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Serum "big insulin-like growth factor II" from patients with tumor hypoglycemia lacks normal E-domain O-linked glycosylation, a possible determinant of normal propeptide processing

WILLIAM H. DAUGHADAY*, BAKULA TRIVEDI*, AND ROBERT C. BAXTER†

*Division of Endocrinology, Diabetes, and Metabolism, Department of Medicine, Washington University School of Medicine, St. Louis, MO 63110; and †Department of Endocrinology, Royal Prince Alfred Hospital, Camperdown, New South Wales, Australia

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ABSTRACT **The insulin-like growth factor II (IGF-II) gene is overexpressed in many mesenchymal tumors and can lead to non-islet-cell tumor hypoglycemia (NICTH). ProIGF-II consists of the 67 aa of IGF-II with a carboxyl 89-aa extension, the E domain. A derivative of proIGF-II containing only the first 21 aa of the E domain [proIGF-II-(E1–21)] has been isolated by others from normal serum and has O-linked glycosylation. We found that the "big IGF-II" of normal serum, as detected by an RIA directed against residues 1–21 of the E domain of proIGF-II, was reduced in size by treatment with neuraminidase and O-glycosidase. The big IGF-II, which is greatly increased in NICTH sera, was unaffected by neuraminidase and O-glycosidase treatment. We have also shown that big IGF-II from normal serum is retained by jacalin lectin columns and that big IGF-II from NICTH serum was not retained, indicating that it lacked O-glycosylation. Normal O-linked glycosylation may be required for proper peptidase processing of proIGF-II. The lack of normal O-linked glycosylation by tumors may explain the predominance of big IGF-II in NICTH sera. In normal serum, most of the IGF-II is present in a 150-kDa ternary complex with IGF-II binding protein (IGFBP) 3 and α subunit. In NICTH serum, however, the complexes carrying big IGF-II are <50 kDa. We investigated whether big IGF-II of NICTH was responsible for this abnormality. Tumor big IGF-II and IGF-II were equally effective in forming the 150-kDa complex with purified IGFBP-3 and ^{125}I-labeled α subunit. Both ^{125}I-labeled IGF-II and ^{125}I-labeled proIGF-II-(E1–21), when incubated with normal serum, formed the 150-kDa complex as detected by Superose 12 exclusion chromatography. We conclude that the nonglycosylated big IGF-II of NICTH serum can form normal complexes with serum IGFBPs. The defective binding in NICTH is attributable to defective IGFBP-3 binding.**

Insulin-like growth factor (IGF) II is synthesized as proIGF-II, which consists of the 67 aa of IGF-II and an 89-aa carboxyl-terminal extension, the E domain (1). Processing of proIGF-II normally occurs in a stepwise fashion, and cleavage after the single lysine at position 21 of the E domain yields a 10.5-kDa peptide [proIGF-II-(E1–21)] that has been isolated from serum (2). A larger variant of proIGF-II-(E1–21), with O-linked glycosylation on threonine at position 8 of the E domain, has also been isolated from serum (3).

Many mesenchymal and some renal, adrenal, and hepatic cell tumors have increased expression of the IGF-II gene and synthesize and secrete IGF-II peptides (4). In some patients with large tumors, the secreted IGF-II peptides can produce hypoglycemia. However, measurement of these peptides with the IGF-II RIA has given inconsistent results (5, 6). Two recent findings provided an explanation for these inconsistencies. The predominant form of IGF-II in tumors and sera of patients with non-islet-cell tumor hypoglycemia (NICTH) is bigger than IGF-II by gel chromatography. Tumor "big IGF-II" is incompletely recognized by IGF-II RIA. In addition, big IGF-II is carried in plasma as binary complexes of <50 kDa with binding proteins rather than as the ternary complex with IGF-II binding protein (IGFBP) 3 and α subunit, which predominates in normal sera (7, 8). Because the smaller complexes traverse the capillary membrane readily to reach target tissues, the associated big IGF-II exerts a greater biologic effect than normal IGFs associated with the ternary complex.

