A soluble form of the insulin-like growth factor II/mannose 6-phosphate receptor (sIGF-II/MPR) is present in fetal bovine serum and carries mature 7.5-kDa insulin-like growth factor II (IGF-II) and at least 12 different high molecular weight (Mr) IGF-II isoforms (Valenzano, K. J., Remmler, J., and Lobel, P. (1995) J. Biol. Chem. 270, 16441–16448). In this study, we used gel filtration and anion exchange chromatographies to resolve the isoforms into eight fractions that were characterized with respect to their biochemical, biophysical, and biological properties. Each fraction contained one to three major protein species with apparent sizes ranging from 11 to 17 kDa by SDS-polyacrylamide gel electrophoresis. The 11-kDa species contains no post-translational modifications and consists of an extended IGF-II backbone terminating at Gly-87. The remaining high Mr IGF-II isoforms are also composed of an 87-amino acid IGF-II peptide backbone but contain increasing amounts of sialated, O-linked sugars. Plasmon resonance spectroscopy experiments revealed that all the high Mr isoforms and mature 7.5-kDa IGF-II bound to immobilized recombinant soluble human IGF-I receptor, recombinant human IGF-binding protein 1, and sIGF-II/MPR with similar kinetics. In addition, radiolabeled tracer experiments demonstrated that both mature and high Mr IGF-II isoforms have similar binding profiles in fetal bovine serum and have similar affinities for IGF-II-binding proteins secreted from human fibroblasts. Finally, the biological activity of high Mr IGF-II was shown to be similar to or slightly better than mature IGF-II in stimulating amino acid uptake in fibroblasts and in inducing myoblast differentiation.

Insulin-like growth factor II (IGF-II)1 is a peptide hormone related to insulin that is present at high levels during fetal development. Genetic evidence suggests that IGF-II plays an important role in prenatal growth and that its actions are mediated through the IGF-I receptor and another yet characterized receptor (1–3). In addition, IGF-II also binds to the IGF-II/mannose 6-phosphate receptor (IGF-II/MPR). This protein has two distinct functions: first, it mediates the biosynthetic targeting of mannose 6-phosphate containing lysosomal enzymes to the lysosome (for review, see Ref. 4); second, it mediates endocytosis of IGF-II, resulting in its delivery to the lysosome and subsequent degradation (5, 6).

The availability of IGF-II for interaction with its receptors is regulated by its association with binding proteins. To date, six different IGF-binding proteins have been characterized and have molecular masses ranging from 22.8 to 31.3 kDa (for review, see Refs. 7 and 8). In addition, a soluble form of the IGF-II/MPR (sIGF-II/MPR) has been detected in serum and urine (9–13). This protein retains IGF-II and Man-6-P binding activities and is abundant (~5 μg/ml) in fetal bovine serum (FBS) (14). In FBS, ≤5% of IGF-II is in the free state, with the remainder circulating as high molecular weight (Mr) complexes with the binding proteins and sIGF-II/MPR (14). Complex formation with the binding proteins has been shown to both inhibit and potentiate IGF-II’s interactions with its receptors as well as increase the serum half-life of the ligand (for review, see Ref. 8).

IGF-II is synthesized as a preproprotein (15). After cleavage of its signal sequence, the COOH-terminal 88 residues (E-peptide) of the proprotein are removed to yield the 67-residue, mature 7.5-kDa IGF-II. The mature polypeptide can be subdivided into four domains (B, C, A, and D domains) that are homologous to the B and A chains of mature insulin and the B, C, A, and D domains of IGF-I. In addition, the existence of extended forms of IGF-II has been documented (for review, see Refs. 16–18), although their physiological role in the biology of IGF-II is not clear.

To date, high Mr isoforms of IGF-II have been detected in human serum (19–21), cerebrospinal fluid (22), and malignant tissue extracts (23–25). Human IGF-II variants that are substituted at Ser-29 and Ser-33 with Arg-Leu-Pro-Gly and Cys-Morpholinoethanesulfonic acid, respectively, with or without E-peptide extensions, have also been identified (21, 26). In addition, two IGF-II species with apparent Mr values of 15,000 and 11,500 were

7.5-kDa IGF-II; rhIGF-II(1–88), rhIGF-II extended to Lys-88; rhIGF-I, recombinant human insulin-like growth factor I; sIGF-IR, soluble IGF-I receptor; IGFBP-1, insulin-like growth factor binding protein 1; FBS, fetal bovine serum; BSA, bovine serum albumin; HBS, HEPES-buffered saline; PBS, phosphate-buffered saline; AEX, anion exchange; AIB, a-aminoisobutyric acid; MES, 2-(N-morpholino)ethanesulfonic acid; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

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# The abbreviations used are: IGF-II, insulin-like growth factor II; IGF-II/MPR, insulin-like growth factor II/mannose 6-phosphate receptor; sIGF-II/MPR, soluble IGF-II/MPR; rhIGF-II, recombinant human insulin-like growth factor II; rhIGF-II(1–88), rhIGF-II extended to Lys-88; rhIGF-I, recombinant human insulin-like growth factor I; sIGF-IR, soluble IGF-I receptor; IGFBP-1, insulin-like growth factor binding protein 1; FBS, fetal bovine serum; BSA, bovine serum albumin; HBS, HEPES-buffered saline; PBS, phosphate-buffered saline; AEX, anion exchange; AIB, α-aminoisobutyric acid; MES, 2-(N-morpholino)ethanesulfonic acid; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.
purified from human serum Cohn fraction IV, and represent 87–88-amino acid polypeptides that are glycosylated at Thr-75 (18).

We recently described the isolation of a high M, IGF-II fraction from FBS (14). In this study we have separated the high M, IGF-II isoforms into eight different fractions. Each fraction is composed of a COOH-terminally extended IGF-II that terminates at Gly-87 and contains variable amounts of O-linked sugars. Our studies indicate that all high M, isoforms are similar to 7.5-kDa IGF-II in terms of both receptor/binding protein interactions and biological activity.

