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Compartmentalization, processing and redistribution of the plasma membrane protein CE9 on rodent spermatozoa

Relationship of the annulus to domain boundaries in the plasma membrane of the tail

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SUMMARY

Western blotting, immunofluorescence and immunogold electron microscopy were used to examine the compartmentalization, processing and redistribution of the integral plasma membrane protein CE9 on the spermatozoa of rats, mice and hamsters. In each species examined, spermatozoal CE9 was found to undergo endoproteolytic processing followed by a net redistribution from the posterior-tail domain into the anterior-tail domain of the plasma membrane during epididymal maturation. Compared to spermatozoa of the rat and mouse, those of the hamster were found to express a greater proportion of their CE9 within the anterior-tail plasma membrane domain at all stages of maturation. As a consequence, CE9 was judged to be a suitable marker for two different spermatozoal plasma membrane domains: the posterior-tail plasma membrane domain (spermatozoa from the testis and caput epididymidis of the rat and mouse) and the anterior-tail domain (spermatozoa from the cauda epididymidis of the hamster). Immunogold electron microscopy was used to pinpoint the positions of the boundaries of these CE9-containing plasma membrane domains at a high level of resolution. In each case, the position of the CE9 domain boundary was found to be strongly correlated with that of the subplasmalemmal electron-dense ring known as the annulus. The precise spatial relationship between the CE9 domain boundary and the annulus was, however, found to differ significantly among species and/or as a function of maturation.

Key words: spermatozoon, plasma membrane, membrane domain, membrane protein, domain boundary, annulus, immunogold electron microscopy, rodent

INTRODUCTION

Cells exhibit the ability to organize their plasma membrane constituents laterally into specialized domains. This aspect of cellular structure has been especially well characterized for the integral plasma membrane proteins of cells that display a striking morphological asymmetry, such as epithelial cells, neurons, flagellated protists and spermatozoa (reviewed by Rodriguez-Boulan and Powell, 1992; Bloodgood, 1988; Bearer and Friend, 1990). The compartmentalization of integral plasma membrane proteins by these grossly asymmetrical cells appears to be maintained in part through the action of interregional barriers to lateral diffusion. These barriers to lateral diffusion are believed to operate at domain boundaries that themselves appear to coincide roughly with specialized regions of the plasma membrane: the tight junction of the epithelial cell (reviewed by Schneeberger and Lynch, 1992), the axon hillock/initial segment of the neuron (Dotti and Simons, 1990; Dotti et al., 1991; Peters et al., 1991), the flagellar bracelet of the flagellated protists (Weiss et al., 1977; Musgrave et al., 1986; Bloodgood, 1988; Hunnicutt et al., 1990) and the equatorial segment, posterior striated ring and annulus of the spermatozoon (Myles et al., 1984; Cowan et al., 1987; Nehme et al., 1993; reviewed by Friend, 1989). Although each of these specialized regions contains a specific arrangement of intramembranous particles and/or a subplasmalemmal electron-dense plaque, the precise spatial relationships between these ultrastructural landmarks and the corresponding domain boundaries remain largely unknown. Here we address this issue as it pertains to a domain-specific integral plasma membrane protein of the rodent spermatozoon called CE9.

CE9 is a Type-Ia transmembrane glycoprotein and a member of the immunoglobulin superfamily (Nehme et al., 1993). The protein is compartmentalized to the posterior-tail plasma membrane domain of spermatozoa obtained from the testis or caput epididymidis of the rat (Petruszak et al., 1991). Remarkably, however, the localization of rat spermatozoal CE9 is not fixed: a significant fraction of the protein redistributes laterally into the anterior-tail plasma membrane domain as spermatozoa undergo maturation in the epididymis (Petruszak et al., 1991). As a prelude to this redistribution, a peptide comprising the amino-terminal 75 amino acids of the extracellular tail of rat CE9 is removed by endoproteolytic cleavage as the spermatozoa are propelled through the proximal caput epididymidis

(Petruszak et al., 1991; Nehme et al., 1993). The maturation of spermatozoa in the epididymis is correlated with the acquisition of the capacities to fertilize and to exhibit forward-directed motility (Bedford, 1975; Orgebin-Crist et al., 1975; Fournier-Delpech and Courot, 1987). In light of this correlation, it is tempting to speculate that it is the redistribution of the truncated form of CE9 into the plasma membrane of the midpiece that will prove to be relevant to the functioning of the mature spermatozoon and, therefore, to fertilization. If so, then one might expect to see evidence of a generality in the patterns of compartmentalization, endoproteolytic processing and redistribution when comparing the life cycle of rat spermatozoal CE9 to those of its homologues on the spermatozoa of other species. On the basis of comparisons of cDNA sequence, putative homologues of CE9 have already been identified in mice (Altruda et al., 1989; Miyauchi et al., 1990), chickens (Schlosshauer and Herzog, 1990; Seulberger et al., 1990) and humans (Miyauchi et al., 1991; Kasinrerk et al., 1992). But to our knowledge there have as yet been no attempts to localize these homologous proteins on the spermatozoa of these other species. To test for generality in the life cycle of spermatozoal CE9, we have used affinity-purified rabbit anti-rat CE9 antibodies to compare the localization and processing of the CE9 homologues on the spermatozoa of two other species of rodent, the mouse and the hamster.

Although the physiological significance of the complex life cycle of spermatozoal CE9 remains to be determined, we have already begun to obtain some insight into the basis for the compartmentalization of the protein. When examined by fluorescence-recovery-after-photobleaching, CE9 proved to be highly mobile within the posterior-tail plasma membrane domain of the rat testicular spermatozoon (recovery=105±12%, diffusion coefficient=$(1.1\pm0.3)\times10^{-9}$ cm^2/s; Nehme et al., 1993). From this observation, we inferred that CE9 was confined to the posterior-tail plasma membrane domain through the action of an interregional barrier to lateral diffusion. And given the localization pattern observed for CE9 on testicular and caput epididymidal spermatozoa of the rat by immunofluorescence (Petruszak et al., 1991; Nehme et al., 1993), the most likely place for this diffusion barrier is at the relevant domain boundary, i.e. somewhere near the junction of the midpiece and principle piece of the spermatozoal tail. This would coincide roughly with the position of that enigmatic ultrastructural entity known as the annulus (Fawcett, 1970; Friend and Fawcett, 1974). To pinpoint the position of the domain boundary of spermatozoal CE9 at a higher level of resolution and to determine whether there is indeed a spatial correlation between the domain boundary and the annulus, we have used immunogold electron microscopy to localize CE9 on oriented preparations of rodent spermatozoa.

