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Characterization of Fucosyltransferase Activity during Mouse Spermatogenesis: Evidence for a Cell Surface Fucosyltransferase†

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ABSTRACT: Fucosyltransferase activity was quantified in mouse germ cells at different stages of spermatogenesis. Specifically, fucosyltransferase activities of pachytene spermatocytes, round spermatids, and cauda epididymal sperm were compared. Fucosyltransferase activity of mixed germ cells displayed an apparent V_{max} of 17 pmol (mg of protein)$^{-1}$ min^{-1} and an apparent K_m of approximately 13 μM for GDP-L-[^{14}C]fucose in the presence of saturating amounts of asialofetuin at 33 °C. Under these conditions, cellular fucosyltransferase activity was found to increase during spermatogenesis. In agreement with assays of intact cells, examination of subcellular fractions indicated that a large fraction of fucosyltransferase activity was associated with the cell surface. The fraction of fucosyltransferase activity that was associated with the cell surface progressively increased throughout spermatogenesis and epididymal maturation so that nearly all of the fucosyltransferase in epididymal sperm was on the cell surface. Specifically, by comparison of activities in the presence and absence of the detergent NP-40, the fraction of fucosyltransferase activity that was associated with the cell surface in pachytene spermatocytes, round spermatids, and epididymal sperm was 0.36, 0.5, and 0.85, respectively. These results suggest that a cell surface fucosyltransferase may be important during differentiation of spermatogenic cells in the testis as well as during epididymal maturation and fertilization.

Spermatogenesis involves complex morphological and biochemical events leading to the development of mature spermatozoa from proliferating germ cells. The development of a mature sperm from its stem cell, the spermatogonium, can be divided into four phases: (1) mitotic proliferation of stem cells, (2) meiosis, (3) the formation of highly specialized spermatozoa (spermiogenesis), and (4) the release of sperm into the lumen of the seminiferous tubule (spermiation). Further maturational changes of spermatozoa occur during epididymal transit. Although relatively little is known about the biochemical events that regulate spermatogenesis, macromolecules at the cell surface, many of which are glycosylated, likely play a prominent role in the development of germ cells.

Glycosylation of proteins occurs via specific glycosyltransferases, and, with few exceptions, the Golgi apparatus appears to be the site of the oligosaccharide elongation reactions. Developing spermatogenic cells represent an interesting situation since after meiosis the Golgi apparatus of the newly formed round spermatid undergoes impressive morphological

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changes. In the rat, spermiogenesis can be divided into 19 distinct steps on the basis of changes taking place in the Golgi apparatus and nucleus (Leblond & Clermont, 1952). During spermiogenesis, some of the Golgi attach to a region of the nucleus that will later define the anterior pole of the spermatozoa. These Golgi elements form the sperm acrosome. The remaining Golgi appear to migrate distally along the flagellum and eventually regress by step 16 of spermiogenesis. The Golgi apparatus of early spermatids clearly contributes to the substantial glycosylation of the developing acrosome constituents (Leblond & Clermont, 1952; Clermont & Leblond, 1955; Burgos & Fawcett, 1955; Sandoz, 1970; Susi et al., 1971). For example, glycoproteins in early spermatids were shown by using autoradiography to be synthesized by the Golgi and delivered to the acrosome (Sandoz, 1972; Sandoz & Roland, 1976; Tang et al., 1982). Studies have also shown that particular carbohydrates are attached to the cell surface of developing spermatogenic cells, even after the Golgi apparatus has coalesced to form the acrosome.

Of particular interest are carbohydrates containing terminal L-fucose. In older rat spermatids, the residual Golgi apparatus does not contribute nascent glycoproteins to the developing acrosome, but the acrosome itself is able to incorporate radioactive fucose (Tang et al., 1982; Clermont & Tang, 1985). In addition, experiments on cultured mouse spermatogenic cells have shown that fucose can be incorporated into at least 15 different macromolecules by both pachytene spermatocytes and round spermatids (Grootegoed et al., 1982; Gerton & Millette, 1986). Moreover, Gerton and Millette (1986) demonstrated by cellular fractionation that all of the components detected represented plasma membrane glycoconjugates. Further, eight of the fucosylated proteins were specific to the membranes of pachytene spermatocytes while two fucosylated proteins were specific to round spermatids. These results imply that either there is a difference in fucosyltransferase specificity or there is a difference in the substrates available to these fucosyltransferases at different stages of spermatogenesis.

Since specific fucosylation events may occur during differentiation, we decided to assay fucosyltransferase activity at different stages of spermatogenesis. In addition, we fractionated both pachytene spermatocytes and round spermatids to localize the enzyme at a subcellular level. We report here that cellular fucosyltransferase activity changes during spermatogenesis and remains at high levels in fully developed spermatozoa. In addition, an increasing amount of fucosyltransferase activity becomes associated with the cell surface during spermatogenesis and epididymal maturation.

