Journal of Cellular Biochemistry 56:262–273 (1994)

Identification of the Major Sites of Phosphorylation in IGF Binding Protein-3

Wolfgang G. Hoeck and Venkat R. Mukku

Department of BioAnalytical Technology, Genentech, Inc., South San Francisco, California 94080

Abstract Insulin-like growth factor binding protein-3 (IGFBP-3) is the major carrier of insulin-like growth factor I and II in the circulation. IGFBP-3 is secreted by various tissues and cell lines as a glycosylated phosphoprotein. We have identified two major serine phosphorylation sites located at amino acids 111 and 113 of the human protein. These serine residues and neighboring amino acids potentially involved in defining a protein kinase recognition sequence were mutated to alanine using PCR. Single and double point mutants were stably transfected into CHO-cells and analyzed for their level of phosphorylation. Mutation of both serines reduced phosphorylation by >80% in the full-length protein and completely abolished phosphorylation in a 17 kDa IGFBP-3 fragment, derived from digestion with EndoProteinase Lys-C. The 17 kDa fragment contained serines 111 and 113. S111A/S113A, a double serine-to-alanine mutant at positions 111 and 113, showed a strongly reduced glycosylation pattern that appears to be the result of amino acid substitutions rather than lack of phosphorylation. Mutant S111A/S113A, despite being non-phosphorylated and non-glycosylated, is functionally similar to the wild-type IGFBP-3 in terms of IGF-I binding. These results enhance our understanding on the functional role of glycosylation and phosphorylation of IGFBP-3.

Key words: IGF, IGFBP-3, mutagenesis, phosphorylation, growth factor

Insulin-like growth factors I and II (IGF-I/II) are polypeptide growth factors that interact with cell surface-bound receptor proteins to stimulate cell proliferation. Their mitogenic actions are important during embryonic and postnatal development and some fall under growth hormone regulation during postnatal growth [Baker et al., 1993]. In bodily fluids and in conditioned medium of cell lines IGF-I and -II are complexed with binding proteins, referred to as insulin-like growth factor binding proteins or IGFBPs [Guidice, 1992; Rosenfeld et al., 1990, and references therein]. Six IGFBPs have thus far been identified and their cDNAs have been cloned. IGFs and IGFBPs are synthesized and secreted by a variety of cell types in vivo and in vitro [Delhanty et al., 1993]. They are reported to act both locally (autocrine or paracrine) as well as remotely (endocrine) by modulating the mitogenic effects of IGFs [Guidice, 1992]. IGFBP-3 is the most abundant binding protein found in the circulation and represents the major regulator of IGF-actions [Wood et al., 1988]. Its cDNA sequence has been determined from human, pig, rat, and bovine sources [Albiston and Herington, 1990; Sommer et al., 1991; Wood et al., 1988]. IGFBP-3 can be found in the circulation primarily in the form of a ternary complex consisting of IGF-I/II, IGFBP-3, and an acid labile subunit (ALS) [Leong et al., 1992]. IGF-I/II must first form a binary complex with IGFBP-3 before the third component, ALS, can be bound [Baxter and Martin, 1989]. Complex formation between the growth factor, IGFBP-3 and ALS increases the half-life of all its components, thereby regulating the availability of IGFs to act on target tissues. The molecular nature of the interaction between the three protein components is currently unknown. Besides the described endocrine role, IGFBP-3 is implicated in modulation of IGF-dependent mitogenic effects in a paracrine/autocrine fashion [Blum et al., 1989; Conover, 1992; Elgin et al., 1987; Liu et al., 1992; Tesch et al., 1993].

Protein phosphorylation has proven to be a rapid and efficient means of controlling the interaction and function of proteins across all species. Recent highlights are control of DNA-binding and transactivation functions of transcription factors [Hunter and Karin, 1992; Jackson, 1992] and the phosphorylation-depen-

Received January 19, 1994; accepted March 30, 1994.

Address reprint requests to Dr. Venkat R. Mukku, Department of BioAnalytical Technology, Genentech, Inc., 460 Pt. San Bruno Boulevard, South San Francisco, CA 94080.

dent protein stability of recombination activator protein RAG-2 [Lin and Desiderio, 1993]. Even proteins responsible for adding or removing phosphate from amino acid residues on proteins, protein kinases and phosphatases, are controlled by phosphorylation [Crews and Erikson, 1993]. IGF binding proteins appear to be no exception. IGFBP-1 has been described as a phosphoprotein [Frost and Tseng, 1991; Jones et al., 1991]. Phosphorylation occurs on multiple serine residues in vivo [Jones et al., 1993] and modulates its biological effects by increasing the affinity to IGF-I by 6-fold. In vitro, Casein kinase II and cAMP-dependent protein kinase are able to phosphorylate IGFBP-1 [Frost and Tseng, 1991]. Recently we have described phosphorylation of IGFBP-3 on serine residues of endogenously or recombinantly expressed protein [Mukku et al., 1994]. In these studies in vitro dephosphorylation on purified recombinant IGFBP-3 suggested that phosphorylation might be involved in modulating protein-protein interactions and therefore biological functions of IGFBP-3.

Here we describe the methods used to identify potential phosphorylation sites on human IGFBP-3. Single and double serine-to-alanine mutants were generated by PCR and stably transfected into CHO-cells. Mutant proteins showed a > 80% reduced incorporation of ^{32}P-orthophosphate. In preliminary experiments the mutants were found to be functionally indistinguishable from the wild-type protein with respect to their binding to IGF-I.

MATERIALS AND METHODS

Cell Lines

Chinese Hamster Ovary (CHO-DUKX) cells stably transfected with the proinsulin gene (CHO DP-12) were grown in a 1:1 mixture of F12/DMEM supplemented with 5% Fetal Bovine Serum. CHO DP-12 cells were used for all mutational studies. Stable transfections of CHO DP-12 cells with wild-type or mutated IGFBP-3 cDNA plasmid and DHFR-selection marker [Wurm et al., 1992] were performed using the calcium-phosphate precipitation technique essentially as described previously [Wurm, 1994]. Transfectants were selected in a 1:1 mixture of F12/DMEM supplemented with 5% Fetal Bovine Serum and 1 × GHT (glycine, hypoxanthine, and thymidine).

