Biosynthesis and Molecular Cloning of Sulfated Glycoprotein 1 Secreted by Rat Sertoli Cells: Sequence Similarity with the 70-Kilodalton Precursor to Sulfatide/G_{M1} Activator†

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ABSTRACT: Sulfated glycoprotein 1 (SGP-1) is one of the abundant proteins secreted by rat Sertoli cells. Pulse–chase labeling shows that SGP-1 is synthesized as a cotranslationally glycosylated 67-kilodalton (kDa) precursor which is posttranslationally modified to a 70-kDa form before secretion to the extracellular space. A plasmid cDNA library was constructed from immunopurified mRNA, and two overlapping clones coding for the entire protein coding sequence were isolated. The cDNAs represent 27 nucleotides of 5′ noncoding sequence, 1554 nucleotides of coding sequence, and 594 nucleotides of 3′ noncoding sequence. The derived SGP-1 sequence contains 554 amino acids and has a molecular weight of 61 123. Four potential N-glycosylation sites occur within the sequence. An internal region of SGP-1 shows 78% sequence identity with the 67 N-terminal amino acids described for human sulfatide/G_{M1} activator (SAP-1). Sequence comparisons suggest that SGP-1 is the precursor to sulfatide/G_{M1} activator; however, the secretion of the protein from Sertoli cells is distinct from the proteolytic processing and lysosomal compartmentalization which have been described for human fibroblasts. The presence of internal sequence similarity suggests that three additional binding sites may occur in SGP-1. Northern blots show similar levels of expression for the 2.6-kilobase SGP-1 mRNA in all tissues examined. The site of SGP-1 synthesis in testis was localized to Sertoli cells by immunofluorescence and in situ hybridization.

Sertoli cells are the nondividing somatic epithelial cells located within the wall of the seminiferous tubule. Because of their close association with developing germinal cells and their secretory nature, Sertoli cells are thought to provide both physical and biochemical support to the process of spermatogenesis. The formation of tight junctional complexes between adjacent Sertoli cells results in a partitioning of the seminiferous tubule into two compartments (Fawcett, 1975). The outer "basal" compartment is in contact with the vascular supply and contains spermatogonia and preleptotene spermatocytes while the inner "adluminal" compartment contains meiotic germinal cells and spermatids. The tight junctions and secretory products of Sertoli cells therefore help to create a unique, serum-free environment in which spermatogenesis and meiosis can occur. The importance of Sertoli cells in the regulation of spermatogenesis is further supported by evidence that they are the primary target cells for follicle-stimulating hormone (FSH)[1] and testosterone (Fritz, 1978; Ritzen et al., 1981).

Sertoli cell secreted proteins which have been identified to date include testicular transferrin and ceruloplasmin, major secreted proteins which probably facilitate iron and copper transport to developing germinal cells (Skinner & Griswold, 1980, 1983). Androgen binding protein (ABP) has also been identified as a Sertoli cell secreted protein and may be responsible for androgen transport to tissues peripheral to the testis (French & Ritzen, 1973).

An analysis of proteins secreted by cultured rat Sertoli cells suggests that the majority of secreted protein synthesis is directed toward the production of two sulfated glycoproteins referred to as SGP-1 and SGP-2 [previously band 4 and DAG proteins (Kissinger et al., 1982; Sylvester et al., 1984)]. SGP-2 is a disulfide-linked heterodimer with subunit molecular weights of 47K and 34K. After secretion, SGP-2 becomes associated with the acrosome and distal tail portions of spermatozoa (Sylvester et al., 1984). Although the amino acid sequence of SGP-2 shows little sequence identity with other known proteins, a distant relationship with apolipoprotein A-I from human has been suggested (Collard & Griswold, 1987).

SGP-1 is also an abundant protein secreted by cultured rat Sertoli cells and like SGP-2 can be metabolically labeled with [^{35}S]sulfate. SGP-1 has a molecular size of 70 kDa and shows a high degree of charge heterogeneity, with an average p*I* of ~4.6 (Kissinger et al., 1982). In this paper, we describe the biosynthesis and secretion of SGP-1 by cultured rat Sertoli cells and the localization of SGP-1 and its mRNA in whole testis. Molecular cloning and sequencing of a cDNA to SGP-1 identify it as the precursor to the sulfatide/G_{M1} activator protein (also sphingolipid activator protein or SAP-1) which is a necessary component in the lysosomal degradation of glycosphingolipids (Stevens et al., 1981; Inui et al., 1983).

Materials and Methods

Cell Culture and Pulse–Chase Analysis. The procedures used for cell culture, immunoprecipitation, SDS–PAGE, and pulse–chase labeling were identical with those previously de-

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[1] Abbreviations: GbOse$_3$Cer, Galα1→4Galβ1→4Glc→Cer; G_{M1}, Galβ1→3GalNAcβ1→4[NeuAcα2→3]Galβ1→4Glc→Cer; PTH, phenylthiohydantoin; THF, tetrahydrofuran; SGP-1, sulfated glycoprotein 1; SGP-2, sulfated glycoprotein 2; cAMP, adenosine cyclic 3′,5′-phosphate; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; IPTG, isopropyl β-D-thiogalactopyranoside; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; NBRF, National Biomedical Research Foundation; PIR, Protein Identification Resource; kDa, kilodalton(s); FSH, follicle-stimulating hormone; ABP, androgen binding protein; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; kb, kilobase(s).

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scribed (Wilson & Griswold, 1979; Collard & Griswold, 1987).

