The Primary Structure of Glycoprotein III from Bovine Adrenal Medullary Chromaffin Granules

SEQUENCE SIMILARITY WITH HUMAN SERUM PROTEIN-40,40 AND RAT SERTOLI CELL GLYCOPROTEIN 2

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Glycoprotein III (GpIII) was purified from the soluble fraction of bovine chromaffin granules, the secretory vesicles of the adrenal medulla, by chromatography using wheat germ agglutinin-Sepharose followed by reverse-phase high performance liquid chromatography (HPLC). Characterization of this glycopeptide by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, reverse-phase HPLC, amino acid analysis, and partial NH₂-terminal sequence analysis indicated that GpIII was a disulfide-linked heterodimer with 37-kDa subunits. Analysis of in vitro translation products of adrenal medullary poly(A)+ RNA by immunoprecipitation using an anti-GpIII serum and sodium dodecyl sulfate-polyacrylamide gel electrophoresis suggested that both subunits are synthesized from a single precursor. Partial NH₂-terminal sequence analysis allowed construction of oligonucleotides which were used as primers for a polymerase chain reaction to generate a GpIII-specific DNA probe. This probe was used to isolate a cDNA clone encoding the GpIII precursor from a bovine adrenal medullary cDNA library. The predicted amino acid sequence of GpIII has >80% similarity to human serum protein-40,40, a protein implicated in the complement system, and to a major secretory product of Sertoli cells, glycoprotein 2, which is thought to play a role in spermatogenesis. Northern blot analysis confirmed that RNA encoding GpIII is also abundant in liver, tests, and brain.

Chromaffin granules are subcellular organelles of the adrenal medulla with specialized functions for the biosynthesis, storage, and secretion of catecholamines and some peptides and proteins, such as enkephalins and chromogranins. These vesicles are relatively easy to purify in good yield and have been used extensively as model secretory granules. Considerable information is available on the composition of these vesicles (see Ref. 1 for a review).

Most of the information available regarding chromaffin granule membrane proteins is based on electrophoretic techniques. Over 40 membrane proteins have been identified (2), many of which are glycosylated. Five major membrane glycoproteins, termed GpI-V, have been identified by lectin binding (3). Subsequently, improvements in analysis using twodimensional gel electrophoresis with lectin binding and membrane fractionation using Triton X-114 have enabled the identification of at least 20 membrane glycoproteins (4, 5). While the composition of chromaffin granule membranes has been increasingly well studied there is little detailed structural information available for the membrane proteins.

Three glycoproteins, GpI (dopamine β-hydroxylase) GpII, and GpIII, have been purified from chromaffin granule membranes using sequential lectin affinity chromatography (6). These workers determined that GpIII had a high carbohydrate content (32%), with the major sugars being galactose, N-acetylglucosamine, sialic acid, and mannose. The carbohydrate moieties are exposed in the inner or matrix side of the chromaffin granule membrane (3). GpIII was later shown to also be a component of the soluble contents of chromaffin granules (7). No differences were found between the membrane and soluble forms of GpIII. Both are present as acidic glycoproteins which migrate in Laemmli gels as proteins of relative molecular mass 74,000 under non-reducing conditions and 37,000 under reducing conditions (7).

There are a number of reasons which make GpIII interesting. An antiserum raised against this glycoprotein has been used to show that the chromaffin granule membrane is returned to the Golgi following exocytosis and recycled into new secretory granules (8). It has been demonstrated immunologically that GpIII is also present in pituitary tissue (7), suggesting that GpIII may have a general role in secretory vesicles. Finally, GpIII, like dopamine β-hydroxylase and carboxypeptidase H, has membrane and soluble forms for which neither the structural basis or functional significance is known (9–11).

In the present paper we report a purification and characterization of soluble GpIII. The complete amino acid sequence has been deduced from cloned cDNA. GpIII was found to have sequence similarity to human serum protein-40,40 (12), a glycoprotein associated with complement components, and rat Sertoli cell glycoprotein 2 (13), a glycoprotein which may be important in spermatogenesis.

