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Multivalent Cations Depress Ligand Affinity of Insulin-Like Growth Factor-Binding Proteins-3 and -5 on Human GM-10 Fibroblast Cell Surfaces

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Abstract The effect of multivalent cations on [^{125}I]-IGF binding to cell-associated IGFBPs was investigated using human fibroblasts. The major cell-associated binding site for [^{125}I]-IGF-I is IGFBP-3 and for [^{125}I]-IGF-II are IGFBP-3 and IGFBP-5. Lanthanum and chromium did not affect either [^{125}I]-IGF-I or [^{125}I]-IGF-II binding to cell-associated IGFBPs. By contrast, zinc (Zn^{2+}), gold (Au^{3+}), and cadmium (Cd^{2+}) depressed binding of both ligands. Ligand binding resulted in nonlinear Scatchard plots. Assuming a pre-existent asymmetric model with high- (K_{aHi}) and low- (K_{aLo}) affinity sites, Zn^{2+} lowered both K_{aHi} and K_{aLo}. Au^{3+} eliminated K_{aHi}. Assuming that the nonlinear plots were caused by ligand-induced negative cooperativity, Zn^{2+} and Cd^{2+} lowered both K_e and K_f (affinity of unoccupied and saturated IGFBPs, respectively). Au^{3+} eliminated K_e and reduced K_f. Zn^{2+} was active at serum levels in lowering IGF binding. Zinc, gold, and cadmium bind to similar regions within proteins (a zinc-binding motif) indicating similar mechanisms of action. A zinc-binding motif is present in the IGFBPs, but not in the IGFs. We demonstrate for the first time that the trace nutrient zinc and related multivalent cations decrease IGF binding to fibroblast-associated IGFBPs by lowering the affinity of the IGF–IGFBP interaction. J. Cell. Biochem. 69:364–375, 1998.

Key words: IGF; IGFBP; zinc; IGFBP-3; IGFBP-5

IGF-I and IGF-II stimulate a variety of anabolic effects and are important growth factors for a variety of cells [Clemmons et al., 1991; Cohick and Clemmons, 1993]. Although the IGFs are the active agents, IGFBPs are important modulators of metabolism because of their ability to bind IGF-I and IGF-II with high affinity. IGFBPs in extracellular fluids decrease IGF activity, an effect whose mechanism is easy to explain. Simply, soluble IGFBPs delay IGFs from activating the type 1 IGF receptor on the cell surface. IGFBPs can also intensify IGF activity apparently involving IGFBP association with the cell surface [Jones and Clemmons, 1995].

The mechanism(s) controlling the distribution of the IGFBPs between the extracellular fluids and the cell surface is poorly understood, although we have shown this to be cation dependent [McCusker and Clemmons, 1997]. Even less is known about the control of IGF distribution between cell-associated IGFBPs and the type 1 IGF receptor. Cell-associated IGFBPs probably form a reservoir of bound IGF in the pericellular milieu. This in itself may act to enhance IGF activity by partitioning more IGF to the cell surface. However, the physiological release mechanism to free the IGF for receptor activation is undefined. Release of IGFs from IGFBPs may involve proteolysis of the soluble IGFBPs found in serum and other extracellular fluids [Camacho-Hubner et al., 1992; Conover and Kiefer, 1993; Fowlkes et al., 1994; Kajimoto et al., 1995; Kanzaki et al., 1994]. Proteolysis of IGFBP-3 lowers ligand affinity [Baxter and Skriver, 1993; Blat et al., 1994; Lamson et al., 1993], thus releasing IGFs. However, cell-associated IGFBPs appear to be resistant to proteolysis [Jones et al., 1993]. Here we describe cation-induced changes in IGFBP affinity that would serve to release IGFs from cell-associated IGFBPs. The physiologically relevant cation involved in this action is Zn^{2+}.

Zinc-deficient animals stop growing compared with those provided with sufficient di-

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etary Zn^{2+} [Roth and Kirchgessner, 1994]. Zn^{2+} has a variety of intracellular functions that may produce this effect, including a requirement for optimal activity of DNA binding proteins and numerous enzymes [Vallee and Falchuk, 1993]. However, growth retardation occurs in parallel with depressed extracellular (serum) Zn^{2+} levels before changes in tissue (intracellular) Zn^{2+} levels take place [Brandaoneto et al., 1995]. Another aspect of the growth regulatory activity of Zn^{2+} includes controlling IGF levels in serum [Dorup et al., 1997; Droke et al., 1993; Mcnall et al., 1995; Ninh et al., 1995, 1996; Roth and Kirchgessner, 1994], although depressed IGF-I levels may be secondary to the low food intake of Zn^{2+}-deficient animals [Clegg et al., 1995]. Again, depressed IGF levels and food intake still indicate alterations in physiological responses before tissue Zn^{2+} concentrations change. Together these findings hint at an extracellular mode of action, as extracellular (serum) levels of Zn^{2+} drop early upon zinc removal from the diet. Also, Zn^{2+} enhances the metabolic and mitogenic activity of IGF-1, but not insulin [Matsui and Yamaguchi, 1995]. An intracellular mechanism of action was proposed. If so, Zn^{2+} should have enhanced the activity of insulin, as insulin and IGF share common intracellular signaling mechanisms [Prager and Melmed, 1993]. Thus, an extracellular mode of action for Zn^{2+} in regulating IGF activity is again implicated.

The current work was designed to test for a new mechanism whereby multivalent cations, especially the trace nutrient Zn^{2+}, interact with the IGF system. The objective was met by studying [^{125}I]-IGF binding to cell-associated IGFBPs using cells grown in zinc-deficient conditions with and without cation supplementation during the binding assay. We report that several cations are capable of reducing the binding affinity of both IGF-I and IGF-II with cell-associated IGFBP-3 and -5 on human fibroblasts. We have previously demonstrated that IGFBP-3 released from GM-10 cell surfaces have a 10-fold enhancement in ligand affinity over that on the cell surface [McCusker et al., 1990; McCusker and Clemmons, 1997]. Also, La^{3+} and Zn^{2+} prevent the loss of cell-associated IGFBP-3 and IGFBP-5 and, like IGFBP-3, soluble IGFBP-5 has a higher affinity than cell-associated IGFBP-5 [McCusker and Clemmons, 1997]. In the current work, we find that ligand affinity of cell-associated IGFBPs is further depressed ≈40% with the addition of Zn^{2+}. Hence, there is a stepwise change in ligand affinity with relative binding affinities of approximately 10:1:0.6, respectively:

K_a ranking; soluble IGFBPs
⩔
cell-associated IGFBPs
⋁
cell-associated IGFBPs + zinc

MATERIALS AND METHODS

Cell Culture

GM-10 (human fibroblasts) cells were acquired from the NIGMS Human Mutant Genetic Cell Repository (Camden, NJ). Cells were passaged once a week as previously described [McCusker et al., 1990]. Cells were grown in Eagle's minimum essential medium (EMEM) plus 10% calf serum (GIBCO-BRL, Grand Island, NY), 100 µg/ml pyruvate, 30 µg/ml asparagine, 21 µg/ml serine, 10 U/ml penicillin and 10 µg/ml streptomycin (Sigma, St. Louis, MO). EMEM does not contain zinc or any of the multivalent cations tested in this study; thus, the cells were maintained in 10% circulating levels of zinc supplied by the calf serum before the binding assays (zinc-deficient conditions). Zinc (Zn^{2+}), lanthanum (La^{3+}), chromium (Cr^{3+}), cadmium (Cd^{2+}), and gold (Au^{3+}) were purchased as chloride salts from Sigma.

