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Immunoassay of insulin-like growth factor binding protein-1

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Accurate measurement of insulin-like growth factor (IGF) binding protein-1 (IGFBP-1) is important for precise definition of its physiological roles and potential diagnostic values. Because altered phosphorylation results in altered IGFBP-1 immunoreactivity, current assays may significantly underestimate or fail to detect physiological changes in the IGFBP-1 concentrations. We developed three ELISAs (ELISA 1–3) using a common capture but three different detection antibodies. IGFBP-1 in serum, synovial fluid (SF), cerebrospinal fluid (CSF), and amniotic fluid (AF) were measured before and after treatment with alkaline phosphatase (ALP). Among the methods, only ELISA-1 was unaffected by IGFBP-1 phosphorylation and generated identical results before and after ALP treatment. The serum and SF values by ELISA-2 and -3 were lower by ~4- to 10-fold, but increased after ALP treatment to within 66–98% of those by ELISA-1. The medians in AF, and to a lesser extent in CSF, by all methods were similar and did not change significantly after dephosphorylation. ELISA-1 showed excellent correlation with ELISA-2, ELISA-3, and a commercial IGFBP-1 IRMA only after ALP-treated samples were analyzed by the comparative methods. ELISA-1 is highly specific for IGFBP-1 and demonstrated acceptable analytical performance characteristics.

INDEXING TERMS: insulin-like growth factors • insulin-like growth factor binding proteins • phosphorylation • ELISA

Insulin-like growth factors (IGF-I and IGF-II) belong to a family of peptides that mediate a broad spectrum of growth hormone-dependent as well as -independent mitogenic and metabolic actions *[1–4]*.[4] Unlike most peptide hormones, IGFs in circulation and other physiological fluids are associated with a group of high-affinity binding proteins (IGFBPs) that specifically bind and modulate their bioactivity at the cellular level. Six structurally homologous IGFBPs with distinct molecular size, hormonal control, and tissue expression and functions have been identified *[4–9]*.

IGFBP-1, synonymous with placental protein-12 *[10]* and the pregnancy-associated endometrial α_1-globulin *[11]*, is a 25-kDa protein expressed and secreted by hepatocytes, ovarian granulosa cells, and decidualized endometrium *[12–16]*. IGFBP-1 is present in serum, is the predominant binding protein in amniotic fluid, and is the major IGFBP in fetal and maternal circulation *[12, 17–19]*. Because of the reported inverse relation between birth weight and both fetal and maternal IGFBP-1 concentrations, a critical role for IGFBP-1 in fetal growth and development has been suggested *[12, 19]*. In normal individuals, circulating IGFBP-1 concentrations fluctuate rapidly by 10-fold or more in response to the acute changes in insulin concentrations that have been reported to inhibit IGFBP-1 gene transcription and, thus, its production by the liver *[12, 13]*. Because of the apparent regulation of IGFBP-1 production by insulin and other glucoregulatory hormones, a significant role for IGFBP-1 in glucose counterregulation has been also proposed *[12, 20]*.

A number of reports have shown that IGFBP-1 is capable of both inhibition as well as augmentation of the IGFs' actions *[4, 21–24]*. These observations may be explained by the findings that differential phosphorylation of IGFBP-1 could significantly alter its affinity for the IGFs *[25]* and, therefore, its capacity to influence their cellular responsiveness. Analysis of IGFBP-1 by anion-exchange chromatography and nondenaturing polyacrylamide gel

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[4] Nonstandard abbreviations: IGF, insulin-like growth factor; IGFBP, insulin-like growth factor-binding protein; LS, Laron syndrome; AF, amniotic fluid; SF, synovial fluid; CSF, cerebrospinal fluid; HRP, horseradish peroxidase; TMB, tetramethylbenzidine; sulfo-SMCC, sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate; ALP, alkaline phosphatase; BSA, bovine serum albumin; NBCS, newborn calf serum; and DEA, diethanolamine.

electrophoresis has identified up to five IGFBP-1 variants, differing only in their degree of phosphorylation. Various cell types such as Hep G2, decidual, and liver cells were found to secrete predominantly phosphorylated forms, whereas amniotic fluid and fetal serum contained substantial amounts of nonphosphorylated and lesser-phosphorylated variants *[4, 16, 25, 26]*. In contrast, in the sera of normal adults only a single highly phosphorylated variant of IGFBP-1 has been detected *[27]*. The IGFBP-1 profile of normal adult serum may, however, change under certain conditions such as during pregnancy or in subjects with Laron syndrome (LS), when nonphosphorylated and lesser-phosphorylated variants are also expressed *[20, 27]*. Dephosphorylation of IGFBP-1 has been proposed as a possible mechanism for increasing IGF bioavailability *[4, 27]*. The nonphosphorylated IGFBP-1 isoforms have reportedly four- to sixfold lower binding affinity for IGF-I as compared with the phosphorylated variants *[25]*. The stimulation and inhibition of the IGF actions have been found in association with the nonphosphorylated and phosphorylated IGFBP-1 variants, respectively *[4]*.

Changes in the phosphorylation state of IGFBP-1 have also been reported to result in significant change in its immunoreactivity *[27]*. As a result of differential recognition of the various IGFBP-1 phosphoforms by different antibodies, up to 11-fold differences in the circulating concentrations of IGFBP-1 in normal adults were observed. However, in serum samples from pregnant subjects the antibodies demonstrated only twofold variations in the mean IGFBP-1 concentrations *[27]*. Because variable antibody recognition of the IGFBP-1 isoforms may obviously result in false estimates of actual concentrations or inappropriate interpretations of the IGFBP-1 determinations, development of immunoassays unaffected by the state of IGFBP-1 phosphorylation is urgently needed.

We report for the first time development and performance characteristics of a novel noncompetitive ELISA for total IGFBP-1 that is unaffected by changes in its states of phosphorylation. The distinguishing feature of the proposed assay has been established by showing that the measured IGFBP-1 concentrations in various biological fluids remain virtually unchanged after sample pretreatment with alkaline phosphatase (ALP), which is known to readily dephosphorylate IGFBP-1 *[16, 25, 27]*. Comparison of the present total IGFBP-1 ELISA (ELISA-1) with other ELISAs (ELISA-2 and ELISA-3) that appear to measure a subset of nonphosphorylated or lesser-phosphorylated IGFBP-1 isoforms and a commercially available IGFBP-1 IRMA is also presented.