EXPERIMENTAL PROCEDURES

Materials—FBS was obtained from Inovar. Yeast phosphomannan was kindly provided by Dr. M. E. Slodki (U.S. Department of Agriculture, Peoria, IL). Phosphomannan affinity resin was prepared by coupling cyanogen bromide-activated Sepharose CL-6B (Pharmacia) to phosphomannan core as described previously (27). Recombinant human IGF-IIs with and without a 21-amino acid E-peptide extension (rHIGF-IIepe and rHIGF-II), respectively, were as described (28, 29). Recombinant human IGF-I was obtained from either Pharmacia or ProPepTech, Inc. (Rocky Hill, NJ). 125I-Labeled IGF-I (specific activity 120–125.7 μCi/μg) was prepared as described previously (30). Soluble recombinant human IGF-II receptor (sIGF-IR) and recombinant human IGF-binding protein-1 (IGFBP-1) were kindly provided by M. Jansson and C. Dyring (Pharmacia Upjohn, Stockholm, Sweden) and will be described elsewhere. α-(methyl)-[3H]Aminoisobutyric acid (8 Ci/mmol) was purchased from DuPont NEN. Reagents for surface plasmon resonance spectroscopy, including sensor chips CM5 (certified), Surfactant P20, N-hydroxysuccinimide, N-ethyl-N′-[3-dimethylaminopropyl]carbodiimide hydrochloride, and ethanolamine-HCl were from Pharmacia Biosensor AB (Uppsala, Sweden). All other chemicals were reagent grade.

Buffers—HEPES-buffered saline 1 (HBS-1): 150 mM NaCl, 2 mM EDTA, 20 mM HEPES, pH 7.2; HBS-2: 150 mM NaCl, 3.4 mM EDTA, 0.05% Surfactant P20, 10 mM HEPES, pH 7.2; acid buffer: 60 mM ammonium acetate, 340 mM acetic acid, pH 4.0; propion buffer: 20 mM 1-propanol, 8 mM ammonium acetate, 42 mM acetic acid, pH 4.0; anion exchange (AEX) buffer. After addition of 0.1 volumes 10 M NaCl, fractions were pooled as indicated under “Results,” and desalted as described above. Proteins were rechromatographed on the anion exchange column as described above and fractions pooled as indicated under “Results.” Some fractions were further purified by anion exchange chromatography using a different buffer system. Here, samples were exchanged into propanol/acetate, dried, and resuspended in AEX buffer 2 (100 μl). Anion exchange chromatography was as described above but used a linear gradient of 8–28% AEX buffer 2B (80–280 mM NaCl) over 19 min, and 20 μl fractions were collected. Appropriat fractions were pooled as indicated under “Results,” exchanged into propanol/acetate, and stored at −80 °C.

Radiolabeling of IGF-II—Peaks A–I representing the different bovine IGF-II isoforms and 7.5-kDa rhIGF-II were iodinated using Na125I (NEZ-033A, DuPont NEN) and lactoperoxidase (Boehringer Mannheim) as described previously (31) except the reactions were quenched with tyrosine. Radiolabeled high M, IGF-II was separated from free iodine using prepacked G-25 columns (PD10, Pharmacia) with PBS as the mobile phase. BSA was included as a carrier in the column buffer of early experiments, but later omitted due to its uptake of label. As each of the radiolabeled high M, IGF-II isoforms eluted as a single peak after analytical gel filtration chromatography on a Superose 12 column (1.6 × 85 cm), these were used without further purification (data not shown). Radiolabeled 7.5-kDa IGF-II (peak A and rhIGF-II) was applied to a Sephadex G50 fine column (Pharmacia, 0.7 × 28 cm) using PBS as the mobile phase. Three radioactive peaks were detected, and fractions comprising the second peak (IGF-IImonomer) were pooled. The specific activities ranged from 120 to 770 Ci/mmol for high M, IGF-II and was −25 Ci/mmol for rhIGF-II.

Deglycosylation of High M, IGF-II—All digests were carried out at 37 °C. For the experiment shown in Fig. 5, samples of iodinated high M, IGF-II and rhIGF-II (~30,000 cpm/reaction) were incubated with either 2.5 milliunits of recombinant neuraminidase (Glyko), 1 milliunit of recombinant O-glycosidase (Glyko), or both enzymes in a total volume of 10 μl. Digests were carried out for 1 h. For double digests, samples were first incubated with neuraminidase for 1 h before addition of O-glycosidase. Digested samples were resolved by 12.5–17.5% polyacrylamide gel under nonreducing conditions. For the isoelectric focusing experiment shown in Fig. 6, approximately 80,000 cpm of radiolabeled IGFs were used per reaction. Samples were digested with 10 milliunits of neuraminidase (Arthrobacter; Boehringer Mannheim) for 2 h followed by the addition of 2 milliunits of O-glycosidase (Oxford GlycoSystems) and incubation for 18 h. Digests were carried out in a total volume of 48 μl of glycosidase buffer B. Both untreated and digested samples were resolved by isoelectric focusing on precast gels (pH 3–7) according to the manufacturer (Novex). Isoelectric gels were fixed in 3.5% sulfosalicylic acid, 11.5% trichloroacetic acid for 30 min prior to staining.

Surface Plasmon Resonance Spectroscopy—A Pharmacia BIAcore system was used to monitor interaction of the high M, IGF-II isoforms with the sIGF-II/MPR, sIGF-IR, and IGFBP-1. Details of the instrumentation and analytical methods have been described in several recent reports (32, 33). Briefly, standard amine coupling procedures were used for the immobilization of ligand (sIGF-II/MPR, sIGF-IR, or IGFBP-1) to the dextran matrix of the sensor chip surface (34). Surfaces were activated with a 35-μl injection of a 0.05 M N-hydroxysuccinimide, 0.2 M N-ethyl-N′-[3-dimethylaminopropyl]carbodiimide hydrochloride mixture according to the manufacturer using a flow rate of 5 μl/min HBS-2. sIGF-II/MPR and IGFBP-1 were diluted to a final concentration of 5 μg/ml in 20 mM sodium acetate, pH 4.7, before injection over the activated dextran matrix. sIGF-IR was diluted to a final concentration
Characterization of High Mr, IGF-II

of 30 µg/ml in 0.2 M acetic acid before injection. Typically 2000–4000 resonance units of sIGF-II/MPR or sIGF-IR or 300–600 resonance units of IGFBP-1 were immobilized per experiment. Unreacted carbonyl groups on the dextran matrix were blocked with a 35 mM solution of 0.2 M solution of ethanolamine. Sensorgrams were generated using a flow rate of 2 ml/min. Typically, 2-µl dilutions of each analyte (rIGF-I, rIGF-II, and different high Mr, IGF-II isoforms) were used in each experiment. The concentrations ranged from 10 to 80 nM for analyte passed over sIGF-II/MPR and IGFBP-1 surfaces. For sIGF-IR surfaces, 25–200 nM or 12.5–100 nM of the ISO-II isoforms or of rIGF-II, respectively, were used. Analytes were applied for 3 min to measure association kinetics and then washed out using PBS–B (0.15 M NaCl, 1 mM CaCl2, 25–100 mM NaHCO3/1 mM CaCl2 on sIGF-IR surfaces.

