Identification of the Sites of Phosphorylation in Insulin-like Growth Factor Binding Protein-1

REGULATION OF ITS AFFINITY BY PHOSPHORYLATION OF SERINE 101

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Serine phosphorylation of insulin-like growth factor binding protein-1 (IGFBP-1) has been shown to alter its affinity for the insulin-like growth factors (IGF-I and IGF-II) and to modify its capacity to modulate cellular responses to the IGFs. Because of this, we determined the sites of serine phosphorylation. Purification of [32P]-labeled IGFBP-1 was followed by digestion with trypsin and endoproteinase Glu-C and radiodequencing of labeled peptides. Three serines were found to be phosphorylated, with Ser101, Ser110, and Ser119 containing 70%, 5%, and 25% of the incorporated [32P] respectively. A mutated IGFBP-1, substituting alanine for serine at positions 98 and 101, was expressed in CHO cells. On nonnadenaturing gels, the wild type protein migrated as five isoforms (one nonphosphorylated and four phosphorylated). However, in the mutated protein, the most rapidly migrating band (a phosphorylated form) was not present. The cells containing the mutated cDNA incorporated 60% less [32P] into immunoprecipitable IGFBP-1. The mutated protein had a 3-fold reduction in affinity for IGF-I compared to the wild type protein. We conclude that Ser101 represents the major site of phosphorylation containing 63% of the total [32P] incorporated and that phosphorylation of Ser110 is important for maintenance of high affinity binding for this growth factor.

The insulin-like growth factors, IGF-I and -II, are present in extracellular fluids bound to specific high affinity binding proteins (1, 2). Our laboratory has previously demonstrated that during purification of IGFBP-1 from human amniotic fluid two forms were identified following anion exchange chromatography. One form is capable of potentiating the effects of IGF-I on cell growth (3) while the other is inhibitory.

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‡ The abbreviations used are: IGFBP-1 and -II, insulin-like growth factors I and II; IGFBP, insulin-like growth factor binding protein; BSA, bovine serum albumin; CHO, Chinese hamster ovary; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresia; PCR, polymerase chain reaction; PVDF, polyvinylidene difluoride.

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Amersham Corp. The Bluescript plasmid was obtained from Stratagene. Trypsin treated with 1-(tosylamido-2-phenyl)ethyl chloromethyl ketone was from Worthington Biochemical. Calf intestinal alkaline phosphatase and endoproteinase Glu-C (from Staphylococcus aureus V8) were purchased from Boehringer Mannheim. DNA restriction enzymes were purchased from Promega. Other chemicals except those listed were purchased from Sigma. Sequelon discs were obtained from Milligen.

Tissue Culture—CHO cells were maintained in α-modified Eagle’s minimal essential medium containing 10% fetal bovine serum and 50 μg/ml gentamicin. An expression vector containing the cDNA encoding human IGFBP-1 was constructed as described previously (11) into a PUC18-derived plasmid (pNUT, a gift of Richard Palmiter, University of Washington) as previously described (5). Transcription of the cDNA was driven by a mouse metallothionine promoter utilizing polyadenylation signals and insertion sites derived from the human growth hormone gene. pNUT contains the gene for dihydrofolate reductase driven by an SV-40 promoter allowing selection of successfully transfected cells 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 by passaging in diazyl bovine serum and 50 μM methotrexate by increasing the concentration of methotrexate in the medium at regular intervals. The cell line designated CHOBP1-D6 secreting the greatest amount of human IGFBP-1 was used as a source of phosphorylated IGFBP-1 in all of the experiments. The cell line designated CHOBP1-SA 98101-1 contained the same expression vector with the serine to alanine mutations described below. After selection, both cell lines were stably maintained in α-modified Eagle’s minimal essential medium supplemented with 10% diazyl fetal bovine serum and 50 μM methotrexate.

Preparation of Radiolabeled IGFBP-1. The radiolabeled conditioned medium was prepared by labeling each of the two transfected cell lines, grown to confluency in 175-cm² tissue culture dishes. The cultures were incubated with 2 μCi/ml bovine trypsin in 0.13 M sodium phosphate buffer, pH 6.6, over 30 min (16). The fractions containing IGFBP-1 were concentrated by lyophilization and loaded onto an IGF affinity column. The remaining purification steps were as described for radiolabeled IGFBP-1. Purity was determined by SDS-PAGE with silver staining (17). IGFBP-1 secreted by HepG2 cells was purified as described previously (19). These fragments each extended over the reverse phase HPLC (C4) column as described previously (13).

