Characterisation of recombinant glycosylation variants of insulin-like growth factor binding protein-3

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Abstract

There are three potential *N*-glycosylation sites in the non-conserved central region of the insulin-like growth factor binding protein-3 (IGFBP-3) sequence ($N^{89}AS$, $N^{109}AS$, $N^{172}FS$). IGFBP-3 exists as two glycoforms which reduce to a single form on enzymatic deglycosylation. To determine the functional significance of the carbohydrate chains, the *N*-glycosylation sites were mutated singly and in combinations by substituting Asn residues with Ala. Each recombinant glycoform was detected by radioimmunoassay, indicating that glycosylation is not essential for secretion in Chinese hamster ovary cells. Ligand blotting of the conditioned media using [^{125}I]IGF-I indicated that all seven mutants are active. On the basis of the number and molecular masses of the bands detected for each glycoform, there is approximately 4, 4·5 and 5 kDa of carbohydrate on Asn^{89}, Asn^{109} and Asn^{172} respectively, with variable occupancy of Asn^{172}. Ternary complex formation by the glycovariants in the presence of ALS and excess IGF-I was not significantly different from that of fully glycosylated recombinant human (rh)IGFBP-3 [K_a (fully glycosylated)=12·5 ± 4·1 l/nmol; mean K_a (all mutants)=22·1 ± 3·0 l/nmol]. In contrast, Asn to Asp substitutions decreased acid-labile subunit (ALS) binding activity. Cell-surface association experiments indicate that glycosylation may influence the partitioning of IGFBP-3 between the extracellular milieu and the cell surface. Therefore, while the carbohydrate units appear to be non-essential to ALS or IGF binding, they may modulate other biological activities of IGFBP-3.
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Introduction

Insulin-like growth factor binding protein-3 (IGFBP-3) is a member of a family of homologous proteins that bind the insulin-like growth factors, IGF-I and IGF-II, with high affinities. When bound to either IGF-I or IGF-II, IGFBP-3 can form a high-molecular-weight complex with the acid-labile subunit (ALS), which represents a tightly regulated source of circulating IGFs (Rajaram *et al.* 1997). IGFBP-3 has also been shown to have cellular activities such as stimulation and inhibition of cell proliferation (Jones & Clemmons 1995), induction of apoptosis (Gill *et al.* 1997, Rajah *et al.* 1997) and nuclear translocation (Jaques *et al.* 1997, Li *et al.* 1997, Schedlich *et al.* 1998), and it is thought that many of these cellular functions may require the cell-surface association of IGFBP-3.

IGFBP-3 is known to undergo post-translational modification by phosphorylation and glycosylation. Analysis of the human (h)IGFBP-3 cDNA sequence (Wood *et al.* 1988) predicts the core protein to be 264 amino acids with a molecular mass of approximately 29 000. The amino acid sequence also predicts three potential *N*-glycosylation sites (Asn-X-Ser/Thr) located at Asn^{89}, Asn^{109} and Asn^{172} (sites 1, 2 and 3 respectively) in the central region, which is not conserved among the IGFBPs. Native hIGFBP-3 is usually found as a characteristic doublet of approximately 40–45 kDa, from both cellular and plasma sources. The two natural forms of hIGFBP-3 were demonstrated to be glycoproteins by precipitation with concanavalin A and by positive staining with periodic acid–Schiff's reagent (Martin & Baxter 1986). The same characteristic doublet is seen for recombinant protein, rhIGFBP-3, expressed in Chinese hamster ovary (CHO) cells which upon treatment with N-glycanase under reducing conditions produces a single band of approximately 30 000 Da (Tressel *et al.* 1991). This led to the speculation that the doublet consists of two differentially glycosylated forms but the data did not indicate which of the three potential sites were utilised as oligosaccharide acceptor sites. Non-glycosylated rhIGFBP-3 has also been expressed in *Escherichia coli*, and was shown to be identical to fully glycosylated rhIGFBP-3 from CHO cells in its IGF-I binding activity (Sommer *et al.* 1993). Although non-glycosylated rhIGFBP-3 from *E. coli* has been used extensively in various studies as a pure form of the protein, the role of the carbohydrate chains, either in total or individually, has not been studied

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extensively, and the non-glycosylated protein has not been compared with glycosylated IGFBP-3 from the same cellular source.

The general function of *N*-glycosylation is to aid in the *in vivo* folding and assembly of the nascent polypeptide chain, maintaining its conformation and directing its ultimate secretion from the cell (Rademacher *et al.* 1988, Lis & Sharon 1993). More specifically, carbohydrates on glycoproteins can modulate physicochemical properties and biological activities. For example, it is now well established that correct and complete *N*-glycosylation of the common α subunits of gonadotrophic hormones such as chorionic gonadotrophin, luteinizing hormone and follicle-stimulating hormone is critical for signal transduction; in the case of human chorionic gonadotrophin, deglycosylated forms bind with greater affinity to the cognate receptor, but are unable to activate adenylate cyclase (Lis & Sharon 1993, Thotakura & Blithe 1995).

