The *in vivo* Phosphorylation and Glycosylation of Human Insulin-like Growth Factor-binding Protein-5*[

Mark E. Graham†‡§, Dean M. Kilby§¶, Sue M. Firth¶, Phillip J. Robinson‡, and Robert C. Baxter¶

Mass spectrometry is often used to determine post-translational modifications by analysis of tryptic digests of proteins. Here we demonstrate that the analysis of tryptic peptides together with analysis of the full-length protein provided optimal characterization of insulin-like growth factor-binding protein-5 (IGFBP-5) phosphorylation and glycosylation. IGFBP-5 binds insulin-like growth factors with high affinity and has important roles in cell survival, differentiation, and apoptosis. Until now, the primary structure of IGFBP-5 has been incompletely defined. We analyzed human IGFBP-5 from T47D cells by mass spectrometry to determine all of the *in vivo* post-translational modifications. In full-length IGFBP-5, 31% of the protein was unmodified, 37% was monophosphorylated, and 4% was diphosphorylated with no other modification. The remaining 27% was glycosylated, more than half of which was also monophosphorylated. The major phosphorylation site was Ser*[^266]* in the central domain, and a minor phosphorylation site was Ser*[^248]* near the C terminus. Neither site was phosphorylated *in vitro* by casein kinase 2, ruling it out as the *in vivo* kinase. An *in vivo* phosphorylation site was also found in IGFBP-2 at an analogous position, Ser[^106]. IGFBP-5 was heterogeneously O-glycosylated mainly by sialylated core 1 type glycans. The most abundant structure contained N-acetylhexosamine, hexose, and two N-acetylneuraminic acid carbohydrates. A small amount of sialylated core 2 type glycans was also present. Phosphorylation and O-glycosylation both affected IGFBP-5 binding to heparin but not insulin-like growth factor binding or ternary complex formation with the acid-labile subunit. The results reveal the first description of the *in vivo* phosphorylation of IGFBP-5 and its glycan composition. *Molecular & Cellular Proteomics* 6:1392–1405, 2007.

Insulin-like growth factor (IGF)*[^1]*-binding protein-5 (IGFBP-5) is one of a family of six structurally related proteins with high binding affinity for IGF-I and IGF-II (1). These proteins are all found in the extracellular environment. Functional roles inside the cell have also been described, including the translocation of IGFBP-3 and IGFBP-5 to the cell nucleus and their interaction with nuclear receptors of the retinoid receptor superfamily (2). All six IGFBPs are released into the bloodstream where they are believed to circulate either free or complexed to IGF-I or IGF-II. In the case of IGFBP-3 and IGFBP-5, binary complexes with IGFs can associate with a third protein, the acid-labile subunit (ALS), to form high molecular weight ternary complexes that act as relatively stable reservoirs of circulating IGF-I and IGF-II.

In the cellular environment, IGFBPs can modulate IGF access to the IGF-I receptor, either inhibiting or enhancing IGF-I receptor signaling, and can also act in ways that appear to be independent of their IGF binding activity. Paradoxically IGF-dependent actions include both the inhibition and the potentiation of IGF signaling, depending on the cellular context (2). For example, IGFBP-5 has been described as an inhibitor of the cell survival function of IGF in mammary gland (3) but as a potentiator of IGF action in stimulating osteoblast proliferation (4) in addition to its IGF-independent stimulatory action (5).

Post-translational modification of members of the IGFBP family is common. Several IGFBPs are secreted as phospho-proteins. In cell culture, IGFBP-3 shows constitutive phosphorylation of serine-containing motifs similar to consensus casein kinase 2 (CK2) phosphorylation sites. Serine phosphorylation of IGFBP-3 by CK2 *in vitro* affects its cell surface association and susceptibility to proteolysis but not its IGF binding affinity (6). In contrast, phosphorylation of IGFBP-3 by DNA-dependent protein kinase abolishes its IGF binding, increases its nuclear retention (7, 8), and has been reported to be essential for the apoptotic and growth-inhibitory effects of IGFBP-3 (8). The ability of IGFBP-1 to potentiate or inhibit IGF action appears to depend on its phosphorylation status. Highly phosphorylated IGFBP-1 has high binding affinity and is inhibitory to IGF action, whereas the hypophosphorylated protein has lower activity and potentiates IGF action (9). A

BP, IGF-binding protein; ALS, acid-labile subunit; Hex, hexose; HexNAc, N-acetylhexosamine; NeuAc, N-acetylneuraminic acid; CK, casein kinase; RIA, radioimmunoassay; DRB, 5,6-dichloro-1-β-o-ribofuranosylbenzimidazole; S/N, signal to noise.

[^1]: The abbreviations used are: IGF, insulin-like growth factor; IGF-
single preliminary report over a decade ago suggested that IGFBP-5 is secreted as a phosphoprotein, but nothing is known about the phosphorylation sites, the protein kinases involved, or the effect of phosphorylation on IGFBP-5 function.

IGFBP-3 and IGFBP-4 have established N-glycosylation sites. N-Linked carbohydrate in IGFBP-3 is slightly inhibitory to cell surface association but has no other known function (10). Although the structure of N-linked oligosaccharides on IGFBP-4 has been described, their function is unknown (11). O-Linked glycosylation has been found in IGFBP-5 (12) and IGFBP-6 (13) and also has been noted in an early description of IGFBP-1 (14). Like the N-linked carbohydrate in IGFBP-3 (15), the O-glycosylation of IGFBP-6 is inhibitory to cell surface binding (13).

Part of the tertiary structure of IGFBP-5 has been described. Kalus et al. (16) characterized the N-terminal domain by NMR and revealed two disulfide bond pairs at Cys47 to Cys50 and Cys54 to Cys80. Potentially IGFBP-5 has nine disulfide bonds using all of the highly conserved cysteines of the IGFBP family. Ständker et al. (17) determined by Edman sequencing that IGFBP-5 was O-glycosylated at Thr152; however, the structure of the glycans is unknown. The central domain that contains the O-glycosylation is also the predicted location of phosphorylation sites because other IGFBP family members are phosphorylated in this domain. This has not been investigated for IGFBP-5.

