Affinity-labeled Plasma Somatomedin-C/Insulinlike Growth Factor I Binding Proteins

Evidence of Growth Hormone Dependence and Subunit Structure

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Abstract

By using disuccinimidyl suberate, we have covalently cross-linked ^{125}I-labeled somatomedin-C (Sm-C)/insulinlike growth factor I to specific binding proteins in human plasma. In unfractionated plasma samples from normal and acromegalic donors, ^{125}I-Sm-C binding-protein complexes with relative molecular weights (M_r) of 160,000, 135,000, 110,000, 80,000, 50,000, 43,000–35,000, and 28,000–24,000 were consistently observed. In contrast, the 43,000–35,000-mol wt species were frequently the only specific complexes observed in hypopituitary plasma and were consistently more intensely labeled in such samples. Reduction of samples with β-mercaptoethanol did not alter the electrophoretic pattern of these ^{125}I-Sm-C binding-protein complexes. All Sm-C binding proteins, with the exception of the 43,000–35,000-mol wt complex, were adsorbed by concanavalin A-Sepharose. When acromegalic or normal plasma was fractionated on a Sephadex G-200 column and affinity labeled, the same complexes that were adsorbed by concanavalin A were found in fractions that eluted near the γ-globulin peak. On the other hand, the 43,000–35,000-mol wt complex consistently eluted in size-appropriate fractions near the albumin peak. These data suggest that the growth hormone (GH)-dependent Sm-C binding protein, represented by the 160,000-mol wt complex, is in some way composed of smaller species, i.e., the 135,000-, 110,000-, 80,000-, 50,000-, and 28,000–24,000-mol wt complexes. Acid incubation of plasma prior to Sephadex G-200 chromatography results in the elimination of specific ^{125}I-Sm-C binding-protein complexes which elute near γ-globulin and a concurrent increase in the labeling intensity of the 28,000–24,000-mol wt complexes. We speculate, therefore, that each of the GH-dependent Sm-C binding-protein complexes represents an oligomer composed of 28,000–24,000-mol wt protomers. The 43,000–35,000-mol wt species is not dependent upon GH and appears to represent a different type of Sm-C binding protein.

Introduction

The molecular weight of somatomedin-C/insulinlike growth factor I (Sm-C/IGF-I)[1] is 7,649 (1, 2), but it circulates in the blood at much higher relative molecular weights (M_r ~ 150,000 and 40,000) because it is complexed with binding proteins (3–15). The γ-globulin-sized (~150,000 mol wt), somatomedin binding protein is growth hormone (GH) dependent and is associated with the majority of circulating somatomedin (6–9, 11, 16–19). It is thought to be composed of at least two subunits (8); however, neither its structure nor that of the smaller (~40,000 mol wt) Sm-C binding protein is known.

Virtually all published studies have approached the characterization of plasma somatomedin binding proteins in one or both of two ways: evaluation of the elution pattern of radioactivity from gel filtration columns after incubation of labeled somatomedin and serum (3–12, 14–16, 18–21), or assessment of binding activity by activated charcoal separation of free and bound labeled peptide after its incubation with serum (3, 6, 9–13, 15, 16, 18, 19, 21). We have developed a technique to affinity-label circulating Sm-C binding-proteins using the cross-linking agent disuccinimidyl suberate (DSS) (22, 23) and now report its utility in studies of these proteins in postnatal human plasma. This technique utilizes sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) to separate the covalently cross-linked ^{125}I-Sm-C binding-protein complexes, and hence provides greater resolution of Sm-C binding moieties than has heretofore been available. New insights resulting from this study have led us to propose that the GH-dependent, γ-globulin-sized plasma Sm-C binding protein is an oligomer composed of identical subunits. The apparent M_r of the ^{125}I-Sm-C monomer complex is 28,000–24,000.

Methods

Plasma samples. All blood samples were collected between 0900 and 1200 h by venipuncture into EDTA vacutainers (Becton-Dickinson & Co., Rutherford, NJ) from individuals on regular diets. They were immediately chilled on ice, separated by centrifugation within 2 h, and frozen at −20°C until use (usually within 4 wk). Immunoreactive Sm-C was determined on each plasma sample by a modified (7, 20) nonequilibrium technique (24). Studies of normal plasma were performed on samples collected from healthy adult males. Acromegalic plasma samples were obtained from a number of individuals, but all the studies reported here represent samples obtained from a 12-yr-old white boy whose diagnosis was based on a basal GH level of 11 ng/ml, a peak GH of 20 ng/ml 30 min after thyrotropin-releasing hormone infusion, and plasma immunoreactive Sm-C concentrations of between 8.1 and 11.2 U/ml. Transphenoidal exploration revealed an adenoma 8 mm in diameter. Hypopituitary plasma was obtained from a number of prepubertal children between the ages of 2-7/12 and 18-9/12 yr (four boys and four girls). All were significantly below the 3rd centile for height (2.9–6.0 SD below 50th centile), had growth

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1. *Abbreviations used in this paper:* BS^3, *bis*(sulfosuccinimidyl)suberate; Con A, concanavalin A; DMS, dimethyl suberimidate; DSS, disuccinimidyl suberate; DTSSP, 3,3′-dithio*bis*(sulfosuccinimidyl propionate); GH, growth hormone; hGH, human growth hormone; IGF-I, insulinlike growth factor I; PAGE, polyacrylamide gel electrophoresis; Sm-C, somatomedin-C.

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velocities of <4.00 cm/yr (1.9–3.7 cm/yr), lacked a significant GH response to two different provocative stimuli (usually insulin-induced hypoglycemia and arginine), and had basal plasma Sm-C concentrations of <0.25 U/ml (0.03–0.23 U/ml). Collection of blood was approved by the Committee for Protection of Human Subjects, University of North Carolina at Chapel Hill.

Reagents. ^{125}I-labeled somatomedin-C (^{125}I-Sm-C) had a specific activity of 200 μCi/μg and was judged by multiple criteria to be no less than 90% pure (25). The ^{125}I-Sm-C was stored at −20°C in 2% bovine serum albumin. For studies of binding specificity, purified Sm-C was used. When an excess of Sm-C was required, partially purified preparations obtained after isoelectrofocusing were used (25). Monocomponent porcine insulin (lots 615-07J-50) was a gift from Eli Lilly Co (Indianapolis, IN). DSS, *bis*(sulfosuccinimidyl)suberate (BS^3), sulfosuccinimidyl 6-(4'-azido-2'-nitrophenylamino)hexandate (Sulfo-Sanpah), 3,3'-dithio*bis*(sulfosuccinimidyl propionate) (DTSSP), and dimethyl suberimidate (DMS) were all purchased from Pierce Chemical Co, Rockford, IL. All chemicals used were reagent grade.