We have previously shown that the ligand-binding activities of CHO-derived wild-type rhIGFBP-3 are similar to those of pure, plasma-derived hIGFBP-3 (Firth *et al.* 1998). In a preliminary report, we described a panel of IGFBP-3 cDNA mutants encoding proteins that have lost one, combinations of two or all three of the potential *N*-glycosylation sites and the expression of the proteins in CHO cells (Firth & Baxter 1995). In this study, we have compared these recombinant IGFBP-3 glycoforms with fully glycosylated rhIGFBP-3 (also derived from CHO cells) for their IGF, ALS and cell-surface binding abilities.

Materials and Methods

Materials

All radiolabelled proteins used were prepared as described previously (Baxter & Martin 1986, Baxter *et al.* 1992). Oligonucleotides were synthesised on an Oligo 1000 DNA Synthesiser (Beckman Instruments, Palo Alto, CA, USA). Restriction and modifying enzymes were purchased from Promega Corp. (Madison, WI, USA) and the T7 Sequencing Kit was purchased from Pharmacia Biotech Inc. (Uppsala, Sweden). Hexadimethrine bromide (Polybrene), dexamethasone, hypoxanthine, xanthine, thymidine, and mycophenolic acid were from Sigma Chemical Co. (St Louis, MO, USA) and aminopterin was purchased from Life Technologies Inc. (Gaithersburg, MD, USA). Nucleoside-free α-modified Eagle's medium (α-MEM) and foetal calf serum were from Cytosystems (North Ryde, NSW, Australia). The human IGFBP-3 radioimmunoassay (RIA) has been described previously (Baxter & Martin 1986). Antiserum R-100 has a higher titre, but similar specificity, compared with the antiserum R1–4.

Site-directed mutagenesis and construction of expression vectors

The cloning of IGFBP-3 cDNA into both the mutagenesis vector, pSELECT (Promega Corp.), and the expression vector, pMSG (Pharmacia Biotech Inc.), has been described elsewhere (Firth *et al.* 1998). Using the pSELECT-hIGFBP-3 plasmid as the mutagenesis vector, various combinations of oligonucleotides were used to introduce single, double and triple mutations *in vitro*, as described previously (Firth *et al.* 1998). The following oligonucleotides were used for substituting Asn^{89} with Ala or Asp: 5′-GCGGGCTCTGCGTCgcCGCTAGTGCC GTC or 5′-GCGGGCTCTGCGTCgACGCTAGTGC CGTC; substituting Asn^{109} with Ala or Asp: 5′-CGCC AGCTCCAGGAgcTGCTAGTGAGTCG or 5′-CGC CAGCTCCAGGAgATGCTAGTGAGTCG; and substituting Asn^{172} with Ala or Asp: 5′-GCACAGATACCC AGgcCTTCTCCTCCGAG or 5′-GCACAGATACCC AGgACTTCTCCTCCGAG. Nucleotides that differ from the IGFBP-3 sequence are indicated in lowercase letters. The hIGFBP-3 coding sequences of each construct were verified by plasmid DNA sequencing (Tabor & Richardson 1987), following which each cDNA was amplified and cloned into pMSG as described for wild-type hIGFBP-3 cDNA previously (Firth *et al.* 1998).

Cell culture and transfection

CHO cells were grown in α-MEM supplemented with 10% (v/v) foetal calf serum at 37 °C. For transfection, the cells were plated out at 6×10^5 cells per 75 cm^2 flask, incubated for 24 h and then transfected with 20 μg each rhIGFBP-3 expression plasmid or pMSG in the presence of 100 μg polybrene (Chaney *et al.* 1986). The plasmids contain the guanine phosphoribosyltransferase (GPT) gene, *gpt*, which confers resistance to mycophenolic acid. The transfected cells were cultured in GPT selection medium for 21 days to select for stable transfectants. The medium for GPT selection consisted of α-MEM supplemented with 10% (v/v) foetal calf serum, 250 μg/ml xanthine, 25 μg/ml mycophenolic acid, 2 μg/ml aminopterin, 10 μg/ml thymidine and 15 μg/ml hypoxanthine. Expression of the recombinant proteins is driven by the mouse mammary tumour virus long terminal repeat (MMTV LTR) promoter on pMSG, which has a glucocorticoid-responsive element, hence expression is inducible by dexamethasone. Following the selection period of 21 days, the mixed population of each transfected cell line was grown to confluence and the media changed to serum-free α-MEM supplemented with 0·1% (w/v) BSA and 10 μM dexamethasone. After 72 h, the conditioned media were collected and stored at −15 °C, before being assayed for rhIGFBP-3 by RIA. Where required, the conditioned media were concentrated by centrifugation through Centriprep-10 concentrators (Amicon Inc., Beverley, MA, USA). At the end of some experiments, total DNA in the cell monolayer was measured by fluorimetry using Hoechst 33258 dye (Calbiochem, La Jolla, CA, USA), as described previously (Labarca & Paigen 1980).