We analyzed IGFBP-5 by mass spectrometry to determine the in vivo post-translational modifications. Using 32P-labeling of T47D cells and tryptic digestion of IGFBP-5, we identified two phosphorylation sites. We also determined the identity of the glycans at Thr152. By analyzing the intact protein we obtained quantitative data on the amount of phosphorylation and glycosylation of IGFBP-5, thereby providing information on the amount of protein in different pools of modified IGFBP-5. We also investigated CK2 as a candidate for the protein kinase that might phosphorylate the observed sites and explored the functional relevance of these modifications.

**EXPERIMENTAL PROCEDURES**

**Metabolic 32P Labeling and Purification of IGFBP-5 from T47D Human Breast Carcinoma Cells**—The method of in vivo radio labeling of IGFBP-5 was similar to that used by Pattison et al. (17). T47D breast carcinoma cells were cultured to subconfluence in RPMI 1640 medium containing 20 mM HEPES, 10% FCS, 10 mg/liter bovine insulin, and 4 mg/ml glucose. Cells were then incubated overnight in a similar but serum-free medium containing 0.1% BSA before a 1-h phosphate washout period using phosphate-free Dulbecco’s modified Eagle’s medium also containing BSA, insulin, and glutamine. Cells were then incubated for 24 h in fresh phosphate-free medium, as described above, with [32P]orthophosphoric acid (100 μCi/ml) and the anti-proteolytics a2-macroglobulin (5 μg/ml), leupeptin (0.5 μg/ml), and aprotonin (Trasylo, Bayer Pharmaceuticals, 1:20 dilution). Radioactive media were collected, and EDTA was added to a final concentration of 0.5 mg/ml. Initial experiments were performed in 12-place multiwells alongside non-radioactive replicate plates, and media were collected at various time points for analysis by radioimmunoassay (RIA) and Western ligand blot after precipitation using IGFl-1-conjugated agarose. Kinase inhibitor studies were similarly performed, and resultant comparisons were made between control wells and those containing the CK2 inhibitor 5,6-dichloro-1-β-d-ribofuranosylbenzimidazole (DRB) (Calbiochem) at 0.01 and 0.1 μm. For peptide mapping experiments, larger scale preparations of [32P]-labeled IGFBP-5 were made in 420-cm² flasks, and IGFBPs were semipurified from clarified medium by affinity chromatography using IGFl-1-conjugated agarose. Bound IGFBPs were washed with 50 mM sodium phosphate, pH 6.5, and eluted using 1 μl acetic acid. Radioactive fractions were pooled, lyophilized, and stored at –80 °C until used. Non-radiolabeled T47D cell-derived IGFBP-5 was purified from conditioned serum-free RPMI 1640 medium as described above. IGFl-1 affinity chromatography followed by reverse-phase HPLC yielded pure, intact IGFBP-5 free from IGFBP-2 and IGFBP-4 contamination as determined by mass spectrometry. Recombinant human IGFBP-5 (AdrIGFBP-5) was expressed in 911 human retinoblastoma cells using an adenviral vector as described previously (18).

**Tryptic Digestion and HPLC**—Approximately 15 μg of purified 32P-labeled IGFBP-5 from T47D cells was prepared and purified by HPLC as described previously (19). The protein was dissolved in Laemmli sample buffer and heated to near boiling for 5 min. Reduced cysteines were alkylated by addition of acrylamide to a final concentration of 2% (v/v) for 45 min prior to SDS-PAGE. The gel was zinc-stained (20), and the IGFBP-5 band was excised and diced before being destained (20) and washed three times with 1 ml of water. The gel pieces were digested with 250 ng of trypsin in 75 μl of 50 mM ammonium bicarbonate for 16 h at 37 °C. A volume of acetonitrile equal to half of the total volume was added to the solution, and the tryptic peptides were extracted after vortexing for 15 min. A further extraction was obtained with 40 μl of 5% formic acid solution after 15 min of vortexing. The combined extract was dried down to ~4 μl. This solution was made up to 50 μl with 0.1% TFA and injected into a HPLC system (SMART system, GE Healthcare). The peptides were separated by reverse-phase chromatography using a 2.1 × 100-mm column (μRPC C2/C18, 3 μm, 120 Å, GE Healthcare) at 100 μl/min. The gradient was from 0% to 40% acetonitrile in 0.1% TFA in 25 min and then to 100% acetonitrile, 0.1% TFA in 10 min. Fractions (50 μl) were collected every 30 s, dried in a rotational vacuum concentrator (ALPHA-RVC IR, Martin Christ GmbH, Osterode, Germany), and dissolved in 5 μl of 10% acetonitrile solution. A 0.5-μl aliquot of each solution was spotted onto nitrocellulose and allowed to dry. The radioactive spots on the nitrocellulose were detected by a PhosphorImager (Storm 860, GE Healthcare) after exposure for 2 weeks.

**Alkaline Phosphatase Treatment and Mass Spectrometry**—A 0.5-μl aliquot of each radioactive fraction was mixed with a 2-μl solution containing 50 mM ammonium bicarbonate, 2 mM magnesium chloride, and 0.3 units/μl Antarctic phosphatase (New England Biolabs) and incubated at 22 °C for 3 h. The phosphate-treated sample was desalted using graphite or Poros R2 microcolumns as described previously (21). Graphite microcolumns were specifically used to trap hydrophilic peptides eluting in the first few fractions. The peptides were eluted from these columns with matrix solution (0.3% TFA, 60% acetonitrile aqueous solution containing 10 mg/ml α-cyano-4-hydroxycinnamic acid and 10 mM ammonium dihydrogen phosphate) directly onto the MALDI plate. The phosphate-treated sample was compared with an equal amount of untreated sample. The MALDI spots were analyzed using a Voyager DE-Pro MALDI mass spectrometer (Applied Biosystems, Boston, MA) in positive and negative linear modes. External calibration was used. Phosphopeptides in the remaining sample were sequenced by tandem mass spectrometry using a QSTAR XL quadrupole-TOF mass spectrometer (Applied Biosystems/MD Sciex) using the NanoSpray™ electrospray ion source (ESI-MSMS). Samples analyzed using the NanoSpray were concentrated using microcolumns and centrifuged into
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nanoelectrospray capillaries (Proxeon, Odense, Denmark) in 0.1% formic acid, 50% acetonitrile aqueous solution and were sprayed at 800–1,200 V. Argon was used as the collision gas. Tandem mass spectra of phosphopeptides were acquired in multiple channel acquisition mode for 5–15 min and were manually interpreted with the aid of Analyst 1.1 and BioAnalyst 1.1 (Applied Biosystems). Mass accuracy was between 20 and 60 ppm. No effort was made to distinguish between Glu/Lys and Leu/Ile. Phosphopeptides were distinguished from sulfated peptides (none found) by use of 32P-labeling and phosphatase treatment.

