RECEPTOR-MEDIATED ENDOCYTOSIS OF TESTICULAR SULFATED GLYCOPROTEIN-1 (SGP-1) BY THE NONCILIATED CELLS OF THE RAT DUCTULI EFFERENTES

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This work is dedicated to my family with love, respect and gratitude

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ABSTRACT

The present study examines the endocytosis of testicular sulfated glycoprotein-1 (SGP-1) by the nonciliated cells of the efferent ducts. SGP-1 is a 70 KDa protein secreted by the Sertoli cells. Once secreted in the seminiferous lumen, the protein binds to the tail of spermatozoa. In the efferent ducts, it is endocytosed by the nonciliated cells, presumably via a receptor-mediated process. Because the initial steps of receptor-mediated endocytosis result from the binding of a ligand's terminal oligosaccharide to a receptor on the cell surface, several monosaccharides were injected into the lumen of the rete testis to study their effect on the endocytosis of SGP-1 in the efferent duct. A quantitative electron microscopic immunocytochemical approach was used. The labeling density (which represents the number of gold particles indicating anti-SGP-1 immunoreactive sites/μm^2 profile area) of various endocytic compartments (including microvilli, endocytic vesicles, and early and late endosomes) was estimated and compared in untreated and treated animals with various sugars. The following sugars were tested: glucose, galactose, mannose, mannose 6-phosphate, N-acetylglucosamine, N-acetylgalactosamine, and sialic acid. Experiment 1 showed that sialic acid caused the greatest significant decrease in the labeling density of each endocytic compartment and that mannose 6-phosphate also caused significant decreases to a lesser extent. Experiment 2 studied various concentrations of sialic acid (0.02 mM, 0.2 mM, 2 mM, 20 mM, and 200 mM) and showed that most of these concentrations

produced a significant (>50%) decrease in the labeling density in the three endocytic compartments studied (endocytic vesicles and early and late endosomes). Moreover, a western blot, followed by a glycan differentiation procedure, revealed that SGP-1 has sialic acid residues that are terminally linked α(2-6) to galactose or N-acetylgalactosamine residues. Previous studies have shown that SGP-1 possesses a sialic acid binding domain. Therefore, these findings suggest that, via its sialic acid binding domain, SGP-1 may bind to glycolipids on the tail of spermatozoa, remove them from the membrane forming a lipo-protein complex. The complex may then be endocytosed by the nonciliated cells, via a receptor that would recognize SGP-1's terminal sialic acid residues, and be delivered to the lysosomes to be degraded.

RESUME

Cette étude porte sur l'endocytose de la glycoprotéine-1 sulfatée (GPS-1) testiculaire par les cellules non-ciliées du canal efférent. La GPS-1 est une protéine de 70 kDa sécrétée par la cellule de Sertoli. Une fois sécrétée, elle se lie à la queue du spermatozoïde. Dans les canaux efférents, elle subit une endocytose par les cellules non-ciliées agissant probablement par l'intermédiaire d'un récepteur. Comme la liaison d'un oligosaccharide terminal de coordinat à un récepteur trouvé sur la surface de la cellule survient à des étapes initiales de l'endocytose par médiation d'un récepteur, plusieurs monosaccharides furent injectés dans la lumière du rete testis afin d'étudier leurs effets sur l'endocytose de la GPS-1 dans le canal efférent. Nous avons utilisé les méthodes immunohistochimiques quantitatives avec microscope électronique: la densité de marquage (qui représente le nombre de particules d'or indiquant les sites d'anti-GPS-1 immunoréactifs par μm^2) de divers compartiments endocytiques, y compris les microvillosités, les vésicules endocytiques et l'endosome précoce et tardif, fut évaluée et comparée entre les animaux traités et non traités avec divers sucres. Les sucres suivants furent testés: glucose, galactose, mannose, mannose 6-phosphate, N-acétylglucosamine, N-acétylgalactosamine et l'acide sialique. Les premières mesures ont montré que l'acide sialique entraîne la diminution la plus importante de la densité de marquage de chaque compartiment endocytique, et que le mannose 6-phosphate causait

lui aussi une diminution notable, mais moindre. Dans une deuxième série d'évaluation, diverses concentrations (0,02 mM, 0,2 mM, 2 mM, 20 mM, 200 mM) d'acide sialique furent utilisées, et il fut montré que la plupart de ces concentrations produisent une diminution importante (> 50%) dans la densité de marquage pour les trois compartiments endocytiques étudiés (vésicules endocytiques, endosomes précoces et tardifs). De plus, une empreinte Western suivie d'un procédé de différentiation glycan a révélé que la GPS-1 présente des résidus d'acide sialique qui sont liés aux extrémités α(2-6) au galactose ou à la N-acétylgalactosamine. Des études précédentes ont montré que la GPS-1 possède un domaine de liaison avec l'acide sialique. Par conséquent, ces résultats suggèrent que la GPS-1 pourrait, par l'intermédiaire de son domaine de liaison avec l'acide sialique, se lier aux glycolipides se trouvant sur la queue du spermatozoïde. Ceci a pour effet d' enlever les glycolipides de la membrane, formant un complexe lipo-protéinique. Le complexe pourrait alors subir une endocytose par les cellules non-ciliées agissant par l'intermédiaire d'un récepteur qui reconnaîtrait les résidus terminaux d'acide sialique de la GPS-1, et être expédié aux lysosomes pour être dégradé.

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INTRODUCTION

Sulfated glycoprotein-1 (SGP-1) is one of the major secretory products of rat Sertoli cells in culture (Sylvester et al., 1984; Collard et al., 1989). This heavily glycosylated and sulfated protein of Mr 70,000 shares substantial sequence similarity with human prosaposin, the precursor of human sphingolipid activator proteins (SAPs) (O'Brien et al., 1988; Sylvester et al., 1989). Prosaposin is proteolytically cleaved into four smaller proteins referred to as saposins A, B, C, and D (Morimoto et al., 1989; O'Brien and Kishimoto, 1991). Saposins are small, heat-stable lysosomal proteins that are instrumental in lipid degradation. They act upon the glycolipids, which are integral components of mammalian membranes, by solubilizing the glycolipids from the lipid bilayer, thus permitting the water-soluble lysosomal hydrolases to come into contact with these glycolipids (Fisher and Jatzkewitz, 1978; O'Brien et al., 1988; O'Brien et al., 1991; Hiraiwa et al., 1992). Deficiencies in levels of these proteins have been associated with several diseases, namely, metachromatic leukodystrophy (Wenger et al., 1989), Gaucher's disease (Christomanou et al., 1986) and Tay-Sachs disease (O'Brien and Kishimoto, 1991).

In vivo, SGP-1 is secreted by the Sertoli cell into the lumen of the seminiferous tubules, where it then binds preferentially to the tail of the spermatozoon (Sylvester et al., 1989). The latter travels through the excurrent duct system, where it acquires maturity, motility, and fertility. In the efferent ducts, SGP-1 dissociates from the spermatozoon and is endocytosed by the

nonciliated cells of the efferent ducts, possibly by means of receptor-mediated endocytosis (Hermo et al., 1992; Igdoura et al., 1993). Once internalized, SGP-1 is targeted to the lysosome (Hermo et al., 1992). Different forms of SGP-1, such as the 65 KDa protein and the 15 KDa related proteins, are also present in secondary lysosomes of hepatocytes, fibroblasts, and other human cells (Li and Li, 1976; Mraz et al., 1976; Wenger and Fujibayashi, 1986) and in lysosomes of Sertoli cells (Igdoura and Morales, 1991).

Certain functions can be attributed to SGP-1, owing in part to its substantial sequence homology with prosaposin (O'Brien et al., 1988) and its subcellular location in the lysosomes (Hermo et al., 1992). Therefore, the 65 KDa and 15 KDa proteins found in the lysosomes are indicators of possible saposin-like activity (Sylvester et al., 1989). The 70 KDa, found on tails of spermatozoa, may play a role in the maturation of these cells (Hermo et al., 1992).

The main objective of the present work is to identify the specific mechanism used by the nonciliated cells of the efferent ducts to endocytose testicular SGP-1. The conjugation of N-linked oligosaccharides to proteins allows the latter substances to achieve unique conformations that facilitate receptor binding (Drickamer et al., 1991). By introducing specific sugars into the lumen of the efferent ducts to displace SGP-1 from its putative receptor on the apical plasma membrane of nonciliated cells and by using a quantitative electron microscopic immunocytochemical approach, one can determine the terminal saccharide responsible for binding SGP-1 to the binding site of the receptor. For this purpose, the labeling densities (which will represent the

number of gold particles indicating anti-SGP-1 immunoreactive sites/μm2 profile area within various endocytic compartments in untreated animals and in animals treated with specific doses of different saccharides) will be estimated and compared. The result that gives the lowest labeling density within various endocytic compartments will reflect the saccharide responsible for the binding to the receptor because this saccharide will, by competitive inhibition, effectively hinder the endocytosis of SGP-1 by the nonciliated cells.

LITERATURE REVIEW

Section 1) The Male Reproductive System

A) General Outline of the Male Reproductive System

The male reproductive system consists of two testes, which function as an exocrine gland that produces the male germ cells and as an endocrine gland that manufactures the male sex hormone, testosterone; a copulatory organ, the penis, which is responsible for the delivery of the male germ cell from the body and for the excretion of urine; a duct system, which permits the passage of the male germ cells from the testes to the copulatory organ; and the male accessory glands (the seminal vesicles, the prostate, and the bulbourethral glands), all of which contribute fluid secretions to the semen (a composite of the male germ cell and a fluid vehicle) upon ejaculation (Fig. 1).

B) The Testes

The testes are suspended in the scrotum and encapsulated by a thick fibrous capsule. Any cross-section of the testis is composed of the seminiferous tubules and the interstitium. The wall of the tubules consists of a limiting membrane composed of one or two layers of myoid cells, as well as a lining epithelium and a central lumen. Between each tubule is the interstitium, which

consists of blood vessels, lymphatics, connective tissue, fibroblasts, mast cells, macrophages, mesenchymal cells, and Leydig (testosterone-producing) cells.

1) The Seminiferous Tubules

Each seminiferous tubule, collected from the rat, measures 32.2 cm in length and 350 μm in diameter (Clermont and Huckins, 1961). The tubules are lined by a complex stratified epithelium consisting of two cell types: 1) a somatic, nonproliferative population, the Sertoli cells and 2) a spermatogenic proliferative population of germinal cells that undergo spermatogenesis, a complex differentiation process (Clermont, 1962).

2) The Germinal Cells

The germinal cells undergo spermatogenesis, the process by which diploid spermatogonial stem cells divide and differentiate to give rise to haploid spermatozoa (Clermont, 1962). Morphologically, the germinal cells can be classified into three types: the spermatogonia, which include a subpopulation of stem cells that rest on the basal membrane and undergo mitotic division; the primary and secondary spermatocytes, both of which undergo meiotic divisions; and the spermatids, which undergo a long process of maturation known as spermiogenesis, to become mature spermatozoa. The spermatozoa are released into the tubular lumen, in a process known as spermiation (Clermont, 1962).

3) The Sertoli Cell

In the adult, the Sertoli cell is a nondividing epithelial cell. Morphologically, it is a tall (75 to 100 μm), columnar, stellate cell whose base is solidly attached to the basement membrane of the tubules and whose apex reaches the tubular lumen (Fawcett, 1975). It contains numerous lateral and apical veil-like processes that extend between and around all the germ cells (Webber et al., 1983; Wong and Russel, 1983). Histologically, the Sertoli cell contains a large polymorphous nucleus with pale dusty chromatin and a large nucleolus (Fawcett, 1975); an extensive and continuous system of rough endoplasmic reticulum cisternae (Fawcett, 1975); a large perinuclear and supranuclear Golgi apparatus that forms a continuous network (Fawcett, 1975; Rambourg et al., 1979); polymorphous mitochondria (Fawcett, 1975); an impressive endocytic apparatus consisting of endosomes, multivesicular bodies, and lysosomes (Dietert, 1966; Fawcett, 1975; Lalli et al., 1984; Morales et al., 1985, 1986); and an extensive cytoskeleton with microtubules and microfilaments, and lipidic inclusions (Fawcett, 1975). Furthermore, the Sertoli cell has unique lateral processes, which form plate-like connections (separated by a space of 2 nm) with adjacent Sertoli cells, thereby forming tight junctions (Dym and Fawcett, 1970). The function of these junctions is to create two distinct compartments within the seminiferous epithelium: 1) the adluminal compartment, where the meiotic germ cells and spermatids are found and 2) the basal compartment, where the spermatogonia and the early preleptotene spermatocytes reside (Dym and Fawcett, 1970). The basal compartment has

access to a vascular supply, whereas the adluminal compartment is deprived of one (Dym and Fawcett, 1970). These junctional complexes establish a blood-testis barrier, which is responsible for the differences in chemical composition of the tubule fluid and blood plasma (Fawcett, 1975).

The Sertoli cell has several functions (Fig. 2). It has a supportive, or sustentacular, role in maintaining germinal cells in the adluminal compartment during their maturation (Fawcett, 1975). It participates in endocytosis (Clermont et al., 1987), more specifically, phagocytosis by internalizing the residual bodies left behind by the mature spermatozoa (Morales et al., 1986; Hermo et al., 1994). It is also involved in pinocytosis or fluid-phase absorption, which takes place at the luminal surface, and receptor mediated endocytosis, which takes place at the cell's base (Morales and Hermo, 1983; Morales and Clermont, 1985, 1986).

One of the major functions of the Sertoli cell is the maintenance of spermatogenesis (see review by Griswold et al., 1988). That is, it plays a role in lifting the early meiotic germ cells from the basal to the adluminal compartment and releasing the late spermatids into the tubular lumen (Dym and Fawcett, 1970). It also nourishes the germ cells by supplying them with sugars, amino acids, lipids, and metals. From both its apical and basal poles, the Sertoil cell secretes water, ions, proteins such as androgen binding protein (ABP), sulfated glycoprotein 1 and 2 (SGP-1, SGP-2), and metallic carriers such as transferrin and ceruloplasmin, as well as proteases, inhibitors, hormones, and growth and paracrine factors (Griswold, 1988).

Finally, the tight junctions found on the cell's lateral processes serve as a

blood-testis barrier that prevents large molecules from penetrating the tubular lumen (Dym and Fawcett, 1970); ensures a varied composition of fluid, proteins, and ions between the plasma and the seminiferous tubules, thereby helping to create a special environment for the germ cells to undergo meiosis (Tuck et al., 1970; Skinner and Griswold, 1983; Setchell, 1980); and prevents the immune system of the body from contacting proteins produced by haploid spermatids and spermatozoa (Setchell, 1980).

C) The Excurrent Duct System

1) The Rete Testis

At the posterior margin of the testis, the tunica albuginea, which encapsulates the seminiferous tubules, thickens and forms the mediastinum through which ducts, blood vessels, lymphatics, and nerves enter and leave the testis. As the convoluted seminiferous tubules approach the mediastinum, their contents empty into the rete testis, which is a plexus of anastomosing channels lined by a cuboidal epithelium. These channels possess an elaborate endocytic apparatus and are actively involved in fluid-phase and adsorptive endocytosis (Morales et al., 1984).

2) The Ductuli Efferentes

The ductuli efferentes emerge from the mediastinum and connect the rete

testis with the ductus epididymis. There are 4 to 20 coiled ducts (Hemeida et al., 1978), which are lined by an alternating epithelium of tall ciliated cells and low columnar nonciliated cells (Robaire and Hermo, 1988). In earlier studies (Van Wagener, 1925; Wagenseil, 1928; Young, 1933; Mason and Shaver, 1952), it was shown that this epithelium has the capacity to absorb material from the lumen.

a) The Nonciliated Cells

The nonciliated cells are the most numerous cells of the efferent duct. These columnar cells have short microvilli, canalicular invaginations, and an extensive endocytic apparatus (Hermo, 1994). They are involved in fluid-phase and adsorptive endocytosis (Robaire and Hermo, 1988). Endocytosis of proteins by these cells includes SGP-1 (Igdoura et al., 1993), SGP-2 (Hermo et al., 1991), and androgen binding protein (Pelliniemi et al., 1981).

b) The Ciliated Cells

Like the nonciliated cells, these are tall columnar cells extending from the basement membrane to the lumen. However, the ciliated cells are less numerous than the nonciliated ones. They are recognized on the basis of their numerous cilia, few microvilli, and an apical nucleus (Robaire and Hermo, 1988). The beating of the cilia is thought to be involved in the transport of spermatozoa and luminal fluid through the efferent ducts (Robaire and Hermo,

1988). These cells have also been shown to perform fluid-phase and adsorptive endocytosis, even though they possess a less elaborate endocytic apparatus than the nonciliated cells (Hermo et al., 1985). They also modify the luminal composition by means of endocytosis (Robaire and Hermo, 1988).

3) The Ductus Epididymis

The ductus epididymis is a single, highly tortuous tube, which delivers the spermatozoa to the vas deferens (Robaire and Hermo, 1988). The ductus epididymis is lined by a pseudostratified columnar epithelium composed of principal columnar cells and clear, narrow, halo, and round basal cells (Reid and Cleland, 1957). The ductus can be separated into five major regions: the initial segment, the intermediate zone, the head or caput, the body or corpus, and the tail or cauda (Robaire and Hermo, 1988; Hermo et al., 1991). The spermatozoa acquire unidirectional motility and fertilizing capacity in the tail of the ductus epididymis.

Section 2) Sulfated Glycoprotein-1

A) Biochemistry

An analysis of protein secreted by cultured rat Sertoli cells has shown the existence of two major sulfated glycoproteins referred to as SGP-1 and SGP-2 (Kissinger et al., 1982; Sylvester et al., 1984; Griswold, 1988). It has been

shown that SGP-1 exists in different forms: a secreted form with a molecular weight (MW) of 70 KDa (Sylvester et al., 1984; Collard et al., 1988; Sylvester et al., 1989); a precursor form with a MW of 55 KDa; a lysosomal transient form with a MW of 65 KDa; and lysosomal end products of the 65 KDa form with a MW of 15 KDa (Igdoura and Morales, 1994). SGP-1's deduced protein sequence, studied by Collard et al (1988), involves 554 amino acids with a MW of 61,123 Da. The first 16 amino acids represent a hydrophobic signal sequence, which is atypical due to its shortness and lack of positively charged residues following the initiator methionine residue (Collard et al., 1988). When SGP-1 (the 70 KDa form) is treated with endoglycosidase F, it produces a protein with a MW of 57 KDa on SDS PAGE, suggesting that extensive cotranslational glycosylation occurs during its biosynthesis (Collard et al., 1988). SGP-1 consists of four domains that share sequence similarity with each other (Sylvester et al., 1989). Between the third and fourth domain, there is a proline-rich region, normally observed in secretory proteins, that may explain the existence of the secreted form of SGP-1 (Collard et al., 1988; Sylvester et al., 1989). The SGP-1 mRNA, which has a molecular size of 2600 bases, has been observed in the rat testis, epididymis, liver, brain, kidney, spleen, and ovary (Collard et al., 1988).

B) Similarities with Prosaposin

Rat SGP-1 shares sequence similarity with the 70,000 Mr precursor of human sphingolipid activator protein (SAP), prosaposin (O'Brien et al., 1988;

Sylvester et al., 1989). Prosaposin is proteolytically cleaved in the lysosome into four subunits, SAPs A, B, C, and D (Morimoto et al., 1989; O'Brien and Kishimoto, 1991). Prosaposin is found in high concentration in the testes, seminal plasma, cerebral grey matter, and human milk and in low concentration in the liver and spleen (O'Brien and Kishimoto, 1991). Saposins are small heat-stable proteins required for the hydrolysis of sphingolopids by specific lysosomal hydrolases (O'Brien and Kishimoto, 1991). The c-DNA fragments encoding 67 amino acids of SAP-1 have 83% similarity with the corresponding segment of SGP-1 cDNA (Collard et al., 1988). Furthermore, SGP-1 has a distant relationship with proteins classified in the proline-rich superfamily (Collard et al., 1988).

C) Glycolipids

Glycolopids are integral components of the outer leaflet of mammalian membranes. Their function is to confer structural rigidity to the lipid bilayer and to mediate a number of cellular events such as cell-cell recognition, contact inhibition, cell differentiation, and oncogenesis (Hakomori 1981; Yogeeswaran, 1983). The saposins activate the degradation of glycolipids by solubilizing the glycolipids from the lipid bilayer, thus allowing interaction between the glycolipid substrate and water-soluble hydrolases (Fisher and Jatzkewitz, 1978).

D) Saposins

Deficiencies in saposins have an extreme impact on lysosomal function. Massive accumulation of saposin A has been found in the brains of patients with Tay-Sachs disease and infantile Sandhoff disease (O'Brien and Kishimoto, 1991). Saposin B activates the hydrolysis of cerebroside sulfate by arylsulfatase A, of GM1 ganglioside by acid-b-galactosidase, and of globotriaosylceramide by a-galactosidase (O'Brien and Kishimoto, 1991). Cerebroside sulfate and glycolipid accumulation results in a disease known as metachromatic leukodystrophy (Stevens et al., 1981; Inui et al., 1983; Hyde et al., 1992). Saposin C, and in part saposin A, activates the hydrolysis of glucocerebroside by glucosylceramide b-glucosidase (O'Brien and Kishimoto, 1991). Deficiencies in saposin C result in Gaucher's disease (Wenger and Williams, 1991). Finally, saposin D is involved in sphingomyelinase activity (O'Brien and Kishimoto, 1991).