Protein Purification and Sequencing. SGP-1 was purified from Sertoli cell culture medium by two consecutive reverse-phase HPLC runs as previously described (Griswold et al., 1986). Cyanogen bromide cleavage of the purified SGP-1 was performed by a protocol modified from Givol (1965) as follows: the purified SGP-1 was cleaved with a 250-fold excess (w/w) of cyanogen bromide in 70% formic acid for 24 h with gentle agitation under argon in the absence of light (Givol & Porter, 1965). The resulting solution of SGP-1 peptides generated by the cleavage was lyophilized to remove excess volatile reagents and byproducts and was subsequently desalted over a Bio-Gel P-2 gel filtration column (Bio-Rad). The desalted cyanogen bromide peptides were then separated by C-4 reverse-phase HPLC in solvent A (0.1% TFA in H_2O) with an increasing gradient of solvent B (0.1% TFA in acetonitrile) from 10% to 100% in 60 min as previously described (Griswold et al., 1986). The peak eluting at 45% B was collected, lyophilized, and subsequently resuspended in a minimal volume of (1:1:1) acetonitrile/tetrahydrofuran/water in preparation for analysis by Edman degradation. The amino-terminal sequence of this peptide and purified SGP-1 was obtained by using an Applied Biosystems Model 470-A gas-phase protein sequenator in conjunction with on-line analysis of the PTH-amino acid derivatives with an Applied Biosystems Model 120-A PTH analyzer. Gas-phase Edman degradation was performed according to the standard sequencing program 03RPTH; standard protocols provided by the manufacturer were followed.

Antibody Production. Purified SGP-1 which gave a single band by PAGE analysis was used for two subcutaneous injections into a rabbit. Approximately 500 μg of the protein in Freund's complete adjuvant was used for the first injection. This was followed 3 weeks later by an injection of 300 μg of protein in Freund's incomplete adjuvant.

Immunopurification of SGP-1 mRNA. Polysomes were isolated from homogenates of adult rat testes by the Mg^{2+} precipitation method of Palmiter (1974). Antibodies to SGP-1 were purified, and polysomes were immunoadsorbed to protein A–Sepharose (Sigma) as described by Kraus and Rosenberg (1982). Poly(A+) RNA was then isolated from the eluted polysomes as previously described (Huggenvik et al., 1987).

cDNA Synthesis and Selection of Recombinants. Immunopurified mRNA was used as a template for cDNA synthesis by the method of Land et al. (1981). The cDNA was cloned into the *Pst*I site of pTZ18U (U.S. Biochemical) by G-C tailing and then transfected into *Escherichia coli* JM105 (Viera & Messing, 1982). Plasmid DNA was purified from white colonies selected on X-gal/IPTG indicator plates and was sized by agarose gel electrophoresis. Plasmids containing large (>1 kb) inserts were immobilized on nitrocellulose and were used to hybrid select mRNA from total Sertoli cell mRNA (Gurney et al., 1982).

After identification of pSGP-1A by hybrid selection, the clone was used as a hybridization probe to screen approximately 1000 additional colonies. No additional 3′ clones were found. pSGP-1B was obtained by rescreening of the colonies with a human SAP-1 cDNA probe which was specific for the 3′ end of the mRNA (kindly provided by Dr. J. O'Brien, University of California, San Diego). Upon sequencing, it was found that pSGP-1B overlapped with pSGP-1A and extended the 3′ sequence through the end of the SGP-1 coding region.

SP6 Plasmid Construction, DNA Sequencing, and Northern Blot Analysis. The *Hin*dIII/*Nsi*I restriction fragment from pSGP-1A was subcloned into the *Hin*dIII/*Pst*I restriction site of pSP65 (Promega Biotech), and cRNA (anti-sense) was synthesized from purified plasmid using SP6 polymerase and biotinylated UTP as previously described (Morales et al., 1987). SGP-1 cDNA was sequenced by the dideoxy chain termination method as modified for use with reverse transcriptase (Sanger et al., 1977; Duncan, 1985) as previously described (Collard & Griswold, 1987). Northern blot analysis was conducted as previously described (Huggenvik et al., 1986) with the exception that nylon membranes (Micron Separations Inc.) were used in place of nitrocellulose.

Endoglycosidase F Digestion and in Vitro Translation. Immunoprecipitates of SGP-1 were eluted from pansorbin (Calbiochem-Behring) pellets by heating at 95 °C for 5 min in 60 μL of 0.17% SDS, 100 mM sodium acetate, pH 6.0, and 1.25% Triton X-100. After centrifugation, the supernatant was divided into equal portions and then incubated 24 h at 37 °C in the absence or presence of 0.4 unit of endoglycosidase F (New England Nuclear). For in vitro translation, approximately 1 μg of poly(A+) RNA was translated in a rabbit reticulocyte system (Bethesda Research Laboratories) supplemented with 50 μCi of [^{35}S]methionine per 30-μL reaction volume.

Immunofluorescence and in Situ Hybridization. The detection of SGP-1 protein by indirect immunofluorescence was similar to what we have previously described for SGP-2 (Sylvester et al., 1984). In situ hybridization using biotinylated cRNA probes on fixed testis was also carried out as previously described (Morales et al., 1987).

Computer Analysis. The NBRF/PIR computer programs and programs obtained from the Wisconsin Genetics Group (Devereux et al., 1984) were run on a VAX 11/785 computer (Digital Equipment Corp.). The NBRF/PIR protein data base (release 12), the GenBank data base (release 52), and the EMBL data base (release 12) were used in a sequence comparisons. The RELATE program (Dayhoff et al., 1983) was used to compare SGP-1 to PIRT3 (NBRF accession code), a proline-rich protein secreted by rat parotid gland (Ziemer et al., 1984). The program was run by using the mutation data scoring matrix and a fragment length of 26. Segment comparison scores were calculated from 100 runs of the randomized sequences.

RESULTS

Biosynthesis and Secretion of SGP-1. Primary cultures of rat Sertoli cells were grown in medium supplemented with dibutyryl-cAMP and were labeled with [^{35}S]methionine after 4 days of culture. Proteins isolated from the cultures were analyzed by SDS–PAGE under reducing conditions followed by fluorography. The majority of the [^{35}S]methionine-labeled protein secreted by cultured Sertoli cells after a 24-h labeling period is represented by the reduced 47- and 34-kDa subunits of SGP-2, and the 70-kDa subunit of SGP-1 (Figure 1, lane 1) (Kissinger et al., 1982). The other prominent Sertoli cell secreted protein visible is transferrin (76 kDa) (Skinner & Griswold, 1980).