Materials and Methods

Samples and materials

Serum samples, amniotic fluids (AF), synovial fluids (SF), and cerebrospinal fluids (CSF) were obtained from the clinical laboratory at Mount Sinai Hospital, Toronto, ON, Canada. The samples were residuals from routine clinical test samples from an adult population. After collection, blood samples were allowed to clot and were then separated. After clinical testing, the residuals were stored at −20 °C and used within 4 weeks after collection. AF, SF, and CSF were stored at −20 °C for <4 weeks before use. Timed serum samples from two individuals (one male and one female) who had undergone a 75-g oral glucose tolerance testing were obtained from the clinical biochemistry laboratory at Mount Sinai Hospital, Toronto. These samples were also residuals from routine clinical test samples collected by the laboratory and were tested in the IGFBP-1 ELISAs within 2 weeks after collection.

Horseradish peroxidase (HRP) was obtained from Scripps Labs., San Diego, CA. Tetramethylbenzidine (TMB) microwell peroxidase substrate system was from Kirkegaard and Perry Labs., Gaithersburg, MD. Sulfosuccinimidyl4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC) and 2-iminothiolane were purchased from Pierce, Rockford, IL. Enzyme immunoassay-grade ALP was obtained from Boehringer Mannheim, Indianapolis, IN. All other chemical reagents were of highest quality and were obtained from Sigma Chemical, St. Louis, MO or Amresco, Solon, OH. Microtitration strips and frames were products of Costar, Cambridge, MA.

Recombinant human IGF-I and IGF-II were obtained from GroPep, Adelaide, Australia (distributed by DSL, Webster, TX) and recombinant nonglycosylated IGFBP-3 from Celtrix Pharmaceuticals, Santa Clara, CA. Recombinant human IGFBP-2 and IGFBP-4–6 were purchased from Austral Biologicals, San Roman, CA. IGFBP-I, purified from human amniotic fluid according to previously described methods *[13, 28]*, was obtained from DSL. The preparation was calibrated against pure recombinant human IGFBP-1.

Five different IGFBP-1 mouse monoclonal antibodies and an affinity-purified goat polyclonal anti-IGFBP-1 antibody were obtained from DSL.

IGFBP-1 ELISA

The IGFBP-1 assay buffer was 0.05 mol/L Tris-maleate, pH 7.0, 9 g/L NaCl, 20 g/L bovine serum albumin (BSA), and 0.1 g/L thimerosal. The antibody-HRP conjugate buffer was 0.02 mol/L sodium phosphate, pH 7.0, 9 g/L NaCl, 1 g/L $CaCl_2$, 5 g/L BSA, and 0.1 g/L thimerosal. The stopping solution was 2 mol/L sulfuric acid in deionized water. The composition of the coating and blocking buffers, as well as the wash solution, were as described previously *[29]*.

IGFBP-1 antibody coating to microtiter wells was performed at a concentration of 0.25–20 mg/L by using previously published methods *[29]*. In brief, 0.1 mL of the capture antibody solution was added to each microwell and allowed to incubate overnight at room temperature. The wells were then washed with the wash solution and 0.2 mL/well of the blocking solution was added and allowed to incubate for 1 h as above. The wells were washed again before use.

The IGFBP-1 detection antibodies were coupled to HRP as previously described *[30]*. The coupling reaction involved activation of the enzyme with sulfo-SMCC and its subsequent conjugation to the anti-IGFBP-1 detection antibody, which had been activated by 2-iminothiolane. The stock HRP-conjugated antibody solution was diluted at least 1000-fold before use.

IGFBP-1 calibrators were prepared by dissolving precalibrated IGFBP-1 from amniotic fluid in a protein-based buffer matrix (0.05 mol/L sodium phosphate, pH 7.4, 9 g/L NaCl, 1 g/L BSA, 0.1 g/L thimerosal) or in newborn calf serum (NBCS) containing 0.1 g/L thimerosal. The preparation, diluted in the standard matrix to give IGFBP-1 values of ~0.5, 1.0, 2.0, 10, 40, 80, and 160 μg/L, was stable for up to 4 weeks at 4 °C and ≥6 months at −20 °C or lower. The NBCS was chosen as standard matrix because calf serum IGFBP-1 did not demonstrate any cross-reactivity or interferences with IGFBP-1 measurement by the present methods; human IGFBP-1 serially diluted in the protein-based buffer matrix or NBCS generated identical assay response (data not shown). The quality-control samples were prepared by supplementing NBCS at four different IGFBP-1 concentrations (~3–50 μg/L). The nominal concentrations of the control samples were established by analyzing them in the IGFBP-1 ELISAs.

IGFBP-1 ELISA protocol

The IGFBP-1 ELISA was performed according to previously described procedures after determination of optimal assay conditions *[31, 32]*. Calibrators or samples (0.025 mL) were added in duplicate to the precoated wells, followed by addition of the assay buffer (0.05 mL) and 1 h of incubation at room temperature with continuous shaking. The wells were washed four times and incubated with 0.1 mL/well of the appropriate anti-IGFBP-1 antibody–HRP conjugate (diluted in assay buffer to ~0.1–0.25 mg/L) for 30 min at room temperature. The wells were washed five times with the wash solution and 0.1 mL of the TMB/H_2O_2 substrate solution was added for an additional 10 min of incubation at room temperature. Stopping solution (0.1 mL) was then added and absorbance measured by dual wavelength measurement at 450 nm with background wavelength correction set at 620 nm. Absorbance measurements for all ELISAs were performed with the Labsystems Multiskan Multisoft microplate reader (Labsystems, Helsinki, Finland).