In addition to serving as a lysosomal precursor to the saposins, prosaposin may play a secondary role in cellular adhesion, since glycolipids mediate cell contact and adhesion (Roberts et al., 1988). In addition, prosaposin may be capable of binding to sialic acid residues, since domain B, which corresponds to SAP-B, has sequence homology with influenza virus neuraminidase (Potier et al., 1988, 1990b).

E) Immunolocalization of SGP-1 in the Male Reproductive System of the Rat

After light and electron microscopic immunocytochemistry studies (Hermo et al., 1992; Sylvester et al., 1989), the specific localization of SGP-1 in the male reproductive system was determined and was subsequently analyzed for possible roles that it might play therein. Recent studies using immunoperoxidase labeling have shown that the basal and middle region of the Sertoli cell is reactive (Hermo et al., 1992). These results further substantiate the findings of Griswold (1988) and Skinner et al. (1980), which showed that the major secreted glycoproteins in the medium of cultured Sertoli cells were SGP-1 and SGP-2.

Immunogold labeling of SGP-1 in the rete testis and efferent ducts have demonstrated a reaction in the endocytic apparatus. In contrast, the cells of all the regions of the epididymis showed no reaction in the early endocytic apparatus; nevertheless a weak reaction was observed in the multivesicular bodies and lysosomes. In addition to these studies, spermatozoa in the tubular lumen and rete testis lumen showed reactivity; however, they were devoid of labeling in the lumen of the epididymis.

To further investigate the specific location of the synthesis of SGP-1, Northern blot analysis was performed (Hermo et al., 1992). A radioautograph of a 2.6-Kb band, which reflects the mRNA of SGP-1, was observed in the epididymis, efferent ducts, and testes.

These data suggest that SGP-1 is synthesized in the Sertoli cells, the efferent ducts, and the epididymis and that it is endocytosed by the epithelial

cells of the rete testis and the nonciliated cells of the efferent ducts. Therefore, SGP-1 is secreted by the Sertoli cell into the lumen of the seminiferous tubules, where it binds to the tail of the spermatozoa. Subsequently, in the lumen of the rete testis and the efferent ducts, SGP-1 dissociates from the spermatozoa to be endocytosed by the epithelial cells lining these structures. This explains why SGP-1 is neither found on the tail of spermatozoa in the lumen of the epididymis nor endocytosed by the cells of the epididymis. In summary, SGP-1 can follow two major intracellular routes: a secretory pathway or a lysosomal pathway. These two alternate pathways explain the variations in molecular weight observed in different locations within the cell. When found in lysosomes, SGP-1 has a MW of 65 KDa and proteolytically processed forms of 15 KDa (Igdoura and Morales, 1994). When found in the luminal compartment of the rete testis and efferent ducts, SGP-1 is a protein with a MW of 70 KDa (Sylvester et al., 1989; Hermo et al., 1992).

Igdoura and Morales (1994) subjected Sertoli cells to metabolic labeling with ^{35}S-cysteine, followed by immunoprecipitation and SDS-PAGE. They demonstrated that the precursor form of SGP-1 (MW, 55 KDa) was posttranslationally modified to the 65 KDa protein within 30 minutes. After 3 hours, the 70 KDa form was apparent, while the 65 KDa form was still maintained. After 7 hours, a 15 KDa band was detectable. Western blot analysis revealed that testicular fluids contained one major band of 70 KDa MW corresponding to the luminal SGP-1 and that Sertoli cell lysosomal lysates contained a major band of 65 KDa MW, as well as a 15 KDa band (Igdoura and Morales, 1994). Therefore, it can be postulated that the 55 KDa form is

synthesized in the rER and is modified and glycosylated in the Golgi apparatus to produce the 65 KDa, which in part is further modified to give rise to the 70 KDa. The latter protein is secreted into the lumen and endocytosed by other cells. Meanwhile, the 65 KDa is targeted to the lysosomes and is proteolytically processed to produce the 15 KDa forms, which are homologous to the four saposins (Fig. 3)

F) Potential Functions of SGP-1

Certain functions can be attributed to SGP-1 because of its location in the lysosomes and its sequence homology with prosaposin, which has a substantial role in lipid metabolism. In spermiogenesis, at stage VIII of the cycle (Leblond and Clermont, 1952), before the release of step 19 spermatid into the tubular lumen, the residual bodies are unreactive to SGP-1. It is only later, when the residual bodies are phagocytosed by the Sertoli cells and fuse with the lysosomes, that they become reactive to SGP-1 (Igdoura and Morales, 1994). Therefore, SGP-1 may play a role in the degradation of glycolipids of the residual bodies.

The high concentration of SGP-1 in the reproductive tract suggests an important role in this compartment. Because spermatozoa traveling in the duct system are devoid of cytoplasmic organelles and lose their capacities for lipid synthesis and modification (Nikolopoulou et al., 1985; Parks et al., 1985), they need another mechanism to accomplish the modifications of membranes and lipids during maturation in the epididymis. This process may be facilitated by

SGP-1.

In addition, SGP-1 shares structural similarities with a number of immunoregulating agents such as C3B inhibitor (Griswold, 1988). These immunosuppressive agents are known to protect spermatozoa throughout the reproductive tract from attack by the immune system (Griswold, 1988).

Section 3) Endocytosis

A) Classification of Different Types of Endocytosis

The general term endocytosis refers to the process by which the cell ingests extracellular material by trapping it within infoldings of the plasma membrane and pinching it off from the surface to form intracellular plasma membrane-derived vesicles and vacuoles (Goldstein, 1979). This process allows the cell to utilize an extracellular source of nutrients. These endocytosed materials may be soluble substances, particulate materials such as cell debris and microorganisms, macromolecules such as proteins and complex sugars, and low-MW molecules such as vitamins and simple sugars (Anderson, 1992). The cell needs different mechanisms to internalize these diversified nutrients. Molecules smaller than 1000 da. are able to pass freely through the membrane barrier (Anderson, 1992). However, larger molecules are internalized via fluid-phase endocytosis or pinocytosis; phagocytosis; adsorptive endocytosis; or receptor-mediated endocytosis.

Pinocytosis or fluid-phase endocytosis involves the nonselective uptake

of fluid by the cell (Hermo and Morales, 1984). It requires no binding to the cell membrane. The molecules utilized by this mechanism are small particles, soluble molecules, and low-molecular-weight solutes.

Adsorptive endocytosis involves the binding of particles or molecules to nonspecific sites on the cell membrane. The cells of the efferent ducts utilize fluid-phase and adsorptive endocytosis to remove substances that do not bind or that bind nonspecifically to the cell surface. (Hermo et al., 1984). These internalized substances follow the endocytic route and are targeted to the lysosomes to be degraded. Because these cells are known to utilize these mechanisms frequently, it is postulated that the cells have a functional role in determining the amount and composition of the luminal fluid that carries the spermatozoa.

Phagocytosis is defined as the uptake of large particulates, which involves close apposition of a segment of the plasma membrane to the particulate's surface (Silverstein, 1977).

Receptor-mediated endocytosis denotes the uptake of substances by means of binding to specific receptors on the cell surface before internalization (Hermo and Morales, 1984). Because the cell surface has a limited number of receptors, the molecules bind with high affinity, and this binding requires specificity (Anderson, 1992). By this mechanism, the cell can take up nutritional and regulatory proteins from the external fluid. These substances include plasma transport proteins (LDL), polypeptide hormones (insulin), epidermal growth factor, asialoglycoproteins, and lysosomal enzymes (Goldstein, 1979).

B) Endocytic Apparatus of the Nonciliated Cells of the Efferent Ducts and Receptor-Mediated Endocytosis of SGP-1

At least 13 systems have been described in which non-phagocytic cells use endocytosis to internalize proteins that have become bound to surface receptors (Goldstein et al, 1979). Receptor-mediated endocytosis is characterized by four steps (Goldstein et al, 1979; Wileman et al, 1985):

1) The binding cornponent on the surface of the cell, the receptor, binds an endogenous ligand to achieve a physiological effect.

2) Internalization is coupled to binding. Once the protein is bound, the half-time for internalization is less than 10 minutes.

3) Receptor-bound proteins enter the cell via coated pits. Two alternative theories explain the latter concept: either receptors spontaneously localize in these coated regions, or binding of the ligand induces migration of the receptors to the coated pits.

4) Internalized proteins are delivered to the lysosomes for degradation.

In terms of the endocytic apparatus, the nonciliated cells of the efferent ducts can be divided into three regions (Hermo and Morales, 1984): the apical region, which consists of tubulovesicular profiles, tubular coated pits, apical tubules in abundance, mitochondria, and endoplasmic reticulum; the supranuclear region, which contains endosomes with an electron-lucent matrix, multivesicular bodies with an electron-dense matrix, lysosomes, and the Golgi apparatus; and the basal region, where the ovoid nucleus and lipid droplets are located (Hermo and Morales, 1984).

Binding and internalization of receptors are mediated by specialized plasma membrane regions termed coated pits (Goldstein et al, 1979). Receptors segregate in these coated regions, and the binding process induces clustering of the binding sites and formation of a coat. Once the ligand is bound to the receptor, the coated pits invaginate, pinch off the membrane, and give rise to coated vesicles (Gruenberg and Howell, 1989). Pearse (1975) isolated these coated vesicles and observed that the coat was composed of a single protein with a MW of 180,000, identified as clathrin. It is noncovalently bound to the surface of the membrane, because it can be removed with urea or protonated amines (Goldstein et al, 1979). Two active sites on the receptor have been postulated: the binding site (on the external surface) and the internal site (on the cytoplasmic surface). Interaction of the internal site with clathrin fixes the receptor in the coated pit (Goldstein et al, 1979).

Further studies by Hermo and Morales (1984) show that tubular coated pits invaginate to give rise to apical tubules, not vesicles. Apical tubules were observed in other cell types associated with endocytic activity: proximal convoluted tubules of the kidney (Graham and Karnovsky, 1966), endothelial cells lining the sinuses of the liver (De Bruyn et al, 1983), proximal small intestinal cells (Rodewald, 1973), and other regions of the male excurrent duct system (Morales et al., 1984).

Coated vesicles or apical tubules fuse with each other or with preexisting early endosomes (Gruenberg and Howell, 1989). Early endosomes are large, spherical, irregular membranous vesicular elements with a diameter of 250 to 400 nm (Helenius, 1983; Hermo et al, 1988). The early endosomal lumen is

characterized by an electron-lucent matrix with flocculent materials. In the endosome, the pH decreases sharply, causing the receptor-ligand complex to dissociate because, at an acidic pH, binding affinity decreases, and a conformationai change occurs that causes uncoupling (Helenius, 1983). Therefore, the molecules are sorted out, and ligands either 1) are routed back to the plasma membrane or Golgi complex or 2) are targeted to the lysosomes, whereas receptors are recycled back to the plasma membrane or Golgi apparatus (Gruenberg and Howell, 1989).

From the early endosome, ligands then travel through the late endosome. These two structures differ from each other morphologically (Gruenberg and Howell, 1989). Early and late endosomes can be separated with a density gradient centrifuge. Late endosomes are much larger, have a denser matrix, and exhibit a more complex organization of internal membranes. In early endosomes, immunocytochemical studies have indicated that ligands are located at the periphery; they are closely associated with their receptors and the plasma membrane, as they have not yet fully dissociated from these structures. In late endosomes, ligands are randomly distributed throughout the matrix, as they are no longer associated with their receptors.

From the late endosomes, the ligands proceed through a series of pale to dark multivesicular bodies. They finally end up in the lysosomes, which are membrane-bound, electron-dense vesicles that are acid phosphatase-positive and are characterized by an acidic pH (<5.0) (Hermo et al., 1988). The acidity in these vesicles is caused by a proton gradient generated by a vacuolar ATPase pump across the lysosomal membrane (Yamashiro et al., 1983;

Gruenberg and Howell, 1989).

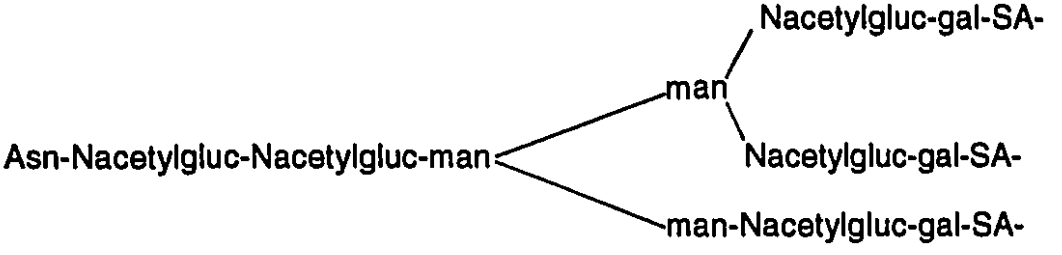
Whereas the apical tubules mediate the transport of external cell markers into the early endosomes, it is not yet clear how these markers are transported from the early to the late endosomes and thence to the lysosomes. It is known, however, that microtubules are essential for the movement of endocytic vesicles to the perinuclear region, because depolarization of microtubules reduces the number of endocytic vesicles traveling to this region (Gruenberg and Howell, 1989). To explain the intracellular transport from the early to the late endosome and thence to the lysosome, two models have been proposed (Helenius, 1983; Gruenberg and Howell, 1989; Dunn et al., 1992). The first, suggested by Palade (1975), refers to the vesicular shuttle transport and states that early endosomes, late endosomes, and lysosomes are long-lived preexisting organelles that contain resident proteins. Ligands are transported from one to the other by carrier vesicles, which bud off from one organelle and fuse with another. The second proposal, the maturational model, suggests that endocytic vesicles fuse with early endosomes; these mature and translocate to the supranuclear region to become late endosomes and then mature into lysosomes.

Section 4) Glycoprotein Receptors

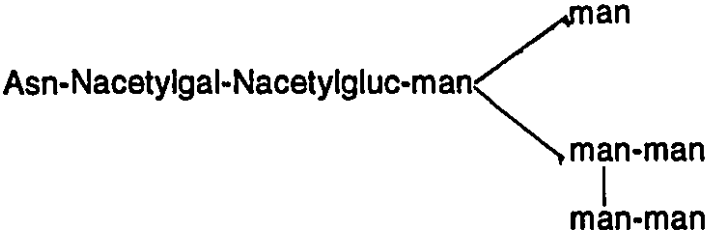
A) Purpose of Selective Glycosylation for the Clearance of Glycoproteins

Glycoproteins are synthesized in the rough endoplasmic reticulum (rER), modified in the Golgi apparatus, and destined for transport to either the lysosomes, the plasma membrane, or the extracellular space. Preformed oligosaccharides composed of N-acetylglucosamine, mannose, and glucose residues are transferred en bloc to proteins in the rER. The oligosaccharides are transferred to a side NH2 group of an asparagine amino acid residue of the protein. The N-linked oligosaccharides are then trimmed while still in the rER and undergo further modification in the Golgi apparatus. Two broad classes of oligosaccharides attach to mammalian glycoproteins, the complex type and the high mannose type. Heterogeneous and hybrid oligosaccharides also exist.

The Complex Type:



The High Mannose Type:



A multitude of carbohydrates are associated with proteins and have significant effects on the function and half-life of these glycoproteins (Ashwell and Harford, 1982; Drickamer et al., 1991). The conjugation of saccharides to proteins allows proteins to achieve a unique conformation that facilitates their intrinsic activities such as catalysis, ligand binding, and receptor binding (Stoolman, 1983; Drickamer et al., 1991). Moreover, recent studies suggest that selective glycosylation may have evolved as a means of controlling the persistence of proteins in circulation (Drickamer et al., 1991). For example, desialylated glycoproteins are rapidly removed from the circulation via the hepatic asialoglycoprotein receptor (Ashwell and Harford, 1982). In order to obtain desialylated glycoproteins, the action of neuraminidase is needed to cleave the sialic acid residues off the glycoproteins (Ashwell and Harford, 1982; Drickamer et al., 1991). Therefore, glycoproteins with more N-linked glycosylation sites would be a better target for neuraminidase and should clear faster than glycoproteins with fewer glycosylation sites.

With respect to intracellular transport and processing, different receptor-ligand systems and cell types vary markedly. There are four general categories of receptor-ligand systems (Wileman et al., 1985). When the ligand-receptor complex dissociates in the endosomes, the ligand is targeted to the lysosomes, and the receptor can be recycled back to the plasma membrane or the Golgi apparatus. Examples are the LDL (Goldstein et al., 1979), the asialoglycoprotein (Schwartz et al., 1984), the mannose 6-phosphate (Fisher et al., 1980), and the mannose receptors (Stahl et al., 1980). Alternatively, the

receptor is not recycled but is degraded. Examples include the epidermal growth factor (Carpenter and Cohen, 1976) and insulin receptors (Kasuga et al., 1981). In some cases such as those involving the IgA receptor, the ligand is not targeted to the lysosome, and the receptor is not recycled (Kuhn and Kraehenbuhl, 1979). Finally, ligand-receptor complex can remain attached during its transit through the cell, to be recycled back to the same pole or to the pole opposite that where it was endocytosed; transferrin and IgG-receptor complex follow these processes (Abrahamson and Rodewald, 1981), which are referred to as transcytosis (Mostov and Simister, 1985).

Because this project focuses on the identity of the receptor responsible for the uptake of SGP-1, a study of the major glycoprotein receptors is essential. The major receptors of interest in this project are the asialoglycoprotein (ASGP), mannose 6-phosphate, sulfated N-acetylgalactosamine, and mannose receptors.

B) The Asialoglycoprotein Receptor

Ashwell and Morell (1974) discovered the receptor for ASGPs while studying the serum glycoprotein, ceruloplasmin. They noticed that, after enzymatic removal of sialic acid residues from this glycoprotein, there was a rapid disappearance of ceruloplasmin from the circulation of rabbit serum. Because almost all serum glycoproteins are secreted with sialic acid-terminated N-linked oligosaccharide, neuraminidase activity is required before the glycoprotein can be cleared. Ashwell and Morell (1974) used this model to

study the ASGP receptor, which is an integral membrane glycoprotein expressed exclusively in parenchymal hepatocytes (Spiess, 1990; Steer and Ashwell, 1990). The receptor mediates specific recognition and uptake of glycoproteins with terminal galactose or N-acetylgalactosamine (Steer and Ashwell, 1990; Schwartz et al., 1984). It is water-soluble, and 10% of its dry weight is constituted of carbohydrates (Steer and Ashwell, 1990).

Receptor-ligand binding requires calcium and is effective only at a pH greater than 6.5 (Spiess, 1990). Once the receptor-ligand complex reaches the early endosome, it dissociates because of the low pH therein. The majority of dissociated ASGPs reach the lysosomes within 5 to 15 minutes, while the receptor recycles back to the plasma membrane (Spiess, 1990).

The rat ASGP receptor is derived from two separate genes, which have 53% homology (Steer and Ashwell, 1990; Spiess et al., 1990; Geffen et al., 1992). These two genes are responsible for producing three proteins, RHL-1, RHL-2, and RHL-3. These proteins have been isolated by affinity chromatography and by SDS-PAGE. The latter technique identified them as having MWs of 42, 49, and 54KDa, and showed that they exist in a ratio of 8:1:1, respectively (Spiess et al., 1990). The receptor, being an integral membrane protein, spans the lipid bilayer once and exposes a small amino-terminal segment to the cytoplasm and a large carboxy-terminal, of which a portion is exposed to the exoplasmic milieu (Spiess et al., 1990). The cytoplasmic domain of the ASGP receptor plays a crucial role in the segregation of the endocytosed proteins (Spiess et al., 1990). A deletion in this domain results in a reduction of the internal rate of ASGPs. The cytoplasmic tail is responsible for

catalyzing the formation of coated pits. Associated with coated pits and vesicles are accessory or adaptor protein complexes (Pearse, 1988; Smythe et al., 1991). The latter are known to recognize the cytoplasmic tail of receptors and to induce the formation of coated pits (Spiess et al., 1990). Pearse (1988) observed a direct interaction between the cytoplasmic tail of the LDL receptor and one of the adaptor proteins.

Immunohistochemical studies (O'Brien et al., 1989) show that the minor form of the rat ASGP receptor, i.e. RHL-2 and RHL-3, is found in Sertoli cells, spermatogenic cells, and epididymal sperm. In addition, it was recently observed that RHL-1 is localized to the late-stage spermatids (Huber et al., 1992). The significant implication of the latter observations is that serum ASGPs that are efficiently removed by parenchymal hepatic ASGP do not concentrate in the testicular and epididymal compartments because of the blood-testis barrier. Therefore, ASGP receptors may play an intrinsic role in spermatogenesis or fertilization.

C) The Mannose 6-Phosphate Receptor

Lysosomes are acidic cytoplasmic vacuoles that contain many hydrolytic enzymes important to the degradation of endocytosed and endogenous molecules. The cell targets Golgi-derived enzymes or endocytosed enzymes to lysosomes via the mannose 6-phosphate receptor system (Kornfeld, 1992). Endogenous enzymes acquire phosphomannosyl residues and can bind with a greater affinity to the mannose 6-phosphate receptor, which will target the

enzymes to the lysosomes (Kornfeld, 1992). Most of the endocytosed enzymes dissociate from their receptors in the endosomes and are destined to the lysosome by the mannose 6-phosphate receptor (Kornfeld, 1992).

