Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 7481–7485, September 1991
Medical Sciences

Phosphorylation of insulin-like growth factor (IGF)-binding protein 1 in cell culture and *in vivo*: Effects on affinity for IGF-I

(Chinese hamster ovary cells/hepatoma cells/decidual cells/amniotic fluid/non-SDS polyacrylamide gel electrophoresis)

JOHN I. JONES*, A. JOSEPH D'ERCOLE†, CECILIA CAMACHO-HUBNER*, AND DAVID R. CLEMMONS*‡

Departments of *Medicine and †Pediatrics, University of North Carolina School of Medicine, Chapel Hill, NC 27599-7170

Communicated by Dale E. Bauman, May 23, 1991 (received for review January 21, 1991)

ABSTRACT **The insulin-like growth factors (IGF-I and IGF-II) are present in extracellular fluids bound to specific IGF-binding proteins (IGFBPs). We and others have reported varying biologic activity of different preparations of IGFBP-1 that appeared to have identical amino acid sequences and molecular sizes. This observation prompted us to determine whether IGFBP-1 undergoes posttranslational modifications. Immunoprecipitation was used to show that Chinese hamster ovary cells (transfected with a human IGFBP-1 cDNA construct) and human hepatoma (HepG2) cells secrete ^{32}P-labeled IGFBP-1 following incubation with [^{32}P]orthophosphate. Phospho amino acid analysis of ^{32}P-labeled IGFBP-1 revealed only phosphoserine residues. A method was developed that could separate nonphosphorylated IGFBP-1 from four or five phosphorylated isoforms. Using this technique we demonstrated that human amniotic fluid and human fetal serum contain a large proportion of nonphosphorylated IGFBP-1, as well as phosphorylated forms. In contrast, HepG2 cells and human decidual cells secrete predominantly the phosphorylated isoforms. These observations suggest that IGFBP-1 is secreted as a phosphoprotein and is subsequently dephosphorylated *in vivo*. Binding studies showed that the phosphorylated IGFBP-1 secreted by HepG2 cells has a 6-fold higher affinity for IGF-I than it does after dephosphorylation. We conclude that IGFBP-1 is phosphorylated and that this phosphorylation is a physiologically important posttranslational modification.**

The insulin-like growth factors (IGF-I and IGF-II) are present in plasma and other extracellular fluids bound to specific IGF-binding proteins (IGFBPs). Our laboratory has demonstrated that a specific form of IGFBP derived from human amniotic fluid, designated IGFBP-1 (1), potentiates the mitogenic activity of IGF-I in smooth muscle cells and fibroblasts in culture (2). Not all of our IGFBP-1 preparations have demonstrated equivalent biologic activity, and other laboratories, using various biologic test systems, have reported that IGFBP-1 inhibits the effects of IGF-I. Specifically, IGFBP-1 has been found to inhibit IGF-I stimulation of cell growth (3, 4) or differentiated cellular functions, such as amino acid transport (4). In addition, differing estimates of the affinity of IGFBP-1 for IGF-I have been reported (5–7), suggesting that the purified preparations have not been truly homogeneous. It has been proposed that an unidentified posttranslational modification may account for these differences in biologic activity and binding characteristics (6). To date the only posttranslational modification reported has been the capacity of IGFBP-1 from amniotic fluid to form disulfide-linked multimers (7). The current studies were initiated to determine whether IGFBP-1 might be phosphorylated, whether phosphorylated IGFBP-1 could be detected in cell culture supernatants and in physiologic fluids, and whether phosphorylation resulted in alterations of the binding affinity of IGFBP-1 for IGF-I.

MATERIALS AND METHODS

Materials. Chinese hamster ovary (CHO-K1) cells and human hepatoma (HepG2) cells were obtained from the American Type Culture Collection. Conditioned medium from primary cultures of human decidual cells (8) was a gift from Stuart Handwerger. IGFBP-1 was purified from human amniotic fluid (6). Recombinant human IGFBP-1 from a bacterial source was a gift from Synergen (Boulder, CO). Human fetal serum was obtained at 20 weeks of gestation (9). Rabbit antiserum specific for human IGFBP-1 was prepared as described (10). Recombinant IGF-I was a gift from Genentech. [^{32}P]Orthophosphoric acid (8500–9120 Ci/mmol; 1 Ci = 37 GBq) was purchased from NEN and [γ-^{32}P]ATP (3000 Ci/mmol) from Amersham. Poly(vinylidene difluoride) (PVDF) transfer membranes were obtained from Millipore. All other chemicals were purchased from Sigma.