IGFBP-1 ELISA validation procedures

The lower limit of detection (sensitivity) was determined by interpolating the mean plus 2SD of 12 replicate measurements of the zero calibrator (NBCS). The intraassay CVs were determined by replicate analysis (n = 12) of four samples at IGFBP-1 concentrations of ~3–50 μg/L in one run and interassay CVs by duplicate measurement of the samples in 9–12 separate assays. Recovery was assessed by adding 25 μL of exogenous IGFBP-1 diluted in the standard matrix (NBCS) to 225 μL of three serum samples and analyzing the supplemented and unsupplemented samples. Percent recovery was determined by comparing the amount of added IGFBP-1 with the amount measured after subtracting the endogenous IGFBP-1 concentrations. Linearity was tested by analyzing three serum samples serially diluted (2- to 32-fold) in the zero calibrator of the assay.

IGFBP-1 IRMA

IGFBP-1 concentrations were also determined by using an IGFBP-1 IRMA (DSL). Calibrators or samples (0.1 mL) and ^{125}I-labeled anti-IGFBP-1 antibody were added to anti-IGFBP-1-coated tubes. The tubes were incubated at room temperature for 16–20 h, washed, and counted (LKB 1275 Minigamma counter; Pharmacia LKB Biotechnology, Uppsala, Sweden). Calibrators ranged from 0.8 to 200 μg/L. Intra- and interassay CVs are ~3.4–6% within the assay range. The assay has no cross-reactivity with other human IGFBPs.

Molecular sieve chromatography

A fresh serum (0.2 mL) was subjected to size-exclusion chromatography on a precalibrated 600 × 7.5 mm Bio-Sil SEC-250 HPLC column (Bio-Rad Labs., Richmond, CA) as described *[30, 32]*. The column was preequilibrated and eluted with 0.05 mol/L Tris-HCl, pH 7.2, 9.0 g/L NaCl at 0.5 mL/min with collection of 1.0-mL fractions. Distribution of IGFBP-1 immunoreactivity was determined by IGFBP-1 ELISAs.

Dephosphorylation of IGFBP-1

Dephosphorylation of IGFBP-1 was achieved by sample pretreatment with ALP by using a similar procedure described previously *[16, 25, 27]*. Briefly, ALP dissolved in 10 μL of 1 mol/L diethanolamine (DEA), pH 9.5, containing 0.5 mmol/L $MgCl_2$ was added to a 200-μL aliquot of the sample, mixed, and incubated at room temperature for 2 h. The untreated control aliquot received 10 μL of the DEA buffer and was similarly incubated. ALP-treated and untreated samples were then analyzed.

Data analysis

IRMA and ELISA data were analyzed with data reduction packages included with the respective instrumentation, both based on cubic spline (smoothed) curve fit. Other statistical analyses were performed with the Statworks statistical software package (Starlight Network, Mountain View, CA) on an Apple Macintosh SE computer. Descriptive data are presented as the mean, median, and SD unless otherwise specified. Linear regression analysis was performed by the least-squares method and correlation coefficients were determined by the Pearson method.

Results

ANTIBODY EVALUATION

We evaluated various combinations of six different anti-IGFBP-1 antibodies (one polyclonal and five monoclonal) in a two-step ELISA format. The best calibration curve characteristics were obtained with a coating monoclonal antibody paired with a polyclonal (ELISA-1) or three of the remaining four monoclonal antibodies (ELISA-2–4) used for detection.

Because of the possible antibody recognition of antigenic determinants at or near the ligand (IGF-I or IGF-II) binding site of IGFBP-1, ELISA-1–4 were tested for potential interferences by free IGFs. In these experiments, IGF-I was added to a buffer-based IGFBP-1 preparation (~8 μg/L) to ensure efficient IGF-I–IGFBP-1 complex formation rather than IGF-I binding to other IGFBPs that would be also present if serum had been used. As shown in Table 1, IGF-I at concentrations up to 200 μg/L did not interfere with IGFBP-1 measured by ELISA-1 but showed some interferences in ELISA-3 at relatively high concentrations (≥50 μg/L). In contrast, ELISA-2 showed significant interferences by free IGF-I at concentrations ≥5 μg/L, and ELISA-4 falsely overestimated the expected IGFBP-1 concentrations and was therefore discontinued. In ELISA-2, the interfering effects of the free IGF-I added to actual serum samples occurred at significantly higher concentrations (≥10 μg/L). Because these concentrations of free IGFs may be above the expected endogenous concentrations available for binding to IGFBP-1, ELISA-2 was further evaluated. However, the accuracy of results obtained by ELISA-2 may be questionable, as the assay is incapable of detecting IGFBP-1 molecules that may be present in association with IGF-I or IGF-II.

IGFBP-1 ELISA

The IGFBP-1 ELISA protocols were optimized for analytical performance. This was easily achievable, as the ELISA-1–3 are based on a common monoclonal capture antibody and a common two-step immunoassay protocol that allows detection of the bound IGFBP-1 by each of the three different detection antibodies in a separate step (i.e., the second step).

The protocol optimization was based on the initial evaluation of a number of factors that could potentially affect detection limit, dynamic range, precision, and delayed sample addition. The best performances were obtained with a coating antibody concentration of 5 mg/L (500 ng/0.1 mL per well), a detection antibody concentration of ~0.1–0.25 mg/L (10–25 ng/0.1 mL per well), a sample size of 0.02 mL, a first- and second-step room temperature incubation of 60 and 30 min, respectively, and a 10-min substrate development step. With this protocol, the differences in assay results caused by 1–20 min delays between addition of the same samples into the coated wells was <10%.

The standard range and performance characteristics of ELISA-1–3 are summarized in Table 2. Analysis of IGFBP-2 and IGFBP-4–6 (up to 500 μg/L), IGFBP-3 (up to 4.2 mg/L), and insulin (up to 200 μg/L) added to the zero calibrator or a calibrator preparation of ~8 μg of IGFBP-1/L did not show any cross-reactivity or interferences in IGFBP-1 ELISA-1–3. Addition of IGF-I (up to 200 μg/L) and IGF-II (up to 120 μg/L) to the assay zero calibrator followed by IGFBP-1 analysis by ELISA-1–3 did not show any cross-reactivity (data not shown).