Sertoli cell cultures were pulse-labeled with [^{35}S]methionine for 1 h, after which the medium was removed. Cells were then immediately lysed or incubated an additional 4 h with medium containing an excess of nonradioactive methionine. At the end of the chase period, both cells and medium were collected for immunoprecipitation with a polyclonal antibody raised to purified SGP-1 (see Materials and Methods). A single 67-kDa protein was immunoprecipitated from Sertoli cells that had been labeled for 1 h (Figure 1A, lane 2). After a 4-h chase, the 67-kDa precursor decreased in intensity while a second immunoprecipitable protein of 70 kDa became apparent

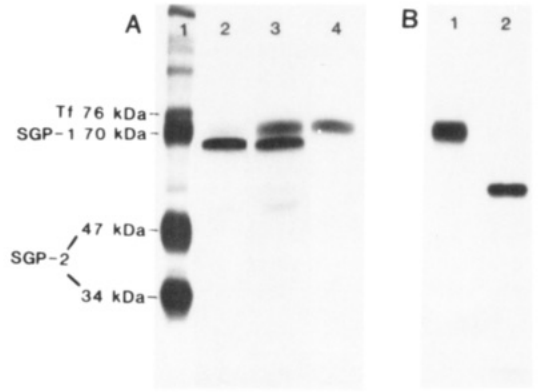

FIGURE 1: Pulse–chase analysis and endoglycosidase F digestion of SGP-1 synthesized by cultured rat Sertoli cells. The figure is a fluorograph of [^{35}S]methionine-labeled proteins separated on SDS–10% polyacrylamide gels under reducing conditions. (A) Lane 1 depicts the proteins secreted into culture medium after a 24-h labeling period. Lane 2 is an immunoprecipitation of SGP-1 from Sertoli cell lysates after a 1-h pulse-labeling period. Lanes 3 and 4 are immunoprecipitations of SGP-1 from cell lysates and medium (respectively) after a 4-h chase with unlabeled methionine. (B) SGP-1 was immunoprecipitated from culture medium and was then incubated for 24 h at 37 °C in the absence (lane 1) or presence (lane 2) of endoglycosidase F.

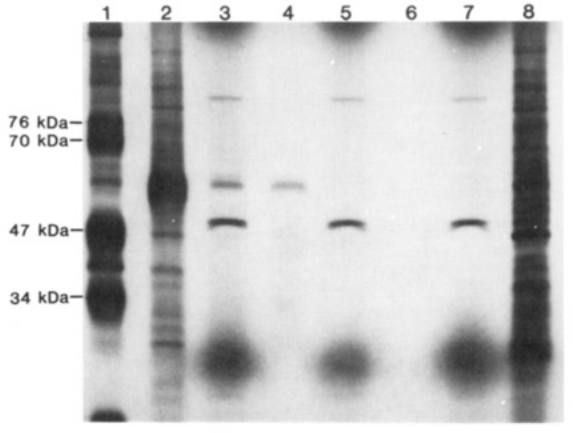

FIGURE 2: Fluorograph of [^{35}S]methionine-labeled proteins synthesized by in vitro translation. Proteins were separated on an SDS–10% polyacrylamide gel under reducing conditions. Lane 1, Sertoli cell secreted proteins (as markers); lane 2, translation products produced by immunopurified SGP-1 mRNA; lanes 3 and 5, translation products of mRNA selected from total Sertoli cell poly(A+) RNA by hybridization to immobilized pTZ18U plasmid containing SGP-1 cDNA (lane 3) or no insert (lane 5); lanes 4 and 6, immunoprecipitations (using anti-SGP-1) of 2 × the reaction volumes shown for lanes 3 and 5 (respectively); lane 7, control translation with no added RNA; lane 8, translation products of total Sertoli cell poly(A+) RNA.

(Figure 1A, lane 3). An analysis of the culture medium suggested that the majority of the 70-kDa protein was being secreted and was not being processed to smaller polypeptides (Figure 1A, lane 4). The change in SDS–PAGE mobility between the 67-kDa SGP-1 precursor and the mature 70-kDa secreted product is similar to what has been observed for SGP-2 upon sialation and sulfation of carbohydrate moieties (Collard & Griswold, 1987). Removal of carbohydrate from mature SGP-1 by digestion with endoglycosidase F produced a protein with an SDS–PAGE mobility of 57 kDa (Figure 1B), suggesting extensive cotranslational glycosylation during the biosynthesis of SGP-1.

Construction and Sequence of SGP-1 cDNA. Anti-SGP-1 serum was used to enrich for SGP-1 mRNA sequences by immunopurification. Polysomes prepared from whole rat testis by Mg^{2+} precipitation (Palmiter, 1974) were incubated with anti-SGP-1 and then adsorbed to a protein A–Sepharose column. After the column was extensively washed, enriched polysomes were eluted, and poly(A+) RNA was isolated. In vitro translation of the RNA produced a major 58-kDa protein product (Figure 2, lane 2) which was similar in its SDS–PAGE mobility to that of SGP-1 which had been treated with endoglycosidase F (Figure 1B, lane 2). The SGP-1-enriched RNA was used to construct an enriched plasmid library, and a clone of 1555 bases (designated pSGP-1A) was initially verified by hybrid-select translation of SGP-1 mRNA from total *Sertoli* cell mRNA. mRNA selected by hybridization with pSGP-1A produced a 58-kDa protein which was immunoprecipitable with anti-SGP-1 (Figure 2, lanes 3 and 4). No additional translation products were observed in selections which used control plasmid (Figure 2, lanes 5 and 6) when compared to translation reactions containing no added mRNA (Figure 2, lane 7). A second overlapping clone of 637 nucleotides (designated pSGP-1B) was isolated and provided the remainder of the SGP-1 coding sequence (Figure 3). The sequencing strategy for SGP-1 is shown in Figure 3, and the complete nucleotide sequence of the two overlapping cDNAs is shown in Figure 4. An open reading frame begins with the first ATG codon at nucleotide position 28 and continues through nucleotide position 1690, where it terminates with a single TAG codon. The deduced protein sequence contains 554 amino acids and has a calculated molecular weight of 61 123. The first 16 amino acids represent a hydrophobic signal sequence that is somewhat atypical due to its short length, its lack of a positively charged residue directly following the initiator methionine, and the large number of hydrophobic residues throughout the entire length of the signal (von Heijne, 1985). However, the translation initiation site conforms well to the consensus sequence described by Kozak (1986), and the proteolytic processing site for the signal sequence was confirmed by N-terminal gas-phase sequencing of purified SGP-1 (see Materials and Methods). Protein sequencing of a peptide obtained by cyanogen bromide cleavage of SGP-1 provided additional confirmation of the deduced protein sequence