EXPERIMENTAL PROCEDURES

Preparation and Characterization of GpIII

Preparation of Chromaffin Granules and Chromaffin Granule Lysate—Chromaffin granules were prepared from bovine adrenal medulla by the method of Clyne et al. (14) with the following modifications.

The abbreviations used are: Gp, glycoprotein; bp, base pairs; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; SP2, rat Sertoli cell glycoprotein 2; SP-40,40, human serum protein-40,40; WGA, wheat germ agglutinin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
Adrenal Medullary Chromaffin Granule Glycoprotein III

dulla (55 g) as described (14) except that all sucrose solutions were buffered with 10 mM Hepes-NaOH, pH 7.0 (15) and that the final purification of chromaffin granules involved sedimentation through a 1.7 M sucrose cushion (Beckman Ti 70, 50,000 rpm, 60 min) at 4 °C. Chromaffin granules were lysed by suspension in 5 mM Tris-acetate, pH 7.0, 0.2 M NaCl and incubated with 1 M ethanalamine-HCl, pH 9.0, for 1 h at room temperature. Finally the WGA-Sepharose was washed with 500 ml of 0.05 M sodium phosphate, pH 7.0, 0.2 M NaCl and stored in 0.05 M sodium phosphate, pH 7.0, 0.2 M NaCl containing 0.02% (w/v) sodium azide.

WGA-Septarose Chromatography—This was based on the method described in Ref. 7. Chromaffin granule lysate (150 ml, obtained from 110 g of adrenal medulla) was dialyzed overnight at 4 °C against 5 mM Tris-succinate, pH 5.9 (15 volumes), diluted with an equal volume of 0.1 M sodium phosphate, pH 7.0, 0.4 M NaCl, and applied to a 1 x 5 cm WGA-Sepharose column, previously equilibrated with 0.05 M sodium phosphate, pH 7.0, 0.2 M NaCl (sodium phosphate buffered saline), at 16 ml/h. The column was washed with phosphate-buffered saline for 3 h at 20 ml/h and eluted with 30 ml of 0.3 M N-acetylglucosamine in phosphate-buffered saline at 12 ml/h. The chromatography was performed at 4 °C. The eluate was reduced to 1-2 ml using an Amicon ultrafiltration apparatus with a PM-10 membrane and subjected to reverse-phase HPLC.

Reverse-Phase HPLC—Reverse-phase HPLC was performed using a Vydac C18 column (25 x 0.46 cm), with 0.1% aqueous trifluoroacetic acid and 80% acetonitrile, 0.1% trifluoroacetic acid (solvent B) as solvents. The column was eluted with a linear gradient of 10% to 80% solvent B over 80 min, at 1 ml/min. Absorbance was monitored at 214 nm. Peaks were collected by hand and immediately neutralized with 1 M NH4HCO3.

SDS-Polyacrylamide Gel Electrophoresis—SDS-PAGE was performed using the buffer system of Laemmli (18).

Reduction and Carboxymethylation—GpIII was reduced in 6 M guanidinium hydrochloride, 0.3 M Tris-HCl, pH 8.5, 2 M EDTA, and 10 mM dithiothreitol for 3 h at 37 °C and carboxymethylated by the addition of iodoacetic acid to 40 mM and incubation for 45 min at room temperature, in the dark. The reaction was terminated by the addition of dithiothreitol to 110 mM.

Trypsin Digestion—Samples were dissolved in 2 ml urea, 2 mM CaCl2, 100 mM NH4HCO3 and incubated with L-1-tosylamido-2-phenylethyl chloromethyl ketone-trypsin (substrate/enzyme, 50:1) for 2 h at 37 °C. The tryptic peptides were separated by reverse-phase HPLC using a Vydac C8 column (25 x 0.46 cm) using similar conditions to those described above.

Protein Sequencing—Sequence analysis was performed using a gas-phase sequencer (470A) equipped with an on-line phenylthiohydantoin-amino acid analyzer (120A) with chemicals and the program, 03RPTH, supplied by the manufacturer (Applied Biosystems).

Isolation of RNA and in Vitro Translation—Total cellular RNA was isolated from bovine tissues by the guanidinium thiocyanate-CsCl method (19). Poly(A) RNA was isolated from total RNA by chromatography on oligo(dT) cellulose (20). Bovine adrenal medullary poly(A) RNA was translated in vitro using a commercial wheat germ system (Promega) with [35S]methionine as label.