The 150-kDa ternary complex is formed stepwise. First, the IGF must be bound to IGFBP-3 before binding to an acid-labile protein (α subunit) can occur (8). The failure to form the 150-kDa complex in the sera of patients with NICTH might be attributable to each of the components of the ternary complex (i.e., tumor big IGF-II, IGFBP-3, or α subunit). In an earlier paper (9), we (R.C.B. and W.H.D.) reported that the mean concentration of IGFBP-3 in the sera of five patients with NICTH was 63% of that of normal controls by RIA and that the mean concentration of α subunit was 38% of that of controls. Despite the reduced concentration of serum α subunit in NICTH, this could not be responsible for the failure to form 150-kDa complexes, because it is normally present in considerable excess over that required for formation of the 150-kDa complex. Moreover, incubation of sera from patients with NICTH with ^{125}I-labeled IGF-I (^{125}I-IGF-I) cross-linked to purified IGFBP-3 formed the 150-kDa complex normally.

In this paper we show that the small amount of big IGF-II in normal serum has O-linked glycosylation, but this was not demonstrable in big IGF-II of NICTH serum. We present evidence that the abnormality in serum binding of big IGF-II does not reside in the abnormal peptides but is attributable to abnormal function of IGFBP-3.

MATERIALS AND METHODS

Materials. A peptide with the first 21 aa of the E domain of proIGF-II, hereafter identified as E1–21, was synthesized by the Protein Chemistry Laboratory (Gregory Grant) of the Washington University School of Medicine. Recombinant IGF-II was purchased from Bachem. Unglycosylated recombinant proIGF-II-(E1–21) was kindly provided by Bjorn Hammarberg *et al.* (10). Tumor big IGF-II was prepared from 17 ml of serum from a NICTH patient with a mesothelioma. This serum contained 819 μg of IGF-II per liter by RIA and 37 μg of IGF-I per liter by RIA. The serum was acidified and passed in four separate runs through a 2.5 × 95 cm Bio-Gel P-60 column using 0.5 M acetic acid/0.075 M NaCl as the

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Abbreviations: E1–21, the first 21 amino acids of the E domain of proIGF-II; IGF, insulin-like growth factor; IGFBP, IGF-II binding protein; NICTH, non-islet-cell tumor hypoglycemia.

eluent. This column permits the separation of big IGF-II from 7.5-kDa IGF-II (11). The tubes containing big IGF-II were pooled, and the big IGF-II was recovered by absorption and elution from C_{18} cartridges (Sep-Pak; Waters) (11). A total of 9 μg of big IGF-II (by RIA) was obtained.

Neuraminidase (from *Arthrobacter*) was from Boehringer Mannheim, and endo-*N*-acetylgalactosaminidase (O-glycosidase) was from Genzyme. Sephadex G-50-40 and the Superose 12 10/30 fast performance liquid chromatography column were obtained from Pharmacia. Beads of 4% agarose containing 4 mg of covalently coupled jacalin lectin per ml were purchased from Vector Laboratories. This lectin has high affinity for O-linked α-galactopyranosides (12). All other reagents were obtained from Sigma.

Sera. Normal sera were obtained from healthy young students and laboratory personnel. Sera from a patient with NICTH from a hemangiopericytoma were used in the studies shown in Fig. 3, and serum from a patient with a fibrosarcoma was used in Fig. 6. Both sera were kindly provided by Kirstin Hall (Stockholm). The sera had been lyophilized in Stockholm and shipped at ambient temperature. After reconstitution with distilled water, the sera were stored at −17°C until studied.

Removal of O-Linked Sugars. The IGF peptides were extracted from 1 to 2 ml of acidified serum with Sep-Pak cartridges (13). The alcohol extracts were lyophilized. Lyophilized peptides were taken up in 20 mM phosphate buffer (pH 6.0) with 10 milliunits of neuraminidase and 10 milliunits of O-glycosidase in a total volume of 0.5 ml. The mixture was incubated overnight at 37°C.

Chromatographic Methods. Sephadex G-50-40 exclusion chromatography was done in a 1.5 × 85 cm column in 0.1 M acetic acid. The sample was passed in a descending direction with a buffer flow rate of 4.6 ml/hr, and 30-min fractions were collected. The total column volume of each run was identified with $CoCl_2$. The column was calibrated with ^{125}I-labeled E1–21 (2.4 kDa), ^{125}I-IGF-I (7.5 kDa), cytochrome *c* (12.4 kDa), and ferritin (44.8 kDa).

The fast performance liquid exclusion chromatography was done with a Superose 12 column using a Beckman 344 gradient liquid chromatograph. Three hundred microliters of serum was loaded on the column, and the column was washed with phosphate-buffered saline (pH 7.4). The flow rate was 0.5 ml/min, and 1-min fractions were collected.