Intact IGFBP-5 protein, either AdIGFBP-5 or protein purified from T47D cell medium, was reconstituted in formic acid and diluted to 0.1% formic acid. It was then concentrated and desalted using a Poros R2 microcolumn to a final concentration of 20 μM and sprayed into the QSTAR XL mass spectrometer using a nanoelectrospray capillary in 0.5% formic acid, 50% acetonitrile aqueous solution. AdIGFBP-5 was dephosphorylated by incubation with 0.3 units/μL Antarctic phosphatase as above except that 10 mM magnesium chloride was used. Fresh phosphatase was added after 3 h, and the reaction was continued for another 3 h. The IGFBP-5 was purified from the Antarctic phosphatase by precipitation on IGF-I-agarose beads. A residual amount of bovine serum albumin from the precipitation was removed by loading it onto a Poros R3 microcolumn and eluting in steps of 10% acetonitrile. The purest IGFBP-5 fraction, which eluted at 30% acetonitrile, was concentrated to 20 μM and analyzed by ESI-MS. Mass spectra were deconvoluted using the Bayesian Protein Reconstruct tool of BioAnalyst 1.1. Mass spectra were first background subtracted using BioAnalyst 1.1 with a window width setting of z 4. A molecular mass spectrum in the range 25,000–35,000 Da was produced from the m/z range of 850–2,000 with a step mass of 0.5 Da, a signal-to-noise threshold of 1.5, and a minimum intensity of 0.01% for 20 iterations. We have reported the apex molecular mass. Each spectrum was acquired immediately after calibration with [Glu1]-fibrinopeptide B (Sigma-Aldrich). All molecular masses were reproducible to within ±1 Da under three repeated analyses.

Phosphorylation in Vitro with CK2—A reaction volume of 40 μl was used to phosphorylate purified AdIGFBP-5. The reaction contained 3 μg of dephosphorylated IGFBP-5, 10 mM magnesium sulfate, 20 mM Tris, pH 7.4, 25 units of CK2 (Sigma-Aldrich), and 40 μM ATP with 10 μCi of [γ-32P]ATP. This reaction was allowed to progress for 5 min. Another three lots of 3 μg of IGFBP-5 were phosphorylated with CK2 without 32P label for 30 min. These samples were also phosphorylated with extra magnesium (20 mM) and ATP (400 μM). After SDS-PAGE and tryptic digestion, prior to HPLC (see above), the three non-radioactive samples were added to the radioactive sample to boost the concentration of phosphopeptides, thereby improving the signal in the mass spectral analysis.

Binding Assays—Non-phosphorylated and non-glycosylated samples for ligand binding comparisons were prepared by enzymatic treatment of AdIGFBP-5. Twenty micrograms of AdIGFBP-5 were dephosphorylated using Antarctic phosphatase as described above. Deglycosylated IGFBP-5 was achieved by incubation of AdIGFBP-5 (20 μg) with neuraminidase (50 milliunits) and O-glycosidase (10 milliunits) (Calbiochem) together overnight at room temperature in 50 mM sodium phosphate, pH 7.5, including the protease inhibitors α2-macroglobulin (5 μg/ml), leupeptin (0.5 μg/ml), and aprotinin (Trasylo1, 1:20 dilution). Purification from the treatment enzymes was achieved using IGF-I affinity chromatography.

Heparin binding analysis was performed by manually loading IGFBP-5 (1 μg) onto a heparin sulfate Hi-Trap microcolumn (Amersham Biosciences) in 10 mM sodium phosphate, pH 7.5, 0.1% BSA. A linear 30-ml gradient (0–1 M NaCl in binding buffer) was then used to elute the bound IGFBP-5 at a flow rate of 0.5 ml/min. Fractions of 1 ml were collected from which 50 μl were assayed by RIA.

Binding of IGFBP-5 to IGF-I and IGF-II was performed by an adaptation of a method described previously (18). Briefly IGFBP-5 (0.025–5 ng) was incubated with [125I]-IGF-I or [125I]-IGF-II (20,000 cpm/100 μl) in a final volume of 250 μl of buffer containing 50 mM sodium phosphate, pH 6.5, 0.25% BSA for 2 h at 22 °C. Cold γ-globulin (0.25%, 250 μg/ml) was added, and binary complexes were precipitated using polyethylene glycol 6000 (25%, 500 μl) followed by centrifugation. Ternary complex formation assays were performed using (125I)- AL3 (10,000 cpm/100 μl) to which IGFBP-5 (0.01–5 ng) was allowed to bind in the presence of either IGF-I (10 ng) or IGF-II (10 ng) in a final volume of 300 μl of buffer containing 50 mM sodium phosphate, pH 6.5, 0.25% BSA for 2 h at 22 °C. In-house IGFBP-5 antiserum (SFK Y1, 5 μl) was added and incubated overnight at 4 °C, and complexes were precipitated using sheep anti-chicken γ-globulin (2 μl) followed by 1 ml of 60 g/liter polyethylene glycol 6000 in 0.15 M NaCl and centrifugation.

RESULTS

IGFBP-5 is Secreted as a Phosphoprotein—Metabolic labeling with 32P, indicated that IGFBP-5 is secreted as a phosphoprotein by T47D human breast carcinoma cells. Proteins were precipitated from conditioned medium on IGF-I-agarose beads. Autoradiography demonstrated a single diffuse band corresponding to IGFBP-5 (Fig. 1a); however, the possibility that other IGFBPs secreted by these cells, IGFBP-2 and IGFBP-4 as shown by ligand blot (Fig. 1c, inset), also carry a low...
level of labeled phosphate cannot be excluded. The relative production of IGFBP-5 and IGFBP-2 by these cells was ~2:1 as determined by RIA (Fig. 1b). In a larger scale purification, ~700 ml of culture medium, heavily conditioned by 5 × 10^8 T47D cells, contained 420 μg of IGFBP-5, determined by RIA, that after IG-I affinity chromatography and reverse-phase HPLC gave a yield of ~300 μg of pure IGFBP-5. Fig. 1c shows that after IG-I affinity chromatography, reverse-phase HPLC clearly separated IGFBP-5 from IGFBP-2 and IGFBP-4. Peaks at 27 and 36 min were immunologically confirmed as IGFBP-5 and IGFBP-2, respectively. The peak at 29 min was confirmed as IGFBP-4 by N-terminal sequencing (data not shown).

