Physiological Differences in Insulin-Like Growth Factor Binding Protein-1 (IGFBP-1) Phosphorylation in IGFBP-1 Transgenic Mice

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Insulin-like growth factor binding protein (IGFBP)-1 has been shown to alter cellular responses to insulin-like growth factor 1 (IGF-1). Human IGFBP-1 undergoes serine phosphorylation, and this enhances both its affinity for IGF-1 by six- to eightfold and its capacity to inhibit IGF-1 actions. To investigate the physiological role of IGFBP-1 in vivo, transgenic mice have been generated using either the human IGFBP-1 or rat IGFBP-1 transgene. Both lines of mice expressed high concentrations of IGFBP-1 in serum and tissues; however, human IGFBP-1 transgenic mice did not show glucose intolerance and exhibited no significant intrauterine growth retardation, whereas rat IGFBP-1 transgenic mice showed fasting hyperglycemia and intrauterine growth restriction. The aim of this study was to investigate the physiological differences in the phosphorylation state of human IGFBP-1 and rat IGFBP-1 in these transgenic mice. The phosphorylation status of IGFBP-1 in transgenic mouse serum was analyzed by nondenaturing PAGE. Almost all of the IGFBP-1 in serum from the human IGFBP-1 transgenic mice was present as a nonphosphorylated form. Most of the rat IGFBP-1 in the serum of the mice expressing the rat IGFBP-1 was phosphorylated. Immunoprecipitation showed that mouse hepatoma (Hepa 1–6) cells (exposed to $[^{32}P]H_3PO_4$) secrete ^{32}P-labeled IGFBP-1. When the human IGFBP-1 transgene was transfected into Hepa 1–6 cells, all of the IGFBP-1 was secreted in the nonphosphorylated form. However, when the rat IGFBP-1 transgene was transfected into these cells, phosphorylated forms of IGFBP-1 were secreted. To confirm this result, the mouse hepatoma cell protein kinase was partially purified. This kinase activity phosphorylated mouse and rat IGFBP-1 in vitro, but it did not phosphorylate human IGFBP-1. Scatchard analysis showed that the affinity of phosphorylated rat IGFBP-1 for IGF-1 was 3.9-fold higher than that of nonphosphorylated human IGFBP-1. We conclude that the mouse IGFBP-1 kinase activity cannot phosphorylate human IGFBP-1, whereas it can phosphorylate rat IGFBP-1. The phosphorylation state of human IGFBP-1 may account for part of the phenotypic differences noted in the two studies of transgenic mice, and it is an important determinant of the capacity of human IGFBP-1 to inhibit IGF-1 actions in vivo.
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Insulin-like growth factor binding proteins (IGFBPs) that are present in extracellular fluids have been shown to modulate the biological activity of IGFs (1,2). Human IGFBP-1, IGFBP-3, and IGFBP-5 are phosphorylated on serine residues. When human IGFBP-1 purified from amniotic fluid or obtained from HEP G-2 cell culture supernatants is analyzed by nondenaturing gel electrophoresis, one nonphosphorylated and four phosphorylated isoforms can be identified (3). Anion exchange chromatography can also be used to separate these phosphoisoforms on the basis of their degree of phosphorylation (3,4). Three phosphorylation sites have been identified in human IGFBP-1 (Ser 101, 119, and 169) (5). In human hepatoma cells, casein kinase II and an unknown kinase that is similar but not identical to casein kinase I have been shown to phosphorylate human IGFBP-1 (6). Rat IGFBP-1 that is synthesized by H-4-II-EC3 rat hepatoma cells is phosphorylated at two sites that have been determined to be Ser 107 and Ser 132 (7). Unlike human IGFBP-1, dephosphorylation of rat IGFBP-1 does not affect its affinity for IGF-1, and dephosphorylated rat IGFBP-1 retains its capacity to inhibit DNA synthesis in 3T3 cells (7).

Phosphorylation of human IGFBP-1 enhances both its affinity for IGF-1 by six- to eightfold and its capacity to inhibit IGF-1 action (4,5,8,9). This difference in affinity has been shown to correlate with the ability of human IGFBP-1 to alter IGF-1 actions (10). Numerous in vitro studies indicate that human IGFBP-1 may either potentiate or inhibit IGF-1–stimulated biological activity (1,2,5). The differences in IGFBP-1 actions appear to be due in part to differences in the phosphorylation state of IGFBP-1. For example, phosphorylated human IGFBP-1 incubated with cultured cells inhibits IGF-1–stimulated DNA synthesis, as well as wound healing in experimental animals. In contrast, nonphosphorylated human IGFBP-1 has been shown to enhance IGF-1 actions in these test systems (4,11). One study administered nonphosphorylated IGFBP-1 in vivo to hypophysectomized rats and showed that a molar excess neutralized the effect of concomitantly administered IGF-1 (12). However, the role of IGFBP-1 phosphorylation in vivo has not been definitively determined. To investigate the function of IGFBP-1 in vivo,

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DMEM, Dulbecco's modified Eagle's medium; DTT, diothiothreitol; hBP, human binding protein; HPLC, high-performance liquid chromatography; IGFBP, insulin-like growth factor binding protein; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene difluoride; rBP, rat binding protein.

transgenic mice have been generated using human and rat IGFBP-1. D' Ercole et al. (13) generated mice carrying a human IGFBP-1 transgene driven by mouse metalothionein-I promoter. Rajkumar et al. (14) generated a mouse line carrying the rat IGFBP-1 transgene driven by mouse phosphoglycerate kinase promoter. Both strains of transgenic mice expressed high concentrations of IGFBP-1 in several tissues, and each had similar concentrations of IGFBP-1 in their serum. The human IGFBP-1 transgenic mice, however, did not develop glucose intolerance, had normal intrauterine growth, and had postnatal growth rates that were similar to control animals (13). In contrast, the mice expressing rat IGFBP-1 had glucose intolerance and significant intrauterine growth retardation (14). The phosphorylation state of IGFBP-1 was not determined in either study; therefore, we speculated that the status of IGFBP-1 phosphorylation might differ between these two lines of transgenic mice and might partially explain the phenotypic differences between these lines.