Jacalin lectin chromatography was performed in 0.7 × 4 cm columns with 2 ml of jacalin-agarose beads. The samples were applied in 150 mM Tris (pH 7.4) and eluted with 8 ml of 150 mM Tris with 20 mM α-methylgalactopyranoside as described by Hortin and Trimpe (12).

IGFBP-3 Binding Studies. The affinity of tumor big IGF-II for purified IGFBP-3 was measured by competition with ^{125}I-labeled IGF-II (^{125}I-IGF-II) as described (14). Separation of the binary complex from free ^{125}I-IGF-II was by immunoprecipitation using IGFBP-3 rabbit antiserum R-7.

The ability of recombinant human IGF-II and tumor big IGF-II to form the 150-kDa ternary complex was measured in a reaction mixture containing ^{125}I-labeled α subunit (^{125}I-α-subunit) (≈10,000 cpm, 1 ng) and 10 ng of purified human IGFBP-3. Bound tracer was separated from free by immunoprecipitation with 1 μl of IGFBP-3 antiserum R-7 and 5 μl of goat anti-rabbit immunoglobulin (for further details see ref. 14).

The binding of human ^{125}I-IGF-II and ^{125}I-labeled proIGF-II-(E1–21) [^{125}I-proIGF-II-(E1–21)] to serum IGFBPs was carried out by incubating 1 ml of serum with one of the two labeled peptides in the presence of 100 kallikrein units of aprotinin and 1 mM phenylmethylsulfonyl fluoride at 37°C for 17 hr. Separation of 150 kDa and <50 kDa bound from free labeled IGF peptide was by Superose 12 exclusion chromatography.

Iodinations. E1–21 was iodinated with Iodobeads (Pierce) to a specific activity of 50–70 μCi/μg (1 Ci = 37 GBq). ProIGF-II-(E1–21) and recombinant human IGF-II were iodinated using a soluble lactoperoxidase method (15).

RIAs. IGF-II was measured by RIA using the Amano monoclonal antibody (Amano Pharmaceutical, Troy, VA) (16). Recombinant IGF-II was used as a standard. In the IGF-II RIA, recombinant proIGF-II-(E1–21) had a potency of 30% compared to that of recombinant human IGF-II peptide. The RIA for E1–21 peptide was conducted with a polyclonal rabbit antibody raised in this laboratory against synthetic E1–21, and this peptide was used as a standard (17). After addition of rabbit gamma globulin to a final concentration of 0.5%, free ^{125}I-labeled E1–21 was separated from that bound to antibody by polyethylene glycol (M_r 8000) at a final concentration of 12.5%. In this assay, recombinant proIGF-II had a potency of 35% of that of the E1–21 standard.

RESULTS

We characterized the size of the IGF-II-related peptide reacting with our E1–21 RIA after gel elution from a Sephadex G-50 column in 0.1 M acetic acid. In Fig. 1 are shown the combined results of the study of three normal sera. Two major peaks of E1–21 immunoactivity are evident. The first was eluted slightly before the cytochrome *c* 12.4-kDa marker, and the second peak was eluted after the ^{125}I-IGF-I 7.6-kDa marker and in front of the ^{125}I-labeled E1–21 2.5-kDa marker.

Treatment of normal serum with neuraminidase and O-glycosidase led to a shift to a smaller size of both the first and second peaks of E1–21 immunoactivity (Fig. 2). The shift of the second peak of the E1–21 immunoactivity appeared to be incomplete. When the extracted IGFs from serum of a patient with NICTH were incubated with or without neuraminidase and O-glycosidase, the major peak of immunoactivity was eluted from the column in the same fraction (Fig. 3).