MATERIALS AND METHODS

Animals and materials

Sexually mature, male Fischer F344 rats (250-350 g), Balb/c mice (20-25 g), and Syrian Golden LVG hamsters (125-150 g) were purchased from either Charles River Breeding Laboratories (Wilmington, MA) or Harlan Sprague-Dawley (Indianapolis, IN). Tetramethylrhodamine isothiocyanate-labeled goat anti-rabbit IgG was from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA). The 5 nm diameter colloidal gold goat anti-rabbit IgG conjugate was from Polysciences, Inc. (Warrington, PA). Bovine serum albumin (BSA; Fraction V) was from ICN Biochemicals (Cleveland, OH). The polyclonal anti-CE9 antibody was prepared in rabbits and affinity-purified on columns of rat liver CE9-Sepharose as described by Bartles and Hubbard (1986). The sources of other reagents may be found in earlier articles from this laboratory (Petruszak et al., 1991; Nehme et al., 1993).

Collection of spermatozoa

Spermatozoa were collected by mincing the testes or specific regions of the epididymides at room temperature in either modified Dulbecco's phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 8.1 mM NaP_i, 1.5 mM KP_i, pH 7.4) or, when no immunolabeling was required, in modified Hanks' buffer (95 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 5.6 mM D-glucose, 21.6 mM lactic acid, 0.5 mM sodium pyruvate, 50 μg/ml streptomycin sulfate, 50 units/ml sodium penicillin G, and 0.4% (w/v) BSA, adjusted to pH 7.4 by equilibration with 5% CO_2) (Petruszak et al., 1991).

Western blotting and immunofluorescence

The preparation of gel samples from testicular or epididymal spermatozoa, SDS-polyacrylamide gel electrophoresis, Western blotting with affinity-purified rabbit polyclonal anti-rat CE9 antibody and ^{125}I-Protein A, and immunofluorescent labeling of 2% paraformaldehyde-fixed spermatozoa in the presence of 0.01% (w/v) saponin, either in agarose squares (for quantification of distributions) or on poly-L-lysine-coated slides, have all been described previously (Petruszak et al., 1991).

Immunogold labeling and electron microscopy

Spermatozoa were fixed and dried on poly-L-lysine-coated coverslips to help orient the cells in a single plane. Suspensions of spermatozoa that were to undergo immunogold labeling were mixed with equal volumes of 2× concentrated fixative in modified Dulbecco's phosphate-buffered saline to achieve final concentrations of 2% paraformaldehyde and either 0.05% (hamster and rat) or 0.01% (mouse) glutaraldehyde. Samples of this mixture were applied directly to coverslips and, after 5 minutes, the coverslips were tipped vertically, the excess suspension was decanted, and the remaining thin film of adherent suspension was allowed to just reach dryness by incubation at room temperature for an additional 5-10 minutes. Immunolabeling of spermatozoa was carried out at room temperature by sequential incubation in the following solutions: 0.25% (w/v) NH_4Cl in PBS/sap (0.15 M NaCl, 20 mM NaP_i, 3 mM NaN_3, pH 7.4, containing 0.01% (w/v) saponin)(3× 5 minutes), 0.5% (w/v) BSA in PBS/sap (8 minutes), affinity-purified polyclonal rabbit anti-rat CE9 antibody in PBS/sap with 0.5% (w/v) BSA (30-40 minutes), PBS/sap (3× 5 minutes), 0.5% (w/v) BSA in PBS/sap (5 minutes), 5 nm diameter colloidal gold goat anti-rabbit IgG conjugate in PBS/sap with 1.0% (w/v) BSA (60 minutes), and PBS/sap (5× 5 minutes). The saponin was included as a membrane-permeabilizing detergent (Bartles et al., 1985). To decrease nonspecific labeling, the 5 nm colloidal gold-antibody conjugate was preabsorbed overnight by incubation at 4°C with an excess of fixed-quenched spermatozoa that had been embedded in agarose and cut into squares (Bartles et al., 1985). Specimens were post-fixed with 2.5% (w/v) paraformaldehyde, 1.5% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4, for 30 minutes at 4°C and 1% OsO_4 in 0.1 M sodium cacodylate, pH 7.4, for 1 hour at 4°C. After staining with 0.5% (w/v) uranyl acetate for 1 hour at 22°C, the specimens were dehydrated with a graded series of ethanol solutions and infiltrated with a graded series of Polybed 812 solutions (Polysciences, Inc., Warrington, PA) in propylene oxide. Beem capsules containing fresh Polybed 812 were inverted onto horizontal coverslips, and the resin was polymerized at 68°C for 48 hours. Polymerized specimens were dipped briefly into liquid nitrogen to facili-

tate the removal of the coverslip. Then 80 to 90 nm thick sections were cut with a diamond knife, collected on uncoated 200-mesh square copper grids and stained with uranyl acetate and lead citrate in preparation for viewing and photography at ×27,000 on a Jeol JEM-100CXII electron microscope. The analyses of immunogold-labeled samples was performed on the electron microscope negatives with the aid of a reticle scaled with divisions equivalent to 1.8 nm. To ensure that measurements of annular thickness were taken parallel to the longitudinal axis of the spermatozoon, we only considered those cells that were sectioned within an estimated ±12° of the longitudinal plane as judged by the presence of the spermatozoal tail over extended distances within the plane of section. This variation in plane of section would theoretically be expected to result in a margin of error of less than 2% in our measurements of annular thickness. All immunogold data are reported as mean ± s.d. for the indicated number of independent annular profiles examined: rat (95), mouse (10) and hamster (19).