Phosphorylation Sites of IGFBP-5 from T47D Cells—Purified IGFBP-5 from 32P-labeled T47D cells was reduced, alkylated with acrylamide, and resolved by SDS-PAGE (Fig. 2a). Three micrograms of the sample was visualized by autoradiography (lane 1) and stained with Coomassie Blue, which also revealed a minor BSA band (lane 2). The remainder of the sample was zinc-stained (20), and the entire ~34-kDa band was excised and digested with trypsin. The trypptic digest was applied to a reverse-phase column, and peptides were collected in fractions. The radiation detected from each fraction was measured (Fig. 2b). Each radioactive fraction was subjected to Antarctic phosphatase treatment and compared with an untreated portion of the fraction using MALDI-TOF MS. The treated and untreated spectra were examined for signals that were a result of reductions in mass equal to multiples of 80 Da, demonstrating enzymatic dephosphorylation. The molecular mass of the signals identified as phosphopeptides by mass spectrometry were matched to theoretical molecular masses of IGFBP-5 tryptic peptides and are listed in Table I and indicated on Fig. 2b. Examples of the MALDI-TOF MS spectra are shown in Supplemental Fig. S1, a–h.

Of the eight sequence assignments in Table I, three are tentative at this point because peptide sequencing data are required to rule out contamination by an unknown protein, however improbable. Note that many of the detected phosphopeptides contained Met and Cys and so were detected as mono- and dioxidized phosphopeptides (+16 or +32 Da), increasing the evidence for their identification. Generally the more oxidized phosphopeptides eluted earlier (fractions 18–20; Fig. 2b) in the HPLC than the non-oxidized phosphopeptides (fractions 21 and 22), resulting in a small broad peak before a sharper peak. The relatively large amount of starting protein enabled the detection of low abundance, incompletely cleaved tryptic phosphopeptides using the more sensitive modes of MALDI-TOF MS detection for phosphopeptides, i.e. linear mode with positive or negative ion detection, rather than the less sensitive reflectron mode with positive ion detection (Table I). Phosphopeptides detected in these modes were at or near the limit of detection. These phosphopeptides should be regarded as either “difficult” to detect (e.g. large phosphopeptides) or so low in abundance that their physiological relevance is questionable.

Most of the phosphopeptides detected were from the same sequence of the IGFBP-5, 88EQVKIERDSREHEEPTTSEMAEETYSPKFIRPK120, with up to three missed trypsin cleavages (Fig. 2c). In assigning the 32P radioactive peaks, these phosphopeptides were found to contain the majority of radiolabel. Fractions 3 and 18–22 contained 88.5% of the total measured radiation (Fig. 2b). The remaining phosphopeptides from IGFBP-5 were from the C-terminal sequence 227YGKMLPGMEYDGFQCHTFDSSNV125, with up to three missed trypsin cleavage sites in Table I. Phosphopeptides detected in these modes were at or near the limit of detection. These phosphopeptides should be regarded as either “difficult” to detect (e.g. large phosphopeptides) or so low in abundance that their physiological relevance is questionable.
phosphopeptide matching IGFBP-2-(100–142). However, there is sufficient resolution of the two phosphopeptides, in fractions 29, 30, and 31, to conclude that phospho-IGFBP-5-(231–252) contributed between 4.5 and 7.1% of the total radiation.

The isolation and detection of an IGFBP-2 phosphopeptide was a serendipitous finding. Most of the IGFBP-2 was excluded in the purification of IGFBP-5 (Fig. 1c), and a significant signal (1%) for IGFBP-2 was undetectable in subsequent ESI-MS analysis of all the intact protein in our preparation (data not shown). Nevertheless a tryptic phosphopeptide was detected (Supplemental Fig. S1, g and h).

Due to the abundance of important fragment ions, we localized a phosphorylation site for IGFBP-2. The phosphorylated serine was detected between b6 and b7 as a dehydroalanine residue (resulting from neutral loss of H3PO4 from a phosphoserine residue) and again as the intact phosphoserine residue between y36 and y37 (Fig. 3). Therefore, Ser106 is an in vivo phosphorylation site for IGFBP-2.

Only a subset of the phosphopeptides shown in Table I was suitable for sequencing by tandem MS. The small, hydrophilic phosphopeptide detected in fraction 3 (Fig. 2b) at m/z 855.40 was sequenced by MALDI-TOF MSMS (data not shown). The sequence was confirmed as IGFBP-592IERDSR97 where Ser96 is phosphorylated. The location of the phosphoserine at Ser96 explains the strong MALDI-TOF MS signal we observed (data not shown) for phospho-IGFBP-592IERDSREHEEPTTSEMAEETYSKP115. It is likely that Ser96 phosphorylation contributed to the inhibited trypsin cleavage after Arg94 and/or Arg97. Sequencing of phospho-IGFBP-5-(92–115) revealed that Ser96 was also the phosphorylation site on this peptide (Fig. 4a). The phosphorylated Ser96 was detected by a transition from the b4 fragment ion to the b5 as a dehydroalanine residue. The C-terminal part of the sequence is mainly described by abundant y type ions that have no additional phosphate. However, there were some fragment ions, detected with low relative abundance, that show there was another phosphoamino acid residue near the C terminus of this phosphopeptide (data not shown). The fragment ions that attest to an alternative sequence were: y14, S/N 4 (which was phosphorylated) and b9, S/N = 3 and b10, S/N = 16.