Because the determination of the size of big IGF-II of other normal and NICTH sera before and after deglycosylation proved inconsistent, we have also characterized the O-glycosylation state by absorption and elution from jacalin lectin columns. In these studies, IGF Sep-Pak extracts of sera were subjected to Sephadex G-50 chromatography in 0.1 M acetic acid. The big IGF-II was identified by E1–21 RIA and pooled. The lyophilized residue was taken up in 150 mM Tris buffer and absorbed and eluted from the jacalin columns as described in *Materials and Methods*. We found that with four

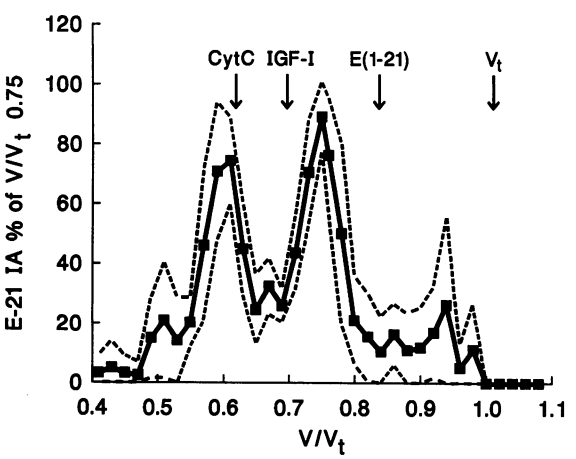

FIG. 1. IGFs were extracted from normal sera with C_{18} cartridges and subjected to gel-filtration through Sephadex G-50 in 0.1 M acetic acid. E1–21 immunoactivity (E-21 IA) was determined by RIA. For graphic purposes, the results were normalized to 100% for the 5.5-kDa peak. The solid line is the mean, and the dashed lines are ±1 SD. CytC, cytochrome *c*; V_t, total volume; V, elution volume.

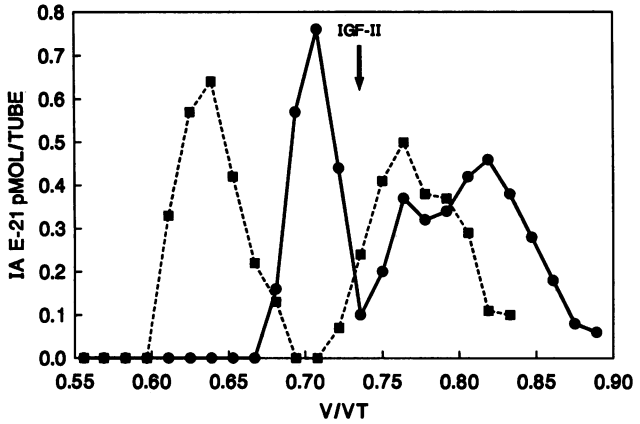

FIG. 2. IGFs were extracted from a normal serum with a C_{18} cartridge (■) and were incubated for 17 hr with neuraminidase and O-glycosidase (●). The extracts were then passed through a Sephadex G-50 column in 0.1 M acetic acid, and E1–21 immunoactivity (IA E-21) was measured in each fraction by RIA. VT, total volume; V, elution volume.

normal sera 3.70 ± 0.53 (mean ± SE) pmol was absorbed by the column and 0.8 ± 0.36 pmol was not absorbed. In contrast, with three NICTH sera, only 1.03 ± 0.83 pmol was absorbed by the column and 15.13 ± 1.72 pmol was not retained. These results confirm that normal big IGF-II is largely O-glycosylated, whereas big IGF-II from NICTH serum was nearly entirely lacking in O-glycosylation.

We wished to determine whether the failure to form the 150-kDa complex was a property of big IGF-II or of IGFBP-3. First, the ability of tumor big IGF-II and recombinant human IGF-II to compete for binding in a system containing ^{125}I-IGF-II and purified human IGFBP-3 was examined. After immunoprecipitation of the ^{125}I-IGF-II·IGFBP-3 complex, it was found that both tumor big IGF-II and recombinant human IGF-II were equally potent in displacing ^{125}I-IGF-II from its binding site on IGFBP-3 (Fig. 4).

Using essentially the same specific IGFBP-3 immunoprecipitation system as above, we found that ^{125}I-IGF-II was bound by the IGFBP-3 of both normal and NICTH serum that had been stripped of IGFs by Bio-Gel P-60 acid chromatography. The binding by different aliquots of the two stripped sera was parallel, and the IGFBP-3 potency of the NICTH serum was 53% of that of the normal serum (data not shown).