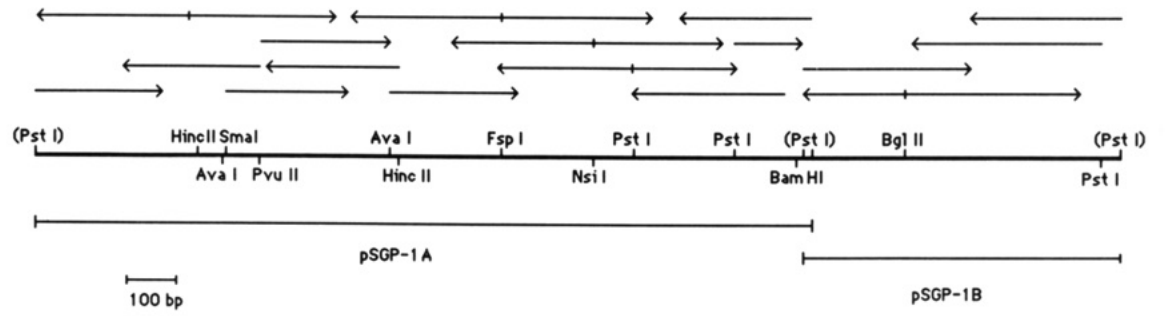

FIGURE 3: Sequencing strategy for SGP-1. Nucleotide sequence was determined from two overlapping cDNA clones by the dideoxy chain termination method. The arrows represent the strand and the extent of the sequence determinations for each subcloned DNA fragment. Restriction sites in parentheses were generated at the cDNA termini during the cloning procedure.

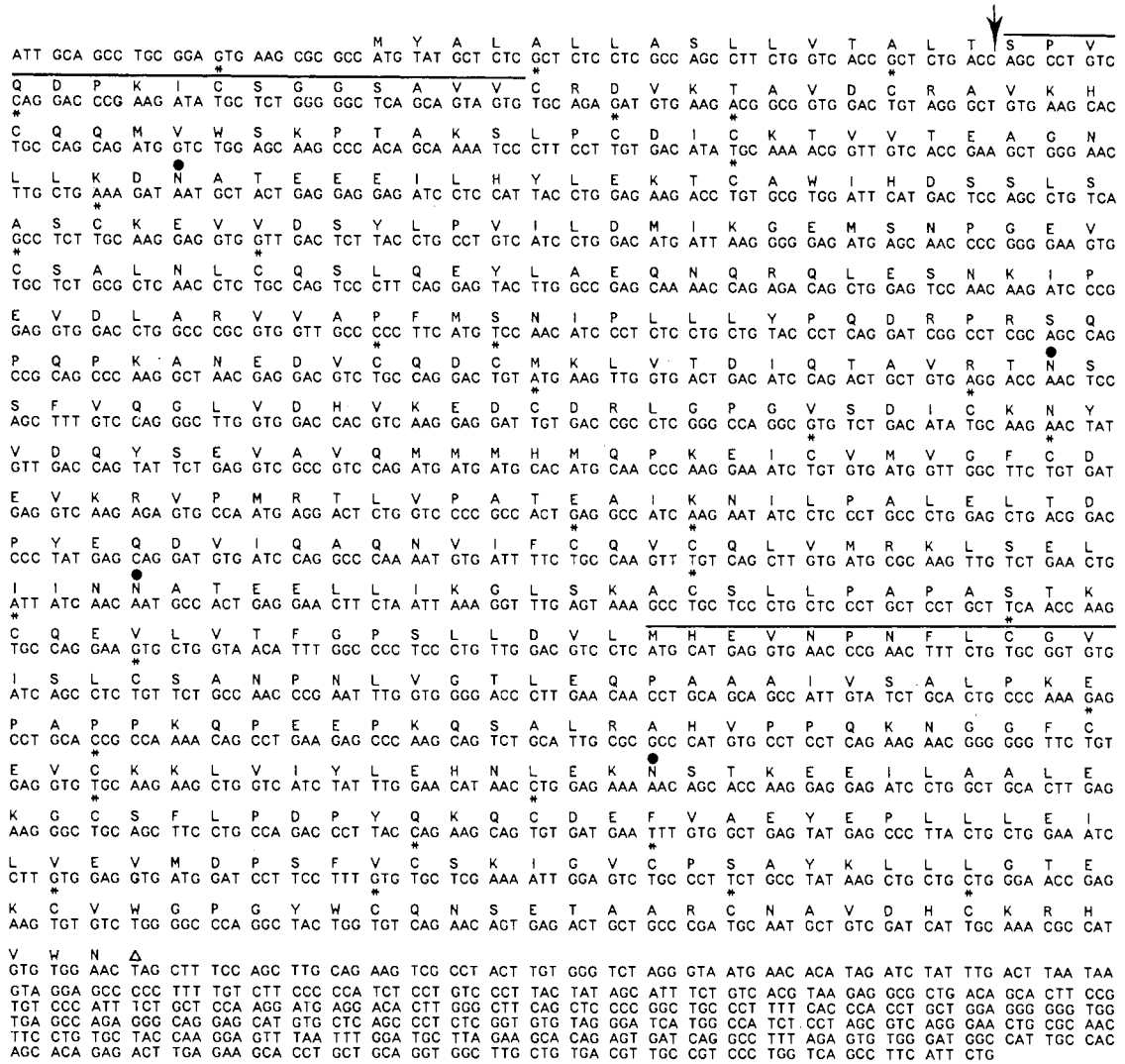

FIGURE 4: Nucleotide and deduced amino acid sequence of rat SGP-1 cDNA. Nucleotide and amino acid numbering occurs in the margins. Regions of amino acid sequence confirmed by protein sequencing are indicated by overlines. An arrow indicates the site for proteolytic processing of the leader peptide, and an open triangle identifies the stop codon used for termination of translation. Asterisks denote cysteine residues while closed circles indicate potential N-glycosylation sites.