Affinity-labeling technique. Aliquots of plasma (10 or 50 μl) diluted 1:10 in 0.05 M Na phosphate buffer (pH 7.4) were preincubated on ice for 10–30 min in the presence of 250,000 cpm (110 fmol) of ^{125}I-Sm-C. Cross-linking of ^{125}I-Sm-C to plasma proteins was accomplished by the addition of 10 μl of 10 or 50 mM DSS dissolved in dimethyl sulfoxide to give a final concentration of 1 mM DSS, followed by incubation at room temperature for 10 min.

When 50 μl of normal plasma dissolved in 0.5 ml of 0.05 M Na phosphate buffer (pH 7.4) was incubated with as little as 5 ng of unlabeled Sm-C in addition to ^{125}I-Sm-C, labeling of specific Sm-C binding proteins was significantly reduced. Incubation with 100 ng of unlabeled Sm-C was sufficient to obliterate completely all specific labeling, whereas as much as 1 mg of insulin had no effect. Labeled bands were judged to be specific Sm-C binding proteins if they were abolished as a result of incubation with an excess of unlabeled Sm-C (25 ng/10 μl plasma in a 1:10 dilution with buffer).

The parameters for incubation of ^{125}I-Sm-C with plasma and the cross-linking procedure were determined experimentally by altering variables individually at first and later in concert to determine the conditions that consistently resulted in intensely labeled, specific bands and minimalized nonspecific labeling. The parameters studied were the dilution of plasma (1:1, 1:5, 1:10, 1:20 and 1:50), time (0.5 min to 18 h) of incubation, and concentration of DSS (0.25–5.0 mM), and temperature of cross-linking (0, 4, and 22–24°C). >1:10 dilution of whole plasma samples generally decreased the intensity of specifically labeled bands; on the other hand, plasma dilutions of <1:10 dramatically increased nonspecific labeling without an increase in the intensity of specifically labeled bands. The time of incubation of ^{125}I-Sm-C and plasma at either 0° or 4°C did not alter results, but incubation at 22°–24°C increased nonspecific labeling. Concentrations of DSS between 0.5–1.0 mM maximized the intensity of specifically labeled bands. Higher DSS concentrations consistently resulted in an increase in nonspecific cross-linking and protein precipitation. The time allowed for the reaction was not critical, because the cross-linking reaction goes to completion in minutes. Plasma, rather than serum, was used in this study because the Sm-C binding proteins appeared to be more stable over time in plasma; however, no differences were observed when fresh plasma and serum were compared. Collection of plasma in Aprotinin 5–10 TIU/ml (Sigma Chemical Co., St. Louis, MO) or 0.1 mM phenylmethylsulfonyl fluoride did not alter results.

The cross-linking agents BS^3, Sulfo-Sanpah, DTSSP, and DMS were tested under similar experimental conditions to explore the possibility that binding proteins could exist which were not affinity labeled by DSS, but might be labeled when an alternate cross-linker was used. Of those tested, BS^3 and DTSSP were effective and produced electrophoretic patterns of cross-linked ^{125}I-Sm-C binding-protein complexes identical to those generated by DSS.

PAGE and autoradiography. SDS-PAGE was performed on 7–15% linear gradient 16 × 17-cm slab gels according to the method of Janis et al. (26) with minor modifications. Gels were preelectrophoresed for at least 2 h with a constant power of 10 W/gel before loading samples. Electrophoresis was performed using the Tris-glycine buffer system of Laemmli (27). Electrophoresis of samples was accomplished overnight at room temperature with constant voltage of 110–120 V. Samples were prepared for electrophoresis by the addition of 0.005% bromophenol blue in 0.015 M Tris-HCl (pH 8.8). The standard molecular weight markers used in these experiments were myosin (200,000), phosphorylase *b* (94,000), bovine serum albumin (67,000), ovalbumin (45,000), carbonic anhydrase (30,000), chymotrypsinogen (25,000), soybean trypsin inhibitor (20,000), myoglobin (17,800), and cytochrome *c* (12,400) (Sigma Chemical Co.). After electrophoresis, gels were stained with Coomassie Blue (Serva Fine Biochemicals Inc., Garden City Park, NY) in 10% acetic acid:25% 2-propanol and destained with a 10% acetic acid:25% 2-propanol solution. After hydration, gels were dried and exposed to Kodak XAR-5 or XRP-1 X-ray film (Eastman Kodak Co., Rochester, NY) at −70°C for 2–6 or 7–21 d, respectively.

Efficiency of affinity labeling. To evaluate the efficiency of cross-linking, ^{125}I-Sm-C (1,500,000 cpm) was incubated with 1 ml of normal human plasma and fractionated on a Sephadex G-200 column (see below). Equivalent aliquots (100 μl) were taken from the two peaks of ^{125}I-SM-C that eluted with the plasma proteins (the γ-globulin and the near-albumin peaks) and subjected to SDS-PAGE either directly (uncross-linked) or after cross-linking with 1 mM DSS. Gels were fixed, sliced into 2-mm fragments, and counted in a gamma counter (Packard Instrument Co., Downers Grove, IL). Because free ^{125}I-Sm-C migrates as a single peak on the gel, and cross-linking decreases the amount of free ^{125}I-Sm-C present in this peak by the amount cross-linked, the efficiency of cross-linking was defined as number of counts per minute lost from the peak of free ^{125}I-Sm-C as a result of the cross-linking procedure divided by the total number of counts per minute present in the free ^{125}I-Sm-C peak when the samples were not cross-linked. Efficiencies of cross-linking were 1% in the γ-globulin fractions and 20% in the near-albumin fractions. Of the counts per minute calculated to be cross-linked, >90% migrated in peaks representing specific ^{125}I-Sm-C binding proteins.

We also compared the number of counts per minute specifically bound to Sm-C binding proteins in normal plasma by both a charcoal binding assay (21) and by cross-linking with DSS. ^{125}I-Sm-C (100,000 cpm) was incubated with 5 μl of normal plasma in the absence and presence of unlabeled Sm-C (25 ng), cross-linked with 1 mM DSS, and subjected to SDS-PAGE. In parallel, 5 μl of normal plasma was incubated with 100,000 cpm ^{125}I-Sm-C and the apparent bound and unbound counts per minute were separated with activated charcoal. Although only 7.9% of the total counts per minute added were cross-linked to bands representing specific Sm-C-binding-protein complexes, these specifically bound counts per minute exceeded the counts per minute that could be interpreted as bound in the charcoal binding assay by about threefold (7,800 cpm vs. 2,200–2,600).