Materials and Methods

Materials. All chemicals were purchased from Sigma Chemical Co. unless otherwise stated. Sodium chloride and potassium chloride were purchased from Baker Chemical Co. (Phillipsburg, NJ). Essential and nonessential amino acids were purchased from GIBCO (Grand Island, NY). Collagenase (CLS II; EC 3.4.24.3) was purchased from Cooper Biomedical (Malvern, PA). GDP-[^{14}C]fucose (262 mCi/mmol) and UDP-[^{3}H]galactose (12.9 Ci/mmol) were purchased from New England Nuclear (Boston, MA).

Enriched Krebs–Ringer bicarbonate medium (EKRB) contained 120.1 mM NaCl, 4.8 mM KCl, 25.2 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4 \cdot 7H_2O$, 1.3 mM glucose, 1 mM glutamine, 10 mL/L essential amino acids, 10 mL/L nonessential amino acids, 100 μg/mL streptomycin sulfate, and 60 μg/mL penicillin G (K^+ salt) as described by Romrell et al. (1976). The pH was adjusted between pH 7.2 and 7.4 with 5% CO_2 in air.

A protease inhibitor cocktail (PIC) contained 1 mg/mL leupeptin, 2 mg/mL antipain, 10 mg/mL benzamidine, 1 mg/mL chymostatin, and 1 mg/mL pepstatin.

Fucosyltransferase buffer (FT buffer) contained 100 mM NaCl, 50 mM 4-morpholinepropanesulfonic acid (Mops), 10 mM $MnCl_2$, and 0.4% bovine serum albumin (BSA, fraction V) adjusted to pH 6.5. To prevent proteolysis, PIC was diluted 1:1000 in FT buffer. In experiments where disruption of cells was desired, 2% Nonidet P-40 (NP-40) was added to FT buffer.

Animals and Cell Preparation. Adult, 12–16-week male Swiss–Webster mice were purchased from Taconic Farms (Germantown, NY) and sacrificed by cervical dislocation. Spermatogenic cells were isolated by using previously described procedures (Bellvé et al., 1977a,b; Romrell et al., 1976). Testes were removed, decapsulated, and immediately placed into enriched EKRB as originally formulated by Romrell et al. (1976). Seminiferous cell suspensions were prepared by using sequential incubations in 0.5 mg/mL collagenase and 0.5 mg/mL trypsin (bovine pancreas, type III; EC 3.4.21.4). Action by these enzymes was stopped by using 0.5 mg/mL soybean trypsin inhibitor. Protease inhibitor cocktail (PIC) was subsequently added to all cellular suspensions (1:1000) to prevent further proteolysis from occurring.

Individual spermatogenic cell types were fractionated and concentrated from a crude testicular cell suspension by unit gravity sedimentation according to published procedures (Romrell et al., 1976; Bellvé et al., 1977a,b). Pachytene spermatocytes and round spermatids were obtained in greater than 90% purity from a mixed germ cell population that contained primarily germ cells with only a small contaminating population of either Sertoli cells or Leydig cells. Cell purities were determined under a microscope using a hemacytometer. Morphological criteria for the identification of isolated mouse spermatogenic cells were according to Bellvé et al. (1977a,b). Morphometric determinations of cell dimensions were performed by using a light microscope with differential interference contrast optics and a stage micrometer as a reference. For surface area calculations, both pachytene spermatocytes and round spermatids were assumed to be spheres (surface area = $4\pi r^2$) whereas cauda epididymal spermatozoa were taken to be cylinders about the length and radius of the tail (l_t, r_t) and the midpiece (l_m, r_m) with the head taken as a cone using the length and radius at the base of the head (l_h, r_h) to calculate the surface area [total surface area = $\pi[r_h(r_h^2 + l_h^2)]^{1/2} + 2r_m l_m + 2r_t l_t$].

Subcellular fractionations of these cell types were performed according to procedures outlined by Millette et al. (1980). After hypotonic lysis and subsequent cell homogenization, the supernatant from a 1000*g* spin was centrifuged at 80000*g* for 90 min in a discontinuous sucrose gradient. A fraction that has been shown to be enriched in plasma membrane proteins migrated to the 30%/40% sucrose interface (band 2) and was collected. In addition, a fraction that was enriched in cytosolic proteins (including endoplasmic reticulum, Golgi, and submitochondrial markers) migrated to the 40%/45% sucrose interface (band 3) and was also collected. Bands 2 and 3 were then frozen at −70 °C until future use.

Mature sperm were collected from the cauda epididymides of mice by clamping the proximal portion of the organ with a hemostat and puncturing the distal part of the organ with a 26-gauge needle and collecting the expelled fluid with a capillary tube. The sperm were then washed 3 times by centrifuging them at 700*g* for 5 min and resuspending them before each spin.