Binding Assay

Binding assays were performed with confluent 7- to 8-day cultures. Cultures were rinsed three times with phosphate-buffered saline (PBS) and then incubated with Earl's balanced salt solution (EBSS) at 4°C for 3 h to allow release of loosely attached IGFBPs from the cell, minimizing IGFBP release during the subsequent incubation [McCusker et al., 1990]. Cells were then rinsed twice with PBS and once with EBSS and incubated at 4°C with assay buffer consisting of EMEM without sodium bicarbonate, with 20 mM HEPES, with 1% bovine serum albumin (BSA) at pH 7.4. Radiolabeled IGF-I or IGF-II (70,000–80,000 cpm, ≈0.6 ng/ml) was added with varying doses of unlabeled IGF-I or IGF-II (1,000 ng/ml for non-specific binding) to a final volume of 250 µl. All cations were added at 200 µM, except in one experiment as indicated. Cations were added as chlo-

ride salts diluted in water and the same amount of water (5 µl) was added to control wells. The addition of the cations did not affect the pH of the assay buffer. As all assays are performed at 4°C, the cations did not affect cell number/well, cell viability, or protein content, and none of the treatments detached cells from the dish. All wells contained 10 µg/ml sodium–insulin (GIBCO-BRL) to eliminate binding to the type 1 IGF receptor [McCusker et al., 1990], except in part of the affinity-labeling experiment. After 3 h at 4°C (confirmed to be at equilibrium by preliminary studies), the assay buffer (AB) was collected into 12 × 75-mm tubes. The cells were rinsed with PBS, and 0.3 M NaOH was added for 1 h to solubilize the cells. The cells were then transferred to tubes and counted to quantify cell surface bound [^{125}1]-IGF.

To quantify [^{125}I]-IGF bound to IGFBPs released into the assay buffer, bound and free [^{125}I]-IGF were separated by precipitation of bound ligand with polyethylene glycol (PEG) [McCusker et al., 1990]. The results of the PEG precipitation assay are not presented, but were conducted for all assays. Less than 2% of the added tracer was specifically bound to released IGFBPs.

Affinity Labeling

Affinity labeling was performed with disuccinimidyl suberate (Pierce, Rockford, IL) as previously described [McCusker et al., 1990]. Samples (30 µl) of cells solubilized directly in Laemmli buffer (50 µl/cm^2) with 6% β-mercaptoethanol were electrophoresed through 5–18% gradient sodium dodecyl sulfate–polyacrylamide gels and then exposed to PhosphoImager Screens (Molecular Dynamics, Sunnyvale, CA).

^{125}Iodine Labeling

IGF-I, IGF-II (Bachem, Torrance, CA), and R^3-IGF-I (Gro-Pep, Adelaide, Australia) were iodinated by incubating 5 µg of peptide with 2 mCi [^{125}I] (Amersham, Arlington Heights, IL) and 12 µg/ml chloramine-T in 0.5 M sodium phosphate buffer (pH 7.4). Free iodine was separated from the ^{125}I-labeled IGF with Millipore (UFC3 LGC 25) 10,000 kDa nominal molecular-weight cutoff filter units. These filter units retain more than 95% of labeled peptide with more than 85% recovery of ^{125}I-labeled IGF. Specific activities ranged between 150 and 300 µCi/µg, as determined by trichloroacetate (TCA) precipitability. The manufacturers claim that R^3-IGF-I binds poorly to IGFBPs, but with normal affinity to type 1 IGF receptors, although this has not yet been published.

Data Analysis

Scatchard analysis [Scatchard, 1949] was performed using a previously described program [McCusker and Clemmons, 1997]. Hill coefficients were determined as described by De Lean and Rodbard [1979]. Hill coefficients deviating from 1 indicate the degree of nonlinearity of the Scatchard plots, with values of <1 indicating possible negative cooperativity. Fractional occupancy of the binding sites and average affinity at each point along the competition curve were determined [Demeyts and Roth, 1975]. These two measurements were imported into the Sigmaplot 2D curve fit program (Jandel Scientific Software, San Rafael, CA). Average affinity and fractional occupancy data were fit to gaussian cumulative formula 8012 to calculate K_e and K_f. K_e and K_f are the estimated affinities (equivalent to K_a, not K_d) at 0% and 100% of fractional binding site occupancy, respectively. Thus, K_{aHi} and K_{aLo} are affinity binding constants to describe nonlinear Scatchard plots caused by the presence of two distinct binding sites with pre-existent differences in affinity (pre-existent asymmetric model), whereas K_e and K_f represent the high- and low-affinity binding constants, assuming that the nonlinear plots are a result of a ligand-induced decrease in affinity (ligand-induced sequential model), in which B is bound, F is free, and R_o is receptor number. Graphically, $\bar{y}$ is presented as log (B/R_o) and average affinity for each point (K_a) along the competition curve was calculated as

$$(B/F)/(R_0 - B)$$

as described by DeMeyts and Roth [1975]. Data were further analyzed using SAS (Statistical Analysis Software, Cary, NC) and the general linear models procedure. Means were compared by Duncan's multiple range test.

Similar cation-induced findings on binding constants resulted independent of the mode of analysis (pre-existent versus ligand-induced); that is, if K_{aHi} was affected, so was K_e. Thus, several points should be made regarding the presentation of both analyses. The binding constants for the low-affinity sites (K_{aLo} and K_f) were similar, independent of the model and, indeed, should be [Demeyts and Roth, 1975]. However, K_{aHi} and K_e differed by three- to six-fold (K_{aHi} should always be greater than K_e with

negative cooperativity). Thus conclusions drawn from comparisons among IGFBPs or between binding to IGFBPs and receptors with other manuscripts will depend on the type of analysis performed and on whether other assays detect nonlinear Scatchard plots. Until the cause of the nonlinear Scatchard plots is defined, both analyses are necessary for comparisons and for future use of the data.