RESULTS

Western blot analysis

The endoproteolytic processing of spermatozoal CE9 that takes place during epididymal maturation in the rat (Petruszak et al., 1991) is readily apparent upon Western blot analysis: the partially resolved multiplet of bands comprising the glycosylation variants of rat CE9 shows a decrease in apparent molecular mass from 40-48 kDa (Fig. 1A, lane T) to 23-33 kDa (Fig. 1A, lanes P and D) coincident with the entry of the spermatozoa into the epididymis. A single broad protein band or multiplet of closely spaced bands in the range of 40-45 kDa was also found to react specifically with the affinity-purified rabbit anti-rat CE9 antibody when testicular spermatozoa of the mouse and hamster were examined in parallel (Fig. 1B,C, lanes T). This value for apparent molecular mass agreed with that expected for the mouse homologue of CE9 (Altruda et al., 1989; Miyauchi et al., 1990), suggesting that the affinity-purified anti-rat CE9 antibody reacted specifically with CE9 homologues on the spermatozoa of mice and hamsters. As was observed for spermatozoal CE9 in the rat, broad bands or multiplets of crossreactive bands were detected at a lower apparent molecular mass (23-33 kDa) on spermatozoa collected from the epididymides of mice and hamsters (Fig. 1B,C, lanes P and D). These data suggested that the endoproteolytic processing observed for spermatozoal CE9 during epididymal maturation in the rat was also experienced by CE9 homologues on the spermatozoa of the mouse and hamster.

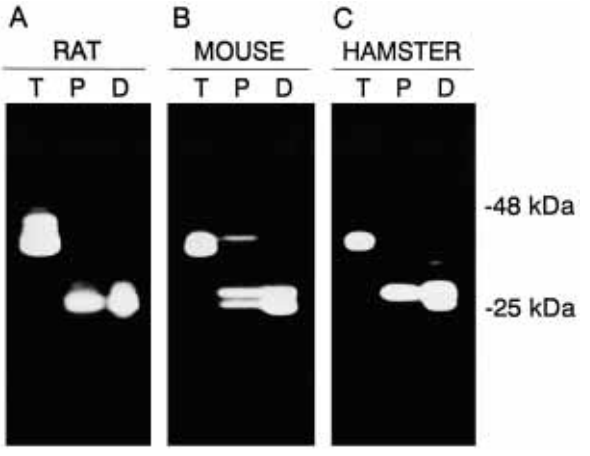

Fig. 1. Western blots of homogenates prepared from the testis (T), caput epididymidis (P, for proximal) or cauda epididymidis (D, for distal) of a rat (A), mouse (B) or hamster (C).

Immunofluorescence analysis

We next used the affinity-purified rabbit anti-rat CE9 antibody in immunofluorescence experiments to compare the localizations of CE9 and its homologues on spermatozoa collected from the testes and different segments of the epididymides of rats, mice and hamsters. The patterns of specific labeling observed for spermatozoa of the mouse proved to be quite similar to those of the rat (Petruszak et al., 1991). That is, the vast majority of spermatozoa collected from the caput epididymidis (Fig. 2A,B (rat) and Fig. 2E,F (mouse)) or testis (immunofluorescence data not shown) showed labeling only over the posterior-tail domain (Fig. 3A,B). Then, coincident with maturation in the epididymis, a significant fraction of the labeling was found to become associated with the anterior-tail domain. As a consequence, by the time the spermatozoa of the rat and mouse had reached the cauda epididymidis (Fig. 2C,D (rat) and Fig. 2G,H (mouse)), the vast majority of the cells had more labeling within the anterior-tail domain than within the posterior-tail domain (Fig. 3A,B). When the percentages of spermatozoa exhibiting the different localization patterns were measured at each stage, a clear trend of redistribution of labeling from the posterior-tail domain to the anterior-tail domain was evident for spermatozoa of the rat (Fig. 3A) and mouse (Fig. 3B). The situation observed for the spermatozoa of the hamster was significantly different, however, in that considerably more labeling was found to be localized to the anterior-tail domain at all stages of maturation (Figs 2 and 3). Thus, the vast majority of spermatozoa collected from the caput epididymidis (Fig. 2I,J) or testis (immunofluorescence data not shown) of the hamster had labeling that was more equally distributed between anterior-tail and posterior-tail domains (Fig. 3C). And by the time the spermatozoa had reached the cauda epididymidis of the hamster (Fig. 2K,L), the vast majority of the cells had labeling that was concentrated to an unusually high degree within the anterior-tail plasma membrane domain (Fig. 3C). Despite these differences, there was still evidence of a clear redistribution of labeling from the posterior-tail domain to the anterior-tail domain during epididymal maturation in the hamster (Fig. 3C). These data suggested that the net redistribution of spermatozoal CE9 from the posterior-tail domain to the anterior-tail domain during epididymal maturation in the rat was also experienced by CE9 homologues on the spermatozoa of the mouse and hamster.

Immunogold analysis

By virtue of the sharpness of the boundaries observed between the brightly labeled and unlabeled regions of the spermatozoal tails when examined by immunofluorescence (Fig. 2), it appeared that CE9 and its homologues could serve as markers for two different spermatozoal plasma membrane domains: the posterior-tail plasma membrane domain (spermatozoa from the testis and caput epididymidis of the rat and mouse; e.g. Fig. 2A,B and 2E,F) and the anterior-tail domain (spermatozoa from the cauda epididymidis of the hamster; Fig. 2K,L). Furthermore, on the basis of the position of the boundaries