Transfection of CHO Cells. An expression vector containing the cDNA for the protein-coding region of human IGFBP-1 was constructed by ligating a human IGFBP-1 cDNA (bases 1–933; ref. 11) into a pUC18-derived plasmid, pNUT (the generous gift of Richard D. Palmiter, University of Washington, Seattle), so that the cDNA was driven by the mouse metallothionein promoter and utilized polyadenylylation signals and sites derived from the human growth hormone gene. pNUT also contains the gene for dihydrofolate reductase driven by the simian virus 40 promoter, allowing selection of cellular transfectants with methotrexate. CHO cells were transfected with the expression vector by calcium phosphate precipitation followed by glycerol shock (12). Stable methotrexate-resistant cell lines were established. The cell line (designated CHOBP1-A6) secreting the greatest amount of human IGFBP-1 was used in phosphorylation experiments.

Phosphorylation and Immunoprecipitation of IGFBP-1. Transfected CHOBP1-A6 cells and HepG2 human hepatoma cells were grown to confluency on 10-cm tissue culture dishes. The cell monolayers were incubated for 5 hr in 5 ml of phosphate-free Eagle's minimal essential medium (MEM) containing 250 μCi of $^{32}P_i$, and conditioned media were collected. The cells were extracted for 30 min at 25°C with 0.5% Triton X-100 in 5 ml of phosphate-buffered saline with 5 mM ATP, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and 1 mM benzamidine hydrochloride. Aliquots (1 ml) of conditioned media and cell lysates were incubated in siliconized tubes at 4°C with a 1:1000 dilution of rabbit polyclonal antiserum to cell human IGFBP-1. After overnight incubation, 8 μl of an ovine antiserum to rabbit IgG was added to all tubes and incubated 1 hr at 4°C. Antibody-bound

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: IGF, insulin-like growth factor; IGFBP, IGF-binding protein; PVDF, poly(vinylidene difluoride).

‡To whom reprint requests should be addressed.

IGFBP-1 was separated by centrifugation at 8000 × g for 20 min. The immunoprecipitated proteins were resolved by SDS/12.5% PAGE.

To determine whether an ectokinase was present on cell surfaces, confluent monolayers of transfected CHOBP1-A6 cells in 2-cm^2 wells were incubated in 0.5 ml of MEM (not phosphate-free) with test substances and 5 μCi of [γ-^{32}P]ATP, 0.01% bovine serum albumin, 1 mM $MnCl_2$, and 20 mM Hepes (pH 7.4). After 30 min at 37°C, phosphorylation was terminated by the addition of 0.25 ml of phosphate-buffered saline at 4°C with 2 mM unlabeled ATP. Immunoprecipitation was immediately performed at 4°C for some media samples as described above, and other samples were precipitated at 4°C with 15% (wt/vol) trichloroacetic acid.

PAGE in the Absence of SDS. Non-SDS PAGE was performed in 5 cm × 8 cm × 1.5 mm slab gels in a minigel apparatus (Hoefer). A discontinuous buffer system was used, with acrylamide concentrations of 10% in the resolving gel and 4% in the stacking gel. The resolving gel was buffered to pH 7.5 with 100 mM Tris·HCl, and the stacking gel to pH 5.5 with 70 mM Tris·HPO_4. Both the stacking and the resolving gel contained a nonionic detergent, *n*-octyl glucoside, at 20 mM. Sample buffer (170 mM Tris·HPO_4, pH 5.5/90 mM *n*-octyl glucoside/40% glycerol/0.008% bromphenol blue) was added to all samples in a 1:3 ratio. Electrophoresis was performed at 25°C with tap water cooling. The cathode buffer contained 3 g of Tris and 2 g of glycylglycine per liter (pH 8.1), and the anode buffer contained 50 mM Tris·HCl (pH 8.1). The gels were run at constant current: 10 mA per gel while stacking and 25 mA per gel while resolving. The proteins were transferred to PVDF membranes by electroblotting (13). The transfer membranes were then subjected to autoradiography, in the case of ^{32}P-containing samples, and/or Western blotting by probing with either ^{125}I-labeled IGF-I (150,000 cpm/ml, visualized by autoradiography) or rabbit antiserum to human IGFBP-1 (1:1000 dilution, visualized using an alkaline phosphatase-linked anti-rabbit IgG from Sigma and a phosphatase-dependent color development system from Promega) as described (10, 13, 14).

Assay of Binding of IGFBP-1 to IGF-I. To determine the binding affinities of phosphorylated and nonphosphorylated IGFBP-1 for IGF-I, ^{125}I-labeled recombinant IGF-1 (15) (25,000 cpm; specific activity, 300 μCi/μg) was incubated with IGFBP-1 (4 ng/ml) from three sources. IGFBP-1 was purified from a recombinant bacterial source, HepG2 conditioned medium, and human amniotic fluid (6). The HepG2-derived IGFBP-1 was purified from conditioned medium by phenyl-Sepharose chromatography followed by HPLC (Vydac C_4 column). Two micrograms of the purified HepG2 protein was dephosphorylated by incubation with 12 units of *Escherichia coli* alkaline phosphatase (Sigma) at 37°C for 30 hr. The dephosphorylated protein was repurified by HPLC on a C_4 column. All four preparations of IGFBP-1 were shown to be homogeneous by SDS/PAGE with silver staining. IGFBP-1 and labeled IGF-I were incubated in triplicate overnight at 4°C in 0.25 ml of 0.1 M Hepes/44 mM NaH_2PO_4/0.01% Triton X-100/0.1% bovine serum albumin/0.02% NaN_3, pH 6.0), in the presence of unlabeled recombinant IGF-I (0–40 ng/ml). Bound label was separated from free by precipitation in 12.5% polyethylene glycol (6, 16). Nonspecific binding was determined by measuring bound ^{125}I-IGF-I in the presence of unlabeled IGF-I (1 μg/ml). Nonspecific binding constituted <25% of bound radiolabel in the absence of unlabeled IGF-I and <15% of the total amount of radiolabel added to each tube.