There exist two types of mannose 6-phosphate receptors, both of which are integral membrane glycoproteins. The first type has a MW of 275 KD and does not require divalent cations. In addition to binding to mannose 6-phosphate residues like the second type, it binds to IGF II (Kornfeld, 1992). Binding of ligands is maximal at a neutral pH and drops sharply at a pH of <6.0 (Steer and Ashwell, 1990). The second type of receptor has a MW of 46KD and is dependent on divalent cations (Kornfeld, 1992).

The mannose 6-phosphate-IGF II receptor contains four stuctural domains: an amino-terminal signal sequence, an extracytoplasmic domain, a single transmembrane domain, and a carboxy-terminal cytoplasmic domain (Kornfeld, 1992). The cytoplasmic domain has four potential binding sites for protein kinases: protein kinase C, cAMP-dependent protein kinase, and casein kinase I and II (Kornfeld, 1992).

The 46KD is synthesized in a precursor form with a signal peptide, a single membrane-spanning domain, an extracytoplasmic domain, and a cytoplasmic stretch (Steer and Ashwell, 1990).

Brown and Farquhar (1984) localized mannose 6-phosphate receptors to the coated pits, coated vesicles, endosomes, multivesicular bodies, lysosomes, and cis-Golgi stacks. Receptor synthesis, in the Golgi complex, is constitutive. The receptor also cycles repeatedly among the Golgi complex, endosomes, and plasma membrane (Kornfeld, 1992). The 46KD mediates the transport of

endogenous ligands only (Steer and Ashwell, 1990). However, the 275KD functions in the sorting of newly synthesized lysosomal enzymes and endocytosed extracellular lysosomal enzymes (Kornfeld, 1992).

D) The Sulfated N-acetylgalactosamine Receptor

A newly described receptor has been identified in hepatic endothelial and Kupffer cells, which binds oligosaccharides that terminate with the sequence sulfated-4N-acetylgalactosamine-β1, 4N-acetylglucosamine-β1,2-mannose-α (SO4-4GalNAcβ1, 4GlcNAcβ1,2-Manα (S4GGnM)) (Fiete et al., 1991). This receptor can account for the rapid removal of the pituitary glycoprotein hormone lutropin, which contains Asn-linked oligosaccharides terminating with S4GGnM (Green et al., 1986; Green and Baenziger, 1988a, 1988b). This receptor mediates binding at the cell surface and transports the ligand to the lysosomal compartment. The receptor-ligand complex dissociates at a pH of <5.0 (Fiete et al., 1991).

Experiments were done on Chinese hamster ovary (CHO) cells, which contain recombinant lutropin (LH) terminating with the sequence sialic acid-Galβ1,4GlcNAcβ1,2Manα (Smith et al., 1990). The latter substance lacks the sulfated sugar normally present on lutropin. The presence of sulfated oligosaccharides on LH reflects the activity of a glycoprotein-hormone specific GalNAc-transferase (Smith and Baenziger, 1988, 1990, 1991) and a sulfotransferase (Green et al., 1985), neither of which is expressed in CHO cells (Smith and Baenziger, 1988). When the internalization of LH was studied, it

was found that native LH bearing the sulfated oligosaccharides was removed from the circulation four to five times more rapidly than recombinant LH bearing the sialylated oligosaccharides (Baenziger et al., 1991). Therefore, this finding suggests the existence of a receptor which specifically recognizes oligosaccharides that terminate with the S4GGnM sequence and which is responsible for the rapid clearance of native LH (Fiete et al., 1991).

E) The Mannose Receptor

The mannose receptor was identified as a 175 KDa cell-surface transmembrane glycoprotein in macrophages, where it functions both as a mediator of the uptake of mannose-rich glycoproteins and as a phagocytic receptor for bacteria, yeasts, and other pathogenic microorganisms bearing high mannose-type carbohydrate chains (Harris et al., 1992; Otter et al., 1992). The receptor is also involved in the rapid clearance of tissue-type plasminogen activator from the circulation (Otter et al., 1992).

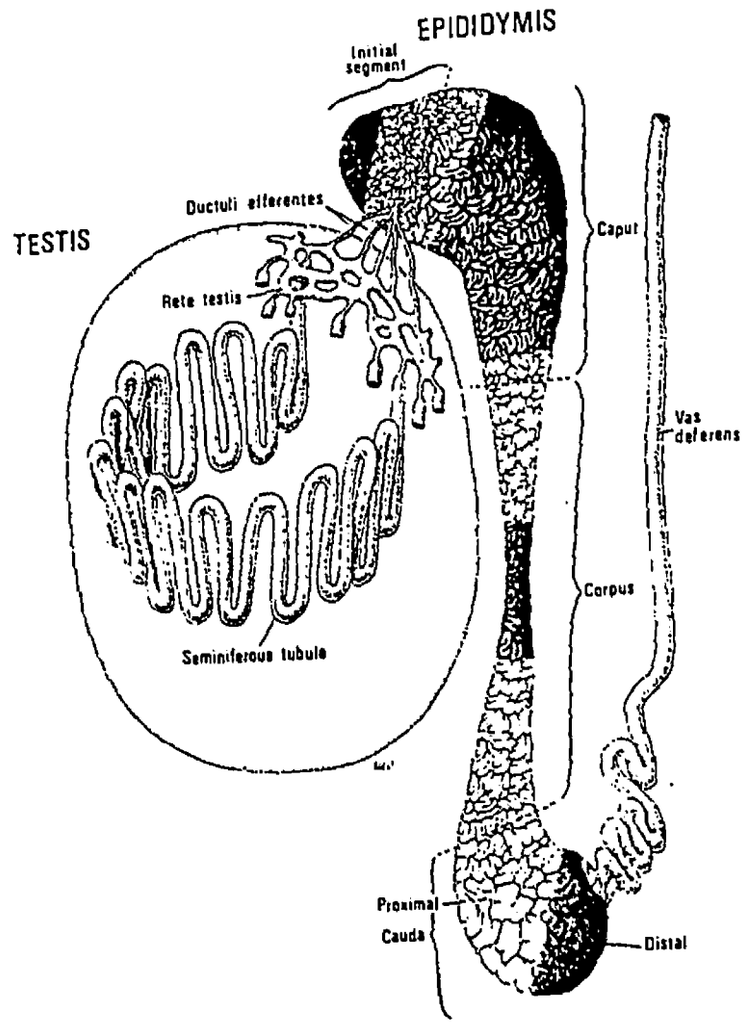
The cDNA of the mannose receptor predicts a protein of 1,456 amino acids, with an extracellular domain containing an N-terminus that is cysteine-rich, followed by a fibronectin type II domain and eight carbohydrate recognition domains (CRDs) (Harris et al., 1992; Taylor et al., 1992). The extracellular domain is linked to a hydrophobic transmembrane region, followed by a 46-amino acid cytoplasmic tail (Harris et al., 1992). Endocytosis of mannose-terminated glycoproteins is mediated by the extracellular domain but more specifically by the carbohydrate recognition domains (CRDs) (Taylor et

al., 1992). The cysteine-rich domain and the fibronectin type II domain are not essential for endocytosis. The carbohydrate binding activity resides in CRDs 4 to 8, which show the highest affinity binding, and has multispecificity for a variety of monosaccharides (Taylor et al., 1992).

FIGURES AND LEGENDS

FIGURE 1

Schematic representation of the testis and excurrent duct system of the adult rat. The testis consists of seminiferous tubules that empty their content in the rete testis. The latter is connected to the ductuli efferentes which are themselves connected to the epididymis. The shaded regions represent the different segments of the epididymis, i.e., the initial segment, caput, corpus, and proximal and distal cauda. The vas deferens is an extension of the epididymis.


EPIDIDYMIS
Initial segment
Ductuli efferentes
TESTIS
Rete testis
Caput
Vas deferens
Corpus
Seminiferous tubule
Proximal
Cauda
Distal

①

FIGURE 2

Diagrammatic representation of the structure of the rat seminiferous epithelium. The base of the seminiferous epithelium consists of layers of myoid cells known as the limiting membrane. Resting on the limiting membrane are various types of spermatogonial stem cells and non-proliferating Sertoli cells. Adjacent Sertoli cells are linked by tight junctional complexes that divide the seminiferous epithelium into two compartments: a basal compartment, where spermatogonial stem cells reside, and an adluminal compartment, where the meiotic germinal cells and spermatids are found. The Sertoli cell has several functions. It is involved in the formation of the blood-testis barrier and in the nutrition of germinal cells; it has a sustentacular role; it secretes water, ions, and proteins; it is involved in spermiation, pinocytosis, phagocytosis, and receptor-mediated endocytosis; and it plays a role in the maintenance of spermatogenesis by lifting the early meiotic germ cells from the basal to the adluminal compartment. A = spermatogonial stem cell; eSptd = elongating spermatid; M = myoid cell; N = Sertoli cell nucleus; PL = preleptotene spermatocyte; RB = residual body; SER = Sertoli cell; Sptc = spermatocyte; Sptd = spermatid; Tjn = tight junctional complexes.

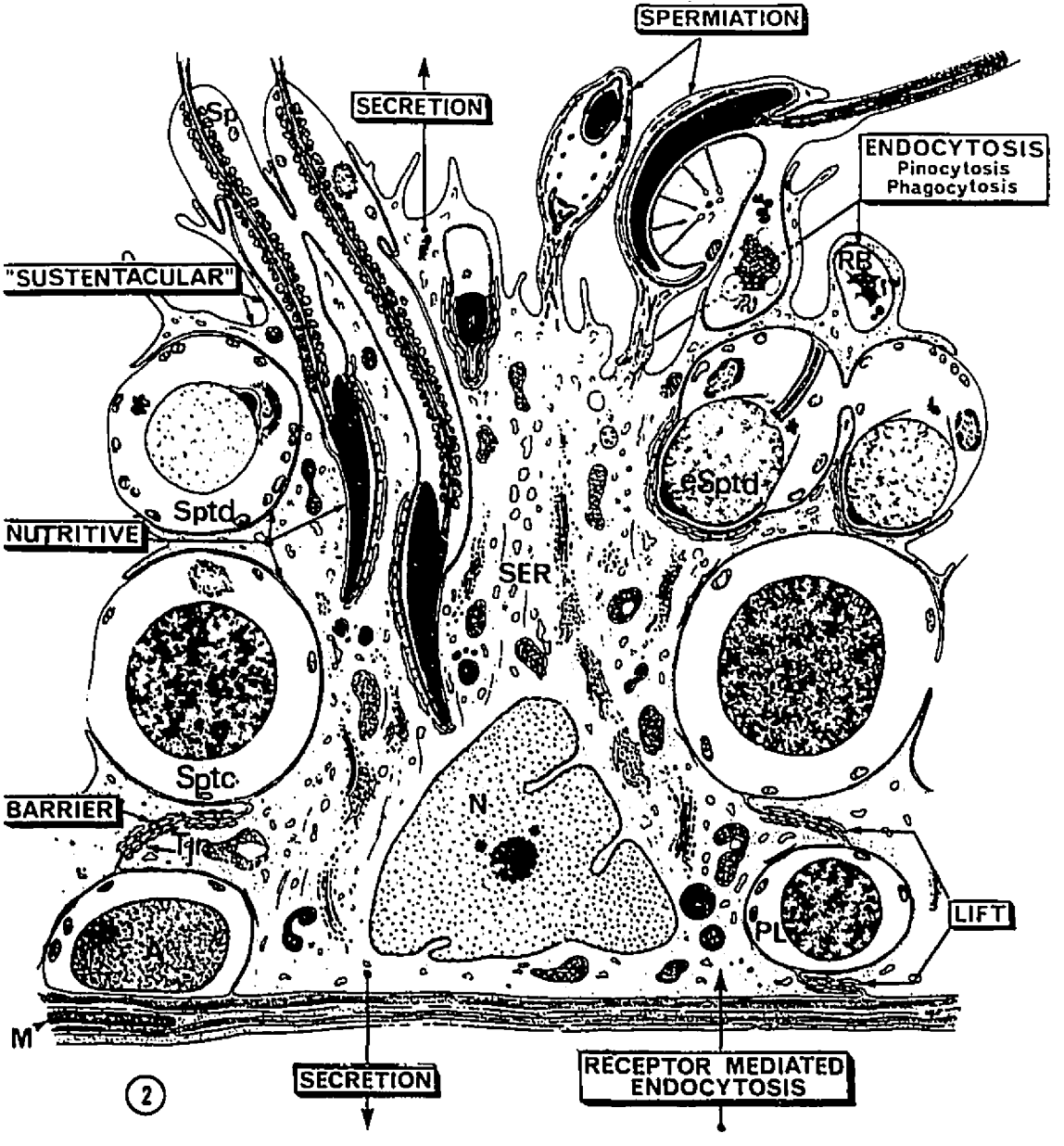
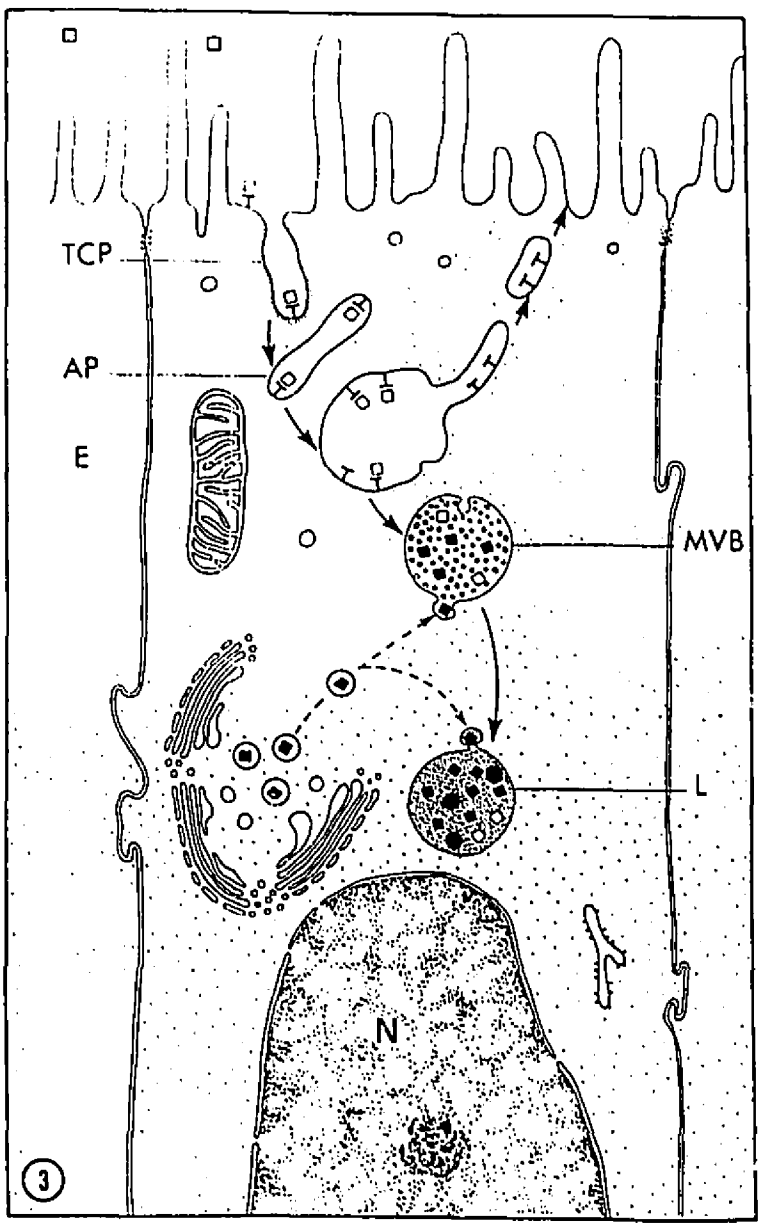

SPERMIATION
SECRETION
Sp
ENDOCYTOSIS
Pinocytosis
Phagocytosis
"SUSTENTACULAR"
RB
eSptd
Sptd
NUTRITIVE
SER
Sptc
BARRIER
N
LIFT
PL
M
SECRETION
RECEPTOR MEDIATED
ENDOCYTOSIS
2

FIGURE 3

Diagrammatic representation of the events taking place in the nonciliated cell of the efferent ducts. Testicular SGP-1 (t-SGP-1, 70 KDa) synthesized by the Sertoli cells are endocytosed, possibly via receptor-mediated endocytosis, by the nonciliated cells and appears in the endocytic apparatus, which includes tubular coated pits, apical tubules, endosomes, multivesicular bodies, and lysosomes. Acidification of the endosomes causes the dissociation of t-SGP-1 from its putative receptor. The latter is recycled back to the plasma membrane. Endogenous SGP-1 (65 KDA) is synthesized in the endoplasmic reticulum, modified and glycosylated in the Golgi apparatus, and targeted via small vesicles to the lysosome, where it is possibly processed to smaller proteins, the saposins (15 KDa). AP = apical tubules; E = endosomes; L = lysosomes; MVB = multivesicular body; N = nucleus of the nonciliated cells; TCP = tubular coated pits; □ = t-SGP-1; T = receptor; □ = endogenous SGP-1; O = saposins.


TCP
AP
E
MVB
L
N
3

MATERIALS AND METHODS

1) EXPERIMENT 1: The Effect of Different Sugars on the Endocytosis of SGP-1 by the Nonciliated Cells.

A) Experimental Procedure

Four adult male Sprague Dawley rats (275 to 375 g), obtained from Charles River Canada LTD., were anesthesized with 0.4 ml of sodium pentobarbital (Somnitol), injected intraperitoneally. The testes were exposed through an abdominal incision. A given volume of 200 µl of different sugar solutions was injected into the lumen of the rete testis using a 28-1/2-gauge needle. Each sugar solution was tested on one testis. After an interval of 15 minutes, the animals were sacrificed. Their efferent ducts were removed and fixed with 4% paraformaldehyde and 0.25% glutaraldehyde in 0.1 M phosphate buffer saline (PBS) at a pH of 7.4 for 2 hours at room temperature. After this fixation period, the efferent ducts were trimmed into three pieces and were fixed for another hour. The tissues were then washed three times for 10 minutes with PBS and were left overnight in PBS. After fixation, the tissues were dehydrated in graded methanol up to 90% and were embedded in Lowicryl K4M, as described by Hermo et al. (1988) and Oko (1988). Ultra-thin sections were mounted on 300 mesh Formvar-coated nickel grids.

B) The Sugar Solutions

Each sugar solution injected into the rete testis contained a sugar residue with a concentration of 20 mM in 10 times PBS (100 µl) and Nile Blue (900 µl). The different sugar residues used and their exact amounts were:

- glucose (3.604 mg)
- mannose (3.604 mg)
- mannose 6-phosphate (7.910 mg)
- sialic acid (6.186 mg)
- galactose (3.604 mg)
- N-acetylgalactosamine (4.400 mg)
- N-acetylglucosamine (4.400 mg)

An equal amount of PBS was used as the control.

C) Immunocytochemistry

Each grid was floated for 1 hour on a 20 µl drop of 20 mM Tris-Hcl buffer saline (TBS) and 10% goat serum. It was then incubated for 1 hour on a 20 µl drop of SGP-1 antibody diluted 1:50 in TBS. Sections were washed four times for 5 minutes each in TBS containing 0.2% Tween-20. They were then transferred to a 20 µl drop of TBS with 10% goat serum for 15 minutes and were incubated for 1 hour on a 20 µl drop of colloidal gold (10 nm) conjugated goat anti-rabbit IgG (Zollinger Incorporated, Montreal). After being washed four times for 5 minutes each in TBS with 0.2% Tween-20, sections were washed

twice for 5 minutes each in distillled water. They were counterstained with uranyl acetate in 30% ethanol for 2 minutes, followed by lead citrate for 30 seconds. Electron micrographs were taken on a Philips 400 electron microscope.

D) Quantitative Electron Microscopic Analysis

A quantitative analysis was performed on the endocytic apparatus of the nonciliated cells of the efferent ducts. Profile areas of the endocytic vesicles, early endosomes, late endosomes, and perimeter of microvilli were calculated with a Zeiss MOP-3 image analyzer (Carl Zeiss, Inc. Montreal, Quebec). The immunogold labeling density, expressed as the mean number of gold particles per μm^2 profile area, was calulated from 30 microvilli, 100 endocytic vesicles, 30 early endosomes, and 30 late endosomes for each sugar treatment. The endocytic structures of the control group were compared with the corresponding endocytic structures undergoing different sugar treatments, and the differences in labeling densities were analyzed with a one-way ANOVA test ($p=0.05$).

2) EXPERIMENT 2: The Effect of Different Sialic Acid Concentrations on the Endocytosis of SGP-1 by the Nonciliated Cells

A) Experimental Procedure

Three adult male Sprague Dawley rats (275 to 375 g), obtained from Charles River Canada LTD., were anesthesized with sodium pentobarbital (Somnitol). The same procedure was used as in Experiment 1, except that the solutions injected in the rete testis were increasing concentrations of sialic acid: 0.02 mM, 0.2 mM, 2 mM, 20 mM, and 200 mM, each in 100 µl of PBS and 900 µl of Nile Blue. The control was an injection of PBS and Nile Blue in the rete testis. The procedure for electron microscopy and immunocytochemistry was the same as in Experiment 1.

b) Quantitative Electron Microscopic Analysis

Quantitative electron microscopic analysis was performed on the endocytic compartments of the nonciliated cells. Profile areas of endocytic vesicles, early endosomes, and late endosomes were calculated with a Zeiss MOP-3 image analyzer. The immunogold labeling density was calculated for 50 endocytic vesicles, 15 early endosomes, and 15 late endosomes for each of the concentrations of sialic acid. The control group was used as a reference point. The differences in labeling densities were analyzed with a one-way

ANOVA test (p=0.050).