Preparation of [Ala98-101]IGFBP-1 Expression Vector—Two mutations (Ser98 and Ser101 to alanine) were introduced into human IGFBP-1 by PCR as previously described (14). In addition, the material that was not retained by the C-8 column was reapplied to a HPLC, C-18 column. Six peaks were eluted using a linear acetonitrile gradient from 0 to 60% in 0.4% trifluoroacetic acid (TFA) in water, over 30 min (16). The fractions containing IGFBP-1 were concentrated by lyophilization and loaded onto an IGF affinity column. The remaining purification steps were as described for radiolabeled IGFBP-1. Purity was determined by SDS-PAGE with silver staining (17). IGFBP-1 secreted by HepG2 cells was purified as described previously (19). The protein content of the purified forms of IGFBP-1 was determined by radioimmunounassay of IGFBP-1 (10).

Preparation of [Ala98-101]IGFBP-1 Expression Vector—Two mutations (Ser98 and Ser101 to alanine) were introduced into human IGFBP-1 using the PCR strategy described by Higuchi (18). A 26-base DNA oligonucleotide (5′-GGAATTCC CAT CTG GTT TCA GTT TTG-3′, designated GW19), and a 26-base oligonucleotide (5′-GGAATTCC CTG GAG ATG TCA GCC ACG GAG  ATA AC-3′, designated GW21) and a 26-base oligonucleotide (5′-GGAATTCC CTG GAG ATG TCA GCC ACG GAG  ATA AC-3′, designated GW21) were designed to be complementary to the IGFBP-1 sequence at bases 112–135 in the 5′ to 3′ orientation and bases 909–929 in the 3′ to 5′ orientation, respectively, and each contained an additional 8-base pair sequence that contains an EcoRI restriction site (italicized). Two 29-base primers (5′-CCG GTA GGC CCC CCA GAG GAG GTA ATG GAG GAC GGC CTT GG-3′ and 5′-ACT GGG GCC TCT TGG GGC TTC AGG GCC TCT TGG GGG GG-3′) were designed to be complementary to the IGFBP-1 sequence at bases 481–509 in the 5′ to 3′ orientation and bases 506–478 in the 3′ to 5′ orientation, respectively, except that they contained mutations that change amino acids 98 and 101 from serine to alanine (mutations italicized).

By using GW19 with GW22 and GW20 with GW21 in separate PCR reactions with the wild type IGFBP-1 cDNA ligated into pBS as a template, we amplified 392- and 432-base pair fragments, respectively. To obtain those products, 5 μl of template was amplified by PCR in a 50 μl reaction containing 1× PCR buffer, 200 μM dNTPs, 2.5 units of Taq DNA polymerase, and 20 μM Tris-HCl, pH 8.8, 2 mM MgSO4, 0.1% Triton X-100, 0.1 mg/ml BSA, 0.8 mM concentration of each dNTP, 0.1 μM concentration of each primer. The reaction mixture was covered with mineral oil and heated for 5 min at 97 °C, and 1 unit of Vent DNA polymerase was added. Amplification was performed with a denaturing temperature of 97 °C. The remaining 21 cycles were done with a denaturing temperature of 95 °C. Annealing and extension temperatures were 55 °C and 72 °C, respectively, in all cycles.

The two DNA fragments that were prepared were analyzed and purified. These fragments were sequenced to verify that they were derived from the outside primers to the regions of the mutations, where they each contained the Alasubstitution and overlapped 26 bases. The products were then mixed together in equal amounts (100 ng each), and PCR was performed under reaction conditions as described above.
using primers GW19 and GW20. Twenty-one cycles were completed with denaturing, annealing, and extension temperatures of 97 °C, 50 °C, and 72 °C, respectively.