Chromatography. Gel filtration chromatography was performed using a 1.6 × 90-cm (bed volume = 180 ml) Sephadex G-200 Superfine (Pharmacia Fine Chemicals, Piscataway, NJ) column. Plasma samples (0.5 ml) were eluted at 4°C in 0.1 M Na phosphate buffer (pH 7.2) containing 0.01 M NaCl and collected in 1-ml fractions which were monitored for protein by determination of optical density at 280 nm. Aliquots (200 μl) taken from selected column fractions (usually every other) were affinity labeled with ^{125}I-Sm-C (150,000 cpm) after chromatography in the presence or absence of unlabeled Sm-C as described above, and subjected to SDS-PAGE. In other experiments, 0.5-ml plasma samples were incubated with ^{125}I-Sm-C (60,000 cpm) before chromatography. Fractions were counted in a gamma counter and aliquots (50–100 μl) assayed to determine the migration of endogenous immunoreactive Sm-C. In one experiment, a 0.5-ml sample of normal plasma was affinity labeled with ^{125}I-Sm-C (2.5×10^6 cpm) in a total volume of 5.0 ml before gel chromatography and 100-μl aliquots from selected fractions were subjected to SDS-PAGE. In another experiment, 4 ml of normal plasma was dialyzed against 0.1 M glycine-HCl, pH 3.6, for 72 h at room temperature before chromatography on a 2.6

× 70-cm (bed volume = 372 ml) column equilibrated in 0.05 M $NaPO_4$, pH 7.4.

Concanavalin A (Con A)-Sepharose (Pharmacia Fine Chemicals) affinity chromatography was performed on a 1.2 × 15-cm (bed volume = 10 ml) column. Plasma samples (0.5 ml) were eluted at 4°C in 0.05 M Na phosphate buffer (pH 7.0) containing 0.1 M NaCl (40 ml). The column was washed with an additional 40 ml of buffer, and the adsorbed proteins were subsequently eluted in the starting buffer containing 0.5 M α-methyl-D-mannoside (Sigma Chemical Co.). Aliquots (200 μl) from the Con A fractions were affinity labeled with ^{125}I-Sm-C (150,000 cpm) after chromatography in the presence or absence of excess unlabeled Sm-C as described above. In other experiments, plasma samples were affinity labeled with ^{125}I-Sm-C (3×10^6 cpm) before Con A fractionation.

Results

Affinity-labeled Sm-C binding-protein complexes in whole plasma

When whole plasma samples were incubated with ^{125}I-Sm-C and cross-linked, a number of specifically labeled Sm-C binding-protein complexes were observed (Fig. 1 shows a representative autoradiogram). In acromegalic plasmas, the most intensely labeled complexes migrated as doublets with apparent M_r of 28,000–24,000 and 43,000–35,000. More faintly labeled complexes were observed with apparent M_r of 50,000, 80,000, 110,000, 135,000, and 160,000. In normal plasmas, the pattern of specific ^{125}I-Sm-C binding-protein was similar; however, the labeling of the 28,000–24,000-mol wt doublets and the complexes with M_r of 80,000 and greater was less intense. The 43,000–35,000-mol wt complexes appeared as triplets and were more intensely labeled than in the acromegalic samples. In hypopituitary plasmas, the 28,000–24,000-mol wt doublets and the complexes M_r of 80,000 and greater were either only faintly visible or not observed. These plasmas exhibited the most intensely labeled 43,000–35,000-mol wt complexes, which again appeared as triplets. The 50,000-mol wt complexes were somewhat less intensely labeled than in acromegalic and normal plasma samples.

When the incubation of ^{125}I-Sm-C and plasma was carried out in the presence of an excess of unlabeled Sm-C, a number of autoradiographic bands were neither abolished nor diminished in intensity. Bands near the bottom of all lanes (M_r of 14,000) probably represent dimers of the ^{125}I-Sm-C molecule resulting from the iodination procedure, because they were also observed when ^{125}I-Sm-C alone was subjected to SDS-PAGE. Diffuse and noncompetitive labeling was observed in the regions between 50,000 and 70,000 mol wt and between 135,000 and 160,000 mol wt (Figs. 1 and 2). Much of the labeling in these areas apparently represented nonspecific interaction of ^{125}I-Sm-C and scavenger proteins because they either appeared or increased in intensity when ^{125}I-Sm-C was displaced from its specific binding proteins by unlabeled Sm-C.

Reduction of samples with 2% β-mercaptoethanol prior to SDS-PAGE did not alter the electrophoretic pattern of the specifically labeled plasma Sm-C binding-protein complexes. When whole plasma samples (diluted 1:10 in $NaPO_4$ buffer, pH 7.4) were heated at temperatures of up to 60°C for 20 min, the specific Sm-C binding proteins retained the capacity to bind ^{125}I-Sm-C; however, temperatures of 65°C and greater for 20 min destroyed the binding activity.

Inasmuch the GH secretory status of the blood donor appeared to be an important determinant of which ^{125}I-Sm-C binding-protein complexes were observed, the influence of GH secretory status was further studied. Plasma samples from hypopituitary children obtained before and during human growth hormone (hGH) treatment were affinity labeled and subjected to SDS-PAGE (Fig. 2). In plasmas from untreated

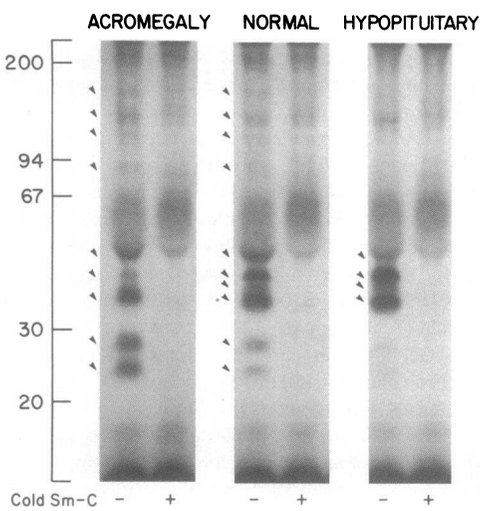

Figure 1. Electrophoretic analysis and autoradiography of the specifically labeled Sm-C binding-protein complexes in acromegalic, normal, and hypopituitary whole plasma. Samples of each plasma were affinity labeled with ^{125}I-Sm-C in the absence (cold Sm-C, − in left lane of each pair) or presence (cold Sm-C, + in right lane of each pair) of excess unlabeled Sm-C as described in Methods. Arrowheads indicate the bands that were abolished when the incubation was performed in the presence of an excess of unlabeled Sm-C. In hypopituitary plasma, arrowheads are omitted for the faintly labeled 135,000- and 28,000–24,000-mol wt bands, because these bands were not invariably observed in such plasma. The vertical axis shows molecular weights ($\times 10^{-3}$) of standards.