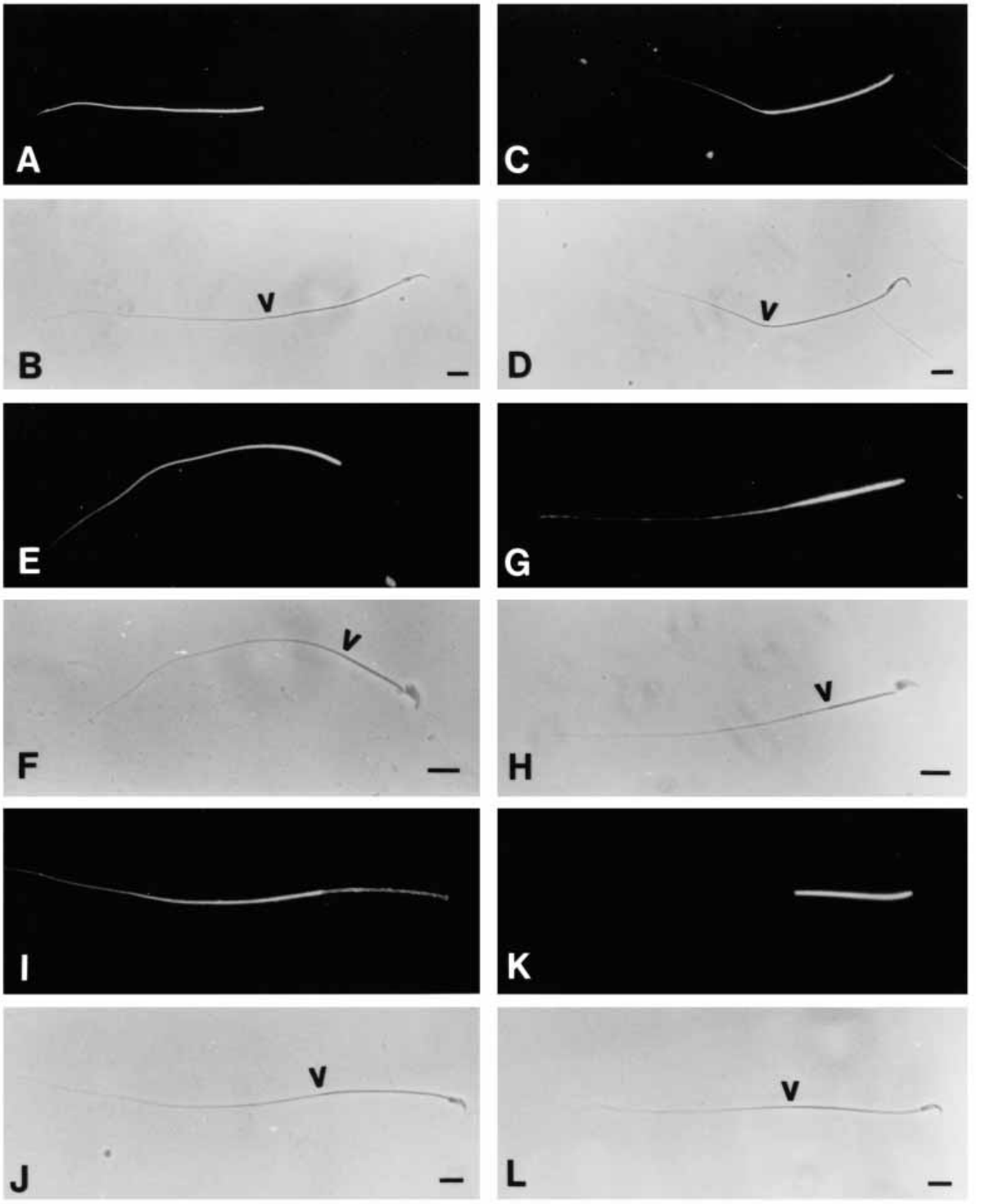

Fig. 2. Immunofluorescence of spermatozoa collected from the caput epididymidis (left panels) or cauda epididymidis (right panels) of the rat (A-D), mouse (E-H) or hamster (I-L). (B,D,F,H,J and L) Phase-contrast. Arrowhead, approximate position of junction between midpiece and principal piece of tail. Bars, 10 μm.

between the brightly labeled and unlabeled regions, both the posterior-tail and anterior-tail plasma membrane domains as defined by CE9 immunofluorescence appeared to have boundaries that coincided roughly with the junction of the midpiece and principal piece of the spermatozoal tail, i.e. near the position of the ultrastructural entity known as the annulus. Invisible by phase-contrast microscopy, the annulus is comprised of a ~200 nm thick ring of electron-dense material of unknown composition (Fawcett, 1970; Friend and Fawcett, 1974). It is positioned immediately subjacent to the plasma

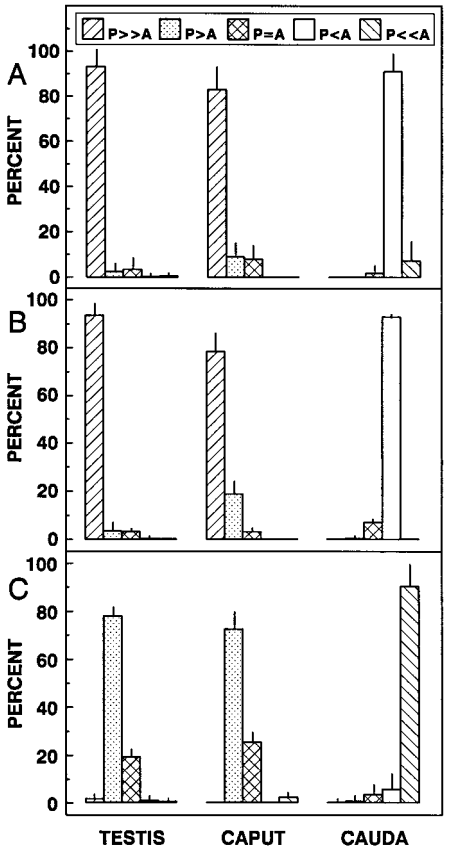

Fig. 3. Quantification of immunofluorescence patterns observed for spermatozoa collected from the testis, caput epididymidis or cauda epididymidis of the rat (A), mouse (B) or hamster (C). The immunofluorescence patterns observed were divided into categories on the basis of the relative labeling intensities of posterior-tail (P) and anterior-tail (A) domains. The percentages of spermatozoa in each category were plotted as mean ± s.d. for 3-15 experiments; 100 spermatozoa were analyzed in each experiment.

membrane between the last mitochondrion of the midpiece and the anterior-most element of the fibrous sheath and appears roughly triangular in cross-section in spermatozoa of the three species of rodent under examination (e.g. see Fig. 4A). To determine whether there was in fact any spatial correlation between the domain boundaries of spermatozoal CE9 and the annulus, we used immunogold electron microscopy to localize CE9 and its homologues on saponin-permeabilized, fixed spermatozoa obtained from rats, mice and hamsters. To help increase the likelihood of finding longitudinal views of the annular region of the spermatozoal tail, cells were fixed, immunolabeled, processed and embedded while oriented in a single plane on poly-L-lysine-coated coverslips.