RESEARCH DESIGN AND METHODS

Materials. Mouse hepatoma (Hepa 1–6) cells and human hepatoma (Hep G2) cells were obtained from the American *Type Culture* Collection (Rockville, MD). Penicillin, streptomycin, and geneticine (G418) were purchased from GibcoBRL (Grand Island, NY). [^{32}P]H_3PO_4 (400–800 mCi/ml) and [γ-^{32}P]ATP (4,000 mCi/mmol) were obtained from ICN Biochemical (Costa Mesa, CA). Polyvinylidene difluoride (PVDF) transfer membranes (Immobilon-P) were obtained from Millipore (Bedford, MA). The Eukaryotic TOPO TA cloning kit was obtained from Invitrogen (Carlsbad, CA). Talon metal affinity resin was purchased from Clontech (Palo Alto, CA). Centricon-10 microconcentrators were obtained from Amicon (Beverley, MA). Phenyl-Sepharose CL-4B were obtained from Pharmacia LKB Biotechnology (Piscataway, NJ). Calf intestinal alkaline phosphatase was obtained from Boehringer Mannheim (Indianapolis, IN). All other chemicals were purchased from Sigma Chemical (St. Louis, MO).

Tissue culture. Hepa 1–6 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal calf serum (GibcoBRL) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). The cells were grown in 5% CO_2:95% O_2 at 37°C and passaged using a split ratio of 1:4 after trypsinization.

^{32}P labeling and immunoprecipitation of mouse IGFBP-1. Hepa 1–6 cells were grown to confluency on 10-cm tissue culture dishes (no. 3003; Falcon Labware). The cells were rinsed with serum-free Eagle's minimum essential medium without sodium-phosphate and were then incubated for 16 h in 5 ml of the same medium in the presence of 250 µCi [^{32}P]H_3PO_4, before the conditioned media were collected. Aliquots of conditioned media were incubated with 1:500 dilution of rabbit polyclonal antiserum to human IGFBP-1 (15) overnight at 4°C. The immune complexes were incubated with protein-A Sepharose at 4°C for 2 h. Immobilized protein-A was centrifuged at 7,000*g* for 1 min, and the immunoprecipitated proteins were resuspended in a Laemmli sample buffer or a nondenaturing PAGE sample buffer, as described below.

Nondenaturing PAGE. Nondenaturing PAGE was performed to separate phosphoisoforms of IGFBP-1, as described previously (16). The samples were mixed with an equal volume of sample buffer (125 mmol/l Tris HCl, 20 mmol/l CHAPS [3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate], 20% glycerol, and 0.01% bromphenol blue, pH 6.8) and loaded onto a discontinuous polyacrylamide gel using pH 6.8 in the stacking gel and pH 8.2 in the resolving gel. Both gels contained 10 mmol/l CHAPS, and the electrode buffer (pH 8.2) contained 1 mmol/l CHAPS. The proteins were transferred onto a PVDF membrane (0.45 µm pore size), and the membranes were probed with ^{125}I-labeled IGF-1 (100,000 cpm/ml) and visualized by autoradiography or with rabbit antiserum to human IGFBP-1 (1:1,000 dilution, visualized using an alkaline phosphatase–conjugated anti–rabbit IgG and a phosphatase-dependent color development system) as described previously (17,18). In the case of ^{32}P-containing samples, the gels were dried and visualized by autoradiography. Sera (1.0 µl) from transgenic mice were analyzed by n-octyl glucoside PAGE and immunoblotted as described previously (5,8).

Partial purification of protein kinases and in vitro protein kinase assay. Protein kinase activity from Hepa 1–6 cells was partially purified as described previously (6). In brief, cells were grown to confluence in 10-cm dishes, and 1 ml of lysis buffer (100 mmol/l HEPES, pH 7.5, 100 mmol/l NaCl, 1% [vol/vol] Triton X-100, 1 mmol/l diothiothreitol [DTT], 10 mmol/l EDTA, 1 mmol/l EGTA, 1 mmol/l phenylmethylsulfonyl fluoride [PMSF], 1 µg/ml leupeptin, and 1 µg/ml pepstatin) was added to each dish. The insoluble material was removed by centrifugation at 13,000*g* for 15 min, and the supernatant was added to phosphocellulose (10 mg protein/ml resin) equilibrated with 100 mmol/l HEPES, pH 7.5, and 100 mmol/l NaCl. The phosphocellulose bound protein was eluted in elution buffer (100 mmol/l HEPES, pH 7.5, 1 mol/l NaCl, 10 mmol/l $MgCl_2$, 1 mmol/l DTT, and 10% [vol/vol] glycerol) and was concentrated by centrifugation using Centricon-10 microconcentrators.