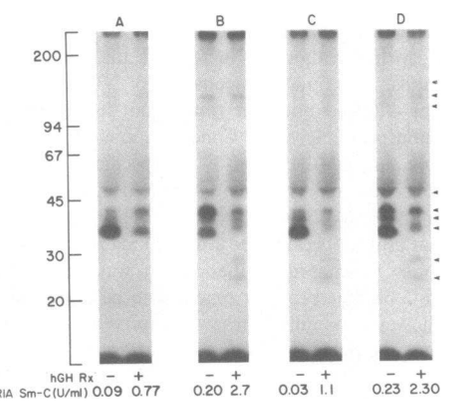

Figure 2. Electrophoretic analysis and autoradiography of the specifically labeled Sm-C binding-protein complexes in the whole plasma of hypopituitary children before and during hGH treatment. Plasma samples from hypopituitary children not receiving GH treatment (left lane of each pair) and during hGH treatment (right lane of each pair) were affinity labeled with ^{125}I-Sm-C as described in Methods. Arrowheads indicate specific ^{125}I-Sm-C binding-protein complexes. Immunoreactive Sm-C concentrations for each plasma appear at the bottom of the lanes. The vertical axis shows molecular weights ($\times 10^{-3}$) of standards.

GH-deficient children, an intensely labeled triplet complex with an M_r of 43,000–35,000 was consistently observed. In samples from the same children obtained during hGH treatment, the labeling intensity of the 43,000–35,000-mol wt complex invariably diminished and, in most, faintly labeled 28,000–24,000-mol wt complexes were observed. The 160,000-, 135,000-, 110,000-, 80,000-, and 50,000-mol wt complexes were not consistently affected.

Sm-C binding-protein complexes in fractionated plasma

Sephadex G-200 elution of plasma incubated with ^{125}I-Sm-C. When acromegalic plasma was incubated with ^{125}I-Sm-C and subjected to Sephadex G-200 chromatography, two major peaks of ^{125}I-Sm-C binding activity were observed (Fig. 3, see vertical arrows). The first peak, which co-migrated with the vast majority of the endogenous Sm-C, eluted in fractions containing proteins slightly smaller than the γ-globulin peak. The second peak eluted from the column just after the albumin peak. The migration of the ^{125}I-Sm-C binding activity peaks, as well as that of endogenous immunoreactive Sm-C, during Sephadex G-200 fractionation of normal plasma was indistinguishable from acromegalic plasma (Fig. 4, vertical arrows). In contrast, Sephadex chromatography revealed that hypopituitary plasma contained only a single peak of ^{125}I-Sm-C binding activity, which eluted slightly after the albumin peak and co-migrated with the endogenous Sm-C (Fig. 5, vertical arrow).

Affinity-labeled ^{125}I-Sm-C binding-protein complexes in Sephadex G-200 fractions of plasma. When acromegalic plasma fractions eluting identically to the peaks of the ^{125}I-Sm-C binding activity were affinity labeled, those eluting near the γ-globulin peak contained not only intensely labeled γ-globulin-sized bands (apparent M_r of 160,000 and 135,000), but also

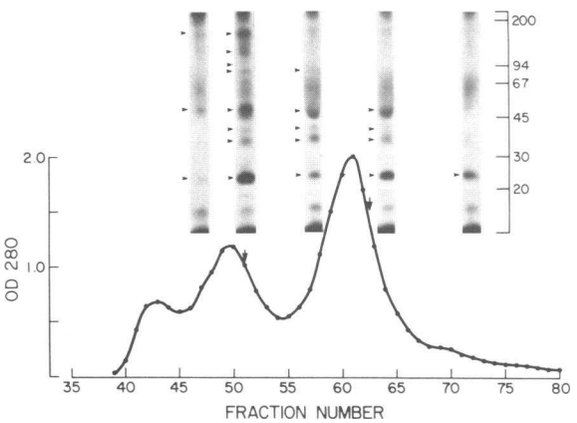

Figure 3. Sephadex G-200 fractionation of acromegalic plasma. Plasma (0.5 ml) was fractionated on a 1.6 × 90-cm Sephadex G-200 Superfine column, as described in Methods. The protein profile, as measured by optical density of 280 nm, is displayed. Vertical arrows at fractions 51 and 63 indicate the peaks of ^{125}I-Sm-C binding activity observed when plasma was incubated with ^{125}I-Sm-C before Sephadex chromatography. The arrow at fraction 51 also denotes the elution peak of immunoreactive Sm-C. Electrophoretic analysis and autoradiography of representative fractions appear in the inset above the protein profile. Horizontal arrowheads indicate the bands that were abolished in the presence of an excess of unlabeled Sm-C (lanes not shown), i.e., specifically labeled Sm-C binding-protein complexes. The vertical axis shows molecular weights ($\times 10^{-3}$) of standards.