In support of a surface localization for CE9 and its homologues, we found the specifically bound gold particles to be localized preferentially to the extracellular face of the spermatozoal plasma membrane irrespective of species (Fig. 4A-C). Furthermore, in support of the relatively high degree of domain specificity inferred from the results of our immunofluorescence experiments, the number of specifically bound gold particles per unit length of plasma membrane was typically found to be greater than 8- to 20-fold higher within those plasma membrane domains that showed a strict concentration of CE9 by immunofluorescence. In the course of our immunogold labeling experiments, we noted that we were able to achieve a very close packing of the 5 nm diameter gold particles over the posterior-tail plasma membrane domain of spermatozoa obtained from the caput epididymidis of the rat (Fig. 4A). When averaged over the 95 independent annular profiles examined, the average density of gold particles along the plasma membrane of the posterior-tail domain was found to be 14.8±2.2 particles/100 nm (range: 10.5-20.0 particles/100 nm). This amounted to an average spacing between gold particles of only 1.75 nm or 0.35 gold-particle diameters. These results suggested that we might be able to assign the position of the relevant domain boundary to the site where the dense gold labeling stopped, at least within the limits of resolution afforded by the overall length contributions of the antibodies and gold particles. Of course, since the primary and secondary antibodies each contribute a linear extension of about 11 nm (Sarma et al., 1971), we acknowledge that the position of a given domain boundary could actually reside anywhere within a space 11 nm + 11 nm + 2.5 nm = 24.5 nm (or 4.9 gold-particle diameters) on either side of the center of the last gold particle in a series.

In spermatozoa collected from the caput epididymidis of the rat, the thickness of the annulus at its maximum (i.e. subjacent to the plasma membrane) was found to be 178±29 nm. When we examined the distribution of immunogold labeling on these cells, we found evidence of a strong correlation between the position of the domain boundary and that of the annulus. A total of 90 out of 95, or 94.8%, of the independent annular profiles examined had a domain boundary - as defined by the center of the last gold particle in the series - that was positioned on the plasma membrane directly over the annulus (Figs 4A and 5A). The remaining 5 annular profiles out of the 95 examined had domain boundaries in the immediate vicinity; all were positioned within a distance of less than 0.1 units of annular thickness ahead of the anterior margin of the annulus (Fig. 5A). Thus, on average, the domain boundary was found to be positioned over the middle of the annulus, 0.59±0.25 units of annular thickness from its posterior margin (Fig. 5A).

The results of this immunogold localization study suggested that there was in fact a strong spatial correlation between the domain boundary of spermatozoal CE9 and the annulus, at least for spermatozoa collected from the caput epididymidis of the rat. To ascertain whether there were any detectable alterations in the ultrastructure of the annulus that accompanied the redistribution of spermatozoal CE9 from the posterior-tail domain to the anterior-tail domain during epididymal maturation, we performed electron microscopy on oriented preparations of spermatozoa taken from the caput, corpus, and cauda regions of the rat epididymis. The annuli of 50 sperm from each region were examined. Irrespective of region, greater than 98% of the spermatozoa showed no significant alteration in annular ultrastructure, suggesting that there were no such changes that accompanied epididymal maturation in the rat (data not shown).

We next wanted to compare the localizations of the domain boundaries on spermatozoa of the mouse and hamster. Unfortunately, in these two cases, the density of immunogold labeling was generally lower than that observed on the sper-