To evaluate the distribution and molecular mass of immunoreactivity detected, a fresh serum sample was fractionated by molecular sieve HPLC and fractions were assayed for IGFBP-1. As expected, a major peak at ~30 kDa, corresponding to the reported molecular mass of IGFBP-1 *[12]*, was detected by ELISA-1–3. However, there appear to be significant differences in the peak IGFBP-1 intensity measured by the three methods (Fig. 1).

It is now well established that production of IGFBP-1 by the liver is inversely regulated by insulin so that in normal individuals, serum IGFBP-1 should fall rapidly after a meal, whereas increases should occur during fasting *[12, 13]*. To evaluate the assay response to meal-induced IGFBP-1 suppression, timed serum samples taken from two apparently normal individuals during glucose tolerance testing were assayed. As shown in Fig. 2, the expected postmeal decline in IGFBP-1 was clearly registered by ELISA-1–3, further confirming the specificity of the assays for IGFBP-1. However, in another sample, only ELISA-1 was able to demonstrate decreasing IGFBP-1 concentrations. The IGFBP-1 concentrations measured by ELISA-2 and ELISA-3, on the other hand, did not show the expected fall in values. This observation might be related to changes in the phosphorylation status of IGFBP-1, which could alter its molecular structure and, thus, its binding to different IGFBP-1 antibodies. (see below).

Table 1. IGFBP-1 ELISA binding interference with IGF-I.

	IGFBP-1 measured, μg/L[a]			
IGF-I added, μg/L	ELISA-1	ELISA-2	ELISA-3	ELISA-4
0	7.3	7.2	7.6	7.7
5	7.4	5.3	7.3	NT
50	7.8	1.9	6.2	19.6
200	7.8	1.3	6.0	22.0

[a] Expected IGFBP-1 concentration = 8.0 μg/L
NT, not tested.

Table 2. IGFBP-1 ELISA-1–3 validation data.

Assay parameter	ELISA-1	ELISA-2	ELISA-3
Detection limit, μg/L	0.30	0.12	0.07
Standard range, μg/L	1–180	0.45–80	0.45–80
Intrassay CV, %	2.9–5.8	2.2–5.3	3.3–5.3
Interassay CV, %	5.4–8.4	5.9–7.4	4.5–5.6
Recovery of added IGFBP-1, %	92 ± 5.1	92 ± 3.2	91 ± 6.2
Recovery after dilution, %	106 ± 6.1	87 ± 7.5	90 ± 8.6

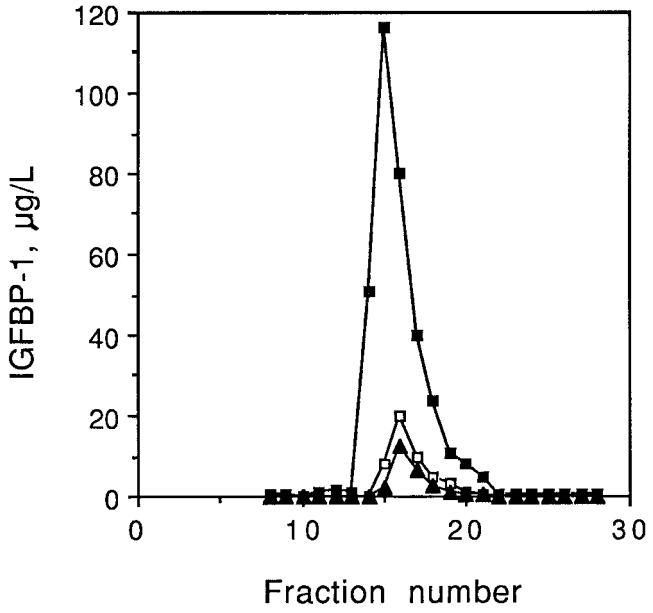

Fig. 1. HPLC profile of immunoreactive IGFBP-1.

A fresh serum sample was fractionated on a precalibrated Bio-Sil Sec-250 HPLC column. Fractions were analyzed by ELISA-1–3. The column was calibrated with molecular mass calibrators from Bio-Rad Labs. eluting at fractions 9 (660 kDa), 12 (160 kDa), 15 (44 kDa), 17 (17 kDa), and 21 (1.4 kDa). ■, ELISA-1; □, ELISA-2; ▲, ELISA-3.

IGFBP-1 concentrations in physiological fluids

The IGFBP-1 ELISA-1–3 measured considerably different concentrations in randomly selected serum samples and SF (Table 3). The median serum value measured by ELISA-1 was 3.3- and 13-fold higher than the concentrations measured by ELISA-2 and ELISA-3, respectively. Furthermore, regression analysis of values by ELISA-2 and -3 showed relatively poor correlations in comparison with ELISA-1, with significant scattering of data points around the regression line (Fig. 3). The median IGFBP-1 concentrations by ELISA-1–3 in SF were 19.5, 3.5, and 2.1 µg/L, respectively. In contrast, the concentrations of IGFBP-1 measured by ELISA-1–3 in AF and CSF were relatively similar (Table 3).

Effect of IGFBP-1 dephosphorylation

To investigate whether the apparent discrepancy in IGFBP-1 measurement was caused by differential antibody recognition of the IGFBP-1 variants, the effect of IGFBP-1 dephosphorylation on the response of ELISA-1–3 was examined.

Experiment 1. Seven serum samples (200 µL) were incubated as described with increasing concentrations of ALP (0–100 U). The enzyme-treated and untreated samples were then analyzed by ELISA-1–3. As represented in Fig. 4, there were no significant differences in the concentrations of IGFBP-1 measured by ELISA-1 before and after ALP treatment ($P = 0.337$ by a two-way ANOVA), suggesting equivalent recognition of all IGFBP-1 variants (nonphosphorylated and phosphorylated). However, concentrations detected by ELISA-2 and ELISA-3 increased progressively in response to increasing amounts of ALP treatment and reached a plateau at concentrations close to the amount measured by ELISA-1. The increases in IGFBP-1 concentrations detected by ELISA-2 and ELISA-3 were highly significant ($P < 0.001$ by ANOVA). In this and subsequent experiments, ELISA-3 initially detected only 5–10% of IGFBP-1 measured by ELISA-1 but almost 80% of the total concentrations after IGFBP-1 dephosphorylation. Analysis of 100–1000 U of ALP added to IGFBP-1 calibrators did not show any cross-reactivity or interferences in ELISA-1–3.