Kinetic constants were determined using the BLAEvaluation software (version 2.0). Both the association and dissociation phases of the binding curves were fit with simple bimolecular interaction assumptions, using the resonance unit signal as a relative measure of the ligand-analyte complex concentration with respect to time. The association phase of different sensorgrams was fit directly with a nonlinear least squares iterative curve fitting function. The association constants obtained with the four different analyte concentrations were averaged. Dissociation rates were calculated from the highest analyte concentrations used to minimize signal contributions due to rebinding of analyte to ligand at the surface.

Analysis of IGF-II-binding Proteins in FBS—Binding of radiolabeled bovine 7.5-kDa (peak A) or the different high Mr, IGF-II isoforms (peaks B–I) to serum-binding proteins was determined as described previously (14). Briefly, the different radiolabeled IGFs (30,000 cpm) were each incubated with FBS (HyClone; 1 ml). Time course experiments indicated that a 24-h incubation at room temperature was sufficient for both mature and high Mr, IGF-II radiotracers to reach equilibrium with serum-binding proteins (see Ref. 14 and data not shown), thus reflecting the distribution of endogenous IGF-II. The serum was applied to a Superose 12 column (1.6 × 85 cm) and eluted at 1 ml/min with PBST at room temperature as indicated. Fractions (2 ml) were counted for 1 min in a Packard Cobra auto γ-counter.

AIB Uptake Studies—Filoblasts from a normal 18-year-old man (WUMS1) were isolated at Washington University School of Medicine, St. Louis, MO (35). Uptake of AIB in human filoblasts was measured as described previously (36) using an assay volume of 500 µl.

C2I Myoblast Differentiation—The C2I subclone of C2 myoblasts (37) that differ only in insulin or IGF-I to differentiate in vitro was kindly provided by Dr. Peter Rotwein at Washington University School of Medicine, St. Louis, MO. Differentiation in C21 myoblasts was induced as described by Rotwein et al. (38) except that assays were performed in 24-well clusters, using an assay volume of 1.0 ml. After ~70 h, cells were washed and lysed by incubation with 100 µl of Tris/75% for 15–20 min at room temperature. Creatine kinase activity was determined as described and normalized for total protein content, using the BCA protein assay (Pierce). IGF-binding proteins secreted during C2I myoblast differentiation were analyzed by ligand blotting as described previously (35).

Competition Binding Studies—To compare binding of IGF-I, IGF-II, and the different high Mr, IGF-II isoforms to the IGF-binding proteins secreted by human filoblasts, conditioned buffer was collected from WUMS1 filoblasts after incubation in AIB assay buffer for 3 h and clarified by centrifugation. Competition binding studies were performed as described previously (35) with minor modifications. Briefly, filoblast-conditioned buffer was incubated for 1 h at 37 °C with 125I-labeled IGF-I and increasing amounts of unlabeled IGF-I, IGF-II, or high Mr, IGF-II in AIB assay buffer (final volume, 250 µl). AIB assay buffer rather than 0.1 M HEPES, pH 8.0, with 44 mM NaH2PO4, 0.01% Triton X-100, 1 mM NaCl, and 1.0% BSA, and 0.02% NaN3, (35) was used to order to reproduce more closely the conditions under which IGFs and secreted binding proteins interact during the AIB uptake assay. Complexes of binding protein and IGF were precipitated with the addition of 750 µl of 25% polyethylene glycol, 4.0 mg/ml bovine γ-globulin (21). Binding data were analyzed with LIGAND, a computer program developed by Munson and Rodbard (39).

To compare binding of IGF-I, IGF-II, and the different high Mr, IGF-II isoforms to the human IGF-I receptor, IGF-I receptor was purified from human placenta using wheat germ agglutinin-Sepharose chromatography, insulin affinity chromatography, and IGF-I affinity chromatography (30). Competition binding studies were performed as described previously (30). Briefly, IGF-I receptor was incubated overnight at 4 °C with 125I-labeled IGF-I and increasing amounts of unlabeled IGF-I, IGF-II, or high Mr, IGF-II in imidazole binding buffer. Receptor-bound IGF-I was precipitated by the addition of 900 µl of 33.3% polyethylene glycol, 1.5 mg/ml bovine γ-globulin (1:1). Binding data were analyzed by LIGAND.

Other Methods—sIGF-II/MPR concentrations were determined by a two-antibody sandwich enzyme-linked immunosorbent assay as described previously (40). Protein concentrations were determined with BSA standards using the Lowry method (41) adapted to microtiter plates. Molecular concentrations were calculated using the following protein Mr, values: rIGF-I, 7639; rIGF-II, 7475; 7.5-kDa bovine IGF-II (peak B–I), 9578; SDS-PAGE was performed as described (42). Gels were stained with 0.2% Coomassie Brilliant Blue R-250 (Bio-Rad) and/or silver using the Novex Silver Xpress silver staining kit as indicated. Dried radioactive gels were exposed to a phosphor storage screen, scanned, and quantitated using a Molecular Dynamics PhosphorImager 400 and ImageQuant 3.15 software. The molecular mass of the different IGF-II isoforms was determined using both a 292Cf plasma desorption time-of-flight mass spectrometer Bio-Ion 20 (Applied Biosystems, Gothenburg, Sweden) and a VG Quattro electrospray mass spectrometer (Fisons Instruments, Altrincham, United Kingdom). For laser desorption mass spectrometry, samples were dissolved in 0.1% trifluoroacetic acid, applied to nitrocel lulose-coated foil (Applied Biosystems), dried, and analyzed in a positive-ion mode for 1 h with an acceleration voltage of 18 kV. Samples for electrospray mass spectrometry were applied in 50% methanol, 1% acetic acid at a flow rate of 5 µl/min. Amino acid analyses were performed at the Cornell Biotechnology Analytical/Synthesis Facility (Ithaca, NY). Amino-terminal sequencing was performed using automated Edman degradation on a Hewlett-Packard G1000A protein sequencer or a Milligen Biosearch Prosequencer type 6600. Predicted isoelectric points were calculated from amino acid compositions using the University of Wisconsin GCG program (43). The contribution of glycosylation to pI (isoelectric point) was estimated using a pH 2 of 2.6 for sialic acid (44), and ionizable cysteines were excluded from the analysis as all six cysteines present in IGF-II are disulfide bonded.