^{32}P-Cell Labeling and Immunoprecipitation

A stable cell clone transfected as above with wild-type IGF BP-3 plasmid (BP3-32) was used as a source for the preparation of recombinant BP-3. Cells were grown in 75 cm^2 tissue culture flasks to a density of 1×10^7/ml. For radioactive labeling cells were concentrated by centrifugation and incubated in 1 ml phosphate-free medium plus 2 mCi ^{32}P-orthophosphate inside a microfuge tube. After incubation for 4 h at 37°C, cells were pelleted and supernatant was transfered to a fresh microfuge tube containing 1 μl preimmune serum and 40 μl Protein A-Sepharose (PAS). One hour later, PAS was pelleted and the supernatant was transferred to a fresh microfuge tube containing 1 μl of a rabbit polyclonal antibody directed against IGFBP-3 and 40 μl of PAS; 1.5 h later antigen-antibody complexes were pelleted and washed five times with 1 ml RIPA-buffer (50 mM Tris-HCl, pH 7.8, 1% Triton X-100, 0.5% Na-deoxycholate, 0.1% SDS, 250 mM NaCl, and 5 mM EDTA). Antigen was released by adding 25 μl TE-buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), 2 μl 20% SDS, and 1 μl β-mercaptoethanol and 5 min boiling. PAS was then pelleted and the supernatant transferred to a fresh microfuge tube containing 12.5 μl TE-buffer and 12.5 μl 7.5% Triton X-100.

Deglycosylation and Proteolysis

The released antigen antibody mixture was treated with 1 μl N-Glycanase (GenZyme, Cambridge) overnight at 37°C. Then 10 μl of freshly prepared Endoproteinase Lys-C (sequencing grade, Boehringer Mannheim, IN) was added for further 4 h incubation.

Non-Radioactive Western Blot Analysis

IGFBP-3 was detected using a rabbit polyclonal antibody MU72A (Mukku V., unpublished) directed against recombinant IGFBP-3 expressed in and purified from A-293 cells [Mukku et al., 1989]. Antibody-antigen sandwich was further detected using an horseradish peroxidase conjugated goat anti-rabbit antibody (Tago Immunologicals, Burlingame, CA). Enhanced chemiluminescence (ECL) detection (Amersham, Arlington Heights, IL) was employed for visualization of secondary/primary antibody-antigen complexes on Immobilon-P membranes (Millipore, Bedford, MA).

Computational Biology Tools

Prediction of proteolytic fragments of IGFBP-3 and multiple sequence alignments were performed using default parameters in GeneWorks 2.2 for the Apple Macintosh (Intelligenetics, Mountain View, CA). GeneWorks was also used for the graphical display of hydrophobicity [Kyte and Doolittle, 1982], hydrophilicity [Hopp and Woods, 1981], and surface probability [Janin et al., 1978] protein plots. The comparison of the IGFBP-3 protein sequence against the PROSITE protein pattern database [Bairoch, 1991] was performed using MacPattern 2.1 [Fuchs, 1991].

PCR Mutagenesis

Single and double point mutations were introduced into the IGFBP-3 cDNA by using a three step PCR reaction utilizing PCR primers containing the appropriate base changes [Higuchi et al., 1988; Ho et al., 1989]. The plasmid pSV16B.ibp1.1 containing the complete IGFBP-3 cDNA under the control of an SV40 promoter/enhancer combination [Wood et al., 1988] served as a template. The PCR reaction was performed in a Perkin Elmer 9600 Thermocycler using the following primer sequences and temperatures for denaturation, annealing, and extension: PCR #1 (5′-GCTAGCTTGGGCTGCATCGATTG-3′ and 5′-GAAATTGCTAGTGAGgcgGAGG-3′) used 94/58/72°C, PCR #2 (5′-CCTCcgcCTCACTAGCATTTCC-3′ and 5′-AGCTTCTGCAGGTCGACTCTAGAGG-3′) used 94/65/72°C, and PCR #3 (5′-GCTAGCTTGGGCTGCATCGATTG-3′ and 5′-AGCTTCTGCAGGTCGACTCTAGAGG′-3′ plus purified products from PCR #1 and #2) used 94/64/72°C, respectively. Primer sequences for other mutants can be derived from Figure 3. All reactions were run for 25 cycles and used Pfu DNA polymerase (Stratagene, La Jolla, CA) as the thermostable enzyme with the inclusion of 10% DMSO in the reaction buffer. Optimal primer pairs and annealing temperature conditions were determined using Oligo 4.0 (National Biosciences, Plymouth, MA) and Amplify 1.2 (Bill Engels, University of Wisconsin, Madison, WI), PCR primer analysis programs for the Apple Macintosh.

IGF-I Competitive Displacement Assay

Equal amounts (2 ng; 0.14 nM) of wild-type and S111A/S113A mutant IGFBP-3 were incubated with ^{125}I-labeled IGF-I (20,000 cpm, specific activity 64 μCi/μg) in the presence or absence of increasing amounts of unlabeled IGF-I in 500 μl total reaction volume. After incubation at 4°C for 18 h, rabbit anti-IGFBP-3 serum was added, followed by further incubation for 3 h. IGF/IGFBP/antibody complexes were bound to Immunobeads (Bio-Rad, Richmond, CA) containing immobilized goat anti-rabbit IgG and washed by centrifugation. Bound radioactivity was counted in a gamma scintillation counter (Packard, Palo Alto, CA).

RESULTS

IGFBP-3 Digestion With Endoproteinase Lys-C

IGFBP-3 appears as a glycosylated polypeptide with a molecular weight of 43 kDa on a reducing SDS-PAGE. The protein core predicts a molecular weight of 29 kDa; the rest consists of sugar residues. The entire protein contains 27 serine residues which could serve as phosphoacceptor sites. In order to reduce the size of the protein and therefore the number of serines to study IGFBP-3 phosphorylation we searched for a protease that would a) generate a smaller sized fragment of IGFBP-3 and b) generate a fragment which contained the majority of IGFBP-3 phosphorylation sites. An analysis of more than 20 protease specificities and the resulting digestion patterns suggested the use of endoproteinase Lys-C. Computer-simulated endoproteinase Lys-C digestion predicted the generation of an undigested 17 kDa fragment (EndoL-fragment) that corresponds to the amino-terminal sequence of human IGFBP-3. The remainder of IGFBP-3 is digested into small peptides due to the presence of a large number of lysines that serve as cleavage sites (Fig. 1A). To make all potential protease cleavage sites accessible, IGFBP-3 purified by immunoprecipitation (see Methods) was first denatured by treatment with SDS and β-mercaptoethanol. Complete denaturation was followed by deglycosylation with N-Glycanase. Finally, endoproteinase Lys-C was added to obtain the 17 kDa EndoL-fragment. Each of the reaction products was separated on a reducing SDS-PAGE and visualized by autoradiography or Western blotting followed by chemiluminescence detection (Fig. 1B and next paragraph).