![FIG. 3. IGFBP-2 was co-purified with IGFBP-5 from 32P-labeled T47D cells, and a phosphorylation site was found. Using tandem MS, the quadruply charged parent ion at m/z 1104.7 from fraction 29 was selected for fragmentation. The sequence describes IGFBP-2-(100–142) where Ser106 is phosphorylated. Met131 and Met133 were oxidized (shown underlined). The Asn113 predicted by the sequence (Swiss-Prot accession number P83628) was detected as Asp (or iso-Asp), indicating that deamidation had occurred. The parent ion has been truncated, and the m/z range from 950 to 1,650 has been multiplied by a factor of 7 to improve the clarity of the fragment ions in this region of the spectrum.](image-url)

### Table I

**IGFBP-5 sequences matching the molecular mass of phosphopeptides detected by MALDI-TOF mass spectrometry**

Phosphopeptides were detected as positive (+ve) and negative (−ve) charged ions using reflectron and linear TOF modes and were confirmed by Antarctic phosphatase treatment. p, phospho.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Fractions</th>
<th>Experimental m/z</th>
<th>Theoretical m/z</th>
</tr>
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<tbody>
<tr>
<td>IGFBP-5</td>
<td>p92–97</td>
<td>855.4a</td>
<td>855.4</td>
</tr>
<tr>
<td></td>
<td>p95–115</td>
<td>2,533.0a,b</td>
<td>2,530.0</td>
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<td></td>
<td>p95–115</td>
<td>2,614.8, diphospho0</td>
<td>2,614.5</td>
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<td></td>
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<tr>
<td></td>
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<tr>
<td></td>
<td>p231–252d</td>
<td>2,643.0a</td>
<td>2,642.8</td>
</tr>
<tr>
<td></td>
<td>p100–142</td>
<td>29 and 30</td>
<td>4,384.3a</td>
</tr>
</tbody>
</table>

* a These phosphopeptides were also sequenced by mass spectrometry.

b IGFBP-5-(95–115) was detected as a mono- and diphasphopeptide; IGFBP-5-(92–115) was detected as a mono-, di-, and triphosphopeptide.

c Only the oxidized form of this phosphopeptide was detected.

d The cysteine in this phosphopeptide is alkylated with acrylamide (see "Experimental Procedures").
(which were not phosphorylated). Collectively these data suggest that there was an alternative phosphorylation site within IGFBP-5-(103–113). The alternate sequence data was between 9- and 70-fold less intense than the dominant sequence. Therefore, any extra phosphorylation sites would have a very low stoichiometry. This matches the MALDI-TOF MS data in which low abundance di- and triphosphorylated signals were detected for IGFBP-5-(92–115) and -(95–115), and a low intensity monophosphorylated signal was detected for IGFBP-5-(98–115) (Table I). Ser96 was also phosphorylated when IGFBP-5-(95–115) was sequenced (data not shown). There was no evidence for an alternative phosphorylation site. Therefore, Ser96 is undoubtedly the major phosphorylation site in IGFBP-5, but there may be nearby phosphorylation sites of very low stoichiometry.

Phospho-IGFBP-5-(231–252) was sequenced, and it was found that Ser248 was phosphorylated (Fig. 4b). The phosphorylation site, detected as a dehydroalanine residue, spanned between b17 and b18. Therefore, the 32P labeling and MSMS sequencing showed that IGFBP-5 had a major phosphorylation site at Ser96, accounting for 88.5% of the incorporated phosphate, and a minor phosphorylation site at Ser248.

Glycosylation Sites of IGFBP-5—Signals for glycopeptides (showing neutral loss of carbohydrate) were detected in fractions 15 and 16 (Fig. 2b) when analyzed by MALDI-TOF MS. A base peak signal at m/z 1,255.6 was detected in the same spectra, matching the molecular mass of IGFBP-5-(145–156). This peptide was identified previously as containing an O-linked glycosylation site at Thr152 (12). However, in that study the constituents of the O-linked glycan were not determined.
The glycopeptide fractions were combined and further analyzed using tandem MS (ESI-MSMS). Several related glycopeptides were detected as shown in Fig. 5. Each glycopeptide was fragmented, and the carbohydrate constituents were determined. An example of a fragmentation product spectrum is shown in Fig. 5b. Because only molecular mass information was revealed, only the size of the carbohydrates, but not the structure of the glycan, could be determined. However, it was clear that the glycans were combinations of hexose (Hex), N-acetyllactosamines, (HexNAc), and N-acetylneuraminic acid (NeuAc, or sialic acid). Each glycopeptide contained some y and b type ions as well (Fig. 5b) that were a result of the fragmentation of the IGFBP-5-(145–156) sequence conjugated to the glycan. The non-modified signal was also fragmented confirming that the peptide was IGFBP-5-(145–156) or studied in any detail.

The glycopeptide fractions were combined and further analyzed using tandem MS (ESI-MSMS). Several related glycopeptides were detected as shown in Fig. 5a. Each glycopeptide was fragmented, and the carbohydrate constituents were determined. An example of a fragmentation product spectrum is shown in Fig. 5b. Because only molecular mass information was revealed, only the size of the carbohydrates, but not the structure of the glycan, could be determined. However, it was clear that the glycans were combinations of hexose (Hex), N-acetyllactosamines, (HexNAc), and N-acetylneuraminic acid (NeuAc, or sialic acid). Each glycopeptide contained some y and b type ions as well (Fig. 5b) that were a result of the fragmentation of the IGFBP-5-(145–156) sequence conjugated to the glycan. The non-modified signal was also fragmented confirming that the peptide was IGFBP-5-(145–156).
IGFBP-5 Phosphorylation and Glycosylation