This partially purified protein kinase activity was used in the in vitro protein kinase assays. The reaction was initiated by the addition of 100 µmol/l [γ-^{32}P]ATP and incubated at 30°C for 15 min in 100 mmol/l HEPES, pH 7.5, 100 mmol/l NaCl, 10 mmol/l $MgCl_2$, and 1 mmol/l DTT (25-µl final volume). The assays were terminated by the addition of 4 × Laemmli buffer (200 mmol/l Tris-HCl, pH 6.8, 8% [vol/vol] SDS, 400 mmol/l DTT, and 40% [vol/vol] glycerol) and boiling for 10 min. The samples were analyzed by 12.5% SDS-PAGE and autoradiography.

Transfection of Hepa 1–6 cells. Rat or human total RNA was isolated from rat liver or Hep G2 cells using the guanidinium isothiocyanate method (19). A rat IGFBP-1 cDNA fragment, spanning nucleotides +121 to +936, was cloned by reverse transcription–polymerase chain reaction. The cDNA was cloned into the expression vector pcDNA 3.1/V5/His-TOPO. In the same manner, a human IGFBP-1 cDNA fragment spanning nucleotides +151 to +909 was cloned into the same expression vector according to the manufacturer's instructions. Each plasmid produces a product that has a six–amino acid polyhistidine sequence fused to the NH_2-terminus of each protein. Each plasmid was transfected into Hepa 1–6 cells using calcium phosphate precipitation (20). Samples of 5 µg of each plasmid was precipitated and added to 2×10^5 cells, and then positive clones were selected using 1.5 mg/ml G-418. The clones were maintained in DMEM containing 750 µg/ml G-418 and designated Hepa 1–6 human binding protein (hBP)-1 and Hepa 1–6 rat binding protein (rBP)-1.

Purification of IGFBP-1. Mouse IGFBP-1 was purified from conditioned medium of Hepa 1–6 cells. The medium (1,000 ml) was loaded onto a phenyl-Sepharose (CL-4B) column, and the proteins were eluted with a 0–15% acetonitrile gradient. The fractions containing IGFBP-1 (as determined by immunoblotting) were loaded onto an IGF-1 affinity column and eluted with acetic acid. This sample was applied to a reverse-phase high-performance liquid chromatography (HPLC) column and eluted using a linear acetonitrile in 0.4% trifluoroacetic acid as described previously (4). Purity was determined by SDS-PAGE with silver staining. Polyhistidine tagged human and rat IGFBP-1 from conditioned medium of Hepa 1–6 hBP-1 and Hepa 1–6 rBP-1 were purified by Talon metal affinity resin, according to the manufacturer's instructions. Before use in the IGF-1 binding experiments, the polyhistidine tag was removed from rat and human IGFBP-1 with enterokinase (Sigma), according to the manufacturer's recommendation. The absolute amounts of human, rat, and mouse IGFBP-1 were determined by amino acid composition analysis.

Binding assay of IGFBP-1 for IGF-1. To determine the binding affinity of phosphorylated rat and dephosphorylated human IGFBP-1 for IGF-1, we used a polyethylene glycol precipitation method (5). Dephosphorylated human IGFBP-1 was obtained by incubating IGFBP-1 (20 µg) that had been purified from Hep G2 cell–conditioned medium with alkaline phosphatase (50 U) for 18 h at 37°C. The IGFBP-1 was separated from alkaline phosphatase by reverse-phase HPLC using a C-4 column. Rat IGFBP-1 was purified from Hepa 1–6 transfected cells as described above. ^{125}I-labeled IGF-1 (25,000 cpm/tube) was incubated overnight at 4°C with rat IGFBP-1 (8 ng) or dephosphorylated human IGFBP-1 (8 ng), and increasing concentrations of unlabeled IGF-1 in 0.25 ml of 100 mmol/l HEPES, 44 mmol/l NaH_2PO_4, 0.01% Triton X-100, 0.1% bovine serum albumin, and 0.02% NaN_3, pH 6.0. The bound and free labeled IGF-1 were separated by precipitation in 12.5% polyethylene glycol. Nonspecific binding was determined measuring 1 µg/ml unlabeled IGF-1. Affinity was determined by Scatchard analysis.

Radioimmunoassay of rat and human IGFBP-1. The concentrations of total human and rat IGFBP-1 were determined by radioimmunoassay (14,15).

RESULTS

Phosphoisoforms of IGFBP-1 in transgenic mouse serum. IGFBP-1 phosphoisoforms in the transgenic mouse serum were analyzed by n-octyl glucoside PAGE and Western immunoblotting (Fig. 1). Almost all of the IGFBP-1 in the serum from mice carrying the human transgene was in the nonphosphorylated form, and it migrated through the gel identically with the nonphosphorylated human IGFBP-1 from Hep G2 cells. In contrast, analysis of IGFBP-1 in serum from mice carrying the rat IGFBP-1 transgene contained