^{32}P-Phosphate Labeling of IGFBP-3

BP3-32 cells, a CHO cell clone stably transfected with an IGFBP-3 expression plasmid and

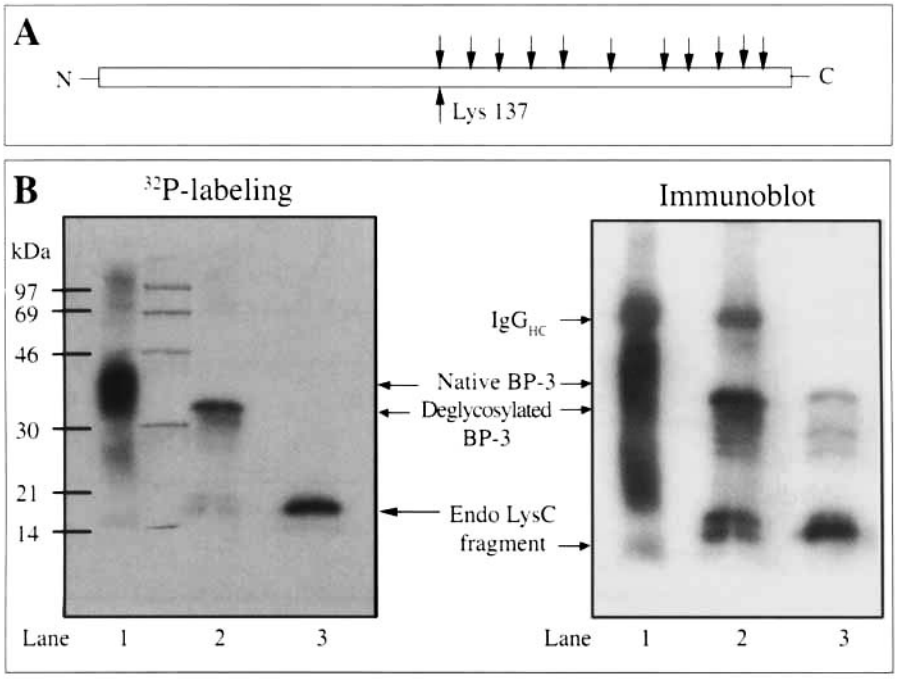
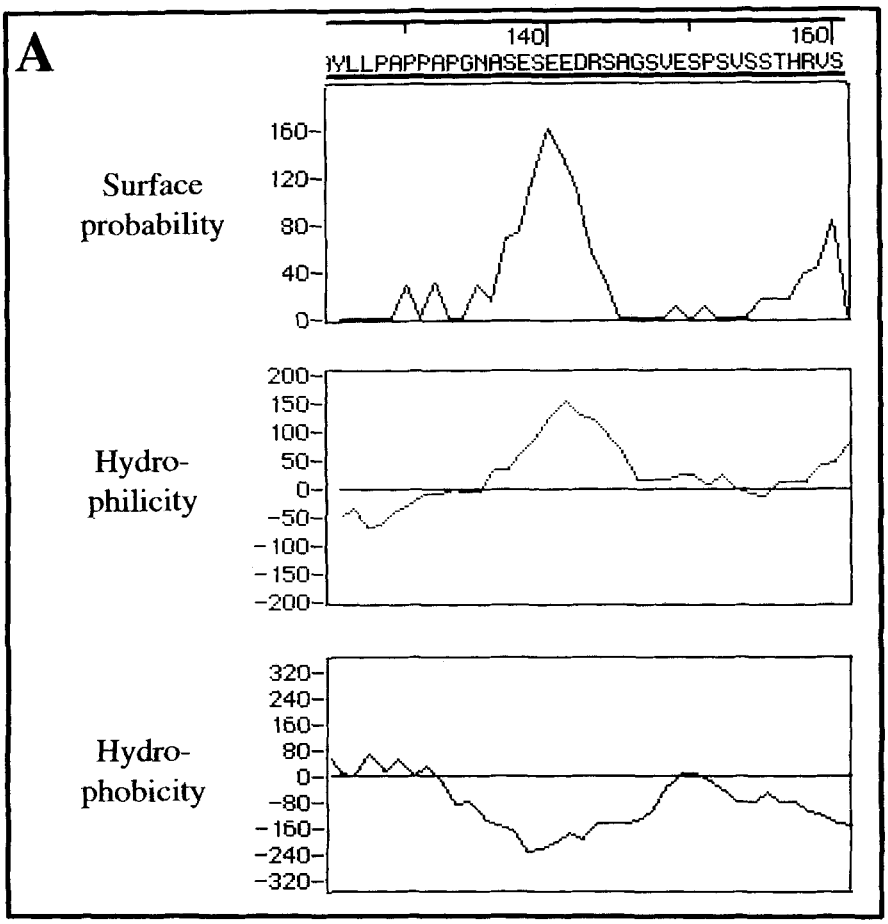


Fig. 1. Endoproteinase Lys-C digestion of IGFBP-3. **A:** Predicted cleavage pattern of endoproteinase Lys-C along the linear IGFBP-3 sequence. Arrows indicate cleavage sites with lysine 137 being the first; **B:** Autoradiography and chemiluminescence immunoblot of wild-type IGFBP-3 (lane 1), ^{14}C molecular weight markers, deglycosylated IGFBP-3 (lane 2) and deglycosylated and endoproteinase Lys-C treated IGFBP-3 (lane 3) from ^{32}P-labeled conditioned medium (left panel) or unlabeled conditioned medium detected by antibody (right panel). IgG_{HC} indicates the location of the immunoglobulin heavy chain of the first antibody used to immunoprecipitate IGFBP-3. Separation was performed on a 16% SDS-PAGE.

optimized for high level protein production (T. Etcheverry, unpublished), were labeled with ^{32}P-orthophosphate. IGFBP-3 was isolated from the conditioned medium by immunoprecipitation using a rabbit polyclonal antiserum directed against purified CHO-derived IGFBP-3. Equal amounts of protein were separated on SDS-PAGE. Immunopurified IGFBP-3 was subjected to deglycosylation and protease treatment as outlined. Figure 1B (left panel) shows an autoradiogram of ^{32}P-labeled IGFBP-3 untreated (lane 1), treated with N-Glycanase (lane 2) and treated with N-Glycanase and endoproteinase Lys-C (lane 3). As a control for equal loading of reaction samples the SDS-polyacrylamide gel was transfered to Immobilon-P membrane and incubated with IGFBP-3 antiserum for chemiluminescence detection of IGFBP-3 polypeptides. Figure 1B (right panel) shows that equal amounts of IGFBP-3 products were loaded in each lane. Comparison of the intensity of the phosphorylation signal of deglycosylated wild-type protein with that of the EndoL-fragment suggested that the 17 kDa fragment contains the majority of IGFBP-3 phosphorylation sites.