Ligand blotting

Each protein was purified by immunoprecipitation with IGFBP-3 specific antibody bound to Protein-A Sepharose CL-4B (Pharmacia Biotech, Uppsala, Sweden) before electrophoresis. Briefly, approximately 50 ng each protein were incubated with the antibody–Protein-A Sepharose mixture for 2 h at room temperature. Following extensive washes with 50 mM sodium phosphate buffer (pH 6·5), each sample was resuspended in 50 μl Laemmli sample buffer (0·01 M Tris, pH 6·8, 1% (w/v) SDS, 10% (v/v) glycerol), heated at 95 °C for 5 min and then electrophoresed on a 12% SDS–polyacrylamide gel and electroblotted onto nitrocellulose. The blot was incubated with [^{125}I]IGF-I (1×10^6 c.p.m./50 ml) for 16 h at 22 °C and washed as described previously (Firth *et al.* 1998). The blot was placed against a Storage Phosphor Screen for 16 h and the image scanned and analysed on a PhosphorImager SP (Molecular Dynamics, Sunnyvale, CA, USA).

ALS binding assays

ALS binding in the presence of wild-type or variant rhIGFBP-3 was measured as described previously (Baxter *et al.* 1989). Briefly, [^{125}I]ALS (10 000 c.p.m.) was incubated with mixtures of IGF-I (50 ng) and varying concentrations of rhIGFBP-3 or variants (over the range 0–10 ng) in a total volume of 0·3 ml 50 mM sodium phosphate containing 1% (w/v) BSA at 22 °C for 2 h. Ternary complexes were immunoprecipitated with 0·5 μl IGFBP-3 antibody and 2·5 μl goat anti-rabbit immunoglobulin in the presence of a 4% final concentration of polyethylene glycol.

The affinity of ALS binding to the rhIGFBP-3–IGF-I binary complex was determined as described previously (Baxter *et al.* 1992), except that the concentrations of rhIGFBP-3 analogues (1 ng) and IGF-I (50 ng) were held constant, whereas unlabelled ALS was added over the concentration range 2·5–200 ng in a total volume of 0·3 ml. Bound tracer was separated from free tracer as described above. Scatchard analysis was performed as previously described (Baxter *et al.* 1992).

Detection of cell-associated IGFBP-3

Cell-surface association of endogenous rhIGFBP-3 produced by the transfected cell lines was measured by a previously described immunological assay (Martin *et al.* 1992). Briefly, cells were plated out at 2×10^4 cells per well in 24-well plates for 48 h. Cultures were changed to serum-free media supplemented with 0·1% (w/v) BSA and incubated for a further 48 h. The cell monolayers were then washed and incubated with either anti-hIGFBP-3 (R-100) or normal rabbit serum (as control for non-specific effects) diluted 1/5000 in 0·5 ml media. After a 16-h incubation at 22 °C, the cell monolayers were washed again before incubation with [^{125}I]Protein A (20 000 c.p.m. in 0·5 ml media) for 2 h. Excess tracer was removed by washing and the cells were solubilised with 0·5% (w/v) sodium dodecyl sulphate. The cell lysates were collected and radioactivity was determined in a γ-counter.

Statistical analysis

Statistical analysis was carried out using StatView 4·02 (Abacus Concepts Inc., Berkeley, CA, USA). Differences between groups of data were evaluated by Fisher's Protected Least Significant Difference test after analysis of variance.

Results

Construction and expression of IGFBP-3 glycosylation variants

A panel of seven IGFBP-3 analogues was constructed such that the Asn residue of each potential *N*-glycosylation site (Asn-X-Ser/Thr) in IGFBP-3 was substituted with Ala at one, combinations of two, or all three sites, as shown in Fig. 1A. CHO cells were transfected with the plasmid vector, pMSG, or each of the recombinant plasmids. After selection, the transfected cells were expanded and mixed populations of stable transfectants were established. Serum-free media conditioned for 72 h by each cell line were collected and assayed for hIGFBP-3 by RIA. Although the levels of expression were variable, all seven IGFBP-3 glycosylation variants were detectable (Fig. 1B), suggesting that the process of glycosylation is not required for the secretion of these proteins from CHO cells. The detection of all the variant proteins by RIA also indicated that our specific polyclonal hIGFBP-3 antibody can recognise both non-glycosylated hIGFBP-3 and its glycoforms. We have previously shown that this antibody recognises an epitope in the amino-terminal portion of IGFBP-3 (Firth *et al.* 1998), a region without *N*-glycosylation sites. In the IGFBP-3 RIA, whereas rhIGFBP-3 displayed a displacement curve parallel to that of the standard (pure, human serum-derived IGFBP-3), all the glycoforms showed parallel displacement curves up to 1–2 ng/tube, but lost parallelism at greater concentrations (data not shown). Non-parallel displacement curves in the RIA have also been observed for IGFBP-3 fragments (Firth *et al.* 1998). As the RIA was the only method available for quantifying the proteins, all samples were repeatedly assayed near the midpoint (1·2 ng/tube) of the standard curve.