Construction of a GpIII-specific cDNA Probe and Adrenal Medullary cDNA Library: Isolation and Sequence Analysis of cDNA Clones

Preparation of the cDNA Probe—First strand cDNA was transcribed from bovine adrenal medullary poly(A) RNA as described (21). The RNA was then hydrolyzed with NaOH, and the cDNA was amplified with Taq polymerase using a Perkin-Elmer Cetus kit. The reaction mixture, containing 2.7 μg of each primer, was subjected to repeated cycles of 95 °C for 2 min, 42 °C for 2 min, and 55 °C for 5 min, by 50°C, and transferred from a heating block and waterbaths set at these temperatures.

Construction of the cDNA Library—Bovine adrenal medullary poly(A) RNA was transcribed and converted into double-stranded cDNA using a modification of the Gubler and Hoffman (21) method recommended by Promega. The cDNA ends were blunted using T4 DNA polymerase and ligated to EcoRI adaptors (Promega). This material was fractionated on a Sepharose CL4B column, and cDNA longer than 500 bp was phosphorylated and ligated to dephosphorylated AgI10 arms (Promega). Finally, the DNA was packaged using a commercial kit (Promega). The library was amplified once using Escherichia coli C600rifl as host.

Library Screening and Northern Blot Analysis—To screen the cDNA library, phage were grown using E. coli c600rifl as host and the viral plaques transferred to nitrocellulose (22). For Northern blot analysis, total RNA was fractionated using formaldehyde-agarose gels containing 0.66 M formaldehyde and transferred to nitrocellulose (23). The PCR product was 32P-labeled by primer extension using both PCR primers and avian myeloblastosis virus reverse transcriptase. The cloned GpIII cDNA was labeled by nick translation. Hybridizations were carried out overnight at 42 °C in 6 x SSC (0.9 M NaCl, 0.9 M sodium citrate), 50% formamide, 0.2% SDS, 5 x Denhardt’s, and 100 μg/ml yeast tRNA. The filters were washed twice for 10 min at 65 °C in 0.1 x SSC, 0.2% SDS.

DNA Sequencing—Specific restriction fragments of the GpIII cDNA and the PCR product were cloned into Bluescript (Stratagene). Deoxyxyst chain termination sequencing was carried out using double-stranded DNA templates with the T7 and T3 sequencing primers and commercial kits (KiloBase, Bethesda Research Laboratories and Sequenase 2.0, United States Biochemical). One sequence was obtained using an internal primer. [35S]dATP (800 Ci/mmol, Du Pont-New England Nuclear) was used as label. One compression was resolved using 7-deaza-dGTP in place of dGTP.

RESULTS

Purification and Characterization of GpIII—GpIII was purified approximately 150-fold from chromaffin granule lysate by affinity chromatography on WGA-Sepharose (approximately 2 mg of GpIII was recovered from 300 mg of lysate protein). This glycoprotein was identified in the column eluate as the major component by SDSPAGE with Coomassie Blue staining, with relative molecular mass 74,000 under nonreducing conditions and 37,000 under reducing conditions (Fig. 1). A major peak of GpIII was recovered following further purification of this material by reverse-phase HPLC (Figs. 1 and 2A). Automated NH2-terminal sequence analysis of HPLC-purified GpIII revealed two sequences in approximately equal yield. When GpIII was reduced and alkylated and subjected to reverse-phase HPLC, two major peaks were isolated (Fig. 2B). The NH2-terminal sequence of the earlier eluting peak, termed the A chain, was Ile-Ser-Asp-Lys-Glu-Leu-Glu-Glu-Met-Ser-Thr-Glu-Gly and the NH2-terminal sequence of the later eluting peak, termed the B chain, was Asn-Val-Met-Pro-Phe-Pro-Leu-Leu-Glu-Glu-Pro-Leu-Val-Phe-Gln-Pro. The two sequences agreed with the results obtained with GpIII prior to reduction.