RESULTS

Secretion of Phosphorylated IGFBP-1 by Cells in Culture. The CHOBP1-A6 transfected cell line secreted human IGFBP-1 as determined by RIA and Western blotting. Human IGFBP-1 was present at a concentration of 2 μg/ml in 48-hr conditioned medium. Ligand blotting showed that nontransfected CHO cells secreted a M_r 24,000 IGFBP. This protein did not react with IGFBP-1 antibody (data not shown). The transfected CHO cells secreted an additional M_r 29,000 form of IGFBP that reacted strongly with the anti-human IGFBP-1 antibody.

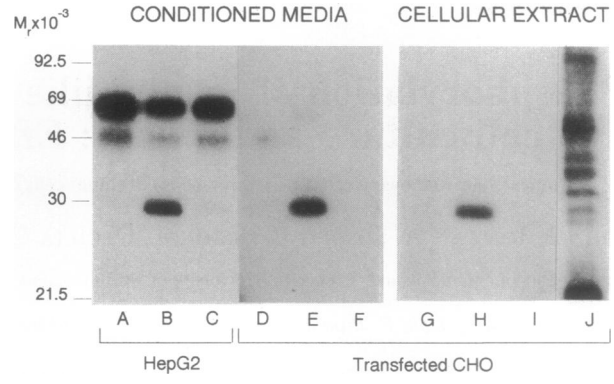

FIG. 1. Immunoprecipitation of IGFBP-1 from ^{32}P-labeled conditioned media of CHOBP1-A6 cells (lanes D–F) and of HepG2 cells (lanes A–C) and from ^{32}P-labeled CHOBP1-A6 Triton cell lysates (lanes G–I). Samples (200 μl) were immunoprecipitated with nonimmune rabbit serum (lanes A, D, and G), IGFBP-1 antiserum (lanes B, E, and H), or antiserum preincubated with unlabeled IGFBP-1 at 1 μg/ml (lanes C, F, and I). The immunoprecipitates were analyzed by SDS/PAGE with autoradiography of the dried gels. A specifically immunoprecipitated M_r 29,000 band of phosphorylated IGFBP-1 is apparent in lanes B, E, and H. ^{32}P-labeled CHOBP1-A6 cell layers were also extracted directly with Laemmli sample buffer containing 2% SDS (lane J), to demonstrate total cell-associated phosphoproteins.

Immunoprecipitation of media conditioned by CHOBP1-A6 cells and by HepG2 hepatoma cells in the presence of $^{32}P_i$ demonstrated a M_r 29,000 ^{32}P-labeled protein (Fig. 1, lanes B and E). The immunoprecipitation of this band was specific, since band intensity was diminished when an excess of unlabeled IGFBP-1 was used (lanes C and F). This phosphoprotein was not present in medium conditioned by nontransfected CHO cells (data not shown). To determine which amino acid(s) was phosphorylated, analysis of the immunoprecipitated ^{32}P-labeled IGFBP-1 was performed by two-dimensional electrophoresis on cellulose TLC plates (17). Only phosphoserine residues were evident (data not shown).

A phosphoprotein with identical apparent molecular weight was also immunoprecipitated from the Triton lysate of CHOBP1-A6 cells (Fig. 1, lane H). The location of the phosphoprotein in the Triton lysate could have been either in the intracellular compartment or on the external surface of the cell membrane, and therefore the kinase responsible for its phosphorylation could have been in either location. Kinases that are similar to casein kinase II and cAMP-dependent protein kinase and are associated with external cell surfaces (ectokinases) have been described in both CHO and hepatoma cells (18, §). To determine whether an ectokinase might phosphorylate IGFBP-1, the transfected CHO cells were incubated with [γ-^{32}P]ATP and exogenous unlabeled, nonphosphorylated IGFBP-1. No IGFBP-1 was phosphorylated under these conditions, whether secreted by the cells during the incubation (Fig. 2, lane E) or added exogenously at the beginning of the incubation period (lanes F–H). This was true despite the demonstration of the presence of casein kinase activity on the surfaces of the cells under our

§Vilgrain, I. & Baird, A., Endocrine Society, 72nd Annual Meeting, June 23, 1990, p. 1327 (abstr.).