Estimated molecular masses of the carbohydrate chains on IGFBP-3

Ligand blotting of the conditioned media using [^{125}I]IGF-I as the ligand indicated that all seven mutants are capable of

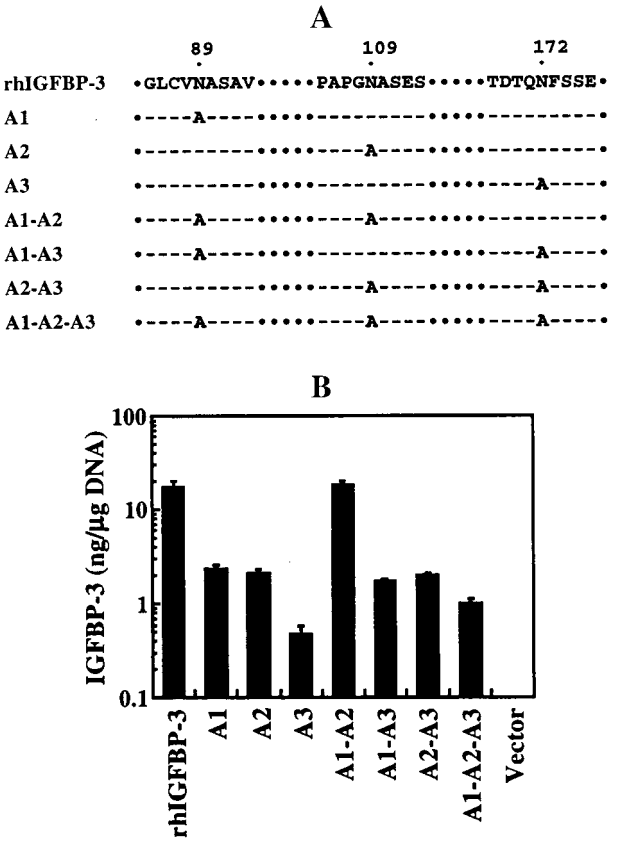

Figure 1 Amino acid sequence of IGFBP-3 *N*-glycosylation sites and the expression of recombinant IGFBP-3 glycoforms. (A) The sequences surrounding the three potential *N*-glycosylation sites of IGFBP-3 are shown, with amino acid numbers above. The corresponding sequence of the series of mutants constructed, as described in Materials and Methods, is shown below with the Asn (N) to Ala (A) substitution shown. Dashes indicate residues identical to those of rhIGFBP-3. Nomenclature for the mutant proteins are A1 (Asn^{89} substituted with Ala), A2 (Asn^{109} substituted with Ala) and A3 (Asn^{172} substituted with Ala). (B) Media conditioned by transfected CHO cells for 72 h were assayed for IGFBP-3 by RIA. IGFBP-3 concentrations are expressed after correction for DNA content of cells.

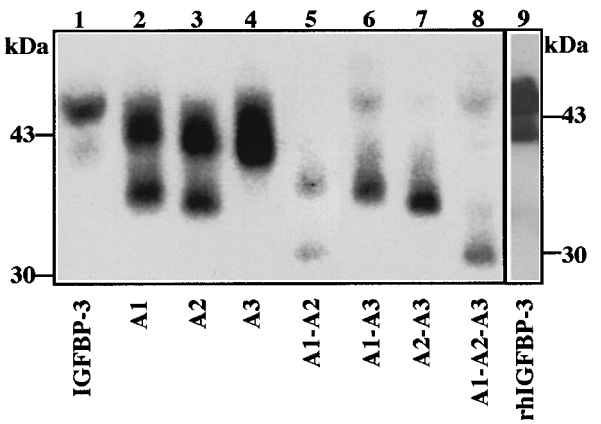

Figure 2 [^{125}I]IGF-I ligand blotting of IGFBP-3 glycoforms. After immunoprecipitation of 50 ng each sample, as described in Materials and Methods, the proteins were electrophoresed on a 12% SDS–polyacrylamide gel and then transferred to nitrocellulose. The blot was processed for ligand blotting and analysed on a phosphorimager. Samples are purified, serum-derived IGFBP-3 (lane 1), A1 (lane 2), A2 (lane 3), A3 (lane 4), A1–A2 (lane 5), A1–A3 (lane 6), A2–A3 (lane 7) and A1–A2–A3 (lane 8). Relative migration distances of molecular mass standards are indicated on the left of the panel. Purified rhIGFBP-3 (lane 9) was electrophoresed on a separate gel and relative migration distances of molecular mass standards are indicated on the right of the panel. The faint bands seen at about 45 kDa in lanes 6 and 8 are believed to be artefactual, resulting from the very high degree of concentration of culture medium required for these samples.