Fig. 1. Analysis of the WGA-Sepharose column eluate and HPLC-purified GpIII by SDS-PAGE. Lanes 1 and 2 are samples of the WGA-Sepharose column eluate (approximately 7 μg of protein). Lanes 3 and 4, GpIII purified by HPLC (approximately 20 μg of protein). Lanes 5 and 6 contain the A and B chains of GpIII (approximately 20 μg of each protein). Samples were run under non-reducing (lanes 1 and 3) or reducing (lanes 2, 4-6) conditions. Proteins were visualized by staining with Coomassie Blue.
FIG. 2. Purification GpIII by reverse-phase HPLC (A) and analysis of reduced and alkylated GpIII by reverse-phase HPLC (B). Both separations were performed using a Vydac C4 column. Each trace corresponds to 330 μg of protein recovered from the WGA-Sepharose column eluate. The horizontal bars indicate the material that was collected.

FIG. 3. Identification of the GpIII amino acid precursor. Adrenal medullary poly(A)+ RNA (1 μg) was translated in vitro in the presence of [35S]methionine. The translation products were subjected to immunoprecipitation using an anti-GpIII serum in the absence (lane 1) or presence (lane 2) of added non-radioactive GpIII (approximately 1 μg). The immunoprecipitated products were subjected to SDS-PAGE, using a 12% gel, followed by fluorography.

Analysis of the isolated A and B chains by SDS-PAGE showed that they have a similar relative molecular mass (Fig. 1). Both chains contain carbohydrate which can be removed with glycopeptidase F (data not shown). Presumably, microheterogeneity within the carbohydrate moieties gives rise to the appearance of GpIII in SDS-PAGE as a broad band. The amino acid composition of the two chains, while not identical, was similar (data not shown). The A and B chains were compared further by tryptic digestion and peptide mapping by reverse-phase HPLC (data not shown). The two chains appeared to have no tryptic peptides in common, indicating that GpIII is a heterodimer containing disulfide-linked sub-units.

Immunization of rabbits with purified GpIII gave an antiserum which recognized the isolated A and B chains before and after treatment with glycopeptidase F (data not shown). Adrenal medullary poly(A)+ RNA was translated in vitro, and an immunoprecipitation was carried out using the anti-GpIII serum. Fig. 3 shows that a single translation product was specifically immunoprecipitated, suggesting that the A and B chains are derived from a single precursor.

Isolation and Characterization of a GpIII-specific cDNA Clone—A DNA probe to GpIII was generated by mixed oligonucleotide-primed amplification of cDNA (24). The primers were based on the NH2-terminal sequence of the B chain and an internal sequence obtained from a cyanogen bromide fragment of this chain (Fig. 4A). These primers were used to amplify single-stranded cDNA prepared from adrenal medullary poly(A)+ RNA. After 25 cycles of amplification a single DNA product of approximately 650 bp was obtained (Fig. 4B).

When the PCR product was used as a probe for a Northern blot analysis of total adrenal medullary RNA, a single RNA species of approximately 2000 bp was detected (Fig. 4C).

A cDNA library was constructed in λgt10 from adrenal medullary poly(A)+ RNA and screened using the PCR product.

Fig. 4. Synthesis of a GpIII-specific cDNA probe using the polymerase chain reaction and characterization of GpIII mRNA by Northern blot analysis. A, the NH2-terminal sequences of the B chain (sense primer) and a CNBr fragment of the B chain (antisense primer) are shown with the regions used for primer construction underlined. The primers are shown below the corresponding amino acid sequences with the restriction sites underlined. An asterisk indicates positions where not all of the possible codons are represented. An NH2-terminal methionine (') was assumed. B, the products of the PCR (25 μg) were analyzed after 25 cycles by electrophoresis on a 1.5% agarose gel followed by staining with ethidium bromide (lane 2). Lane 1 contains size markers (Bethesda Research Laboratories). C, Northern blot analysis of total adrenal medullary RNA (25 μg). The RNA was fractionated on a 1.4% agarose gel and the nitrocellulose blot was hybridized with the radioactively labeled PCR product.
Adrenal Medullary Chromaffin Granule Glycoprotein III as probe. Forty positive clones were detected from approximately 20,000 plaques. A number of the clones were plaque-purified and characterized by restriction mapping. The longest clone contained a 1700-bp insert which was recovered as three EcoRI fragments which were cloned into Bluescript and sequenced, as outlined in Fig. 5A. The alignment of the three EcoRI fragments was deduced from the sequence obtained from the cloned PCR product. Fig. 5B shows the nucleotide sequence of the cDNA. The predicted amino acid sequence of the GpIII precursor is also shown in Fig. 5B and appears to be complete. The deduced sequence is in complete agreement with amino acid sequence results, containing the regions corresponding to the NH2-terminal sequence of the A and B chains as well as NH- terminal sequences obtained from CNBr fragments.