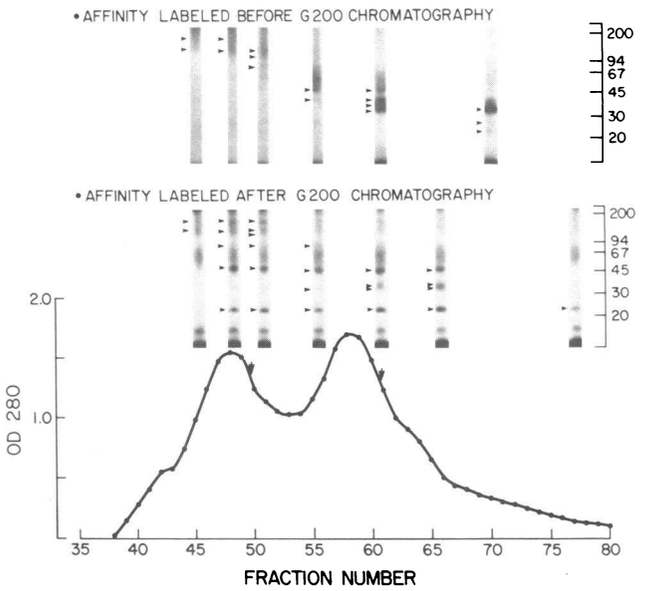

Figure 4. Normal plasma affinity labeled with ^{125}I-Sm-C before and after Sephadex G-200 chromatography. Fresh plasma (0.5 ml) alone and an equivalent volume of plasma affinity labeled with ^{125}I-Sm-C before chromatography were fractionated, as described in Methods. The protein profile, optical density of 280 nm, is displayed. Vertical arrows at fractions 50 and 61 indicate the ^{125}I-Sm-C binding-activity peaks observed when normal plasma was incubated with ^{125}I-Sm-C before chromatography. The arrow at fraction 50 also denotes the elution peak of immunoreactive Sm-C. Electrophoretic analysis and autoradiography of representative fractions which were affinity labeled with ^{125}I-Sm-C before G-200 chromatography (*top inset*) or after fractionation (*bottom inset*) appear as insets above the protein profile. Horizontal arrowheads indicate the specifically labeled ^{125}I-Sm-C binding-protein complexes. Vertical axes on the right show molecular weights ($\times 10^{-3}$) of standards.

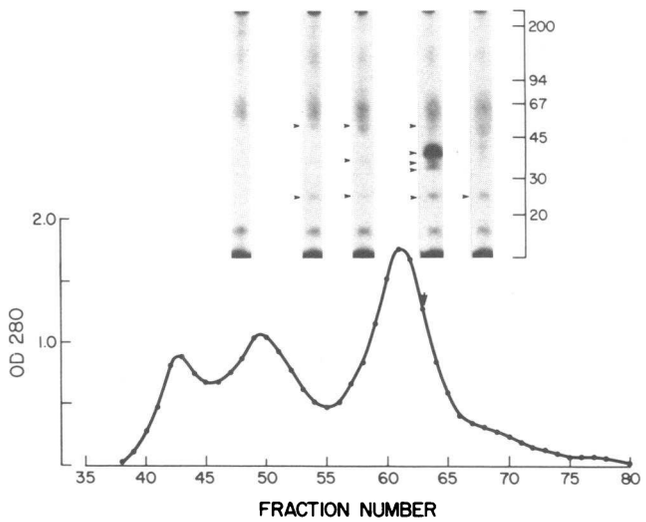

Figure 5. Sephadex G-200 fractionation of hypopituitary plasma. Plasma (0.5 ml) was fractionated as described in Methods. The protein profile, optical density of 280 nm, is displayed. The vertical arrow at fraction 63 indicates the peak of ^{125}I-Sm-C binding activity observed when plasma was incubated with labeled Sm-C before Sephadex chromatography. Electrophoretic analysis and autoradiography of representative fractions appears in the inset above the protein profile. Horizontal arrowheads indicate the specifically labeled bands. The vertical axis shows molecular weights ($\times 10^{-3}$) of standards.

^{125}I-Sm-C binding-protein complexes with apparent M_r of 110,000, 80,000, 50,000, 43,000–35,000, and 24,000 (Fig. 3). Of these complexes, the 160,000-, 50,000-, and 24,000-mol wt species were the most intensely labeled. The smaller complexes, i.e., the 50,000-, 43,000–35,000-, and 24,000-mol wt species, were also observed in size-appropriate column fractions (i.e., their migration during gel filtration was consistent with their apparent M_r as determined by SDS-PAGE) and account for the second peak of ^{125}I-Sm-C binding activity. The 50,000- and 24,000-mol wt species had two distinct peaks of labeling intensity which were coincident with the ^{125}I-Sm-C binding activity peaks (Fig. 3, insets; contrast the labeling intensity of these species in fractions 51 and 64 with their labeling in fraction 58). On the other hand, the 43,000–35,000-mol wt species eluted as a broad peak with the most intense labeling occurring near the albumin peak (Fig. 3, insets; note the similar labeling intensity of the 43,000–35,000-mol wt species in fractions 51, 58 and 64; fractions 60–62, not shown, contained the most intensely labeled 43,000–35,000-mol wt species).

When normal plasma was fractionated, the elution pattern of the specific Sm-C binding-protein complexes was similar to that of acromegalic plasma (Fig. 4, insets; affinity labeled after G-200 chromatography). However, in normal plasma labeled 43,000–35,000-mol wt complexes were nearly absent in fractions from the peak of ^{125}I-Sm-C binding activity eluting near γ-globulin, but were intensely labeled in fractions eluting just after the albumin peak. Together with the 50,000- and 24,000-mol wt species, these binding proteins appear to account for the second peak of ^{125}I-Sm-C binding activity observed in both acromegalic and normal plasma. That a portion of the 43,000–35,000-mol wt binding species eluted with the γ-globulin-sized binding proteins in acromegalic plasma is not readily apparent, but may be due to nonspecific protein–protein associations.

The single ^{125}I-Sm-C binding activity peak of hypopituitary plasma that eluted slightly after albumin contained intensely labeled 43,000–35,000-mol wt complexes and very faintly labeled bands representing the 50,000- and 24,000-mol wt complexes (Fig. 5). The higher molecular weight ^{125}I-Sm-C binding-protein complexes present in the γ-globulin fractions of both acromegalic and normal plasma were not observed in hypopituitary plasma; however, very faintly labeled 50,000- and 24,000-mol wt complexes were detected in these fractions.

Because all labeled Sm-C binding-protein complexes did not migrate in a size-appropriate fashion during Sephadex G-200 column chromatography, normal plasma was affinity labeled before Sephadex G-200 chromatography to determine if the affinity-labeled complexes migrate aberrantly under these conditions as well. Cross-linked ^{125}I-Sm-C binding-protein complexes eluted predominantly in size-appropriate Sephadex column fractions (Fig. 4, top inset: affinity labeled before G-200 chromatography); specifically, γ-globulin-sized column fractions contained only very faint bands of <80,000-mol wt cross-linked complexes. These results imply that cross-linked ^{125}I-Sm-C binding-protein complexes do not migrate aberrantly on Sephadex G-200 columns. In addition, these results suggest that covalent cross-linking of each entity comprising the 160,000-mol wt complex is necessary for this complex to remain intact during SDS electrophoresis. SDS-PAGE may not by itself, however, account for the <80,000-mol wt complexes observed in γ-globulin-sized column fractions, in that events occurring during or after column chromatography, but before cross-linking, such as dilution or proteolysis, could result in the dissociation of the 160,000-mol wt complex.