Identification of Potential Phosphorylation Sites

An examination of the amino acid sequence of the human IGFBP-3 17 kDa EndoL-fragment is predicted to have 16 serines which could potentially serve as phosphoacceptor sites. To eliminate serines that are unlikely to be phosphorylated we performed the following computational analyses: a) multiple sequence alignment of IGFBP-3 protein sequence from four different species; b) hydrophilicity, hydrophobicity, and surface probability plots on the human IGFBP-3 sequence; and c) comparison of human IGFBP-3 sequence against the protein pattern database PROSITE [Bairoch, 1991]. Based on the assumption that phosphorylation sites critical for IGFBP-3 function are conserved among species we looked for conserved residues in human, rat, bovine, and pig IGFBP-3. Multiple sequence alignment identified five conserved serine residues (data not shown). Further computational analysis of the location of the five serines in the human 17 kDa fragment, however, identified serine 111 and serine 113 as most likely residues that occur in hydrophilic regions and have a



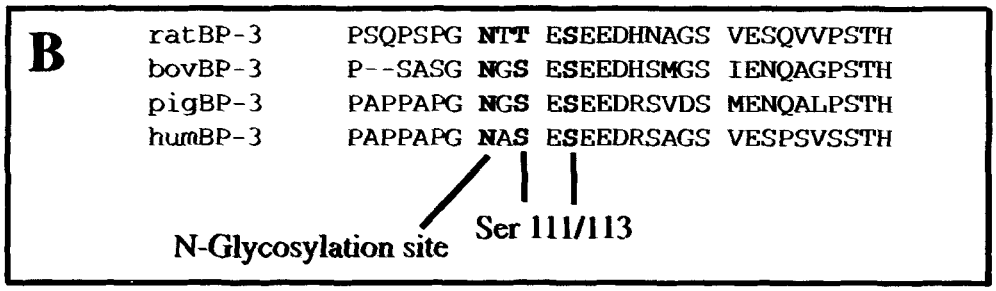


Fig. 2. Computational analysis of IGFBP-3 sequences: **A:** Protein analysis plots for hydrophobicity, hydrophilicity and surface probability profiles of a section of the human IGFBP-3 sequence and **B:** multiple sequence alignment of IGFBP-3 sequences from rat, bovine, pig, and human origin. Potential sites for glycosylation and phosphorylation are indicated.

high probability to be on the surface of the protein (Fig. 2A). Furthermore, analysis of the PROSITE protein pattern database suggested serines 111 and 113 as potential targets for Casein kinase II phosphorylation (data not shown). Figure 2B summarizes the results and highlights the putative phosphorylation sites within the context of the amino acid sequence of the four species.

PCR-Mediated Site-Directed Mutagenesis

Following the computer predictions described above serines 111 and 113 were targeted for site-directed mutagenesis to alanine (S111A/

wildtype	**AAT**	**GCT**	**AGT**	GCG	TCG	GAG	GAA	GAC	CGC	AGC	GCC
	N	**A**	**S**	E	S	E	E	D	R	S	A
S113A					G						
	N	**A**	**S**	E	A	E	E	D	R	S	A
S111A/S113A			**GC**		G						
	N	**A**	**A**	E	A	E	E	D	R	S	A
E114A/E115A						C	C				
	N	**A**	**S**	E	S	A	A	D	R	S	A
D116A								C			
	N	**A**	**S**	E	S	E	E	A	R	S	A

Fig. 3. Mutagenesis of putative phosphorylation sites on IGFBP-3 using PCR. A comparison of wild-type to mutant sequences is shown with base changes indicated on a separate line and amino acid changes underlined. Bold faced letters indicate the presence of a putative N-glycosylation sequence.

S113A and S113A). Neighboring glutamic acid residues 114 and 115 (E114A/E115A) and aspartic acid 116 (D116A) were also included in the mutagenesis with the assumption that they might define the kinase recognition sequence. A three step PCR-reaction was used to generate mutated DNA-fragments using oligonucleotides which incorporated single and double base changes at the IGFBP-3 cDNA level (Materials and Methods and Fig. 3). Mutated IGFBP-3 sequences were subcloned into an IGFBP-3 expression plasmid, confirmed by sequencing, and stably transfected into CHO DP-12 cells using a dihydrofolate reductase selection marker. Pools of transfected cells were isolated and analyzed for IGFBP-3 expression (data not shown).

^{32}P-Labeling of Stable Cell Pools

To analyze the effect of mutagenesis on IGFBP-3 phosphorylation mock-transfected cell pools, wildtype IGFBP-3 and mutant IGFBP-3 cell pools were incubated with 2 mCi ^{32}P-orthophosphate for 4 h. IGFBP-3 was isolated from the conditioned medium by immunoprecipitation with a rabbit polyclonal antiserum as described. In one experiment IGFBP-3 was denatured and deglycosylated (Fig. 4). In another experiment IGFBP-3 was further digested with endoproteinase Lys-C (Fig. 5). All samples were separated on a reducing SDS-PAGE and subsequently blotted onto Immobilon-P membrane. The membrane was first exposed for autoradiography. Wild-type IGFBP-3 (Fig. 4, ^{32}P-labeling, lane 1) shows a strong phosphorylation signal, whereas S113A (lane 2) and E114A/E115A (lane 4) show a 50% reduced, S111A/S113A (lane 3) a greater than 80% reduced phosphorylation signal, as determined by densitometric scanning. The phosphorylation signal obtained for D116A (lane 5) appears to be stronger than wild-type. Amounts of IGFBP-3s, as judged by the signal on the immunoblot (Fig. 4, Immunoblot), were comparable in all cases except for D116A (lane 5) which showed increased intensity. This could explain the increased ^{32}P-signal of D116A over wild-type. The difference in ^{32}P-signal intensity between S113A and S111A/S113A can be explained by the elimination of one versus two phosphorylation sites in S113A and S111A/S113A, respectively. The fact that there is still some residual phosphorylation detectable even in S111A/S113A (Fig. 4, lane 3) argues for further minor phosphorylation sites. A lower molecular weight IGFBP-3 fragment that is strongly phosphorylated appears to be the result of nonspecific proteolysis during the deglycosylation reaction.