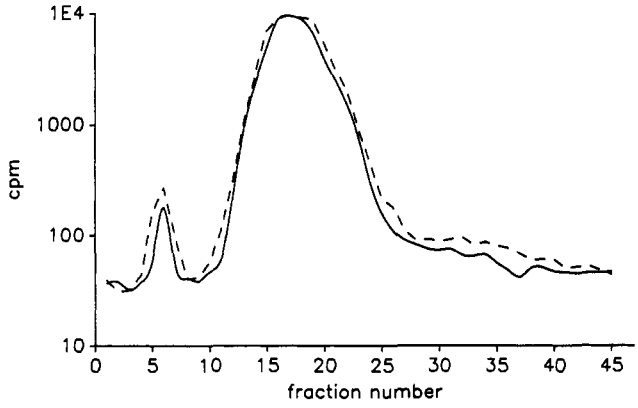

FIGURE 1: Chromatograms from a Bio-Gel P-10 size-exclusion column. Pachytene spermatocytes (—) or round spermatids (---) were placed in 10 μL of fucosyltransferase buffer containing 60 μM GDP-L-[^{14}C]fucose and 20 mg/mL asialofetuin. The reaction was stopped after 15 min. The extract was then diluted to a final volume of 150 μL and loaded on the column. Fractions of 200 μL were collected and quantified in a scintillation counter. High molecular weight radiolabeled product eluted in the void volumn (V_o) while unreacted radiolabeled substrate eluted as V_i. Alternatively, high molecular weight product was trapped on size-exclusion filters in a micropartition system as described in the text.

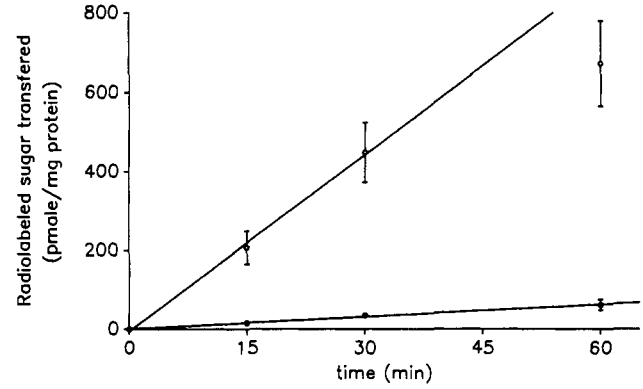

FIGURE 2: Time course of fucose transfer to asialofetuin using solubilized mixed spermatogenic cells. With 60 μM GDP-L-[^{14}C]fucose (open circles) and 20 mg/mL asialofetuin, L-[^{14}C]fucose incorporation into asialofetuin was linear up to 30 min. For this reason, all assays performed under saturating conditions were stopped after 15 min, and activity was determined at that time. Alternatively, sperm were incubated with UDP-[^{3}H]galactose (closed circles) and asialofetuin. The rate of incorporation of radiolabeled galactose was approximately 30 times less than radiolabeled fucose to asialofetuin. Data points represent the average of triplicate determinations from four groups of animals with error bars representing the standard error of the mean for these averages.

Fucosyltransferase Assays. Fucosyltransferase activity was assayed by using a procedure modified from Letts et al. (1974b). In experiments where total fucosyltransferase activity was assayed, spermatogenic cells were placed into FT buffer containing 2% NP-40 at a final concentration of 10^7 cells/mL. In experiments where cell surface activity was assayed, the FT buffer contained no NP-40. Total protein concentrations were determined by the method of Lowry et al. (1951) using a modification for cellular extracts in high concentrations of detergent (Wessel & Flugge, 1984). Extracts disrupted with NP-40 either were used immediately for fucosyltransferase assays or were frozen at −70 °C for future use. Spermatogenic cell extracts that were frozen and thawed 4 times showed no detectable difference in fucosyltransferase activity.

Ten microliters containing predetermined amounts of asialofetuin, GDP-L-[^{14}C]fucose, and 3.5 mg/mL 5′-AMP was placed into a 0.4-mL microcentrifuge tube and dried in a Speedvac (Savant Instruments) for 30 min. A cell suspension containing approximately 10^4 cells in FT buffer was then added to the microcentrifuge tube. After incubation at the desired temperature for a predetermined time, the reaction was stopped by adding cold 50 mM EDTA to the reaction mixture to a final volume of 60 μL. The entire sample was then frozen immediately in an acetone dry ice bath and stored at −70 °C.