(Figure 4). The purification of SGP-1 has been previously described (Griswold et al., 1986).

SGP-1 Contains Four Repeats Which Are Similar to the Sulfatide/G_{M1} Activator from Human. The SGP-1 cDNA and protein sequences were screened against nucleic acid and protein data bases using the FASTN and FASTP homology search programs (Wilbur & Lipman, 1983; Lipman & Pearson, 1985). A cDNA fragment which encodes 67 amino acids of the human sulfatide/G_{M1} activator (also referred to as SAP1, GenBank accession code M12710) was found to have a high degree of similarity (83%) to a corresponding segment of SGP-1 cDNA. Further comparison of the derived protein sequences showed that amino acids 194–261 of SGP-1 were 78% identical with the 67 N-terminal amino acids which have been published for human sulfatide/G_{M1} activator (Figure 5). This similarity is increased to about 97% when conservative substitutions are taken into account.

An extensive amount of internal sequence similarity is observed when SGP-1 is compared to itself using dot matrix analysis (Figure 6). This analysis suggests that 4 regions of approximately 100 amino acids are repeated along the 554 amino acid length of SGP-1 (see arrows, Figure 6). Interestingly, we observed that one of these repeats (arrow 2, Figure 6) delimits a region that is continuous with the amino acid sequence shown to be similar to the sulfatide/G_{M1} activator

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SGP-1   191 KANEDVCQDCMKLVTDIQTAVRTNSSFVQGLVDHVKEDCDRLGPGVSDIC 240
            ||||||   |||||||||||| ||| || |||| |||||||  |||
SAP-1     1 ...GDVCQDCIQMVTDIQTAVRTNSTFVQALVEHVKEECDRLGPGMADIC 47

          241 KNYVDQYSEVAVQMMMHMQPKEICVMVGFCDEVKRVPMRTLVPATEAIKN 290
              |||  |||  | ||||||||
           48 KNYISQYSQIAIQMMMHMQP..................................  67
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FIGURE 5: Alignment of SGP-1 and SAP-1 amino acid sequences. Amino acid numbering occurs in the margins, and identical residues are noted by bars. SAP-1 is the nomenclature used by Dewji et al. (1986) in their sequence description of the 67 N-terminal amino acids for mature sulfatide/G_{M1} activator.

(Figure 5). Iterative use of a gapping algorithm on the four repeats shown in Figure 6 results in the multiple sequence alignment shown in Figure 7. Several distinct aspects of this alignment include a complete conservation in the alignment position of six cysteine residues and one proline residue in each of the repeated regions. An alignment of the four potential N-glycosylation sites occurring in SGP-1 is also observed. A consensus sequence for two or more identical amino acids occurring in the same position of the alignment suggests that a high degree of similarity is present between the four repeated regions in SGP-1.

Further screening of SGP-1 against the sequence data bases suggests a more distant relationship with several proteins classified to the proline-rich superfamily. The sequence in SGP-1 responsible for this similarity was limited to a region contained approximately within amino acids 395–430. When

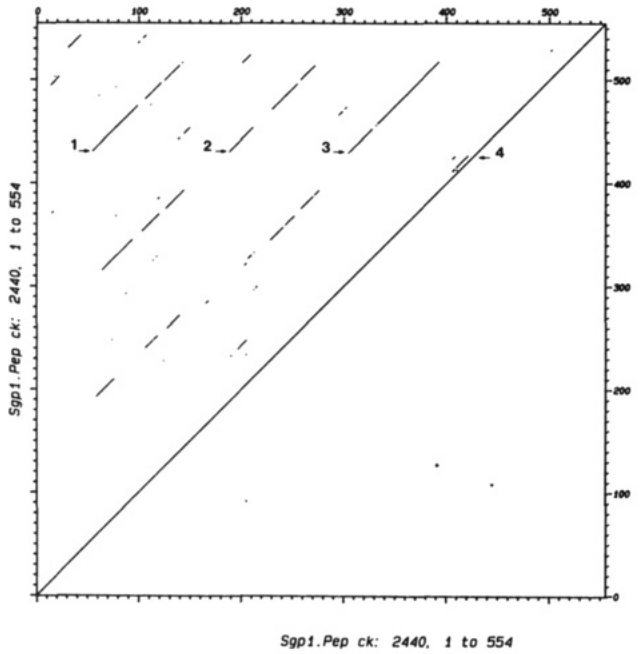

FIGURE 6: Dot matrix comparison of SGP-1 against itself. The computer programs COMPARE and DOTPLOT of the Wisconsin Genetics Group utilize the method of Maizel and Lenk (1981) and were run using a window length of 24 and a stringency of 25. The PIR Mutation Data Matrix (Dayhoff et al., 1983) was used as the scoring matrix. The arrows indicate four repeated regions in SGP-1.

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 60-140  .SLPCDICKTVVT..EAGNLLKDNAT.EEEILHY..LEKTCAWIHDSSLS
194-272  EDV.CQDCMKLVTDIQTA..VRTNSSFVQGLVD..HVKEDCDRL.GPGVS
311-389  VIF.CQVCQLVMRKL..SELIINNAT..EELL.IKGLSKACSLLPAPA.S
437-514  .GF.CEVCKKLVIYLEH.NLEK.NST.KEEIL..AALEKGCSFLPDP...
                 ▲  ▲                        ▲
Consensus  --F-CQVCKKVVT-LE--NL-K-NAT--EELL----LEK-CS-LPDP-LS

 60-140  AS....CKEVVDSYLPVILD.MIKGE.MSNPGEVCSALNLCQS
194-272  ....DICKNYVDQYSEVAVQMMM...HM.QPKEICVMVGFCDE
311-389  T....KCQEVLVTFGPSLLDVLM..HEV.NPNFLCGVISLCSA
437-514  ..YQKQCDEFVAEYEPLLLEILV..EVM.DPSFVCSKIGVCPS
            ▲                    ▲  ▲  ▲
Consensus  ------CKEVVD-Y-PVLLD-MM--E-M-NP-FVCS-IGLC-S
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FIGURE 7: Multiple alignment of the internal repeats occurring in SGP-1. The Wisconsin GAP program utilizes the procedure of Needleman and Wunsch (1970) and was used sequentially to provide a gapped alignment of the SGP-1 amino acid regions indicated. The program was run by using the mutation data scoring matrix. The numbers in the left margin refer to the total region being compared. A consensus sequence is given where two or more identical amino acids align. Closed triangles represent residues which align in all four regions while the bar indicates alignment of the four potential N-glycosylation sites occurring in SGP-1.