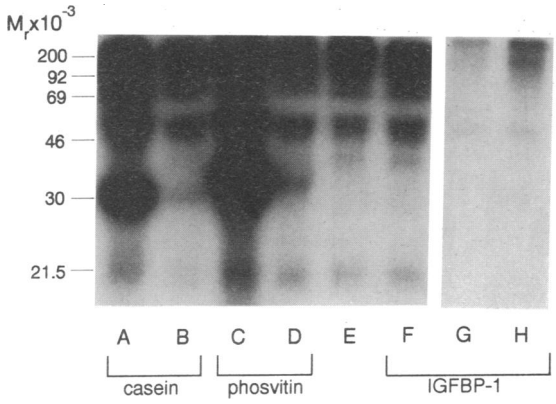

FIG. 2. An ectokinase on the surface of CHOBP1-A6 cells phosphorylates casein and phosvitin but not IGFBP-1. Cells were incubated for 30 min with [γ-^{32}P]ATP and 50 μg of casein (lanes A and B), 50 μg of phosvitin (lanes C and D), 100 ng of bacterial recombinant IGFBP-1 (lanes F–H), or with no other additives (lane E), and the media were collected for analysis. For lanes A–F, proteins were precipitated with 15% trichloroacetic acid; lanes G and H contained immunoprecipitates with IGFBP-1 antiserum. Phosphorylated casein (M_r 30,000) is demonstrated in lane A and phosphorylated phosvitin (M_r 36,000) in lane C. Heparin (50 μg) was included in lanes B and D and resulted in attenuation of casein and phosvitin phosphorylation. Other proteins in the media were phosphorylated by ectokinase activity, as demonstrated by the presence of multiple other ^{32}P-labeled bands. No phosphorylated IGFBP-1 (M_r 29,000) was detected by either acid precipitation (lane F) or immunoprecipitation (lanes G and H). cAMP (100 μM) was added to the sample for lane H. The addition of cAMP (10 μM), cGMP (10 or 100 μM), or dibutryl-cAMP (10 or 100 μM) resulted in results similar to lane H (data not shown).

experimental conditions, as has been described for CHO cells (19). We observed phosphorylation of casein (Fig. 2, lane A) and phosvitin (lane C) that was suppressed by coincubation with heparin (lanes B and D), as expected for this type of ectokinase (20). We incubated the transfected CHO cells with 10–100 μM cAMP (lane H) as well as cGMP and dibutryl-cAMP (lanes not shown but similar to lane H) in the presence of [γ-^{32}P]ATP and nonphosphorylated IGFBP-1 and were unable to demonstrate phosphorylation of IGFBP-1 by a cAMP-dependent ectokinase. We conclude that the phosphorylated IGFBP-1 we find in the cell supernatants is phosphorylated by an intracellular kinase.

Separation of Nonphosphorylated IGFBP-1 from Phosphorylated Isoforms. Although immunoprecipitation demonstrated the presence of phosphorylated IGFBP-1, this technique does not permit evaluation of the relative amounts of phosphorylated and nonphosphorylated IGFBP-1. PAGE was performed in the absence of SDS in order to alter protein migration on the basis of molecular charge as well as mass, thus permitting separation of phosphorylated from nonphosphorylated IGFBP-1. In addition, the pH during the resolving phase of electrophoresis was lowered to 8.3. The phosphorylated and ^{32}P-labeled IGFBP-1 from CHOBP1-A6 cells resolved into four bands that could be visualized by both autoradiography and immunoblotting. A single band of nonphosphorylated IGFBP-1 was visualized by immunoblotting but not by autoradiography, because it contained no ^{32}P (Fig. 3). Following exposure to alkaline phosphatase, the intensity of the nonphosphorylated band was increased and that of the phosphorylated bands was diminished.

Detection of Phosphorylated and Nonphosphorylated IGFBP-1 in Human Physiologic Fluids. To determine whether physiologic fluids and purified preparations of IGFBP-1 contained phosphorylated or nonphosphorylated forms of IGFBP-1, other nonlabeled samples were evaluated by non-SDS PAGE and immunoblotting (Fig. 4). Both phosphorylated and nonphosphorylated forms of IGFBP-1 were demonstrated. The immunoblots showed that recombinant IGFBP-1 derived from a bacterial expression system represented a homogeneous nonphosphorylated form, whereas IGFBP-1 secreted by HepG2 cells consisted primarily of four or five isoforms of phosphoprotein and very little of the nonphosphorylated protein. Human decidual cells secreted only phosphorylated isoforms. These isoforms were converted into more slowly migrating isoforms by 2 hr of treatment with alkaline phosphatase as shown. Prolonged treatment (8 hr) was required for complete dephosphorylation (data not shown). Human fetal serum and human amniotic fluid contained significant amounts of both phosphorylated and nonphosphorylated IGFBP-1 but minimal amounts of the rapidly migrating phosphorylated isoforms. The ligand blot (Fig. 4) demonstrated that both nonphosphorylated and phosphorylated isoforms were capable of binding ^{125}I-IGF-I.