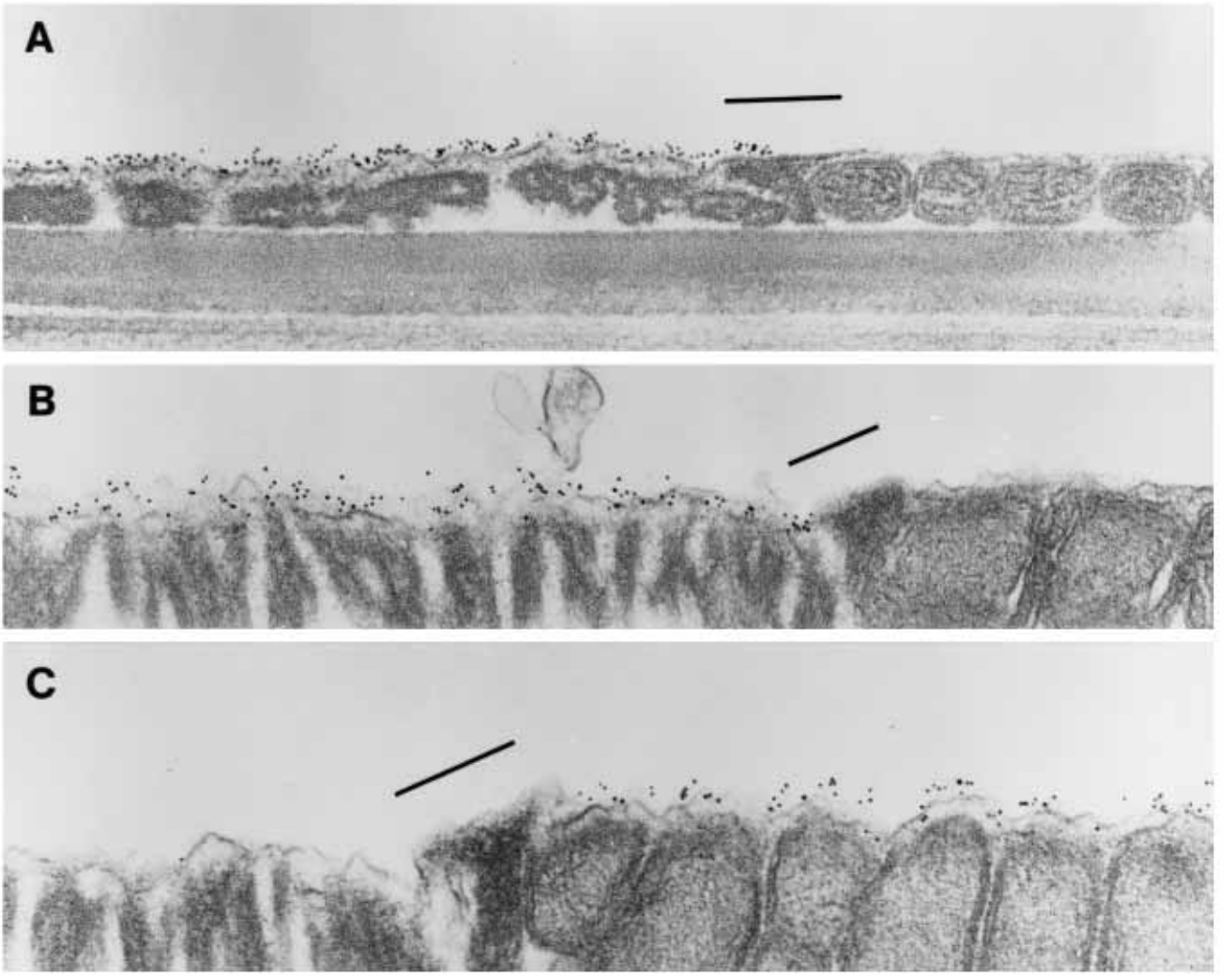

Fig. 4. Immunogold localization of CE9 on oriented spermatozoa collected from the caput epididymidis of the rat (A), caput epididymidis of the mouse (B) or cauda epididymidis of the hamster (C). Longitudinal views of spermatozoal tails depicting the region of the annulus are shown. The bar above each annulus designates annular thickness (193 nm (A), 170 nm (B) and 263 nm (C)). The anterior-tail domain is to the right (note mitochondria), and the posterior-tail domain is to the left (note fibrous sheath). The sections shown in (B) and (C) were cut in planes more tangential to the spermatozoal surface than the section shown in (A).

matozoa of the rat (e.g. cf. Fig. 4B,C to Fig. 4A), presumably because the antibody was raised against rat CE9. In addition, it was difficult to obtain as many spermatozoa from the caput epididymidis of the mouse simply because of the smaller size of the organ. Nevertheless, in an attempt to maximize the reliability of comparable measurements of the positions of domain boundaries on spermatozoa of the mouse and hamster, we only scored those subsets of independent annular profiles (10 for the mouse and 19 for the hamster) that showed greater than 8.5 gold particles/100 nm of plasma membrane in the vicinity of the annulus. This amounted to a maximum average spacing between gold particles of only 5.75 nm or just slightly greater than 1 gold-particle diameter. On spermatozoa collected from the caput epididymidis of the mouse, the position of the domain boundary - again, as defined by the center of the last gold particle in the series - was found to lie close to the posterior margin of the annulus (Figs 4B and 5B (crosshatched)). On average, the thickness of the annulus in sections of these mouse spermatozoa was found to be 170±30 nm, and the domain boundary was found to be 0.01±0.14 units of annular thickness behind the posterior margin of the annulus (Fig. 5B (crosshatched)). Conversely, the position of the domain boundary on spermatozoa obtained from the cauda epididymidis of the hamster was found to lie just in front of the anterior margin of the annulus (Figs 4C and 5B (stippled)). On average, the length of the annulus in these hamster spermatozoa was found to be 230±40 nm, and the domain boundary was found to be 1.05±0.11 units of annular thickness from the posterior margin of the annulus (Fig. 5B (stippled)). When the means were compared according to Student's *t*-test, the locations of these domain boundaries as measured by immunogold labeling were found to be significantly different ($P<0.001$) among all three experimental groups, suggesting that there may be interspecies and/or maturation-dependent differences in the position of the CE9 domain boundary in relation to the annulus.

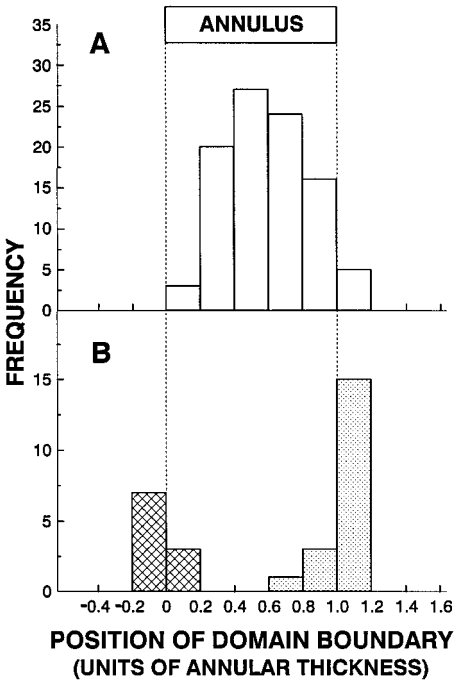

Fig. 5. Localization of CE9 domain boundaries on spermatozoa collected from the caput epididymidis of the rat (A), the caput epididymidis of the mouse (B, crosshatched) or the cauda epididymidis of the hamster (B, stippled) using immunogold electron microscopy. The position of a given domain boundary was taken as the center of the last gold particle in the densely packed series (see text). The distance of the domain boundary from the posterior margin of the annulus was measured and normalized on the basis of annular thickness. The frequency with which the domain boundary fell within increments of 0.2 units of annular thickness in either direction from the posterior margin of the annulus was plotted for the designated number of independent annular profiles examined: rat (95), mouse (10) and hamster (19). The posterior margin of the annulus lies at 0, and the anterior margin of the annulus lies at 1.0 (dashed lines).

DISCUSSION

The mammalian spermatozoon displays multiple plasma membrane domains that differ in their distributions of intramembranous particles, lectin binding sites, lipids, and/or protein antigens (e.g. see Koehler, 1978; Primakoff and Myles, 1983; Holt, 1984; Peterson and Russell, 1985; Eddy et al., 1985; Jones, 1987; Bearer and Friend, 1990). What is most remarkable is that the pattern of plasma membrane organization is not static: a number of spermatozoal surface proteins redistribute within the plasma membrane as a function of posttesticular maturation during epididymal transit, capacitation or the acrosome reaction (Myles and Primakoff, 1984; Jones et al., 1990; Phelps et al., 1990; Cowan et al., 1991; Petruszak et al., 1991). Why go to all this trouble? Cowan and Myles (1993) have postulated that it is the time of synthesis during spermatogenesis that determines the localization of a plasma membrane protein to a particular plasma membrane domain. If correct, then the redistribution of spermatozoal plasma membrane proteins might be one way to overcome the limitations on compartmentalization imposed by this mechanism in the absence of de novo synthesis. Additionally, we must entertain the possibility that the existence of complex protein life cycles - like that observed for spermatozoal CE9 - might reflect a deliberate attempt to keep molecules out of specific plasma membrane domains until the appropriate time during posttesticular maturation. Whatever the reason, there is mounting evidence that these redistributional events position spermatozoal surface proteins at the sites where they can carry out their functions for the mature spermatozoon (Primakoff et al., 1985; Primakoff et al., 1987). In the case of CE9, however, no such function has yet been identified. Nevertheless, the development of anti-CE9 antibodies that crossreact with homologues on the spermatozoa of hamsters and mice has allowed us to identify those aspects in the life cycle of the plasma membrane protein CE9 that are common to spermatozoa of multiple species and are, therefore, likely to be of functional significance.