The final product extended the length of the IGFBP-1 cDNA (795 base pairs, including the entire protein coding region) and was cloned by EcoRI and gel-purified as described previously (19). The overhangs were then filled in with the Klenow fragment of DNA polymerase I to create blunt ends in a 4.0-µl reaction volume containing 50 mM NaCl, 10 mM Tris-Cl, pH 7.5, 10 mM MgCl₂, 1 mM dithiothreitol, 0.05 mM dATP, 0.05 mM dTTP, and 5 U units of Klenow fragment. After incubation for 10 min at 70 °C, the DNA was ligated directly into pNUT, which had been linearized with Smal and dephosphorylated (19). The blunt end ligation was performed at 16 °C with a vector-to-insert molar ratio of 1:4. Ligation, transfection of Escherichia coli (strain DH-5α), and analysis of clones was performed as described previously (19).

The enzymes used for determining orientation of the inserts were EcoRI/BamHI, and this was carried out as previously described (19). The clones containing the correctly oriented inserts were sequenced using the dye-terminator sequencing method (20). The final product contained the correct mutation while the rest of the sequence was identical with the sequence published for human IGFBP-1 (11). A clone containing the correct sequence in pNUT was transfected into CHO cells, and the IGFBP-1 expressing clones were selected with methotrexate as described above.

Anion Exchange Chromatography—To determine the degree of phosphorylation in wild type and mutated IGFBP-1, each of the transfected cell lines was incubated in phosphate-free Eagle's minimal essential medium containing 50 µCi of [32P]orthophosphate for 6 h. Duplicate wells were treated identically except that 32P was omitted and the amount of IGFBP-1 contained in the conditioned media was determined by radioimmunoprecipitation (10). These results were used to estimate the volume of 32P-labeled conditioned media that contained 100 ng of IGFBP-1, and this volume was transferred to siliconized tubes. The volume in each tube was adjusted to 1.0 ml with a buffer containing 30 mM NaF, pH 7.5, 50 mM sodium EDTA, 0.1% BSA, 0.01% Tween 20, and 0.1 µM of rabbit polyclonal anti-human IGFBP-1 antisera was added. After overnight incubation at 4 °C, 8 µl of ovine antiserum to rabbit IgG was added to each tube, and they were incubated for 1 h at 4 °C, followed by the addition of 2 µl of normal rabbit serum and incubation for a 1 h more. Antibody-bound IGFBP-1 was separated by centrifugation at 8000 × g for 20 min. The immunoprecipitated pellets were washed three times, dissolved in Laemmli sample buffer (containing 1% SDS and 0.1 M dithiothreitol), and boiled for 10 min. The immunoprecipitated proteins were resolved by SDS-PAGE, transferred to PVDF filters, and subjected to autoradiography (in the case of 32P-containing samples) or immunoblotting as previously described (5).

Anion Exchange Chromatography—IGFBP-1 isoforms were separated using anion exchange chromatography. Wild type or mutant IGFBP-1 (25 µg) were diluted in equilibrating buffer (20 mM (NH₄)₂CO₃, pH 8.2) and loaded onto the DEAE-anion exchange column. Protein was monitored at 214 nm. After the absorbance returned to baseline, isoforms of IGFBP-1 were eluted with buffer B (20 mM (NH₄)₂CO₃, 1 mM NaCl, pH 6.8) using a linear gradient of 12% B to 100% B over 12 min, followed by a linear gradient to 25% B over 8 min, and a final 10-min linear gradient to 100% B. IGFBP-1 eluted immediately in the linear gradient step, but this fraction was analyzed for various phosphorylated isoforms by nondenaturing gel electrophoresis.

Assay of IGFBP-1 Binding of IGF-I—To determine the binding affinities of the phosphorylated and nonphosphorylated IGFBP-1, 125I-IGF-I was incubated with wild type IGFBP-1 from CHO cells, IGFBP-1 purified from the HepG2 human hepatoma cell-conditioned medium, or [Ala98,x]IGFBP-1. In one experiment, 8 µg of wild type IGFBP-1 from CHO cells and 8 µg of [Ala98,x]IGFBP-1 were dephosphorylated by exposure to 14 units of alkaline phosphatase in 1.0 ml of buffer (0.05 M Tris-HCl, pH 8.5, 0.1 mM EDTA, 1 mM MgCl₂, 0.5 mM ZnCl₂) for 16 h at 37 °C, and the reaction products were purified by reverse phase HPLC (C-4 column). Ten ng of each of the purified forms of IGFBP-1 were added to 0.25 ml of binding assay buffer containing 0.1 M HEPES, 44 mM sodium phosphate, 0.01% Triton X-100, 0.1% BSA, pH 7.0, in the presence of unlabeled IGF-1 (0–100 ng/ml). The 125I-IGF-I that was bound was separated from unbound by precipitation in 12.5% polyethylene glycol (4). Nonspecific binding was determined in the presence of 1 µg/ml IGF-I and constituted less than 15% of the bound radiolabeled material.