3) EXPERIMENT 3: Dertermination of Terminal Sialic Acid Residues on SGP-1

A) Collection of Testicular SGP-1

In order to obtain enough SGP-1 samples, seven adult male Sprague Dawley rats (250 to 350 g), (Charles River Canada LTD.) were sacrificed by means of CO_2 inhalation, and their testes were removed through an abdominal incision. The testes were decapsulated, diced thoroughly, and rinsed in Hanks Balanced Salt Solution (HBSS). The tissue was then washed two times in 50 ml of HBSS, and the wash was spun down at 3500 RPM for 10 minutes to eliminate the cell component. The supernatant was heated at 90°C for 10 minutes to eliminate proteins that were not heat stable. It was then spun down at 11000 RPM for 20 minutes. Trypsin inhibitors (10 mg for a total volume of 100 ml) and PMSF (phenyl methylsulphonyl fluoride, 100 µl for 100 ml) were added to the supernatant. Dialysis was performed using a membrane with a cutoff point of 12,000 to 15,000 Kb. The solutes in the dialysis membrane were collected, lyophilized, and stored at 4 °C.

b) Affinity Chromatography

Testicular SGP-1 was purified by means of affinity chromatography. Protein A sepharose (Sigma, St. Louis, MO) (200 µl) was added to SGP-1 antibody (25 µl) and left on ice for 1 hour with gentle mixing of the antibody to bind to the protein A. After this incubation, the beads were spun down to settle at the bottom of the tubes, and the supernatant was washed twice with 0.2 M sodium tetraborate (pH, 9.0). The beads were resuspended in 0.2 M sodium tetraborate, and 20 mM methylpimelimidate was added to the bead solution, to allow a covalent link between the protein A and the SGP-1 antibody. After 30 minutes, the beads were washed with 0.2 M ethanolamine (pH, 8.0) and incubated at room temperature in 0.2 M ethanolamine for 2 hours with gentle mixing. The beads were then washed with PBS and were stored in PBS and 0.01% thimerasol (Simaris and Lane, 1985).

Lyophilized testicular SGP-1 collected from seminiferous fluids was dissolved in lysis buffer (10 mM Tris Base, 1% non-idet P40, 2 mM EDTA, 0.15 M NaCl, 50 µg/ml PMSF, 2 µg/ml leupeptin, 1 mg/100 ml apoprotinin). Testicular SGP-1 was added to the beads, which had been spun down to eliminate the supernatant, and the solution was incubated for 30 minutes on ice, with gentle shaking. The beads were then washed three times with 1X TBS with 0.1% Tween-20, and 1 time with 1X TBS without Tween-20. The beads were incubated in 1 ml glycine (ph 2.9) for 1 minute to dissociate the SGP-1 from its antibody. The elutant was then incubated in 1 ml 2X TBS (pH, 9.0) in order to neutralize the solution. The beads were washed once with 2X TBS and once

with 1X TBS with 0.1% thimerasol for storage. The elutant containing SGP-1 was concentrated with centripep-3 microconcentrator (Amicon) during centrifugation at 2500 RPM for 2 hours.

C) Sodium Dodecyl Sulfate (SDS)-PAGE

The concentrated testicular SGP-1 was solubilized in 2% SDS, 5% beta mercaptoethanol for 10 minutes at 100 °C and was run on polyacrylamide gels (12%) according to the SDS-discontinuous system originally described by Laemmli (1970). Apparent molecular weights were determined from the mobility of low molecular weight standards (Pharmacia Electrophoresis Calibration Kits, Piscataway, NJ). The gels were stained with silver nitrate as follows: each gel was incubated in 50% methanol for 60 minutes. It was then incubated for 15 minutes in a solution containing 20% AgNO3 added to a solution of 3.6% NaOH and 25% NH4OH. The gel was washed in distilled water five times for 5 minutes. It was developed in 1% citric acid and 38% formaldehyde for 15 minutes and was washed in distilled water five times for 5 minutes. Development was stopped by placing the gel in a solution containing 45% methanol and 10% acetic acid.

D) Western Blotting

SDS-PAGE proteins were electrophoretically transferred from the gels onto nitrocellulose paper (0.45 μm pore size; Scheicher and Schwell Inc.,

Keene, NH) using a Hoefer Semidry Transphor Apparatus (Hoefer Scientific Instruments, San Fransisco, CA) according to the techniques of Towbin et al. (1979) and Towbin and Gordon (1984). After SDS-PAGE, the gel was rinsed in a solution containing 25 mM Tris-base, 192 mM glycine, and 20% methanol (pH, 8.3). Electrophoretic transfer was carried out at 100 volts for 1 hour.

E) Immunoblotting

The blot was rehydrated in TBS and blocked with TBS and 10% goat serum for 30 minutes. The blot was then incubated with SGP-1 antibody for 2 hours at room temperature. It was washed four times for 5 minutes with TBS and 0.05% Tween-20. It was then blocked with TBS and 10% goat serum for 15 minutes and was incubated in alkaline phosphatase-conjugated goat anti-rabbit IgG, diluted 1:1000 for 45 minutes. Next, the blot was washed four times for 5 minutes with TBS and washed once with a sodium glycinate solution (50 mM Na-glycinate and 0.5% Tween-20, ph 9.6) for 2 minutes. It was then incubated in a solution containing 10 ml of Na-glycinate solution, 1 mg of p-nitro blue tetrazolium chloride, 0.5 mg of 5-bromo-4-chloro-3-indolyl phosphate, and 40 µl of 1M $MgCl_2$ under low light conditions for 10 to 20 minutes or until bands started appearing. The blot was then rinsed in TBS and stored in deionized water or dried on filter paper.

F) HPLC

For further purification, reverse-phase high performance liquid chromatography (HPLC) was performed after affinity chromatography with protein A. The SGP-1 sample was dissolved in 20% acetonitrile and was loaded on a C8 ultrapore reverse-phase HPLC column (4.6 x 250 mm, 5 µm particle size). After a 10-minute run at 100% mobile phase A (20% acetonitrile and 0.1% TFA or trifluoroacetic acid), absorbed molecules were eluted using a 40-min linear gradient of 0% to 100% mobile phase B (80% acetonitrile and 0.1% TFA) using wavelengths of 254 and 280 nm. Two-ml fractions were collected, and aliquots of 50 µl from the different fractions were placed in each well of a micro-ELISA plate in order to determine the immunoreactivity of each peak to anti-SGP-1 antibody.

G) ELISA

The enzyme-linked immunosorbent assay (ELISA) was performed as described by Harlow and Lane (1988). Ten percent goat serum was added to each well for 1 hour. Rabbit anti-SGP-1 antibody was diluted 1:200 in TBS and was added to each well for 1 hour. The wells were washed three times with TWBS (TBS and 0.1% Tween-20) for 5 minutes. The secondary antibody (peroxidase-conjugated, Sigma, St. Louis, MO) goat anti-rabbit, diluted 1:1000 in TBS, was added for 1 hour. The wells were then washed three times for 5 minutes with TWBS. One mM of 2,2'-azino-bis-(3-ethyl-benzthiazoline sulfonic

acid) and 0.3% hydrogen peroxide in 100 mM citrate phosphate buffer, pH 4.2, was used as a developer. The absorbance of the reaction product was determined at 415 nm. The sample with the highest absorbance, which corresponded to the peak eluting at 60% solvent B, was lyophilized, resuspended in distilled water, electrophoresed on a polyacrylamide gel, and stained with silver nitrate.

H) Glycan Differentiation

This method is used to identify carbohydrates, based on the fact that lectins conjugated with the steroid hapten digoxigenin, which enables immunological detection of the bound lectins, can bind specifically to carbohydrate moieties. Therefore, lectins selectively recognize terminal sugars, and carbohydrate structures can be differentiated from one another.

After Western blotting, the blots were trimmed into strips containing the protein SGP-1 and were tested for SGP-1 with Ponceau Rouge staining. The strips were incubated for 30 minutes in a blocking solution containing 0.5 mg of a blocking reagent in 100 ml of TBS (pH, 7.5). They were then washed twice for 10 minutes in TBS and once in a buffer containing TBS, 1 mmol/l of $MgCl_2$, 1 mmol/l of $MnCl_2$, and 1 mmol/l of $CaCl_2$ (pH, 7.5). Each strip was incubated in different lectin solutions for 1 hour. Lectin solution SNA (sambucus nigra agglutinin) identify sialic acid residues linked a(2-6) to galactose or N-acetylgalactosamine, whereas lectin solution MAA (maackia amurensis agglutinin) recognized sialic acid residues linked α(2-3) to galactose. Ten µl of

SNA solution was diluted in 10 ml of buffer 1, and 50 µl of MAA solution was diluted in 10 ml of buffer 1. The strips were then washed three times for 10 minutes with TBS and were incubated in anti-digoxigenin-AP for 1 hour. After being washed three times for 10 minutes with TBS, they were developed with a buffer containing TBS, 0.1 mol/l of Tris HCL, 0.05 mol/l of MgCl2, 0.1 mol/l of NaCl with 4-nitroblue tetrazolium chloride (NBT), and 5-bromo-4-chloro-3-indolyl phosphate until bands appeared on the strips. Finally, the strips were rinsed several times with distilled water and were dried on paper towels.

RESULTS

1) The Immunogold Distribution of SGP-1 in Nonciliated Cells of the Efferent Ducts

Immunogold labeling of SGP-1 in nonciliated cells of the efferent ducts was evident in the endocytic apparatus. These cells displayed gold particles 1) on the surface of microvilli; 2) in the tubular coated pits, which pinch off the plasma membrane to give rise to apical tubules or endocytic vesicles; 3) in the endocytic vesicles, which reach the early endosomes and fuse with them to empty their content; 4) in the early endosomes, where gold particles are preferentially located at the periphery, in association with the endosomal membrane; 5) in the late endosomes, where the labeling was randomly distributed throughout the lumen of the endosomes; 6) in the multivesicular bodies; 7) and in the lysosomes. Labeled small vesicles or clusters of gold particles were seen in the apical and supranuclear regions, in close apposition to the multivesicular bodies and lysosomes. This labeling corresponds with data already published by Igdoura et al. (1993). Figure 4 represents the immunogold pattern observed in nonciliated cells of the efferent ducts.

2) The Effect of Different Sugars on the Endocytosis of SGP-1 by the Nonciliated Cells

Different oligosaccharides including galactose, mannose, mannose 6-phosphate, N-acetylglucosamine, N-acetylgalactosamine, sialic acid, and glucose were tested in order to study their effects on the endocytosis of the nonciliated cells. Animals were 15 minutes after the injection of the various sugars. Figures 6 to 17 and Table 1 show the effect of each of the sugar treatments on the labeling density (mean number of gold particles/μm^2 profile area) in various endocytic compartments, including microvilli, endocytic vesicles, and early and late endosomes. These different endocytic compartments are represented in Figures 5, 5a, and 5b. Statistical evaluation of the data obtained with these oligosaccharide treatments was performed using a one-way ANOVA, with p values set at p=0.05. The control group was taken as a reference point.

A) Microvilli

For microvilli, the labeling density was calculated according to the perimeter instead of the area, because the protein, bound to the receptor, was found only on the surface of the microvilli. Sialic acid was the only sugar that caused a significant effect, i.e., a reduction of 86% in the labeling density as seen in Figure 6. Figure 8 represents microvilli of nonciliated cells treated with

sialic acid. There is an extreme reduction in the labeling density of microvilli in Figure 8 when compared to that of the control in Figure 7.

B) Endocytic Vesicles

Many oligosaccharides produced a statistically significant decrease in the labeling density in the endocytic vesicles. These substances included galactose, mannose, mannose 6-phosphate, N-acetylglucosamine, sialic acid, and glucose (Fig. 9). The decrease in the number of gold particles per unit area was as follows: galactose, 69%; mannose, 43%; mannose 6-phosphate, 83%; N-acetylglucosamine, 45%; sialic acid, 84%, and glucose, 64%. The sugar that seemed to cause the greatest decrease was sialic acid. Figure 10 represents control endocytic vesicles and Figure 11 represents endocytic vesicles treated with sialic acid. The endocytic vesicles seen in Figure 11 are almost completely devoid of gold particles.

C) Early Endosomes

Early endosomes were also affected by many oligosaccharides including galactose, mannose, mannose 6-phosphate, N-acetylglucosamine, sialic acid, and glucose (Fig. 12). All of these sugars produced a statistically significant decrease in the labeling density. Compared to control samples, the mean concentration of gold particles per unit area in early endosomes was 46% less with galactose, 55% less with mannose, 78% less with mannose 6-phosphate,

39% less with N-acetylglucosamine, 95% less with sialic acid, and 78% less with glucose. Sialic acid produced the greatest effect. There is an obvious decrease in the labeling density of the early endosome when the cells are treated with sialic acid, as seen in Figure 15, when compared to the control untreated cells, Figure 14.

D) Late Endosomes

Late endosomes were affected greatly by sialic acid and glucose and to a lesser extent by mannose-6-phosphate (Fig. 13). All three sugars produced a statistically significant decrease in the labeling density: mannose 6-phosphate, 47%; sialic acid, 76%; and glucose, 81%. Glucose produced the greatest decrease in the labeling density. However, as seen in Figure 17, the decrease produced by sialic acid is apparent when compared to the control (Fig. 16).

Table 1 summarizes the results obtained from this experiment. Sialic acid was the oligosaccharide that had the most significant effects on the endocytosis of SGP-1 by the nonciliated cells. In every endocytic compartment, sialic acid caused a greater than 75% decrease in the labeling density. However, glucose and mannose 6-phosphate also produced a substantial decrease in the labeling density in three out of four endocytic compartments. Therefore, these two sugars may also play an important role in the endocytosis of SGP-1.

3) The Effect of Increasing Concentrations of Sialic Acid on the Endocytosis of SGP-1 by the Nonciliated Cells

The previous experiment showed that, of the seven oligosaccharides, sialic acid produced a significant decrease in every instance and seemed to have the most significant effect in almost every endocytic compartment. The following experiment was performed to confirm that administration of sialic acid significantly reduces the endocytosis of SGP-1 by the nonciliated cells. The effect of increasing concentrations of sialic acid on the labeling density in different endocytic compartments was analyzed in order to observe a linear decrease in the amount of endocytosed SGP-1 in the nonciliated cells. Five different concentrations of sialic acid were administered: 0.02 mM, 0.2 mM, 2 mM, 20 mM, and 200 mM, and animals were sacrificed 15 minutes after these injections. The labeling density was calculated in endocytic vesicles and in early and late endosomes. The results were compared with each other and with a control. Figures 18 to 26 and Table 2 show the effect of different concentrations of sialic acid on the labeling density of various endocytic compartments.

A) Endocytic Vesicles

In the endocytic vesicles, all five concentrations of sialic acid produced a significant (>50%) reduction in the labeling density of SGP-1. However, as

Figure 18 shows, the different concentrations of sialic acid did not produce a linear decrease in the labeling density. The decreases caused by each treatment were more or less equivalent to each other, and none was significantly different from any of the others. The 0.02 mM concentration produced the greatest decrease (71%), whereas the 0.2 mM concentration resulted in the lowest (54%). Figure 20 is an electron micrograph of endocytic vesicles in nonciliated cells treated with 0.2 mM sialic acid. The decrease in the labeling density is evident when compared to the control (Fig. 19).

B) Early Endosomes

In early endosomes, each different concentration of sialic acid produced a significant (>60%) decrease in the labeling density, compared to the control group (Fig. 21). The reductions caused by each concentration of sialic acid were more or less equivalent. Therefore, no linear decrease in the labeling density was observed. The highest reduction (76%) was produced by 2 mM sialic acid, and the lowest (63%) was caused by 0.2 mM sialic acid. The decrease caused by the 0.2 mM concentration is illustrated in Figure 24. The early endosome is almost completely devoid of gold particles. Figure 23 serves as a control.

C) Late Endosomes

In late endosomes, only the 0.02 mM, 0.2 mM, and 20 mM concentrations

of sialic acid produced a significant decrease (51%, 52%, and 56%, respectively) in the labeling density (Fig. 22). These three concentrations caused equal reductions in the labeling density of SGP-1. The remaining concentrations, 2 mM and 200 mM, did not have any significant effect, as they caused reductions of only 41% and 39%, respectively. Therefore, no linear decrease in the labeling density was observed. In Figure 26, there are very few gold particles located in the late endosome of nonciliated cells treated with 0.2 mM sialic acid when compared to the control (Fig. 25).

Table 2 summarizes the effect of the different concentrations of sialic acid on the labeling density in the endocytic vesicles and early and late endosomes.

4) Purification of Testicular SGP-1

After being isolated from the testicular fluids, SGP-1 was purified by means of affinity chromatography and was subjected to reverse-phase HPLC. After ELISA testing, samples with the highest immunoreactivity, which corresponded to those whose peak eluted at 60% solvent B on HPLC (Fig. 27), were subjected to a SDS-PAGE and stained with silver nitrate to identify the 70 KDa protein, which corresponded to testicular SGP-1.

Western blot analysis, performed with anti-SGP-1 antibody, recognized the testicular SGP-1, which is represented by the major band of 70KDa seen in Figure 28.

5) Glycan Differentiation

Samples collected from HPLC were subjected to a SDS-PAGE, and a Western blot was used to perform a glycan differentiation procedure. Figure 29 is a blot obtained after the glycan differentiation procedure. It reveals that testicular SGP-1 is reactive to Sambucus nigra agglutinin (SNA) (lane 1). This indicates that SGP-1 contains sialic acid that is terminally linked α(2-6) to galactose or N-acetyl galactosamine. However, the blot was unreactive to Maackia amurensis agglutinin (MAA) (lane 2), thereby indicating that SGP-1 does not possess sialic acid that is terminally linked α(2-3) to galactose.

FIGURES AND LEGENDS

FIGURE 4

Diagrammatic representation of the immunogold pattern of SGP-1 observed in the nonciliated cells of the efferent ducts. SGP-1 appears in the lumen, on the surface of microvilli, at the periphery of tubular coated pits, endocytic vesicles, and early endosomes, and homogeneously scattered in the lumen of late endosomes, multivesicular bodies, and lysosomes in increasing quantity.

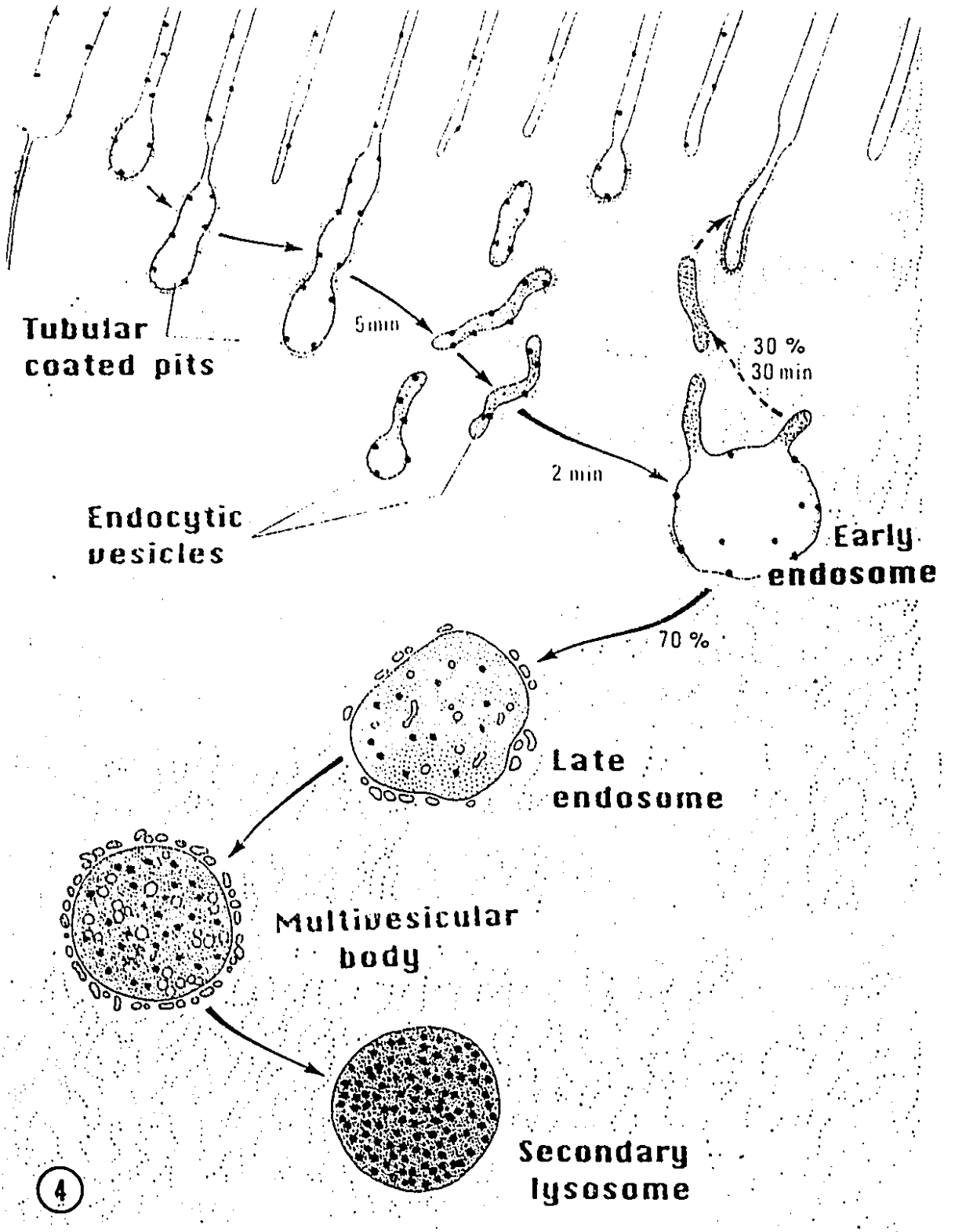

Tubular coated pits
5 min
30 %
30 min
2 min
Endocytic vesicles
Early endosome
70 %
Late endosome
Multivesicular body
Secondary lysosome
4

FIGURE 5

Electron micrograph of a section of an efferent duct showing ciliated and nonciliated cells. The nonciliated cell is characterized by microvilli and an extensive endocytic apparatus including endocytic vesicles, early and late endosomes, and lysosomes. The ciliated cell's endocytic apparatus is less apparent. CIL = ciliated cells; E = early and late endosomes; EV = endocytic vesicles; L = lysosomes; LU = lumen of the efferent duct; M = microvilli; MY = myoid cell; N = nucleus of the nonciliated cell; NONCIL = nonciliated cell. X 3,300.