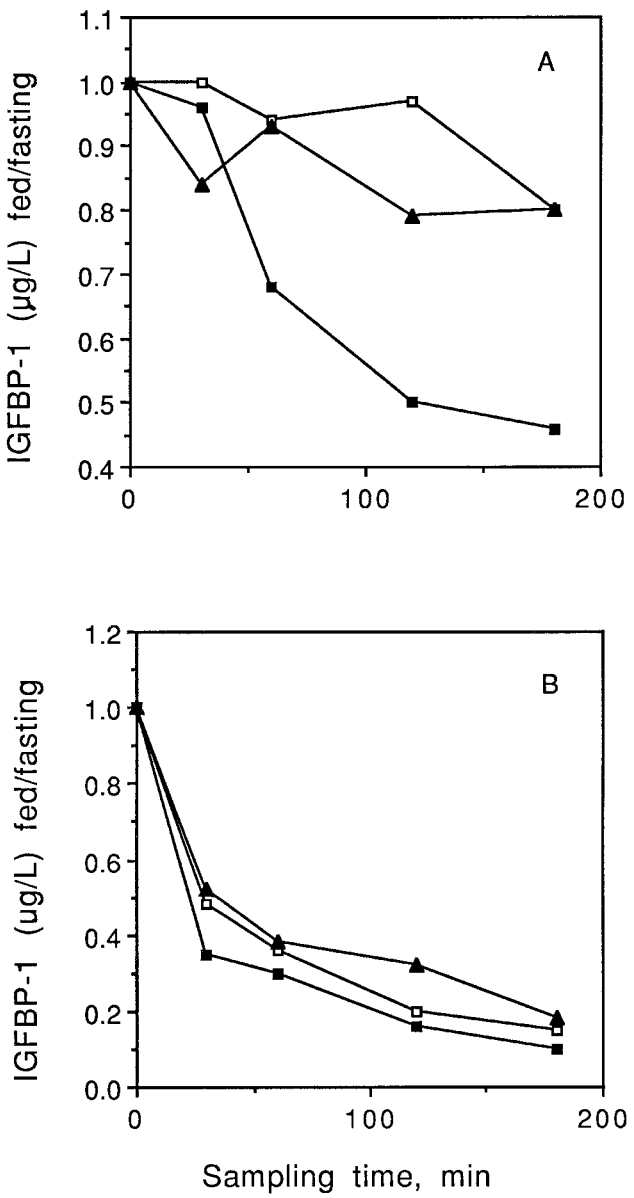

Fig. 2. Serum IGFBP-1 in relation to glucose intake.

IGFBP-1 concentrations in an apparently normal female (*A*) and a normal male (*B*) were measured during a 75-g oral glucose tolerance test. Samples were analyzed by ELISA-1–3. Values (mean of duplicates) by each method plotted as the ratio of IGFBP-1 measured after feeding with the corresponding fasting (0 time) concentrations vs the sampling time. ■, ELISA-1; □, ELISA-2; ▲, ELISA-3.

Table 3. IGFBP-1 concentrations (μg/L) in physiological fluids.

Sample	ELISA	Mean	Median	SEM	Range	n	Ratio[a]
Serum	1	39.0	26.0	5.0	1.2–127	44	1.0
	2	12.9	7.8	2.0	0.1–68	44	3.3
	3	2.74	2.0	0.39	0.08–13	43	13.0
SF	1	18.5	19.2	5.1	2.4–35	6	1.0
	2	5.7	3.5	2.6	0.99–18	6	5.5
	3	2.8	2.1	1.1	0.04–7.5	6	9.1
AF	1	10.7	8.2	2.7	1.7–24	10	1.0
	2	8.3	6.5	1.9	1.9–18.0	10	1.26
	3	10.2	7.4	2.6	1.7–22.8	10	1.11
CSF	1	6.6	3.3	2.8	0.3–25	9	1.0
	2	6.6	2.7	3.0	0.22–27	9	1.2
	3	4.7	1.9	2.1	0.13–18.4	9	1.7

AF was diluted 10 000–20 000-fold before testing.
[a] Ratio of the median values obtained by ELISA-1 relative to those by ELISA-2 and -3.

Experiment 2. To further evaluate the differential antibody recognition of IGFBP-1 by ELISA-1–3, the SF, AF, and CSF samples were treated with ALP (at 0 and 100 U/200 μL) as above and assayed. In SF, the assay responses to IGFBP-1 dephosphorylation were similar to those described in experiment 1 for the serum samples. ELISA-1 appeared to quantify total IGFBP-1 concentrations, as there was no change in the mean ± SD value before (18.45 ± 12.5 μg/L) and after (18.70 ± 13 μg/L) ALP treatment. IGFBP-1 concentrations by ELISA-2 and ELISA-3 increased by ~2.2- and 6.4-fold, respectively, in response to ALP treatment, with concentrations by ELISA-3 approaching those detected by ELISA-1 (data not shown). In contrast, the IGFBP-1 concentrations (mean ± SD) measured in AF by ELISA-1–3 were similar and did not show any significant increases in response to ALP treatment. Similarly, the IGFBP-1 concentrations in CSF demonstrated a relatively smaller response to ALP treatment. Again, the IGFBP-1 values (mean ± SD) measured by ELISA-1 before and after treatment with ALP were identical (Table 4).

