N-Linked Glycosylation and Sialylation of the Acid-labile Subunit

ROLE IN COMPLEX FORMATION WITH INSULIN-LIKE GROWTH FACTOR (IGF)-BINDING PROTEIN-3 AND THE IGFs*

(Received for publication, April 29, 1998, and in revised form, November 20, 1998)

Jackie B. M. Janosi, Sue M. Firth, Judy J. Bond, Robert C. Baxter, and Patric J. D. Delhanty‡

From the Kolling Institute of Medical Research, University of Sydney, Royal North Shore Hospital, St Leonards, New South Wales 2065, Australia

Over 75% of the circulating insulin-like growth factors (IGF-I and -II) are bound in 140-kDa ternary complexes with IGF-binding protein-3 (IGFBP-3) and the 84–86-kDa acid-labile subunit (ALS), a glycoprotein containing 20 kDa of carbohydrate. The ternary complexes regulate IGF availability to the tissues. Since interactions of glycoproteins can be influenced by their glycan moieties, this study aimed to determine the role of ALS glycosylation in ternary complex formation. Complete deglycosylation abolished the ability of ALS to associate with IGFBP-3. To examine this further, seven recombinant ALS mutants each lacking one of the seven glycan attachment sites were expressed in CHO cells. All the mutants bound IGFBP-3, demonstrating that this interaction is not dependent on any single glycan chain. Enzymatic desialylation of ALS caused a shift in isoelectric point from 4.5 toward 7, demonstrating a substantial contribution of anionic charge by sialic acid. Ionic interactions are known to be involved in the association between ALS and IGFBP-3. Desialylation reduced the affinity of ALS for IGFBP-3-IGF complexes by 50–80%. Since protein glycosylation is often modified in disease states, the dependence of IGF ternary complex formation on the glycosylation state of ALS suggests a novel mechanism for regulation of IGF bioavailability.

Insulin-like growth factors (IGF)1 I and II are peptide hormones that regulate the differentiation and proliferation of a large number of cell types and also have a role in glucose homeostasis (1). At least 75% of the total circulating IGFs are carried in 130–150-kDa ternary complexes containing IGF-binding protein-3 (IGFBP-3) (2) and an 85-kDa glycoprotein, the acid-labile subunit (ALS) (3). Recently, IGFBP-3 was also shown to form a similar ternary complex with IGFs and ALS in serum (4). It is thought that the size of the ternary complex restricts the passage of IGFs to target cells, while free IGFs, or IGFs in binary complexes with IGFBPs, can cross the capillary endothelial barrier. Therefore, ALS regulates the hypoglycemic and mitogenic potential of the circulating IGFs via the formation of the ternary complexes. Furthermore, ALS plays a vital role in maintaining a circulating store of the IGFs, IGFBP-3, and possibly IGFBP-5, by significantly increasing their serum half-lives (5, 6).

Despite the importance of the ternary complexes in regulating serum IGF bioactivity, nothing is known about the structural aspects of ALS that enable it to interact with IGFBPs. There are two features of ALS structure that may play a part. First, the protein backbone of ALS is made up of repeating blocks each containing 24 amino acids, of which 6 are typically leucine residues. This places ALS in the leucine-rich repeat superfamily of proteins (7), all of which are involved in protein-protein interactions (8). Second, serum ALS is heavily and heterogeneously glycosylated with N-linked glycan chains (3), and glycosylation is known to influence the interactions of many proteins.

Electrophoretic studies have shown that human ALS circulates as two glycoforms. Serum-purified ALS displays a characteristic doublet on SDS-PAGE at 84–86 kDa, which is reduced to a single band of less than 70 kDa after enzymatic removal of the N-linked sugars (3). There are seven consensus N-linked sugar attachment sites within the amino acid sequence of ALS that are conserved between primate and rodent (7, 9–11). One site occurs almost in the center of the sequence, and a cluster of three sites is found toward each terminus. Between six and seven bands are observed upon partial deglycosylation of ALS derived from serum, suggesting that multiple sites are used (12).

Although there are no studies that directly identify physical features of ALS involved in ternary complex formation, there is evidence that charge-charge interaction exists between ALS and the IGF-IGFBP-3 binary complex. Polyanions, polycations, and increasing ionic strength all decrease the affinity of ALS for IGFBP-3 (13, 14). Recently we have shown that the removal of basic residues in the carboxyl-terminal region of IGFBP-3 decreased its affinity for ALS by 90%, indicating the importance of positive charge in this region (15). From these observations, it is likely that negative charges on ALS may be involved in the interaction. At physiological pH, ALS binds tightly to weak anion exchange columns, indicating that it has a net negative charge (3).

Since carbohydrates are a potential source of negative charge in glycoproteins, as well as being involved in protein interactions, we have investigated whether N-linked sugars on ALS play a role in the formation of ternary complexes between IGFs, IGFBP-3, and ALS.