RESULTS

Isolation and Separation of High Mr, IGF-II—We previously reported the co-purification of sIGF-II/MPR and bound IGF-II from FBS (14). In addition to mature 7.5-kDa IGF-II, we also isolated ~12 different high Mr, IGF-II isoforms that were associated with the soluble receptor in low abundance. In order to investigate the properties of this high Mr, IGF-II fraction in more detail, we modified and scaled-up our original purification scheme to isolate milligram quantities of these IGF-II isoforms (see “Experimental Procedures”). Large-scale isolation of sIGF-II/MPR and bound IGF-II from 50- and 100-liter lots of FBS was achieved by affinity adsorption of receptor to phosphomannan-agarose and elution with mannos 6-phosphate. Mildly acidic gel filtration chromatography was employed to dissociate bound IGF-II from receptor and to resolve high Mr, IGF-II species from sIGF-II/MPR and 7.5-kDa IGF-II. Finally, pooled fractions from the high Mr, IGF-II peak were re-applied to the acidic gel filtration column to remove contaminating sIGF-II/MPR and 7.5-kDa IGF-II. Using this strategy, we have processed ~1000 liters of FBS yielding ~1.5 mg of total high Mr, IGF-II.

The different high Mr, IGF-II isoforms were subfractionated by anion exchange chromatography at pH 8.0. For each 50- or 100-liter preparation, the high Mr, IGF-II peak from the second acid gel filtration step was divided into five to eight fractions, and each was chromatographed on a Mono Q column using an ammonium bicarbonate gradient (Fig. 1). The elution profiles of the different chromatograms were similar, although in general, the eluting fractions (lower Mr) were smaller than the contaminating fractions (larger versus smaller proteins), the earlier eluting gel filtration fractions tended to be biased toward the later (more acidic) eluting ion exchange fractions. The chromatogram depicted in Fig. 1 is of material eluting late in the acidic gel filtration column, and all the peaks of interest are well represented. The anion exchange column fractions were analyzed by SDS-PAGE
and Coomassie staining on 14% gels. The regions labeled A–I contained visualizable protein and sequence analysis on selected peaks (A, B, D, and H) revealed that all contained proteins with the amino-terminal residues AYRPS, identical to 7.5-kDa bovine IGF-II (45).

For further purification, peaks A–I from different anion exchange runs were pooled and rechromatographed (Fig. 2) as described above; fractions were pooled as indicated. In addition, Peaks A, B, D, and H were chromatographed on a Mono Q column at pH 6.0 and eluted with a gradient of sodium chloride and ammonium bicarbonate gradient by a dashed line. The two unlabeled peaks eluting before peak A contained material that appeared similar to peak A by SDS-PAGE but was only faintly detected by Coomassie staining. This was not analyzed further.

The purity of peaks A–I was assessed using SDS-PAGE and silver staining (Fig. 3). Equivalent peaks from different primary anion exchange runs were pooled and re-chromatographed using the conditions described in Fig. 1. Material used for further purification (peaks B, D, and H) or analysis (remaining peaks) is indicated by cross-hatching. Note that peak A was chromatographed using a shallower gradient and is not shown. The absorbance at 280 nm is indicated by a solid line and the ammonium bicarbonate gradient by a dashed line.

Chemical Characterization of High Mr IGF-II—The purity of peaks A–I was confirmed by SDS-PAGE and silver staining (Fig. 4). Peak A consisted of a single protein species that ran identically to the rhIGF-II standard. Subsequent analysis by mass spectrometry and isoelectric focusing (see below) showed that this species represented mature 7.5-kDa bovine IGF-II that was not resolved from the high Mr IGF-II peak by gel filtration chromatography. The remaining peaks contained one to three different protein species with apparent sizes ranging from 11 to 17 kDa.

We previously examined the nature of the increased Mr, seen in the unfractionated high Mr IGF-II pool using a combination of protease and glycosidase sensitivity assays (14). We demonstrated that all proteins comprising this pool contain a common peptide backbone that extends beyond Glu-67 and that this extension is modified with various amounts of sialated, O-linked sugars resulting in the pool’s heterogeneity. To confirm and extend these earlier studies, the fractionated high Mr isoforms in peaks B–I were iodinated and then digested with neuraminidase and/or O-glycosidase to remove terminal sialic acids and O-linked core disaccharides, respectively (Fig. 5). No shift in the electrophoretic mobility of peak B (Fig. 5) or of rhIGF-II standard (data not shown) was detected. In contrast, digestion of peaks C–I using a combination of neuraminidase and O-glycosidase caused a mobility shift in all the isoforms, resulting in the appearance of a single major species that ran identically to both undigested and digested peak B (arrow). These data suggest that peak B represents an extended, non-glycosylated isoform of high Mr IGF-II and that the species contained in peaks C–I have the same peptide backbone as peak B but are further modified with various amounts of O-linked sugars. Interestingly, double digestion of peak F demonstrated the presence of two bands, one that ran identically to peak B and another that migrated slightly slower. This behavior may represent multiple IGF-II species with slight differences in their peptide backbones (i.e. longer extensions and/or variant internal sequences) or the presence of other post-translational modifications.

The high Mr forms of IGF-II were also digested with the two glycosidases separately (Fig. 5). Treatment with neuraminidase alone caused a small but significant increase in the mobility of all bands indicating the presence of sialic acid. In contrast, treatment with O-glycosidase alone had no effect on any of the high Mr IGF-II isoforms. As O-glycosidase only removes the unmodified disaccharide Galβ1–3GalNAc from threonine or serine residues, these data suggest that essentially all of the O-linked sugars in each of the high Mr IGF-II isoforms are protected with sialic acid.