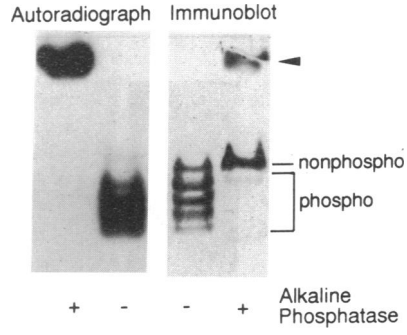

FIG. 3. Non-SDS PAGE of ^{32}P-labeled conditioned medium from CHOBP1-A6 cells. ^{32}P-labeled conditioned medium (20 μl) was analyzed by non-SDS PAGE after incubation for 2 hr at 37°C in the presence or absence of 3 units of bovine intestinal alkaline phosphatase. After electrophoresis the proteins in the gel were transferred to a PVDF membrane. Both an autoradiograph of the membrane and the membrane itself after immunoblotting with IGFBP-1 antiserum are shown. Alkaline phosphatase (arrowhead) is visible as a band on the autoradiograph because it incorporated ^{32}P during the incubation period, and it is visible on the immunoblot because it reacted with the developing reagent.

Effects of IGFBP-1 Phosphorylation on Affinity for IGF-I. To evaluate the physiologic significance of the phosphorylation state of IGFBP-1, we compared the IGF-I binding affinity of phosphorylated IGFBP-1 (purified from HepG2 conditioned medium) with that of nonphosphorylated IGFBP-1 (both the recombinant bacterial material and the dephosphorylated HepG2 IGFBP-1) and with that of IGFBP-1 purified from human amniotic fluid (Fig. 5). Scatchard plots obtained for the phosphorylated and nonphosphorylated forms of IGFBP-1 were linear, indicating the presence of a single binding site. The slopes of these Scatchard plots were significantly different, however, and indicated that the IGF-I affinity constant of the dephosphorylated HepG2 IGFBP-1 ($K_a = 2.8 \pm 0.2 \times 10^9$ M^{-1}) was 6-fold lower than the affinity constant of the mixture of phosphorylated isoforms from HepG2 conditioned medium ($K_a = 1.7 \pm 0.2 \times 10^{10}$ M^{-1}). The affinity of the nonphosphorylated recombinant IGFBP-1 ($K_a = 4.7 \pm 0.4 \times 10^9$ M^{-1}) was 4-fold lower than that of phosphorylated IGFBP-1. In contrast, the Scatchard plot for the IGFBP-1 purified from amniotic fluid was curvilinear, as has been described previously (5, 6). This result might be expected, in light of our findings that amniotic fluid IGFBP-1 is a combination of phosphorylated and nonphosphorylated IGFBP-1 isoforms, with differing IGF-I binding affinities.

DISCUSSION

These studies demonstrate that IGFBP-1 undergoes phosphorylation, an important posttranslational modification.

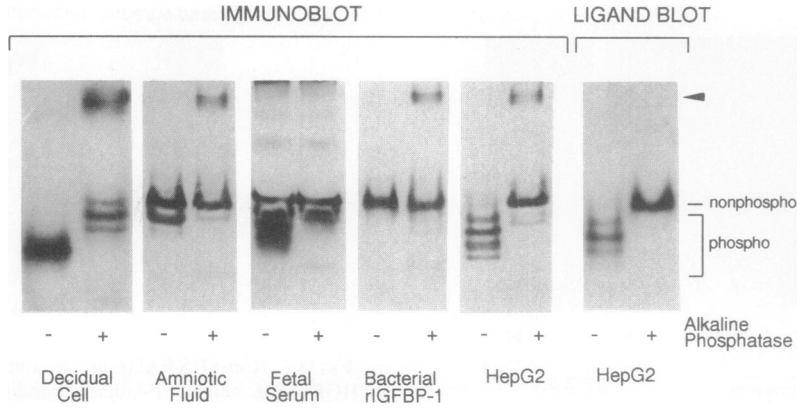

FIG. 4. Non-SDS PAGE of unlabeled samples that contain human IGFBP-1. Decidual cell and HepG2 conditioned media (20 μl), fetal serum (5 μl), recombinant IGFBP-1 (30 ng), and human amniotic fluid IGFBP-1 (30 ng) were analyzed after incubation for 2 hr at 37°C in the presence or absence of 3 units of alkaline phosphatase. The transfer membranes after immunoblotting and an ^{125}I-IGF-I ligand blot of the HepG2 conditioned medium are shown. The alkaline phosphatase band in the immunoblots is shown by the arrowhead. The upper band, which is intensified by incubation with alkaline phosphatase, represents nonphosphorylated IGFBP-1. The lower four or five bands, which are attenuated by incubation with alkaline phosphatase, represent phosphorylated isoforms of IGFBP-1.