RESULTS

To determine the site of serine phosphorylation, wild type CHO cells were incubated with [32P]orthophosphate, and the radiolabeled, secreted protein was purified to homogeneity. The pure product was reduced and alkylated then exposed to trypsin, and the released peptides were separated by HPLC. Three radiolabeled peaks (fractions 19, 32, and 34) were detected containing 2, 10, and 65% of the total radioactivity, respectively. Amino acid sequence analysis showed that fractions 32 and 34 had the same N-terminal sequence. Fraction 19 was completely sequenced and contained 2 serines at positions 169 and 174 (Table I). This experiment was repeated three times, and each time fraction 19 contained radioactivity, but it was consistently <2% of the total incorporated 32P. However, approximately 23% of the total radiolabeled protein did not adhere to the HPLC C-8 column. When this fall-through material was rechromatographed on a C-18 column, a single radiolabeled peak was detected. This peptide was sequenced and shown to contain the same N-terminal sequence as fraction 19 (Table I). Radioscaning of the fall-through peptide showed that 90% of the total radioactivity was released from the filter when Ser169 was cleaved from the peptide. This strongly supports that serine 169 is phosphorylated and is a major site of phosphorylation in IGFBP-1 (i.e., 25% of the total 32P incorporated). Because fraction 34, which contained >70% of the total radioactivity, contained 9 serines, endoproteinase Glu-C digestion was performed, and the endoproteinase Glu-C digestion products were further purified by HPLC. Four radiolabeled peaks were obtained (Fig. 1). All four were N-terminally sequenced and contained serine residues. Three were subjected to radioscanning. One peak (fraction 12) was shown to contain the Ser9-Pro-Glu-Ser89-Pro-Glu-Ser101-Thr-Glu sequence at positions 95–103, and radioscanning showed that the serines at positions 95 and 98 were not labeled. In contrast, the serine at position 101 was shown to have releasable radioactivity after manual Edman degradation. Fraction 11 also contained the Ser9 as its only serine and was intensely labeled. These two peptides containing Ser9 (Fig. 1, fractions 11 and 12) had >85% of the radioactivity in the tryptic fraction 94. This supports the conclusion that Ser9 is a major site of phosphorylation in IGFBP-1. Fraction 24 from the HPLC column of the endoproteinase Glu-C digest contained <8% of the radioactivity. It had only a single serine residue at position 101. This indicates that serine 119 is definitely labeled but represents a minor site of phosphorylation. Fraction 29, which was labeled, contained a mixture of two sequences, one of which contained serine 119. Fraction 25 did not contain radioactivity and contained serines 124, 131, and 136, suggesting that they were not labeled. Fraction 8 was minimally (<2%) labeled and contained Ser83 and Ser92. The phosphorylation state of serines 83 and 86 was further analyzed in a separate digestion.
The amino acid sequences for the three $^{32}$P-labeled tryptic peptides that eluted from the HPLC C-8 column and for the $^{32}$P-labeled peptide isolated from the fall-through material of this column are shown in the upper panel. The first 15 amino acids of peptide 19 and the first 24 amino acids of peptide 32 were determined by sequencing. The lower panel shows the amino acid sequences for eight peptides that were isolated from the digestion of the tryptic peptide in fraction 34 of the upper panel. Peptides containing significant amounts of $^{32}$P are marked with an asterisk. The serine residues that were determined to be phosphorylated are underlined.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Sequences</th>
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<tr>
<td>Fall through*</td>
<td>16A Q E T S G E I S K</td>
</tr>
<tr>
<td>19*</td>
<td>16A Q E T S G E I S K F Y L P N C N K</td>
</tr>
<tr>
<td>32*</td>
<td>17G Q G A C V Q E S D A S A P H A A E A G S P E S P E S T E</td>
</tr>
</tbody>
</table>