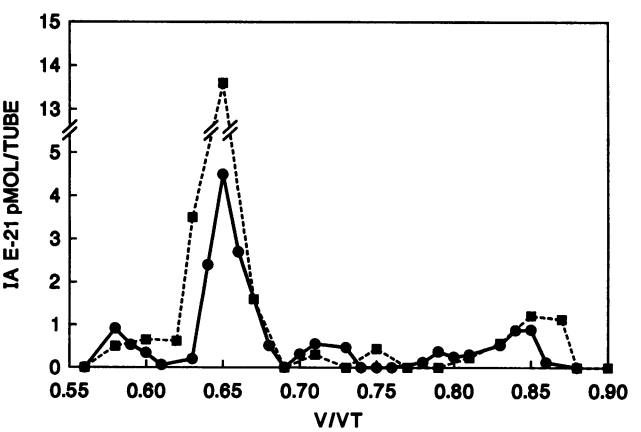

FIG. 3. Sephadex G-50 acid exclusion chromatography was conducted with IGF C_{18} extracts of serum from a patient with NICTH without (■) and with (●) prior treatment with neuraminidase and O-glycosidase. Conditions and abbreviations are as indicated in Fig. 2. The lower recovery of the V/VT peak with neuraminidase and O-glycosidase treatment was not seen in other experiments.

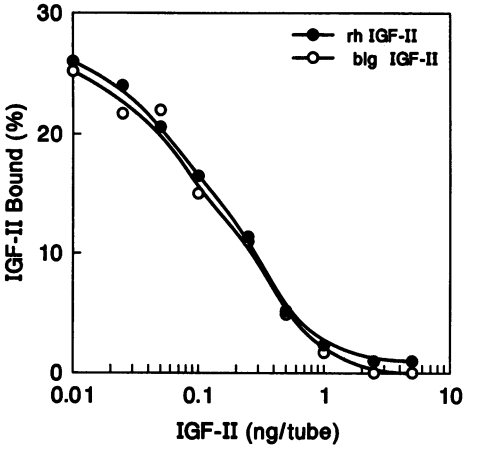

FIG. 4. Competition for the binding of ^{125}I-IGF-II to human IGFBP-3 (0.5 ng) by recombinant human (rh) IGF-II and big IGF-II extracted from serum of a patient with NICTH due to a mesothelioma. The IGFBP-3 complex was immunoprecipitated. The percentage of the total cpm immunoprecipitated is shown on the ordinate.

This is in agreement with earlier measurements of IGFBP-3 by RIA.

We next compared the ability of IGF-II and big IGF-II to form the 150-kDa ternary complex when incubated with ^{125}I-α-subunit and purified IGFBP-3. Bound ^{125}I-α-subunit was precipitated immunologically with antiserum directed against IGFBP-3. Fig. 5 shows that recombinant IGF-II and tumor big IGF-II were both able to form the ternary complex.

We next compared the serum binding profiles of ^{125}I-proIGF-II-(E1–21) and ^{125}I-IGF-II when incubated with normal and NICTH serum. Resolution of the 150-kDa and <50-kDa complexes was achieved by fast performance liquid chromatography through Superose 12 columns in phosphate-buffered saline (pH 7.4). The results obtained with ^{125}I-IGF-II are shown in Fig. 6*A* and those with ^{125}I-proIGF-II-(E1–21) are shown in Fig. 6*B*. In the experiments with normal serum, there are three peaks corresponding to species of 150 kDa, <50 kDa, and free tracers. Both tracers were equally incorporated into the 150-kDa peak. In the incubations with NICTH serum, the 150-kDa species was virtually absent with both ^{125}I-proIGF-II and ^{125}I-IGF-II tracers. Moreover, the concentration of free tracer in the experiments with NICTH

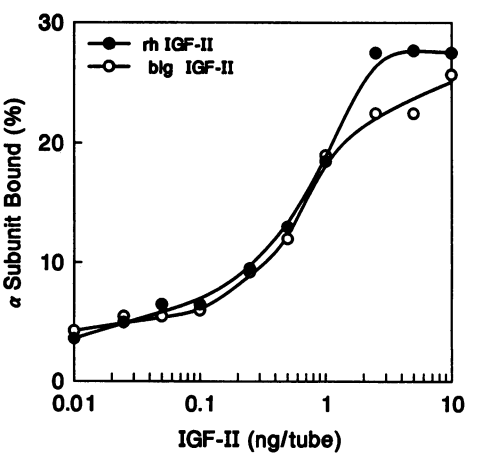

FIG. 5. Formation of the ternary 150-kDa complex in the presence of recombinant human (rh) IGF-II and big IGF-II. The incubation mixture included ^{125}I-α subunit, 10 ng of IGFBP-3, and increasing concentrations of either human IGF-II (●) or big IGF-II (○) from serum of a patient with NICTH from a mesothelioma. The percentage of the total cpm immunoprecipitated is shown on the ordinant.

