were then rinsed three times with EBSS before the addition of assay buffer, plus or minus cations (200 µ*M*) or IGFI (100 ng/mL). GM10 and T98G cells received 10 µg/mL of sodium insulin to minimize receptor binding. Wells received [^{125}I]IGF-II in a final volume of 250 µL. The plates were incubated for various times at 4°C. At the appropriate time, the assay buffer was aspirated and the culture was rinsed three times with PBS. Cells were solubilized with 300 m*M* NaOH, transferred into 12 ↔ 75 mm tubes, and counted. Rate of association was calculated from the binding data *(32)*. During all parts of the assay, plates were placed on a platform rotator to ensure adequate mixing.

Dissociation Assay

Confluent cultures grown on 96-well plates were cooled to 4°C, rinsed three times with PBS, and then incubated for 3 h with EBSS at 4°C. After the incubation, cells were rinsed three times and [^{125}I]IGF-II was added in 20 µL of fresh assay buffer. The cells were incubated for 3 h at 4°C to allow tracer binding. GM10 and T98G cells received 10 µg/mL of sodium insulin to minimize IGF-1R binding. The cells were rinsed three times with PBS to remove unbound tracer, then remained in the cold room while assay buffer and either cations (200 µ*M*) or IGF-I (100 ng/mL) was added to a final volume of 340 µL. During all parts of the assay, plates were placed on a platform rotator at 160 rpm to ensure adequate diffusion of dissociated [^{125}I]IGF-II. Wells were aspirated at various times and then rinsed three times with PBS. After the final timed rinse, all cells were solubilized with 300 m*M* NaOH and transferred to 12 ↔ 75 mm tubes for counting of [^{125}I]IGF-II that remained bound to the cells.

When dissociation was assayed using the protocol above but performed at 37°C, most of the released [^{125}I]IGF-II was precipitated with PEG-8000, indicating that the ligand dissociating from cells was still bound to IGFBPs. Previous work had shown that IGFBPs can dissociate from the cell surface *(24,29)*. Therefore, it is very likely that [^{125}I]IGF-II could be lost from the cell surface via the loss of cell-asso-

ciated IGFBPs, which would prevent calculation of [^{125}I]IGF-II dissociation from the IGFBP. To avoid this complication, dissociation was determined at 4°C and room temperature. At 4°C or room temperature the [^{125}I]IGF-II released from the cell surface was not precipitated by PEG-8000 (not shown) and so was not bound to IGFBP. At room temperature results were identical to that found at 4°C (not shown).

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