* This work was supported by Grants 940447 and 960875 from the National Health and Medical Research Council, Australia. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† To whom correspondence should be addressed. E-mail: delhanty@med.usyd.edu.au.
‡ The abbreviations used are: IGF, insulin-like growth factor; ALS, acid-labile subunit; IGFBP, insulin-like growth factor-binding protein; Endo F, endo-β-N-acetylglucosaminidase; PNGase F, peptide-N-acetylglucosaminidase; MANase III, N-acetylmuraminidase III; O-glycosidase, endo-α-N-acetylgalactosaminidase; DANA, 2,3-dehydro-2-deoxy-N-acetylneuraminic acid; CHO, Chinese hamster ovary; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; α-MEM, α-modified Eagle’s medium; CHAPS, 3-[3-cholamidopropyl]dimethylammoniomethyl]-1-propanesulfonic acid.

© 1999 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in U.S.A.
Experimental Procedures

Preparations of natural human ALS, human IGFBP-3, and rabbit antiserum against IGFBP-3 were similar to those used in previous studies (3, 16). ALS was radioiodinated and purified by ion-exchange chromatography as described previously (17). IGF-I (Genentech, San Francisco, CA) was iodinated and cross-linked to IGFBP-3 as in previous studies (18). Restriction enzymes and materials for site-directed mutagenesis were from Promega Corp. (Madison, WI). Bovine serum albumin (BSA; radiommunossay grade, fraction V), y-globulin, hexamethrine bromide (Polybrene), dexamethasone, hypoxanthine, xanthine, thymidine, and mycophenolic acid were purchased from Sigma. Aminopterin was obtained from Life Technologies Inc.. Nucleoside-free \( \alpha \)-modified Eagle’s medium (\( \alpha \)-MEM) and fetal calf serum were from CytoSystems (New South Wales, Australia). Centricon 30 microconcentrators were obtained from Amicon (Beverly, MA). \( n \)-Octyl glucoside, \( O \)-glycosidase (endo-\( \alpha \)-N-acetylgalactosaminidase), and peptide-\( N \)-glycosidase F (PNGase F; peptide-\( N \)-(acetyl-\( \beta \)-glucosaminyl)-asparagine amide) were obtained from Boehringer Mannheim. \( N \)-Acetylgala-

Enzymatic Deglycosylation and Desialylation of ALS

Characterization of ALS Glycosylation—PNGase F (5 units) and NANase III (25 milliunits) were used to remove \( N \)-linked sugars and sialic acids, respectively. Reactions contained \( \sp{125}I \)ALS (4 \( \times \) \( 10^7 \) cpm, \( \sim 40 \) ng), 50 mM sodium phosphate buffer (pH 6.5), and 0.1\% (w/v) \( \sp{125}I \)ALS in 50 mM sodium phosphate buffer, pH 6.5, containing 0.0005\% (w/v) \( \sp{125}I \)ALS was treated with 640 milliunits of Endo F/\( \sp{125}I \)ALS was incubated for an additional 1–2 h at 37 °C. To investigate O-glycosylation, \( \sp{125}I \)ALS (\( \sim 40 \) ng) was treated at 37 °C with 5 units of PNGase F with the addition of 12.5 milliunits of NANase III after 8 h, then 2 milliunits of \( O \)-glycosidase 3 h later. The mixture was subsequently incubated for an additional 12–16 h at 37 °C.

Enzymatic Deglycosylation for IGFBP-3 Binding Studies—A series of increasingly deglycosylated \( \sp{125}I \)ALS preparations was generated by incubation with 2.3, 11.7, 29.3, and 58.6 milliunits of Endo F/ng of \( \sp{125}I \)ALS in 50 mM sodium phosphate buffer, pH 6.5, containing 0.0005\% (w/v) \( \sp{125}I \)ALS. Reactions were set up without enzyme as controls. To investigate O-glycosylation, \( \sp{125}I \)ALS (10,000 cpm, 0.035 milliunits of NANase III/ng of \( \sp{125}I \)ALS was prepared in 50 mM sodium phosphate buffer (pH 6.5) at 37 °C for 16 h. In experiments where DANA was used, it was added at 1 mmol/millilunit NANase III to specifically inhibit the sialidase activity of NANase III.