binding IGF-I (Fig. 2). Therefore, the carbohydrate chains on IGFBP-3 are not essential for IGF binding. The calculated molecular mass of the core protein of IGFBP-3 is approximately 29 kDa, similar to the estimated mass of the A1–A2–A3 mutant (non-glycosylated), which migrated as a single major glycoform of approximately 31·5 kDa (Fig. 2, lane 8). Mutants A1–A3 and A2–A3 also displayed single glycoforms of apparent molecular masses about 36 and 35·5 kDa respectively (Fig. 2, lanes 6 and 7). This implies that there are approximately 4 kDa and 4·5 kDa of carbohydrate on *N*-glycosylation site 1 (N^{89}AS) and site 2 (N^{109}AS) respectively. In contrast, there were two glycoforms of A1–A2 (approximately 36·5 and 31·5 kDa; Fig. 2, lane 5), suggesting variable occupancy at site 3 (N^{172}FS). On the basis of the molecular weights of these proteins, there is probably about 5 kDa of oligosaccharides in the larger form, and very little or no carbohydrate on the smaller protein. This notion of variable occupancy at site 3 is supported by the observation that there are invariably two glycoforms of any mutants that have the natural site 3, as in both mutants A1 (40·5 and 36 kDa; Fig. 2, lane 2) and A2 (40 and 35·5 kDa; Fig. 2, lane 3) and in natural IGFBP-3 (44·5 and 40 kDa; Fig. 2, lane 1). In contrast, a single glycoform is present when site 3 is mutated as in mutants A3, A1–A3, A2–A3 and A1–A2–A3 (Fig. 2, lanes 4, 6, 7 and 8 respectively). The faint band at about 45 kDa in lanes 6–8 is an artefact (possibly CHO IGFBP-3) resulting from the high degree of concentration of these samples. Thus Asn^{89} and Asn^{109} appear to be invariably occupied by glycan chains, suggesting that the two naturally occurring glycoforms of hIGFBP-3, which are invariably seen in the serum and in cell culture medium, are generated by variable glycosylation of Asn^{172}.

ALS binding characteristics of IGFBP-3 glycoforms

The relative abilities of the IGFBP-3 glycoforms to form ternary complexes with ALS in the presence of 50 ng IGF-I are shown in Fig. 3. The dose–response curves of the various glycoforms indicated that the absence of any

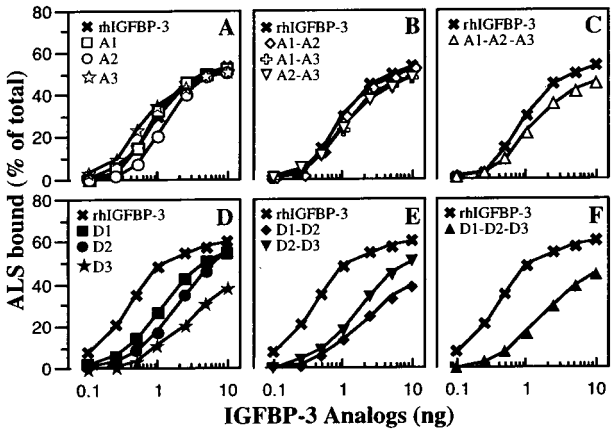

Figure 3 Formation of the ternary complex in the presence of various IGFBP-3 glycoforms. [^{125}I]ALS was incubated with increasing amounts of each IGFBP-3 in the presence of excess (50 ng) IGF-I, as described in Materials and Methods. The upper panels (A, B and C) show binding curves for IGFBP-3 glycoforms in which the Asn residue has been substituted with Ala, and the lower panels (D, E, F) show binding curves for IGFBP-3 glycoforms in which the Asn residue has been substituted with Asp. Nomenclature for the mutant proteins is the same as for Fig. 1, except that D denotes substitution with Asp. The data presented compare single *N*-glycosylation site mutants (panels A and D), double mutants (panels B and E) and triple mutants (panels C and F) with fully glycosylated rhIGFBP-3. Mutant D1–D3 was excluded from the assay because of insufficient protein. Data are the average of duplicate assay determinants and similar results were obtained in at least one other independent measurement for the Ala mutants.

one (Fig. 3A), two (Fig. 3B) or all three (Fig. 3C) *N*-linked carbohydrates had little effect on ALS binding. In contrast, when the Asn residue of the *N*-glycosylation sites was substituted with Asp instead of Ala, there was a marked decrease in ALS binding (Fig. 3D–F). The double mutant, D1–D3, is not shown in Fig. 3E, as there was insufficient recombinant protein for the assay, because of a very low level of expression.

We determined the displacement of [^{125}I]ALS from ternary complexes formed in the presence of 50 ng IGF-I and 1 ng IGFBP-3 glycoforms, by increasing concentrations of unlabelled ALS. Representative Scatchard plots derived from these binding curves are shown in Fig. 4, with slopes essentially identical for all mutants. The relative affinities of ALS are summarised in Table 1. Although the association constants for ALS appeared slightly higher in the glycosylation variants compared with rhIGFBP-3, analysis of variance indicated that the association constant was not significantly changed by any mutation (K_a (rhIGFBP-3)=12·5 ± 4·1 l/nmol; mean K_a (all mutants)=22·1 ± 3·0 l/nmol). There was no statistically significant difference either between rhIGFBP-3 and each glycosylation variant or among the glycosylation variants.