Because Sm-C binding proteins can be freed of the bound peptide by acidification, normal plasma was incubated in 0.1 M glycine-HCl, pH 3.6 (ionic strength 0.05) (20) and then subjected to Sephadex G-200 chromatography under neutral conditions (Fig. 6). Acidification resulted in the apparent elimination of the specifically labeled Sm-C binding-protein complexes with M_r greater than or equal to 50,000, i.e., those complexes which previously eluted with the γ-globulin peak. Acid treatment also resulted in an apparent increase in nonspecific binding of ^{125}I-Sm-C, particularly in the proteins with M_r > 100,000 (see Fig. 6, inset at fraction 90, for example). The specific binding activity of the 43,000–35,000- and 28,000–24,000-mol wt complexes was preserved. The 43,000–35,000-mol wt complexes eluted in a narrow peak immediately after albumin. These complexes, which had appeared as a triplet when nonacid-treated plasma was studied, now appeared on autoradiograms as a broad band with M_r of 45,000–30,000, suggesting that they had sustained some damage as a result of acidification. After acid treatment, the 28,000–24,000-mol wt band, which had eluted in two distinct peaks in nonacid-treated plasma (both with the γ-globulin-sized proteins and in size-appropriate fractions), appeared as a single broad, size-appropriate peak whose labeling intensity was markedly increased. As expected, the immunoreactive Sm-C eluted as the free peptide.

Con A-Sepharose fractionation of affinity-labeled ^{125}I-Sm-C binding-protein complexes. Con A-Sepharose column chromatography effects a fractionation of the specific plasma Sm-

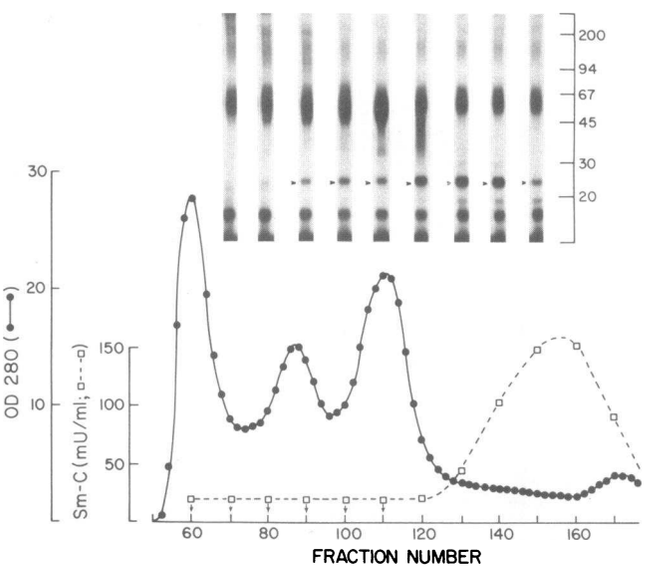

Figure 6. Acid-treated normal plasma affinity labeled with ^{125}I-Sm-C after G-200 chromatography. Normal plasma was dialyzed for 72 h at 22°C against 6 liters of 0.1 M glycine-HCl, pH 3.6, and subjected to Sephadex G-200 chromatography in 0.05 M $NaPO_4$, pH 7.4, as described in Methods. Closed circles and solid lines represent the protein profile (optical density of 280 nm) and open squares and the dotted line represent the elution profile of immunoreactive Sm-C. Electrophoretic analysis and autoradiography of representative fractions that were affinity labeled with ^{125}I-Sm-C after chromatography appear in the inset above. Horizontal arrowheads indicate the specifically labeled GH-dependent 24,000-mol wt complex. The vertical axis on the right shows molecular weights ($\times 10^{-3}$) of standards.

C binding-protein complexes, which is unchanged when cross-linking is performed before chromatography (Fig. 7). In all plasmas studied, the fall-through (i.e., nonadsorbed) fraction contained only the 43,000–35,000-mol wt Sm-C binding-protein complexes. In contrast, when acromegalic or normal plasma were chromatographed, the retentate fraction (i.e., the fraction eluted from the column with 0.5 M α-methyl-D-mannoside) contained all of the previously observed specific Sm-C binding-protein complexes (160,000, 135,000, 110,000, 80,000, 50,000, 43,000–35,000, and 28,000–24,000 mol wt). The 43,000–35,000-mol wt species, however, were faintly labeled compared with their labeling in the fall-through fraction. The Con A retentate fractions of hypopituitary plasma contained only faintly labeled 43,000–35,000-mol wt complexes. Thus, affinity for Con A distinguishes the 43,000–35,000-mol wt species from other Sm-C binding-protein complexes. That some of the 43,000–35,000-mol wt species were observed in the retentate fractions was attributed to inefficient fractionation due to nonspecific interactions among proteins.

Table I. Characteristics of Affinity-labeled ^{125}I-Sm-C Binding-Protein Complexes in Human Plasma

M_r*	GH dependent‡	Elutes with γ-globulin on Sephadex G-200§	Affinity for Con-A‖
160,000	+	+	+
135,000	+	+	+
110,000	+	+	+
80,000	+	+	+
50,000	±	+	+
43,000–35,000	−	−	−
28,000–24,000	+	+	+

* Apparent M_r determined on SDS-PAGE.
‡ Labeling is more intense in acromegalic than normal whole plasma, but faint or absent in hypopituitary plasma.
§ Identified in fractions migrating near γ-globulin when acromegalic or normal plasma is eluted.
‖ Retained by a Con A-Sepharose affinity column.

Discussion

Specific Sm-C binding-protein complexes have been identified in human plasma by affinity labeling with ^{125}I-Sm-C using the homobifunctional cross-linking agent DSS and separating the products by SDS-PAGE. These complexes can be distinguished from each other by several criteria: apparent M_r, GH dependence, behavior on Sephadex G-200, and affinity for Con A-Sepharose (Table I).