SGP-1 was compared to one of the proline-rich proteins from rat (Ziemer et al., 1984) by using the RELATE program of Dayhoff et al. (1983), a score of 7.1 standard deviation units was obtained (see Materials and Methods). This suggests that the probability that these two proteins are related by chance is less than 6.3×10^{-13} (Dayhoff et al., 1983). Similarly high RELATE scores were obtained when other members of the proline-rich superfamily were compared to SGP-1 (data not shown).

Northern Blot Analysis. Expression of SGP-1 mRNA in a variety of tissues was examined by Northern blot analysis. Poly(A+) RNA was isolated from testis, epididymis, liver, brain, kidney, and spleen of adult male rats and from Sertoli cell cultures of 20-day-old male rats. Poly(A+) RNA was also isolated from the ovary and mammary glands of adult female rats. Two micrograms of RNA from the various sources was fractionated on a denaturing 1.5% agarose gel and then transferred to nylon membranes. The blot was hybridized to an SGP-1 cDNA probe which had been radiolabeled with ^{32}P by nick translation. Figure 8A shows an autoradiogram of the nylon blot and indicates the relative levels of SGP-1

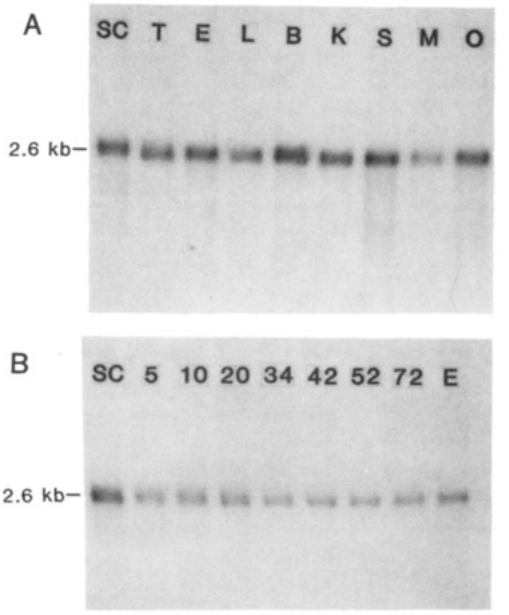

FIGURE 8: Northern blot analysis of SGP-1 mRNA. Two micrograms of poly(A+) RNA was loaded in each lane, and the nylon blots were hybridized with nick-translated pSGP-1A plasmid. (A) RNA isolated from rat Sertoli cell cultures (SC), testis (T), epididymis (E), liver (L), brain (B), kidney (K), spleen (S), mammary gland (M), or ovary (O). (B) RNA isolated from rat Sertoli cell culture (SC), testis from 5-, 10-, 20-, 34-, 42-, 52-, and 72-day-old rats, or rat epididymis (E).

mRNA expression among the various tissues. A single mRNA species with a molecular size of approximately 2600 bases was observed in all of the tissues which were examined. Band intensities are similar among all the tissues, except in mammary gland where mRNA levels appeared slightly depressed.

Expression of testicular SGP-1 mRNA as a function of age was also examined by Northern blot analysis (Figure 8B). Poly(A+) RNA was isolated from the testes of rats varying in age from 5 to 72 days and was analyzed as described above. Similar SGP-1 band intensities are observed in testis from animals 5–20 days-of-age. A slightly lower level of SGP-1 mRNA expression is observed in testis from animals that vary in age from 34 to 72 days-of-age (see Discussion).

Testicular Localization of SGP-1 by Immunofluorescence and in Situ Hybridization. To establish which testicular cell type was responsible for SGP-1 synthesis, testicular cross sections from adult rats were examined by using indirect immunofluorescence as described under Materials and Methods. Fluorescence appeared to occur exclusively in Sertoli cell cytoplasm and processes, while only background fluorescence was observed in other testicular cell types (Figure 9A).

To further characterize the site of testicular SGP-1 synthesis, the location of SGP-1 mRNA synthesis was determined by in situ hybridization. The 1145 base pair *Hin*dIII/*Nsi*I restriction fragment from pSGP-1A was subcloned into pSP65, and biotinylated cRNA probes to SGP-1 were synthesized by using SP6 polymerase and biotinylated UTP. Cross sections of fixed testis were incubated with the probes, and after being extensively washed, regions of tissues to which the cRNA probe hybridized were visualized by treatment with avidin-linked glucose oxidase and an appropriate tetrazolium salt substrate. Figure 9B shows that the majority of the enzymatic reaction is confined to Sertoli cell cytoplasm. Pretreatment of tissue sections with RNase A or hybridization at 0 °C resulted in a total loss of the enzymatic reaction (not shown).