The contribution of oligosaccharide to the net charge of high Mr IGF-II was investigated using isoelectric focusing. The measured pl of recombinant human IGF-II was ~6.85 (Fig. 6, upper panel), close to the pl of 6.80 predicted from its composition. The minor band in the rhIGF-II lane likely represents contaminating BSA introduced from the column buffer as ascertained by comparison of the iodinated preparation with authentic BSA by SDS-PAGE and isoelectric focusing. Peak A gave a pl of ~6.85 (data not shown), similar to the pl of 6.84 predicted for unmodified mature 7.5-kDa bovine IGF-II. Peak B gave a pl of ~4.85 (Fig. 6, upper panel), which is consistent with the isoelectric point of 4.81 predicted for an unmodified 87-residue extended IGF-II. The remaining bands displayed increasingly acidic pl’s, ranging from ~4.6 to 3.5, respectively (Fig. 6, upper panel). Each sialic acid added to the 87-residue peptide is expected to decrease its isoelectric point 0.12 to 0.23
Thus, the decreasing isoelectric points of peaks C–I are consistent with increasing numbers of sialic acid residues decorating the core O-linked disaccharides. Treatment with both neuraminidase and O-glycosidase reduced all of the high Mr IGF-II isoforms to a major species that had a pI of 4.85 (Fig. 6, lower panel), identical to both undigested and digested peak B (Fig. 6, upper and lower panels, respectively). In addition, we noted that the deglycosylated samples contained minor species with pI values of approximately 5.4, 4.7, and 4.5 (Fig. 6, lower panel). These minor species may represent experimentally (iodination)-induced modifications in the peptide backbone as similar variability was not detected by SDS-PAGE of native or radiolabeled samples (see above). Coomassie staining of isoelectric focusing gels (data not shown) or mass spectrometry (see below). Regardless of the identity of the minor species, the important point is that all of the high Mr IGF-II isoforms contain an identical peptide backbone and that peak B represents non-glycosylated, extended bovine IGF-II.

We next determined the molecular mass of the high Mr IGF-II isoforms using mass spectrometry (Table I). Recombinant human IGF-II isoforms were used for method validation. Mass determinations of 7465 and 9802 for rhIGF-II and rhIGF-II_E88 were included as standards. Precise mass determinations on the other high Mr IGF-II peaks were not possible due to the presence of sugar as deglycosylated peak D appeared identical to peak B by both SDS-PAGE and isoelectric focusing (see above). The measured mass of 9572 for peak B is again consistent with this protein terminating at Gly-87, which would give a predicted mass of 9578. Precise mass determinations on the other high Mr IGF-II peaks were not possible due to the presence of sugar.
Characterization of High \( M_r \) IGF-II

Samples were analyzed as described under “Experimental Procedures.” Mass spectrometry analysis of high \( M_r \) IGF-II isoforms have a common peptide backbone of mature IGF-II and that the heterogeneity observed in the different peaks is due to differences in O-linked glycosylation.

Surface Plasma Resonance Studies—The interactions of high \( M_r \) IGF-II with sIGF-II/MPR, a soluble fragment of recombinant human IGF-I receptor (sIGF-IR), and recombinant human IGF-binding protein 1 (IGFBP-1) were compared with those of mature IGF-II by measuring their association and dissociation rate constants using the BIACore system (see “Experimental Procedures”). Either the individual receptors or binding protein were covalently attached to the dextran matrix of the sensor chip and different concentrations of the IGF-II isoforms (analyte) passed through the flow cell. The signal is proportional to the amount of IGF-II bound to the immobilized sIGF-II/MPR. The regions of the sensorgrams following introduction of analyte were used to determine the association and dissociation rate constants, respectively. Kinetic rate constants for all high \( M_r \) IGF-II isoforms with the two receptors and binding protein are presented in Tables II and III. In addition, equilibrium dissociation constants \( (K_d) \) were determined from the kinetic data and are presented in Table IV.

Our data demonstrate that all of the high \( M_r \) IGF-II isoforms bind with similar affinities as those measured for mature IGF-II (both bovine and human) and reflect nearly identical association and dissociation rate constants. Whereas binding to the sIGF-II/MPR and IGFBP-1 was in the low nanomolar range for all species, interaction with the sIGF-IR was of considerably lower affinity \( (K_d > 35 \text{ nM}) \). In contrast, rhIGF-I displayed 6–10-fold higher affinity for the sIGF-IR than any of the IGF-II isoforms. These data suggest that E-peptide extensions and glycosylation do not influence binding of IGF-II to the sIGF-II/MPR, sIGF-IR, or IGFBP-1.

| Table I | Mass spectrometry analysis of high \( M_r \) IGF-II |
|---|---|
| | Molecule | Deduced | Measured | Range | n |
| rhIGF-II | 7475 | 7465 ± 9 | 2 |
| rhIGF-II \( \text{tag} \) | 9807 | 9802 ± 10 | 2 |
| Peak A | 7552 | 7572 ± 3 | 4 |
| Peak B | 9578 | 9572 ± 5 | 5 |
| Peak D | 10854 | 10854 ± 57 | 1 |
| Peak G | 10360 | 10360–13,160 | 1 |
| Peak I | 10462 | 10,462–12,999 | 1 |

| Table II | Receptor/binding protein association rate constants |
|---|---|
| Ligand | sIGF-II/MPR | sIGF-IR | IGFBP-1 |
| rhIGF-I | ND | 5.07 ± 1.77 | ND |
| rhIGF-II | 10.7 ± 2.0 | 1.47 ± 0.5 | 9.4 ± 3.7 |
| Peak A | 6.1 ± 3.8 | 1.03 ± 0.08 | 5.7 ± 3.5 |
| Peak B | 7.7 ± 2.0 | 0.88 ± 0.17 | 6.0 ± 2.6 |
| Peak C | 8.5 ± 2.0 | 1.06 ± 0.08 | 6.6 ± 1.8 |
| Peak D | 9.4 ± 4.1 | 0.95 ± 0.13 | 7.5 ± 3.5 |
| Peak E | 10.7 ± 5.3 | 1.35 ± 0.55 | 9.3 ± 2.4 |
| Peak F | 13.1 ± 3.5 | 0.99 ± 0.24 | 8.7 ± 3.3 |
| Peak G | 7.3 ± 2.3 | 0.88 ± 0.15 | 7.1 ± 1.2 |
| Peak H | 5.9 ± 0.7 | 1.47 ± 0.81 | 5.8 ± 1.9 |
| Peak I | 9.3 ± 1.7 | 0.97 ± 0.11 | 8.0 ± 2.6 |