Site-directed Mutagenesis of ALS—The ALS cDNA was generated by reverse transcriptase-polymerase chain reaction from normal human liver using RNA as described previously (10). This cDNA was inserted into the BamHI/XhoI site of pSELECT (Promega Corp.) for mutagenesis according to the manufacturer’s recommended protocol. Mutagenic deoxyoligonucleotides were used to generate seven different ALS cDNAs, each containing Asn to Ala codon substitutions at one of the seven consensus \( N \)-glycan linkage sites (Asn \( \beta \); Asn \( \gamma \); Asn \( \delta \); Asn \( \epsilon \); Asn \( \zeta \); Asn \( \iota \); Asn \( \kappa \)). Substituted nucleotides are shown in lowercase. Oligonucleotides were synthesized using an Oligo 1000 DNA Synthesizer (Beckman, Palo Alto, CA). The mutations were confirmed by sequencing (T7 sequencing kit; Amersham Pharmacia Biotech), and then the cDNAs were excised from pSELECT and inserted into the Nhel/SalI site of pMSG (Amersham Pharmacia Biotech), an expression vector that contains the constitutively active and glucocorticoid-inducible murine mammary tumor virus promoter.

Cell Culture and Transfections—Chinese hamster ovary (CHO) cells were transfected with either wild-type or mutant ALS expression constructs, or pMSG alone using the Polybrene/MeSO technique (19). pMSG contains the guanine phosphoribosyltransferase gene, which confers resistance to mycophenolic acid. Stable transfecants were selected for 3 weeks in \( \alpha \)-MEM supplemented with 10\% fetal calf serum, 25 \( \mu \)g/ml mycophenolic acid, 2 \( \mu \)g/ml aminopterin, 250 \( \mu \)g/ml xanthine, 15 \( \mu \)g/ml hypoxanthine, and 10 \( \mu \)g/ml thymidine. Some ALS transfec-

SDS-PAGE Analysis—Radioiodinated ALS (5,000 cpm, \( \sim 0.5 \) ng) in Laemmli buffer was loaded, without heat treatment, onto 7.5\% Ready gels (Bio-Rad) and electrophoretically separated under nonreducing conditions. The gels were then dried and exposed to Hyperfilm MP (Amerham, Bucks, UK) overnight at \( \sim 80 °C \).

Isoelectric Focusing of ALS—Preparations of \( \sp{125}I \)ALS (10,000 cpm, \( \sim 1 \) ng), untreated or treated with NANase III, were used to assay for \( \sp{125}I \)ALS containing 4\% (w/v) CHAPS (BDH Ltd, Poole, UK), 10 mM Tris-HCl, pH 7.2, and 2\% (v/v) glycerol and electroforetically separated under nonreducing conditions. The gels were then dried and exposed to Hyperfilm MP (Amerham, Bucks, UK) overnight at \( \sim 80 °C \).

Electrophoretic Analyses

SDS-PAGE Analysis—Radioiodinated ALS (5,000 cpm, \( \sim 0.5 \) ng) in Laemmli buffer was loaded, without heat treatment, onto 7.5\% Ready gels (Bio-Rad) and electrophoretically separated under nonreducing conditions. The gels were then dried and exposed to Hyperfilm MP (Amerham, Bucks, UK) overnight at \( \sim 80 °C \).

Isoelectric Focusing of ALS—Preparations of \( \sp{125}I \)ALS (10,000 cpm, \( \sim 1 \) ng), untreated or treated with NANase III, were used to assay for \( \sp{125}I \)ALS containing 2\% (w/v) CHAPS (BDH Ltd, Poole, UK), 10 mM Tris-HCl, pH 7.2, and 2\% (v/v) glycerol and electroforetically separated under nonreducing conditions. The gels were then dried and exposed to Hyperfilm MP (Amerham, Bucks, UK) overnight at \( \sim 80 °C \).

Isoelectric focusing was performed in a Multiphor II electrophoresis unit (Amerham Pharmacia Biotech). A broad pl isoelectric focusing calibration kit (Amerham Pharmacia Biotech) was run in parallel with the...
ALS Glycosylation and Ternary Complex Formation

ALS samples. The theoretical pl for the human ALS protein backbone was calculated using the program ISOELECTRIC from the Genetics Computer Group, Inc. (Madison, WI).

Binding Assays

Gel Filtration Studies with Mutated ALS—Size fractionation chromatography was used to determine whether the seven site-directed mutant recombinant ALS species lacking individual consensus N-glycosylation sites were able to form ternary complexes (18). Briefly, cross-linked [125I]IGF-IGFBP-3 (1 × 10^6 cpm) was incubated for 30 min at 25 °C with conditioned medium containing 50 ng of mutant ALS equilibrated in 50 mM sodium phosphate buffer (pH 6.5) containing 1% (w/v) BSA, in a total volume of 200 μl. The mixture was then injected into a Superose-12 column (Amersham Pharmacia Biotech), and 0.5-ml fractions of eluate were collected at a flow rate of 1 ml/min. The degree of conversion from binary to ternary complex was evaluated by the shift of radioactivity from fractions corresponding to 50 kDa to those corresponding to 140 kDa (18).