Fucosyltransferase activity was assayed either by using size-exclusion chromatography (Bio-Gel P-10) or by trapping the radiolabeled substrate onto a 10000 molecular weight cutoff size-exclusion filter in an Amicon Centrifree micropartition system as described by Cardullo et al. (1987). Figure 1 shows a typical chromatogram from the size-exclusion column for both pachytene spermatocytes and round spermatids. The area under the void peaks represented the amount of radiolabeled fucose transferred to asialofetuin, and activities were calculated from these. Alternatively, the method employing size-exclusion filters gave results that were not detectably different from size-exclusion chromatography and allowed us to make many more determinations of fucosyltransferase activity in a given time (Cardullo et al., 1987). Initial experiments involving the characterization of fucosyltransferase associated with mixed germ cells primarily used size-exclusion chromatography to quantify overall activity. Subsequently, experiments assaying for activity in isolated cell types and subcellular fractions primarily utilized the micropartition system. However, in each case, the results were checked at least once using size-exclusion chromatography.

Results

Enzyme Characterization. Before meaningful significance could be assigned to fucosyltransferase data, optimal kinetic parameters for the enzyme had to be determined. Fucosyltransferase covalently attaches fucose to an appropriate acceptor, ultimately producing a fucosylated product. In these experiments, radiolabeled GDP-L-fucose was reacted with asialofetuin (ASF) in the following manner:

$$\text{GDP-L-[}^{14}\text{C]fucose} + \text{ASF} \rightarrow \text{ASF–L-fucose} + \text{GDP}$$

Since glycosyltransferase addition involves two different substrates, the concentrations of both asialofetuin and GDP-L-fucose had to be optimized over the time course of a given experiment. At saturating concentrations of asialofetuin (20 mg/mL) and GDP-L-[^{14}C]fucose (60 μM), fucosyltransferase activity was linear over 30 min (Figure 2). These constituted saturating conditions for all cell types, and all subsequent experiments were performed over a 15-min time period. In comparison, identical concentrations of UDP-galactose were only minimally incorporated into asialofetuin over the same time periods (Figure 2).

When the concentration of asialofetuin was varied from 2 to 20 mg/mL, at a fixed concentration of GDP-L-[^{14}C]fucose, there was no significant difference in fucosyltransferase activity although in the absence of asialofetuin, fucosyltransferase activity was not detectable. However, when the concentration of GDP-L-[^{14}C]fucose was varied from 0 to 100 μM, there was a monotonic increase in fucosyltransferase activity that leveled off at approximately 50 μM. Lineweaver–Burk analysis of these data showed that the K_m for GDP-L-fucose was approximately 13 μM with a V_{max} of 17 pmol (mg of protein)$^{-1}$ min^{-1}.

Finally, fucosyltransferase activity was monitored as a function of temperature. Fucosyltransferase activity increased steadily from 20 to 40 °C. In this temperature range, fucosyltransferase exhibited a Q_{10} of 1.7. At temperature above 40 °C, fucosyltransferase activity fell off rapidly and was undetectable at temperatures above 50 °C.

Table I: Fucosyltransferase Activity in the Absence and Presence of Detergent[a]

	fucosyltransferase activity			
	pmol (mg of protein)$^{-1}$ min^{-1}		pmol (10^6 cells)$^{-1}$ min^{-1}	
	no NP-40	+2% NP-40	no NP-40	+2% NP-40
mixed germ cells	6.6 ± 0.7	17.6 ± 2.5		
pachytene spermatocytes	7.9 ± 1.4	22.4 ± 2.4	1.9 ± 0.2 (0.36 ± 0.04)	5.3 ± 0.5
round spermatids	25.4 ± 3.1	52.0 ± 4.2	1.5 ± 0.2 (0.50 ± 0.03)	3.0 ± 0.2
epididymal sperm			2.7 ± 0.3 (0.85 ± 0.05)	3.2 ± 0.2

[a] Enzymatic activity was expressed either in units of picomoles of fucose transferred to asialofetuin per milligram of cellular protein per minute or in picomoles of fucose transferred to asialofetuin per 10^6 cells per minute. In the presence of detergent, the cells were completely solubilized, and total fucosyltransferase activity was measured. In the absence of detergent, the substrates were impermeable to the cell, and hence activities represent only the cell surface component. There was an increase both in the absolute cellular fucosyltransferase activity and in the fraction of cell surface fucosyltransferase activity from pachytene spermatocytes to cauda epididymal sperm. The higher proportion of surface-associated enzyme in both round spermatids and epididymal sperm suggests that this enzyme is being concentrated at the cell surface late in spermatogenesis. Data represent the average of triplicate determinations from at least four groups of animals with error bars representing the standard error of the mean for these averages. Numbers in parentheses represent the fraction of fucosyltransferase activity that is associated with the cell surface.

Using these characterizations from mixed germ cells, we made subsequent determinations of fucosyltransferase activity in isolated cell types using concentrations of asialofetuin and GDP-[^{14}C]fucose of 20 mg/mL and 60 μM, respectively, and at a temperature of 33 °C (the physiological temperature of the mouse testis). Under these conditions, fucosyltransferase activity in all cell types was linear up to 30 min.