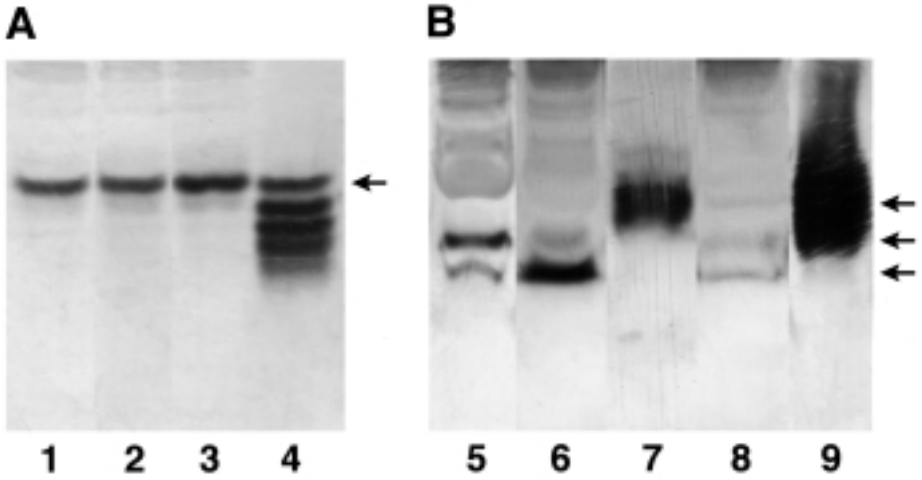

FIG. 1. Phosphorylation status of IGFBP-1 in transgenic mouse sera. Samples of 1 μl human (*A*) and rat IGFBP-1 (*B*) transgenic mouse sera were analyzed by nondenaturing PAGE and immunoblotting using an IGFBP-1 antiserum that recognizes both rat and human IGFBP-1 (15,21). *Lanes 1–3* show individual serum samples from the human IGFBP-1 transgenic mice. *Lane 4* shows the human IGFBP-1 standard. The arrow indicates the position of nonphosphorylated human IGFBP-1. *B*: *Lanes 5* and *6* show two separate pools of rat IGFBP-1 transgenic mouse sera. *Lane 7* contains the same serum sample shown in *lane 5* after exposure to alkaline phosphatase. *Lane 8* shows the control mouse serum, and *lane 9* shows the human IGFBP-1 standard. The two lower arrows indicate the positions of the phosphorylated isoforms of rIGFBP-1, and the upper arrow denotes the nonphosphorylated rIGFBP-1.

several phosphorylated forms. The concentrations of total IGFBP-1 were similar for the two groups of animals: 64 ± 14 ng/ml (n = 7) for the human IGFBP-1 animals and 42 ± 12 ng/ml (n = 6) for rat IGFBP-1. These results suggested that the mouse protein kinase(s) that phosphorylates mouse IGFBP-1 can phosphorylate rat IGFBP-1, but it does not phosphorylate human IGFBP-1.

Phosphoisoforms of mouse IGFBP-1 in Hepa 1–6 conditioned medium. ^{32}P-labeled condition medium from Hepa 1–6 cells (Hepa 1–6 CM) was immunoprecipitated using a rabbit polyclonal anti–human IGFBP-1 antiserum, and the precipitated proteins were analyzed by SDS-PAGE or nondenaturing PAGE followed by autoradiography. A single ^{32}P-labeled 30-kDa IGFBP-1 band was detected (Fig. 2, *lane 1*). In contrast, nondenaturing PAGE showed four IGFBP-1 isoforms (Fig. 2, *lane 2*). These forms were not detected after the incubation for 2 h at 37°C with calf intestinal alkaline phosphatase (*lane 3*). When Hepa 1–6 CM was analyzed by nondenaturing PAGE and Western immunoblotting, four phosphorylated isoforms were demonstrated (*lane 4*). These results demonstrate that Hepa 1–6 cells secrete phosphorylated IGFBP-1 and that these cells contain a kinase that phosphorylates mouse IGFBP-1.

Phosphorylation of human and rat IGFBP-1 in Hepa 1–6 cells. To determine whether mouse liver kinase could phosphorylate human and rat IGFBP-1, mouse hepatoma cells were transfected with either the rat or human transgene. These transfected cells secreted native mouse IGFBP-1 and either polyhistidine-tagged human or rat IGFBP-1 into the conditioned medium. Human and rat IGFBP-1 were purified from the medium using a metal affinity resin to remove the native mouse IGFBP-1. Nondenaturing PAGE and Western ligand blotting analysis of rat IGFBP-1 revealed three phosphoisoforms (Fig. 3, *lane 1*). After incubation with alkaline phosphatase, the three bands were reduced to a single band, demonstrating that rat IGFBP-1 secreted by mouse hepatoma cells was phosphorylated, whereas the human IGFBP-1 secreted by those cells migrated as a single band that was identical to recombinant nonphosphorylated human IGFBP-1 (Fig. 3, *lane 3*). After alkaline phosphatase exposure, there was no change in the migration of this single band (*lane 4*). These results demonstrated that newly synthesized rat IGFBP-1 in Hepa 1–6 cells was phosphorylated, whereas the synthesized human IGFBP-1 was not.