| Table III | Receptor/binding protein dissociation rate constants |
|---|---|
| Ligand | sIGF-II/MPR | sIGF-IR | IGFBP-1 |
| rhIGF-I | ND | 2.74 ± 0.27 | ND |
| rhIGF-II | 3.25 ± 0.12 | 4.77 ± 0.73 | 2.59 ± 1.0 |
| Peak A | 2.73 ± 0.38 | 4.90 ± 0.64 | 2.44 ± 0.93 |
| Peak B | 2.69 ± 0.33 | 4.90 ± 0.37 | 2.32 ± 0.82 |
| Peak C | 2.70 ± 0.10 | 4.57 ± 0.13 | 2.26 ± 0.78 |
| Peak D | 2.56 ± 0.22 | 4.95 ± 0.47 | 2.26 ± 0.81 |
| Peak E | 2.60 ± 0.27 | 4.37 ± 0.32 | 2.23 ± 0.68 |
| Peak F | 2.75 ± 0.14 | 5.11 ± 0.64 | 2.25 ± 0.78 |
| Peak G | 2.58 ± 0.08 | 4.79 ± 0.31 | 2.28 ± 0.84 |
| Peak H | 2.39 ± 0.11 | 4.71 ± 0.23 | 2.32 ± 0.8 |
| Peak I | 2.55 ± 0.06 | 4.71 ± 0.29 | 2.27 ± 0.7 |

| Table IV | Receptor/binding protein dissociation rate constants calculated from kinetic data |
|---|---|
| Ligand | Equilibrium dissociation constants |
| sIGF-II/MPR | sIGF-IR | IGFBP-1 |
| rhIGF-I | ND | 5.9 ± 2.2 | ND |
| rhIGF-II | 3.10 ± 0.50 | 35.2 ± 11.5 | 2.80 ± 0.35 |
| Peak A | 5.45 ± 2.27 | 44.5 ± 5.1 | 4.83 ± 1.25 |
| Peak B | 3.64 ± 0.74 | 57.7 ± 16.4 | 4.00 ± 0.52 |
| Peak C | 3.30 ± 0.78 | 43.3 ± 3.8 | 3.45 ± 0.68 |
| Peak D | 3.18 ± 1.64 | 52.6 ± 9.0 | 3.20 ± 0.61 |
| Peak E | 2.97 ± 1.61 | 36.4 ± 16.5 | 2.40 ± 0.36 |
| Peak F | 2.20 ± 0.53 | 54.8 ± 19.9 | 2.47 ± 0.23 |
| Peak G | 3.43 ± 1.10 | 54.9 ± 6.5 | 3.15 ± 0.80 |
| Peak H | 4.07 ± 0.32 | 38.2 ± 17.6 | 4.63 ± 2.87 |
| Peak I | 2.80 ± 0.53 | 49.3 ± 8.0 | 2.87 ± 0.38 |
Characterization of High Mr, IGF-II

Serum Binding Profiles of High Mr, IGF-II—Radiolabeled tracer experiments were performed to: 1) estimate the fraction of high Mr, IGF-II carried by the sIGF-II/MPR in FBS and 2) compare the endogenous distribution of high Mr, and mature IGF-II among different serum-binding proteins in FBS. Serum was incubated with each of the eight different iodinated high Mr, IGF-II isoforms or mature IGF-II under conditions that allow approach to equilibrium (see Ref. 14; similar kinetics were observed for mature and high Mr, IGF-II) and fractionated by gel filtration chromatography as described under “Experimental Procedures.”

Comparison of mature 7.5-kDa bovine IGF-II (peak A) and two high Mr, IGF-II isoforms (peaks B and E, representing extended, nonglycosylated and extended, glycosylated isoforms, respectively) demonstrated similar binding profiles with essentially no tracer eluting as the free polypeptide (Fig. 7, upper three panels). In addition, ~35% of the tracer in each case was associated with a peak that elutes identically to sIGF-II/MPR standard (Fig. 7, upper three panels, region 1). Likewise, values ranging from 31–99% for radiotracer association with the sIGF-II/MPR peak were determined for the other high Mr, IGF-II isoforms (data not shown). Most of this IGF-II binding activity represents authentic sIGF-II/MPR as receptor-depleted serum has greatly diminished activity (Fig. 7, lower panel, region 1). Interestingly, while enzyme-linked immunosorbent assay analysis demonstrated complete removal of receptor (data not shown), 1.5–5% of the IGF-II tracer still migrated in this region when the different high Mr, IGF-II isoforms were tested (Fig. 7, lower panel and data not shown). Thus, while another protein makes a minor contribution to IGF-II binding in this region, these results clearly demonstrate that the endogenous sIGF-II/MPR carries ~1/3 of the total IGF-II (both mature and high Mr, isoforms) in FBS.

The different radiolabeled IGF-II isoforms also eluted in two other regions. Region 2 represents tracer associated with a binding protein(s) that elutes similarly to the 150-kDa standard and may represent ternary complex formation between tracer, IGFBP-3, and the acid labile subunit (8). Region 3 represents tracer association with any of the five remaining IGF-binding proteins that have apparent sizes ranging from 25 to 40 kDa. The similarity in the binding profiles for the different high Mr, IGF-II isoforms and mature bovine IGF-II suggests that these molecules distribute similarly among different IGF-binding proteins in FBS. It is worth noting that peak E appears to have diminished binding activity in region 2 compared with the other IGF-II isoforms (Fig. 7). While this could be interpreted as reflecting differences in affinity for binding proteins, the apparent decreased magnitude of region 2 may simply represent incomplete resolution of the different peak E-binding protein complexes in regions 2 and 3 on the gel filtration column.

Biological Activity of High Mr, IGF-II—It has been demonstrated that uptake of the amino acid analog, a-oaminosobutyric acid (AIB), is stimulated by IGF-I in human fibroblasts (36). Fig. 8A, shows a representative experiment comparing stimulation of AIB uptake by rhIGF-I, mature bovine IGF-II, and high Mr, IGF-II isoforms C–H. In six experiments, the ED50 of rhIGF-I-stimulated AIB uptake in human fibroblasts was 0.29 ± 0.03 nM, as reported previously (36). Stimulation of AIB uptake by mature and high Mr, IGF-II is summarized in Fig. 8B. The ED50 of IGF-II-stimulated AIB uptake was 1.41 ± 0.06 nM, 4.8-fold higher than that of IGF-I. Surprisingly, the ED50 values for stimulation of AIB uptake by high Mr, IGF-II ranged from 0.59 ± 0.04 nM to 0.96 ± 0.09 nM, suggesting that they were slightly more effective than mature IGF-II in this system. Maximal stimulation of AIB uptake (mean ± S.E.) by rhIGF-I (2.42 ± 0.12-fold, n = 6), mature IGF-II (2.36 ± 0.12-fold, n = 5), and high Mr, IGF-II (2.35 ± 0.16-fold to 2.59 ± 0.10-fold, n = 5) did not differ significantly in these experiments.