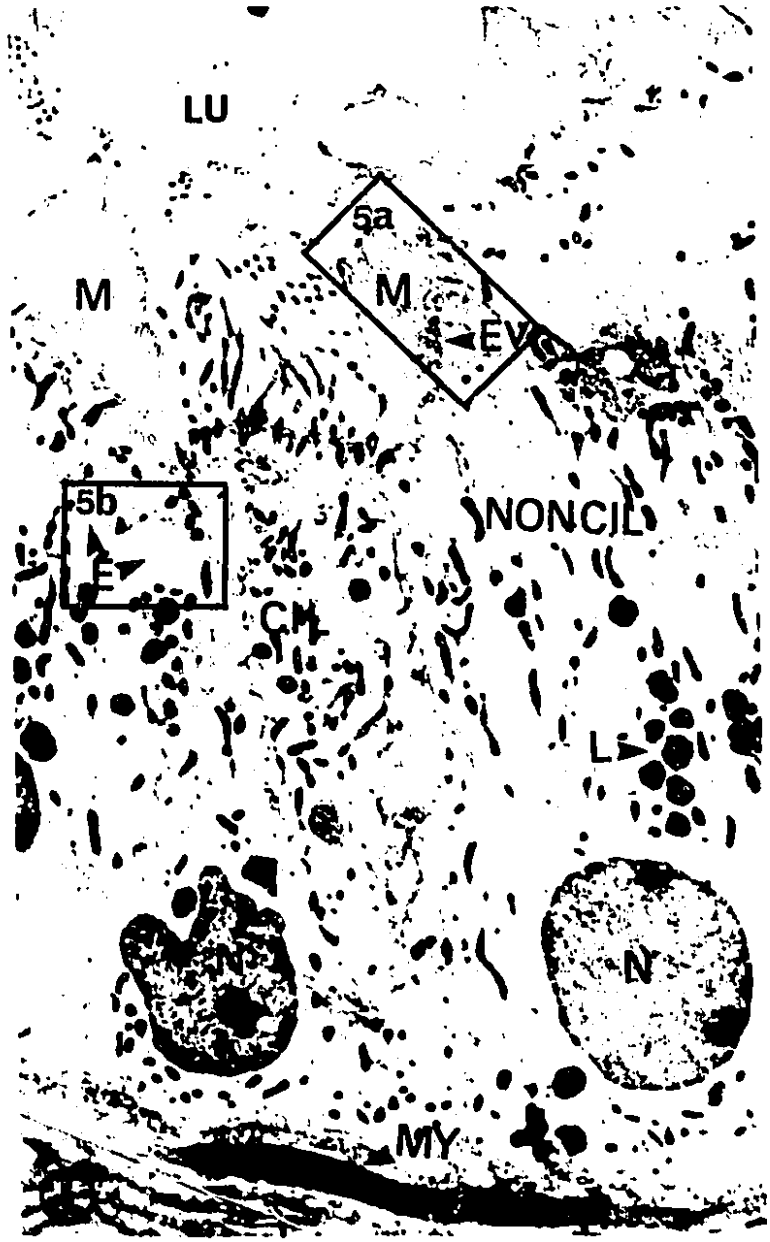

LU
5a
M
M
EV
5b
E
NONCIL
CM
L
N
N
MY

FIGURE 5 a

Electron micrograph showing a portion of the apical region of a nonciliated cell of the efferent ducts of a control (untreated) animal immunolabeled with anti-SGP-1. Gold particles denoting SGP-1 are present in the lumen of the efferent duct, on the surface of the microvilli, and in the endocytic vesicles. EV = endocytic vesicles; LU = lumen of the efferent duct; M = microvilli; arrowhead = gold particles on the microvilli; curved arrows = gold particle in the lumen. X 22,000.

5a

FIGURE 5 b

Electron micrograph of endosomes of nonciliated cells of the efferent ducts of a control (untreated) animal immunolabeled with anti-SGP-1. Gold particles appear at the periphery and in close association with the membrane of the early endosome. Gold particles are homogeneously present in the lumen of the late endosome. EE = early endosome; LE = late endosome; arrowheads = gold particles in the early endosome; curved arrow = gold particles in the lumen of the late endosome. X 25,000.

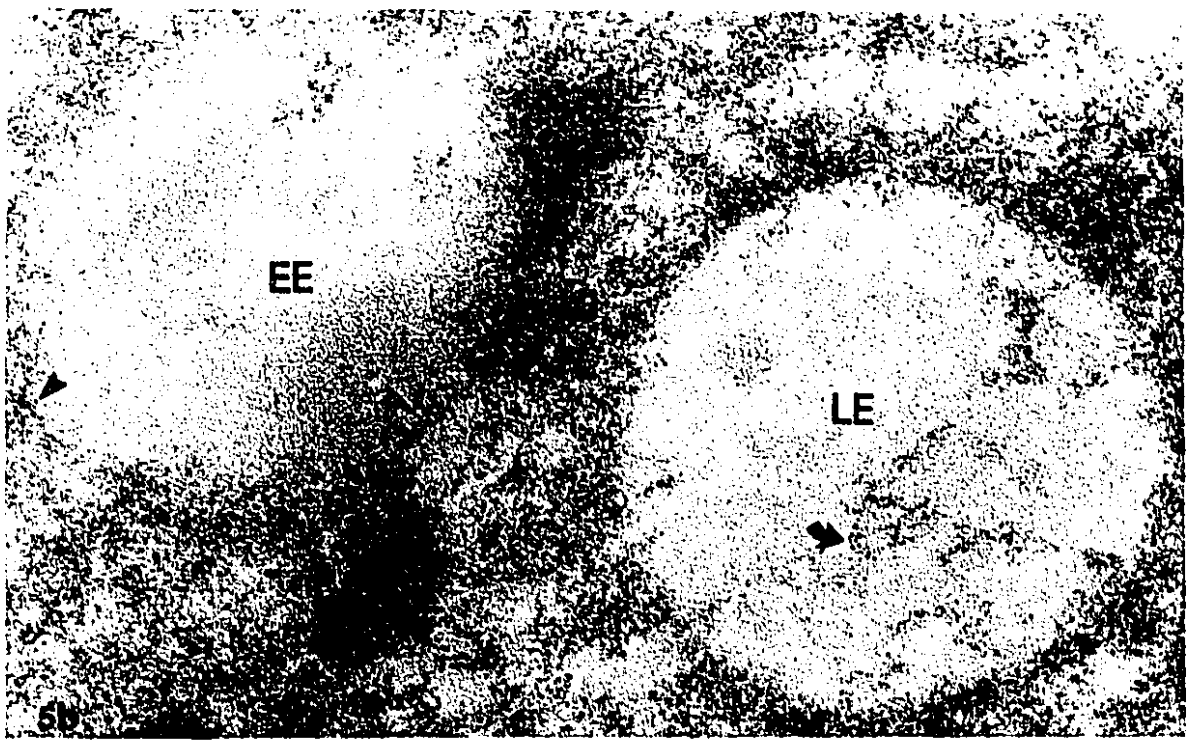

EE
LE
5b

FIGURE 6

Histogram representing SGP-1 labeling density in the microvilli of nonciliated cells subjected to different sugar treatments including galactose, mannose, mannose 6 -phosphate, N-acetylglucosamine, N-acetylgalactosamine, sialic acid, and glucose. The labeling density of microvilli subjected to each treatment was compared to that of a control group. Only sialic acid significantly reduced the labeling density of microvilli as determined by a one-way ANOVA test (with a p level of 0.05). * = caused a significant reduction in the labeling density.

SGP-1 LABELING DENSITY IN THE MICROVILLI UNDER DIFFERENT SUGAR TREATMENTS

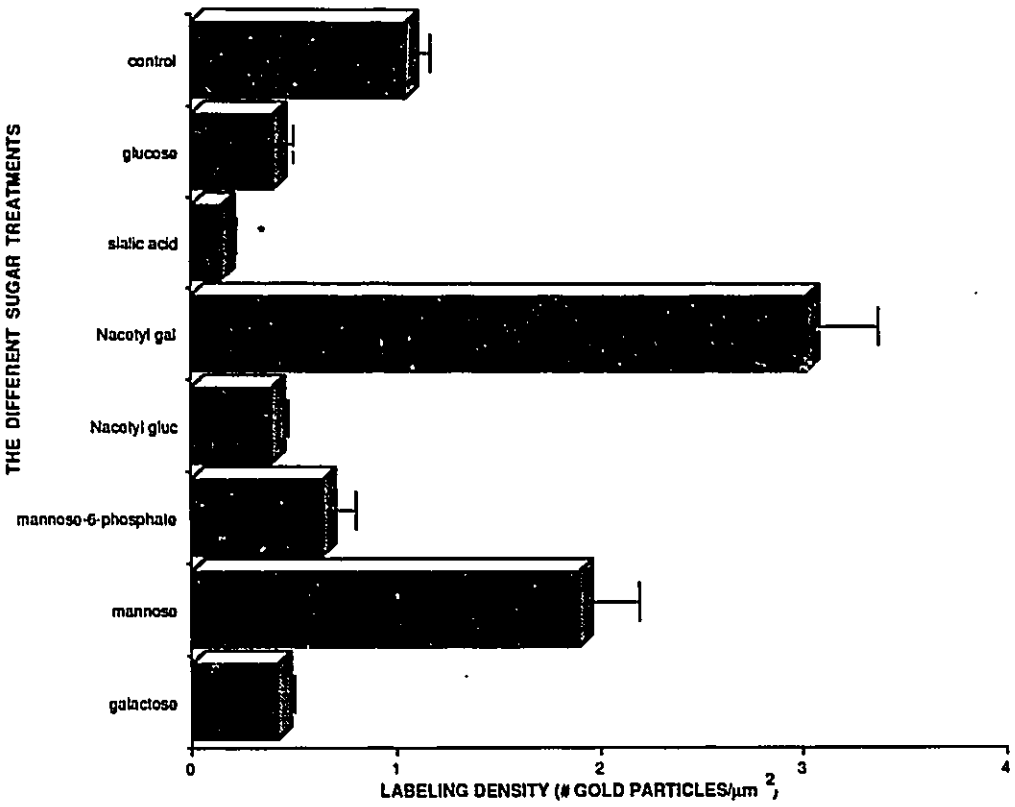


FIGURE 7

Electron micrograph showing microvilli of a nonciliated cell of the efferent ducts of a control (untreated) animal immunolabeled with anti-SGP-1. Gold particles appear in the lumen of the efferent duct and on the surface of microvilli. LU = lumen; curved arrow = gold particles in the lumen. X 25,000.

FIGURE 8

Electron micrograph showing microvilli of a nonciliated cell of the efferent ducts of an animal treated with 20 mM sialic acid, sacrificed 15 minutes later, and immunolabeled with anti-SGP-1. Gold particles appear in the lumen of the efferent duct. The labeling on the microvilli is greatly reduced in comparison to that seen in figure 7. LU = lumen; arrowhead = gold particles. X 22,000.

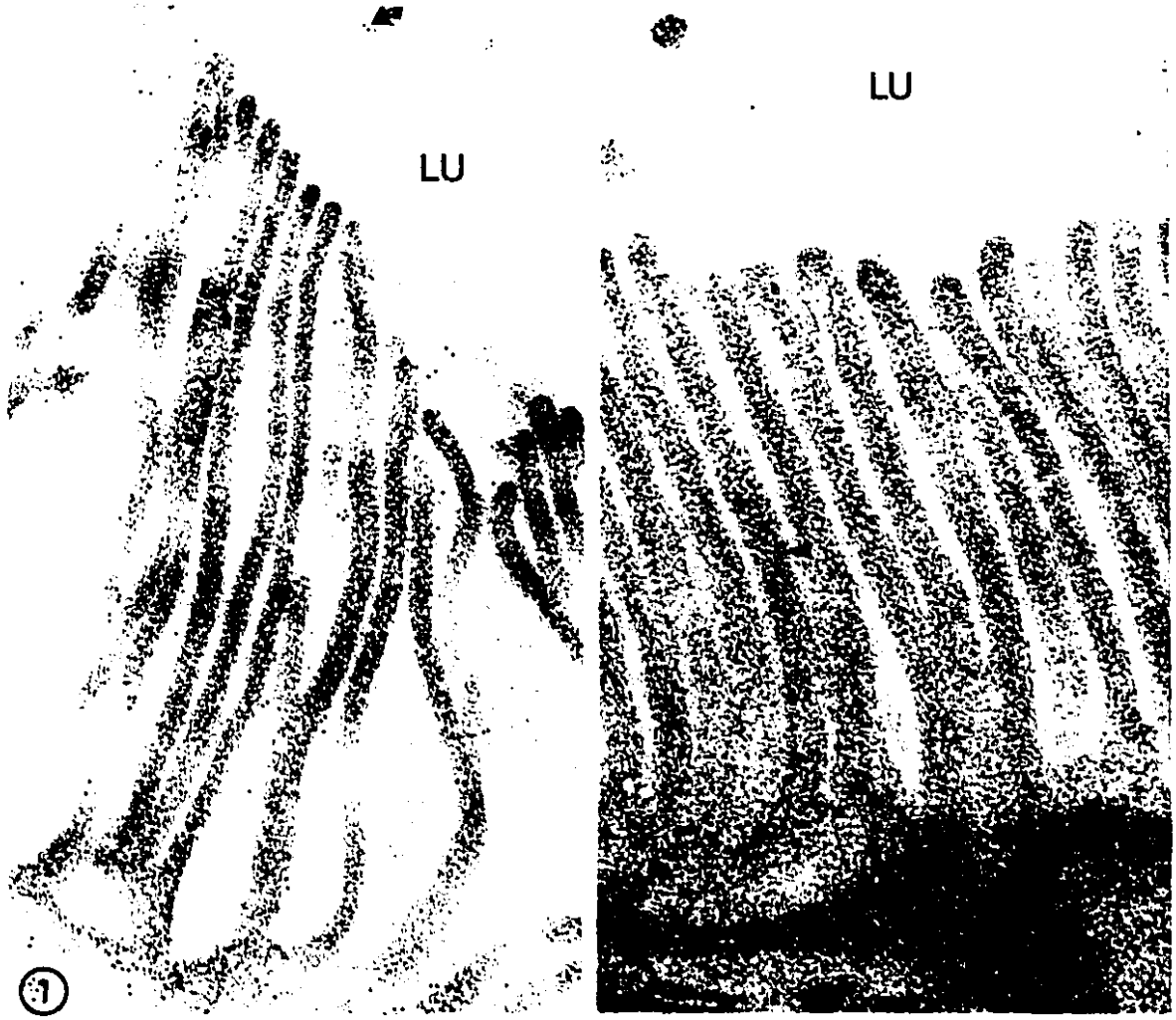

LU
LU
1

FIGURE 9

Histogram representing SGP-1 labeling density in the endocytic vesicles of nonciliated cells subjected to different sugar treatments including galactose, mannose, mannose 6 -phosphate, N-acetylglucosamine, N-acetylgalactosamine, sialic acid, and glucose. The labeling density of endocytic vesicles subjected to each treatment was compared to that of a control group. Glucose, sialic acid, N-acetylglucosamine, mannose 6-phosphate, mannose, and galactose were found to significantly decrease the labeling density of endocytic vesicles as determined by a one-way ANOVA test (with a p level of 0.05). * = caused a significant reduction in the labeling density.

SGP-1 LABELING DENSITY IN THE ENDOCYTIC VESICLES
UNDER DIFFERENT SUGAR TREATMENTS

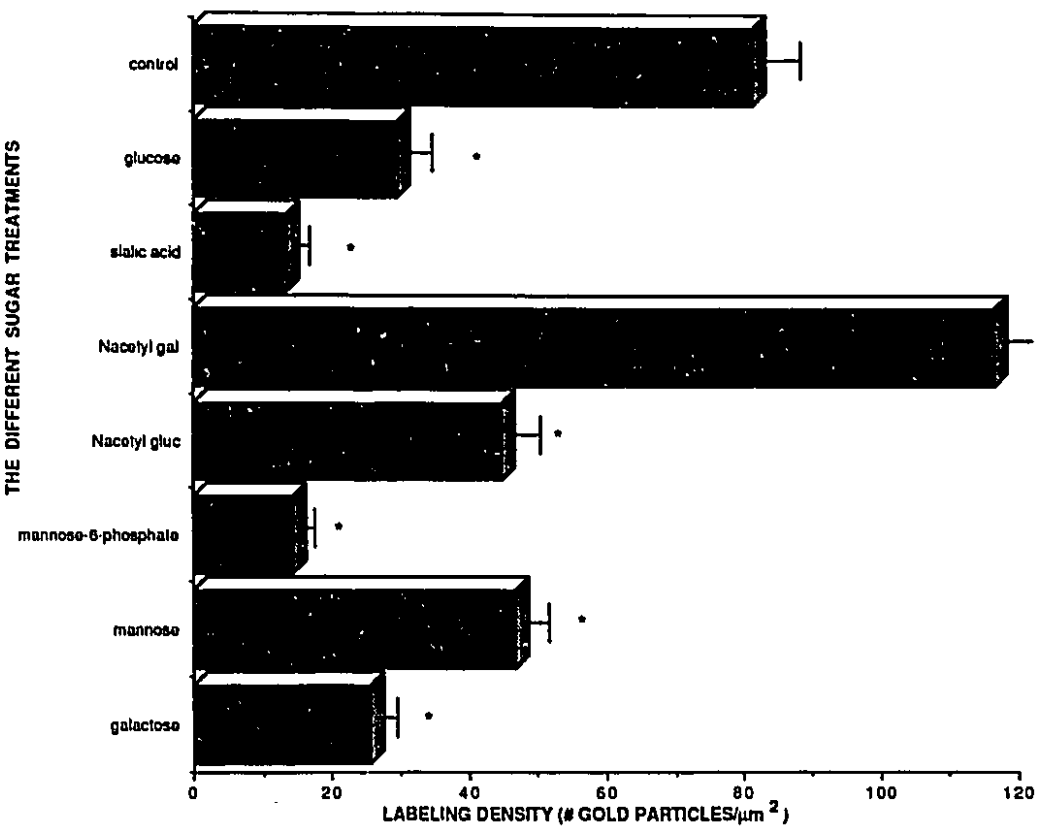


FIGURE 10

Electron micrograph of a portion of the apical region of a nonciliated cell of the efferent ducts of a control (untreated) animal immunolabeled with anti-SGP-1. Gold particles appear in the endocytic vesicles. * = endocytic vesicles. X 22,000.

FIGURE 11

Electron micrograph of a portion of the apical region of a nonciliated cell of the efferent ducts of an animal treated with 20 mM sialic acid, sacrificed 15 minutes later, and immunolabeled with anti-SGP-1. Fewer gold particles appear in the endocytic vesicles than are seen in figure 10. * = endocytic vesicles; arrowheads = gold particles in the endocytic vesicles. X 22,000.