Endoproteinase Glu-C peptides

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>82S D A S A P H A A E</td>
</tr>
<tr>
<td>9</td>
<td>101S T E I T E E</td>
</tr>
<tr>
<td>11*</td>
<td>83G S P E S P E S T E I T E</td>
</tr>
<tr>
<td>12*</td>
<td>109S T E L L D N F H L M A P S E</td>
</tr>
<tr>
<td>22</td>
<td>107E L L D N F H L M A P S E</td>
</tr>
<tr>
<td>25</td>
<td>123H S I L W D A I S T Y D G S K</td>
</tr>
<tr>
<td>29*</td>
<td>109S T E L L D N F H L M A P S E D H S I L W D A I S T Y D G</td>
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**Fig. 1.** HPLC profile of the endoproteinase Glu-C digested peptides. The tryptic peptide that contained >70% of the total radioactivity was subjected to digestion with endoproteinase Glu-C, and the peptides that were generated were separated by HPLC as described under “Materials and Methods.” The fractions that were sequenced are numbered, and those containing $^{32}$P have an asterisk. The absorbance (A$_{214\text{ nm}}$) is shown as a solid line, and the acetonitrile gradient is plotted as a dashed line. No further peptides were eluted after the gradient reached 40% acetonitrile.

**Fig. 2.** Immunoprecipitation of $^{32}$P-labeled IGFBP-1 from transfected CHO cell conditioned media. CHO cells that had been transfected with wild type (lanes 1 and 3) or mutant (lanes 2 and 4) cDNAs were incubated with $[^{32}P]$orthophosphate as described under “Materials and Methods.” An equivalent amount (100 ng) of each radiolabeled protein was immunoprecipitated, and the immunoprecipitates were electrophoresed as described under “Materials and Methods” and then transferred to PVDF filters. The figure shows an autoradiograph of the $^{32}$P-labeled proteins (lanes 1 and 2) and an immunoblot of the identical filter (lanes 3 and 4). The arrow denotes the immunoprecipitated band corresponding to IGFBP-1. Multiple other immunoreactive unlabeled bands are present in lanes 3 and 4, representing rabbit immunoglobulin and other rabbit serum components in the immunoprecipitate that react with the goat anti-rabbit IgG conjugated to horseradish peroxidase that was used for detection.

**Table 1**

**IGFBP-1 peptide sequences**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Sequences</th>
</tr>
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<tbody>
<tr>
<td>19</td>
<td>16A Q E T S G E I S K</td>
</tr>
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</table>

**experiment.** Endoproteinase Glu-C digestion of the whole protein after radiolabeling followed by N-terminal sequencing showed that one fraction contained a peptide with serines at positions 83 and 86. Direct radiosequencing of this peptide showed no releasable radioactivity after Edman degradation. Additionally, the tryptic peptide fraction 34 was radiosequenced and showed no releasable $^{32}$P from the serines at positions 83 and 86. Continued release of amino acids by Edman degradation confirmed the release of radioactivity at the position corresponding to Ser$^{101}$. This residue was changed to alanine by site-directed mutagenesis of the IGFBP-1 expression vector. Because of an ambiguity in the initial phosphopeptide analysis experiments suggesting that Ser$^{101}$ was also phosphorylated (later disproven as described above), we also changed this serine to alanine during the mutagenesis procedure. CHO cells were transfected with this construct containing base substitutions to encode alanines at positions 98 and 101. These cells also secreted phosphorylated IGFBP-1 into their conditioned media, and immunoprecipitation of 100 ng of [Ala$^{98,101}$]IGFBP-1 demonstrated a $^{32}$P-labeled band of identical molecular weight (Fig. 2, lane 1). However, the $^{32}$P-labeled band immunoprecipitated from the media containing [Ala$^{98,101}$]IGFBP-1 was reduced in intensity 58% as determined by scanning densitometry, compared to the band immunoprecipitated from media of CHO cells transfected with the wild type cDNA. Immunoblotted following by scanning densitometry of the chemiluminescence-exposed film showed that 13% more total (labeled plus unlabeled) IGFBP-1 protein was present in the [Ala$^{98,101}$]IGFBP-1 lane. Therefore, there was a net decrease of 63% in $^{32}$P band intensity in the lane containing [Ala$^{98,101}$]IGFBP-1 compared to wild type IGFBP-1. These results strongly suggest that mutagenesis had successfully blocked phosphorylation and that serine 101 was a major site of phosphorylation in IGFBP-
1, since a 63% reduction in phosphate radiolabeling was detected in spite of the fact that similar amounts of protein were immunoprecipitated.