Lectin Solution Binding Assay—A lectin solution binding assay was used to indicate the presence or absence of sialic acids on [125I]ALS after enzymatic desialylation with NANase III and deglycosylation with PNGase F as described above. Identical reactions were set up without enzymes as controls. The assay was modified from that of Abidi et al. (20). Briefly, approximately 35,000 cpm [125I]ALS (3.5 ng), either treated with enzyme or untreated control, was incubated for 1 h with 2 μg of the sialic acid-specific lectin from T. mobilensis in 50 mM sodium phosphate buffer with 0.01% (w/v) BSA, pH 6.5 at 22 °C (final volume 100 μl). [125I]ALS complexed to the lectin was then precipitated using γ-globulin (35 μl) and 6% polyethylene glycol (1 ml) for 10 min at 4 °C. Both BSA and γ-globulin were acid-hydrolyzed and dialyzed against water to remove contaminating sialic acids (21). The tubes were then spun at 3500 rpm in a swing bucket centrifuge for 10 min at 22 °C. The radioactive pellet in each tube was measured as a percentage of the total radioactivity added and these data were used to generate histograms. Nonspecific binding was determined to be the radioactivity measured when no lectin was added to the tube during the 1-h incubation. Control and desialylated forms of [125I]ALS gave nonspecific binding ranging from 10% to 14% of total, and the PNGase F deglycosylated forms of [125I]ALS gave approximately 28% of total.

Solution Binding Assay and Scatchard Analysis—Solution binding assays were carried out as described previously (16). Briefly, 10,000 cpm [125I]ALS, either treated with enzyme or untreated control, was incubated for 2 h with 10 ng of IGF-I or -II and a range from 0 to 10 ng of IGFBP-3 in 50 mM sodium phosphate buffer, pH 6.5, at 22 °C (final volume 0.3 ml). ALS complexed to IGFBP-3 was then precipitated using IGF-I antisera. The radioactivity in each tube was measured as a percentage of the total radioactivity added, and these data were used to generate binding curves. Endo F (58.3 milliunits/ng ALS) was added to a control untreated [125I]ALS preparation during the 2-h incubation to ensure that the presence of the enzyme did not adversely affect complex formation. Nonspecific binding was calculated as the percentage of radioactivity present after precipitation when there was no IGFBP-3 in the reaction mixture. For the Endo F deglycosylation experiments, nonspecific binding ranged from 3% to 18% of total for the partially deglycosylated forms and was approximately 30% of total for the fully deglycosylated forms. For the the NANase III binding curves, nonspecific binding was between 3% and 8% of the total. Scatchard analysis was carried out as described previously (16), except that IGF-I, IGF-II, and IGFBP-3 were held constant at 1 ng/0.3 ml. ALS was added over the range of 0–200 ng/0.3 ml.

Statistical Analyses—Binding curve data were analyzed by repeated measures analysis of variance, followed by Fisher’s protected least significant difference test, using Statview 4.02 (Abacus Concepts Inc., Berkeley, CA). The value was considered significant if the p value was less than 0.05.

RESULTS

Glycosidase Characterization of ALS Carbohydrates—ALS is estimated to have ~20 kDa of N-linked carbohydrate, but the possible presence of O-linked sugars has not been investigated.

![Isoelectric focusing profiles of native and NANase III-treated ALS.](image)

![bind to IGFBP-3.](image)

![Statistical Analyses—Binding curve data were analyzed by repeated measures analysis of variance, followed by Fisher’s protected least significant difference test, using Statview 4.02 (Abacus Concepts Inc., Berkeley, CA).](image)
To address this, [125I]ALS was treated with NAnase III, which removes sialic acid, and O-glycosidase, which hydrolyzes some of the common core sugars of O-linked glycans. SDS-PAGE analysis was then used to identify shifts in the apparent size of treated ALS. Untreated [125I]ALS (Fig. 1, lane 1) appears as a single band of approximately 85 kDa. The diffuse nature of the band may be explained by poor resolution of the 84–86-kDa doublet. The apparent size of ALS after NAnase III treatment (lane 2) was decreased compared with untreated ALS. The approximate size difference is 2–3 kDa, consistent with the removal of 5–15 sialic acid moieties. ALS was also treated with PNGase F, an effective amidase that cleaves all types of N-linked sugars, to show the previously reported size shift to approximately 68–70 kDa (lane 3). This apparent molecular mass is very close to the predicted value of 66 kDa for the amino acid backbone of ALS. However, there is no difference in apparent size between ALS treated with PNGase F alone (lane 3) and ALS treated with PNGase F, NAnase III and O-glycosidase (lane 4). These results suggest that ALS carries sialic acids that are predominantly or entirely attached to the N-linked sugar chains.