Phosphorylation occurs not only in CHO cells transfected with an IGFBP-1 cDNA construct but also in human hepatoma and decidual cells, two naturally occurring cell types.

Phosphorylation of IGFBP-1 results in sufficient alteration in its charge density and/or conformation to result in a change in electrophoretic mobility. Both a nonphosphorylated form and four or five phosphorylated isoforms can be detected. Although the transfected CHO cell line secretes significant quantities of the nonphosphorylated form, HepG2 cells and human decidual cells each secrete primarily phosphorylated forms of IGFBP-1. The immunoblots and ^{32}P autoradiographs of the media conditioned by these cells demonstrate four distinct phosphorylated bands (one of which suggests a doublet). In contrast, human fetal serum and human amniotic fluid contain proportionately more of the nonphosphorylated form and less of the phosphorylated isoforms. Recombinant IGFBP-1 migrates in non-SDS-containing gels as a single nonphosphorylated band, presumably because the bacteria synthesizing it lack the kinase required to phosphorylate the protein. The use of SDS-containing gels to analyze IGFBP-1 in conditioned media and biological fluids explains the failure of prior studies to detect these isoforms.

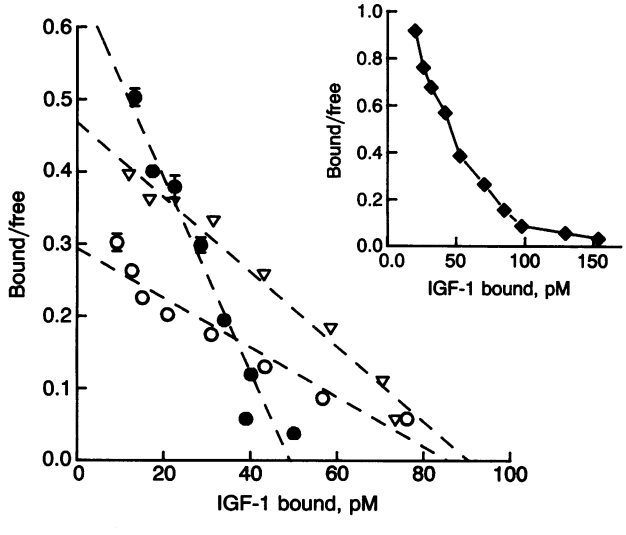

FIG. 5. Scatchard analysis of IGF-I binding to four forms of IGFBP-1: nonphosphorylated recombinant (▽), phosphorylated HepG2-derived before (●) and after (○) dephosphorylation with alkaline phosphatase, and human amniotic fluid-derived (*Inset*, ◆). Equilibrium binding of IGFBP-1 (4 ng/ml) to ^{125}I-IGF-I in the presence of unlabeled IGF-I (0–40 ng/ml) was performed as described in *Materials and Methods*. Symbols designate the mean values and error bars (where larger than the symbols) show the SE for triplicate determinations in a representative experiment. First-order regression lines (dashed) were drawn through the recombinant and HepG2-derived IGFBP-1 plots. The slopes of the regression lines obtained from seven experiments were used to determine mean K_a values (± SE) for HepG2-derived IGFBP-1 before ($1.7 \pm 0.2 \times 10^{10}$ M^{-1}) and after ($2.8 \pm 0.2 \times 10^9$ M^{-1}) dephosphorylation and for recombinant IGFBP-1 ($4.7 \pm 0.4 \times 10^9$ M^{-1}). All three K_a values were statistically different by *t* test ($P < 0.01$).

Dephosphorylation of IGFBP-1 is accompanied by a significant change in its binding affinity for IGF-I. The molecular mechanism accounting for this change remains to be defined. Our observation that the phosphorylated isoforms of IGFBP-1 migrate faster in a non-SDS-containing polyacrylamide gel than does the nonphosphorylated form suggests either that the phosphate groups contribute significantly to the molecular charge of the protein or that phosphorylation results in conformational changes that are neutralized in the presence of SDS. Such conformational changes, or local ionic or steric interactions in the region of the IGF binding site, might explain the 4- to 6-fold higher affinity of the phosphorylated form for IGF-I. A precedent for such an effect of phosphorylation comes from studies of basic fibroblast growth factor (bFGF). Phosphorylation of bFGF in its receptor-binding domain by cAMP-dependent protein kinase results in an increased affinity of the phosphorylated bFGF for the FGF receptor (21).

The biologic significance of the phosphorylation-dependent increase in the affinity of IGFBP-1 for IGF-1 has not been determined. IGFBP-1 preparations that contain predominantly phosphorylated forms inhibit the cell growth response to IGF-I. Likewise, IGFBP-1 can inhibit IGF-I-stimulated growth and other cellular functions, such as amino acid transport (3, 4). Since the affinity of the phosphorylated form of IGFBP-1 for IGF-I ($K_a \approx 10^{10}$) is greater than that of the IGF type 1 receptor ($K_a \approx 10^9$), it is possible that it acts by inhibiting receptor interaction. Because we have shown that multimerization of IGFBP-1 is associated with its capacity to potentiate cellular responses to IGF-I (7), it is also possible that phosphorylation could prevent the intermolecular disulfide bond formation that leads to multimerization.

