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Goat sperm membrane: lectin-binding sites of sperm surface and lectin affinity chromatography of the mature sperm membrane antigens

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The cell surface glycoproteins of goat epididymal maturing spermatozoa have been investigated using lectins as surface probes that interact with specific sugars with high affinity. Concanavalin A (ConA) and wheat-germ agglutinin (WGA) showed high affinity for mature cauda epididymal sperm agglutination, whereas RCA_2, kidney beans lectin and peanut agglutinin caused much lower or little agglutination of the cells. The mature sperm exhibited markedly higher efficacy than the immature caput epididymal sperm for binding both ConA and WGA, as evidenced by sperm agglutination and the binding of the fluorescence isothiocyanate (FITC)-labelled lectins. FITC-ConA binds uniformly to the entire mature sperm surface whereas FITC-WGA binds to the acrosomal cap region of the head. The FITC-RCA_2 mainly labelled the posterior head of mature cauda sperm. However, no WGA-specific glycoprotein receptors could be detected in sperm plasma membrane (PM) by WGA-Sepharose affinity chromatography. The data implied that the epididymal sperm maturation is associated with a marked increase in the ConA/WGA receptors and that WGA receptors may be glycolipids rather than glycoproteins. Analysis of the ConA receptors of cauda sperm PM identified by ConA-Sepharose affinity chromatography and subsequent resolution in SDS-PAGE demonstrated the presence of five glycopolypeptides of different concentrations (98, 96, 43, 27 and 17 kDa) of goat sperm membrane. The immunoblot of these ConA-specific glycopeptides with anti-sperm membrane antiserum showed that 98- and 96-kDa receptors are immunoresponsive.

Introduction

The complex structure of sperm cell plasma membrane (PM) harmonizes reactions with the female egg primarily by initiating flagellar movement and then modulating surface macromolecules such as glycoproteins and glycolipids. The terminal sequences of glycoproteins and glycolipids are important because of the involvement of these cell-surface carbohydrate groups in various cellular interactions. Several lines of evidence indicated that glycoproteins of the maturing sperm cell membrane undergo architectural alteration and chemical turnover during transit through the epididymal tubule [1–5]. Some of these maturational glycoproteins are established as sperm membrane antigens [6,7]. It is important to study the PM antigens as a class, as they may provide the potential immunogens for antifertility vaccine development. In an earlier report [8] we demonstrated the membrane antigens of maturing goat spermatozoa and found that some of these immunoresponsive polypeptides were PAS-positive. In this study, we present the characterization of membrane antigens using lectin as a specific probe. We also report on the lectin-mediated agglutination of cauda and caput epididymal sperm cells and, on the basis of this property, using fluorescein isothiocyanate (FITC)labelled lectin conjugates, examine the surface distribution of lectin receptors. We also demonstrate the D-glucose/D-mannose-linked glycoproteins on the PM and their immunoresponsiveness to the anti-sperm membrane antiserum.

Abbreviations: PAS, periodic acid schiff; PM, plasma membrane; PBS, phosphate-buffered saline; ConA, concanavalin A; FITC, fluorescein isothiocyanate; RCA_2, *Ricinus communis* 2; WGA, wheat-germ agglutinin; NANA, *N*-acetylneuraminic acid.

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Materials and Methods

Collection of spermatozoa

Highly motile spermatozoa were collected from goat cauda epididymides by the procedure described previously [9] in buffer A containing 119 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1.2 mM $MgSO_4$, 10 mM glucose, 16.3 mM potassium phosphate (pH 6.9) and penicillin (50 U/ml). The sperm cells were then sedimented by centrifugation at $500 \times g$ for 5 min at 31 ± 1 °C and the pellet was repeatedly washed to remove the epididymal plasma as much as possible. The viability of the cell was judged under the light microscopy in presence and absence of epididymal plasma. The cells possessing high forward motility in the presence of epididymal plasma were selected for the present study. The pure intact caput spermatozoa were collected by Ficoll gradient. The details of the procedure have been described elsewhere [10].