To further confirm that the cells containing the mutated cDNA secreted a form of IGFBP-1 whose phosphorylation state had been altered, the technique of nondenaturing gel electrophoresis was used. Conditioned medium from cells expressing the wild type protein and the [Ala98**O'']IGFBP-1 protein were electrophoresed using n-octyl glucoside instead of SDS to permit resolution of phosphorylated and nonphosphorylated IGFBP-1. As we have previously shown, IGFBP-1 from CHO cells secreting the wild type protein resolved into five bands (four phosphorylated and one nonphosphorylated) which could be visualized by immunoblotting (Fig. 3). The most rapidly migrating form, which is believed to be the most intensely phosphorylated, was easily detectable. However, when the media from the cells secreting [Ala98**O'']IGFBP-1 were analyzed, only four bands were detected. The most rapidly migrating 5th band was not visualized by immunoblotting (Fig. 3), and the intensity of the 4th band was significantly reduced. In contrast, the intensity of the upper nonphosphorylated band was increased. This suggests that the most heavily phosphorylated band migrates most rapidly through the gel and that it has been eliminated by mutagenesis. HepG2 cell-conditioned media contained an increased amount of the most rapidly migrating bands compared to the media from CHO cells, suggesting that in this cell type phosphorylation is more extensive.

The results were confirmed using anion exchange chromatography. Conditioned media from cells secreting the wild type and mutant proteins were subjected to anion exchange HPLC using a DEAE-anion exchange column as described under "Materials and Methods." The phosphorylated isoforms eluted in four peaks of activity with the first peak containing the dephosphorylated protein and lesser amounts of the three phosphorylated bands. The next three peaks contained a mixture of the three middle bands and lesser amounts of the remaining two bands. The last peak to elute contained the two most rapidly migrating bands. The fractions containing the [Ala98**O'']IGFBP-1 had a major reduction in the amount of protein in the last peak, indicating that loss of the Ser101 phosphorylation site resulted in an alteration in charge (data not shown).

To determine the effect of phosphorylation of Ser101 on the affinity of IGFBP-1 for IGF-I, the [Ala98**O'']IGFBP-1 was purified to homogeneity as described under "Materials and Methods." Homogeneity was proven by SDS-PAGE with silver staining. The capacity of increasing concentrations of IGF-I to compete for binding of 125I-IGF-I to [Ala98**O'']IGFBP-1 and to native IGFBP-1 was compared. Scatchard plots obtained using each protein were linear. The slopes of the two lines were significantly different, however. The association constant (K_a) of the wild type protein for IGF-I was 1.9 X 10^6 M^-1. In contrast, the K_a of the [Ala98**O''] form was 7.2 X 10^5 M^-1 or approximately 3-fold lower (Fig. 4). Furthermore, the affinity of IGFBP-1 purified from HepG2 conditioned media, which contains more of the most rapidly migrating isoforms, was 2.5-fold greater (K_a = 4.8 X 10^6 M^-1) than the wild type CHO protein. To further confirm that serine phosphorylation resulted in changes in affinity, the wild type IGFBP-1 and [Ala98**O'']IGFBP-1 purified from CHO media were each dephosphorylated with alkaline phosphatase, then repurified. IGF-I binding was assessed using both proteins. Scatchard analysis showed that dephosphorylation of wild type IGFBP-1 resulted in a 6-fold reduction in affinity, whereas dephos-
phorylation of [Ala₉₀⁻¹₀₁]IGFBP-1 lowered its affinity by only 2-fold (Fig. 5). After dephosphorylation, both the wild type and [Ala₉₀⁻¹₀₁]IGFBP-1 proteins had identical affinities for IGF-I ($K_c = 3.4 \times 10^8 \text{ M}^{-1}$), indicating that the changes in the affinity for IGF-I as a result were due to differences in phosphorylation and were not due to conformational changes as a result of the replacement of the serines with alanines. The fact that the alanine substitutions did not alter protein conformation was confirmed by the observation that after dephosphorylation both the wild type and the mutated proteins had identical mobilities during nondenaturing gel electrophoresis (inset, Fig. 5, lanes 2 and 4).