Only serine residues in IGFBP-1 appear to be phosphorylated, but it is not known which serine residues are the site(s) of phosphorylation. Since there are four or five phosphorylated isoforms, it is likely that multiple phosphorylation sites

exist. Given the effect phosphorylation has on IGF-I binding affinity, it is also likely that at least one of the phosphorylation sites is in the vicinity of the IGF binding region. We have not determined which of the phosphorylated isoforms are responsible for the increased affinity.

The phosphorylation of intracellular proteins is a well-recognized step in multiple cellular metabolic and signaling pathways; however, the phosphorylation of secreted proteins has only recently been investigated. Several proteins found in the extracellular matrix have been shown to exist in phosphorylated forms. Osteopontin was first identified as a bone matrix phosphorylated glycoprotein involved with cell adhesion. It contains the cell attachment sequence Arg-Gly-Asp (RGD) that is recognized by the integrin family of cell surface receptors (22). The phosphorylated form of osteopontin binds to cells in a heat-stable complex with multimerized fibronectin on the cell surface, while the nonphosphorylated form binds preferentially to soluble fibronectin (23, 24). These findings suggest that phosphorylation of osteopontin has a role in altering cell–matrix interactions. Fibrinogen and vitronectin are other RGD-containing proteins that are phosphorylated on serine residues (25, 26). One-fourth of plasma fibrinogen is phosphorylated in human adults at steady state, but under acute stress conditions and in fetal plasma the relative proportions of phosphorylated fibrinogen increases (27). Phosphorylation of fibrinogen *in vitro* results in decreased susceptibility to plasmin digestion (28) and increased fiber thickness in fibrin clots (29), again suggesting a physiologic function for phosphorylation. The serine phosphorylation of osteopontin, vitronectin, and fibrinogen may be relevant to phosphorylation of IGFBP-1, because IGFBP-1 contains the RGD sequence (11) and can be detected in the extracellular matrix (unpublished findings).

Our studies show that phosphorylated IGFBP-1 is present in cell lysates (Fig. 1) and that an ectokinase is not responsible for its phosphorylation (Fig. 2). These results suggest that IGFBP-1 is secreted after phosphorylation by an intracellular kinase(s). This is analogous to fibrinogen, which is secreted from the liver primarily as a phosphoprotein (30, 31). However, 75% of plasma fibrinogen is dephosphorylated. The sites and regulation of its dephosphorylation, as well as the phosphatase responsible, are unknown. Our results show that IGFBP-1 found in fetal serum and amniotic fluid is relatively nonphosphorylated when compared with that in hepatoma or decidual cell conditioned medium. This suggests that in a similar fashion IGFBP-1, after its secretion as a phosphoprotein, becomes partially dephosphorylated *in vivo*. We have been unable to affect the phosphorylation of IGFBP-1 by cells in culture with IGF-I, insulin, or modulators of either cAMP-dependent protein kinase or protein kinase C. Phosphorylation of IGFBP-1 may be constitutively active and not under tight regulatory control. It may be that dephosphorylation of IGFBP-1 occurring after secretion is a more highly regulated and physiologically important event than phosphorylation.

Because amniotic fluid contains both phosphorylated and nonphosphorylated IGFBP-1, the two forms of IGFBP-1 that were described in an earlier report (6) to have identical mobility in SDS-containing gels but different elution patterns from DEAE-cellulose may have differed only with regard to phosphorylation. If true, this finding is likely to be biologically significant. The form that was previously shown to be eluted from DEAE-cellulose at lower ionic strength could represent the nonphosphorylated form. This form potentiated the effect of IGF-I in stimulating DNA synthesis (6) and multimerized (7). Conversely, an additional form that required higher ionic strength for elution (phosphorylated IGFBP-1) neither potentiated IGF-I action nor formed multimers. In addition, we have observed that recombinant, nonphosphorylated IGFBP-1 potentiates the effects of IGF-I on thymidine incorporation, while the mixture of phosphorylated isoforms of IGFBP-1 from HepG2 cells does not (unpublished observations). The state of phosphorylation of IGFBP-1 therefore may be an important determinant of its biologic activity.

We thank Dr. H. S. Earp for helpful advice and discussions, Ms. Ruth Dy for technical assistance with phospho amino acid analysis, and Dr. W. H. Busby for purifying IGFBP-1 from amniotic fluid and HepG2 conditioned medium. This work was supported by Grants AG02331 (D.R.C.) and HD08299 (A.J.D.) from the National Institutes of Health.