Consistent with the findings of others (6–11, 16–19), our observations suggest that two types of circulating somatomedin binding proteins can be distinguished on the basis of their GH dependence. A GH-dependent somatomedin binding protein has been shown to elute near the γ-globulin peak during gel filtration chromatography (6–11, 16, 18, 19). In these column fractions from either acromegalic or normal plasma, we observed not only specific ^{125}I-Sm-C binding-protein complexes similar in size to γ-globulin (apparent M_r of 160,000 and 135,000), but also smaller complexes with apparent M_r of 110,000, 80,000, 50,000, and 28,000–24,000. These complexes account for the GH-dependent γ-globulin peak of ^{125}I-Sm-C binding activity inasmuch as identical column fractions from hypopituitary plasmas contained either no specifically labeled complexes or only faintly labeled representatives of the 50,000 and 28,000–24,000-mol wt species. The relative labeling intensity of each of these complexes in acromegalic, normal, and hypopituitary whole plasma (more intense labeling in acromegalic than normal plasma, and either faint or no labeling in hypopituitary plasma) and the finding that some of these complexes appear in the plasma of hypopituitary children undergoing GH therapy provides strong evidence of their GH dependency. On the other hand, the 43,000–35,000-mol wt ^{125}I-Sm-C binding-protein complexes did not appear to be GH dependent, in that they were present in all plasma samples regardless of the GH status of the donor. These complexes generally eluted slightly after the albumin peak during gel filtration and did not appear to be associated with the GH-dependent complexes that migrated near the γ-globulin peak. The 43,000–35,000-mol wt complexes account for the non-GH dependent ^{125}I-Sm-C binding activity reported by others (6–11), but in normal and acromegalic plasma the 50,000- and 28,000–24,000-mol wt GH-dependent binding species also contribute to this binding peak. In addition, we were able to separate the two types of Sm-C binding proteins by Con A affinity chromatography. The GH-dependent Sm-C binding complexes (i.e., 160,000, 135,000, 110,000, 80,000, 50,000, and 28,000–24,000 mol wt) were adsorbed by Con A, whereas the non-GH-dependent species (43,000–35,000 mol wt) eluted in the void volume.

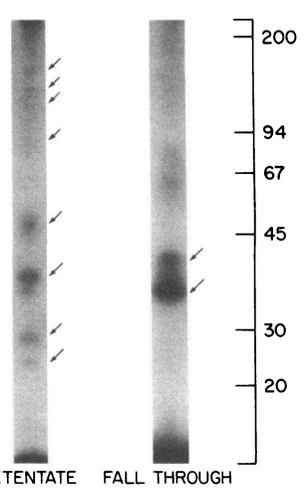

Figure 7. Electrophoretic analysis and autoradiography of ^{125}I-Sm-C affinity-labeled normal plasma fractionated by Con A-Sepharose chromatography. Whole plasma was affinity labeled and fractionated by Con A-Sepharose, as described in Methods. Fall through refers to the column void volume that contained proteins not adsorbed by Con-A. Retentate refers to the fraction that was eluted from the column using 0.5 M α-methyl-mannoside and contained the proteins adsorbed by Con A. Arrows indicate specifically labeled Sm-C binding-protein complexes. The vertical axis on the left shows molecular weights ($\times 10^{-3}$) of standards.

Affinity labeling of specific Sm-C binding-protein complexes is by definition reduced or abolished in the presence of sufficiently high concentrations of unlabeled Sm-C because the binding of ^{125}I-Sm-C to these proteins is largely determined by binding site occupancy. We observed that concentrations of unlabeled Sm-C in the range of those found in plasma (nanomolar) consistently reduce the labeling intensity of all specific ^{125}I-Sm-C binding-protein complexes. This suggests that endogenous plasma Sm-C concentrations influence the labeling intensity of specific binding complexes.

The labeling intensity of the 43,000–35,000-mol wt species is inversely proportional to the plasma Sm-C concentration of the blood donor, i.e., most intense in hypopituitary plasma

and least intense in acromegalic plasma. Furthermore, there is a marked decrease in their labeling intensity after plasma Sm-C concentrations are normalized in hypopituitary children by GH treatment. Nonetheless, the 43,000–35,000-mol wt complexes are intensely labeled in all plasmas studied, suggesting that these species are relatively unsaturated regardless of plasma Sm-C concentrations. This concept is supported by the observations that only a small portion of the endogenous somatomedin migrates with the 43,000–35,000-mol wt species during gel chromatography (6–10, 13, 18, 19), but when ^{125}I-Sm-C is incubated with normal plasma prior to chromatography, the majority of the bound label elutes in the 43,000–35,000 mol wt range.

Consistent with our findings and those of others (5–7, 13, 20, 21), Furlanetto (8) has reported that 95% of the immunoreactive Sm-C in normal serum is associated with GH-dependent, γ-globulin-sized binding activity that is adsorbed by Con A-Sepharose. That the majority of endogenous Sm-C is associated with the GH-dependent binding species strongly suggests that these proteins have a higher affinity for Sm-C than the non-GH-dependent species. This is also consistent with the findings of Binoux et al. (12), who reported that human serum contains two binding proteins that exhibit different affinities for the somatomedins: one exhibiting a higher affinity for Sm-C/IGF-I was the predominant binding species in the 100,000–170,000 mol wt range, whereas a binding protein with a selective affinity for IGF-II predominated in the 40,000–70,000 mol wt range. Confirmation of this hypothesis will require competition studies using homogeneous preparations of the different binding proteins.

There are some problems inherent in studies that employ chemical cross-linking agents (28). Because DSS can covalently cross-link any two free amino groups in the proper orientation and within an 11 Å radius, nonspecific bands resulting from random protein–protein interactions and those representing ^{125}I-Sm-C bound to scavenger proteins (e.g., albumin) were consistently observed, especially after acid treatment of plasma. That the specifically labeled binding proteins with M_r of <50,000 often appeared as doublet or triplet bands may represent an additional problem of this methodology. Because the observed bands represent the M_r of a binding protein plus the cross-linked ^{125}I-labeled moiety, the 28,000–24,000-mol wt doublet may represent the resolution of a single binding protein cross-linked to intact ^{125}I-Sm-C molecules and to ^{125}I-labeled fragments of Sm-C molecules. Svoboda et al. (29) have reported that the purified Sm-C peptide can undergo autolysis, which results in fragments with nearly identical molecular weights (3,000–4,000). On the other hand, the 43,000–35,000-mol wt triplet bands clearly represent a more complicated situation. The appearance of triplet bands could be the result of a combination of factors, such as intact ^{125}I-Sm-C molecule(s) and ^{125}I-labeled fragment(s) of Sm-C molecules cross-linked to a single binding protein and/or to binding proteins that had been modified by glycosylation or proteolysis. Alternatively, these replicate bands could represent unique binding proteins with similar molecular weights.