Figure 5 shows a comparison of EndoL-fragments of ^{32}P-labeled wild-type and mutant IGFBP-3. EndoL-fragments of wild-type IGFBP-3 (lane 4) and D116A (lane 6) show comparable levels of IGFBP-3 expression and ^{32}P-labeling. S113A (lane 2), S111A/S113A (lane 3), and E114A/E115A (lane 5) show virtually no

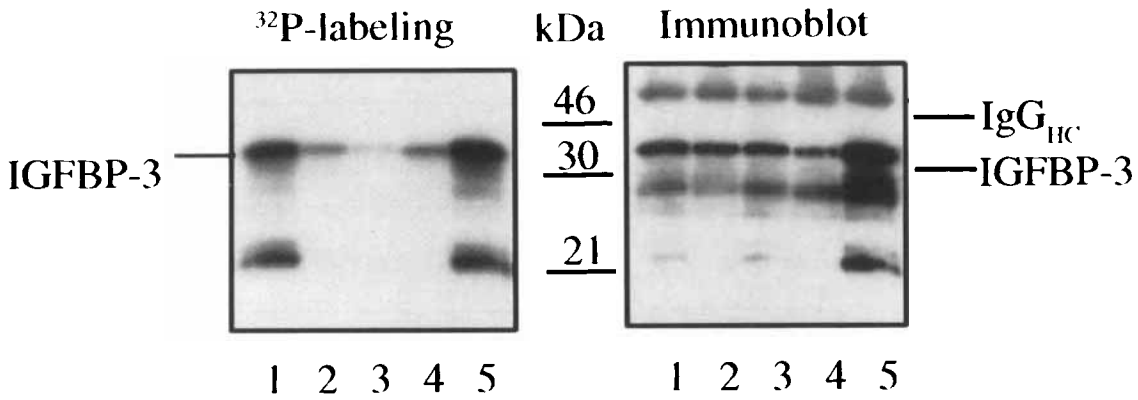


Fig. 4. Phosphorylation of wild-type and mutant IGFBP-3s expressed in transfected CHO-cells. Left panel shows an autoradiograph of an SDS-PAGE separating immunoprecipitated wild-type (lane 1) and S113A, S111A/S113A, E114A/E115A, and D116A (lanes 2 to 5, respectively) IGFBP-3. Right panel shows the corresponding chemiluminescence immunoblot of the identical gel. The positions of full-length IGFBP-3 and IgG_{HC} are indicated.

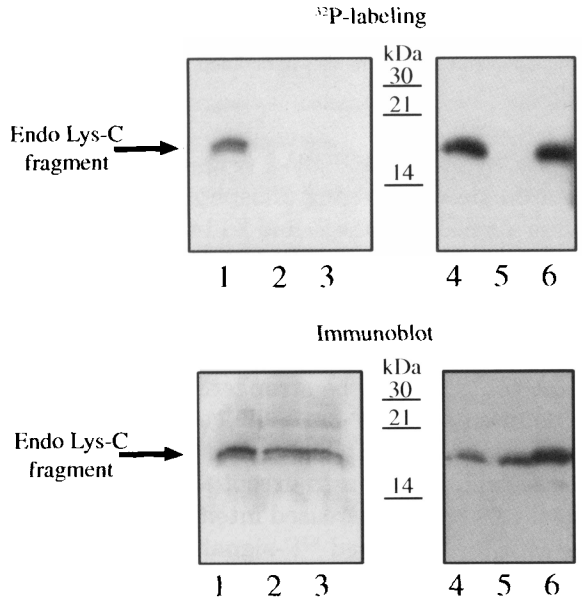


Fig. 5. Phosphorylation of wild-type and mutant IGFBP-3s expressed in transfected CHO-cells: Endoproteinase Lys-C fragment. Top panel shows an autoradiograph of Endoproteinase Lys-C fragments from wild-type (lane 1, 4) and S113A, S111A/S113A, E114A/E115A, and D116A (lanes 2, 3, 5, and 6, respectively) IGFBP-3 polypeptides. Lower panel shows the corresponding chemiluminescence immunoblot of IGFBP-3 Endoproteinase Lys-C fragments.

^{32}P-labeling but the EndoL-fragment is present at comparable levels as shown by immunoblot. A lack of phosphorylation in EndoL-fragments of all mutants indicates that the residual phosphorylation site(s) mentioned in the previous paragraph is located in the COOH-terminal half of the protein.

These results show that serine 111 and serine 113 of human IGFBP-3 are phosphorylated. Mutation of amino acids neighboring those serines, namely glutamic acid 114/115 also abolish phosphorylation. Mutation of aspartic acid 116 has no effect on phosphorylation levels.

Amino Acid Mutation but Not Phosphorylation Abolishes Glycosylation of IGFBP-3

The identified phosporylated residues are close to a potential N-glycosylation site of IGFBP-3 [Wood et al., 1988 and Fig. 3]. Mutation of residues in this region could potentially affect the glycosylation pattern of IGFBP-3. To test this hypothesis we isolated IGFBP-3 by immunoprecipitation from the conditioned medium of wild-type and mutant transfected cells. Samples were split into two equal fractions and either mixed with SDS sample buffer for direct loading on SDS-PAGE or further treated with N-Glycanase for complete deglycosylation. All samples were then separated by reducing SDS-PAGE and blotted onto Immobilon-P membrane. IGFBP-3 was detected via chemiluminescence using monoclonal antibody 1031 which recognizes an epitope in the amino-terminal region of IGFBP-3 (data not shown). Figure 6 shows wild-type IGFBP-3 (lane 1/2) and mutants S111A/S113A (lane 3/4), S113A (lane 5/6), E114A/E115A (lane 7/8), and D116A (lane 9/10) with and without N-Glycanase treatment. Non-treated samples display fuzzy bands characteristic of glycosylated proteins, with the exception of mutant S111A/S113A (lane 3). This mutant displays a distinct band indicating that mutation of serine 111 inhibited glycosylation of IGFBP-3. Serine 111 is part of a putative N-glycosylation site previously identified [Wood et

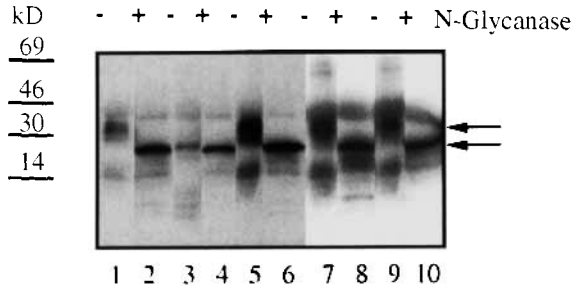


Fig. 6. Glycosylation of wild-type and mutant IGFBP-3 proteins. Immunoblot of wild-type (lane 1/2), S111A/S113A (lane 3/4), D116A (lane 5/6), S113A (lane 7/8) and E114A/E115A mutants (lane 9/10) minus, and plus N-Glycanase treatment. Upper and lower arrows indicate the relative position of glycosylated and non-glycosylated IGFBP-3, respectively.