We were surprised to find that the sequence of GpIII was similar to that found for human serum protein-40,40 (12) and rat Sertoli cell glycoprotein 2 (13). An alignment of the amino acid sequences of these proteins is shown in Fig. 6. Northern blot analysis of RNA prepared from a range of bovine tissues showed that cDNA encoding GpIII hybridized with RNA species of similar size in the adrenal medulla, liver, testis, brain, and spleen (Fig. 7). In testis, a larger and less abundant RNA species was also detected.

**DISCUSSION**

In this paper we describe a purification and characterization of GpIII from bovine adrenal medulla. Several factors were found to have a major influence on the final purity and yield of this glycoprotein. The purity of GpIII following WGA-Sepharose chromatography is greatest when a near saturating...
FIG. 6. Comparison of the predicted amino acid sequences of GpIII, human SP-40,40, and rat SGP2. Identities are indicated with dashes. Gaps are indicated with dots. The numbering refers to the GpIII sequence. Potential N-linked glycosylation sites are shown for GpIII (0). Sites of proteolytic processing are indicated for GpIII above the sequences and for SP-40,40 and SGPZ, which are shown for GpIII above the sequences and below the sequences, by arrowheads.

FIG. 7. Northern blot analysis of GpIII-related RNA in bovine tissues. Total RNA (25 µg) isolated from bovine adrenal medulla (lane 1), liver (lane 2), testis (lane 3), brain (lane 4), and spleen, (lane 5) was fractionated on a 1.4% formaldehyde-agarose gel, and the nitrocellulose blot was hybridized with radioactively labeled GpIII cDNA present in Bluescript. No hybridization was detected when Bluescript DNA alone was used as a probe (not shown). amount of GpIII is applied to the column. Attempts to purify GpIII from smaller amounts of lysate resulted in an increased relative amount of other proteins, particularly dopamine β-hydroxylase in the N-acetylglucosamine eluate. Presumably under these conditions GpIII, which has a relatively high affinity for WGA, displaces more weakly bound proteins from the column. In addition, the recovery of GpIII from HPLC was best when the concentrated WGA-Sepharose eluate was applied to the column at neutral pH. Acidification of the sample resulted in a dramatically decreased yield, presumably because GpIII like secretogranin II, a pituitary secretory granule protein aggregates at low pH (25). To avoid losses, peaks collected from HPLC, containing GpIII, were neutralized immediately.

The predicted amino acid sequence of GpIII precursor confirms the results of earlier protein characterization. The sequences of the A and B chains are contained within a single precursor. The amino acid residues at -3 and -1 suggest a cleavage site for signal peptidase between -1 and +1 (26). Such a cleavage would result in an NH2-terminal sequence which corresponds to the NH2-terminal sequence of the A chain. An additional proteolytic cleavage at an Arg-Asn (position 202-203) would generate an NH2-terminal sequence identical to the NH2-terminal sequence of the B-chain and form the disulfide-linked dimer.

GpIII contains approximately 30% carbohydrate (6) which is consistent with the difference between the calculated protein molecular masses of the A chain (23,620 Da) and the B chain (25,335 Da) and the relative molecular masses of the mature subunits (37,000). Both subunits contain carbohydrate, all of which appears to be N-linked since the relative molecular masses of the subunits following treatment with glycopeptidase F correspond to the calculated protein molecular masses of the two chains (data not shown). There are three potential N-linked glycosylation sites in the predicted sequence of the A chain and five in the B chain. Analysis of partially deglycosylated subunits using glycopeptidase F suggests that most if not all of the potential N-linked glycosylation sites in each chain are utilized.2 The calculated molecular mass of the GpIII precursor (51,100 Da) does not agree with the relative molecular mass (45,000) of the immunoprecipitated in vitro translation product although the reason for this discrepancy is not known.