**DISCUSSION**

These studies demonstrate that IGFBP-1 is phosphorylated on serine residues at positions 101, 119, and 169. Phosphorylation of Ser¹⁰¹ accounts for greater than 60% of the $^{32}$P that is incorporated into the protein. Direct radiosequencing also showed that Ser¹⁰¹ was labeled and accounted for 25% of the $^{32}$P incorporation. Ser¹¹² was not directly radiosequenced due to the small amount (5%) of $^{32}$P incorporated, but complete sequencing of the labeled peptide in fraction 24 after endoproteinase Glu-C digestion showed that it was the only serine present. Further indirect evidence that Ser¹⁰¹ is a phosphorylation site is that it is followed by a sequence that is similar to those following serines 101 and 169 (i.e. Ser-X-Glu). Therefore, we conclude that Ser¹⁰¹ is phosphorylated, but it is a minor site of phosphorylation. Importantly, the serines at positions 83, 86, 96, and 98 were definitively proven to be nonphosphorylated by direct radiosequencing. Likewise, sequencing of several unlabeled peptides showed that several other serines were not labeled. We conclude that these 3 serines are the sites of IGFBP-1 phosphorylation and that serines 101 and 169 account for $>85\%$ of the total $^{32}$P that is incorporated.

Direct radiosequencing showed that the serine at position 101 was a major site of phosphorylation, and the extent of phosphorylation of this residue was confirmed by mutagenesis. Specifically, changing this residue to alanine to prevent its phosphorylation resulted in a 63% reduction in radiolabeling of the immunoprecipitated protein. Western blotting of the immunoprecipitated proteins showed that equivalent amounts of protein were loaded in each gel lane, and therefore the comparison of radiolabeled band intensity is valid. These data taken together confirm that serine 101 is phosphorylated and is a major site of phosphorylation in this protein.

Further definitive evidence that the [Ala₉₀⁻¹₀₁]IGFBP-1 contained an alteration in the degree of phosphorylation of IGFBP-1 was derived from two observations. First, nondenaturing gel electrophoresis showed a significant decrease in the two most rapidly migrating bands containing phosphorylated IGFBP-1 and eliminated the most rapidly migrating band. Reduction in the abundance of this most rapidly migrating band indicates that the rate of migration through the electric field is dependent upon the number of phosphate groups per IGFBP-1 molecule as well as site-specific changes in charge density. This observation supports the hypothesis that different bands detected using this technique are due to differences in the degree of IGFBP-1 phosphorylation and that removal of the most heavily phosphorylated site results in a mobility shift. The results do not definitively determine whether this shift is due to a change in number of serines that are phosphorylated or to the location of specific phosphoserine residues. Second, anion exchange chromatography confirmed these observations. Frost and Tseng (6) have previously shown that this technique will separate the most rapidly migrating bands from the slowest migrating bands. Separation of the isoforms of wild type IGFBP-1 showed that the most rapidly migrating forms eluted from the column at higher salt concentration. The amount of this latter peak of protein was reduced when [Ala₉₀⁻¹₀₁]IGFBP-1 was analyzed, further confirming that mutation had resulted in a reduction in the amount of this phosphorylated isoform.

The physiologic consequences of the mutation of Ser¹⁰¹ were significant. The [Ala₉₀⁻¹₀₁]IGFBP-1 mutant had a 3-fold reduction in affinity for IGF-I, suggesting that phosphorylation of this site is an important means of enhancing IGFBP affinity. The mutation of Ser¹⁰¹ to alanine had no demonstrable effect on the protein, since after dephosphorylation, both the wild type and the mutant proteins had identical affinities for IGF-I and identical mobilities under nondenaturing gel electrophoresis, indicating that the only effect of the two serine-to-alanine mutations was to prevent the phosphorylation of Ser¹⁰¹. Nondenaturing gel electrophoresis is very sensitive in detecting differences in charge or native conformation of proteins, and the identical mobilities of the wild type and mutant IGFBP-1 after dephosphorylation make it extremely unlikely that the amino acid alterations in the mutant protein had any direct effect on conformation. We confirmed our earlier observation that dephosphorylation of IGFBP-1 reduces affinity for IGF-I 6-fold (5). However, preventing phosphorylation of Ser¹⁰¹ by mutagenesis did not completely reduce the affinity of IGFBP-1 to that of the dephosphorylated protein (Fig. 5). This suggests that IGFBP-1 affinity for IGF-I is increased by phosphorylation not only of Ser¹⁰¹ but also of serines 119 and/or 169.