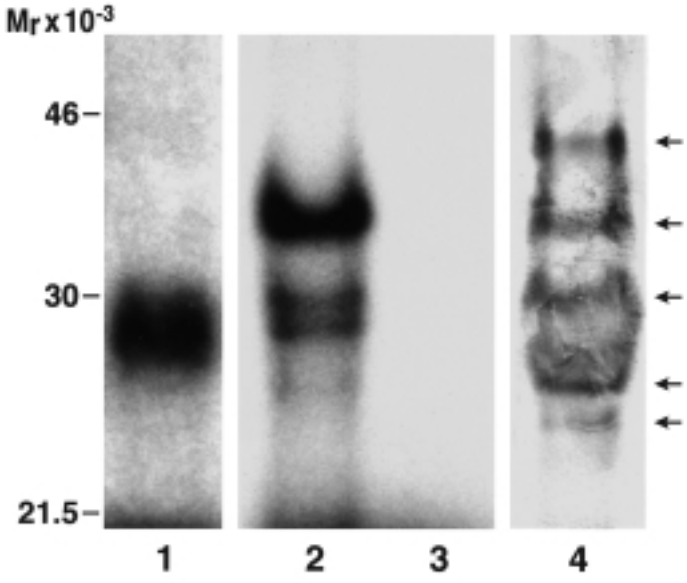

FIG. 2. Phosphorylation of mouse IGFBP-1 in conditioned media of Hepa 1–6 mouse hepatoma cells. Immunoprecipitates of IGFBP-1 from ^{32}P-labeled conditioned media of Hepa 1–6 cells were analyzed by SDS-PAGE (*lane 1*) and nondenaturing PAGE before (*lane 2*) and after (*lane 3*) incubation for 2 h at 37°C with 3 U calf intestinal alkaline phosphatase. The figure shows an autoradiograph of the dried gels. For comparison, conditioned medium from Hepa 1–6 cells was subjected to nondenaturing PAGE, and the phosphoisoforms were identified by Western immunoblotting (*lane 4*). The upper band in *lane 4* is nonphosphorylated, and the four lower bands represent phosphoisoforms. The antibody used for immunoblotting is a rabbit anti–human IGFBP-1 antibody (15) with high cross-reactivity for rat IGFBP-1 (21).

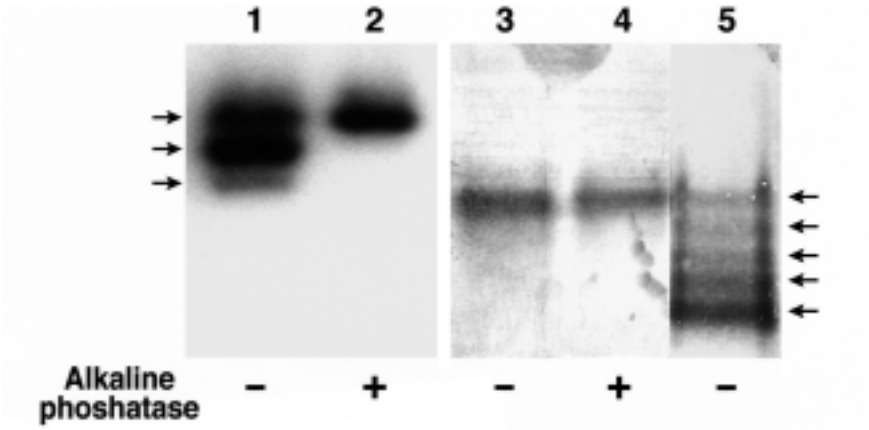

FIG. 3. Phosphorylation status of human and rat IGFBP-1 expressed after transfection into Hepa 1–6 cells. Rat and human IGFBP-1 cDNAs were transfected into Hepa 1–6 cells, as described in RESEARCH DESIGN AND METHODS. Conditioned medium from each transfectant was purified by metal affinity resin and subjected to nondenaturing PAGE. The proteins were transferred to PVDF membranes and visualized by Western ligand blotting using ^{125}I-labeled IGF-1, as described in RESEARCH DESIGN AND METHODS. *Lane 1*: Rat IGFBP-1 from conditioned medium of Hepa 1–6 cells transfected with rIGFBP-1; *lane 2*: the same sample as shown in *lane 1* after exposure to alkaline phosphatase; *lane 3*: human IGFBP-1 from conditioned medium of Hepa 1–6 cells transfected with human IGFBP-1; *lane 4*: the same samples as shown in *lane 3* after exposure to alkaline phosphatase; *lane 5*: conditioned medium of human Hep G2 cells. The arrows on the left denote the position of the nonphosphorylated (upper arrow) and phosphorylated rat IGFBP-1. The arrows on the right indicate the positions of nonphosphorylated (upper arrow) and phosphorylated human IGFBP-1.

In vitro protein kinase assay. To confirm that the failure of Hepa 1–6 cells to phosphorylate human IGFBP-1 was due to the inability of the mouse IGFBP-1 kinase to phosphorylate human IGFBP-1, in vitro protein kinase assays were carried out. Partially purified protein kinase activity from Hepa 1–6 cell extract was incubated with [γ-^{32}P]ATP in the absence and presence of either mouse, human, or rat IGFBP-1. The forms of IGFBP-1 were analyzed by SDS-PAGE in the presence of DTT, followed by autoradiography. When mouse, human, and rat IGFBP-1 were incubated with partially purified Hepa 1–6 cell protein kinase, mouse and rat IGFBP-1 were phosphorylated (Fig. 4, *lanes 1* and *3*). In contrast, this same kinase activity had almost no capacity to phosphorylate human IGFBP-1 (*lane 2*). These results confirmed that the kinase was fully active in phosphorylating rat IGFBP-1, but it phosphorylated human IGFBP-1 minimally.