However, it is possible that, following O-glycosidase treatment, electrophoresis did not resolve the small shift in size predicted if O-linked sugars are a minor component of ALS carbohydrate. To investigate this further, we used a lectin binding assay (Fig. 2) to determine whether the sialic acids present on ALS could be accounted for by the N-linked sugars alone. A sialic acid-specific lectin derived from T. mobilisens was used to precipitate [125I]ALS, which was either untreated or had been desialylated with NAnase III or deglycosylated with PNGase F. Using this assay, we found that desialylation and deglycosylation caused 96 ± 4% and 88 ± 5% loss of binding, respectively. Since there was no significant difference between these values (p = 0.1, t test), we conclude that all of the sialic acids removed by NAnase III treatment are derived from N-linked sugars on ALS.

Charge Contribution of the Sialic Acids to ALS—We used isoelectric focusing to establish the net negative charge that the sialic acids would contribute to ALS. Untreated [125I]ALS and NAnase III-treated [125I]ALS were separated by charge on immobilized pH gradient strips and visualized by autoradiography (Fig. 3). Untreated ALS was found to have at least six distinctly charged isoforms in the pI range of 4.5–5.2. Although the NAnase III-treated ALS was less well resolved than the control, it is clear that the pI of the desialylated [125I]ALS had shifted, as expected, toward a more neutral pI value of approximately 5.5–7.0. Furthermore, the distinct series of bands observed in the control disappeared after NAnase III treatment. Therefore, the six distinct isoforms of ALS are consistent with a series of differentially sialylated ALS molecules.

Complete Deglycosylation of ALS Abolishes Ternary Complex Formation—In order to identify a potential biological role for ALS glycosylation, we investigated the effect that N-linked deglycosylation of ALS has on the formation of ternary complexes between the IGFs, IGFBP-3, and ALS. Enzymatic methods were used to deglycosylate serum-derived ALS. After radiolabeling, [125I]ALS was treated with Endo F, an endoglycosidase mixture able to remove most types of N-linked sugars. The preparation of Endo-F used contained only trace amounts of the common core sugars of N-linked carbohydrates. To limit the potentially confounding effects of converting the carbohydrate-anchoring asparagine to an aspartic acid (22, 23), SDS-PAGE analysis and autoradiography were used to monitor the degree of deglycosylation attained (Fig. 4, A and C), and the resulting preparations were used in a solution binding assay to measure their ternary complex forming ability (Fig. 4, B and D). Fig. 4A depicts the SDS-PAGE analysis of [125I]ALS treated under conditions that fully removed N-linked sugars. The binding curves (Fig. 4B) reveal that there is no specific binding of either the IGF-I-IGFBP-3 or the IGF-II-IGFBP-3 binary complex with the fully deglycosylated ALS preparation. Therefore, the complete removal of N-linked sugars from ALS by Endo F abolishes ternary complex formation.

We also generated a series of partially deglycosylated [125I]ALS preparations (Fig. 4C). As seen in the binding curves depicted in Fig. 4D, the most effectively deglycosylated preparations were the least able to form complexes with IGF-I and IGFBP-3, whereas the preparation with almost fully intact N-glycosylation was virtually indistinguishable from the control. Therefore, although the complete removal of N-linked sugars from ALS abolishes ternary complex formation, ALS with some intact N-linked sugars is able to form the complex.

No N-Linked Sugar Is Solely Responsible for ALS Binding Activity—The binding ability of a protein can be substantially altered by the removal of a single N-linked carbohydrates chain (24). Since we demonstrated that the N-linked sugars on ALS have a role in ternary complex formation, we used site-directed mutagenesis to investigate whether any single glycan chain was solely responsible for the binding of ALS to the IGF-
and IGFBP-3 complex. The primary sequence of ALS has seven consensus NX(S/T) sites for N-linked sugar attachment. Therefore, we constructed a series of seven mutant ALS cDNAs. The mutations were at Asn<sup>37</sup> → Ala, Asn<sup>34</sup> → Ala, Asn<sup>241</sup> → Ala, Asn<sup>448</sup> → Ala, Asn<sup>627</sup> → Ala and Asn<sup>653</sup> → Ala. The mutant ALS cDNAs were then transfected into CHO cells, and the proteins were harvested. All transfections resulted in conditioned medium containing measurable amounts of immunoreactive ALS, as determined by radioimmunoassay (17). From this, we concluded that no single N-linked sugar is an absolute requirement for secretion of ALS by CHO cells.

The conditioned media from the transfectants were then used to determine the ternary complex forming ability of the various glycosylation mutant ALS proteins. Ternary complex formation was evaluated by size shift of a cross-linked [125I]IGF-I-IGFBP-3 complex on a Superose-12 gel permeation column (Fig. 5). The peak radioactivity found in fraction 26 with the pMSG control (Fig. 5A) is consistent with the size of the IGF-1-IGFBP-3 binary complex (18). In contrast, media from wild-type ALS transfected CHO cells caused a clear shift in the peak fraction of radioactivity from fraction 26 to fraction 23 (Fig. 5A). Similar chromatography profiles were obtained for all the mutant ALS forms and are depicted in Fig. 5B, except Asn<sup>448</sup> → Ala, which gave similar results in a separate assay (data not shown). In each case, the shift in peak radioactivity from fraction 26 to fraction 23 is consistent with the formation of ternary complexes. Therefore, we conclude that all of the mutant ALS proteins are able to bind to the IGF-1-IGFBP-3 complex. Hence, no single N-linked sugar of ALS can solely account for the loss of activity observed in the enzymatic deglycosylation experiments.