Fucosyltransferase Activity Changes throughout Spermatogenesis. Table I shows the results of assaying fucosyltransferase activity in homogenized mixed germ cells, pachytene spermatocytes, round spermatids, and epididymal sperm in the presence and absence of 2% NP-40. The detergent, NP-40, totally solubilized the cells and gave maximum activity in all cases examined. Data were expressed in two ways: either on the basis of total cellular protein in these extracts or on a per cell basis. When these activities were compared on the basis of total cellular protein, fucosyltransferase activity was found to increase throughout spermatogenesis with an activity of 17.6 pmol (mg of protein)$^{-1}$ min^{-1} for mixed germ cells, 22.4 pmol (mg of protein)$^{-1}$ min^{-1} for pachytene spermatocytes, and 52.0 pmol (mg of protein)$^{-1}$ min^{-1} for round spermatids. Therefore, fucosyltransferase activity appears to be high in spermatogenic cells with a large proportion of this activity concentrated in pachytene spermatocytes and round spermatids.

When total fucosyltransferase activity was assayed on a per cell basis, however, a different result was obtained (Table I). In this case, the activity of pachytene spermatocytes was 5.3 pmol (10^6 cells)$^{-1}$ min^{-1} while the activity in round spermatids was only 3.0 pmol (10^6 cells)$^{-1}$ min^{-1}. Hence, analyzed in this manner, fucosyltransferase activity appeared to decrease during the transition between pachytene spermatocytes and round spermatids. In addition, the activity in sperm from the cauda epididymidis was not significantly different from that in round spermatids. These cells differ both in their morphometric dimensions and in their total cellular protein content. For instance, whereas pachytene spermatocytes have a mean diameter of approximately 16 μm and a total protein content of 195 pg, round spermatids have a diameter of approximately 10 μm and a protein content of only 58 pg. These differences more than account for the differences in fucosyltransferase activity when analyzed in these two different ways. Since we were interested in seeing how activity changed during development, it was apparent that physiologically meaningful data could only be extracted from the activity assayed on a per cell basis.

Fraction of Cell Surface Associated Fucosyltransferase Activity Increases throughout Spermatogenesis. To see if any activity was associated with the cell surface, spermatogenic cells were also incubated in fucosyltransferase buffer in the absence of detergent (Table I). Surprisingly, a large fraction of activity was detected in the absence of NP-40, and the ratio of total fucosyltransferase activity to that associated with the cell surface increased throughout spermatogenesis. The surface activity of pachytene spermatocytes was 1.9 pmol (10^6 cells)$^{-1}$ min^{-1}, 1.5 pmol (10^6 cells)$^{-1}$ min^{-1} in round spermatids, and 2.7 pmol (10^6 cells)$^{-1}$ min^{-1} in mature sperm taken from the cauda epididymidis. Hence, there is an overall increase in cell surface fucosyltransferase activity during sperm development. Moreover, the ratio of cell surface activity to total activity was 0.36 in pachytene spermatocytes, 0.50 in round spermatids, and 0.85 in mature sperm taken from the cauda epididymidis. Therefore, nearly all of the fucosyltransferase activity in mature sperm appears to be associated with the plasma membrane, and an increasing fraction of this activity becomes associated with the plasma membrane throughout sperm development.

To attach physiological significance to the cell surface fucosyltransferase activity, we calculated the activity on the basis of total cellular surface area. In this manner, activity is best expressed as molecules of fucose transferred per meter squared per second. Analyzed in this way the activity of pachytene spermatocytes was 24.1 molecules μm^{-2} s^{-1}, 47.9 ± 6.4 molecules μm^{-2} s^{-1} in round spermatids, and 23.5 molecules μm^{-2} s^{-1} in epididymal sperm. This calculation assumes that the enzyme is uniformly distributed over the plasma membrane of these cells. However, in a recent report by Scully et al. (1987), a monoclonal antibody directed against a milk-soluble galactosyltransferase recognized a region over approximately a 10 μm^2 area over the sperm head while completely covering the cell surface of both pachytene spermatocytes and round spermatids. If fucosyltransferase were to have a similar distribution, then the surface fucosyltransferase activity of epididymal sperm would be approximately 1500 molecules μm^{-2} s^{-1} instead of our calculated value of 23.5 molecules μm^{-2} s^{-1}. This would represent a 60-fold increase in cell surface fucosyltransferase activity from pachytene spermatocytes to epididymal sperm.