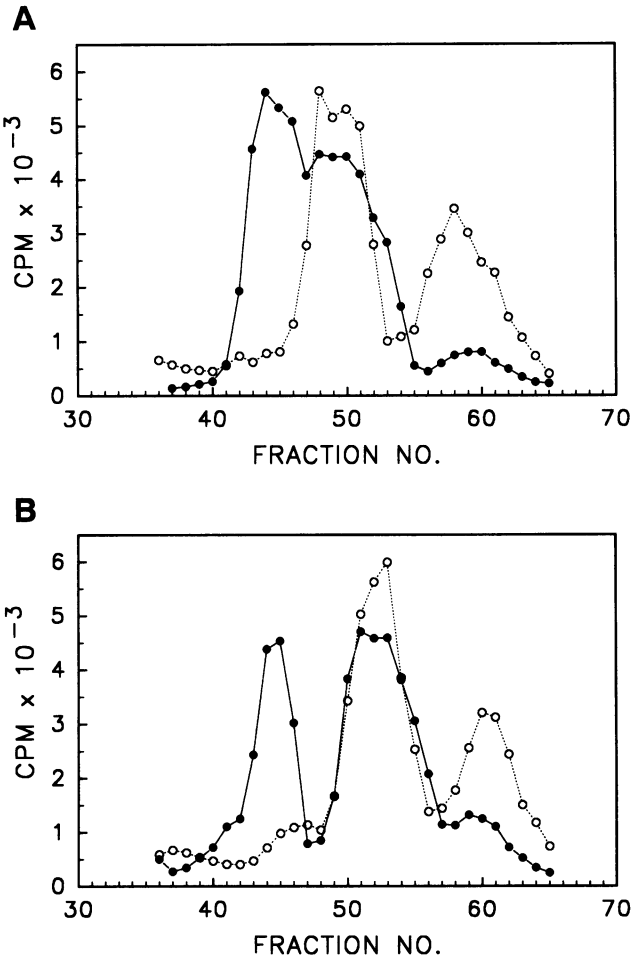

FIG. 6. (*A*) ^{125}I-IGF-II was incubated for 18 hr at 37°C with normal human serum (●) or serum from a patient with NICTH from a hepatoma (○). Separation of the 150-kDa complex (first peak), <50-kDa complexes (second peak), and unbound ^{125}I-IGF-II (third peak) was obtained by fast performance liquid chromatography with a Sepharose 12 column and a neutral buffer. (*B*) Conditions were the same as above except incubations were carried out with [^{125}I]proIGF-II-(E1–21).

serum was higher than that with normal serum, suggesting that the fraction of free IGF-II peptide was increased. Caution in interpreting changes in free tracer is required because the extent of binding of labeled IGF to IGFBPs is greatly influenced by the quality of the tracer.

DISCUSSION

As we have previously reported (18), normal serum contains a small concentration of big IGF-II, detected by the E1–21 RIA, which eluted from our calibrated Sephadex G-50 column in front of our cytochrome *c* 12.4-kDa marker. In addition, there was a second peak of E1–21 immunoactivity with a molecular mass of about 5 kDa, which probably represents glycosylated free E1–21 peptide. Treatment of serum extracts with neuraminidase and O-glycosidase resulted in a shift in elution profile of both peaks, indicating the presence of O-linked oligosaccharides in both peptides (Fig. 2). When sera of patients with NICTH were similarly studied, the first peak of E1–21 immunoactivity was eluted at, or just after, the cytochrome *c* 12.4-kDa marker. There was no significant shift in elution position after treatment with neuraminidase and O-glycosidase (Fig. 3).

We provide further evidence by jacalin lectin absorption chromatography that normal serum big IGF-II is largely O-glycosylated and that NICTH serum big IGF-II is largely unglycosylated.

We conclude from these results that most of the proIGF-II-(E1–21) of normal serum is the O-glycosylated 15.0-kDa form, which has been isolated from normal serum by Hudgins *et al.* (3). In contrast, we found that most of the big IGF-II of NICTH sera lacks the O-linked α-galactopyranoside adduct. The possibility of other O-linked sugars not retained on jacalin columns was not ruled out.