Using the same affinity-labeling technique, we have previously examined serum Sm-C binding proteins in the rat (23) and found that they bear remarkable similarity to those observed in human plasma. Normal rat sera exhibited Sm-C binding species with M_r of 95,000, 26,000–23,000, and possibly a series of complexes ranging from 175,000 to 115,000, none of which were observed in hypophysectomized rat sera. These complexes are analogous to the GH-dependent Sm-C binding proteins in human plasma. Hypophysectomized rat serum contained only a 36,000–33,000-mol wt doublet, which appears homologous to the 43,000–35,000-mol wt non-GH-dependent species observed in human plasma. Using an alternative cross-linking procedure (30), Schwander et al. (31) have reported a 35,000-mol wt IGF-I binding protein in rat liver perfusates, whereas Hossenlopp et al. (32) reported an IGF-I binding protein in both cultured rat and human liver media which migrated as doublet bands between 37,000 and 40,000 mol wt. A multiplication-stimulating activity carrier protein of similar size has been purified from conditioned media of the rat liver cell line, BRL-3A, by Knauer et al. (33). In addition, we have also observed affinity-labeled complexes with apparent M_r of 45,000–35,000 in human cord plasma and amniotic fluid (22).

In the present study, we consistently labeled multiple Sm-C binding proteins (complexes with apparent M_r of 160,000, 135,000, 110,000, 80,000, 50,000, and 28,000–24,000) in fractions that elute near the γ-globulin peak during G-200 Sephadex chromatography. These observations indicate that each of these species is in some way associated with the γ-globulin-sized, GH-dependent ^{125}I-Sm-C binding activity. Indeed, the co-migration of the 160,000-mol wt complex and the smaller species strongly suggests that the native γ-globulin-sized binding protein is a complex that dissociates to generate the pattern of labeled species we have observed on SDS gels. It is not clear whether the 160,000-mol wt complex dissociates as a result of instability in a dilute solution (i.e., dissociation during and after chromatography but before cross-linking) or as a consequence of the SDS treatment followed by electrophoretic separation. These two possibilities are, of course, not mutually exclusive, and we suspect that both occur. The finding that the <160,000-mol wt complexes migrated predominately in size-appropriate column fractions when plasma was labeled before G-200 chromatography suggests that these complexes circulate free in plasma. It seems possible, therefore, that an equilibrium exists in plasma between the 160,000-mol wt complex and the smaller binding species, such that dilution during column chromatography favors a dissociation of the 160,000-mol wt complex into its constituent subunits, as previously suggested by Daughaday et al. (13). Dissociation of subunits could also expose more Sm-C/IGF-I binding sites and, thus, explain the increased binding in the γ-globulin fractions of chromatographed human serum observed by White et al. (19) when they studied binding after elution.

Many studies have documented that acidification of plasma effects a dissociation of Sm-C and its binding proteins (3, 5, 6, 8, 17, 20, 21). Our data, like those of others (8, 18, 33), indicate that acidification dissociates the GH-dependent Sm-C binding protein into subunits. After acidification, normal plasma exhibited only two specific Sm-C binding-protein complexes: the non-GH-dependent 43,000–35,000-mol wt complex and the GH-dependent 28,000–24,000-mol wt species. The migration of the 43,000–35,000-mol wt complex during Sephadex G-200 chromatography was essentially unchanged by acidification, although the diffuse labeling of these complexes indicates that they may be unstable under acidic conditions. On the other hand, the 28,000–24,000-mol wt complex eluted in size-appropriate fractions as a single broad peak. The acid-mediated destruction of the γ-globulin-sized Sm-C binding

protein complex and the concurrent increase in the labeling intensity of the 28,000–24,000-mol wt complexes suggests that the 28,000–24,000-mol wt complex is the Sm-C binding subunit of the 160,000-mol wt, GH-dependent binding-protein complex.

Furlanetto (8) has suggested that the 160,000-mol wt Sm-C binding protein is composed of at least two GH-dependent subunits, one of which does not bind Sm-C. Inasmuch our technique only allows visualization of proteins which are directly associated with ^{125}I-Sm-C, our findings do not preclude this possibility; however, they do suggest an alternative hypothesis. In fractions eluting from Sephadex G-200 columns with γ-globulin-sized proteins and in the retentate fractions of Con A columns, the specifically labeled ^{125}I-Sm-C binding-protein complexes consistently appeared in near multiples of 28,000–24,000 mol wt. This observation suggests that the 160,000-mol wt Sm-C binding-protein complex may be an oligomer composed of noncovalently linked 28,000–24,000-mol wt subunits. The occurrence of six GH-dependent binding species suggests that the native 160,000-mol wt complex is a hexamer and that each of the smaller species represents an oligomer composed of a different number of 28,000–24,000-mol wt protomers covalently cross-linked to one another by DSS. This argument is supported by studies of Davies and Stark (34), who demonstrated that cross-linked oligomers composed of identical subunits can be resolved on SDS polyacrylamide gels to a set of species with molecular weights equal to integral multiples of the protomer's molecular weight. Using DMS to study subunit structure of oligomeric proteins, they clearly showed that the number of principal species observed is identical to the number of protomers in the native oligomer.

The 28,000–24,000-, 50,000-, and 160,000-mol wt complexes that we observed were consistently the most intensely labeled binding proteins eluting in the γ-globulin-sized peak of ^{125}I-Sm-C binding activity, while the 110,000- and 80,000-mol wt species in the same fractions were often only faintly labeled. The precise reason(s) for the differences in labeling intensity among the putative oligomers is unknown. One cause may be the ineffectiveness of DSS in cross-linking protomers that are arranged in certain conformations. For example, some protomers, or the ^{125}I-Sm-C molecules they bind, may not be accessible for cross-linking, resulting in complexes with aggregated but uncross-linked protomers or in complexes not covalently crosslinked to ^{125}I-Sm-C. It has been suggested that a portion of the circulating immunoreactive Sm-C is unavailable for the antibody/antigen reaction because of the manner in which it is complexed with binding proteins (20, 21). Perhaps the same factors that prevent the detection of immunoreactive Sm-C could also prevent cross-linking of the binding protein in certain steric arrangements. This could in turn affect labeling intensity. Another explanation for the labeling differences among the GH-dependent binding species may be that certain complexes, as the 110,000- and 80,000-mol wt species, are intrinsically unstable or labile intermediate forms of the 160,000-mol wt GH dependent complex. Alternatively, each complex may have a unique affinity for Sm-C.

Although consistent with all our findings, our conclusions as to the composition of the GH-dependent, γ-globulin-sized Sm-C binding protein remain speculative. Alternative hypotheses are that the GH-dependent complexes we observed are the result of partial proteolysis of the native 160,000-mol wt binding protein or that each represents a unique binding protein induced by GH. In future experiments, we will attempt to demonstrate involvement of the 28,000–24,000-mol wt subunit in the native 160,000-mol wt Sm-C binding-protein complex by immunologic means.

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