Subcellular Localization of Fucosyltransferase. To check further that fucosyltransferase activity had a cell surface component, spermatogenic cells were fractionated into two fractions that were enriched in either plasma membrane proteins (band 2) or organelle-associated (cytosolic) proteins (band 3). According to the procedures outlined by Millette et al. (1980), fucosyltransferase activities in both band 2 and band 3 were compared for both pachytene spermatocytes and round spermatids. The results from these fractionation procedures are shown in Figure 3. As with solubilized cells, fucosyltransferase activity (picomoles per milligram of protein per minute) was found to be higher in round spermatids than

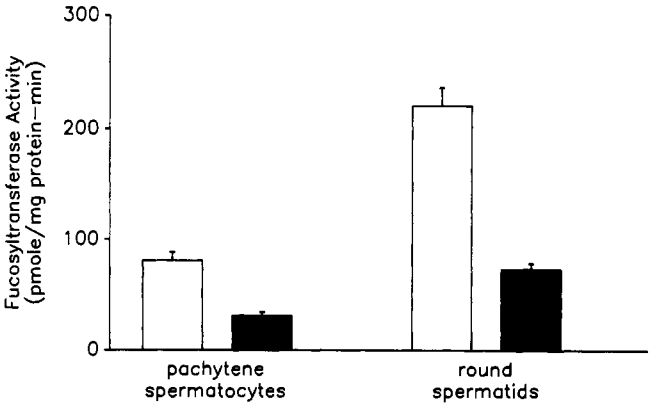

FIGURE 3: Fractionation of spermatogenic cells into a fraction that was enriched in plasma membrane components (band 2, blank box) and a fraction that was enriched in microsomal components (band 3, filled box) showed that fucosyltransferase activity was higher in band 2 than in band 3 in both pachytene spermatocytes and round spermatids. In the absence of a protease inhibitor cocktail, fucosyltransferase activity in fractionated cells was greatly reduced (data not shown), suggesting that fucosyltransferase was becoming degraded subsequent to hypotonic lysis of the intact cells. Data represent the average of triplicate determinations from at least four animals with error bars representing the standard error of the mean for these averages.

in pachytene spermatocytes. In the absence of a protease inhibitor cocktail (PIC), fucosyltransferase activity was low and did not represent an enrichment in activity from solubilized cells (data not shown). However, in the presence of PIC, a large activity was detected in both the plasma membrane fraction and the cytosolic fraction for both pachytene spermatocytes and round spermatids.

In all cases, whether we looked at pachytene spermatocytes or round spermatids, fucosyltransferase activity was 2–3-fold higher in band 2 than in band 3. Since band 2 has been shown to be enriched in a variety of plasma membrane markers (Millette & Moulding, 1981), this result shows that a significant fraction of fucosyltransferase activity is associated with the cell surface. This result is consistent with the experiments using intact cells which also show that a fraction of fucosyltransferase is associated with the cell surface.

Discussion

Experiments performed by Gerton and Millette (1986) identified for the first time stage-specific fucosylated glycoproteins on plasma membranes of pachytene spermatocytes and round spermatids, suggesting that stage-specific fucosyltransferases may exist within germ cells that regulate these fucosylation events. In this report, we have demonstrated directly that there are indeed stage-specific changes in fucosyltransferase activity during spermatogenesis. In addition, we have shown that a significant portion of this activity is associated with a cytosolic fraction that is enriched in Golgi enzymes (Moulding & Millette, 1981). Surprisingly, however, we found that an increasing fraction of this activity became associated with the germ cell surface as spermatogenesis progressed and that essentially all of the fucosyltransferase on mature epididymal sperm was cell surface associated.

Schachter and co-workers (Letts et al., 1974a,b, 1978) provided initial evidence that a variety of glycosyltransferases appear in a stage-specific manner during spermatogensis. These workers showed that there was a dramatic drop in the activity of certain glycosyltransferases during mouse spermatogenesis. The following activities were detected using testis cell suspensions: a UDP-D-galactose–glycoprotein galactosyltransferase, a UDP-*N*-acetyl-D-glucosamine–glycoprotein *N*-acetylglucosaminyltransferase, and a GDP-L-fucose:glycoprotein fucosyltransferase all acting on sialidase- and galactosidase-treated glycoprotein. Using crude cell separation procedures, these authors were able to show that the activity of these enzymes in germ cells was significantly reduced from early to late spermatogenesis. Moreover, no detectable change in glycosyltransferase activity was found between fractions containing pachytene spermatocytes and round spermatids (i.e., during meiosis). The conclusion of this work was that glycoprotein synthesis is "occurring in mouse testis germinal cells during and immediately after meiosis, but that it is turned off in the terminal stages of spermatogenesis" (Letts et al., 1974b).

The techniques used by Letts et al. and described by Lam et al. (1970) to purify populations of germ cells have since been improved so that greater than 90% purity of either pachytene spermatocytes or round spermatids can be achieved (Romrell et al., 1976; Bellvé et al., 1977a,b). Indeed, Letts et al. did not quantify the degree of purification in their cell separation so that significant contamination between cell types could have occurred. We believe that the improved purity of the spermatogenic cell populations used in our study accounts for the observed differences in fucosyltransferase activity between pachytene spermatocytes and round spermatids. Indeed, our measurements of fucosyltransferase activity in mixed germ cell suspensions agree well with the results of Letts et al. (1977b) and only differ significantly when looking at the isolated germ cell populations.