Cell binding characteristics of the IGFBP-3 glycoforms

The interaction between the endogenously produced IGFBP-3 glycoforms and the cell surface of the CHO transfectants was examined (Fig. 5). Cells expressing A3 were excluded because later passage cells were no longer secreting the protein. Cell-surface associated forms of all the various glycoforms tested were detectable. To examine the effect of each carbohydrate chain on the distribution of IGFBP-3 between the cell surface and the culture medium, we expressed cell surface binding of each mutant protein as a ratio of the secreted concentration. When the findings were analysed in this way, all but one glycoform (A1–A2) showed significantly more cell-surface association than fully glycosylated IGFBP-3, and the non-glycosylated protein (A1–A2–A3) showed the greatest cell

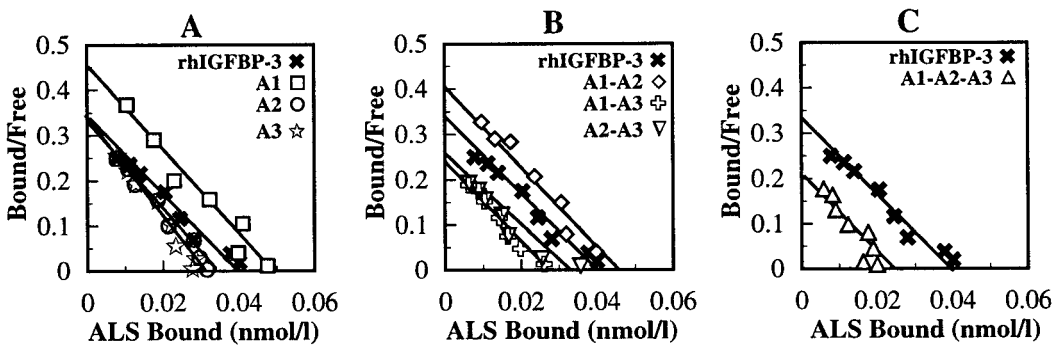

Figure 4 Scatchard plots of ALS binding to various IGFBP-3 glycoforms in the presence of excess (50 ng) IGF-I. Competition for the binding of [^{125}I]ALS to 1 ng IGFBP-3 by increasing concentrations (2·5–200 ng) of ALS was performed as described in Materials and Methods. Representative Scatchard plots derived from these displacement curves are shown, comparing single *N*-glycosylation site mutants (panel A), double mutants (panel B) and triple mutants (panel C). Nomenclature for the proteins is the same as in Fig. 1. The plots shown are representatives of three independent measurement for each protein.

Table 1 Binding parameters for the formation of ternary complexes between IGF-I, ALS, and various glycoforms of IGFBP-3. Ternary complex formation was determined by the binding of [^{125}I]ALS to 1 ng each IGFBP-3 analogue in the presence of 50 ng IGF-I. The molecular weight of ALS was taken as 85 000. Values are means ± S.E.M. for three independent measurements and analysis of variance indicated no significant differences among the various proteins

IGFBP-3 analogue	Association constant (K_a) (l/nmol)
rhIGFBP-3	12·5 ± 4·1
A1	19·6 ± 5·3
A2	24·9 ± 12·9
A3	17·5 ± 2·8
A1–A2	20·1 ± 6·8
A1–A3	22·6 ± 9·1
A2–A3	26·0 ± 15·1
A1–A2–A3	23·8 ± 6·9

binding. We have previously shown that rhIGFBP-3 on the cell surface of CHO transfectants is displaceable by IGF-I (Firth *et al.* 1998); similar displacement of the various IGFBP-3 glycoforms by IGF-I was observed (data not shown).

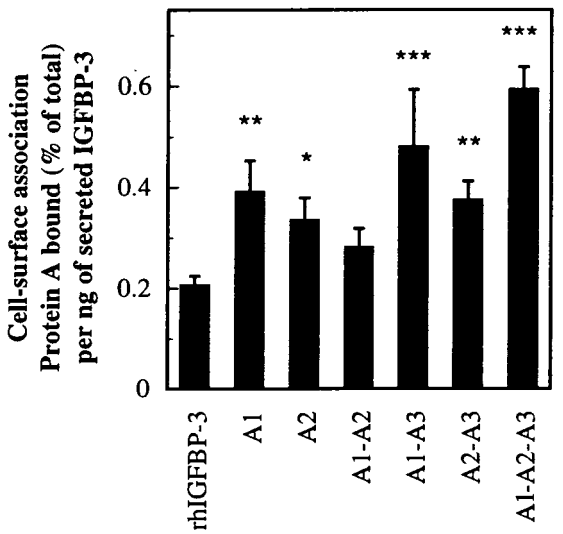

Figure 5 Association of IGFBP-3 glycoforms to the cell surfaces of transfected CHO cells. Serum-free media conditioned by the cultures of transfected cells were collected and soluble IGFBP-3 determined by RIA. The monolayer cultures of these cells were then incubated with IGFBP-3 antiserum or normal rabbit serum, followed by [^{125}I]Protein A incubation, as described in Materials and Methods. The cells were solubilised and the radioactivity in the cell lysates determined. Specific [^{125}I]Protein A binding (minus non-specific binding in the presence of normal rabbit serum) expressed as a percentage of total counts added is indicative of cell-surface associated IGFBP-3. Data shown are ratios of cell-surface associated forms to soluble forms of IGFBP-3 and are the mean ± S.E. of two independent experiments with triplicate measurements each. Significant effects compared with rhIGFBP-3: *P<0·05, **P<0·005 and ***P<0·0001.