Compartmentalization, endoproteolytic processing and redistribution of spermatozoal CE9: conservation among rats, mice and hamsters

Western blotting suggested that CE9 homologues in the mouse and hamster had apparent molecular masses similar to those of their counterparts in the rat. Judging from the observation that proteins of smaller apparent molecular mass first appeared on spermatozoa in the caput epididymidis, we suspect that the CE9 homologues on the spermatozoa of mice and hamsters also undergo an endoproteolytic processing event in the proximal regions of the caput epididymidis. Although the sequence of the hamster homologue is yet to be determined, the sequence of the mouse homologue of CE9 shows 85% amino acid sequence identity with the rat protein and includes the relevant arginine at the cleavage site in the extracellular tail (Altruda et al., 1989; Miyauchi et al., 1990; Nehme et al., 1993). The generality of this endoproteolytic processing event among species, coupled with the demonstration of related proteolytic events associated with the rearrangement of other protein antigens on the surface of the spermatozoon (Phelps et al., 1990; Blobel et al., 1990), suggests that endoproteolytic cleavage could serve as a trigger that unleashes a preset program of protein redistributional events within the plane of the spermatozoal plasma membrane.

Although a redistribution of spermatozoal CE9 from the posterior-tail plasma membrane domain into the anterior-tail plasma membrane domain proved to be characteristic of epididymal maturation in all three rodent species examined, there were some notable differences in the patterns of localization observed for the spermatozoa of the hamster. Specifically, in each segment of the reproductive tract examined, hamster spermatozoa exhibited a higher proportion of CE9 immunolabeling on their anterior-tail plasma membrane domains. One possible explanation is that the hamster homologue of CE9 does not become compartmentalized to the same degree during spermatogenesis. Alternatively, the hamster homologue may simply redistribute prematurely. If the latter explanation applied, then the results of the Western blotting suggest that the endoproteolytic cleavage of the hamster homologue of CE9 is neither domain specific nor a prerequisite for any such premature redistribution. Whatever the explanation for this difference, one unexpected outcome was that spermatozoa

collected from the cauda epididymidis of the hamster were found to have their CE9 homologues strictly compartmentalized to the anterior-tail plasma membrane domain. We have never observed such a strict compartmentalization of rat or mouse spermatozoal CE9 within the anterior-tail plasma membrane domain, even for spermatozoa collected from the vas deferens (Petruszak et al., 1991; and our unpublished results). This unanticipated finding for spermatozoa of the hamster gave us the opportunity to examine the boundaries of two different plasma membrane domains by immunogold localization of a single protein.

Localization of the domain boundaries of spermatozoal CE9: spatial correlation with the annulus

An immunogold electron microscopic analysis of densely labeled preparations of oriented spermatozoa allowed us to pinpoint the position of the domain boundary of the posterior-tail domain-specific integral plasma membrane protein CE9 at a high level of resolution. On spermatozoa collected from the caput epididymidis of the rat, the domain boundary was found to exhibit a strong spatial correlation with the annulus. On the vast majority of annular profiles examined, the domain boundary was found to be situated directly over the annulus, not at its anterior or posterior margin. The strength of this correlation strikes us as quite remarkable, considering that the annulus occupies less than 0.1% of the length of the rat spermatozoon. Although hampered somewhat by a lower density of gold labeling and a smaller sample size, the results of our immunogold studies of the spermatozoa of the mouse and hamster suggested that the location of the domain boundary differed significantly depending on the species and/or state of maturation in question. The domain boundary of posterior-tail CE9 on mouse spermatozoa was found on average to be positioned just behind the posterior margin of the annulus. On the basis of the immunogold labeling experiments of Friend (1989), one can infer a similar position for the domain boundary of the PT-1 antigen on spermatozoa collected from the cauda epididymidis of the guinea pig. In contrast, the domain boundary of anterior-tail CE9 on hamster spermatozoa was found on average to be positioned just ahead of the anterior margin of the annulus. Of course, we would have liked to have been able to compare the domain boundaries of posterior-tail and anterior-tail CE9 on spermatozoa collected from a single species. But, unfortunately, there are as yet no species identified that display such strict patterns of compartmentalization within both anterior-tail and posterior-tail domains.

Prediction of annular substructure: possible role for collections of intramembranous particles

One way to account for the observation that the domain boundaries of CE9 appear to take up such specific positions in relation to the annulus is to suppose that there are discrete interactions between the annulus and its overlying plasma membrane and that both entities have a significant substructure. One indication of the existence of such substructure is the presence of densely packed collections or concentric rows of intramembranous particles observed by freeze-fracture electron microscopy in the plasma membrane overlying the annuli of spermatozoa from the mouse, guinea pig, boar, goat, opossum and rat (Friend and Fawcett, 1974; Stackpole and Devorkin, 1974; Olson et al., 1977; Koehler, 1983; Kan and Pinto da Silva, 1987; Bains et al., 1993). But because freeze-fracture does not afford a direct view of the underlying electron-dense ring that makes up the annulus, the precise spatial relationships between these collections of intramembranous particles and the corresponding annuli remain somewhat uncertain. By analogy to structures like the tight junction of the epithelial cell (reviewed by Schneeberger and Lynch, 1992), the posterior striated ring of the spermatozoon (reviewed by Fawcett, 1975; Holt, 1984; Friend, 1989) and the flagellar bracelet of *Chlamydomonas* (Weiss et al., 1977; Hunnicutt et al., 1990), these collections of intramembranous particles in the plasma membrane overlying the annulus have been hypothesized to act as barriers to lateral diffusion (Myles et al., 1984; Holt, 1984; Friend, 1989; Petruszak et al., 1991). In the case of spermatozoal CE9, we know that such a barrier to lateral diffusion is involved in the compartmentalization of the protein to the posterior-tail plasma membrane domain of rat testicular sperm (Nehme et al., 1993). And one of the most likely places to find such a diffusion barrier is at the domain boundary that we have now determined to be situated directly over the annulus. We have not yet performed diffusion studies to determine whether some of the other domain boundaries we have pinpointed on the spermatozoa of other species might also act as diffusion barriers.