RESULTS

Affinity Labeling

GM-10 cell surface binding sites were affinity-labeled with [^{125}I]-IGF-I, [^{125}I]-IGF-II and [^{125}I]-R^3-IGF-I (Fig. 1). In the presence of sodium (Na)-insulin (10 µg/ml), [^{125}I]-IGF-I binds only to one protein, forming a 46,000-M_r band representing an IGF–IGFBP-3 complex (Fig. 1A). In the presence of insulin, [^{125}I]-IGF-II binds to proteins forming 46,000 M_r (IGF-IGFBP-3) and 37,000 M_r (IGF-IGFBP-5) bands (Fig. 1B). Specificity of binding is indicated by competition with the homologous peptide. The identity of these IGFBPs as IGFBP-3 and IGFBP-5 has been previously determined [Camacho-Hubner et al., 1992]. Insulin prevented binding of [^{125}I]-IGF-I and [^{125}I]-IGF-II to the type 1 IGF receptor (Fig. 1A,B). In the absence of Na-insulin, both [^{125}I]-IGF-I and [^{125}I]-IGF-II label the type 1 IGF receptor and, when directly compared, the IGFBPs are much more intense with [^{125}I]-IGF-II than with [^{125}I]-IGF-I (D). The presence of type 1 IGF receptors is also shown by the prominent labeling of 306,000 M_r and 116,000 M_r bands with [^{125}I]-R^3-IGF-I in the absence of insulin (Fig. 1C). [^{125}I]-R^3-IGF-I did not bind well to cell-associated IGFBPs, although there is a faint indication that the peptide may recognize IGFBP-3.

Na-insulin (10 µg/ml) was added to all wells in all subsequent assays, to prevent binding to the type 1 IGF receptor. Thus, IGFBP-3 and IGFBP-3/5 are the only detectable binding sites for [^{125}I]-IGF-I and [^{125}I]-IGF-II, respectively, in all subsequent assays. There was no evidence of

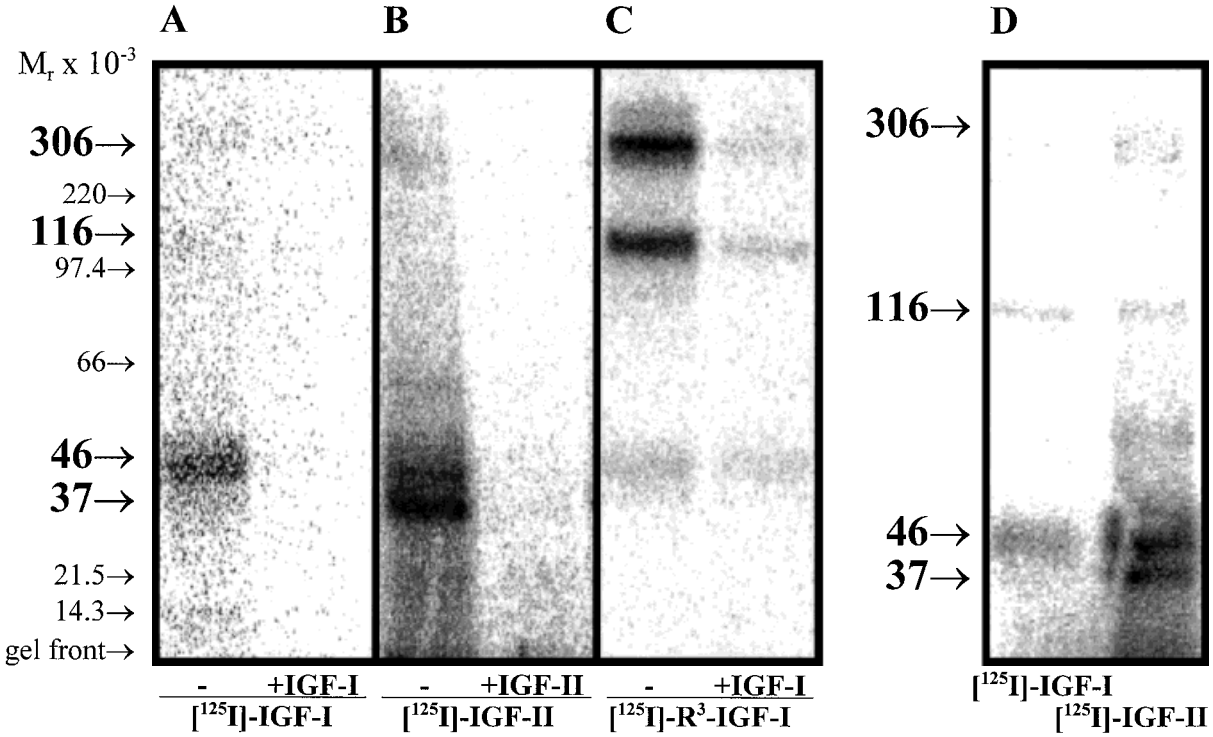

Fig. 1. Affinity labeling of GM-10 cell surfaces with [^{125}I]-IGF. GM-10 cells were affinity-labeled with either [^{125}I]-IGF-I (A, D), [^{125}I]-IGF-II (B, D), or [^{125}I]-R^3-IGF-I (C). Sodium insulin (10 µg/ml) was present during the binding of [^{125}I]-IGF-I (**A**) and [^{125}I]-IGF-II (**B**), but not [^{125}I]-R^3-IGF-I (**C**). Unlabeled IGFs (+IGF-I or +IGF-II) were added at 200 ng/ml. For direct comparison, samples labeled with [^{125}I]-IGF-I or [^{125}I]-IGF-II were run in parallel lanes. **D:** Insulin was not added. Samples were electrophoresed through gradient SDS-polyacrylamide resolving gels. Molecular-weight markers were run in parallel lanes to determine molecular weights of the proteins that bind the IGFs. Size of IGF-specific affinity-labeled bands are indicated by large bold letters and the location of molecular-weight markers by small letters.