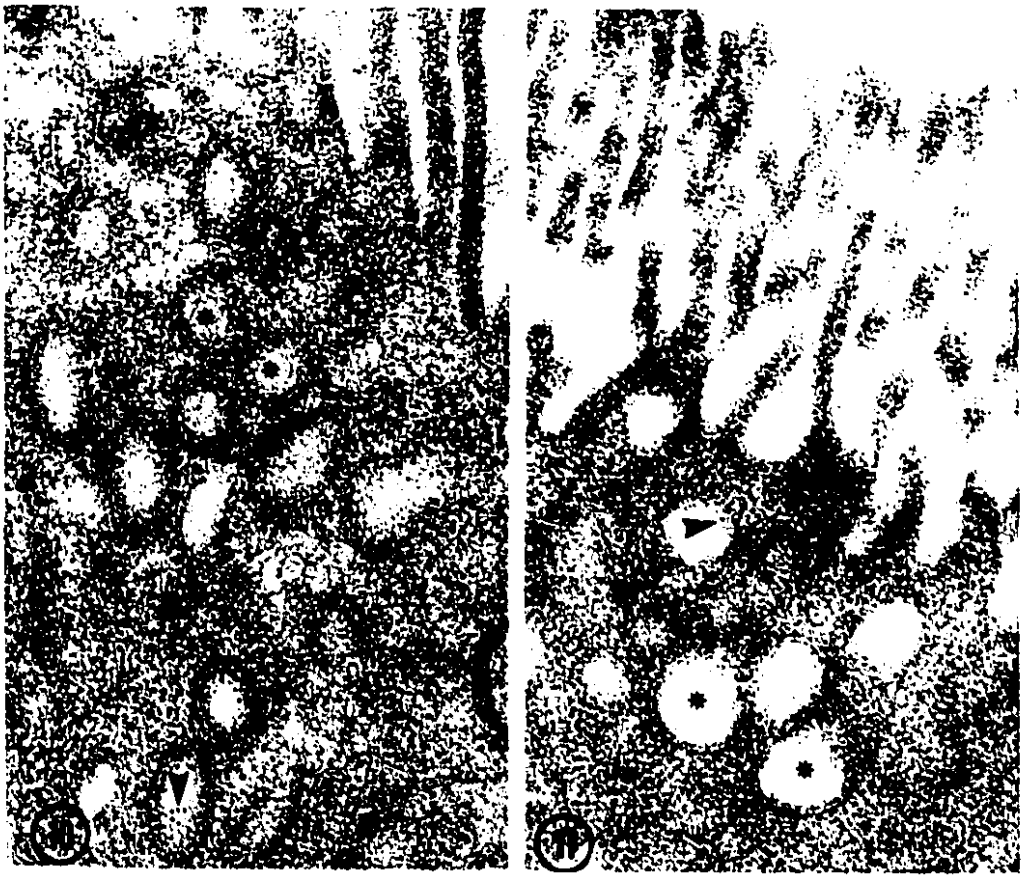


FIGURE 12

Histogram representing SGP-1 labeling density in the early endosomes of nonciliated cells subjected to different sugar treatments including galactose, mannose, mannose 6 -phosphate, N-acetylglucosamine, N-acetylgalactosamine, sialic acid, and glucose. The labeling density of early endosomes subjected to each treatment was compared to that of a control group. Glucose, sialic acid, N-acetylglucosamine, mannose 6-phosphate, mannose, and galactose were found to significantly decrease the labeling density of early endosomes as determined by a one-way ANOVA test (with a p level of 0.05). * = caused a significant reduction in the labeling density.

SGP-1 LABELING DENSITY IN THE EARLY ENDOSOMES
UNDER DIFFERENT SUGAR TREATMENTS

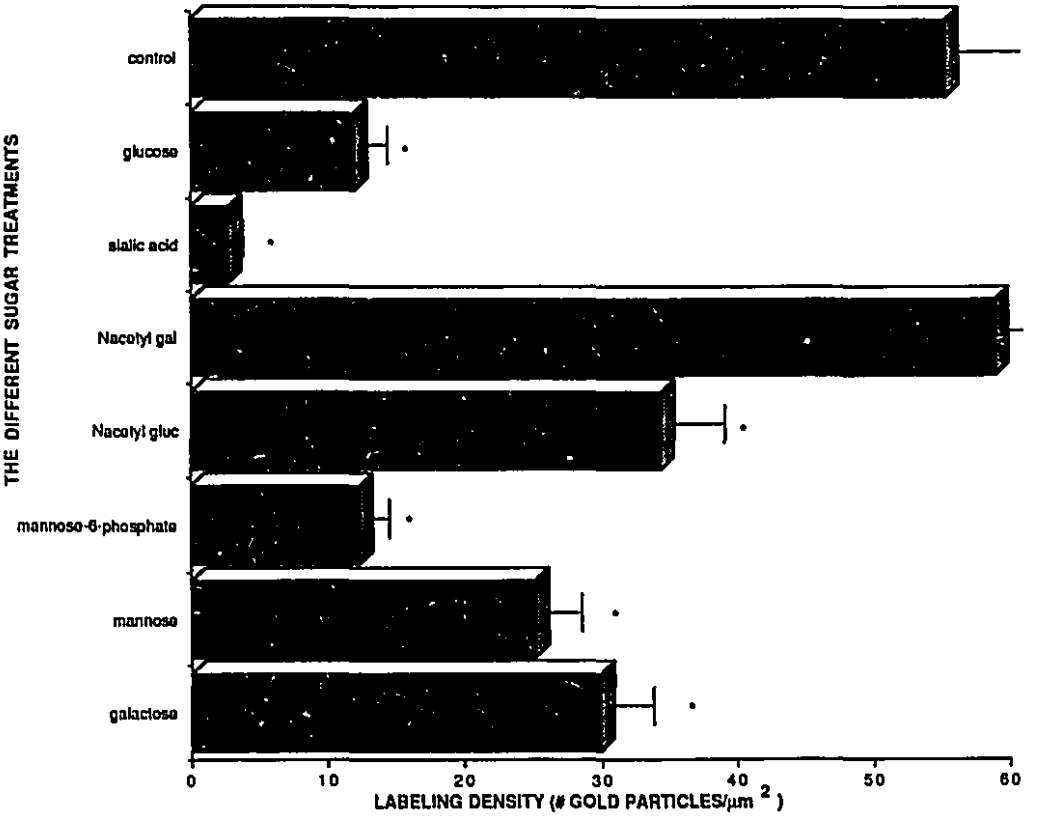


FIGURE 13

Histogram representing SGP-1 labeling density in the late endosomes of nonciliated cells subjected to different sugar treatments including galactose, mannose, mannose 6 -phosphate, N-acetylglucosamine, N-acetylgalactosamine, sialic acid, and glucose. The labeling density of late endosomes subjected to each treatment was compared to that of a control group. Glucose, sialic acid, and mannose 6-phosphate were found to significantly reduce the labeling density of late endosomes as determined by a one-way ANOVA test (with a p level of 0.05). * = caused a significant reduction in the labeling density.

SGP-1 LABELING DENSITY IN THE LATE ENDOSOMES
UNDER DIFFERENT SUGAR TREATMENTS

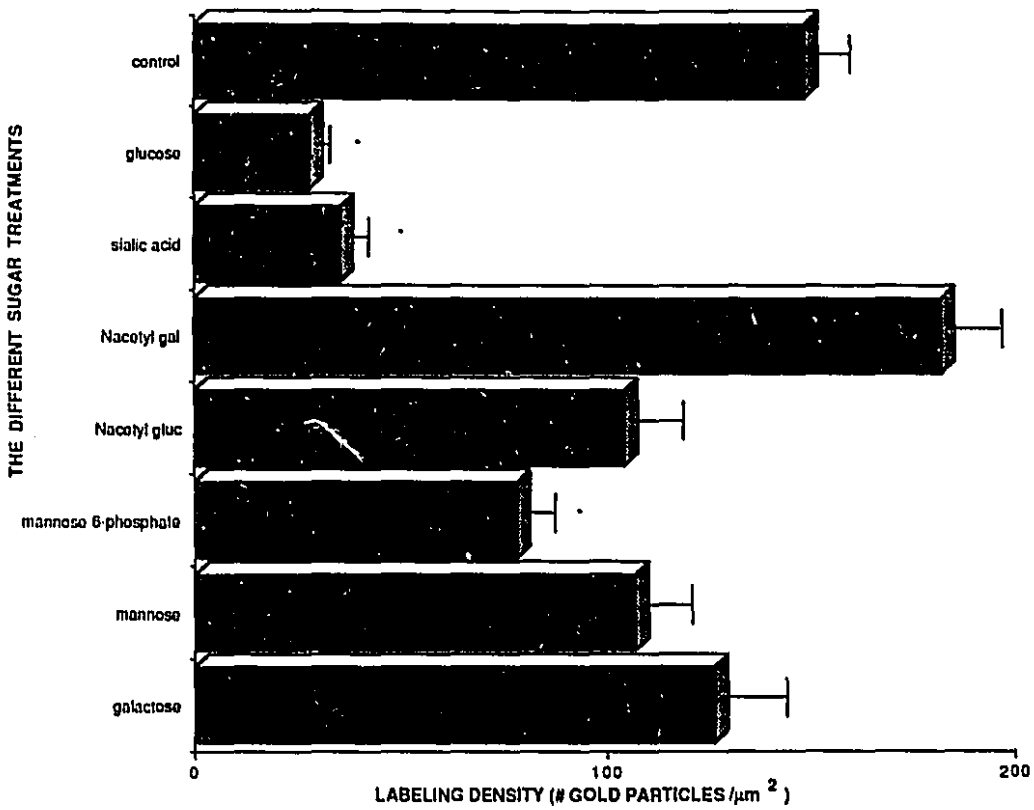


FIGURE 14

Electron micrograph of an early endosome in a nonciliated cell of the efferent ducts of a control (untreated) animal immunolabeled with anti-SGP-1. Gold particles are mostly seen at the periphery and in close association with the endosomal membrane. Acidification of the endosomre causes the protein to dissociate from its receptor, therefore accounting for the few gold particles seen in the center of the endosome. EE = early endosomes; arrowheads = gold particles. X 22,000.

FIGURE 15

Electron micrograph of an ealy endosome in a nonciliated cell of the efferent ducts of an animal treated with 20 mM sialic acid, sacrificed 15 minutes later, and immunolabeled with anti-SGP-1. The early endosome is very weakly labeled and contains few gold particles. EE = ealry endosomes; arrowheads = gold particles. X 22,000.

FIGURE 16

Electron micrograph of a late endosome in a nonciliated cell of the efferent ducts of a control (untreated) animal immunolabeled with anti-SGP-1. Gold particles appear scattered throughout the late endosome. LE = late endosomes. X 22,000.

FIGURE 17

Electron micrograph of a late endosome in a nonciliated cell of the efferent ducts of an animal treated with 20 mM sialic acid, sacrificed 15 minutes later, and immunolabeled with anti-SGP-1. Note the reduction of labeling in the late endosome compared to the control in figure 16. LE = late endosomes; arrowheads = gold particles. X 22,000.

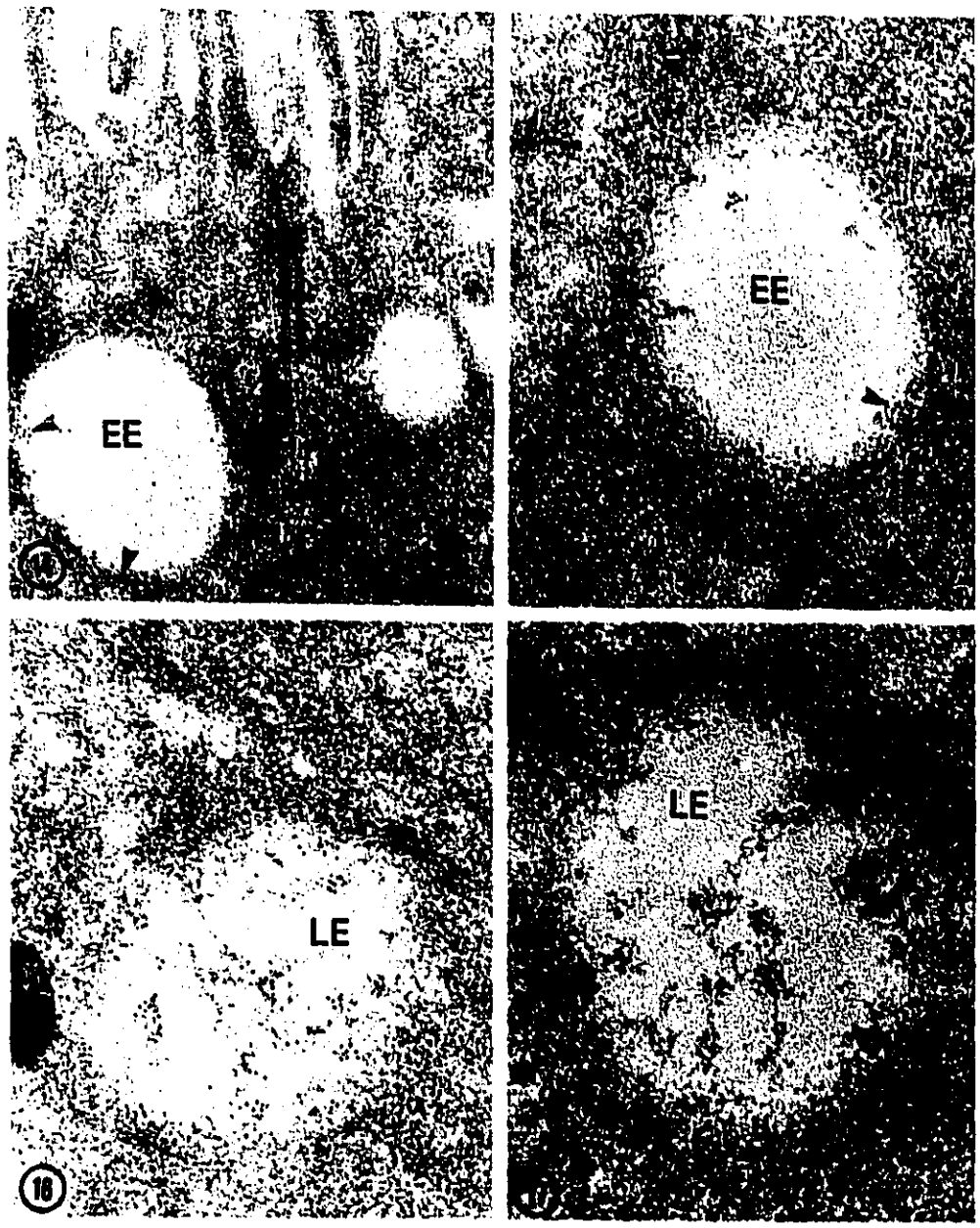

EE
EE
14
LE
LE
16

TABLE 1

The effect of the different sugar treatments on the SGP-1 labeling density in each endocytic compartments of the nonciliated cells. Sialic acid is the only sugar that causes a significant reduction in the labeling density of every endocytic compartment. * = caused a significant reduction in the labeling density.

TABLE 1. The Effect of Different Sugar Treatments on the Labeling Density, Representing Endocytosed SGP-1, in Various Endocytic Compartments of the Nonciliated Cells

	GALACTOSE	MANNOSE	MANNOSE-6-PHOSPHATE	NACETYL GLUCOSAMINE	NACETYL GALACTOSAMI	SIALIC ACID	GLUCOSE
ENDOCYTIC VESICLES	•	•	•	•		•	•
EARLY ENDOSOMES	•	•	•	•		•	•
LATE ENDOSOMES			•			•	•
MICROVILLI						•	

FIGURE 18

Histogram representing the SGP-1 labeling density in the endocytic vesicles of nonciliated cells subjected to different sialic acid concentrations, 0.02 mM, 0.2 mM, 2 mM, 20 mM, and 200 mM. The labeling density of endocytic vesicles subjected to each concentration of sialic acid was compared to that of a control group. Each of the sialic acid concentrations significantly reduced the labeling density of endocytic vesicles as determined by a one-way ANOVA test (with a p level of 0.05). * = caused a significant reduction in the labeling density.

SGP-1 LABELING DENSITY IN THE ENDOCYTIC VESICLES
UNDER DIFFERENT SIALIC ACID CONCENTRATIONS

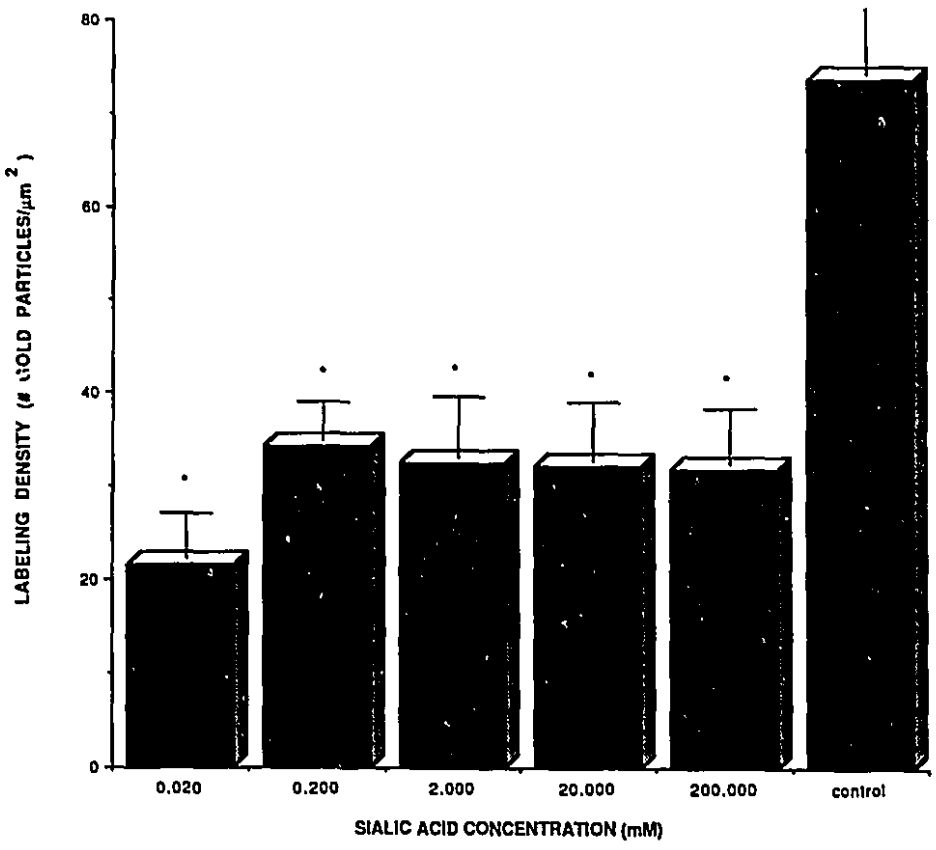


FIGURE 19

Electron micrograph of the apical region of a nonciliated cell of the efferent ducts of a control (untreated) animal immunolabeled with anti-SGP-1. Gold particles appear in the lumen of the efferent duct and in the endocytic vesicles. LU = lumen of the efferent duct; * = endocytic vesicles. X22,000.

FIGURE 20

Electron micrograph of the apical region of a nonciliated cell of the efferent ducts of an animal treated with 0.2 mM sialic acid, sacrificed 15 minutes later, and immunolabeled with anti-SGP-1. Compared to Figure 19, this figure shows a reduction in the number of gold particles in the endocytic vesicles. * = endocytic vesicles; arrowheads = gold particles on the membrane of the endocytic vesicle. X 22,000.

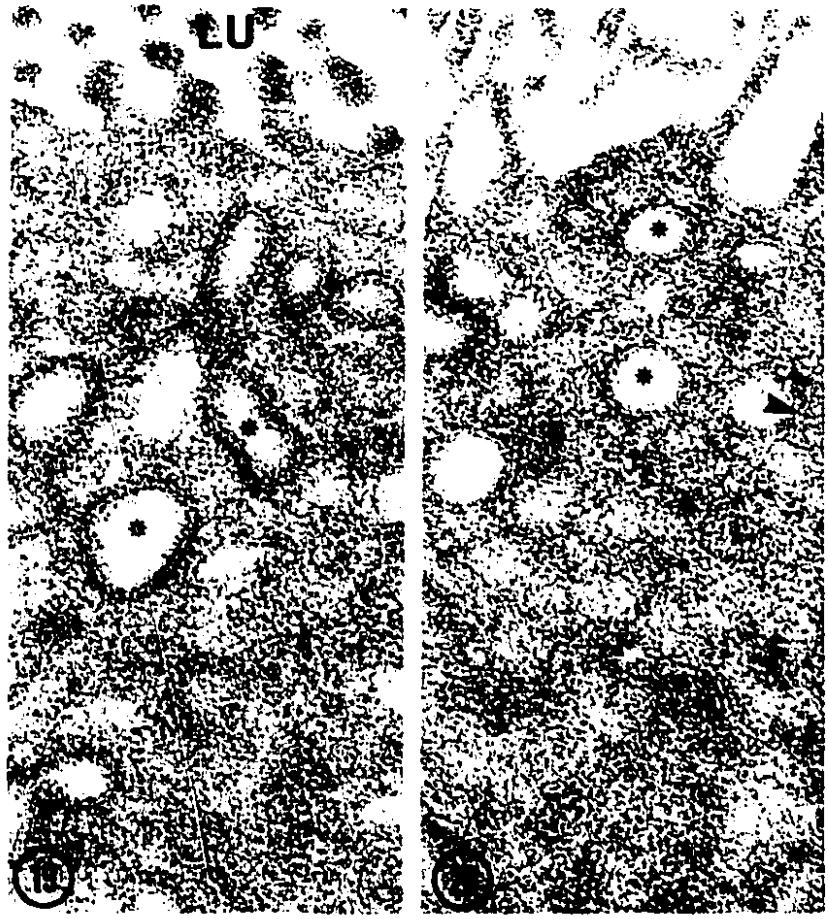

LU
(B)

FIGURE 21

Histogram representing SGP-1 labeling density in the early endosomes of nonciliated cells subjected to different sialic acid concentrations, 0.02 mM, 0.2 mM, 2 mM, 20 mM, and 200 mM. The labeling density of early endosomes subjected to each concentration of sialic acid was compared to that of a control group. Each of the sialic acid concentration significantly reduced the labeling density of early endosomes as determined by a one-way ANOVA test (with a p level of 0.05). * = caused a significant reduction in the labeling density.