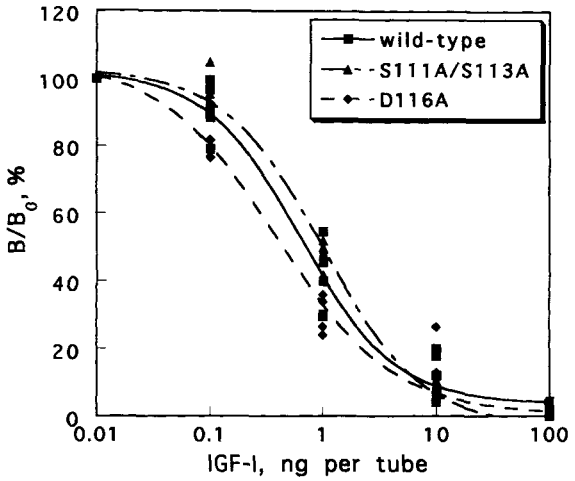


Fig. 7. IGF-I binding assay. Equal amounts of wild-type, S111A/S113A and D116A mutant IGFBP-3 were incubated with ^{125}I-labeled IGF-I in the presence or absence of increasing amounts of unlabeled IGF-I. After incubation with rabbit anti-IGFBP-3 serum immune complexes were bound to Immunobeads containing immobilized goat anti-rabbit IgG. Bound radioactivity was counted and is expressed as percent of total binding to wild-type IGFBP-3 in the absence of competitor. Data at each point were derived from four determinations from two experiments.

al., 1988 and Fig. 3, bold-faced letters]. Analysis of all samples in the presence of N-Glycanase shows that all mutants consist of the same size protein core as wildtype IGFBP-3. Differences in migration are therefore due to differences in the glycosylation pattern and not in the protein backbone.

Effect of Mutagenesis on IGF-I Binding Activity

In preliminary experiments wild-type IGFBP-3, the non-phosphorylated mutant S111A/S113A, and the phosphorylated mutant D116A were analyzed for their ability to bind IGF-I in an ^{125}I-labeled IGF-I competitive displacement assay. Figure 7 shows a comparison of the displacement curves. The IC_{50} calculated for the wild-type IGFBP-3 was 0.17 nM, whereas that for S111A/S113A and D116A was 0.27 nM and 0.1 nM, respectively.

DISCUSSION

We have described here the identification by site-directed mutagenesis of two major phosphorylation sites, namely, serines 111 and 113 of human IGFBP-3, which account for greater than 80% of total phosphorylation. Expression of serine-to-alanine mutants in transfected CHO-cells revealed a) no direct effect of phosphorylation on expression and secretion of IGFBP-3 mutants, b) reduction/elimination of glycosylation as the result of mutation but not phosphorylation, and c) no change in binding to IGF-I due to lack of phosphorylation. Our results here confirm earlier observations with enzymatically dephosphorylated, recombinant IGFBP-3 derived from the A293 cell line, which suggested that phosphorylation does not effect IGFBP-3s binding to IGF-I [Mukku et al., 1994]. These findings are, however, in striking contrast to results obtained for IGFBP-1. There increased phosphorylation correlates with increased binding to IGF-I [Jones et al., 1991, 1993]. Highly phosphorylated IGFBP-1 binds IGF-I with high affinity and appears to inhibit IGF-I mitogenic activity [Frost and Tseng, 1991; Jones et al., 1991]. On the contrary, non-phosphorylated IGFBP-1 still binds IGF-I, albeit with lower affinity, and is believed to stimulate IGF-I mitogenesis [Elgin et al., 1987]. IGFBP-3 exhibits similar activities as IGFBP-1 [Bicsak et al., 1990; Blat et al., 1989; Cohen et al., 1993; Imbenotte et al., 1992; Tesch et al., 1993]. Our results here would suggest that those effects are independent of the phosphorylation and glycosylation state of IGFBP-3, unlike in the case of IGFBP-1. Modulation of mitogenic activity by IGFBP-3 might more likely depend on the cellular context rather than posttranslational modifications [Cohen et al., 1993; Imbenotte et al., 1992; Liu et al., 1992].

IGFBP-3 is secreted as a phosphoprotein from HS-68 cells when expressed from the endogenous gene [Mukku et al., 1994 and data not shown] as well as from stably transfected cell

lines when expressed from a recombinant construct [Mukku et al., 1994 and this paper]. IGFBP-3, therefore, does not appear to be fortuitously phosphorylated in the milieu of a transfected cell line but rather occurs naturally as a phosphoprotein. We have attempted to use a previously described native gel system [Frost and Tseng, 1991; Jones et al., 1991] for the study of IGFBP-3 phosphorylation under non-radioactive conditions, but without success. The lack of a suitable assay for the analysis of phosphorylated and nonphosphorylated IGFBP-3 under non-radioactive conditions makes it impossible to ascertain the phosphorylation state of IGFBP-3 in the circulation, using the methods described in this paper. The use of modern analytical methods, for instance a combination of liquid chromatography and mass spectrometry might be a solution to the analysis of IGFBP-3 phosphorylation states under non-radioactive circumstances.

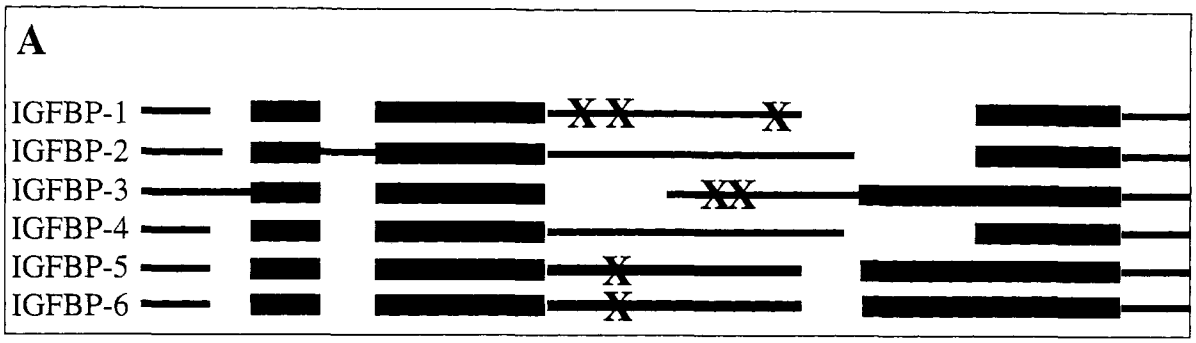
The physical location of the phosphorylation event, whether it occurs inside the cell before secretion or extracellulary after the secretion process, has not been determined explicitly. However, we have found phosphorylated IGFBP-3 intracellularly after ^{32}P-labeling of CHO-cells (data not shown), indicating phosphorylation inside the cell and involvement of an intracellular kinase. Several reports have described phosphorylation of secreted proteins [Feige and Baird, 1989; Rashidbaigi et al., 1985; Tabibzadeh et al., 1989], and in all cases phosphorylation appears to take place intracellularly. This is also demonstrated by the ability of intracellular kinases, such as Protein Kinase A and Casein Kinase II, to phosphorylate these proteins in vitro [Feige and Baird, 1989; Frost and Tseng, 1991; Jones et al., 1993].