It is known that GpIII is present in chromaffin granules in both a membrane-bound and soluble form (6, 7). The membrane-associated species behaves as an integral membrane protein, requiring high concentrations of detergent to affect solubilization (6) and is prone to aggregation.2 Several possible mechanisms of membrane attachment appear unlikely. The predicted amino acid sequence of GpIII contains no obvious hydrophobic segment other than the leader peptide which is capable of transversing the membrane. Anchorage by an uncleaved leader peptide can be excluded since NH2-terminal sequence analysis of the membrane-bound form of GpIII revealed the same two NH2-terminal sequences obtained with the soluble form.2 It is also of interest to note that the unreduced membrane-bound and soluble forms of GpIII elute at the same position from HPLC and that they both bind detergent when analyzed by charge-shift electrophoresis.5 It is possible that a conformational change may convert GpIII from a soluble to a membrane-associated species as has been established for complement protein C9 (27). A number of other chromaffin granule components, most notably dopamine β-hydroxylase are present in both membrane and soluble forms. The presence of an NH2-terminal hydrophobic se-

2 D. J. Palmer and D. L. Christie, unpublished observations.
quence and a covalent glycosyl phosphatidylinositol anchor have recently been excluded as possibilities for dopamine β-hydroxylase (26, 27).

When we began this work it seemed likely that GpIII was a secretory granule component specific to endothelial tissues as it has been detected using immunological methods in anterior and posterior pituitary but not in liver, pancreas, or parotid gland (7). It was therefore surprising to find that GpIII shared a high degree of sequence similarity with a human serum glycoprotein, SP-40,40 (12), and a rat Sertoli cell glycoprotein, SGP2 (13) also termed clusterin. Comparison of the amino acid sequence of GpIII with SP-40,40 revealed a 72% level of identity and with SGP2 a 67% level of identity, suggesting that these proteins are species counterparts. In support of this, identical RNA species were detected by Northern blot analysis in adrenal, liver, and testis.

Alignment of the amino acid sequences of GpIII, SP-40,40, and SGP2 (Fig. 6) shows a number of interesting features. While the sequence identity is 61%, in some regions and in particular two cysteine-rich motifs (residues 77–104 and 258–286) the identity is striking. The first of these regions (residues 77–104) is related to sequences found in several terminal complement components (12) and is likely to be an important structural feature of these proteins. GpIII contains eight potential N-linked glycosylation sites whereas SP-40,40 and SGP2 each contain six, all of which correspond to potential sites in GpIII. The three proteins differ in the length of the leader sequences and at the NH₂ termini of the subunits equivalent to the A chain of GpIII. The two-chain structure of the mature glycoproteins however, results from cleavage at a similar site in all three proproteins.

It is difficult to compare the physiological functions of GpIII, SGP2, and SP-40,40. SGP2 is a major secretory product of rat Sertoli cells and is identical to rat and equivalent to the adrenal medullary protein, proenkephalin is also present in the testis and secreted by Sertoli cells (32, 33). Human SP-40,40 was discovered in association with terminal complement components in immune deposits in a patient suffering from glomerular nephritis, and it is also found associated with the spleen, kidney, and mammary tissue. It is likely that the liver is also known to be secreted (13). It would therefore be of interest to determine the sorting and secretion of this protein in a cell line such as mouse AT-20 cells which have well characterized constitutive and regulated pathways (37).

One common feature of GpIII, SGP2/clusterin, and SP-40,40 is their hydrophobicity, exhibited by their retention on reverse-phase HPLC, susceptibility to aggregation, ability to bind to cells, membranes, and hydrophobic proteins. It is possible that these properties may contribute toward a function in a variety of tissues. In this respect it will be important to understand the molecular basis for the presence of both soluble and membrane forms of this protein in chromaffin granules.

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D J Palmer and D L Christie

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