Sperm agglutination

The highly pure, washed sperm cells from caput and cauda epididymides were suspended in buffer A containing 0.05% BSA, and 25 μl of the sperm suspension (10^{10} cells/ml) were mixed in the small round-bottomed wells of the microtiter plates with equal volumes of lectin at different dilutions. The last two wells in each set of experiments contained only cell suspensions in buffer A, and B containing BSA, respectively. The microtiter plate was agitated for 10 min at 28 °C, kept for 30 min, and the sperm cell mixture was then examined microscopically. The agglutination of the cell in the presence and absence of lectin was recorded on a qualitative scale from 0 (no agglutination) to 4^+ (maximum agglutination), according to the procedure of Nicholson and Yanagimachi [11].

For the Ouchterlony double-diffusion experiment, a gel slide was prepared with 1% agarose in 0.9% NaCl containing 0.1% sodium azide. The central well contained the solubilized membrane protein of either caput of cauda sperm and the surrounding wells contained different lectins. The slide was incubated for 24 h at 28 °C in a humid atmosphere. After incubation the slide was dried and the precipitin line stained with 0.04% Coomassie blue, 25% methanol and 10% acetic acid.

FITC-lectin conjugation and fluorescence microscopy

The method for the coupling of isothiocyanate fluorescein to lectins was as described previously [13]. The lectins were dialysed for 16 h at 4 °C against a solution of 100 μg fluorochrome/ml of 0.05 M bicarbonate-buffered saline (pH 9.2). The reaction was stopped by exchanging the dialysing buffer for 0.02 M phosphate-buffered saline (pH 7.0). Finally, the conjugated sample was chromatographed in a Bio-Gel P2 column, equilibrated with 0.02 M phosphate-buffered saline (pH 7.0).

These FITC-labelled lectins were used separately to localize the binding sites on the plasma membrane of caput and cauda epididymal spermatozoa. The pure intact caput and high-forward-motility cauda sperm were incubated in PBS containing 0.1% BSA at 28 °C with dilutions of different lectins conjugated at concentrations ranging from 0.2 to 2 mg/ml protein. After 10 min of incubation in the dark, the labelling solution was diluted 10-fold and pelleted by centrifugation at $300 \times g$. The labelled sperm was then gently washed $4 \times$ with PBS. The control samples contained α-methyl-D-mannoside (FITC-ConA), NANA (FITC-WGA) and *N*-acetylgalactosamine (FITC-RCA_2) in the incubation and washing buffers at a final concentration of 100 mM. After the final washing the labelled sperm cells were suspended in a minimal volume of PBS buffer and an aliquot was placed on the clean side. The sample was then air-dried and mounted with mineral oil (Sigma). The cells were viewed with a Leitz Diaplan fluorescence microscope and photographs were taken with a Kodak camera.

Isolation of membrane and preparation of anti-sperm-membrane antiserum

The sperm cell membrane was isolated in an aqueous two-phase polymer system consisting of 252 kDa dextran (5.5%) and 20 kDa polyethylene glycol (4.2%). The membrane enrichment was judged by measuring the marker enzymes in the isolated membrane and in the cell debris. Details of the procedure were described previously [12]. The antibody was raised by the immunization of two rabbits, each receiving six subcutaneous injections of purified PM emulsified with adjuvant 15-day intervals. 12 days after the last injection, the titre was measured and blood was collected by heart puncture. The immunoglobulin of the immune serum was precipitated twice with 50% ammonium sulfate and then dialysed against 0.02 M PBS (pH 7.4) containing 1 mM PMSF. This partially-purified polyclonal antibody was used in our immunoblot experiments.