Quantification of phosphorylated and glycosylated forms of IGFBP-5

<table>
<thead>
<tr>
<th>Molecular mass</th>
<th>ΔMassa</th>
<th>Modificationsb,c</th>
<th>Percentage of total protein</th>
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</thead>
<tbody>
<tr>
<td>Da</td>
<td>Da</td>
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<td></td>
</tr>
<tr>
<td>28,556, 28,571, and 28,586</td>
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<td>28,716 and 28,734</td>
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<td>29,001</td>
<td>444</td>
<td>HPO3 + HexNAc and Hex</td>
<td>1.5</td>
</tr>
<tr>
<td>29,047</td>
<td>491</td>
<td>HexNAc and NeuAc or HPO3 + (HexNAc)2</td>
<td>0.9</td>
</tr>
<tr>
<td>29,212 and 29,228</td>
<td>656 and 672</td>
<td>HexNAc, Hex, and NeuAc</td>
<td>2.8</td>
</tr>
<tr>
<td>29,294 and 29,309</td>
<td>738, 753, and 771</td>
<td>HPO3 + HexNAc, Hex, and NeuAc</td>
<td>3.7</td>
</tr>
<tr>
<td>29,505, 29,520, and 29,535</td>
<td>949, 964, and 979</td>
<td>HexNAc, Hex, and (NeuAc)2</td>
<td>6.3</td>
</tr>
<tr>
<td>29,583, 29,602, and 29,617</td>
<td>1,027, 1,046, and 1,061</td>
<td>HPO3 + HexNAc, Hex, and (NeuAc)3</td>
<td>9.7</td>
</tr>
</tbody>
</table>

a Masses are relative to the unmodified IGFBP-5, i.e., 28,556 Da.
b Identity of post-translational modifications were determined by phosphatase treatment (Fig. 5b) and by sequencing of modified peptides (Figs. 3 and 4).
c Modification by reduction of disulfide bonds or oxidation not listed.

(data not shown).

Structures based on common core types are shown for each glycopeptide in Fig. 5a. Structures with alternative branching are equally possible except for the rule that Thr152 is always O-linked to HexNAc. Also NeuAc is normally a terminating carbohydrate. The most abundant signal was detected as an intense triple charged ion as well as a double charged ion (Fig. 5a). Tandem MS of this abundant signal revealed a glycopeptide with HexNAc, Hex, and two NeuAc carbohydrates (Fig. 5b). An unconfirmed signal for this glycosylation was detected previously by MS (12). Mono- and trisialylated forms of this core 1 type structure were also detected. A glycan with only two HexNAc carbohydrates was relatively abundant, whereas the larger glycans built on the di-HexNAc structure (sialylated core 2 type) were in low abundance. Note that these comparisons of signal intensity are only qualitative. In conclusion, IGFBP-5 is heterogeneously modified. From the molecular masses of the modified intact IGFBP-5, and only four to five disulfide bonds were preserved after phosphatase treatment. The small signals at 15–18 and 30–36 Da larger than the five most prominent signals in the spectrum (Fig. 6a) were probably contributions from completely disulfide reduced and/or oxidized IGFBP-5. The phosphatase-treated sample was probably also affected by oxidation to a greater extent as shown by the presence of strong signals at 15 and 31 Da higher than the dominant signal (Fig. 6b).

To arrive at a measure of the amount of modified and unmodified protein, we continued with the assumption that the signal at 28,556 Da was intact and unmodified IGFBP-5 (except for disulfide bonds). Also we grouped oxidized/disulfide reduced signals with the non-oxidized/disulfide bonded signals. From the molecular masses of the modified intact IGFBP-5 and the peptide sequencing data (Figs. 4 and 5) we were able to quantify the degree of phosphorylation as well as the degree and type of glycosylation. Table II shows the quantification of phosphorylated and glycosylated forms of IGFBP-5 from Fig. 6a. Approximately 31% of the protein was unmodified, 37% was monophosphorylated without any other modification, and 4% was diphosphorylated. IGFBP-5 con-
IGFBP-5 Phosphorylation and Glycosylation

taining at least one phosphate represented 56% of the total protein. The full-length IGFBP-5 data supports the findings from $^{32}$P labeling (see above and Fig. 2b) that there was one major phosphorylation site and one minor phosphorylation site. Although there is some evidence of as yet unidentified phosphorylation sites in IGFBP-5-(98–115) (see above), the vast majority of the monophosphorylated IGFBP-5 is undoubtedly phosphorylated on Ser$^{248}$, and the second, low abundance site is mostly Ser$^{248}$.

Overall approximately one-quarter of the protein was glycosylated, and more than half of this pool was also monophosphorylated. The most abundant glycan, 16% of the total, was a combination of HexNAc, Hex, and two NeuAc carbohydrates. A monosialylated form of this structure was 6.5% of the total. The remainder of the glycans were generally in low abundance. These results are in agreement with the glycopeptide data for adenoviral vector-derived (data not shown) and T47D cells (Fig. 5a). Both analyses of peptides and full-length IGFBP-5 indicate that there is a single glycosylation site containing a mixture of mainly core 1 type glycans.

We also analyzed IGFBP-5 from T47D cells to determine whether endogenously expressed IGFBP-5 has a similar profile of post-translational modification. Fig. 6c shows the molecular mass spectrum of IGFBP-5 from T47D cells. This spectrum differs from the adenoviral vector-derived IGFBP-5 in that there are signals showing a loss of 113 Da in addition to the main signals of normal mass. A likely reason for this is the absence of Leu$^{1}$ from the mature IGFBP-5 chain (Fig. 2c).

Signals matching the molecular mass of 1LGSFVHCEPCDEK$^{13}$ and 2GSFVHCEPCDEK$^{13}$ were both detected by MALDI-TOF MS. This suggests some variability in the site of cleavage for the signal peptide from the IGFBP-5 precursor. It is notable that the IGFBP-5 with loss of 113 Da is not phosphorylated to the same extent as the full-length protein. Apart from the loss of 113 Da, the state of post-translational modification of the main signals is very similar to the AdIGFBP-5. In both signal strength and the extent of modification, there is generally similar phosphorylation and glycosylation. One clear exception is that the signal for monosialylated HexNAc + Hex (+656 Da) is slightly more intense in IGFBP-5 from T47D cells (Fig. 6c) than in AdIGFBP-5 (Fig. 6a).