**NANase III-treated ALS Has a Reduced Affinity for IGFBP-3**—Having established that ALS glycosylation has a role in ternary complex formation, we investigated whether negatively charged sialic acid moieties may be specifically involved in ALS binding to the IGF-IGFBP-3 complex. Binding curves for the interaction of NANase III-treated ALS and untreated ALS with IGFBP-3 are depicted in Fig. 6. In the presence of IGF-I, desialylation of ALS significantly shifted the binding curve to the right (p = 0.02) (Fig. 6B), indicative of a decrease in ALS binding. A similar result was obtained in the presence of IGF-II (p = 0.005) (Fig. 6C). Therefore, ternary complex formation in the presence of either IGF-I or IGF-II is reduced after enzymatic desialylation of ALS. One of the desialylated preparations used in this study was also used in the IEF depicted in Fig. 3, demonstrating an increase in pI compared with control [125I]ALS and demonstrating that the preparation was fully desialylated under the conditions used.

The specificity of NANase III treatment was tested by blocking its sialidase activity with the sialic acid analog DANA. ALS treated with NANase III in the presence of DANA showed IGFBP-3 binding activity that was not significantly different from the untreated protein (Fig. 7). This suggested that NANase III specifically removed the sialic acid moieties from ALS and that these sialic acid moieties were necessary for normal ternary complex formation.

Finally, Scatchard analysis was used to determine the difference in affinities of NANase III-treated ALS and untreated ALS for IGF-I-IGFBP-3 and IGF-II-IGFBP-3 complexes. Representative Scatchard plots for IGF-I ternary complex formation are shown in Fig. 8, indicating a decrease in affinity of ALS for IGFBP-3 after desialylation. Representative association constants derived from two experiments are shown in Table I. These experiments show that desialylated ALS has a 50–80% reduction in affinity for IGFBP-3 in the presence of IGF-I or IGF-II compared with normally sialylated ALS.
FIG. 8. Reduced affinity of desialylated ALS for ternary complex formation. Representative Scatchard analyses comparing the affinities of untreated (panel A) and NANase III-treated (panel B) ALS for 1 ng of IGFBP-3 in the presence of 10 ng of IGF-I.

Table I
Summary of affinities of untreated and NANase III-treated ALS for IGF-IGFBP-3 complexes

| IGF       | Untreated | NANase III-treated |
|-----------|-----------|--------------------|
|            | Ka (nM⁻¹) | Ka (nM⁻¹)           |
| IGF-I      | 7.4       | 3.4                |
| IGF-II     | 7.1       | 2.7                |
| IGF-II     | 4.6       | 2.3                |
| IGF-II     | 5.8       | 1.1                |

DISCUSSION

The interaction of ALS with IGF-IGFBP-3 complexes in the serum is believed to regulate both the function and the stability of the bound IGFs and IGFBP-3. Previous reports indicate that the affinity of IGFBP-3 for its ligands may be affected by post-translational modifications such as limited proteolysis (25–27). In this study, we found that the carbohydrate chains on ALS play an influential role in determining its interaction with IGFBP-3. Our data therefore suggest that modifications to ALS as well as IGFBP-3 may be important in fine-tuning the bioavailability of the IGFs.

In addition to the seven putative N-linked carbohydrate attachment sites (NXS/T) in the ALS sequence, there are two putative O-linked glycan sites. The first, at Ser⁴⁶⁰, conforms to a mucin-type O-glycosylation site as predicted by NetOglyc 2.0 (28) and is part of the N-linked carbohydrate attachment site at Asn⁵⁸. The second, FT³⁴⁸PQP, corresponds to the XTPXP sequence recently described as the minimal requirement for O-linked carbohydrates (29) and lies adjacent to the putative N-linked glycosylation site, Asn⁴⁴⁸. Treatment of ALS with O-glycosidase, which hydrolyzes Galβ1-3GalNAc, a common core structure of many O-linked glycans (30), had no effect on the molecular mass of ALS as judged by SDS-PAGE analysis. While it is possible that fucose residues may have interfered with the enzyme, or that a shift in size was below the level of detection, the putative O-linked sites do not appear to be occupied by sugar side chains with the common core structure described above. Furthermore, a lectin binding assay that was specific for sialic acids, a common residue on O-linked carbohydrates, also failed to provide evidence for O-linked sugars on ALS.