Experiment 3. To confirm that phosphorylation of IGFBP-1 was responsible for the significantly lower IGFBP-1 concentrations measured by ELISA-2 and -3, as well as the scattering of values around the regression line in comparison with ELISA-1, fresh serum samples (n = 21) were treated with ALP (100 U/200 μL) and then assayed. Regression analysis showed excellent correlation between IGFBP-1 concentrations measured by ELISA-1 in untreated samples in comparison with the corresponding values obtained by ELISA-2 and ELISA-3 after ALP treatment (Fig. 5). The IGFBP-1 concentrations by ELISA-2 and ELISA-3 in the treated samples increased to within ~70–80% of those measured by ELISA-1. As expected, ALP treatment had no significant effect on IGFBP-1 concentrations measured by ELISA-1 in ALP-treated (y) and untreated (x) samples ($y = -1.18 + 1.07x$, $r = 0.986$, $S_{y|x} = 0.04$, $P = <0.001$).

comparison with commercial igfbp-1 irma

ELISA-1 was compared with a commercially available IGFBP-1 IRMA. Samples (n = 21) before and after ALP treatments were analyzed by both methods. Regression analysis showed about two- to sevenfold higher IGFBP-1 concentrations by ELISA-1, which resulted in significant scattering of values in comparison with those measured by the IGFBP-1 IRMA. However, the correlation parameters improved significantly when the IGFBP-1 concentrations in untreated samples by ELISA-1 were compared with the concentrations in ALP-treated samples measured by the IRMA (Fig. 6). The median values by the IRMA in untreated and ALP-treated samples were 6.8 and 20.5 μg/L, whereas the corresponding values by ELISA-1 remained unchanged at 23.4 and 24 μg/L, respectively.

Discussion

Changes in the phosphorylation state of IGFBP-1, a proposed mechanism for regulation of the IGFs' actions *[4, 16, 25–27]*, appear to be also responsible for a significant change in its immunoreactivity. Methods of IGFBP-1 analysis, which are unaffected by such modifications, are important for reliable determination of its total concentrations when phosphorylation of IGFBP-1 is altered. The latter, resulting in variable expression of the various IGFBP-1 isoforms, reportedly occurs in different biological fluids and in response to different physiological stimuli *[4, 16, 20, 25–27]*. Measurement of subfractions of IGFBP-1 (e.g., nonphosphorylated and lesser-phosphorylated forms) may also be of value in investigations of their origin or the mechanism that regulates them. Finally, the ability to determine quantitative changes in the concentrations of the IGFBP-1 subfractions relative to its total concentration may allow a more precise definition of the physiological functions and potential diagnostic values of this important binding protein.

Although various competitive and noncompetitive immunoassays for IGFBP-1 have been available for almost two decades *[12, 17, 33]*, a method unaffected by the state

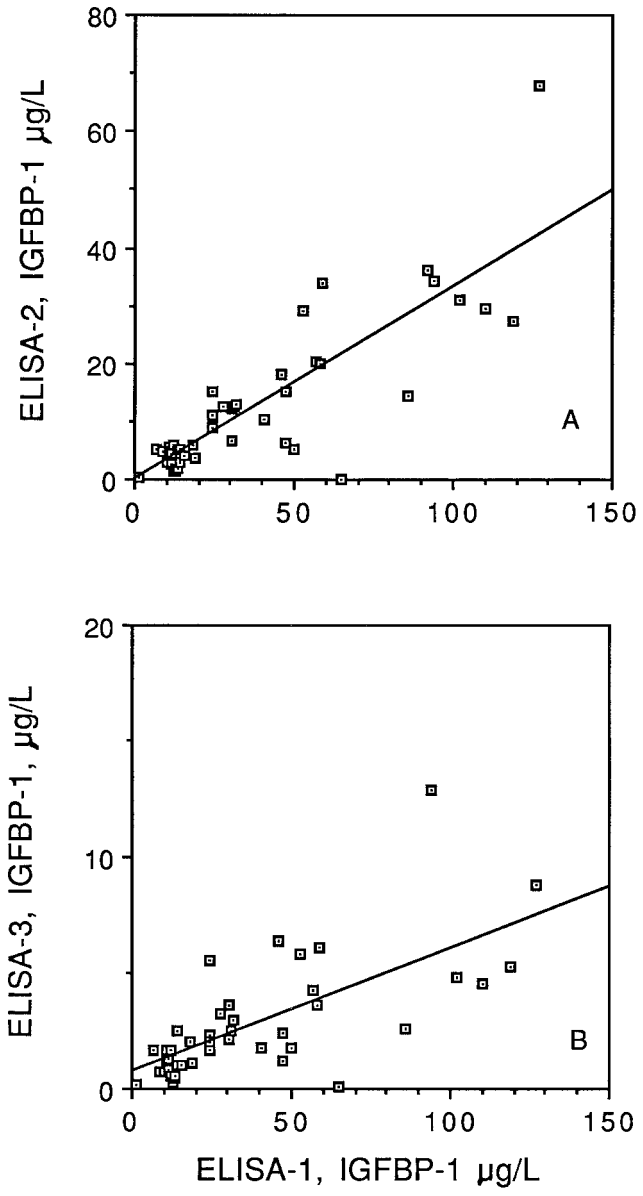

Fig. 3. Comparison of ELISA-1 with ELISA-2 and ELISA-3.

Correlation of IGFBP-1 values by ELISA-1 vs those determined by ELISA-2 (*A*; $y = -0.008 + 0.33x$, $r = 0.83$, $S_{y|x} = 0.03$) and ELISA-3 (*B*; $y = 0.071 + 0.055x$, $r = 0.69$, $S_{y|x} = 0.01$) are shown. Values represent mean of duplicate measurements.

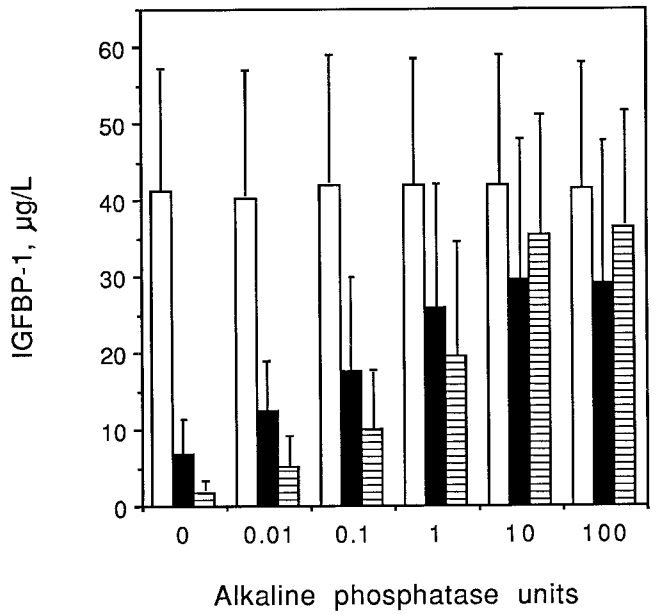

Fig. 4. Effect of IGFBP-1 dephosphorylation on ELISA-1–3.