Nondenaturing gel electrophoresis of IGFBP-1 purified from various sources demonstrates a correlation between the relative abundance of the most rapidly migrating (and most heavily phosphorylated) IGFBP-1 band and relative affinity for IGF-I. When IGFBP-1 purified from the conditioned media of CHO cells is compared to that of HepG₂ cells, the HepG₂ protein contains much more of the most rapidly migrating isoform (Fig. 3). Likewise when the affinity of the HepG₂ IGFBP-1 is compared to the wild type CHO protein, it is consistently 2-3-fold greater (Fig. 4). Thus, the abundance of this most rapidly migrating isoform correlates well with changes in the net affinity of IGFBP-1 for IGF-I. Phosphorylation of Ser¹⁰¹ is necessary for this isoform to be present, since it is absent in the [Ala₉₀⁻¹₀₁]IGFBP-1 mutant (even though at least 2 other serines are phosphorylated), and this is consistent with our observation that the mutant protein has a lower affinity for IGF-I. Taken together with the finding that IGFBP-1 secreted by HepG₂ cells has the highest relative affinity, we conclude that both the degree of phosphorylation of Ser¹⁰¹ and the total number of phosphates per molecule contribute to an increase in IGFBP-1 affinity for IGF-I.

Our finding that the principal site of phosphorylation is contained in a Ser-X-Glu-X-X-Glu-Glu-Glu sequence and that two other sites contain the Ser-X-Glu sequence with other acidic residues located on their C-terminal side of the phosphorylation site suggests that a member of the casein kinase family could be the kinase responsible for phosphorylation of IGFBP-1. Phosphorylation by casein kinase II depends upon acidic residues on the C-terminal side of the phosphorylated serines, and this enzyme has been reported to phosphorylate protein phosphatase inhibitor-2 which contains a Ser-X-Glu sequence (21). Other known serine/threonine kinases do not recognize this motif and neither Ser¹⁰¹, Ser¹¹², nor Ser¹⁰⁹ represents consensus phosphorylation sites for other known protein kinases. We have confirmed the finding reported by Frost and Tseng (6) that casein kinase II...
can phosphorylate IGFBP-1 in vitro, but we have not proven that this is the kinase in CHO cells that phosphorylates IGFBP-1. An important objective of future studies will be to identify the intracellular kinase since it appears to be able to modulate the affinity of IGFBP-1 for IGF-I.

In previous studies, we have demonstrated that nonphosphorylated IGFBP-1 purified from amniotic fluid is a potent potentiator of IGF's effects on porcine aortic smooth muscle cells. Specifically, IGFBP-1 added under these conditions potentiated smooth muscle cell response 5.5-fold, and this response exceeded that of 10% human serum (3). In a recent in vivo assay, we demonstrated that nonphosphorylated IGFBP-1 potentiates wound healing in rats, leading to a 37% increase in hydroxyproline content reflecting increased collagen synthesis, and histologic analysis shows that the wounds that have received nonphosphorylated IGFBP-1 plus the IGF-I have increased cellularity (7). All of these responses are consistent with increased trophic action of this growth factor being potentiated by nonphosphorylated IGFBP-1. In contrast, direct addition of phosphorylated IGFBP-1 resulted in no increase in breaking strength or in hydroxyproline content (7). Therefore, phosphorylated IGFBP-1 not only appears to inhibit cell growth in culture, but also appears to inhibit the wound healing response to IGF-I in an in vivo model test system. Physiological physiological regulation of the kinase that phosphorylates IGFBP-1 in situations of diminished growth is further substantiated by our finding that when newborn pigs are starved for 48 h, IGFBP-1 becomes heavily phosphorylated (22). Therefore, it is possible that phosphorylated IGFBP-1 can act as a growth inhibitor and that metabolic conditions which are associated with growth inhibition result in increased phosphorylation of this protein. Likewise, our data obtained in pigs suggest that phosphorylation of IGFBP-1 may be an important means for inhibiting the insulin-like actions of IGF-I in response to fasting.

In summary, our findings show that Ser1131 is an important phosphorylation site in IGFBP-1. Phosphorylation at this residue markedly alters affinity of the binding protein for the growth factor. Future studies should enable us to determine the effect of substitutions at positions 119 and 169 on the functional activity of IGFBP-1, and these mutants may be useful in confirming the identity of the kinase that phosphorylates IGFBP-1.

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