SGP-1 LABELING DENSITY IN THE EARLY ENDOSOMES
UNDER DIFFERENT SIALIC ACID CONCENTRATIONS

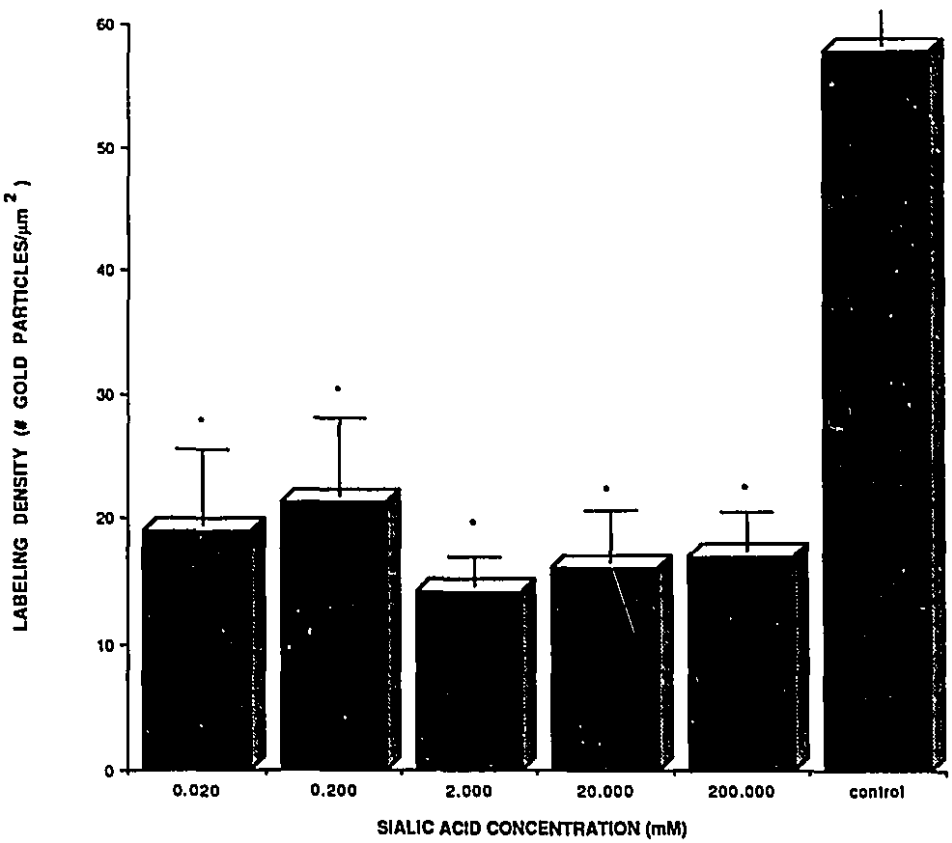


FIGURE 22

Histogram representing SGP-1 labeling density in the late endosomes of nonciliated cells subjected to different sialic acid concentrations, 0.02 mM, 0.2 mM, 2 mM, 20 mM, and 200 mM. The labeling density of late endosomes subjected to each concentration of sialic acid was compared to that of a control group. Only the 0.02 mM, 0.2 mM, and 20 mM concentrations significantly reduced the labeling density of late endosomes as determined by a one-way ANOVA test (with a p level of 0.05). * = caused a significant reduction in the labeling density.

SGP-1 LABELING DENSITY IN THE LATE ENDOSOMES
UNDER DIFFERENT SIALIC ACID CONCENTRATIONS

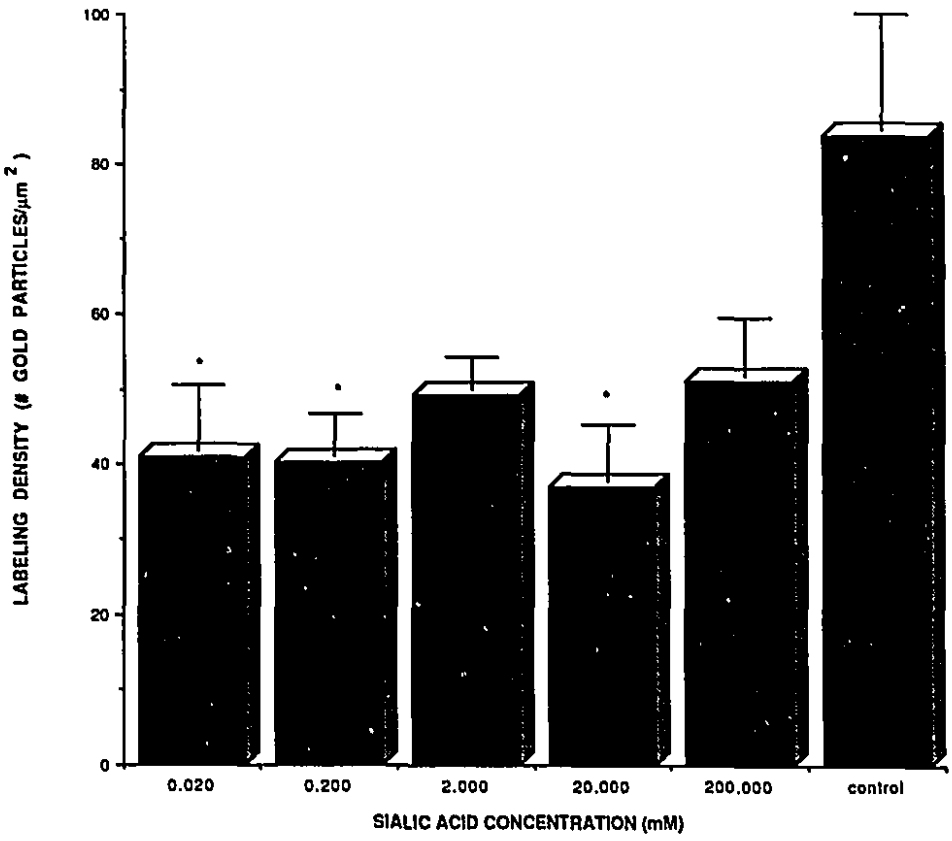


FIGURE 23

Electron micrograph of an ealy endosome in a nonciliated cell of the efferent ducts of a control (untreated) animal immunolabeled with anti-SGP-1. Gold particles are seen at the periphery, and in close association with the endosomal membrane, and in the center of the endosomal lumen. EE = early endosomes. X 25,000.

FIGURE 24

Electron micrograph of an early endosome in a nonciliated cell of the efferent ducts of an animal treated with 0.2 mM sialic acid, sacrificed 15 minutes later, and immunolabeled with anti-SGP-1. Compared to the control sample in Figure 23, this sample shows fewer gold particles in the early endosome. EE = early endosome; arrowheads = gold particles. X 25,000.

FIGURE 25

Electron micrograph of a late endosome in a nonciliated cell of the efferent ducts of a control (untreated) animal immunolabeled with anti-SGP-1. Gold particles are scattered over the lumen of the late endosome. LE = late endosome; curved arrow = a small cluster of gold particles, which may represent labeled vesicles derived from the Golgi apparatus. X 25,000.

FIGURE 26

Electron micrograph of a late endosome in a nonciliated cell of the efferent ducts of an animal treated with 0.2 mM sialic acid, sacrificed 15 minutes later, and immunolabeled with anti-SGP-1. Compared to the control sample in Figure 25, this sample has a reduced number of gold particles in the lumen of the late endosomes. LE = late endosome; arrowhead = gold particles. X 25,000.

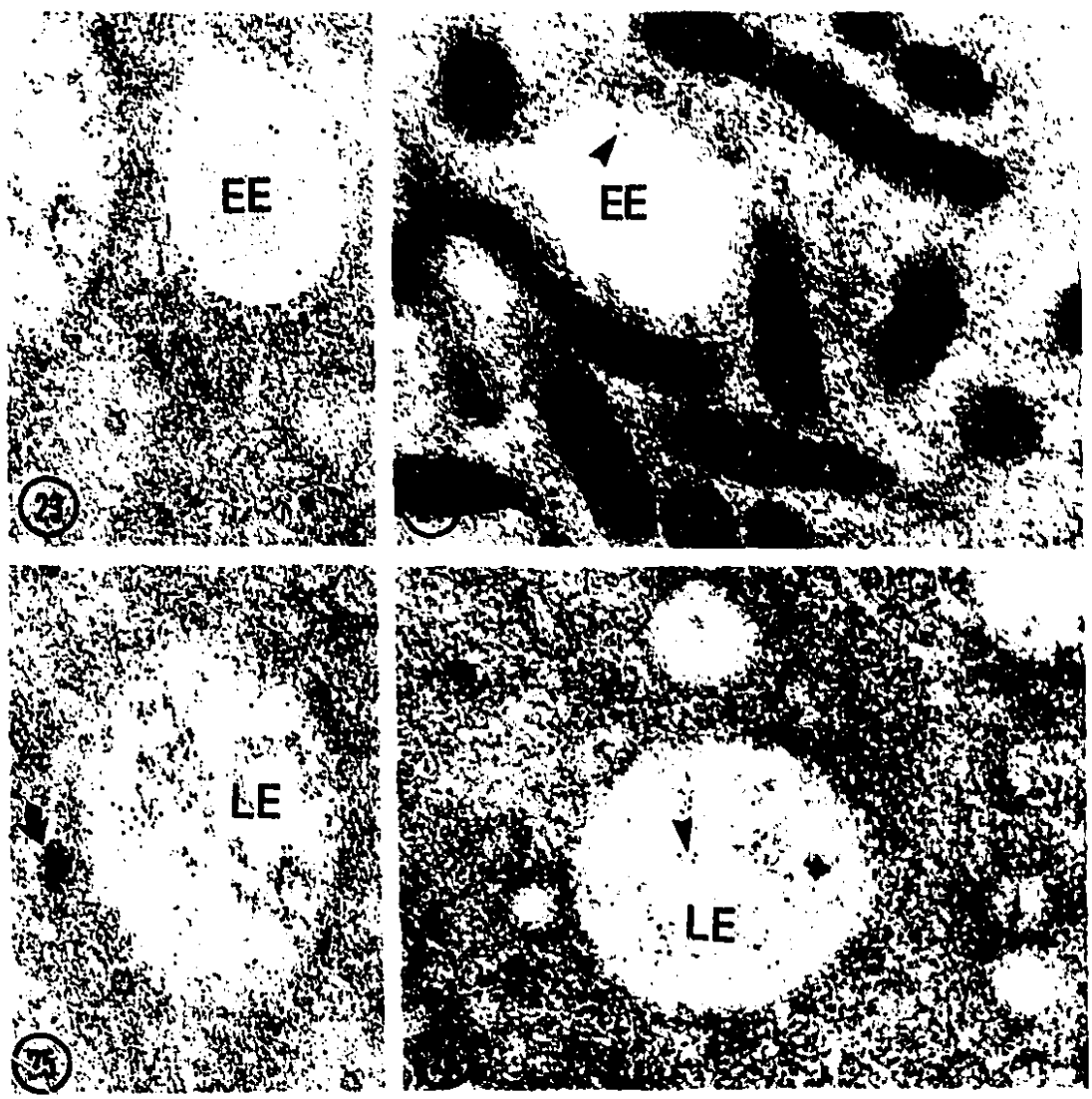

EE
EE
LE
LE

TABLE 2

The effect of different sialic acid concentrations on the SGP-1 labeling density in endocytic compartments including endocytic vesicles and early and late endosomes of the nonciliated cells. * = caused a significant reduction in the labeling density.

TABLE 2. The Effect of Different Sialic Acid Concentrations on the Labeling Density, Representing Endocytosed SGP-1, in Various Endocytic Compartments of the Nonciliated Cells

	0.02 mM	0.2 mM	2 mM	20 mM	200 mM
ENDOCYTIC VESICLES	•	•	•	•	•
EARLY ENDOSOMES	•	•	•	•	•
LATE ENDOSOMES	•	•		•	

FIGURE 27

High performance liquid chromatography (HPLC) elution profile of immunourified testicular SGP-1 (t-SGP-1). After a 10-min run at 100% mobile phase A (20% acetonitrile and 0.1% trifluoracetic acid), absorbed molecules were eluted using a 40-min linear gradient of 0% to 100% mobile phase B (80% acetonitrile and 0.1% TFA) and wavelenghts of 280 nm (absorbance A) and 254 nm (absorbance B). The profile reveals a major peak, which showed immunoreactivity with the antibody to SGP-1 upon Western blot analysis (figure 28).

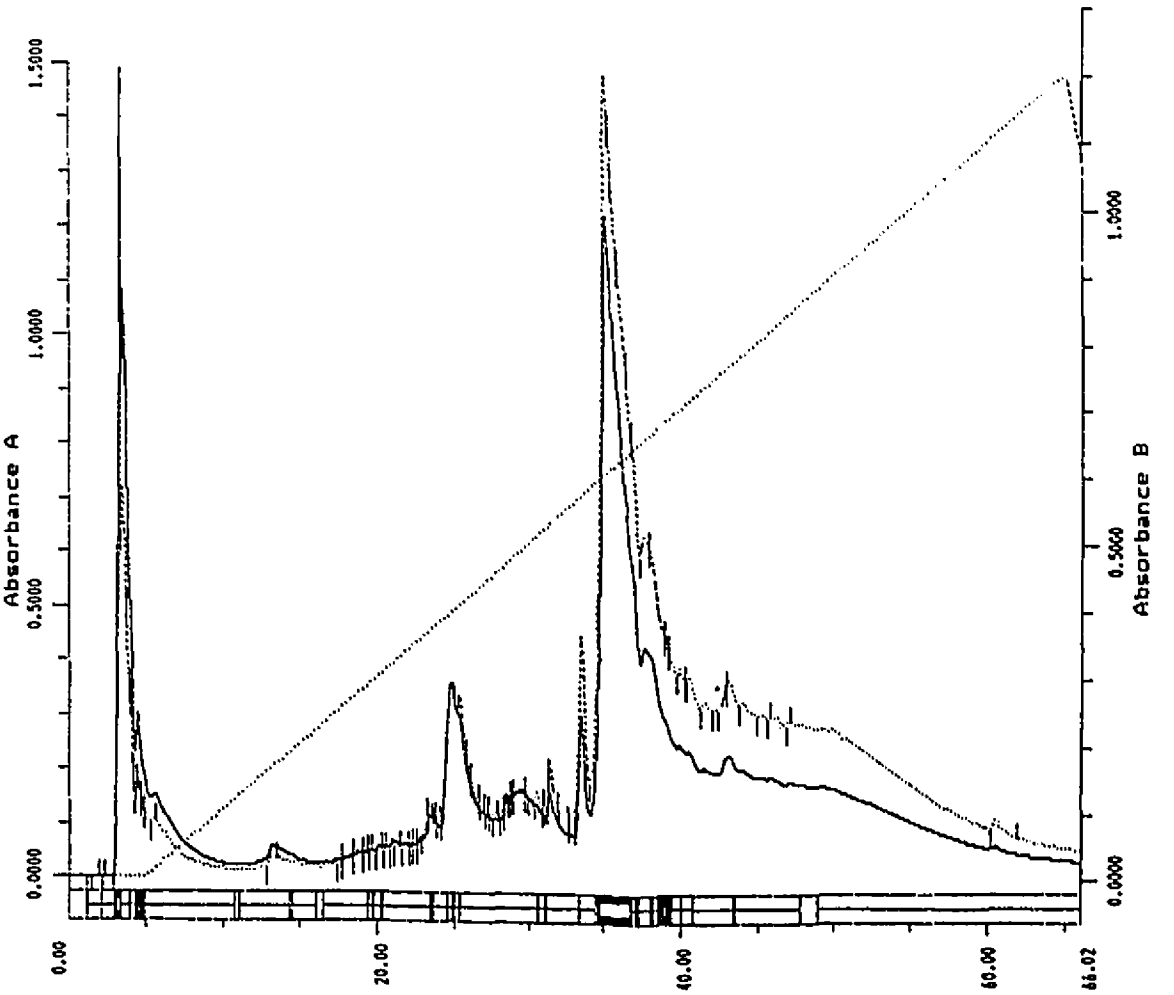

Absorbance A
1.5000
1.0000
0.5000
0.0000
Absorbance B
1.0000
0.5000
0.0000
0.00
20.00
40.00
60.00
66.02

FIGURE 28

Western blot analysis of testicular SGP-1 after affinity chromatography and HPLC. The anti-SGP-1 antibody recognizes the 70 KDa protein.

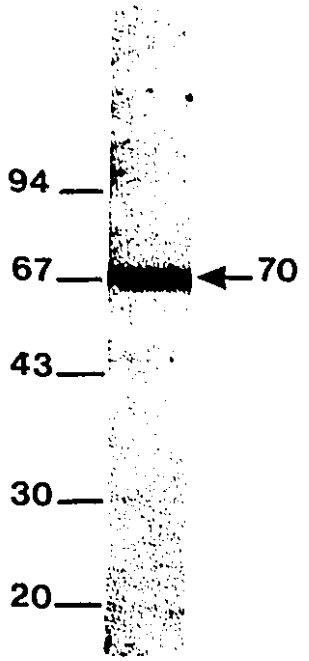
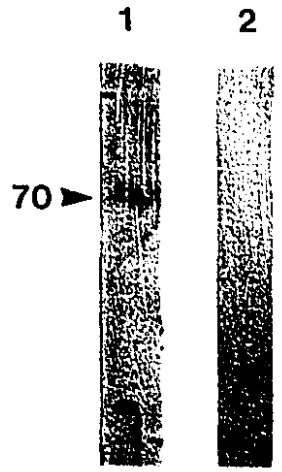

94
67
70
43
30
20

FIGURE 29

Western blot analysis of testicular SGP-1 (t-SGP-1) after affinity chromatography and HPLC. T-SGP-1 showed reactivity to lectin solution sambucus nigra agglutinin (SNA), which recognizes sialic acid linked α(2-6) to galactose or N-acetylgalactosamine (lane 1). However, t-SGP-1 did not show reactivity to lectin solution maackia amurensis agglutinin (MAA), which recognizes sialic acid linked α(2-3) to galactose (lane 2).


1
2
70

DISCUSSION

Sulfated glycoprotein-1 (SGP-1), which exists as a lysosomal protein (Collard et al., 1988), is also secreted by rat Sertoli cells in the lumen of the seminiferous tubules and is eventually endocytosed by the nonciliated cells of the efferent ducts (Sylvester et al., 1989; Hermo et al., 1992; Igdoura et al., 1993). The 70KDa SGP-1 found in the lumen of seminiferous tubules has been shown to possess substantial sequence similarity to human prosaposin (O'Brien et al., 1988). When proteolytically cleaved, prosaposin gives rise to four smaller proteins, which are referred to as sphingolipid activator proteins (SAPs) A, B, C, and D (Morimoto et al., 1989; O'Brien and Kishimoto, 1991). It is not known whether the 70 KDa form of SGP-1 is proteolytically processed into four smaller activator proteins.

Saposins, which are small heat-stable proteins with an Mr of around 10,000, promote the degradation of glycolipids by solubilizing these substances and permitting them to be hydrolyzed by lysosomal hydrolases (Ho et al., 1971; Fisher et al., 1978; O'Brien et al., 1988; O'Brien et al., 1991; Hiraiwa et al., 1992). Saposin A has been characterized as a b-glucosidase activator protein (Morimoto et al., 1989). Saposin B is known as the activator protein of arylsulfatase A, β-galactosidase, and a-galactosidase (O'Brien et al., 1988). Saposin C is characterized as the β-glucosidase activator (Kleinschmidt et al., 1987), and saposin D is a sphingomyelinase activator protein (Morimoto et al., 1988). Saposin B deficiency causes a lysosomal storage disease known as

metachromatic leukodystrophy (Stevens et al., 1981; Inui et al., 1983; Hyde et al., 1992). Saposin C deficiency results in Gaucher's disease (Christomanou et al., 1986; Wenger et al., 1991).

After light and electron microscopic immunocytochemistry studies (Hermo et al., 1992; Sylvester et al., 1989), SGP-1 was localized to the middle and basal region of Sertoli cells. This finding is consistent with results presented by Griswold (1988) and by Skinner et al. (1980), who showed that SGP-1 is a major glycoprotein secreted by Sertoli cells. SGP-1 was also found in the endocytic apparatus of cells of the rete testis and of nonciliated cells of the efferent ducts, as well as on tails of spermatozoa. The cells of the epididymis sowed no reaction to SGP-1 in the early endocytic apparatus, but a weak reaction was observed in the multivesicular bodies and lysosomes. Western blot analysis showed that SGP-1 exists in the lysosomes as a 65 KDa protein and as smaller 15 KDa proteins derived from the 65 KDa protein; it is also found in the testicular fluids as a secreted form of 70KDa MW (Sylvester et al., 1989; Igdoura and Morales, 1994). Northern blot analysis revealed a 2.6Kb band corresponding to the mRNA of SGP-1 in the epididymis, efferent ducts, and testis (Hermo et al., 1992). These data suggest that SGP-1 is synthesized in the Sertoli cell and that it can take one of two pathways, a lysosomal or a secreted route. Once secreted, SGP-1 binds to the tails of spermatozoa and eventually dissociates from them, to be endocytosed by the cells of the rete testis and of the efferent ducts. Between the third and fourth domains of SGP-1 is a proline-rich region characteristic of secretory proteins (Collard et al., 1988). The latter finding may explain the existence of a secreted form of SGP-1.