Serines 111 and 113 of IGFBP-3 are located in a highly divergent domain of IGF binding proteins that is located in-between an amino-terminal and carboxy-terminal region of high sequence homology (Fig. 8A). This is also the case for IGFBP-1 phosphorylation sites (serines 101/119/168). A comparison of the amino acid sequences neighboring the phosphorylated serines shows a relatively high concentration of acidic residues in both IGF binding proteins (Fig. 8B). Traditionally this has pointed towards a casein type kinase (e.g., Casein Kinase II), since these kinases have a demonstrated preference for acidic residues around phosphorylation targets [Bairoch, 1991; Pearson and Kemp, 1991]. In vitro kinase experiments with IGFBP-1 demonstrated that at least the sites in IGFBP-1 can be phosphorylated by Casein Kinase II. Examination of the amino acid sequence of IGFBP-5 and -6, using the methods outlined, suggests another phosphorylation site, with a similar concentration of acidic amino acids around a potential serine phosphoacceptor site (Fig. 8B). Whether Casein Kinase II is the bona fide kinase that phosphorylates all IGF binding proteins remains to be established.

Identification of phosphorylation sites in IGF binding proteins is only partially complete. Only the major phosphorylation sites have been identified in IGFBP-1 [Jones et al., 1993] and in IGFBP-3, described in this paper. One or more minor phosphorylation sites are suspected to be found in the COOH-terminal half of IGFBP-3 for the following reasons. Analysis of phosphorylation from full-length double serine-to-alanine S111A/S113A gave a minor signal on the autoradiograph (Fig. 4) indicating that site-directed mutagenesis eliminated most but not all phosphorylation sites. The analysis of the corresponding endoproteinase Lys-C fragment (Fig. 5) gave no signal at all. The difference between full-length IGFBP-3 and the endoproteinase Lys-C fragment is the absence of the COOH-terminal half of the protein due to the presence of multiple cleavage sites for endoproteinase Lys-C. This region of human IGFBP-3 contains 11 serines, all of which are conserved among species, and could well serve as phosphoacceptor sites.

Preliminary results (Fig. 7) suggest that abolition of phosphorylation at serines 111 and 113 does not have considerable effect on IGF-I binding. Comparison of the IC_{50} values in the competition experiments showed a decrease of about 0.6-fold in IGF-I binding for the mutant that lacks phosphorylation. This is in contrast to IGFBP-1 where the dephosphorylated form has a 6-fold decrease in IGF-I binding. Mutation of aspartic acid to alanine at position 116 appears to cause a 0.4-fold increase in IGF-I binding. Considering experimental variability we believe that the observed differences are not biologically significant. However, more detailed analysis of binding experiments in progress should enable us to draw firmer conclusions.

Mutations in S111A/S113A but not in S113A abolished glycosylation of IGFBP-3 as judged



B

IGFBP-1	98	S	P	E	**S**	T	E	I	T	E	E	E		108
IGFBP-1	116	M	A	P	**S**	E	E	D	H	S	I	L		126
IGFBP-1	165	Q	E	T	**S**	G	E	E	I	S	K	F		175
IGFBP-3	108	G	N	A	**S**	E	S	E	E	D	R	S		118
IGFBP-3	110	A	S	E	**S**	E	E	D	R	S	A	G		120
IGFBP-5	94	E	R	D	**S**	R	E	H	E	E	P	T		104
IGFBP-6	95	E	R	D	**S**	R	E	H	E	E	P	T		105

Fig. 8. Comparative analysis of phosphorylation sites in IGFBP-1, -3, and -5/6: **A:** *Location of IGFBP-3 phosphorylation* sites relative to those known for IGFBP-1. Highly conserved regions are displayed as thick black lines. Non-conserved regions are shown as thin lines. Crosses (x) represent the approximate location of phosphorylation sites. **B:** Comparison of sequences around the phosphorylation sites in IGFBP-1 and -3. *Phosphorylated serines are highlighted, acidic residues are* underlined. The serine phosphorylation site for IGFBP-5/6 is putative and not based on experimental evidence (see Discussion).

by a shift in the migration of S111A/S113A on SDS-PAGE when compared to wild-type IGFBP-3 (Fig. 6). Serine 111 is part of a putative glycosylation site (Fig. 3), one out of three previously identified putative sites [Wood et al., 1988]. Which one of these three potential glycosylation sites are actually utilized has not been determined conclusively yet. The results described suggest that the site containing serine 111 is the major if not the only site used for N-glycosylation of IGFBP-3.

In conclusion, we have identified by site-directed mutagenesis that serines 111 and 113 are the major phosphorylation sites in IGFBP-3. Abolition of phosphorylation at these sites does not appear to have significant effect on the binding of IGF-I. Serine to alanine double mutation also generated an aberrant glycosylation pattern, making this mutant an ideal tool for further studies on the role of posttranslational modifications of IGFBP-3.

ACKNOWLEDGMENTS

The authors thank Florian Wurm and Adriana Johnson for DHFR-selection plasmids and transfection protocols. Furthermore, the contributions of purified IGFBP-3 and BP3-32 cells by David Reifsnyder and Tina Etchevarry and the IGFBP-3 expression plasmid by William I. Wood, respectively, and the assistance of Cynthia Lybarger in the preparation of the manuscript are greatly appreciated. Helpful discussions were provided by Denise Harrison, Paul Fielder, and Mickey Williams.

REFERENCES

Albiston AL, Herington AC (1990): Cloning and characterization of the growth hormone-dependent insulin-like growth factor binding protein (IGFBP-3) in the rat. Biochem Biophys Res Commun 166:892–7.

Bairoch A (1991): PROSITE: A dictionary of sites and patterns in proteins. Nucleic Acids Res 19:2241–2245.

Baker J, Liu J-P, Robertson EJ, Efstratiadis A (1993): Role of insulin-like growth factors in Embryonic and Postnatal Growth. Cell 75:73–82.

Baxter RC, Martin JL (1989): Structure of the Mr 140,000 growth hormone-dependent insulin-like growth factor binding protein complex: Determination by reconstitution and affinity-labeling. Proc Natl Acad Sci USA 86:6898–6902.

Bicsak TA, Shimonaka M, Malkowski M, Ling N (1990): Insulin-like growth factor-binding protein (IGF-BP) inhibition of granulosa cell function: Effect on cyclic adenosine 3′,5′-monophosphate, deoxyribonucleic acid synthesis, and comparison with the effect of an IGF-I antibody. Endocrinology 126:2184–2189.

Blat C, Delbe J, Villaudy J, Chatelain G, Golde A, Harel L (1989): Inhibitory diffusible factor 45 bifunctional activity: As a cell growth inhibitor and as an insulin-like growth factor I-binding protein. J Biol Chem 264:12449–12454.

Blum WF, Jenne EW, Reppin F, Kietzmann K, Ranke MB, Bierich JR (1989): Insulin-like growth factor I (IGF-I)-binding protein complex is a better mitogen than free IGF-I. Endocrinology 125:766–772.