Lectin affinity chromatography

The lectins were separately coupled to cyanogen-bromide-activated Sepharose, as described elsewhere [14]. The required amount of CNBr-activated Sepharose 4B was washed and reswelled in 1 mM HCl to protect the activated groups of the gel. Finally, the gel was suspended in 0.01 M $NaHCO_3$ buffer (pH 8.8). To this suspension, 4 mg/ml of ConA, WGA or RCA_2 was quickly added prior to incubation for 30 min with either α-methyl-D-mannoside, *N*-acetylglucosamine, or *N*-acetylgalactosamine, respectively, and mixed in an 'end-over-end' mixer for 5.5 h at 22 °C. The super-

natant was removed and the lectin-coupled gel was treated with 1 M ethanolamine. The free lectins were removed by washing with coupling buffer (pH 8.2) followed by acetate buffer (pH 4.0) containing 0.5 M NaCl. This washed Sepharose 4B coupled with either ConA or WGA was used to prepare a column of 4 ml bed volume and equilibrated with Tris-HCl buffer (pH 7.2) containing 1 mM EDTA and 0.5 mM PMSF (A). The membrane was solubilized in the same buffer (A) containing 0.5% deoxycholate and, after sedimentation of deoxycholate-insoluble material at $800 \times g$ for 20 min, the supernatant was subsequently passed through the lectin-Sepharose affinity column. The glycoprotein bound to the affinity matrix was eluted with buffer A containing either α-methylmannoside, sialic acid, or *N*-acetylgalactosamine from the respective lectin affinity column. In the case of the ConA affinity column, buffer A contained 1 mM each of $CaCl_2$ and $MnCl_2$. The eluted material was concentrated and analyzed on 10% SDS-polyacrylamide gel.

SDS-PAGE electrophoresis and immunoblotting

The membrane proteins before and after the lectin affinity chromatography were separated on 10% SDS-polyacrylamide gel by the method of Laemmli [15]. For immunoblotting, the electrophoresed proteins were transferred onto nitrocellulose and probed with partially-purified anti-sperm-membrane antiserum according to the method described by Burnette [16].

Results

Lectin-mediated agglutination

The patterns of agglutination of maturing sperm cells in response to treatment with different lectins are presented in Table I. When the washed spermatozoa from caput and cauda epididymides were mixed with ConA, WGA, RCA_2, kidney bean and peanut agglutinins, diluted sequentially, strong agglutination occurred with ConA and WGA and to less extent with RCA_2. However, the degree of agglutination differs between caput and cauda epididymal sperm cells. The cauda epididymal sperm, which is motile and functionally mature, agglutinates more efficiently with ConA and WGA in comparison to caput epididymal sperm, which is an immotile and functionally immature cell. The minimum concentrations on ConA, WGA and RCA_2 required to agglutinate the mature sperm are 2 μg/ml, 8 μg/ml and 30 μg/ml, respectively, and for kidney bean lectin the concentration is 125 μg/ml. These results suggest that the motile mature cell from the cauda epididymis is more sensitive to agglutination by ConA and WGA than the immotile immature cell from the caput epididymis. Agglutination was also found to be inhibited by appropriate saccharide inhibitors.

We also examined the formation of the precipitin line on agglutination of solubilized membrane protein

TABLE I

Agglutination of goat spermatozoa by different lectins

[a] The caput and cauda epididymal spermatozoa were collected from four animals and pooled. [b] The score of agglutination of sperm cell after incubation with different lectins was determined as described previously [16] on a qualitative scale from 4 (no single spermatozoa; maximum agglutination) to 0 (all single spermatozoa). The data shown here were repeated 4×with different sets of animals and the results were essentially same.

Lectin	Inhibitor 100 mM	Epididymal source of spermatozoa [a]	Concentration of lectin (μg/ml) [b]								
			250	125	60	30	15	8	4	2	1
ConA	none	caput	4+	3+	3+	3+	2+	2+	+	±	±
	none	cauda	4+	4+	4+	3+	3+	3+	2+	2+	±
	α-methyl mannoside	cauda	+	+	+	±	±	0	0	0	0
WGA	none	caput	3+	3+	±	±	0	0			
	none	cauda	4+	3+	3+	3+	3+	2+	±	±	0
	N-acetyl-neuraminic acid	cauda	+	±	±	0	0	0			
RCA_2	none	caput	+	+	±	0	0				
	none	cauda	3+	2+	2+	2±	+	+	±	0	0
	N-acetyl-galactosamine	cauda	+	0	0						
Kidney beans lectin	none	caput	0	0	0						
	none	cauda	2+	2+	0						
Peas Agglutinin	none	caput	0	0							
	none	cauda	0	0							