DISCUSSION

Glycolipids are integral components of the outer leaflet of

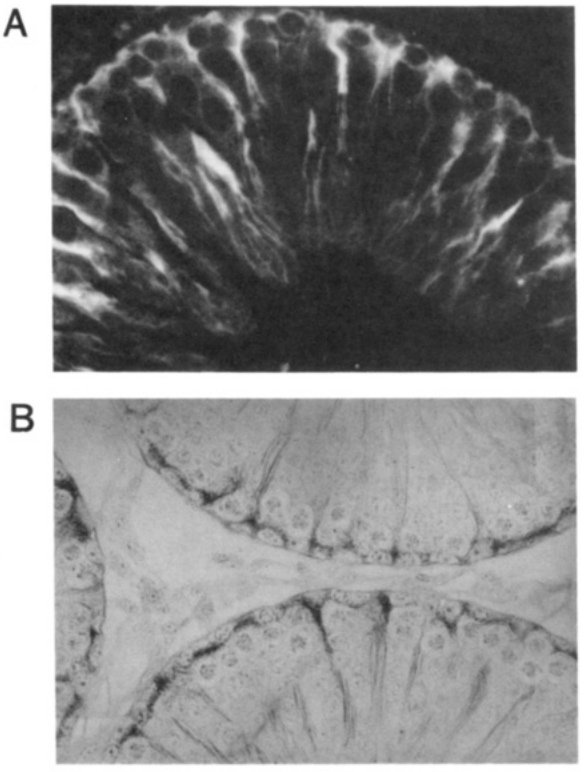

FIGURE 9: Testicular localization of SGP-1 protein and mRNA. (A) Fixed testicular cross sections were processed for indirect immunofluorescence of SGP-1 protein using SGP-1 antiserum, biotinylated second antibody, and fluorescein-conjugated avidin. Sertoli cell cytoplasm produces the majority of the signal. (B) Fixed testicular cross sections were hybridized to a biotinylated cRNA probe for SGP-1 and were then incubated with avidin-linked glucose oxidase followed by a tetrazolium salt substrate. Deposition of the enzyme-catalyzed insoluble product results in heavy staining of Sertoli cell cytoplasm.

mammalian membranes. In addition to conferring structural rigidity to the lipid bilayer, glycolipids are thought to mediate a number of cellular events including cell–cell recognition, contact inhibition, cell differentiation, and oncogenesis [for reviews, see Hakomori (1981) and Yogeeswaran (1983)].

Degradation of glycolipids occurs within the lysosomes of cells through the sequential hydrolysis of saccharides by lysosomal exohydrolases. Although many of the hydrolases required for sphingolipid degradation are membrane bound, in recent years it has been shown that a number of relatively low molecular weight cofactors are present (termed "activators") which promote glycolipid hydrolysis by water-soluble hydrolases (Li & Li, 1986; Furst et al., 1986; Wenger & Fujibayashi, 1986). The mechanism by which these protein cofactors activate degradation of glycolipids is thought to occur through the solubilization of glycolipid from the lipid bilayer, thus allowing interaction of glycolipid substrate with water-soluble hydrolase (Fischer & Jatzkewitz, 1978). The sulfatide/G_{M1} activator (also called sphingolipid activator protein 1, SAP-1, cerebroside sulfatase activator, or dispersin) was first described by Mehl and Jatzkewitz (1964) and was eventually characterized in liver as a 22-kDa heat-stable acidic glycoprotein (Fischer & Jatzkewitz, 1975). Deficiencies in sulfatide/G_{M1} activator result in a variant form of metachromatic leukodystrophy (Stevens et al., 1981; Inui et al., 1983), and genetic studies of these lipid storage diseases have led several investigators to suggest that a single protein activates the hydrolysis of sulfatide, G_{M1}, and $GbOse_3Cer$ (Li et al., 1985; Furst et al., 1986).

Pulse–chase studies on human fibroblasts have shown that sulfatide/G_{M1} activator is synthesized as a 70-kDa precursor which upon treatment with endoglycosidase F is converted to a protein of about 53 kDa (Wenger & Fujibayashi, 1986). The 70-kDa precursor is proteolytically processed to mature species which range in size from 8 to 11 kDa when measured by SDS–PAGE. The protein is thought to be present in liver lysosomes as a 22-kDa homodimer. In this paper, we present evidence that in rat Sertoli cells, SGP-1 is synthesized as a cotranslationally glycosylated 67-kDa precursor which is posttranslationally modified to a 70-kDa form before secretion to the extracellular space. Previously, we have suggested that the native secreted form of SGP-1 occurs as a noncovalent homodimer of 140 kDa (Griswold et al., 1986).

In addition to the similarity in size between SGP-1 and the precursor to sulfatide/G_{M1} activator, there is a high degree of amino acid conservation (78%) between an internal region of SGP-1 and the N terminus of mature sulfatide/G_{M1} activator from human (Figure 5). Additional cDNA sequence analysis of the precursor to human sulfatide/G_{M1} activator indicates that this conservation of sequence similarity (76%) is maintained at the nucleic acid level over the majority of the SGP-1 cDNA coding sequence (John S. O'Brien, personal communication). It therefore seems likely that SGP-1 is the precursor for sulfatide/G_{M1} activator in rat.

SGP-1 displays a high degree of internal sequence similarity which is represented by four repeated regions in the amino acid sequence (Figure 6). One of these regions (amino acids 194–272) represents a slight carboxyl extension of the SGP-1 protein region which is collinear with sulfatide/G_{M1} activator (Figure 5). The partial sequence for sulfatide/G_{M1} activator (Dewji et al., 1986) gives a deduced peptide size of 7.6 kDa. Although the carboxyl terminus of sulfatide/G_{M1} activator has not been defined, this peptide is likely to represent the majority of the mature activator sequence since the size for the glycosylated activator subunit is between 8 and 11 kDa (Wenger & Fujibayashi, 1986). The three other amino acid repeats in SGP-1 show a high degree of similarity with the region which is collinear with sulfatide/G_{M1} activator (Figure 7). Each of these regions defines peptides of about 8 kDa (unglycosylated) that show remarkable conservation in the alignment of cysteine residues and potential N-glycosylation sites. Wynn (1986) has proposed that glycosylation may play an important role in the binding of glycolipids to activator proteins. In consideration of the sequence similarity, our data suggest that SGP-1 may contain three additional binding sites for glycolipids. Although it is generally believed that a single protein promotes the hydrolysis of sulfatide, G_{M1}, and $GbOse_3Cer$, these conclusions have been based on the copurification of these activities from complex mixtures or by immunological techniques (Li et al., 1985; Furst et al., 1986). It is interesting to note that four distinct bands can be observed in immunoprecipitations of mature sulfatide/G_{M1} activator from normal human fibroblasts [see Figure 2 in Wenger and Fujibayshi (1986)]. Although it has been suggested that sulfatide/G_{M1} activator size heterogeneity is due to differences in carbohydrate structure (Inui & Wenger, 1984), it has not been conclusively demonstrated that all of the activator heterogeneity is due entirely to glycosylation differences. In view of the primary structure of SGP-1, a reexamination of sulfatide/G_{M1} activator processing and its glycolipid specificity may be warranted.