Samples (200 µL) were incubated with 0–100 U of ALP in 10 µL of DEA buffer. The mean IGFBP-1 (n = 7) plus SD by ELISA-1–3 in untreated and treated samples are shown. □, ELISA-1; ■, ELISA-2; ▤, ELISA-3.

of IGFBP-1 phosphorylation, thus allowing determination of its total concentrations, has not been reported. The extent of the problem is such that the validity of the reported IGFBP-1 normal ranges, even in the nonpregnant adult population, which apparently expresses a single IGFBP-1 variant, has been recently questioned *[27]*. The findings of variable expression of the IGFBP-1 isoform in biological fluids *[4, 16, 25, 26]* and changes in its circulating profile could further complicate analysis as well as interpretation of the IGFBP-1 measurements. In this context, IGFBP-1 antibodies have been reported to detect significantly different serum concentrations in nonpregnant subjects (up to 11-fold differences in the mean values), while measuring relatively similar concentrations during pregnancy *[27]*. Similarly, in a recent evaluation of the effect of human IGF-I on IGFBP-1 concentrations in subjects with LS, an inverse relation between insulin and serum IGFBP-1 was detected with one antibody, whereas a direct relation was indicated when an antibody with a different IGFBP-1 specificity was used *[20]*. This is in accordance with the present observation that in one subject, only ELISA-1 was able to clearly demonstrate the expected fall in IGFBP-1 concentrations in relation to a meal.

As we demonstrated in this report, ELISA-1 is capable of accurate determination of total IGFBP-1 concentrations. This was confirmed by demonstrating identical assay

Table 4. IGFBP-1 (µg/L) in AF and CSF before and after dephosphorylation.

	AF, mean IGFBP-1 ± SD			CSF, mean IGFBP-1 ± SD		
ALP,[a] U	ELISA-1	ELISA-2	ELISA-3	ELISA-1	ELISA-2	ELISA-3
0	10.7 ± 8.5	8.29 ± 6.0	10.16 ± 8.2	6.61 ± 8.5	6.68 ± 9.6	4.7 ± 6.2
100	10.7 ± 8.8	7.96 ± 5.7	10.20 ± 8.4	6.50 ± 8.0	8.15 ± 9.4	6.4 ± 7.5

[a] Samples (AF, n = 10 and CSF, n = 9) incubated for 2 h at room temperature with 0 or 100 U.

results in response to increment IGFBP-1 dephosphorylation under the condition that resulted in a progressive increase in IGFBP-1 concentrations measured by two other methods (i.e., ELISA-2 and ELISA-3). Although all three methods are based on a common capture antibody and detect a similar profile in HPLC fractions, the observed quantitative differences may not be surprising if differential antibody recognition of the various IGFBP-1 phosphoforms is considered. ELISA-1 consistently measured the highest concentrations in the various biological fluids examined; the medians in serum and SF were, respectively, ~3.3- and 5.5-fold higher than the corresponding concentrations by ELISA-2-, and ~13- and 9-fold higher than those by ELISA-3. However, the concentrations by ELISA-2 and ELISA-3 increased after ALP treatment to within ~66–98% of those measured by ELISA-1. Linear regression analysis of serum values measured by ELISA-2, ELISA-3, and a commercial IRMA showed a relatively poor correlation, and more importantly scattering of the data points, in comparison with those detected by ELISA-1. Again, excellent correlations

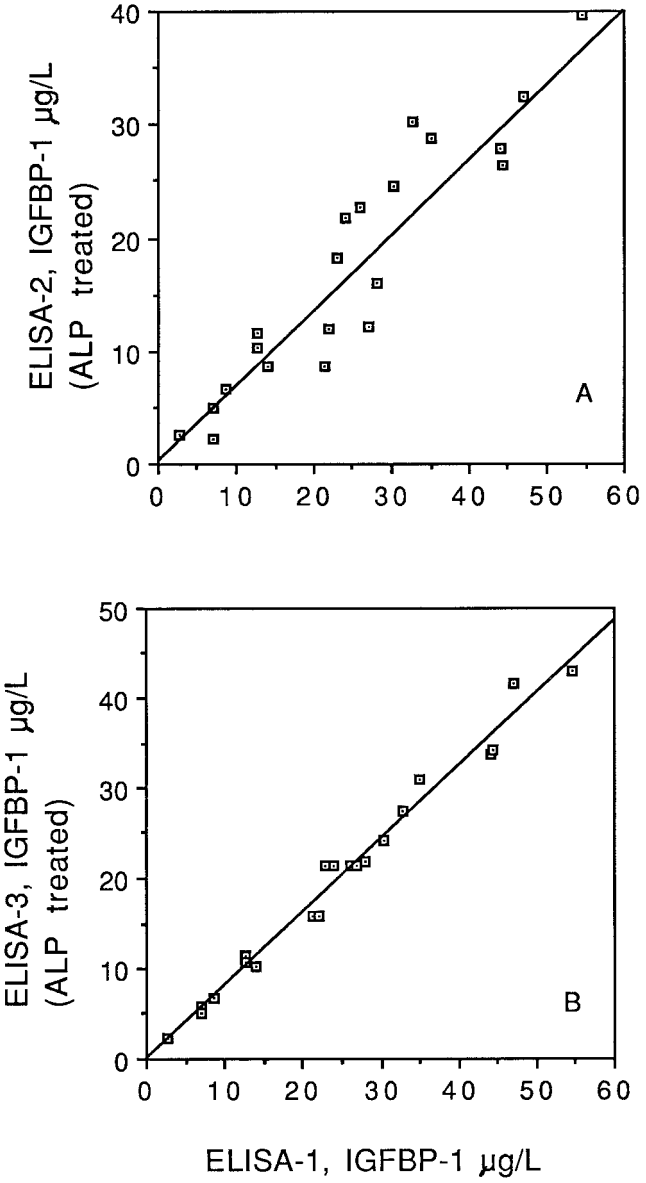

Fig. 5. Comparison of ELISA-1 with ELISA-2 and ELISA-3 after ALP treatment.