Binding affinity of IGFBP-1 for IGF-1. To evaluate the physiological role of rat and nonphosphorylated human IGFBP-1, binding assays were performed, and the data were analyzed according to the method of Scatchard. Human IGFBP-1 (20 µg) was dephosphorylated by incubation with 50 U calf intestinal alkaline phosphatase for 18 h at 37°C, and the reaction products were repurified by HPLC (C-4 column). Rat IGFBP-1 was prepared from Hepa 1–6 transfected cultures, as described in RESEARCH DESIGN AND METHODS. The slope of these Scatchard plots showed that the affinity constant (K_a) of rat IGFBP-1 for IGF-1 (K_a 3.33 $\times$ 10^{-9} mol/l) was 3.9-fold higher than the affinity constant of dephosphorylated human IGFBP-1 (K_a 0.85 $\times$ 10^{-9} mol/l) (Fig. 5).

DISCUSSION

We have demonstrated that serum rat IGFBP-1 expressed in transgenic mice exists in multiple phosphoisoforms, whereas serum human IGFBP-1 from a different line of transgenic mice is not phosphorylated. There are marked phenotypic differences in these lines of mice, despite the fact that they express similar amounts of IGFBP-1 in serum (13,14,22). The rat IGFBP-1 transgenic mice exhibit intrauterine and postnatal growth retardation and glucose intolerance, whereas the human IGFBP-1 transgenic exhibit only modest postnatal growth retardation (except in brain) and normal glucose tolerance. The differences in the phosphorylation state of rat and human IGFBP-1 likely explain a part of the phenotypic differences in the two lines.

Because the liver is the major source of serum IGFBP-1 in vivo, we used mouse hepatocytes to determine if this difference in IGFBP-1 phosphorylation status was due to failure of mouse hepatic kinase activity to phosphorylate human IGFBP-1. Analysis of the forms of rat and human IGFBP-1 secreted by mouse hepatocytes after transfection showed that rat IGFBP-1 was phosphorylated, but human IGFBP-1 was secreted almost solely in the nonphosphorylated form. This effect is attributable to a failure of the hepatic kinase to phosphorylate human IGFBP-1 in vitro, whereas it could phosphorylate rat IGFBP-1.

The specific serines that are phosphorylated in rat IGFBP-1 are different from those that are phosphorylated in human IGFBP-1. Human IGFBP-1 is phosphorylated on serines 101,119, and 169. All of these phosphorylation sites have acidic amino acids in position +2 and/or +3 on the COOH-terminal side of each serine (5). The phosphorylation sites of rat IGFBP-1 have been shown to be Ser 107 and 132, and both are followed by acidic amino acids at the +2 and +3 positions (7). Figure 6 shows three phosphorylation sites of human IGFBP-1, the two phosphorylation sites of rat and mouse IGFBP-1, and the adjacent acidic amino acid residues in each form of IGFBP-1. The primary phosphorylation site in human IGFBP-1, Ser 101, aligns with Ser 114 in rat and mouse IGFBP-1. The rat and mouse Ser 114 residues are followed by the Pro 115–Glu 116 sequence. In human IGFBP-1, two serines that contain a Pro residue in the +1 position were definitively proven to be nonphosphorylated by radiosequencing (5). Therefore, it appears that this proline residue hinders kinase activity, possibly accounting for the lack of Ser 114 phosphorylation in rat IGFBP-1.

Another difference among the species of IGFBP-1 is that human phosphorylation site Ser 169 is not conserved in mouse or rat IGFBP-1. Although the Ser 119 site in human

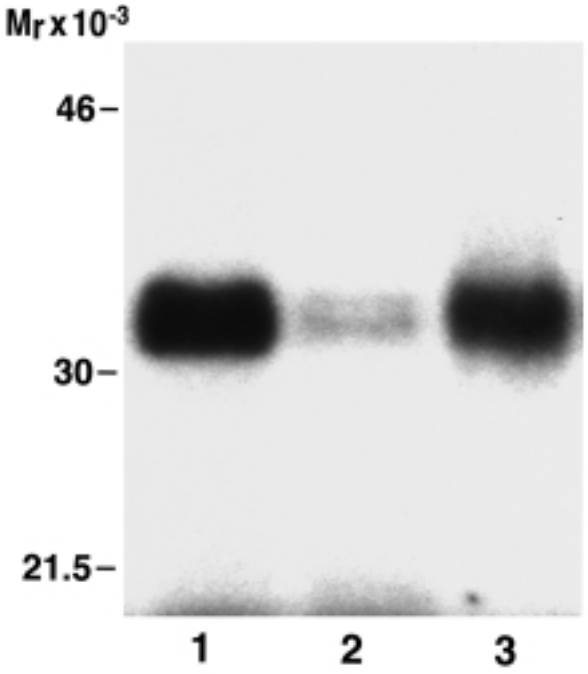

FIG. 4. Phosphorylation of mouse, human, and rat IGFBP-1 by the partially purified kinase from Hepa 1–6 cells. The partially purified protein kinase was incubated with mouse, human, or rat IGFBP-1 (50 ng) and [γ-^{32}P]ATP for 15 min at 30°C. The reaction was terminated after the incubation by the addition of Laemmli sample buffer and boiling for 10 min. The proteins were separated by 12.5% SDS-PAGE and visualized using autoradiography. *Lane 1*, mouse IGFBP-1; *lane 2*, human IGFBP-1; *lane 3*, rat IGFBP-1.