Phosphorylation of IGFBP-5 in Vitro with Casein Kinase 2—Because the phosphorylation sites at Ser$^{246}$ and Ser$^{248}$ are generally located near acidic residues, we sought to determine whether IGFBP-5 was a substrate for phosphorylation by the acidophilic CK2. The metabolic incorporation of $[^{32}]$P phosphate into IGFBP-5 over 16 h in T47D breast cancer cells was significantly inhibited ($p = 0.0075$) by DRB, a CK2 inhibitor. At 0.1 mM, DRB reduced $[^{32}]$P phosphate incorporation by 50% when densitometry analysis was normalized to its own immunostained internal control (Fig. 7, a and b). In vitro assays with $^{32}$P labeling also showed that IGFBP-5 could act as a substrate for CK2 before or after dephosphorylation with Antarctic phosphatase (Fig. 7c). The decreased $^{32}$P incorporation observed in Fig. 7c, lane 4, may be due to some residual phosphatase activity as a result of incomplete Antarctic phosphatase inactivation. Autophosphorylation of the CK2 catalytic subunit (44 kDa) and regulatory subunit (26 kDa), as seen in Fig. 7c, lane 5, is distinct from IGFBP-5 (30–32 kDa). We therefore phosphorylated phosphatase-treated IGFBP-5 with CK2 in vitro with $[^{32}]$PATP, resolved the protein by SDS-PAGE, cut out and digested the band, separated the peptides by HPLC, and analyzed them by MALDI-TOF MS as above for the in vivo phosphorylation site.
analysis. The pattern of radioactive fractions collected from the HPLC was similar (Fig. 8a) with one main group and some low radiation fractions eluting late. However, an important exception was the lack of an early eluting fraction that should have contained phospho-IGFBP-5-(92–97).

Subsequent MALDI-TOF MS and phosphatase analysis...
showed that phosphopeptides similar to the in vivo phosphopeptides were detected, i.e. IGFBP-5-(92–115), -(95–115), -(98–115), and -(231–252) (Fig. 8a, c.f. Fig. 2b and Table I). The strongest signals were for IGFBP-5-(95–115) and -(98–115). This together with the lack of IGFBP-5-(92–97) suggested that Ser249 was no longer inhibiting cleavage after Arg94 and/or Arg97. Sequencing of phosphoIGFBP-5-(95–115) by ESI-MSMS showed that the in vitro phosphorylation was on Thr103 (Fig. 8b). The phosphothreonine residue was detected between fragment ions b16 and b19 or alternatively between y12 and y13. The C-terminal phosphopeptide IGFBP-5-(231–252) was detected with relatively low stoichiometry of incorporation of 32P label similar to the in vivo phosphopeptide. However, when sequenced, it was found that Ser249 was phosphorylated (Fig. 8c) rather than Ser248. The phosphoserine residue was detected between fragment ions b18 and b19 or alternatively between y12 and y13. Both Thr103 and Ser249 are in (S/T)X(E/D) consensus sequences. Nevertheless, the results indicate that although IGFBP-5 is a substrate in vitro for CK2 on Thr103 and Ser249, it is unlikely to be a substrate for this kinase in vivo.

Functional Evaluation of IGFBP-5 Phosphorylation and Glycosylation—To evaluate the functional consequences of IGFBP-5 post-translational modifications, the recombinant protein expressed using an adenoviral vector was dephosphorylated using Antarctic phosphatase, O-deglycosylated using a combination of O-deglycosidase and neuraminidase, and then repurified to remove the enzymes. Fig. 9a shows a Western immunoblot of the purified treated preparations. Dephosphorylation consistently resulted in more highly resolved IGFBP-5 variants after SDS-PAGE (Fig. 9a, lane 2), and deglycosylation produced a single IGFBP-5 band (Fig. 9a, lane 3). The Western ligand blot using 125I-IGF-I band (Fig. 9a, lane 3). The Western ligand blot using 125I-IGF-I demonstrated no resultant major loss of ligand binding activity, and enzymatic treatment had no effect on immunological recognition as determined by RIA (data not shown). Because IGFBP-5 is known to associate with a variety of cell surface and matrix proteins, the effect of treatments on the interaction of IGFBP-5 with heparin (a typical sulfated glycosaminoglycan) was first evaluated. Fig. 9b shows that both enzymatic treatments enhanced the association of IGFBP-5 with heparin; that is, deglycosylated and dephosphorylated IGFBP-5-(95–115) preparations were eluted from a heparin-agarose affinity column at a higher salt concentration than the untreated protein. Despite equivalent loading of the IGFBP-5 preparations onto the heparin column, the treated samples consistently displayed decreased recovery after elution, ~50% compared with untreated IGFBP-5. A second peak at elution fraction 30 was also observed with dephosphorylated IGFBP-5, suggesting a second structural subspecies.

Effects of post-translational modifications on ligand interactions with IGFBP-5 were evaluated by measuring the binding of IGF-I and IGF-II to the treated and untreated preparations as well as the formation of ternary complexes containing IGFBP-5, IGF-I or -II, and ALS. Fig. 9c and d, shows solution binding curves of the IGFBP-5 variants to IGF-I and IGF-II, respectively. Statistical analysis at half-maximal binding (0.5 ng of IGFBP-5) showed no significant difference between the preparations. The ability of dephosphorylated and deglycosylated IGFBP-5 to bind ALS is shown in Fig. 9, e and f. Treatment of IGFBP-5 with Antarctic phosphatase appeared to shift the ALS binding curve to the right in the presence of either IGF-I (Fig. 9e) or IGF-II (Fig. 9f); however, when tested at approximately half-maximal binding (1 ng of IGFBP-5), differences between the two treatment groups (dephosphorylated
and deglycosylated IGFBP-5) and untreated IGFBP-5 were not statistically significant.

DISCUSSION

The strategy used in this study has enabled the first complete structure of IGFBP-5 modifications to be determined. Vital to the strategy was the analysis of both tryptic peptides and the full-length protein, which provided intersecting and complementary data. We demonstrated that IGFBP-5 is secreted as a phosphoprotein and O-linked glycoprotein and that there are two main in vivo phosphorylation sites in human IGFBP-5 at Ser\(^{101}\) and Ser\(^{113}\). Unexpectedly we also demonstrated one in vivo phosphorylation site, Ser\(^{106}\), in IGFBP-2. IGFBP-5 glycosylation on Thr\(^{152}\) was confirmed, and the carbohydrate composition was shown to be a mixture of sialylated and unsialylated glycans.

We determined that the majority of secreted IGFBP-5 was phosphorylated (66%). Using \(^{32}\)P labeling it was evident that most of the phosphorylation was detected at Ser\(^{96}\), and it had the highest turnover of phosphate. Also diphosphorylation was uncommon (4%).