The small, but reproducible, differences between high Mr, IGF-II in stimulating AIB uptake in fibroblasts might be explained by reduced affinity of high Mr, IGF-II for binding proteins secreted by the fibroblasts, resulting in greater availability for IGF receptor binding. To investigate this possibility, binding of rhIGF-I, mature bovine IGF-II, and high Mr, IGF-II isoforms C–H to the IGF-binding proteins secreted by fibroblasts was investigated. The major IGF-binding protein present in fibroblast-conditioned buffer is IGFBP-3 (35). Fig. 9 shows displacement curves from a representative study. The mean dissociation constant derived from LIGAND analysis of IGF-I binding to secreted binding proteins in two independently-performed experiments was 0.21 nM, slightly higher than reported previously (35), IGF-II bound with a very similar affinity, whereas high Mr, IGF-II bound with a similar or slightly higher affinity. These results indicate that the enhanced effectiveness of high Mr, IGF-II in stimulating AIB uptake cannot be explained by reduced affinity for fibroblast IGF-binding proteins.

The biological activity of high Mr, IGF-II was also investigated using the C2I subclone of C2 myoblasts selected by
Pinset et al. (37). This cell line requires only insulin or IGF-I to differentiate in vitro. In preliminary experiments, we found that human or bovine IGF-II also induced terminal differentiation, as evidenced by equivalent maximal induction of creatine kinase activity and myotube formation. Fig. 10 shows a representative experiment comparing the dose-dependent induction of creatine kinase activity in C2I myoblasts by rhIGF-I, mature bovine IGF-II, and high $M_s$ IGF-II isoforms D through G. In three experiments, the ED$_{50}$ of IGF-II-induced differentiation was 2.2–2.8-fold higher than that of rhIGF-I. The high $M_s$ IGF-II isoforms were at least as or slightly more effective than mature IGF-II in this system.

Rotwein et al. (38) have shown that the expression of IGFBP-5 is stimulated during C2I myoblast differentiation. To examine IGF-binding protein expression during differentiation, media were collected from C2I myoblasts after incubation for ~70 h with rhIGF-I, mature bovine IGF-II, or high $M_s$ IGF-II isoforms C–H and analyzed by ligand blotting. As shown in Fig. 11, the major IGF-binding protein stimulated during differentiation migrated with an apparent molecular mass of 23.3 kDa. We confirmed by immunoblot analysis that this binding protein was IGFBP-5 (data not shown). Similar amounts of IGFBP-5 were present in conditioned media from myoblasts incubated with rhIGF-I, IGF-II, and high $M_s$ IGF-II. An IGF-binding protein with an apparent molecular mass of 27.6 kDa was present in conditioned media from myoblasts incubated without IGF or insulin and did not change in amount during differentiation.
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with slightly higher affinity than mature IGF-II, a result which may explain their slightly enhanced biological activity.

DISCUSSION

These studies were initiated to characterize individual high Mr IGF-II isoforms. Preliminary analysis of the unfractionated high Mr IGF-II pool isolated from fetal bovine serum revealed that it contained at least 12 different IGF-II isoforms and that all species contained an identical peptide backbone extended beyond the COOH terminus of mature 7.5-kDa IGF-II (14). In addition, this pool contained a significant amount of sialated, O-linked sugars. In this study, the high Mr IGF-II pool was fractionated into nine distinct fractions to facilitate analysis. Taken together, results from amino-terminal sequence analysis, amino acid analysis, mass spectrometry, glycosidase digestions, isoelectric focusing, and SDS-PAGE experiments demonstrate that all fractions predominantly contain IGF-II species with a 20-amino acid COOH-terminal extension to Gly-87 that is modified with different amounts of sialated, O-linked oligosaccharides.

The presence of O-linked sugars on IGF-II precursors may represent an important modification to promote correct processing to the mature form (46). Mapping of the glycosylation site on an extended IGF-II isoform isolated from human serum indicated that Thr-75 carries this post-translational modification (18). While we did not directly determine the location of the modified residues for the different high Mr IGF-II isoforms, our mass spectrometry and isoelectric focusing results strongly suggest that most isoforms are likely to be glycosylated at multiple positions. The E-peptide extension (residues 68–87) contains five potential O-linked glycosylation sites (two serines and three threonines; Ref. 47). If three sites were modified with a tetrasaccharide consisting of a Galβ1-3GalNAc core modified with two sialic acids, this would give a predicted size of 12,630 and an isoelectric point of 3.9. In comparison, the major species of peak H had an isoelectric point of 3.7 and the Mr range of 10,500–12,500. Similarly, an 87-residue IGF-II with one site modified with the tetrasaccharide would have a predicted isoelectric point of 4.4 and a Mr of 10,600. In comparison, the major species of peak D had an isoelectric point of 4.35 and an Mr of 10,854. Thus, the most likely explanation for the heterogeneity of the different high Mr isoforms is that they contain 1–3 O-linked oligosaccharides, each of which contains one to two sialic acids.

Binding studies with mature 7.5-kDa IGF-II and the high Mr IGF-II isoforms indicate that no substantial differences exist in the association or dissociation rate constants (Tables II and III, respectively) or in the calculated equilibrium dissociation constants (Table IV) for interaction with the sIGF-II/MPR, sIGF-I receptor, or IGFBP-1. In addition, similar distribution profiles among serum-binding proteins (Fig. 7), and similar displacement curves from IGFBP-3 (Fig. 9) and intact human IGF-I receptor (Fig. 12) were observed for both mature and high Mr IGF-II. This suggests that the COOH-terminal extensions do not have adverse effects on the rates or affinities with which these ligands interact with their receptors and binding proteins. This is consistent with other reports. Site-directed mutagenesis studies interpreted in light of the three-dimensional structure of mature IGF-II have implicated three regions of the folded molecule that appear to play important roles in determining protein-protein interactions (48–50). Whereas residues 27 and 43 of the B and A domains, respectively, play key roles in IGF-I receptor interactions, residues 48–50 and 54–55 of the A chain are important in mediating IGF-II/MPR interactions. Likewise, residues 48–50, together with 6–7 of the B domain, are important in IGFBP interactions. More importantly, mutations within, or deletion of, the D domain (comprising the carboxyl-terminal six amino acids of mature IGF-II) has no effect on IGFBP interactions (48). Finally, it has been shown that extended forms of human IGF-II display similar binding affinities for the IGF-II/MPR (20) and the IGF-I receptor (51) as compared with mature IGF-II. Taken together, these data suggest that E-peptide extensions, with or without glycosylation, do not supplant or occlude the important contact regions between IGF-II and the sIGF-II/MPR, IGF-I receptor, or the IGFBPs.