The Golgi apparatus was originally identified as the major site of glycosylation on the basis of autoradiographic studies on tissues taken from animals that had been injected with radioactive sugars such as [^{3}H]glucose or [^{3}H]galactose (Whur et al., 1969). These sugars, however, often get metabolized or are readily converted into hexoses or hexosamines so that they may not give informative incorporation patterns. In contrast, fucose is generally not converted to other sugars (Nwokoro & Schachter, 1975a,b), and more precise incorporation patterns are generally obtained. Further studies have shown that [^{3}H]fucose rapidly localizes to the Golgi with minimal incorporation into other organelles (Haddad et al., 1971). Fucose is considered to be an excellent plasma membrane marker because it shows minimal incorporation at 2–5 min but does begin to appear at later times (Bennett et al., 1974) and has been used with cultured spermatogenic cells for this purpose (Gerton & Millette, 1986). For these reasons, it has been suggested that the Golgi apparatus is the major location of protein fucosylation in the cell and that fucosylated proteins are later incorporated into the plasma membrane. Our results with fractionated spermatogenic cells show that there is significant fucosyltransferase activity in a fraction enriched in intracellular organelles, including Golgi. Moreover, this activity is stage-specific and is in accordance with the results reported by Gerton and Millette (1986) for the incorporation of [^{3}H]fucose into plasma membrane components.

Our finding that some fucosyltransferase activity is associated with the cell surface, and may, in fact, be an ectoenzyme, is the first such report in spermatogenic cells. Although the presence of cell surface glycosyltransferases is rare, it is not unique to these cells. Of particular interest, Shur and workers have identified a galactosyltransferase on the surface of mouse epididymal spermatozoa (Shur & Hall, 1982a; Shur, 1984; Macek & Shur, 1988) and most recently have reported cell surface activity on spermatogenic cells (Scully et al., 1987). These investigators also demonstrated that a monoclonal antibody directed against a soluble milk galactosyltransferase localized to the cell surface of spermatogenic cells and localized

to the acrosomal region of mature spermatozoa (Scully et al., 1987).

Of relevance to our findings regarding fucosyltransferase activity in the testis, other investigators have implicated both glycosyltransferases and glycosylated substrates as important regulators of reproduction in mammals. Shur and colleagues have perhaps provided the best evidence for a cell surface galactosyltransferase involved in fertilization (Shur & Hall, 1982a; Shur, 1984) and sperm capacitation (Shur & Hall, 1982b). These studies have suggested that the cell surface galactosyltransferase is itself the sperm membrane receptor necessary for binding to the zona pellucida of the mouse oocyte. This work nicely complements the work of Wassarman and colleagues (Wassarman & Bleil, 1982; Florman & Wassarman, 1983, 1985; Florman et al., 1984) indicating that the specific sperm receptor on the mouse zona pellucida is the oligosaccharide of a glycoprotein that they have named ZP3.

Other recent data suggest that a variety of cell surface glycosylation events are important during spermatogenesis and epididymal maturation. Fenderson et al. (1984) reported that dramatic alterations in the nature of sialyated cell surface constituents accompany early spermatogenic cell differentiation, implying the involvement of specific sialytransferases. In addition, Cossu and Boitani (1984) have identified lactosaminoglycans on the surface of mouse spermatogenic cells and spermatozoa. These workers have postulated that these lactosaminoglycans are fucosylated via an unknown fucosyltransferase enzyme secreted by the epididymal epithelium. In light of our results showing that epididymal sperm have detectable amounts of fucosyltransferase on their surface, it is possible that the fucosylation of these lactosaminoglycans could be mediated at their own plasma membrane and not require a fucosyltransferase secreted by the epididymal epithelium. Of course, either hypothesis requires the presence of an extracellular sugar–nucleotide, and these have not been reported in the literature.

Another possible role for a cell surface fucosyltransferase may be in mediating cell adhesion events. In this regard, sperm surface fucosyltransferase could act in a manner similar to the galactosyltransferase that functions as the receptor for the zona pellucida identified by Shur et al. (1982b, 1984). Cell surface fucosyltransferases have been shown to mediate cell adhesion in other systems. In particular, Rauvala et al. (1983) presented direct evidence that a purified fucosyltransferase can mediate adhesion of embryonic skin fibroblasts to substrates. Similar processes may well be occurring during spermatogenesis to modulate germ cell–Sertoli cell interactions within the seminiferous epithelium. The fact that fucosyltransferase activity in spermatogenic cells changes throughout development may be important in the adhesion of germ cells to the surrounding Sertoli cell followed by release into the seminiferous tubule during spermiation.