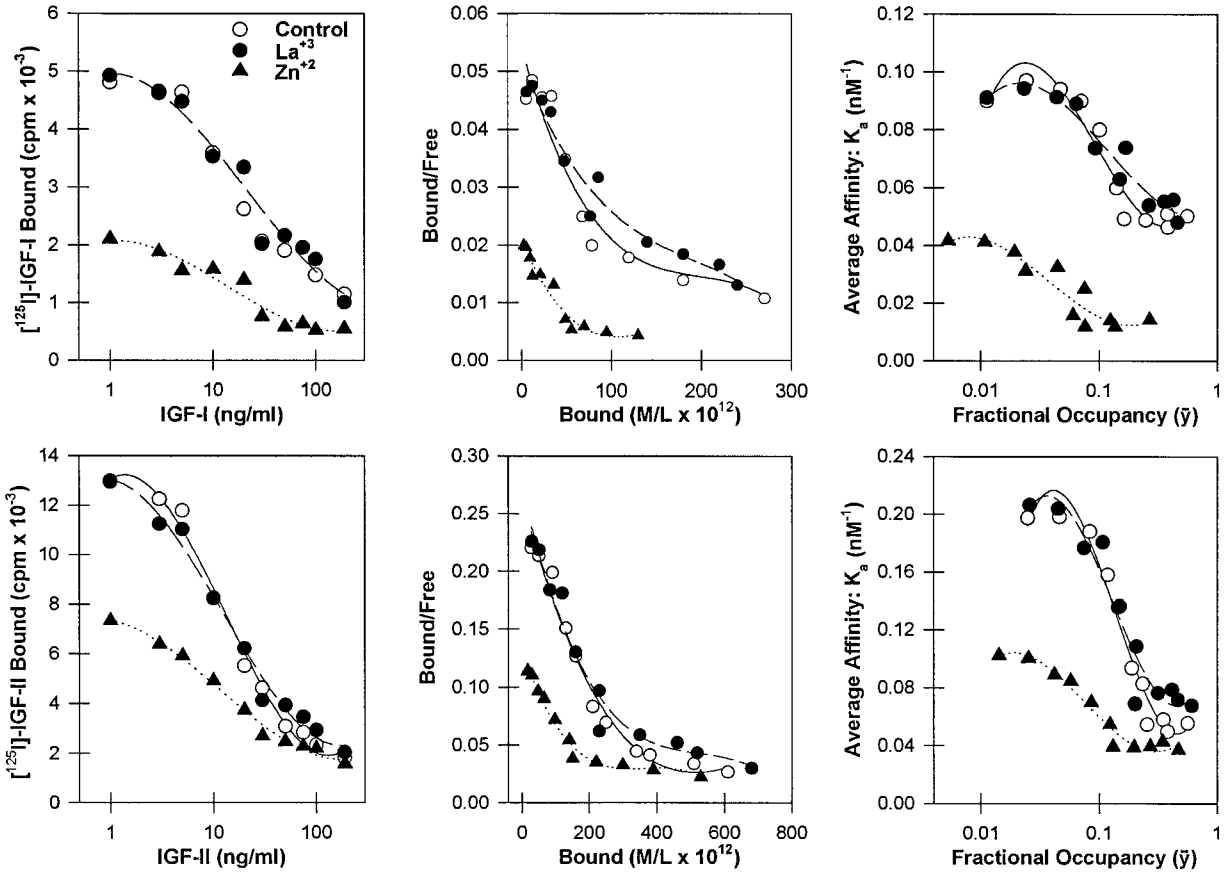

Fig. 2. Effect of La^{3+} and Zn^{2+} on specific binding of [^{125}I]-IGF-I and [^{125}I]-IGF-II to GM-10 cell-associated IGFBPs. La^{3+} (200 μM) or Zn^{2+} (200 μM) were added to cultures; binding was then accomplished by a 3-h incubation with tracer. Binding of [^{125}I]-IGF-I (top) and [^{125}I]-IGF-II (bottom) is shown using homologous ligand competition. Results were taken from a representative assay. Specific binding (**left**), Scatchard plots (**center**), and average affinity plots (**right**) are shown.

a band either the size of the type 2 IGF receptor or a high-molecular weight-band that bound [^{125}I]-IGF-II ≫ [^{125}I]-IGF-I that could represent the type 2 IGF receptor.

Specificity of IGF Binding to Cell Surface IGFBPs

[^{125}I]-IGF-I binding to cell-associated IGFBP-3 was evaluated. [^{125}I]-IGF-I binding was specific, as indicated by competition with unlabeled IGF-I (Fig. 2, top left). La^{3+} (200 μM) did not effect binding, whereas Zn^{2+} (200 μM) decreased binding. [^{125}I]-IGF-II binding (Fig. 2, bottom left) to cell-associated IGFBP-3/5 was specific, as indicated by competition with unlabeled IGF-II. Again, La^{3+} did not effect binding, but Zn^{2+} decreased binding. From repeated assays (Table I), La^{3+} did not affect, but Zn^{2+} decreased, [^{125}I]-IGF-I and [^{125}I]-IGF-II binding by ≈40%. GM-10 cell-associated IGFBPs bind approximately ≈4-fold (Table I) more [^{125}I]-IGF-II than [^{125}I]-IGF-I in agreement with the increased IGFBP band intensities as shown by affinity labeling and labeling of both IGFBP-3 and IGFBP-5 with [^{125}I]-IGF-II versus only IGFBP-3 with [^{125}I]-IGF-I.

TABLE I. Effect of La^{3+} and Zn^{2+} on IGF Binding to Cell-Associated IGFBP-3 and IGFBP-5†

Treatment	[^{125}I]-IGF-I	[^{125}I]-IGF-II
Control	100 (3,854 ± 308)	100 (16,405 ± 1,080)
La^{3+}	108 ± 7	103 ± 7
Zn^{2+}	61 ± 0.4*	63 ± 7*

†Mean ± SE of specific [^{125}I]-IGF binding. Data are expressed as a percentage of control and represent 11 experiments per mean for [^{125}I]-IGF-I and 6 for [^{125}I]-IGF-II. Average specific binding (cpm) of control cultures shown in parentheses. Control cultures specifically bound approximately 5% and 21% of the total counts for [^{125}I]-IGF-I and [^{125}I]-IGF-II, respectively. NSB range, 1–2.5%. Both cations were added at 200 μM.
*$P < 0.05$ vs control.