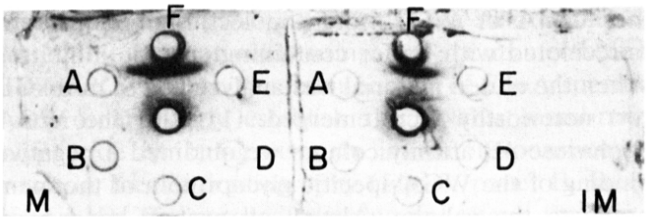


Fig. 1. Ouchterlony double-diffusion test using different lectins. Central well, solubilized PM from mature (M) and immature (IM) sperm. Each central well contains 50 μg of protein. Different lectins used at 20 μg per well. (A, D) and WGA, RCA_2; (C) peanut agglutinin; (E) kidney been lectin; (F) ConA.

of caput and cauda epididymal sperm with different lectins in an Ouchterlony double-diffusion system. After 24 h incubation of the agarose plate at 4 °C a distinct precipitin line appeared only with ConA out of the lectins examined. Coomassie staining (Fig. 1) of this precipitin line, formed with ConA and membrane proteins, clearly indicates the presence of glucose/mannose-specific glycoproteins in the PM of both mature and immature sperm cells.

Localization of lectin-binding sites

Since the results from the agglutination experiment with whole sperm indicated that the carbohydrate groups linked with protein/lipids are present on the PM of goat spermatozoa, the distribution of accessible lectin-binding sites over the surface of mature and immature sperm cells was explored. The results show that the labelling of 4 × washed cauda-epididymal mature sperm with FITC-ConA conjugate at 30 °C achieved uniformly dense labelling which fluoresces in the entire head region. The mid-piece and tail region of the mature sperm were also uniformly labelled but the intensity of this fluorescence was comparatively lower (Fig. 2A). The intensity of fluorescence of entire caput-epididymal immature sperm after labelling with FITC-ConA conjugate is in low profile and comparatively less uniform (Fig. 2B). A significant difference was found in the labelling of FITC-WGA conjugate between sperm from caput and cauda epididymides. Caput sperm exhibited a weak binding over the entire sperm; because of the low fluorescence we were unable to present the photograph of the weakly labelled caput sperm. However, this labelling pattern dramatically