Tryptic peptides provided information on the type of glycans at a specific site. Analysis of the full-length protein confirmed the distribution of the types of glycans and also ruled out any significant amount of alternative post-translational modifications (e.g. acetylation or myristoylation). However, we clearly observed a variability in the site of cleavage of the signal peptide with IGFBP-5 from T47D cells with Leu\(^{1}\) (Leu\(^{21}\) of the IGFBP-5 precursor) being cleaved more often than not. Apart from this variability, there was generally little difference between adenoviral vector-derived IGFBP-5 and IGFBP-5 purified from T47D cells. The similar result for adenoviral vector-derived IGFBP-5 corroborates that we have correctly determined the in vivo phosphorylation and glycosylation state of IGFBP-5.

It should be noted that comparisons of mass spectral signal intensity are only valid when the molecules are alike. Small phosphopeptides are detected with different efficiency compared with the same peptides that lack a phosphate group (22). However, in the case of large phosphopeptides (>8,000 Da), the phosphate group makes no detectable difference (23). This is in agreement with observations that protein phosphorylation often has a much smaller than predicted effect on the pl of the protein (24) and thus its ability to be protonated (or the effect is smaller than the error in the measurement). No comparable studies have been made on the effect of glycosylation on detection efficiency, and therefore it must be noted that the mass spectral analysis of the intact IGFBP-5 may not be strictly quantitative. However, the amount of phosphorylation and glycosylation determined by ESI-MS was clearly in agreement with observed gel staining and \(^{32}\)P distribution (c.f. Fig. 6 and Table II with Fig. 2, a and b).

The major phosphorylation site at Ser\(^{96}\) of IGFBP-5 is at the beginning of the non-conserved central domain. Ser\(^{96}\) on IGFBP-5 shares the same proximity to the N-terminal domain as Ser\(^{101}\) on IGFBP-1 (25), Ser\(^{111}\) and Ser\(^{113}\) on IGFBP-3 (26), and Ser\(^{106}\) on IGFBP-2 (determined in this study). This proximity indicates a possible role in regulating the folding between the N-terminal domain and the central domain or even regulating IGF binding affinity as shown for IGFBP-1 (25). However, this has been shown not to be the case for IGF-I binding to IGFBP-3 (26), and we similarly saw no effect of IGFBP-5 dephosphorylation on IGF-I or IGF-II binding. Alternatively the phosphorylation may regulate binding to the short heparin-binding motif at residues 133–136 (Fig. 2c), although the glycosylation at Thr\(^{152}\) is much nearer to this motif. We found that both deglycosylation and dephosphorylation appeared to enhance the interaction between IGFBP-5 and heparin.

The minor phosphorylation site at Ser\(^{248}\) is five residues from the C terminus (Fig. 2c). It is therefore in a flexible position to simultaneously interact with any protein folds or binding partners near the C terminus. IGFBP-5 has many potential binding partners outside and within the cell, including fibronectin (27), retinoid X receptor-α (28), Ras-association domain family 1 protein (29), four and a half LIM protein 2 (30), and others (2). A flexible C-terminal domain that can be phosphorylated may provide a regulatable docking site for these or other proteins. A C-terminal crystal structure of IGFBP-1 (41) is available for comparison with IGFBP-5, but the final residues after the last conserved cysteine are not clearly defined. In addition to the ligand binding comparisons performed in this study, mutagenesis and other phosphosite-specific studies will be required to advance the understanding of the roles of the two phosphorylation sites determined in this work.

Because both IGFBP-1 and IGFBP-3 are phosphorylated at central domain residues close to the cysteine-rich N-terminal domain, and the kinases involved were considered likely to be CK1, CK2, or a related enzyme (26, 31), we also considered whether CK2 might phosphorylate IGFBP-5. Ser\(^{96}\) was predicted as a potential CK2 phosphorylation site by the kinase specificity prediction program NetPhosK 1.0, and we observed that DRB, a specific inhibitor of CK2, reduced \(^{32}\)P incorporation into IGFBP-5 in cellular metabolic labeling experiments. Despite this, CK2 phosphorylated IGFBP-5 at Thr\(^{103}\) and Ser\(^{249}\) but failed to phosphorylate at Ser\(^{96}\) and Ser\(^{248}\) and is therefore not likely to be an in vivo protein kinase for IGFBP-5. However, there was some evidence that there may be more in vivo phosphorylation sites of very low abundance within the region 103–115. It remains to be determined whether these very low stoichiometry sites can have a significant effect on IGFBP-5 function. Ser\(^{96}\) and Ser\(^{248}\) share a slightly acidic context, DSXXX, and may be phosphorylated by the same in vivo kinase, which also remains to be determined.

Variable glycosylation of IGFBP-5 may differentially affect its susceptibility to different proteases. IGFBP-5 has been...
IGFBP-5 Phosphorylation and Glycosylation

reported to be cleaved C-terminally to residues 138 (32), 143 (33), 188 (12), and 169 (34, 35). This implies that, in various cell types, either N-terminal or C-terminal IGFBP-5 fragments could contain O-glycosylation at Thr$^{152}$. The detection of both core 1 and core 2 type glycans at Thr$^{152}$ indicates that there may be different pools of IGFBP-5 that have different functional glycosylation. This could occur if IGFBP-5 was exposed to different amounts of specific glycosyltransferases. For example, in normal breast, mucin O-glycans are largely core 2 type structures, whereas in breast carcinomas they are often smaller core 1 type structures (36). Differential glycosylation may affect cell association as reported for IGFBP-3 (15) and IGFBP-6 (13).

Variants of IGFBP-5 lacking either covalently linked phosphates or carbohydrates were prepared by treatment with Antarctic phosphatase or neuraminidase and O-glycosidase, respectively. Consistent with original data published by Conover and Kiefer (37), deglycosylation of adenoviral vector-derived IGFBP-5 resulted in a single band resolved after SDS-PAGE. Dephosphorylation of IGFBP-5 was determined to be complete by ESI-MS and produced more tightly resolved isoforms after SDS-PAGE and immunoblotting. Enzymatic treatments had no effect on immunorecognition and therefore allowed accurate quantitation of purified preparations by RIA. The ability of IGFBP-5 to interact with glycosaminoglycans, including heparin sulfate, has been documented previously (38). The enhanced retention of both deglycosylated and dephosphorylated IGFBP-5 preparations by heparin-agarose is consistent with an inhibitory effect of the negative charge which to further investigate the biological consequences of phosphorylation and glycosylation.

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