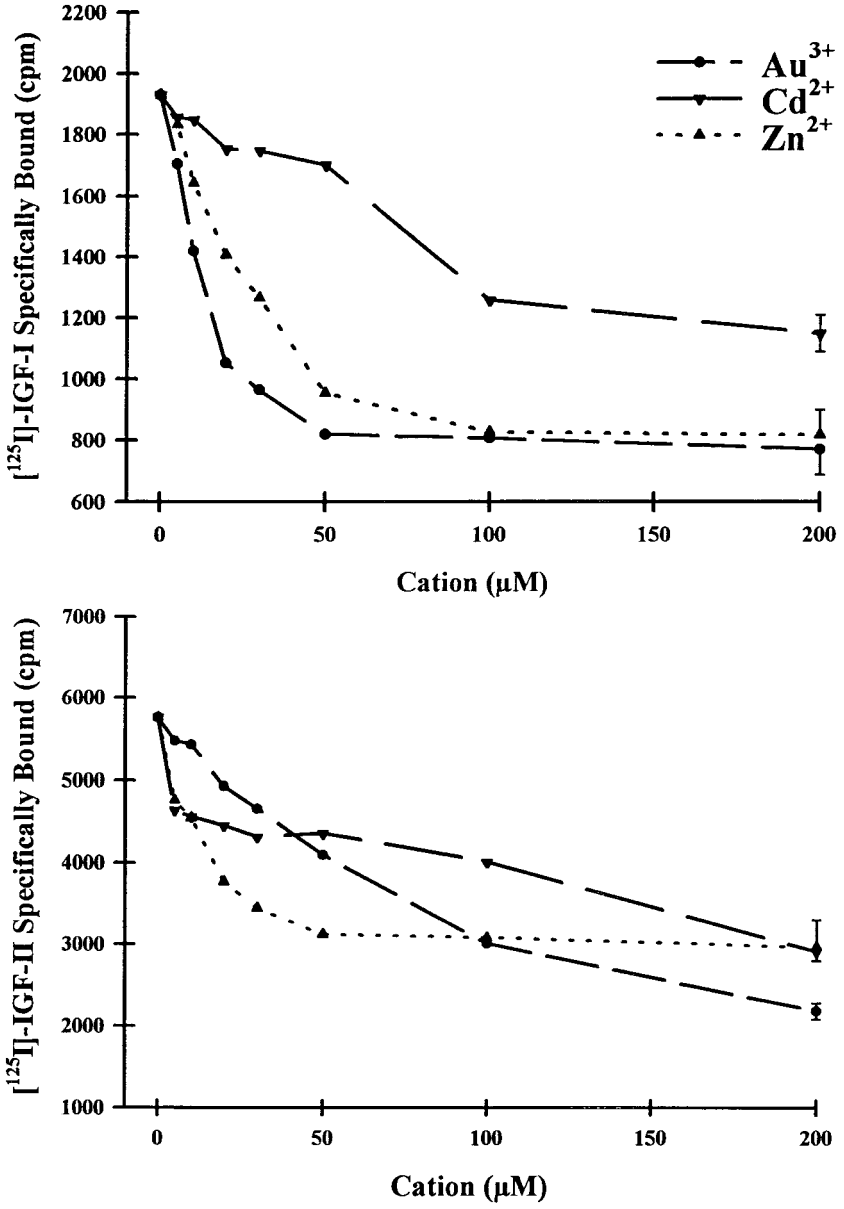

Fig. 3. Dose-dependent effects on IGF binding to GM-10 cell-associated IGFBPs. Varying levels of Zn^{2+}, Au^{3+}, and Cd^{2+} were added to binding assays, using either [^{125}I]-IGF-I (**top**) or [^{125}I]-IGF-II (**bottom**). Specifically bound cpm are presented. NSB was determined for each concentration of each cation and subtracted from B_o to obtain specific binding at each point. SD of the 200 μM duplicate determinations presented to indicate data variation.

Dose Dependence

Three cations were eventually identified that depressed IGF binding to cell-associated IGFBPs. To test their potency, increasing amounts of Zn^{2+}, Au^{3+}, and Cd^{2+} were added to GM-10 cultures (Fig. 3). Independent of the ligand ([^{125}I]-IGF-I, top and [^{125}I]-IGF-II, bottom), all three cations depressed binding. A half-maximal effective dose of Zn^{2+} on [^{125}I]-IGF-I and [^{125}I]-IGF-II binding occurred at ≈20 and 10 μM, respectively, representing physiological (serum) levels of zinc. To determine the mechanism of action on IGF binding, Scatchard analysis was performed both in the absence and in the presence of these three active cations at a maximally active dose of 200 μM and with two other inactive cations (La^{3+} and Cr^{3+}) serving as controls.

Scatchard Plots: Pre-existent Asymmetric Model

[^{125}I]-IGF-I competitive binding data from Figure 2 were subjected to Scatchard analysis. The Scatchard plots (Fig. 2, top middle) were nonlinear (concave-down), indicating the presence of both high- and low-affinity sites. Curvilinear results were found in the absence (control) or presence of either La^{3+} or Zn^{2+}. Scatchard analysis of the [^{125}I]-IGF-II binding data (Fig. 2, bottom middle) again indicated the presence of

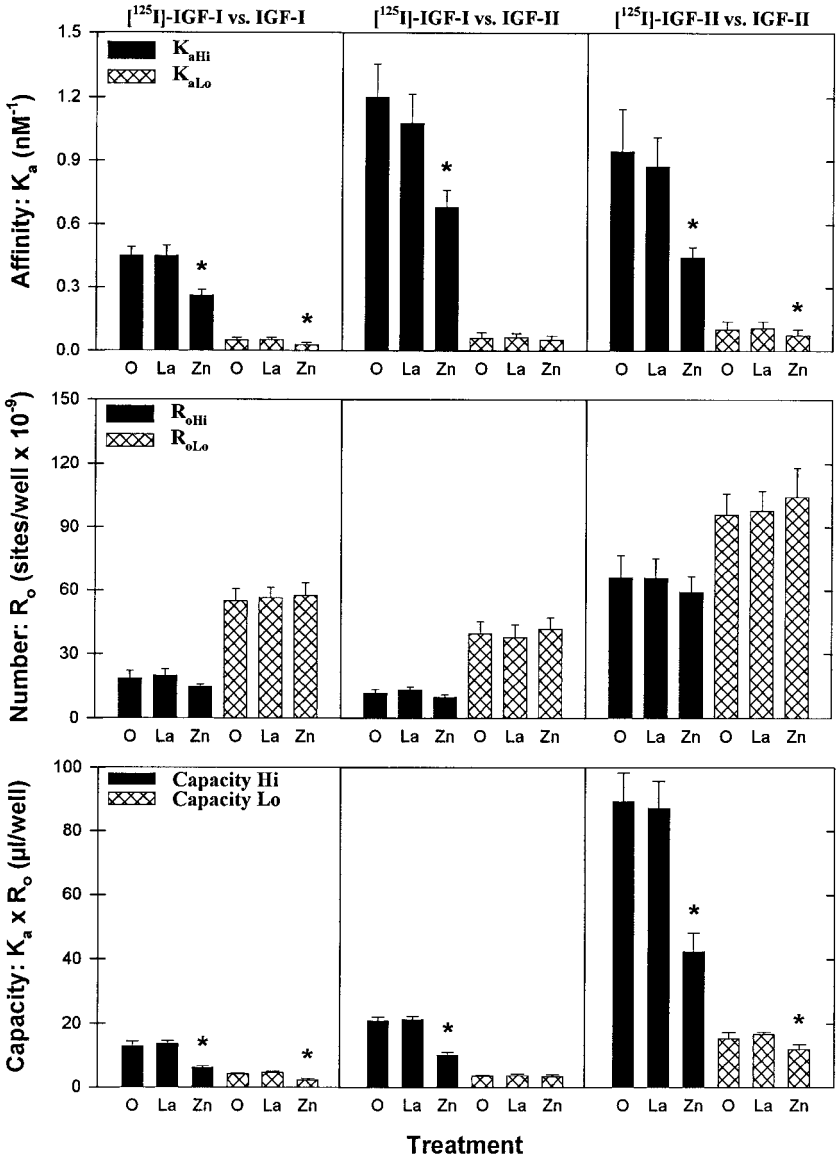

Fig. 4. Effect of La^{3+} and Zn^{2+} on Scatchard analysis binding constants for GM-10 cell-associated IGFBPs. Binding was accomplished through a 3-h incubation with [^{125}I]-IGF-I and [^{125}I]-IGF-II as indicated. Affinity (K_a) for both high- and low-affinity sites are shown (**top**), as are the number of binding sites (**middle**) and their respective binding capacities: $K_a \times R_o$ (**bottom**). All cations were added at 200 µM. Results of each graph are the average of five or six experiments. Values are means ± SD. *Bars within high or low affinity and within ligand combination differ from control, $P < 0.05$.