Cohen P, Lamson G, Okajima T, Rosenfeld RG (1993): Transfection of the human IGFBP-3 gene into Balb/c fibroblasts: A model for the cellular functions of IGFBPs. Growth Reg 3:23–26.

Conover CA (1992): Potentiation of insulin-like growth factor (IGF) action by IGF-binding protein-3: Studies of underlying mechanism. Endocrinology 130:3191–3199.

Crews CM, Erikson RL (1993): Extracellular signals and reversible protein phosphorylation: What to make of it all. Cell 74:215–217.

Delhanty PJD, Hill DJ, Shimasaki S, Han VKN (1993): Insulin-like growth factor binding protein-4, -5 and -6 mRNA's in the human fetus: Localization to sites of growth and differentiation. Growth Reg 3:9–11.

Elgin RG, Busby WJ, Clemmons DR (1987): An insulin-like growth factor (IGF) binding protein enhances the biologic response to IGF-I. Proc Natl Acad Sci USA 84:3254–3258.

Feige J-J, Baird A (1989): Basic fibroblast growth factor is a substrate for protein phosphorylation and is phosphorylated by capillary endothelial cells in culture. Proc Natl Acad Sci USA 86:3174–3178.

Frost RA, Tseng L (1991): Insulin-like growth factor-binding protein-1 is phosphorylated by cultured human endometrial stromal cells and multiple protein kinases in vitro. J Biol Chem 266:18082–18088.

Fuchs R (1991): MacPattern: Protein pattern searching on the Apple Macintosh. Comput Appl Biosci 7:105–106.

Guidice LC (1992): Insulin-like growth factors and Ovarian Follicular Development. Endoc Rev 13:641–669.

Higuchi R, Krummel B, Saiki R (1988): A general method for in vitro preparation and specific mutagenesis of DNA fragments: Study of protein and DNA interactions. Nucleic Acids Res. 16:7351–7367.

Ho SN, Hunt HD, Horton RM, Pullen JK, Pease LR (1989): Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene 77:51–59.

Hopp TP, Woods KR (1981): Prediction of protein antigenic determinants from amino acid sequences. Proc Natl Acad Sci USA 78:3824–3828.

Hunter T, Karin M (1992): The regulation of transcription by phosphorylation. Cell 70:375–387.

Imbenotte J, Desauty G, Harel L (1992): Stimulation by TGF-beta of chick embryo fibroblasts: Inhibition by an IGFBP-3. Exp Cell Res 199:229–233.

Jackson SP (1992): Regulating transcription factor activity by phosphorylation. Trends Cell Biol 2:104–108.

Janin J, Wodak S, Levitt M, Maigrett B (1978): Conformation of amino acid side chains in proteins. J Mol Biol 125:357–386.

Jones JI, Busby WJ, Wright G, Smith CE, Kimack NM, Clemmons DR (1993): Identification of the sites of phosphorylation in insulin-like growth factor binding protein-1: Regulation of its affinity by phosphorylation of serine 101. J Biol Chem 268:1125–11231.

Jones JI, D'Ercole AJ, Camacho HC, Clemmons DR (1991): Phosphorylation of insulin-like growth factor IGF-binding protein 1 in cell culture and in vivo: Effects on affinity for IGF-I. Proc Natl Acad Sci USA 88:7481–7485.

Kyte J, Doolittle RF (1982): A simple method for displaying the hydrophobic character of a protein. J Mol Biol 157:105–132.

Leong SR, Baxter RC, Camerato T, Dai J, Wood WI (1992): Structure and functional expression of the acid-labile subunit of the Insulin-like growth factor-binding protein complex. Mol Endocrinol 6:870–876.

Lin W-C, Desiderio S (1993): Regulation of V(D)J recombination activator protein RAG-2 by phosphorylation. Science 260:953–959.

Liu L, Delbe J, Blat C, Zapf J, Harel L (1992): Insulin-like Growth Factor Binding Protein (IGFBP-3), an Inhibitor of Serum Growth Factors Other Than IGF-I and -II. J Cell Physiol 153:15–21.

Mukku VR, Chu H, Baxter R (submitted): Insulin-like growth factor binding protein-3 is a phosphoprotein: Dephosphorylation influences its binding to the acid-labile subunit and cell surface.

Mukku VR, Courter RG, Gorman C, Parker M, Larsen B, Wood WI (1989): "Expression, Purification, and Characterization of Recombinant BP53, the GH-dependent IGF Binding Protein: Insulin-Like Growth Factor Binding Proteins." New York: Elsevier Science Publishers B.V. (Biomedical Division), pp 65–70.

Pearson R, Kemp BE (1991): Protein kinase phosphorylation site sequences and consensus specificity motifs: Tabulations. Methods Enzymol 200:62–81.

Rashidbaigi A, Kung H-F, Pestka S (1985): Characterization of receptors for immune interferon in U937 cells with ^{32}P-labeled human recombinant immune interferon. J Biol Chem 260:8514–8519.

Rosenfeld RG, Lamson G, Pham H, Oh Y, Conover C, DeLeon DD, Donovan SM, Guidice L (1990): Insulin-like growth factor binding proteins. Recent Prog Hormone Res 46:99–163.

Sommer A, Maack CA, Spratt SK, Mascarenhas D, Tressel TJ, Rhodes ET, Lee R, Roumas M, Tatsuno GP, Flynn JA, Gerber N, Taylor J, Cudny H, Nanney L, Hunt TK, Spencer EM (1991): "Molecular Genetics and Actions of Recombinant Insulin-Like Growth Factor Binding Protein-3: Modern Concepts of Insulin-Like Growth Factors. New York: Elsevier Science Publishing Co., Inc. pp 715–728.

Tabibzadeh SS, Santhanam U, Sehgal PB, May LT (1989): Cytokine-induced production of IFN-beta2/IL-6 by freshly

explanted human endometrial stromal cells. J Immunol 142:3134–3139.

Tesch GH, Cornell HJ, Herington AC, Oakes S (1993): Effects of insulin-like growth factor binding protein complexes on human fibroblast growth. Growth Reg 3:151–159.

Wood WI, Cachianes G, Henzel WJ, Winslow GA, Spencer SA, Hellmiss R, Martin JL, Baxter RC (1988): Cloning and expression of the growth hormone-dependent insulin-like growth factor-binding protein. Mol Endocrinol 2:1176–1185.

Wurm FM (1994): Aspects of Gene Transfer and Gene Amplification in Recombinant Mammalian Cells: "Genetic Manipulation of Mammalian Cells." Berlin: Walter de Gruyter & Co.

Wurm FM, Johnson A, Lie Y, Etcheverry TM, Anderson KP (1992): Host cell derived retroviral sequences enhance transfection and expression efficiency in CHO cells. In "Animal Cell Technology: Developments, Processes and Products." Boston: Butterworth-Heinemann pp 35–41.