Discussion

The post-translational modification of proteins by glycosylation confers heterogeneity resulting in different physical and biochemical properties (for reviews, see Lis & Sharon 1993, Opdenakker *et al.* 1993). Carbohydrate units on glycoproteins can regulate the folding, conformation and intracellular traffic and localisation of the protein; they may act as recognition determinants or contribute to binding sites in protein–protein, protein–cell and cell–cell interactions. In addition, they can confer protection against proteolysis and may act as clearance markers and hence determine the lifetime of the glycoprotein in the circulation.

Although non-glycosylated IGFBP-3 expressed in *E. coli* has been used extensively for various studies, the effect of gycosylation either in total or at any one site has not been examined. In this study, we have compared IGFBP-3 glycoforms derived from the same cellular source. The post-translational process of glycosylation does not appear to have a gross effect on the overall folding or assembly of the nascent IGFBP-3 polypeptide or in directing IGFBP-3 secretion from transfected CHO cells. Although the lack of *N*-glycosylation of IGFBP-3 at any site does not prevent its secretion, subtle effects on secretion cannot be ruled out. Indeed, the difference in expression levels of each recombinant protein may be due to such subtle effects on secretion, differential stability of the recombinant proteins or slight differences in immunoreactivity. However, the levels of recombinant proteins in cell lysates were all lower than the corresponding amount secreted (data not shown), indicating that there is no blockade of secretion.

We have shown that there is approximately 4–5 kDa of carbohydrate at each of the three *N*-glycosylation sites (Asn^{89}, Asn^{109} and Asn^{172}). Consistent with the findings in most glycoproteins, the glycosylation at any one site in IGFBP-3 was not dependent on the occupancy of either or both of the other sites. There is variable glycosylation at Asn^{172}, which was also not influenced by occupancy or non-occupancy of the other sites. Previous studies have shown that hIGFBP-3 treated with *N*-glycanase under non-reducing conditions resulted in a single band of 37 kDa on SDS–PAGE analysis. The size of the protein decreased further to 30 kDa when deglycosylation was carried out under reducing conditions (Tressel *et al.* 1991). On the basis of this observation and our finding that the 40–45 kDa fully glycosylated IGFBP-3 is attributable to variable occupancy of Asn^{172}, it would appear that the carbohydrate attachment site at Asn^{172} is accessible to *N*-glycanase, and that the site at either Asn^{89} or Asn^{109} is not accessible unless the protein is first unfolded.

Table 2 Predicted glycosylation sites in human IGFBPs-1 to -6

	N-glycosylation[a]	*O*-glycosylation[b]
IGFBP-1	—	Thr^{27}, Ser^{95}
IGFBP-2	—	—
IGFBP-3	Asn^{89}, Asn^{109}, Asn^{172}	—
IGFBP-4	Asn^{204}	—
IGFBP-5	—	Thr^{103}, Thr^{104}, Thr^{111}
IGFBP-6	—	Thr^{119}, Thr^{121}, Thr^{122}, Thr^{212} Ser^{120}, Ser^{124}, Ser^{208}

[a]Each IGFBP amino acid sequence was scanned for potential *N*-glycosylation sites (Asn-X-Ser/Thr) using ScanProsite (Appel *et al.* 1994). [b]Potential *O*-glycosylation sites were predicted using NetOGlyc 2.0 (Hansen *et al.* 1998).

Among the IGFBPs, only IGFBP-3 and IGFBP-4 have predicted *N*-glycosylation sites (Table 2). The two forms (24 and 28 kDa) of IGFBP-4, expressed by the rat neuroblastoma cell line, B104, were reduced to 24 kDa when treated with endoglycosidase F (Ceda *et al.* 1991), thus suggesting variable occupancy at the *N*-glycosylation site; similar IGFBP-4 glycoforms are also expressed in human cell lines (Durham *et al.* 1994). Potential *O*-linked attachment sites are predicted for IGFBPs-5 and -6, which are known to be *O*-glycosylated (Bach *et al.* 1992, Conover & Kiefer 1993), and IGFBP-1, which is potentially *O*-glycosylated, as it was reported to contain 4·3% carbohydrate and did not bind to concanavalin A (Lee *et al.* 1993). In contrast, there are no predicted *O*-glycosylation sites on IGFBP-3 and, as the non-glycosylated form (A1–A2–A3) migrated at a molecular mass similar to the predicted mass of the core protein, it appears that the carbohydrate chains of IGFBP-3 are all *N*-linked.

The binding of [^{125}I]IGF-I to all eight IGFBP-3 glycoforms as assessed by ligand blotting suggests that glycosylation *per se* is not essential for IGF-I binding activity. It is interesting to note that previous studies have shown that the IGF-I binding affinity of non-glycosylated, recombinant IGFBP-3 produced by *E. coli* is not significantly different from fully glycosylated, recombinant IGFBP-3 derived from transfected CHO cells (Conover 1991, Sommer *et al.* 1993); therefore, the 'all-or-none' state of glycosylation has no effect on IGF-I binding.