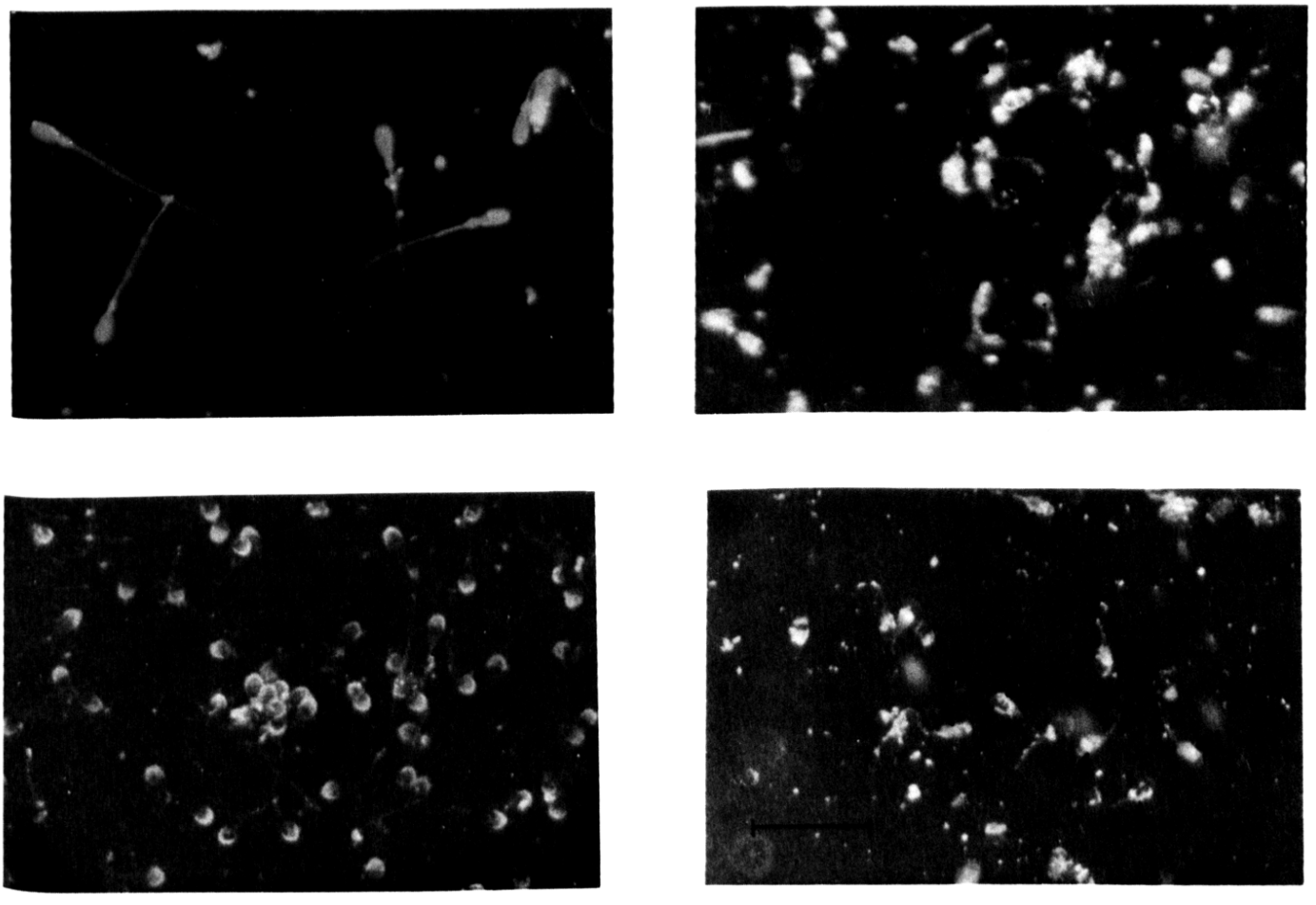
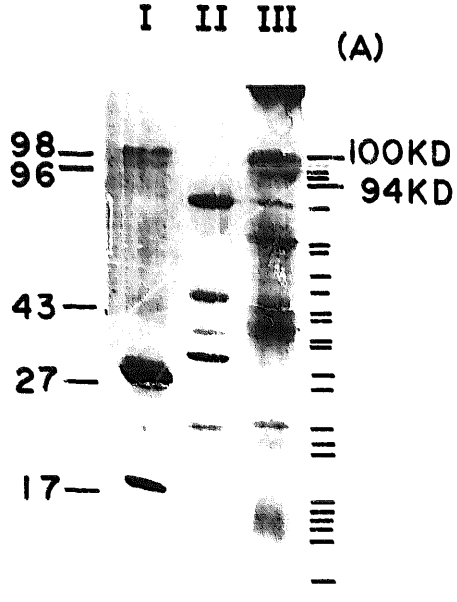


Fig. 2. Lectin binding of goat sperm isolated from caput and cauda epididymal tissue. (a) FITC-ConA to cauda epididymal sperm; (b) FITC-ConA to caput epididymal sperm; arrows indicate weekly labelled immature spermatozoa; (c) FITC-WGA to cauda epididymal sperm; arrows indicate labelled anterior head region; (d) FITC-RCA_2 to cauda epididymal sperm; arrows indicate weekly labelled spermatozoa. Bar, 0.1 μm.

changes as spermatozoa reach the cauda epididymis. The FITC-WGA conjugate binds specifically to the sperm from the cauda epididymal tubule, and as a result a distinct fluorescence appeared in the acrosomal region (Fig. 2C). This clearly suggests the presence of a WGA-specific glycoprotein or glycolipid in the acrosomal cap region of the sperm plasma membrane. The intensity of fluorescence is remarkably low in the post-acrosomal, midpiece and tail regions, indicating that the WGA-specific saccharides are absent in these region. Fig. 2D shows binding of FITC-RCA_2 mainly at the posterior head surface of cauda epididymal sperm. This binding is also characterized by lack of uniformity. The controls, incubated with corresponding inhibitors of the FITC-lectins used, did not fluoresce.