An intriguing question still remaining, therefore, is what is the biological significance of the different IGF-II isoforms? One possible answer is that glycosylation is important for proper folding or export of IGF-II. Evidence suggests that glycosylation of proIGF-II is necessary for proper proteolytic processing to the mature 7.5-kDa form (46). If so, the mechanism may involve binding of glycosylated IGF-II to intracellular lectins that retain and present the glycoprotein to the appropriate processing enzymes (52). In this case, the sIGF-II/MPR-bound high Mr IGF-IIs may simply reflect inefficiencies in this system resulting in the secretion of processing intermediates. Alternatively, the glycosylated IGF-II species may have unique biological functions. This is not unprecedented as N-linked glycosylation of several glycoprotein hormones is necessary for receptor activation but not binding (for review, see Ref. 53). Although no differences in the binding rates or affinities for mature and high Mr IGF-II were observed for the receptors and binding proteins analyzed in this study, this does not preclude

FIG. 11. Ligand blot analysis of the IGF-binding proteins secreted by differentiating C21 myoblasts. Media were collected from C21 myoblasts after incubation for ~70 h without (Control) or with ~26 nM IGF-I, IGF-II, or high Mr IGF-II isoforms C–H, as indicated. Aliquots (22.5 μl) were analyzed by ligand blotting as described under “Experimental Procedures.” The migration of prestained molecular weight standards is indicated.
the possibility that the high Ms isoforms have specialized roles in signal transduction. For instance, the extensions and/or saccharides could: 1) serve as a targeting signal to concentrate IGF-II in specific tissues or cell types; 2) interact with other cellular components, either when free or complexed with receptors or binding proteins, and/or; 3) alter the efficacy/potency of receptor activation after ligand binding. Such possibilities are supported from previous studies that demonstrated that a high Ms form of IGF-II with an apparent Mr of 15,000 was more potent than mature IGF-II in stimulating the replication of fetal dermal fibroblasts (20) and in the clonal expansion and differentiation of peripheral blood granulocyte colony-forming cells (54). Future studies will be required to determine if the high Ms IGF-II glycoproteins have other specialized biological activities or pharmacological properties.

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REFERENCES
1. Filsen, A. J., Louri, A., Efstratiadis, A., and Robertson, E. J. (1993) Development (Camb.) 116, 731–736
2. Baker, J., Liu, J. P., Robertson, E. J., and Efstratiadis, A. (1993) Cell 75, 73–82
3. Liu, J. P., Baker, J., Perkins, A. S., Robertson, E. J., and Efstratiadis, A. (1993) Cell 75, 59–72
4. Korndorf, S. (1992) Annu. Rev. Biochem. 61, 307–330
5. Oka, M., and Czech, M. P. (1986) J. Biol. Chem. 261, 9090–9093
6. Kiess, W., Greenstein, L. A., Miller, B. E., Anders, A. L., Rechler, M. M., and Nissley, S. P. (1987) J. Biol. Chem. 262, 12745–12751
7. Lamson, G., Giudice, L. C., and Rosenfeld, R. G. (1991) Growth Factors 5, 19–28
8. Clemmons, D. R. (1993) Mol. Reprod. Dev. 35, 368–374
9. Kiess, W., Greenstein, L. A., White, R. M., Lee, L., Rechler, M. M., and Nissley, S. P. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 7720–7724
10. Causin, C., Waheed, A., Braulke, T., Junghans, U., Maly, P., Hummel, R. E., and von Figura, K. (1988) Biochem. J. 252, 785–789
11. Gelato, M. C., Kiess, W., Lee, L., Malozowski, S., Rechler, M. M., and Nissley, P. (1988) J. Clin. Endocrinol. & Metab. 67, 669–675
12. Gelato, M. C., Batherford, C., Stark, R. I., and Daniel, S. S. (1989) Endocrinology 124, 2925–2943
13. Li, M., Distler, J. J., and Jourdian, G. W. (1991) Glycobiology 1, 511–517
14. Valenzano, K. J., Remmler, J., and Lobel, P. (1995) J. Biol. Chem. 270, 16441–16448
15. Bell, G. I., Merryweather, J. P., Sanchez-Pescador, R., Stempin, M. M., Priestley, L., Scott, J., and Rall, L. B. (1984) Nature 310, 775–777
16. Tollefsen, S. E., Heath-Monnig, E., Cascieri, M. A., Bayne, M. L., and Daughaday, W. H. (1991) J. Clin. Invest. 87, 1241–1250
17. Honegger, A., and Humbel, R. E. (1986) J. Biol. Chem. 261, 501–507
18. Pinset, C., Montarras, D., Chenewert, J., Minty, A., Barton, P., Laurent, C., and Gros, P. (1988) Differentiation 38, 28–34
19. Rotwein, P., James, P. L., and Kep, K. (1995) Mol. Endocrinol. 9, 913–922
20. Munson, P. J., and Rodbard, D. (1980) Anal. Biochem. 107, 220–239
21. Chen, H. J., Remmler, J., Delaney, J. C. Messner, D. J., and Lobel, P. (1993) J. Biol. Chem. 268, 22338–22346
22. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
23. Laemmli, U. K. (1970) Nature 227, 680–685
24. Devereux, J., Haeberli, P., and Smithies, O. (1984) Nucleic Acids Res. 12, 387–395
25. Paglin, S., and Zapata, M. T. (1975) Acta Physiol. Latinoam. 25, 188–196
26. Honegger, A., and Hummel, R. E. (1986) J. Biol. Chem. 261, 589–597
27. Daughaday, W. H., Trivedi, B., and Baxter, R. C. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 5823–5827
28. Wilson, I. B., Galvez, V., and von Heijne, G. (1991) Biochem. J. 275, 529–534
29. Bach, L. A., Hsieh, S., Sakano, K., Fujinawa, H., Perdue, J. F., and Rechler, M. M. (1994) in Current Direction in Insulin-like Growth Factor Research (LeRoith, D., and Raizada, M. K., eds) pp. 55–61, Plenum Press, New York
30. Oh, Y., Muller, H. L., Zhang, H., Ling, N., and Rosenfeld, R. G. (1994) in Current Direction in Insulin-like Growth Factor Research (LeRoith, D., and Raizada, M. K., eds) pp. 41–54, Plenum Press, New York
31. Schwart, G. N., Hudgins, W. E., and Perdue, J. F. (1993) Exp. Hematol. 21, 1447–1454