Therefore, we focused on the N-linked carbohydrates of ALS and their role in ternary complex formation. Enzymatic removal of the N-linked glycans from ALS decreased its ability to form the complex with IGFBP-3 in a manner that was related to the level of deglycosylation. Intermediate levels of ALS deglycosylation, probably involving the loss of more than a single sugar chain, measurably disrupted ternary complex formation, whereas complete removal of the N-linked glycans abolished complex formation. However, when site-directed mutagenesis was used to mutate each of the N-linked attachment sites individually, no single glycan appeared to have a major impact on ALS binding activity. Therefore, the carbohydrates on ALS clearly influence the affinity that ALS has for the IGF-IGFBP-3 complex, although this influence relies on a number of N-linked sugars rather than any single chain. However, it is not clear whether deglycosylated ALS is less able to form the ternary complex because of conformational changes in ALS induced by removal of the glycans or disruption of interactions between the ALS carbohydrates and the other two proteins.

Sialic acids are common anionic residues that can be attached to both N- and O-linked sugars. They can contribute significant charge to glycoproteins as many sialic acids can be attached to one highly branched N-linked sugar. For example, the acute-phase protein α1-acid glycoprotein has a pl of 2–3 mostly due to the large number of sialic acids attached to its highly branched complex N-linked sugars (31). The calculated pl of ALS, based on its amino acid sequence, is 6.56; however, we observed six discrete isoforms of pl 4.6–5.3 by isoelectric focusing. After NANase III treatment, the pl of ALS increased to between 5.5 and 7, close to the predicted value for the amino acid backbone. The contribution of negative charge by the sialic acid may explain, in part, the high affinity with which ALS binds to weak anion exchange columns used in ALS purification (3). Given that the sialic acid contributes significantly to the negative charge on ALS and that the interactions within the ternary complex are dependent on charge, it could be predicted that the sialic acid on ALS would affect the formation of the complex. Indeed, ALS treated with NANase III to remove sialic acid displayed a 50–80% decrease in affinity for the IGF-I and IGF-II binary complexes compared with that of untreated ALS. However, it is noteworthy that desialylation only lowered the affinity of ALS for the IGF-IGFBP-3 complex unlike deglycosylation, which abolished complex formation. This suggests that the effects of ALS glycosylation on IGFBP-3 binding are not entirely due to the negative charges imparted by sialic acid.

The results from the N-linked glycan studies imply that a number of N-linked sugars are required to act in concert to enable ALS to interact with IGFBP-3 and the IGFs, rather than a specific carbohydrate chain being solely responsible. The placement of the glycans within the tertiary structure of ALS may shed light on this finding. In unpublished studies,³ we have modeled the central leucine-rich repeat region of ALS on the only published crystal structure of a leucine-rich repeat protein, the porcine ribonuclease inhibitor (32). If the modeled

³ J. B. M. Janosi, P. A. Ramsland, M. Mott, S. M. Firth, R. C. Baxter, and P. J. D. Delhanty, unpublished data.
structure is a true representation of ALS, then six out of the seven potential N-linked sugar attachment sites lie very close to each other, suggesting a possible clustering of carbohydrate chains. Therefore, the loss of any single N-linked glycan may be compensated by the potentially large number of other carbohydrates in the vicinity. This model also has implications with regard to the sialic acid moieties that we demonstrated to exist on ALS. A lectin solution binding assay suggested that all the sialic acids on ALS are attached to the N-linked sugars. These sialic acid moieties might therefore result in a region of negative charge where the N-linked sugars are concentrated.

The binding affinity of ALS to the IGF-IGFBP-3 complex is relatively weak, 1–2 orders of magnitude less than the affinity of IGFBP-3 for either of the IGFs (13), and in this sense ALS binding is the limiting step in ternary complex formation. This suggests that modulation of ALS affinity might directly influence complex formation, and thus the bioavailability of IGFs. Isoelectric focusing indicates that ALS purified from normal serum already exists as a number of differently sialylated isoforms. If the degree of ALS sialylation is subject to physiological regulation, as described for other proteins (31), the potential exists for modulation of formation or stability of the ternary complexes. This might occur in addition to the previously described modulation of the circulatory concentration of the ALS protein itself (33) through cytokine suppression (34) or in patients who are critically ill (35) or have hepatic cirrhosis (36).

In summary, we have found that the N-linked carbohydrates on ALS are a requirement for the formation of complexes with IGFBP-3 and IGFs. We have also shown that the removal of sialic acids from ALS significantly reduces the affinity of ALS for binding to IGFBP-3. Since the glycosylation of secreted proteins are often modified in certain physiological and pathological states, the modification of ALS glycosylation has the potential to be an important factor in the regulation of IGF access to the tissues.