sis of the [^{125}I]-IGF-II binding data (Fig. 2, bottom middle) again indicated the presence of high- and low-affinity sites for all three treatments.

Binding Affinity Constants: Pre-existent Asymmetric Model

Affinity (K_a) was determined from Scatchard plots for both high- (K_{aHi}) and low- (K_{aLo}) affinity sites. For [^{125}I]-IGF-I binding to cell-associated IGFBP-3 with IGF-I competition (Fig. 4, top left), K_{aHi} was ≈10-fold that of K_{aLo}. Zn^{2+}, not La^{3+}, decreased both K_{aHi} and K_{aLo} compared with control. For [^{125}I]-IGF-I with IGF-II competition (Fig. 4, top middle), K_{aHi} was ≈8-fold that of K_{aLo} for controls. (Scatchard analysis was designed for homologous ligand combinations. However, in vivo both IGF-I and IGF-II compete for binding. Scatchard analysis was used to estimate the interaction between [^{125}I]-IGF-I and IGF-II interaction in lieu of a better model. Using this analysis, K_a reflects that of the unlabelled ligand, whereas R_o reflects that of the labelled ligand. In other work, IGF-I increases [^{125}I]-IGF-II binding to these cells, rather than depressing the binding and thus this combination of ligands cannot be subjected to Scatchard analysis [McCusker et al., 1990]. ED_{50} (dose at 50% competition) values cannot be used because they assume similar number of binding sites. Homologous ligand comparisons indicate that this is not the case with binding sites being [^{125}I]-IGF-II > [^{125}I]-IGF-I.) In the present work, Zn^{2+}, not La^{3+}, decreased K_{aHi}

compared with control for IGF-II competition with [^{125}I]-IGF-I. K_{aLo} was not significantly affected. For [^{125}I]-IGF-II binding to cell-associated IGFBP-3 and IGFBP-5 with IGF-II competition (Fig. 4, top right), K_{aHi} was ≈9-fold that of K_{aLo}. Zn^{2+}, not La^{3+}, decreased both K_{aHi} and K_{aLo} compared with control. Thus, Zn^{2+} had a similar effect on K_a, independent of the ligand combination. Using homologous ligand combinations, K_{aHi} and K_{aLo} for [^{125}I]-IGF-II binding are double that for [^{125}I]-IGF-I.

Number of Binding Sites: Pre-existent Asymmetric Model

Scatchard analysis was also used to estimate the number of binding sites (R_o). Neither La^{3+} nor Zn^{2+} altered R_{oHi} or R_{oLo} (Fig. 4, middle row), indicating that the cations do not release IGFBPs from the cell surface or detach cells from the wells. R_{oLo} is greater than R_{oHi} for all three ligand combinations. Also, the number of sites ($R_{oHi} + R_{oLo}$) estimated from data for [^{125}I]-IGF-II binding was ≈2-fold higher than that for [^{125}I]-IGF-I binding (with IGF-I competition). The greater number of binding sites for [^{125}I]-IGF-II compared with [^{125}I]-IGF-I coincides with [^{125}I]-IGF-II binding to both IGFBP-3 and IGFBP-5.

Binding Capacity: Pre-existent Asymmetric Model

$K_a \times R_o$ equals binding capacity and was calculated for both high- and low-affinity sites (Fig. 4, lower row). Zn^{2+}, not La^{3+}, depressed the binding capacity of both high- and low-affinity sites. Only low-affinity sites using the nonhomologous ligand pairing was unaffected. Binding capacity of the high-affinity site was three- to fivefold that of the low-affinity site for controls. Total binding capacity for [^{125}I]-IGF-II was approximately 5-fold that of [^{125}I]-IGF-I (homologous combinations).

Binding Affinity Constants: Ligand-Induced Sequential Model

Concave-down Scatchard plots are the result of either two independent pre-existent binding sites or of ligand-induced negative cooperativity. The appropriate model cannot be distinguished by equilibrium studies [Wang and Pan, 1996]. Thus, the binding constants, K_e and K_f, were calculated from average affinity data, as described under Data Analysis. The average affinity decreased as fractional occupancy of the binding sites increased (Fig. 2 right) for both [^{125}I]-IGF-I (top) and [^{125}I]-IGF-II (bottom). Zn^{2+} depressed both K_e and K_f (Fig. 5). All measurements, except K_f for [^{125}I]-IGF-I versus IGF-II, were lowered by Zn^{2+}. La^{3+} did not affect either K_e or K_f. K_f and K_e were ≈2-fold higher for IGF-II than for IGF-I (homologous ligand combinations).

Effect of Other Multivalent Cations: Pre-existent Asymmetric Model

Gold (Au^{3+}) and cadmium (Cd^{2+}) were tested because they bind to proteins by Zn^{2+}-binding motifs; chromium (Cr^{3+}) was chosen as an additional control cation (Table II). Au^{3+} decreased [^{125}I]-IGF-I and [^{125}I]-IGF-II binding (cpm), in some cases to the point of nondetectable specific binding. K_{aHi} was absent in the presence of Au^{3+} for all three ligand combinations. Where quantifiable, Au^{3+} decreased K_{aLo} compared with control. Cd^{2+} had a slight inhibitory effect on [^{125}I]-IGF binding (cpm). K_{aLo}, not K_{aHi}, was depressed by Cd^{2+}. Cr^{3+} did not effect binding of either [^{125}I]-IGF-I or [^{125}I]-IGF-II. Mercury, manganese, selenium, and silver (Hg^{2+}, Mn^{2+}, Se^{3+}, and Ag^{1+}), added as chloride salts at 200 μM,