1. Ballard, J., Baxter, R., Binoux, M., Clemmons, D., Drop, S., Hall, K., Hintz, R., Rechler, M., Rutanen, E. & Schwander, J. (1989) *Acta Endocrinol.* **121,** 751–752.
2. Elgin, R. G., Busby, W. H. & Clemmons, D. R. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 3313–3318.
3. Frauman, A. G., Tsuzaki, S. & Moses, A. C. (1989) *Endocrinology* **124,** 2289–2296.
4. Ritvos, O., Ranta, T., Jalkanen, J., Suikkari, A. M., Voutilainen, R., Bohn, H. & Rutanen, E. M. (1988) *Endocrinology* **122,** 2150–2157.
5. Baxter, R. C., Martin, J. L. & Wood, M. H. (1987) *J. Clin. Endocrinol. Metab.* **65,** 423–431.
6. Busby, W. H., Klapper, D. G. & Clemmons, D. R. (1988) *J. Biol. Chem.* **263,** 14203–14210.
7. Busby, W. H., Hossenlopp, P., Binoux, M. & Clemmons, D. R. (1989) *Endocrinology* **125,** 773–777.
8. Thrailkill, K. M., Clemmons, D. R., Busby, W. H. & Handwerger, S. (1990) *J. Clin. Invest.* **86,** 878–883.
9. D'Ercole, A. J., Drop, S. L. S. & Kortleve, D. J. (1985) *J. Clin. Endocrinol. Metab.* **61,** 612–617.
10. Busby, W. H., Snyder, D. K. & Clemmons, D. R. (1988) *J. Clin. Endocrinol. Metab.* **67,** 1225–1230.
11. Brewer, M. T., Stetler, G. L., Squires, C. H., Thompson, R. C., Busby, W. H. & Clemmons, D. R. (1988) *Biochem. Biophys. Res. Commun.* **152,** 1289–1297.
12. Kingston, R. E. (1988) in *Current Protocols in Molecular Biology*, eds. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (Wiley, New York), pp. 9.1.1–9.1.4.
13. Hossenlopp, P., Seurin, D., Segovia-Quinson, B., Hardouin, S. & Binoux, M. (1986) *Anal. Biochem.* **154,** 138–143.
14. McCusker, R. H. & Clemmons, D. R. (1988) *J. Cell. Physiol.* **137,** 505–512.
15. D'Ercole, A. J., Underwood, L. E., Van Wyk, J. J., Decedue, C. J. & Foushee, D. B. (1976) in *Growth Hormone and Related Peptides*, eds. Pecile, A. & Muller, R. (Excerpta Medica, Amsterdam), pp. 190–205.
16. McCusker, R. H., Busby, W. H., Dehoff, M. H., Camacho-Hubner, C. & Clemmons, D. R. (1991) *Endocrinology* **129,** in press.
17. Cooper, J. A., Sefton, B. M. & Hunter, T. (1983) *Methods Enzymol.* **99,** 387–402.
18. Kubler, D., Pyerin, W., Bill, O., Hotz, A., Sonka, J. & Kinzel, V. (1989) *J. Biol. Chem.* **264,** 14549–14555.
19. Kubler, D., Pyerin, W., Burow, E. & Kinzel, V. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 4021–4025.
20. Pyerin, W., Burow, E., Michaely, K., Kubler, D. & Kinzel, V. (1986) *Biol. Chem. Hoppe Seyler* **368,** 215–227.
21. Feige, J. J., Bradley, J. D., Fryburg, K., Farris, J., Cousens, L. C., Barr, P. J. & Baird, A. (1989) *J. Cell Biol.* **109,** 3105–3114.
22. Butler, W. T. (1989) *Connect. Tissue Res.* **23,** 123–136.
23. Nemir, M., DeVouge, M. W. & Mukherjee, B. B. (1989) *J. Biol. Chem.* **264,** 18202–18208.
24. Singh, K., DeVouge, M. W. & Mukherjee, B. B. (1990) *J. Biol. Chem.* **265,** 18696–18701.
25. McGuire, E. A., Peacock, M. E., Inhorn, R. C., Siegel, N. R. & Tollefsen, D. M. (1988) *J. Biol. Chem.* **263,** 1942–1945.
26. Seydewitz, H. H. & Witt, I. (1985) *Thromb. Res.* **40,** 29–39.
27. Seydewitz, H. H. & Witt, I. (1989) *Thromb. Res.* **55,** 785–790.
28. Forsberg, P.-O. & Martin, S. (1990) *Thromb. Res.* **58,** 119–127.
29. Heldin, P., Hessel, B., Humble, E., Blomback, B. & Engstrom, L. (1987) *Thromb. Res.* **47,** 93–99.
30. Kudryk, B., Okada, M., Redman, C. M. & Blomback, B. (1982) *Eur. J. Biochem.* **125,** 673–682.
31. Seydewitz, H. H., Matthias, F. R., Schondorf, T. H. & Witt, I. (1987) *Thromb. Res.* **46,** 437–445.