In a recent abstract, Yang *et al.*‡ suggested that O-glycosylation at Thr-75 of the proIGF-II sequence may be essential for normal peptidase processing of proIGF-II, which results in the 67-aa 7.5-kDa IGF-II. They constructed plasmids containing DNA for normal proIGF-II and for a proIGF-II variant with alanine substituted for Thr-75. When transfected into NIH 3T3 cells, the plasmid of normal proIGF-II resulted in the secretion of 7.5-kDa IGF-II, whereas the plasmid lacking the O-linked oligosaccharide at Thr-75 resulted in the secretion of a 17.0-kDa proIGF-II. These observations suggest that, at least for these cells, O-glycosylation of Thr-75 was essential for normal processing by peptidase. This requirement for O-glycosylation may be restricted to human cells because neither rat nor bovine proIGF-II has a threonine at position 75. Nevertheless, defective tumor glycosylation of proIGF-II provides an attractive hypothesis to explain why tumors secrete mostly unglycosylated big IGF-II.

The question that remains is whether or not the abnormal form of IGF-II can account for the failure to form the 150-kDa binding protein complex in NICTH serum, which is characteristic of the IGF transport in this condition. In this paper we report that the big IGF-II prepared from serum of a patient with NICTH competed equally well with IGF-II for binding to purified IGFBP-3 (Fig. 4) and normally formed the ternary complex when incubated with purified IGFBP-3 and ^{125}I-labeled purified α subunit (Fig. 5).

These results argue against the possibility that big IGF-II itself can explain the binding anomaly. Direct support for this conclusion is provided by the results of incubation of normal and NICTH serum with ^{125}I-IGF-II and ^{125}I-labeled recombinant proIGF-II-(E1–21). When binding complexes were separated by Superose 12 exclusion chromatography, it was found that both IGF-II and proIGF-II formed the 150-kDa complex equally well when incubated with normal serum, but neither IGF-II nor proIGF-II-(E1–21) formed the 150-kDa complex when incubated with NICTH serum (Fig. 6).

As we have previously reported (9), when ^{125}I-IGF-I cross-linked to IGFBP-3 was incubated with two NICTH sera, normal 150-kDa ternary complex was formed, which indicates that these NICTH sera contained sufficient potent α subunit to form the 150-kDa complex. These results point to an abnormality in IGFBP-3 in NICTH serum, which is responsible for the failure of formation of the 150-kDa complex. This could be the result of protease action as reported by Cotterill *et al.* (19). They found that there was loss of the 40.6- and 38-kDa bands on Western blots of NICTH serum and serum from women late in pregnancy. There is, however, a difference between the two types of sera. In pregnancy, serum retains the ability to form the large molecular mass complex (20, 21), but this is lost in sera from patients with NICTH. The abnormality in NICTH IGFBP-3 could involve either defective binding of IGFs or an inability of the IGFBP-3 complex to form the ternary complex. Our finding that IGFBP-3 in NICTH serum retained the ability to bind ^{125}I-IGF-II suggests that the defect in IGFBP-3 of NICTH serum lies in the inability of the binary complex to

‡Yang, C., Zhan, X., Hu, X. & Perdue, J. E., Program of the Annual Meeting of the Endocrine Society, June 24–27, 1992, San Antonio, TX, p. 80 (abstr.).

form the ternary complex. The fact that IGFBP-2 is reported to be increased in NICTH sera could also contribute to difficulty in forming the 150-kDa complex (22).

Zapf *et al.* (23) in a paper that appeared after completion of our studies came to a different conclusion. They incubated sera from a NICTH patient obtained before and after successful removal of an IGF-II hypersecretory tumor with ^{125}I-labeled recombinant human IGFBP-3 (glycosylated). They generated the 150-kDa complex with the postoperative serum but not with the preoperative serum. They concluded "that decreased acid-labile subunit probably accounts for the reduction or disappearance of the 150 kD complex." The reason why their results differ from those that we reported with two patients with NICTH in which the 150-kDa complex was generated after incubation of NICTH sera with ^{125}I-IGF-I cross-linked to IGFBP-3 is not clear (9). It is possible that the failure to form the 150-kDa complex may be attributable to IGFBP-3 function in some patients and to α-subunit activity in others. Further studies to clarify these possibilities are needed.

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