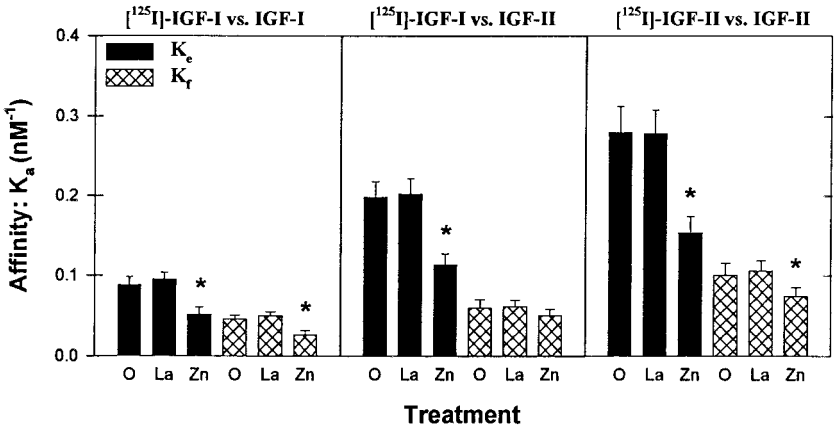

Fig. 5. Effect of La^{3+} and Zn^{2+} on binding affinity constants: Ligand-induced sequential model for negative cooperativity. Average affinity was used to calculate K_e and K_f for all binding assays. K_e and K_f; are the affinity of empty ($\bar{y} = 0$) and filled ($\bar{y} = 1$) binding sites, respectively [Demeyts and Roth, 1975]. Mean values for five or six experiments are presented. All cations were added at 200 μM. Values are means ± SD. *Bars within high or low affinity and within ligand combination differ from control, $P < 0.05$.

TABLE II. Effect of Multivalent Cations on IGF Binding to GM-10 Cell-Associated IGFBP-3 and IGFBP-5: Pre-existent Asymmetric Model and Ligand-Induced Model of Negative Cooperativity*

[^{125}I]-	Cold	Treatment	CPM bound	Pre-existent model: affinity (nM^{-1})		Ligand-induced model: affinity (nM^{-1})	
				K_{aHi}	K_{aLo}	K_e	K_f
IGF-I	IGF-I	Control	3,102	0.543	0.060	0.123	0.050
		Au^{3+}	0	—[a]	—[a]	—[a]	—[a]
		Cd^{2+}	2,102	0.518	0.034	0.081	0.034
		Control	5,930	0.493	0.095	0.209	0.093
		Cr^{3+}	6,226	0.535	0.100	0.211	0.099
IGF-I	IGF-II	Control	7,625	1.228	0.198	0.579	0.166
		Au^{3+}	2,845	—[a]	0.134	—[a]	0.137
		Cd^{2+}	6,745	1.436	0.158	0.488	0.154
		Cr^{3+}	7,728	1.840	0.172	0.606	0.213
IGF-II	IGF-II	Control	19,168	0.518	0.237	0.345	0.212
		Au^{3+}	5,937	—[a]	0.090	—[a]	0.091
		Cd^{2+}	16,600	0.536	0.138	0.243	0.131
		Cr^{3+}	18,065	0.521	0.201	0.297	0.201

*Four binding assays were performed (separated by blank rows) using either IGF-I or IGF-II as indicated. Binding to cell-associated IGFBPs was only performed one time for each combination with the control run simultaneously. All cations were added at 200 μM.
[a]The variable was not present.

were without effect on IGF binding to GM-10 cells (not shown).

Effect of Other Multivalent Cations: Ligand-Induced Sequential Model

K_e was not detected in the presence of Au^{3+} (Table II). Au^{3+} depressed K_f in the two ligand combinations, where quantifiable. Cd^{2+} depressed both K_e and K_f for all three ligand combinations. By contrast, Cr^{3+} did not affect either K_e or K_f.

DISCUSSION

We have identified a novel mechanism by which IGF binding activity is controlled by the modulation of IGFBP affinity. Equilibrium constants (affinity) of both high and low affinity sites for cell-associated IGFBP-3 or IGFBP-3 plus IGFBP-5 are depressed by Zn^{2+}. Zn^{2+} did not alter the number of binding sites (high or low) on cell surfaces. This is important because Zn^{2+} could depress [^{125}I]-IGF binding by causing the loss of high-affinity IGFBPs from the cell surface or, indeed, a loss of cells from the assay wells. Thus, the Zn^{2+} effects appear to be solely due to a reduction in the affinity of fibroblast cell-associated IGFBPs. This findings is entirely new and introduces a novel function for trace nutrients in the regulation of IGF binding to human fibroblasts. Of the cations tested, Zn^{2+} is most likely to play a physiological role to modulate IGF activity. In support of this, Zn^{2+} altered IGFBP binding at physiological levels, i.e., half-maximal effect at serum concentrations.

Scatchard analysis resulted in concave-down curvilinear plots for binding of [^{125}I]-IGF-I to cell-associated IGFBP-3 and [^{125}I]-IGF-II binding to cell-associated IGFBP-3/5. Nonlinear Scatchard plots are either the result of two distinct binding sites coexisting on the cell surface with differing ligand affinities (pre-existent model) or the result of cooperativity of binding (ligand-induced model). It is impossible to distinguish between the two models using equilibrium binding experiments [Wang and Pan, 1996]. Thus binding constants were calculated using both models and similar effects are found for both models.

Similar to previous work [McCusker et al., 1990], the high- and low-affinity constants of cell-associated IGFBPs differ by ≈10-fold. This finding and the presence of concave-down Scatchard plots for cell-associated IGFBPs are similar to results for IGF-I and insulin binding to their appropriate receptors. The low-affinity site is caused by negative cooperativity at high ligand concentrations [Demeyts, 1994]. In fur-

ther support of a cooperative model explaining the kinetics of IGF binding to cell-associated IGFBPs, IGF-II enhances [^{125}I]-IGF-II ligand dissociation from cell-associated IGFBPs [McCusker and Mateski, 1996], evidence for negative cooperativity. In addition, low levels of unlabeled IGF-I do not decrease but increase [^{125}I]-IGF-II binding to GM-10 cells and IGF-I only competes for binding at very high levels [McCusker et al., 1990]. Because of this latter phenomenon, the current work does not contain data for affinity changes using unlabeled IGF-I versus [^{125}I]-IGF-II. This unusual binding phenomenon does indicate, however, that binding cooperativity might be involved and further warrants analysis of data using both types of binding models. IGF-II does compete for [^{125}I]-IGF-I binding sites, again generating nonlinear Scatchard plots. The estimated affinity for this interaction is similar to that for the homologous pairing of IGF-II and [^{125}I]-IGF-II. This indicates that the difference in affinity determined for [^{125}I]-IGF-I and [^{125}I]-IGF-II by homologous ligand pairings is not due to the two ligands binding to completely different binding sites. The data show that IGF-II can compete for the same sites that bind [^{125}I]-IGF-I, but with a higher affinity, and that the competition between the two ligands is affected by Zn^{2+}.