Northern blot analysis (Figure 8) indicates the molecular size of SGP-1 mRNA is approximately 2600 nucleotides. If it is assumed that the mRNA contains 200 bases of polyadenylation, then approximately 200 bases of cDNA sequence were not obtained by our clones. Restriction maps of a third

SGP-1 cDNA clone suggest that no additional sequence occurs 5′ to that shown in Figure 4 (unpublished data). Since no polyadenylation signal and no poly(A) tail are observed in the 594 bases of 3′ noncoding sequence described, the missing sequence most likely occurs 3′ to that shown in Figure 4. Northern analysis also indicates that SGP-1 mRNA is expressed at similar levels (per microgram of total RNA) in all of the tissues which were examined (Figure 8A)9. This is in contrast to SGP-2 mRNA expression, where elevated levels occur in epididymis, liver, testis, and brain (Collard & Griswold, 1987). The presence of SGP-1 mRNA in the testis of animals 5–20 days-of-age (Figure 8B) suggests that SGP-1 is of somatic and not germinal cell origin. The apparent decline of SGP-1 mRNA levels between 34 and 42 days-of-age would be consistent with the pubertal increase of germinal cell mRNA which is lacking in SGP-1 message. Since a constant amount of RNA was used in the Northern blot analysis, an increase in germinal cell mRNA would result in a dilution of somatic SGP-1 mRNA. Although very low levels of SGP-1 expression in other testicular cell types cannot be completely ruled out, it is clear that the majority of SGP-1 mRNA and protein synthesis occurs in Sertoli cells (Figure 9). Unlike SGP-2, we do not see an increase in SGP-1 mRNA expression in cultured Sertoli cells relative to testis (Figure 8). This may indicate that Sertoli cell culture conditions are not optimal for production of SGP-1.

If SGP-1 is indeed the precursor to sulfatide/G_{M1} activator, then the fate of this protein appears to be very different in rat Sertoli cells versus human fibroblasts. Pulse–chase analysis of cultured Sertoli cells shows that the majority of newly synthesized SGP-1 is secreted into culture media while little if any processing to lower molecular weight products is observed (Figure 1). Western blot analysis of tubular fluids suggests that secretion is likely to be the normal physiological event in testis (S. R. Sylvester and M. D. Griswold, unpublished results). The reason why a protein which is normally localized to the lysosomal compartment of cells would be secreted by Sertoli cells is currently not clear but raises several interesting possibilities. Secretion of SGP-1 to the adluminal compartment of the seminiferous tubule might allow membrane modification of germinal cells if glycolipid hydrolases were also present. Alternatively, SGP-1 could act as a glycolipid transfer protein between the two cell types, an activity which has been shown to occur in vitro with liposomes (Conzelman et al., 1982). Since SGP-1 is a major secreted protein, it would seem likely that it may function in a major spermatogenic event. Shabanowitz et al. (1986) have shown evidence that SGP-1 (referred to as S70) is secreted during stage VIII of the spermatogenic cycle. It is during this spermatogenic stage that the tight association of Sertoli cells with spermatids is lost and spermatozoa are released into the lumen of the seminiferous tubule (spermiation). Glycolipids have previously been shown to mediate heterologous cell contacts, including the preferential adhesion of chick neuronal retina cells to the surfaces of intact optic tectum (Marchase, 1977) and also the highly specific recognition process that occurs during the formation of neuromuscular junctions (Obata et al., 1977). While it is conceivable that glycolipid modification of Sertoli cell and germinal cell membranes could induce an event such as spermiation, further investigation will be required before the function of SGP-1 in testis is established.

Added in Proof

During the review of the manuscript, a report describing the full-length sequence of the human sulfatide/G_{M1} activator (SAP-1) was published (Dewji et al., 1988).

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Structure and Stability of γ-Crystallins: Tryptophan, Tyrosine, and Cysteine Accessibility†

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ABSTRACT: The solute perturbation techniques of fluorescence of tryptophan (Trp) and dye-labeled thiol groups of cysteine as well as phosphorescence of tyrosine (Tyr) were utilized to obtain information on the relative solvent exposure and accessibility of these residues in γ-crystallins. Both acrylamide and iodide quenchers were used to evaluate the quenching parameters in terms of accessibility and charge characteristics of the proteins. Stern–Volmer plots reveal the presence of more than one class of Trp residues in γ-III and γ-IV, and these residues in γ-II are least accessible compared to the other two. Both steady-state and lifetime quenching studies of the dye-labeled fluorescence indicate that distinct differences also exist among these crystallins in cysteine (Cys) accessibilities. All three proteins, γ-II, γ-III, and γ-IV, show two distinct lifetime components of the dye-labeled Cys residues. Both components of γ-II undergo dynamic quenching, whereas only the major component of the other two crystallins is affected by the quenchers. Addition of acrylamide causes a decrease in Tyr phosphorescence of γ-III and γ-IV, but no change in the emission of γ-II. The decrease is attributed to the formation of a nonemittive ground-state complex between the acrylamide and Tyr of the proteins; the association constant, K_a, calculated from the emission data, has been considered as a measure of Tyr accessibility. K_a values indicate that Tyr residues in γ-III are most exposed and accessible compared to those in the other two proteins. Results of quenching by iodide ion reveal significant differences in the surface charge of the proteins. This study demonstrates that despite the high degree of sequence homology and similarity in the secondary structure of these proteins, differences exist in the arrangements and microenvironments of Trp, Tyr, and Cys residues, causing significant variation in tertiary structure and charge characteristics. These specific molecular features may be primarily responsible for their remarkable denaturation and cryoprecipitation behavior and photoinduced aggregation.

The α-, β-, and γ-crystallins are the water-soluble proteins of the mammalian lens. The γ-crystallins are low molecular weight (M_r 20 000–21 000), monomeric proteins consisting of several gene products, the major ones being γ-II, γ-III, and γ-IV, with more than 75% sequence homology (Harding & Dilley, 1976; Bloemendal, 1982; Schoenmakers et al., 1984). The essential refractive and accommodative properties of the human eye are derived largely through the ordered distribution of these crystallins (de Jong, 1981). In a properly functioning lens, the crystallins must maintain their short-range ordering (Delaye & Tardieu, 1983). During aging and cataractogenesis, the human lens proteins undergo a number of changes, in-

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