Studies are currently under way to examine directly the possible role of cell surface glycosyltransferases during sperm development and fertilization. In particular, we are examining whether there are further modifications to sperm cell surface fucosyl- and galactosyltransferases during the processes of epididymal maturation and capacitation with regard to activity and substrate specificity.

Acknowledgments

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Registry No. Fucosyltransferase, 9033-08-3; GDP-L-fucose, 15839-70-0.

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A Loop Involving Catalytic Chain Residues 230–245 Is Essential for the Stabilization of Both Allosteric Forms of *Escherichia coli* Aspartate Transcarbamylase†

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ABSTRACT: The allosteric transition of *Escherichia coli* aspartate transcarbamylase involves significant alterations in structure at both the quaternary and tertiary levels. On the tertiary level, the 240s loop (residues 230–245 of the catalytic chain) repositions, influencing the conformation of Arg-229, a residue near the aspartate binding site. In the T state, Arg-229 is bent out of the active site and may be stabilized in this position by an interaction with Glu-272. In the R state, the conformation of Arg-229 changes, allowing it to interact with the β-carboxylate of aspartate, and is stabilized in this position by a specific interaction with Glu-233. In order to ascertain the function of Arg-229, Glu-233, and Glu-272 in the catalytic and cooperative interactions of the enzyme, three mutant enzymes were created by site-specific mutagenesis. Arg-229 was replaced by Ala, while both Glu-233 and Glu-272 were replaced by Ser. The Arg-229 → Ala and Glu-233 → Ser enzymes exhibit 10000-fold and 80-fold decreases in maximal activity, respectively, and they both exhibit a 2-fold increase in the aspartate concentration at half the maximal observed velocity, $[S]_{0.5}$. The Arg-229 → Ala enzyme still exhibits substantial homotropic cooperativity, but all cooperativity is lost in the Glu-233 → Ser enzyme. The Glu-233 → Ser enzyme also shows a 4-fold decrease in the carbamyl phosphate $[S]_{0.5}$, while the Arg-229 → Ala enzyme shows no change in the carbamyl phosphate $[S]_{0.5}$ compared to the wild-type enzyme. The Glu-272 to Ser mutation results in a slight reduction in maximal activity, an increase in $[S]_{0.5}$ for both aspartate and carbamyl phosphate, and reduced cooperativity. Analysis of the isolated catalytic subunits from these three mutant enzymes reveals that in each case the changes in the kinetic properties of the isolated catalytic subunit are similar to the changes caused by the mutation in the holoenzyme. PALA was able to activate the Glu-233 → Ser enzyme, at low aspartate concentrations, even though the mutant holoenzyme did not exhibit any cooperativity, indicating that cooperative interactions still exist between the active sites in this enzyme. It is proposed that Glu-233 of the 240s loop helps create the high-activity–high-affinity R state by positioning the side chain of Arg-229 for aspartate binding while Glu-272 helps stabilize the low-activity–low-affinity T state by positioning the side chain of Arg-229 so that it cannot interact with aspartate. Evidence presented here as well as from previous crystallographic and mutagenesis studies suggests that 240s loop interactions are critical for stabilizing both allosteric forms of the enzyme. A model is proposed that suggests that the stabilization of alternate conformations of the 240s loop, by specific side chain interactions, provides mechanisms by which homotropic cooperativity is manifest, and provides a molecular level explanation for a concerted allosteric transition in aspartate transcarbamylase.

Escherichia coli aspartate transcarbamylase (EC 2.1.3.2) catalyzes the first step in pyrimidine biosynthesis, the reaction of carbamyl phosphate and L-aspartate to form *N*-carbamyl-L-aspartate and inorganic phosphate. The enzyme exhibits a sigmoidal-shaped saturation curve with both of its substrates indicating cooperative interactions between the six active sites of the holoenzyme[1] (M_r 310000). The conversion of the enzyme from a T state having low substrate affinity and low catalytic activity to an R state having high substrate affinity and high catalytic activity has been proposed to occur in a concerted fashion based on kinetic, physicochemical, and structural analyses of both wild-type and mutant versions of the enzyme (Foote & Schachman, 1985; Krause et al., 1987;

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[1] Abbreviations: holoenzyme, native enzyme composed of two catalytic trimers and three regulatory dimers; PALA, *N*-(phosphonoacetyl)-L-aspartate; Tris, tris(hydroxymethyl)aminomethane; T and R states, tense and relaxed conformations of the enzyme having low activity and affinity for substrates and high activity and affinity for substrates, respectively; $[S]_{0.5}$, substrate concentration at half the maximal observed specific activity; 80s loop, flexible loop of the enzyme comprising approximately amino acid residues 76–86 of the catalytic chain; 240s loop, flexible loop of the enzyme comprising approximately amino acid residues 230–245 of the catalytic chain.

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