We have previously reported that a highly basic region in the carboxy-terminal region of IGFBP-3 is essential for ALS binding; mutagenesis of this region of IGFBP-3 reduced its ALS affinity by 90% (Firth *et al.* 1998). In this study, we have shown that the carbohydrate chains on IGFBP-3 do not actively participate in ALS binding. Furthermore, they do not appear to modulate the ALS binding affinity, as all the different glycoforms displayed similar affinities. When the Asn residue of the *N*-linked glycosylation sites was substituted with a negatively charged Asp instead of the non-charged Ala, all the glycosylation variants showed five- to tenfold decrease in ALS binding compared with fully glycosylated IGFBP-3. Given the results obtained with the Ala-containing mutants, this effect is most likely to be due to the introduction of charged Asp residues, rather than to the lack of glycosylation. Assuming that the binding site for ALS is the IGF-I–IGFBP-3 binary complex (Baxter *et al.* 1992), the decreased ALS binding by the Asp-containing mutants may be secondary to a defect in IGF-I binding. However, a direct effect on ALS affinity is more likely, as ALS interacts with basic residues on IGFBP-3 (Firth *et al.* 1998), and the addition of negative charge to the binding protein would tend to inhibit this interaction. Regardless of whether the defect lies in IGF-I or ALS binding, it is evident that replacing any one of Asn^{89}, Asn^{109} or Asn^{172} with Asp causes a relatively minor change in IGFBP-3, resulting in modulation of affinity rather than abolition of function.

In addition to modulating activity, the carbohydrate chains on glycoproteins may act as recognition determinants in protein–protein and protein–cell interactions (Lis & Sharon 1993). Besides binding to its ligands (IGF-I and ALS), IGFBP-3 is also known to associate with cell surfaces, and it has been suggested that this form of IGFBP-3 may be involved in cellular actions that occur independently of the IGF-I receptor. Cell-associated forms of IGFBP-3 were present in CHO cell lines transfected by each glycosylation mutant. This is consistent with our previous observation that an IGFBP-3 variant which has the central, non-conserved region (and hence, the glycosylation sites) deleted is still capable of associating with cell surfaces (Firth *et al.* 1998). A previous report that recombinant non-glycosylated IGFBP-3 expressed in *E. coli* can associate with bovine fibroblast cells (Conover 1991) supports this notion. Although the amount of cell-associated IGFBP-3 detected is variable among the glycoforms, the ratios of cell-surface associated protein to soluble protein (secreted into the medium) of the various IGFBP-3 glycoforms are significantly higher ($P<0·05$) than that of fully glycosylated IGFBP-3 with the exception of A1–A2 ($P=0·16$). This would imply that the proportion of IGFBP-3 that is associated with the cell surface is increased with deglycosylation, as seen by the approximately threefold increase in the proportion of cell-associated forms of the non-glycosylated mutant, A1–A2–A3. Therefore, if the extent of glycosylation on IGFBP-3 varies under different physiological conditions, this may well influence the distribution of IGFBP-3 between the circulation, extracellular milieu and cell surfaces. Further studies with preparations of pure IGFBP-3 glycoforms, which will require higher levels of protein expression than are currently available, may provide more insight into this proposed modulatory role of carbohydrates in IGFBP-3.

Considering that one-third of the molecular mass of natural IGFBP-3 consists of carbohydrates, which would have a considerable surface area compared with the protein backbone, it is perhaps surprising that the overall function

is not affected more substantially by their presence or absence – that is, that they are not absolutely required for any of the functions tested to date. This lack of major effect suggests that the carbohydrates are not essential components of the ligand binding sites on the molecule. In this regard, it is relevant to compare IGFBP-3 with one of the other components of the ternary complex, ALS (Baxter & Martin 1989), which has approximately 20% of its molecular mass contributed by carbohydrate (Baxter *et al.* 1989). We have recently shown that the enzymatic removal of sialic acid from ALS resulted in a two- to threefold decrease in affinity for IGFBP-3, whereas complete deglycosylation of ALS abolished its ability to form ternary complexes (Janosi *et al.* 1999). It is likely that the sialylation status of ALS modulates its binding affinity by varying the overall negative charge of the molecule; it is known that the interaction between IGFBP-3 and ALS involves charge interactions (Baxter 1990). Further studies of ternary complex formation with various combinations of both IGFBP-3 and ALS glycoforms may give us better understanding of the modulating role of carbohydrates in the ternary complexes.

In summary, all three *N*-glycosylation sites on IGFBP-3 are used as oligosaccharide acceptor sites although there is variable occupancy at Asn^{172}; the process of glycosylation adds approximately 4, 4·5 and 5 kDa at Asn^{89}, Asn^{109} and Asn^{172} respectively. Although the carbohydrates are not essential components of the binding sites on IGFBP-3 for IGF-I, ALS or the cell surfaces, the level of glycosylation has the potential to modulate the cell-binding activity of IGFBP-3. It remains to be tested whether the modulatory role of the carbohydrate chains extends to the rates of clearance or protease-susceptibility of IGFBP-3 present in different physiological and pathological conditions.

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