Characterization of membrane polypeptide by lectin affinity column

In view of the agglutination capability in the presence of ConA or WGA and the FITC-lectin-binding property of the cauda epididymal sperm cell, an attempt was made to identify the membrane polypeptides linked with saccharide moieties specific for ConA and WGA. Solubilized membrane proteins from cauda sperm were chromatographed in the affinity column made with Sepharose 4B conjugated with either ConA or WGA, and the lectin-bound proteins were eluted with buffer containing specific inhibitors. When the eluted material was analyzed by SDS-PAGE, two noteworthy facts emerged. (1) With the WGA-Sepharose 4B affinity column we obtained a negative-binding of the WGA-specific glycoprotein of the membrane to the column. Almost all protein bands were recovered in the effluent as analyzed by gel electrophoresis. The authenticity of the WGA affinity column was verified with a known amount of fetuin. These data suggest that the goat sperm membrane receptors for WGA may not be glycoproteins. (2) A number of solubilized membrane proteins were retained by the ConA affinity column as analyzed in 10% SDS-PAGE. Fig. 3 (panel A) shows the Coomassie stain of SDS-PAGE of solubilized membrane in which a cluster of at least five polypeptides are present in the region of 100 to 94 kDa (lane III). From this group of polypeptides only two (98 and 96 kDa) are selectively recognized by the ConA-Sepharose column along with three other membrane polypeptides of lower molecular masses (43, 27 and 17 kDa) with different affinity for ConA as reflected by the Coomassie stain of the gel (lane I). The gel also shows that the 27 kDa membrane polypeptide has been enriched remarkably by this ConA affinity column. In order to establish the antigenicity of



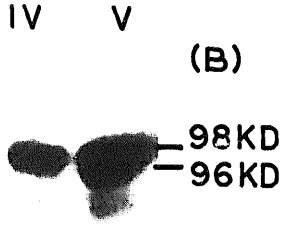


Fig. 3. ConA affinity chromatography of cauda epididymal sperm membrane protein (A) and the immunoblotting analysis of ConA-specific glycopolypeptides with partially purified anti-sperm membrane antiserum (1 : 100 dilution) (B). Lane I: Coomassie blue stain of 10% SDS-PAGE of deoxycholate-solubilized bound membrane proteins (60 μg) eluted from the ConA column with α-methyl mannoside. Lane II: molecular markers (M_r 68000; 45000; 36000; 29000; 24000; 20000; 14200). Identical molecular weights of the membrane proteins before and after the ConA column were also obtained after incorporating phosphorylase B (97400) as an additional molecular marker. Lane III: deoxycholate-solubilized membrane protein before being applied to the ConA column. Lanes IV and V: autoradiography of immunoblot of protein eluted from ConA affinity column. The lanes were loaded with 25 μg and 60 μg protein, respectively.

these membrane glycopolypeptides we next immunoblotted the glycoproteins using anti-sperm-membrane antiserum as probe. The immunoblots of ConA-specific glycoprotein clearly establish the 98-kDa membrane polypeptide as being strongly immunoresponsive, as shown in the autoradiography (Fig. 3; panel B, lanes IV and V). The autoradiography also shows the positive reactivity of a 96 kDa ConA-specific polypeptide towards the antibody. These results clearly demonstrate that these two cauda sperm membrane antigens are D-glucose/D-mannose-linked membrane glycopolypeptides.

Discussion

In this report we present the characterization of the sperm membrane polypeptides using lectin as probe. Each of the lectins used in this study displays a definite saccharide specificity. Changes in the lectin binding or lectin-mediated agglutination patterns reflect the changes in the PM saccharide distribution during the epididymal sperm maturation.