Correlation of IGFBP-1 values in untreated samples by ELISA-1 vs those measured after ALP treatment by ELISA-2 (*A*; $y = 0.12 + 0.70x$, $r = 0.93$, $S_{y|x} = 0.06$) and ELISA-3 (*B*; $y = 0.049 + 0.81x$, $r = 0.99$, $S_{y|x} = 0.04$) are shown. Sample treatment with ALP performed as described in the legend to Table 4.

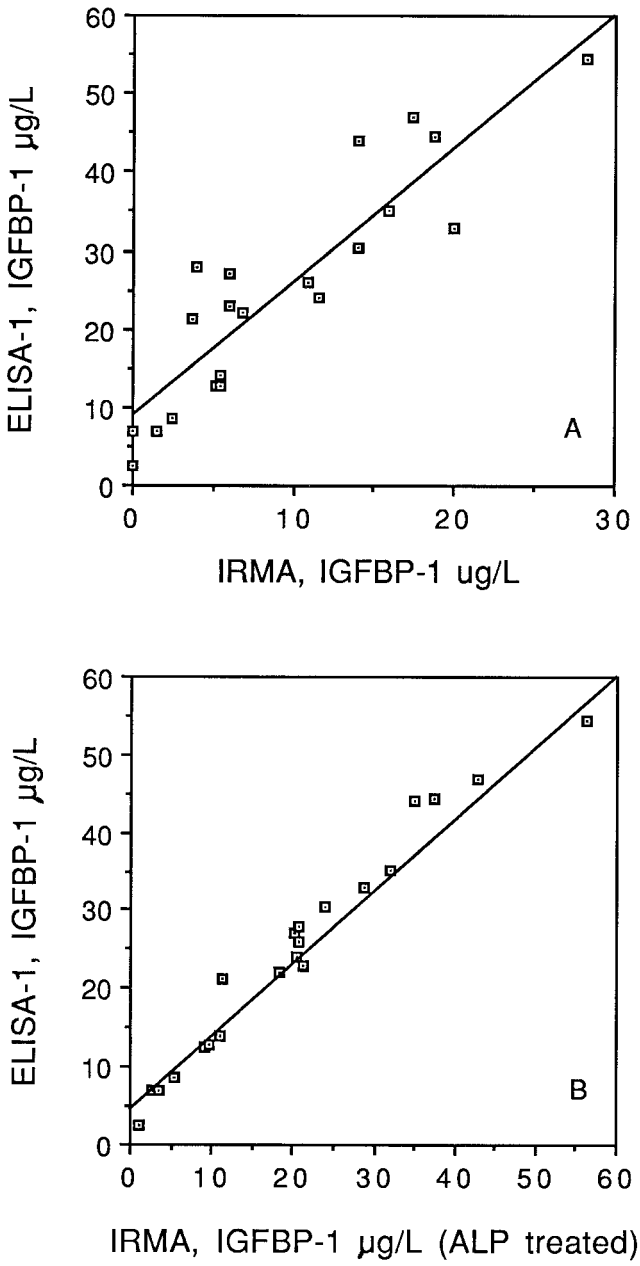

Fig. 6. Comparison of ELISA-1 with IRMA.

Correlation of IGFBP-1 values in untreated samples by ELISA-1 vs those measured by the IGFBP-1 IRMA before (*A*; $y = 8.7 + 1.73x$, $r = 0.90$, $S_{y|x} = 0.19$) and after ALP-treatment (*B*; $y = 4.6 + 0.99x$, $r = 0.98$, $S_{y|x} = 0.04$) are shown. Sample treatment with ALP performed as described in the legend to Table 4.

were obtained only after ALP-treated samples were analyzed by the comparative methods. ELISA-1 was unaffected by IGFBP-1 phosphorylation and generated virtually identical results in response to ALP treatment.

The significant increase in the measured IGFBP-1 values by ELISA-2 and, more pronouncedly, by ELISA-3 after ALP treatment strongly suggests measurement of essentially nonphosphorylated and lesser-phosphorylated IGFBP-1 variants by these methods. This is in accordance with the findings that normal adult serum contains primarily a single highly phosphorylated IGFBP-1 variant that is readily dephosphorylated by ALP *[27]*. As ELISA-3 is capable of a more dramatic response to dephosphorylation, it may be of greater value in monitoring changes in nonphosphorylated and lesser-phosphorylated IGFBP-1 subfractions.

The findings of similar IGFBP-1 concentrations in AF before and after ALP treatment by ELISA-1–3 may be expected, as AF reportedly lacks the highly phosphorylated IGFBP-1 variants *[27]*, but contains predominant concentrations of nonphosphorylated and lesser-phosphorylated forms that are well-recognized by the three methods. The fact that all three ELISAs incorporate the same AF-derived calibrators should further minimize the expected differences. Antibodies that detect significant differences in serum IGFBP-1, but similar concentrations in AF, have been also reported by other investigators *[27]*.

Our data suggest that the IGFBP-1 profile in CSF may be very similar to that present in AF, containing predominantly nonphosphorylated and lesser-phosphorylated variants. Relatively similar IGFBP-1 concentrations in untreated and ALP-treated samples were detected by ELISA-1–3. Again, only ELISA-1 generated identical measures before and after IGFBP-1 dephosphorylation. The higher concentrations of IGFBP-1 measured by ELISA-2 in response to ALP treatment might be due to overexpression of the predominant isoforms that are preferentially recognized by this method.

In summary, we describe the first report on development and validation of a highly specific and simple noncompetitive immunoassay (ELISA-1) for total IGFBP-1 in biological fluids. ELISA-1 is virtually unaffected by the state of IGFBP-1 phosphorylation, which significantly alters its immunoreactivity and thus accuracy of its measurements. Availability of this method should facilitate investigations of the physiological roles and potential diagnostic values of IGFBP-1.

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