REFERENCES
1. Jones, J. I., and Clemmons, D. R. (1995) Endocr. Rev. 16, 3–34
2. Rajaram, S., Baylink, D. J., and Mohan, S. (1997) Endocr. Rev. 18, 801–831
3. Baxter, R. C., Martin, J. L., and Beniac, V. A. (1989) J. Biol. Chem. 264, 11843–11848
4. Twigg, S. M., and Baxter, R. C. (1998) J. Biol. Chem. 273, 6074–6079
5. Guler, H. P., Zapf, J., Schmidt, C., and Froesch, E. R. (1989) Acta Endocrinol. 121, 753–8
6. Lewitt, M. S., Saunders, H., and Baxter, R. C. (1993) Endocrinology 133, 1797–1802
7. Leong, S. R., Baxter, R. C., Camerato, T., Dai, J., and Wood, W. I. (1992) Mol. Endocrinol. 6, 870–876
8. Köbe, B., and Deisenhofer, J. (1995) Curr. Opin. Struct. Biol. 5, 409–416
9. Dai, J., and Baxter, R. C. (1992) Biochem. Biophys. Res. Commun. 188, 304–309
10. Delhanty, P., and Baxter, R. C. (1996) Biochem. Biophys. Res. Commun. 227, 897–902
11. Boisclair, Y. R., Seto, D., Hsieh, S., Hurst, K. R., and Ooi, G. T. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 10028–10033
12. Liu, F., Hintz, R. L., Khare, A., Diaz-Augustine, R. P., Powell, D. R., and Lee, P. D. (1994) J. Clin. Endocrinol. Metab. 79, 1883–1886
13. Holman, S. R., and Baxter, R. C. (1996) Growth Regul. 6, 42–47
14. Baxter, R. C. (1990) Biochem. J. 271, 773–7
15. Firth, S. M., Ganeshrasad, U., and Baxter, R. C. (1998) J. Biol. Chem. 273, 2631–2638
16. Baxter, R. C., Bayne, M. L., and Cascieri, M. A. (1992) J. Biol. Chem. 267, 60–65
17. Baxter, R. C. (1990) J. Clin. Endocrinol. Metab. 70, 1347–1353
18. Baxter, R. C. (1988) J. Clin. Endocrinol. Metab. 67, 265–272
19. Chaney, W. G., Howard, D. R., Pollard, J. W., Sallustio, S., and Stanley, P. (1986) Somat. Cell Mol. Genet. 12, 237–244
20. Abidi, F. E., Bishayee, S., Bachhawat, B. K., and Bhadra, R. (1987) Anal. Biochem. 166, 257–66
21. Dorai, D. T., Bachhawat, B. K., and Bishayee, S. (1961) Anal. Biochem. 113, 130–7
22. Tarentino, A. L., Gomez, C. M., and Plummer, T. J. (1985) Biochemistry 24, 4665–71
23. Maley, F., Trimble, R. B., Tarentino, A. L., and Plummer, T. J. (1989) Anal. Biochem. 180, 195–204
24. Avvakumov, G. V., Warnem-Rodenhiser, S., and Hammond, G. L. (1993) J. Biol. Chem. 288, 862–866
25. Baxter, R. C., and Skriver, L. (1993) Biochem. Biophys. Res. Commun. 196, 1267–1273
26. Bang, P., Brismar, K., and Rosenfeld, R. G. (1994) J. Clin. Endocrinol. Metab. 78, 1119–1127
27. Baxter, R. C., Suikkari, A. M., and Martin, J. L. (1993) Biochem. J. 294, 847–852
28. Hansen, J. E., Lund, O., Telstrup, N., Gosley, A. A., Williams, K. L., and S., B. (1988) Glycoconj. J. 15, 115–130
29. Yoshida, A., Suzuki, M., Ikenaga, H., and Takeuchi, M. (1997) J. Biol. Chem. 272, 18684–18688
30. Umemoto, J., Bharvanandan, V. P., and Davidson, E. A. (1977) J. Biol. Chem. 252, 8609–8614
31. Van Dijk, W., Havenaar, E. C., and Brinkman-van der Linden, E. C. M. (1995) Glycoconj. J. 12, 227–33
32. Kabe, B., and Deisenhofer, J. (1993) Nature 366, 751–756
33. Delhanty, P. J. D. (1998) The Regulation and Actions of ALS: Molecular Mechanisms to Regulate the Activities of Insulin-like Growth Factors (Takano, K., Hizuka, N., and Takahashi, S. I., eds) pp. 135–143, Elsevier Science B. V., Amsterdam
34. Delhanty, P. J. D. (1998) Biochem. Biophys. Res. Commun. 243, 269–272
35. Baxter, R. C., Hawker, F. H., To, C., Stewart, P. M., and Holman, S. R. (1997) Growth Regul. 7, 1–10
36. Donaghby, A. J., and Baxter, R. C. (1996) Baillieres Clin. Endocrinol. Metab. 10, 421–446