Of the lectins tested, the ConA-mediated agglutination and FITC-ConA binding to the goat sperm show little change in the orientation of D-glucose/D-mannose-containing glycocomponents during the epididymal maturation. These data extend the reports of others on the binding of ConA in different species [11,17,20] where little or no change in ConA was also observed. Using isotopically labelled ConA, other groups have attempted to quantify the number of binding sites per sperm cell. Contrary to our results, they have observed that a decrease in this number is associated with the epididymal sperm maturation process [2]. In this study we found a remarkable change in the WGA-mediated agglutination and in the distribution of FITC-WGA binding sites on maturing goat sperm. Weak FITC-WGA binding to the caput epididymal sperm has been replaced by strong binding to the acrosomal region of the cauda epididymal sperm. A progressively increased binding of WGA associated with epididymal transit has previously been reported, but the pattern of binding was different [17–19]. Contrary to our results, others have shown that both WGA-mediated agglutination and binding of WGA to the maturing sperm are decreased along the length of the epididymal tubule [11,20,21].

From the present data, coupled with previously reported data [17,21], it appears that the saccharide moiety linked with PM protein/lipid undergoes modification during epididymal sperm maturation. Therefore, our next approach was to determine the WGA- and ConA-specific membrane polypeptides, if any, and to identify them as sperm membrane antigens. However, with the WGA affinity chromatography we were not able to detect any PM glycopolypeptide, although a distinct binding of FITC-WGA to the acrosomal region of cauda sperm was observed. This finding is in agreement with the observation that sperm PM extract gives no protein precipitin line with WGA in the Ouchterlony diffusion system (Fig. 1). Based on the present data, we tentatively assume that WGA-specific saccharides may be linked with lipids of the cauda sperm PM which are involved in binding with FITC-WGA and WGA-mediated agglutination. Recently, Peterson et al. have also observed that the outer acrosomal membrane has affinity for peanut agglutination and suggested that this region is rich in acidic phospholipid and close to this region the PM is poor in integral membrane protein content [22].

The most noteworthy part of this study is the demonstration of the glucose/mannose-linked glycopolypeptides in mature sperm membrane by ConA affinity chromatography. Using the anti-sperm membrane antiserum we have previously shown that not all but some of the sperm membrane polypeptides posses antigenic determinants [8]. It is evident from the data that the higher-molecular-mass polypeptides acquire antigenicity during the caput to cauda epididymal transit and this finding suggests that these membrane antigens are associated with the sperm maturation process in the epididymal tubule. However, in the present study it was necessary to determine the antigenicity of the major five ConA-specific membrane glycopolypeptides to find out whether these glycoproteins have antigenic determinants and also their position in the groups of sperm membrane antigens already reported [8]. The immunoblot data reported here show that the 98-kDa and 96-kDa polypeptides are immunoreactive membrane glycopolypeptides which appear to belong to the group of higher-molecular-mass sperm membrane antigens [8]. Together, the ConA specificity of some of the membrane polypeptides and their immunoblot data clearly establish that both the 98-kDa and 96-kDa polypeptides are glucose/mannose-linked sperm membrane antigens which may have a functional role in the sperm maturation process in the epididymal tubule. Reports to date appear mainly on the lower-molecular-mass glycoproteins, ranging from 22 to 37 kDa, which may be associated with the sperm maturation process [1–5,23,24] and few of them have antigenic determinants [25]. The detailed mapping of boar sperm membrane polypeptides demonstrates that the greatest number of polypeptides are bound to ConA, of which only 43-kDa glycopolypeptides posses antigenic determinants [7]. However, in the present study the demonstration of glucose/mannose-specific highly immunoresponsive glycopolypeptides (98 and 96 kDa) in goat cauda sperm membrane is of significance for further study to evaluate its role in the epididymal sperm maturation process.

Studies at a number of laboratories indicate that the carbohydrate portion of physiologically active glycoproteins is often important for its biological functions [26]. The mechanism by which the PM antigens having carbohydrate groups undergo modification to serve as potential 'signals' in the maturation process is still not clearly understood. Therefore, the present report will be of value when future studies are designed to learn how these glucose/mannose-containing antigens affect the acrosome reaction and sperm-egg fusion.

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