GM-10 cells synthesize and secrete the 39,000–43,000-M_r IGFBP-3, the 27,000-M_r IGFBP-4, and the 31,000–32,000-M_r IGFBP-5; thus, all three IGFBPs are found in conditioned media [Camacho-Hubner et al., 1992]. However, by affinity cross-linking with [^{125}I]-IGF-I, only one IGFBP (IGFBP-3) is found on the cell surface despite having a curvilinear Scatchard plot. This finding supports our previous work with these cells [McCusker et al., 1990]. Similarly, [^{125}I]-IGF-I binding to IGFBP-3 purified from bovine placental membranes fits a two-site model with high- and low-affinity sites differing in K_a by 10-fold [McCusker et al., 1991]. This is similar to the ratio of K_{aHi}/K_{aLo} for GM-10 cell-associated surface IGFBP-3 in the current study. Thus, the nonlinear Scatchard plots for [^{125}I]-IGF-I binding to GM-10 cells can be explained by the presence of only IGFBP-3. [^{125}I]-IGF-I binding to IGFBP-1 also results in nonlinear Scatchard plots [Jones et al., 1991], an effect attributed to phosphorylation isoforms of the IGFBP [Jones et al., 1991]. Thus a preexistent model explains the binding characteristics for IGFBP-1. However, nonphosphorylated, nonglycosylated IGFBP-3 and native IGFBP-3 have similar affinities for IGF-I [Hoeck and Mukku, 1994]. Thus, the mechanism responsible for curvilinear Scatchard plots with membrane-derived and cell-associated IGFBP-3 remains unknown.

[^{125}I]-IGF-II binds to both cell-associated IGFBP-3 and IGFBP-5 by affinity labeling. IGFBP-5 expressed and secreted from Chinese hamster ovary (CHO) cell conditioned media bind [^{125}I]-IGF-II with a higher affinity than [^{125}I]-IGF-I when measured in solution [Bach et al., 1993], supporting our results. By contrast, IGFBP-5 expressed and purified from yeast extracts bind [^{125}I]-IGF-I and [^{125}I]-IGF-II with similar affinity [Kiefer et al., 1992]. Cell-associated IGFBP-5 produced by GM-10 human fibroblasts in the current work clearly binds [^{125}I]-IGF-II $>$ [^{125}I]-IGF-I. The presence of two affinity states using [^{125}I]-IGF-II could be due to ligand binding to both IGFBP-3 and IGFBP-5. Nonlinear Scatchard plots for purified IGFBP-5 have not been reported. However, using cells with only cell-associated IGFBP-5, Scatchard plots are still nonlinear [Sackett and McCusker, 1998], indicating that the binding kinetics of IGFBP-5 on the cell surface may differ from the secreted soluble form.

Zn^{2+} decreased the affinity of both the high- and low-affinity sites for both [^{125}I]-IGF-I and [^{125}I]-IGF-II for cell-associated IGFBPs. However, Zn^{2+} increased IGF binding to the type 1 IGF receptor [McCusker and Sackett, 1998]. Thus, the inhibitory effect of Zn^{2+} on IGFBPs would not prevent IGF binding to the receptor. Four- to 13-fold higher levels of Zn^{2+} were required to affect receptor binding, indicating that the cations were probably not binding to the IGFs but have differing affinities for their interactions with IGFBPs and the receptor. Lack of a direct interaction of Zn^{2+} with the IGFs is supported by the fact that IGF-I and IGF-II lack the physical properties to either dimerize or bind Zn^{2+} [Zapf and Froesch, 1986: Blundell et al., 1978].

Our current interest is in the mechanism by which multivalent cations affect ligand binding. Zn^{2+} depressed both high- and low-affinity K_as, Cd^{2+} depressed binding only to the low-affinity site, whereas Au^{3+} eliminated binding to a high-affinity site. Although of little physiological relevance, Au^{3+} and Cd^{2+} may prove indispensable in defining the cation effect and mechanism of action. Zn^{2+}, Cd^{2+}, and Au^{3+} can interact with the same cation-binding site on

proteins [Klemba et al., 1995]. Zinc-binding proteins contain a set of motifs residues (e.g., CxxC . . . . . CxxC) that coordinate Zn^{2+} binding [Levenson et al., 1994: Shi and Berg, 1995: Struhl, 1989]. CxxC and CxCCxxC motifs [Bach and Rechler, 1995: Swisshelm et al., 1995] are found near the N-terminal region of IGFBP-1, 2, 3, 4, 5, and 7, whereas CxxC and CxxxxxC sites are found in IGFBP-6. An additional CxC motif is located near the C-terminus of IGFBPs 1–6. Thus, a Zn^{2+} binding site is possible in these seven IGFBPs involving only cysteines, similar to a structure-stabilizing Zn^{2+} binding site [Vallee and Auld, 1990: Vallee and Falchuk, 1993]. The probable conformational changes in the IGFBPs caused by cation binding to these motifs apparently differ for Zn^{2+}, Au^{3+}, and Cd^{2+}. This may indicate that additional untested trace nutrients have the potential to modify IGF binding in distinct ways and thus play divergent roles in regulating IGF activity.

In addition to Zn^{2+} depressing IGFBP affinity, this cation maintains IGFBP-3 and IGFBP-5 on cell surfaces [McCusker and Clemmons, 1997]. Cell-associated IGFBPs are important in the modulation of IGF localization [McCusker et al., 1990]. Soluble IGFBPs, in extracellular fluids, depress the amount of IGF that is available to bind to cell surface binding sites [McCusker et al., 1991]. By contrast, IGFBP-5 added to fibroblast extracellular matrix (ECM) and on the cell surface potentiate the biologic actions of IGF-I [Jones et al., 1993: Mohan et al., 1995]. Similarly, the ability of IGFBP-3 to potentiate IGF-I action may involve cell association [Conover, 1992]. Thus, Zn^{2+} retention of cell–associated IGFBP-3 and IGFBP-5 may provide a mechanism for optimal presentation of IGF-I to the cell.

By retaining IGFBPs on the cell surface and depressing IGFBP affinity, Zn^{2+} may increase the amount of "free" IGF at the cell surface available for receptor activation. Similar to in vivo Zn^{2+} deficiency, in which extracellular (serum) Zn^{2+} levels are low, our cells are maintained in one-tenth serum Zn^{2+} concentrations before the assay. The present work therefore represents a model for Zn^{2+} deficiency and indicates that Zn^{2+} supplementation decreases IGF association with IGFBPs at the cell surface. The results presented in this paper heighten our awareness on how IGFBPs and trace nutrients such as Zn^{2+} interact to control IGF activity.

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