IGFBP-1 is conserved in mouse IGFBP-1 (Ser 132), it is a minor site of human IGFBP-1 phosphorylation (e.g., 5% of total phosphorylation). On the other hand, the phosphorylation site Ser 132 (SRED) in rat IGFBP-1 is conserved in the mouse IGFBP-1, and the rat phosphorylation site Ser 107 (SEDE) is similar to mouse site Ser 107 (SADE). In mice, both sites are flanked by a Glu residue in the +3 position. Neither site is present in human IGFBP-1. Therefore, it appears that the two major phosphorylation sites in human IGFBP-1 (accounting for >95% of the activity) cannot be phosphorylated by mouse protein kinase, whereas mouse and rat IGFBP-1 sequences contain two sites with similar flanking sequences (that are not present in human IGFBP-1), and it is probable that both are phosphorylated.

Because the phosphorylation of human IGFBP-1 increases its affinity for IGF-1 by sixfold, it has been assumed that this property accounts for the differences in human IGFBP-1 effects when it is added to in vitro test systems. Nonphosphorylated IGFBP-1 from human amniotic fluid purified by anion exchange chromatography was shown to potentiate the capacity of IGF-1 to stimulate DNA synthesis in porcine aortic smooth muscle cells and human fibroblasts, whereas the phosphorylated form of IGFBP-1 was inhibitory (4). Changes in phosphorylation status of human IGFBP-1 have also been shown to correlate with changes in endometrial growth. Frost and Tseng (3) demonstrated that cultured endometrial stromal cells secreted primarily dephosphorylated IGFBP-1 during their proliferative phase, whereas lutealized endometrial stromal cells that were treated with medroxyprogesterone acetate secreted predominantly phosphorylated forms. This indicates that the phosphorylation status of human IGFBP-1 correlates negatively with the proliferative response of endometrial stromal cells to IGF-1 (3). Jyung et al. (11) also demonstrated that nonphosphorylated IGFBP-1 potentiates the ability of IGF-1 to stimulate wound healing. Iwashita et al. (16) demonstrated that the proportion of nonphosphorylated IGFBP-1 in serum from appropriate-for-gestational-age fetuses was higher than that in serum from small-for-gestational-age fetuses at term, suggesting that the proportion of human IGFBP-1 phosphoisoforms is an important regulator of fetal growth. In contrast, Cox et al. (12) reported that exogenous administration of recombinant human IGFBP-1 (nonphosphorylated IGFBP-1) inhibited the growth-promoting effects of IGF-1 on hypophysectomized rats. Our studies are more consistent with those of D'Ercole et al. (13), in that the human IGFBP-1 present in transgenic mouse serum is nonphosphorylated and is not associated with significant somatic growth retardation. Those mice, however, did exhibit significant brain growth retardation, though not as marked as that in the rat IGFBP-1 transgenic mice (23,24). Taken together, the findings indicate that endogenous overexpression of nonphosphorylated human IGFBP-1 does not inhibit growth-promoting effects of IGF-1 as markedly as phosphorylated forms, such as the rat IGFBP-1 that caused somatic growth retardation (14). Although rat IGFBP-1 has a greater affinity for IGF-1 than to human IGFBP-1, we cannot exclude the possibility that rat IGFBP-1 has some other intrinsic property that accounts for its inhibitory activity, because the effect of overexpressing a nonphosphorylated rat IGFBP-1 in vivo has not been determined.

The phosphorylation status of IGFBP-1 has been shown to correlate with changes in insulin action. Highly phosphorylated forms of IGFBP-1 occur in conditions such as poorly controlled diabetes and severe trauma with insulin resistance,

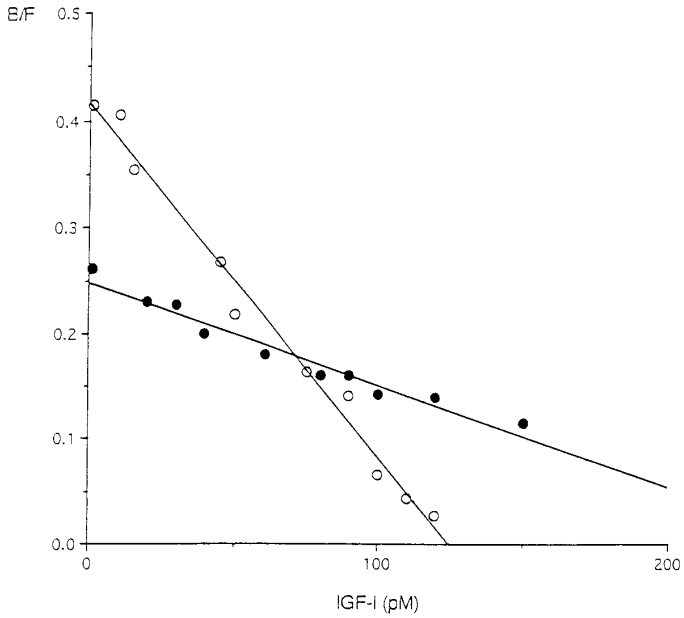

FIG. 5. Scatchard analysis of phosphorylated rat IGFBP-1 and nonphosphorylated human IGFBP-1. Rat IGFBP-1 (○) (purified from Hepa 1–6 transfected cell–conditioned medium) and dephosphorylated human IGFBP-1 (●) were used in the binding assays, as described in RESEARCH DESIGN AND METHODS. The mean affinity constants (K_a) calculated for four experiments were 3.33×10^{-9} mol/l (rat IGFBP-1) and 0.85×10^{-9} mol/l (dephosphorylated human IGFBP-1). B/F, bound/free.