The function of secreted SGP-1 is not yet well established, but this substance may play a role in the seminiferous tubules and efferent ducts. It may modify the spermatozoa plasma membrane by serving as a glycolipid transfer protein or an activator of glycolipid hydrolysis (Collard et al., 1988; Griswold, 1988). Nikolopoulo et al. (1985) studied the changes in the lipid content of the spermatozoa plasma membrane and found that, as the spermatozoa developed fertilizing capacity in the epididymis and as various morphological, physiological, and biochemical changes occurred (Yanagimachi et al., 1981), there was a decrease in sperm lipid content. This decrease has been ascribed to the utilization of lipids as energy sources. Because SGP-1 is homologous to prosaposin, which gives rise to sphingolipid activator proteins involved in the degradation of glycolipids, it may be implicated in the hydrolysis of plasma membrane glycolipids of spermatozoa as they mature through the excurrent duct.

1) Endocytosis of SGP-1 via Sialic Acid Residues

Newly synthesized glycoproteins spend a variable amount of time in the rER (Pelham, 1989) and are then translocated to the Golgi apparatus for further glycosylation and oligosaccharide modification (Farquhar et al., 1991). From the Golgi apparatus, they either are delivered to the lysosomes or are secreted via constitutive or regulated secretory pathways (Varki, 1993). In a few instances, the oligosaccharide modifications on glycoproteins have been shown to direct their subcellular traffic (Varki, 1993). Figure 30 shows the

common subcellular pathways that are followed by glycoproteins and various modifications of N-linked oligosaccharides.

Sugar chains play a considerable role in the trafficking of glycoproteins, especially in the uptake of these substances. The diversity and complexity of the carbohydrate units of glycoproteins suggest that they are rich in information and are functionnally important (Stryer, 1988). In almost all systems, a soluble glycoprotein is picked up by a membrane-bound lectin receptor via recognition of the oligosaccharide chains of the glycoprotein. The complex is then endocytosed in membrane-bound vesicles and is targeted to the lysosmes (Varki,1993). This study focuses on the identity of the terminal sugar essential for the endocytosis of SGP-1 by the nonciliated cells of the efferent ducts. To identify the terminal sugar on SGP-1, we tested different sugars including galactose, mannose, mannose 6-phosphate, N-acetylglucosamine, N-acetylgalactosamine, sialic acid, and glucose. Each sugar solution was infused into the lumen of the rete testis in order to study its effect in the efferent ducts. Quantitative electron microscopic analysis was performed, and one-way ANOVA was used, with p values set at 0.05.

In Experiment 1, sialic acid was the only sugar that produced a significant decrease in the labeling density in the microvilli. In the endocytic vesicles, other sugars produced a significant reduction, but sialic acid had the greatest effect. Similar results were observed in the early endosomes. However, in the late endosomes, even though sialic acid did produce a significant decrease, glucose was the sugar that caused the greatest decrease in the labeling density.

In Experiment 2, 0.02 mM, 0.2 mM, 2 mM, 20 mM, and 200 mM sialic acid produced a significant decrease in the labeling density in the endocytic vesicles and early endosomes; in the late endosomes, however, only the 0.02 mM, 0.2 mM, and 20 mM concentrations produced a significant effect. The increase in sialic acid concentration did not induce a linear decrease in the labeling density in each endocytic compartment; the concentrations of sialic acid were probably too high, and a plateau was already reached. If concentrations lower than 0.02 mM had been chosen, a linear decrease in the labeling density would have been observed.

The Western blot revealed that SGP-1 has sialic acid residues that are terminally linked α(2-6) to galactose or N-acetylgalactosamine residues.

From these findings, it can be concluded that sialic acid is the terminal sugar of SGP-1 that is essential for its endocytosis. Crocker and Gordon (1986) described a lectin-like receptor (originally named sheep erythrocyte receptor, or SER) which is now named sialoadhesin. This substance is expressed in mouse macrophages in the bone marrow (Crocker and Gordon, 1986). It is a 175-185 KDa monomer (Crocker and Gordon, 1989), which binds to sialylated ligands present on developing hematopoietic cells and, through this interaction, influences their growth and differentiation (Crocker et al., 1988). Sialoadhesin exhibits specificity for the terminal oligosaccharide sequence Neu5Acα2-3Galβ1-3GalNAc in sialoglycoproteins and gangliosides (Crocker et al., 1991). These findings and the results of Experiments 1, 2, and 3 indicate that SGP-1 may possess terminal sialic acid residues that are essential for binding to a sialic acid receptor, probably homologous to sialoadhesin. In Figure 30, SGP-1

may adopt the pathway that would give rise to structure J or K, where the terminal sugar residue on the oligosaccharide chain is sialic acid.

2) Endocytosis of SGP-1 via Sialic Acid and Mannose 6-Phosphate Residues

On the other hand, in Experiment 1, mannose 6-phosphate caused a significant decrease in the labeling density in the endocytic vesicles and early endosomes (>75%) and in the microvilli (47%). Experiment 2 showed that even the highest concentration of sialic acid (200 mM) did not completely abolish the endocytosis of SGP-1, therefore suggesting that other receptors are involved in the uptake of SGP-1. Thus, mannose 6-phosphate may also be essential for binding to the receptor.

A major proportion of the mannose 6-phosphate receptor directs newly synthesized enzymes from the Golgi apparatus to the late endosomes; nevertheless, a minor proportion of this receptor is found on the plasma membrane, and follows an endocytic route similar to that of the carbohydrate specific receptors (Rinjboutt et al., 1991). Therefore, this minor proportion of the mannose 6-phosphate receptor may play a substantial role in the endocytosis of SGP-1 and may account for the residual amount of SGP-1 that is bound and taken up when challenged with sialic acid. Rinjboutt et al. (1991) demonstrated that the 73 KDa form of saposin, which corresponds to the 70 KDa form of SGP-1, acquires mannose 6-phosphate residues and that 50% of saposin can be translocated to the lysosomes by the mannose 6-phosphate receptor. The

other 50% is targeted to the lysosomes via another mechanism that is yet unknown. These researcher's findings (1991) and the results obtained in Experiments 1, 2 and 3 indicate that SGP-1 may follow the pathway giving rise to structure F (Fig. 30), thereby acquiring a terminal sialic acid residue and a phosphomannosyl recognition marker. This would explain why both sialic acid and mannose-6-phosphate may have a substantial effect on the endocytosis of SGP-1.

3) Role of N-linked Oligosaccharides in the Endocytosis of SGP-1

Furthermore, because galactose and N-acetylglucosamine also significantly affected the labeling density in some cases and because the blot was reactive to sambucus nigra agglutinin (SNA), which characterizes terminal sialic acid linked to galactose, it is reasonable to state that the entire sugar chain plays a more important role than any one sugar in particular. As Figure 30 shows, SGP-1 may adopt the pathway of structures C, D, E, and F and receive terminal sialic acid residues linked to galactose and N-acetylglucosamine and, on the other chain, a mannose 6-phosphate residue. A protein's oligosaccharide chain probably defines that protein's conformation and facilitates its recognition by the receptor (Varki, 1993). Therefore, in this case, the sugar chain may direct the folding of SGP-1 essential for binding to the receptor; the terminal sugar postulated to be sialic acid may play a substantial role in the actual binding to the receptor, because it produced a greater decrease than all the other sugars.

4) Endocytosis of SGP-1 via Multiple Receptors

Additionally, it has been shown that multiple receptors can participate in the recognition and uptake of glycoproteins. Neoglycoproteins with the terminal sequence Galβ1-4(Fuc α1-3)GlcNAcβ- are recognized by both the asialoglycoprotein receptor and the mannose/fucose receptor of liver cells. Moreover, asialotransferrin is recognized by both the transferrin and the asialoglycoprotein receptors (Stryer, 1988). Therefore, because many sugars affect the endocytosis of SGP-1, this substance may be recognized by multiple receptors including a putative sialic acid receptor, the mannose 6-phosphate receptor, or a receptor that recognizes part or all of the sugar chain.

5) Proposed Function for SGP-1

An alternative explanation is based on data obtained and discussed by Potier et al. (1988, 1990b), who studied the similarity between lysosomal prosaposin and influenza neuraminidase. Potier (1988) reported that lysosomal prosaposin binds to a glycoconjugate affinity column containing terminal sialic acid residues (fetuin-agarose) and is specifically eluted by free N-acetylneuraminic acid. They demonstrated that prosaposin has the capacity to bind to sialic acid (1990b) but lacks the neuraminidase activity that hydrolyzes sialic acid substrates (Paton et al., 1994; Hiraiwa et al., 1992). In studying the structural and functional relationship between neuraminidase and

prosaposin, they observed that saposin B, which is characterized as a sialic acid binding domain, had similarities with β3 and β4 sheets of the influenza neuraminidase (Potier, 1988). To further substantiate their data, they showed that alignment of the amino acid sequence of influenza neuraminidase, prosaposin, and SGP-1 produced an overall homology (Potier, 1988). This evidence suggests that the viral and mammalian proteins share a common ancestor. It also suggests, based on substantial results, that active human neuraminidase (which is a 60 KDa enzyme) is derived from prosaposin through the proteolytic removal of a 10 KDa peptide, which is thought to be the C-terminal end of prosaposin (Potier et al., 1990a). However, a study done by Paton et al. (1994) using cultured fibroblasts of patients with a prosaposin deficiency, showed that neuraminidase activity is not impaired. Furthermore, when fibroblasts from patients with a genetic deficiency of the lysosomal sialidase were used, the biosynthesis and processing of prosaposin was normal. Thus, it is now clear that neuraminidase does not derive from prosaposin.

Hiraiwa et al. (1992) demonstrated that prosaposin binds tightly to gangliosides, which are enriched in neural membranes, and that it transfers gangliosides from donor liposomes to acceptor membranes. These researchers proposed that prosaposin may act as a ganglioside binding and transporting protein *in vivo*. They showed that gangliosides with the greatest number of sialic acid residues had the highest binding affinity for prosaposin; that gangliosides of the "a" series (with terminal sialic acid residues) were bound more avidly than gangliosides of the "b" series (without sialic acids) by

approximately a factor of 3; and that when sialic acid residues were acetylated, the binding affinity was reduced. It is not clear what precise function of SGP-1 may require a sialic acid-binding activity. Nevertheless, this property may be important for the transport of glycolipids or for the recognition of membrane glycolipids of specific organelles such as the lysosomes.

Based on the evidence that SGP-1 is capable of binding to gangliosides (Hiraiwa et al., 1992) and that SGP-1 is the precursor of lysosomal saposins (Igdoura and Morales, 1994), whose function is to hydrolyze glycolipids, it can be proposed that (Fig. 31):

1) SGP-1 is secreted in the seminiferous lumen by the Sertoli cells.

2) In the seminiferous lumen, SGP-1 binds to glycolipids on the plasma membrane of the tail of spermatozoa. The binding is mediated by the sialic acid binding domain found on SGP-1.

3) The lipoprotein complex (glycolipid-SGP-1) is removed from the spermatozoa when SGP-1 is recognized, via its terminal sialic acid residues, by a receptor found on the apical plasma membrane of nonciliated cells of the efferent ducts.

4) The lipoprotein complex is endocytosed by the nonciliated cells of the efferent ducts.

5) In the endosome, due to the low pH, the lipoprotein complex dissociates from itself and from the receptor.

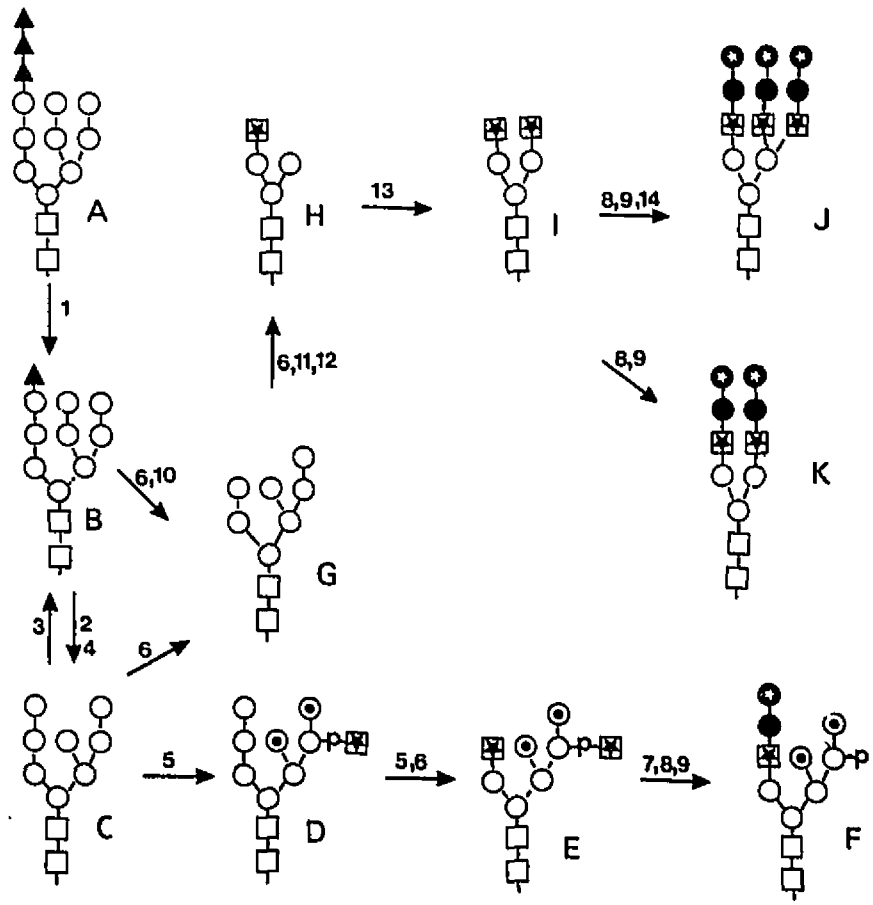
6) In the lysosome, the glycolipids are degraded, and SGP-1 is either degraded or processed into smaller proteins that may act as sphingolipid activator proteins.

In conclusion, SGP-1 may play a double role in the male rat reproductive system. It may assist in the lipid modification of spermatozoa plasma membrane by acting as a glycolipid binding and transport protein. In the lysosome, it may act as a sphingolipid activator protein.

FIGURES AND LEGENDS

FIGURE 30

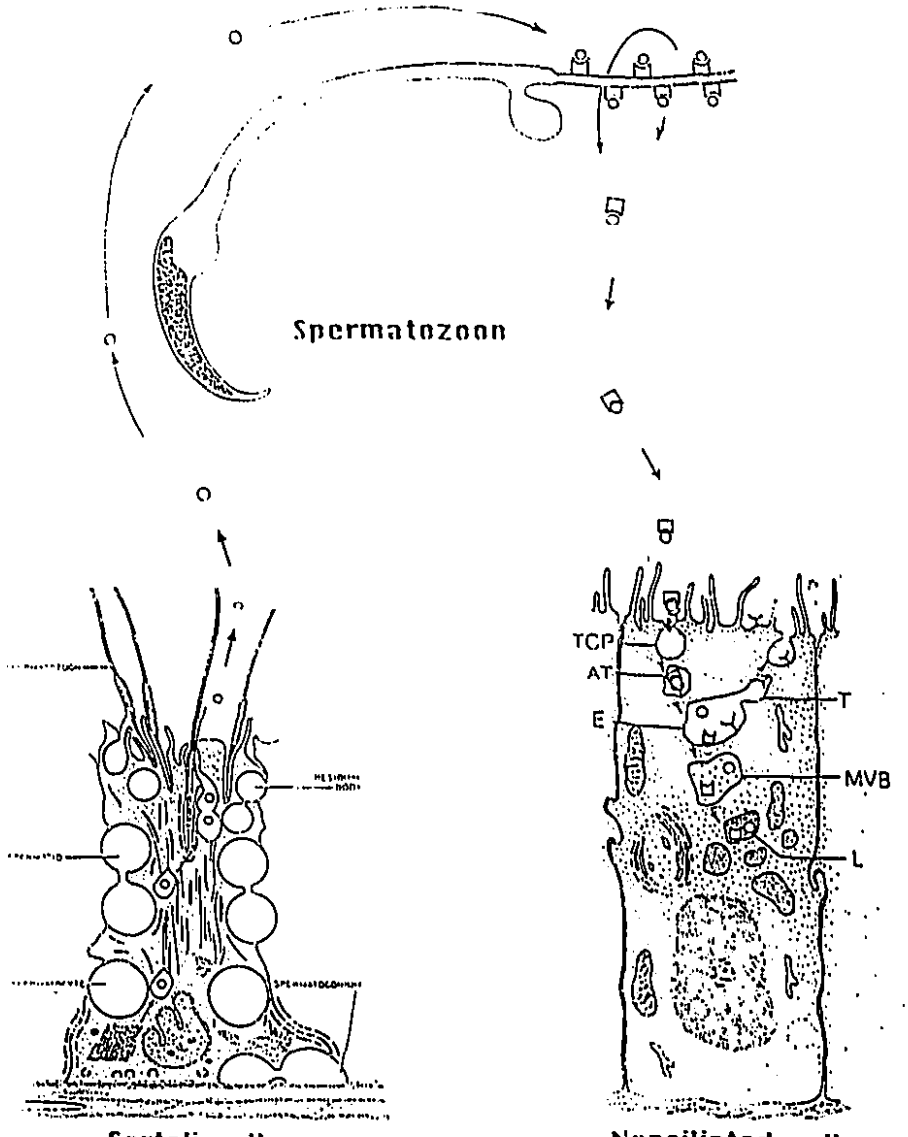
Some pathways for the processing of N-linked oligosaccharides. Structure A, with three glucose residues, nine mannose residues, and two N-acetylgalactosamine residues, is transferred en bloc to an N-asparginyl linkage on newly synthesized glycoproteins. The glucose residues are removed by the sequential action of glucosidase I and II (structures B and C). Structure C is further modified to structure D by the addition of a single N-acetylglucosamine phosphodiester (GlcNAc P) to one of three mannose residues. Structure D can then acquire a second GlcNAc residue (strucutre E). Removal of the outer N-acetylglucosamine residue (GlcNAc) by the phosphodiester glycosidase, and addition of a galactose residue and a terminal sialic acid residue, results in structure F. Another pathway can be adopted by structures B and C, where there is further removal of mannose residues (structure G), addition of GlcNAc residues (structures H and I), and addition of galactose and terminal sialic acid residues (structures J and K). ● = galactose; □ = glucose; ○ = mannose; ⊠ = N-acetylglucosamine; P = pyrophosphate; ✪ = sialic acid; • = alternate location for phosphate residue.



1=Glucosidase I	8=Galactosyl Transferase
2=Glucosidase II	9=Sialyltransferase
3=Glucosyltransferase	10=Endo-mannosidase
4=ER Mannosidase	11=GlcNAc Transferase I
5=GlcNAc Phosphotransferase	12=Mannosidase II
6=Mannosidase I	13=GlcNAc Transferase II
7=Phosphodiester glycosidase	14=GlcNAc Transferase V

FIGURE 31

Diagrammatic representation of a proposed function for testicular SGP-1 (t-SGP-1) in the male reproductive system. T-SGP-1 is secreted by the Sertoli cell into the lumen of the seminiferous tubules. Therein, it binds to glycolipids on the tail of the spermatozoa plasma membrane via its sialic acid binding domain. In the efferent duct, the lipid-protein complex is removed from the spermatozoa and is endocytosed by the nonciliated cells via receptor-mediated endocytosis. This process is possibly mediated by t-SGP-1's terminal sialic acid residues. Once in the endosomes of the nonciliated cells, the acidic pH causes dissociation of the complex from the receptor and dissociation of the protein from the glycolipids. The receptor is recycled back to the plasma membrane, while t-SGP-1 and the glycolipids are delivered to the lysosomes. T-SGP-1 may function as a glycolipid binding and transport protein for spermatozoa and as a sphingolipid activator protein in the lysosome. AT = apical tubules; E = endosome; L = lysosome; MVB = multivesicular body; T = tubules; TCP = tubular coated pits; ○ = t-SGP-1; ⊏ = glycolipids; T = receptor.


Spermatozoon
TCP
AT
E
T
MVB
L
Sertoli cell
Nonciliated cell

FUTURE PERSPECTIVES

The present study showed that endocytosis of testicular SGP-1 by the nonciliated cells of the efferent ducts is mediated, in great proportion, by sialic acid and that SGP-1 has sialic acid residues terminally linked α(2-6) to galactose or N-acetylgalactosamine residues. Further studies on the identity of the receptor may clarify the function that SGP-1 plays in the male reproductive system. By solubilizing the apical plasma membrane of the nonciliated cells and using two affinity columns, one containing SGP-1 and the other sialic acid terminally linked α(2-6) to galactose or N-acetylgalactosamine, the receptor can be isolated, and antibodies can be raised to that receptor for immunocytochemical and biochemical studies.

ORIGINAL CONTRIBUTIONS

1) The demonstration that sialic acid is involved in the receptor-mediated endocytosis of testicular SGP-1 by the nonciliated cells of the efferent ducts.

2) The demonstration that mannose 6-phosphate is also involved to a lesser extent than sialic acid, in the receptor-mediated endocytosis of testicular SGP-1 by the nonciliated cells of the efferent ducts.

3) The demonstration that testicular SGP-1 has sialic acid residues that are terminally linked α(2-6) to galactose or N-acetylgalactosamine residues.

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