											101			
Human	A	E	A	G	S	P	E	S	P	E	**S**	T	E	I
				107										
Rat	A	V	A	**S**	E	D	E	L	A	E	S	P*	E	M
				107							114			
Mouse	A	V	V	**S**	A	D	E	L	S	E	S	P*	E	M
				119										
Human	M	A	P	**S**	E	E	D							
				132										
Rat	M	A	P	**S**	R	E	D							
				132										
Mouse	M	A	P	**S**	R	E	D							
				169										
Human	Q	E	T	**S**	G	E	E							
Rat	Q	Q	K	A	G	D	E							
				182										
Mouse	Q	Q	K	A	G	D	E							

FIG. 6. A comparison of the sequences surrounding the phosphorylation sites of human, rat, and mouse IGFBP-1. The three phosphorylation sites of human IGFBP-1 (Ser 101, Ser 119, and Ser 169) and the two phosphorylation sites of rat and mouse IGFBP-1 (Ser 107 and Ser 132) are shown in bold. The adjacent acidic amino acid residues are underlined, and the prolines that may inhibit phosphorylation are indicated by an asterisk.

suggesting that phosphorylation may be associated with worsening metabolic control (9,25). The kinetics of the association of IGF-1 with its circulating binding proteins is important for understanding the effect of IGFBP-1 effects on glycemic control. Under normal physiological conditions, most plasma IGF-1 is bound to IGFBP-3 and nearly saturates its binding capacity. This IGF-1/IGFBP-3 complex forms a stable pool that shows minimal fluctuation. In contrast, IGFBP-1 and -2 are unsaturated (26). Furthermore, IGFBP-1 concentrations fluctuate sixfold throughout a typical day (15) because IGFBP-1 synthesis is suppressed by insulin (27,28) and is therefore dependent on caloric intake and insulin sensitivity. As a result, free (or unbound) IGF-1 decreases when circulating IGFBP-1 increases. Several lines of evidence indicate that these changes in free IGF-1 lead to increased blood glucose, as indicated by the findings that *1*) acute administration of IGFBP-1 to normal rats causes an increase of plasma glucose levels (29) and *2*) administration of IGF-1 to type 2 diabetics increases insulin sensitivity 3.4-fold (30). Therefore, in the rat IGFBP-1 transgenic mice, it is reasonable to assume that the high IGFBP-1 levels lead to major reductions in free IGF-1 in blood and, in turn, to glucose intolerance. However, because the human IGFBP-1 transgenic mice did not show glucose intolerance, despite similarly elevated blood IGFBP-1 levels, our findings suggest that nonphosphorylated human IGFBP-1 does not lower free IGF-1 concentrations sufficiently to reduce the effect of free IGF-1 on glucose incorporation and that the phosphorylation state of IGFBP-1 is at least as important as its plasma concentration in inducing glucose intolerance.

Recently, Crossey et al. (31) reported that a line of transgenic mice expressing human IGFBP-1 had growth retardation and glucose intolerance. The phenotype human IGFBP-1 transgenic mice showed less glucose intolerance and growth retardation than mice expressing rat IGFBP-1. There are several potential explanations for the phenotypic differences between their mice and our mice that expressed human IGFBP-1. Their mean total IGFBP-1 concentration was 174 ng/ml, which is 2.7-fold higher than our animals that expressed human IGFBP-1. In the mice of Crossey et al., a greater percentage of total human IGFBP-1 was present as lesser phosphorylated forms compared with our animals. For several reasons, however, it is difficult to determine the exact percentage of phosphorylated IGFBP-1 in their animals. The antibody that they used to measure nonphosphorylated IGFBP-1 also measures the lesser phosphorylated forms of IGFBP-1 (32). The degree to which their antibody recognizes phosphorylated mouse IGFBP-1 has not been determined. More importantly, their method of measurement is indirect in that the value that they determined for nonphosphorylated IGFBP-1 is derived by subtracting the values that are obtained with two separate antibodies. In contrast, we have determined the amounts of nonphosphorylated IGFBP-1 in our two groups of mice by direct measurements that yield a more precise determination. Based on our findings, we conclude from Crossley et al.'s study that if sufficient concentrations of non- or lesser phosphorylated forms of human IGFBP-1 are present, then they can inhibit IGF-1 actions; however, the exact concentrations necessary for IGF-1 inhibition have not been established.

Many secreted phosphoproteins exhibit different biological activity than their nonphosphorylated forms. Phosphorylation of osteopontin increases its binding activity for cell surface receptors (33), and phosphorylation of vitronectin enhances the adhesion of bovine aorta endothelial cells (34). Therefore, phosphorylation of IGFBP-1 may lead to changes in its biological actions that are independent of changes in its affinity for IGF-1. Human IGFBP-1 purified from amniotic fluid (predominantly nonphosphorylated IGFBP-1) forms disulfide-linked multimers (35) and associates with cell surfaces (11), presumably by interacting with the $\alpha5\beta1$ integrin on the cell surface (36,37). Similarly, we have determined that nonphosphorylated human IGFBP-1 polymerizes better than phosphorylated IGFBP-1 under certain test conditions (data not shown). The precise mechanisms by which the phosphorylation of IGFBP-1 controls these events have not been elucidated. Because IGFBP-1 has direct effects that are independent of its ability to bind IGF-1, it is possible that phosphorylation plays a role in regulating these events and that changes in its phosphorylation may alter growth or glucose metabolism directly. We conclude that phosphorylation is an important posttranslational modification of human IGFBP-1 that may influence both somatic growth and glucose homeostatic responsiveness to IGF-1 in vivo.

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