Our results are consistent with a model that involves the restriction of the lateral diffusion of posterior-tail CE9 by collections of intramembranous particles situated in the plasma membrane overlying the annulus (Myles et al., 1984; Holt, 1984; Friend, 1989; Petruszak et al., 1991; Nehme et al., 1993). In the context of this model, the position of the diffusion barrier would be determined by interactions between the intramembranous particles and the annulus, and the differential localization of the domain boundaries of CE9 could reflect the percolation of CE9 through these collections of intramembranous particles to differing extents. Alternatively, there could be interspecies variation in the spatial alignment between the collections of intramembranous particles and the annulus. The redistribution of spermatozoal CE9 observed for all three species of rodent during epididymal maturation would presumably require a reorganization of the collection of intramembranous particles to allow CE9 to either percolate or be dragged through into the anterior-tail plasma membrane domain. Observations made on the guinea pig PT-1 antigen during its redistribution from the posterior-tail domain to the anterior-tail domain during capacitation indeed suggest that lateral diffusion through the plasma membrane overlying the annulus is slower than that through the plasma membrane on either side of the annulus (Koppel et al., 1986). Further experiments will be required to determine whether CE9 is also free to diffuse once it reaches the anterior-tail plasma membrane domain. Through the use of a cell culture system, we recently obtained evidence that the redistribution of rat spermatozoal CE9 occurs after a lag period, during which the relevant barrier to lateral diffusion may be modified in what appears to be a temperature- and energy-dependent process (Nehme et al., 1993). In the context of the model, it is tempting to speculate that reorganization of the intramembranous particles in the plasma membrane overlying the annulus will prove to require intracellular ATP and a temperature in the physiological range.

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REFERENCES

Altruda, F., Cervella, P., Gaeta, M. L., Daniele, A., Giancotti, F., Tarone, G., Stefanuto, G. and Silengo, L. (1989). Cloning of cDNA for a novel mouse membrane glycoprotein (gp42): shared identity to histocompatibility antigens, immunoglobulins and neural-cell adhesion molecules. *Gene* **85**, 445-452.

Bains, H. K., Bawa, S. R., Pabst, M. A. and Sehgal, S. (1993). Plasma membrane alterations of maturing goat (Capra indicus) spermatozoa: Lectin-binding and freeze-fracture study. *Cell Tissue Res.* **271**, 159-168.

Bartles, J. R., Braiterman, L. T. and Hubbard, A. L. (1985). Endogenous and exogenous domain markers of the rat hepatocyte plasma membrane. *J. Cell Biol.* **100**, 1126-1138.

Bartles, J. R. and Hubbard, A. L. (1986). Preservation of hepatocyte plasma membrane domains during cell division in situ in regenerating rat liver. *Dev. Biol.* **118**, 286-295.

Bearer, E. L. and Friend, D. S. (1990). Morphology of mammalian sperm membranes during differentiation, maturation, and capacitation. *J. Electron Microsc. Technique* **16**, 281-297.

Bedford, J. M. (1975). Maturation, transport, and fate of spermatozoa in the epididymis. In *Handbook of Physiology*, section 7. Endocrinology, vol. 5 (ed. D. W. Hamilton and R. O. Greep), pp. 303-317. American Physiological Society, Bethesda.

Blobel, C. P., Myles, D. G., Primakoff, P. and White, J. M. (1990). Proteolytic processing of a protein involved in sperm-egg fusion correlates with acquisition of fertilization competence. *J. Cell Biol.* **111**, 69-78.

Bloodgood, R. A. (1988). Gliding motility and the dynamics of flagellar membrane glycoproteins in Chlamydomonas reinhardtii. *J. Protozool.* **35**, 552-558.

Cowan, A. E., Myles, D. G. and Koppel, D. E. (1987). Lateral diffusion of the PH-20 protein on guinea pig sperm: evidence that barriers to diffusion maintain plasma membrane domains in mammalian sperm. *J. Cell Biol.* **104**, 917-923.

Cowan, A. E., Myles, D. G. and Koppel, D. E. (1991). Migration of the guinea pig sperm membrane protein PH-20 from one localized surface domain to another does not occur by a simple diffusion-trapping mechanism. *Dev. Biol.* **144**, 189-198.

Cowan, A. E. and Myles, D. G. (1993). Biogenesis of surface domains during spermiogenesis in the guinea pig. *Dev. Biol.* **155**, 124-133.

Dotti, C. G. and Simons, K. (1990). Polarized sorting of viral glycoproteins to the axon and dendrites of hippocampal neurons in culture. *Cell* **62**, 63-72.

Dotti, C. G., Parton, R. G. and Simons, K. (1991). Polarized sorting of glypiated proteins in hippocampal neurons. *Nature* **349**, 158-161.

Eddy, E. M., Vernon, R. B., Muller, C. H., Hahnel, A. C. and Fenderson, B. A. (1985). Immunodissection of sperm surface modifications during epididymal maturation. *Amer. J. Anat.* **174**, 225-237.

Fawcett, D. W. (1970). A comparative view of sperm ultrastructure. *Biol. Reprod.* (suppl.) **2**, 90-127.

Fawcett, D. W. (1975). The mammalian spermatozoon. *Dev. Biol.* **44**, 394-436.

Fournier-Delpech, S. and Courot, M. (1987). Sperm-zona pellucida binding activity. In *Oxford Reviews of Reproductive Biology*, vol. 9 (ed. J. R. Clarke), pp. 294-321. Oxford University Press, Oxford.

Friend, D. S. and Fawcett, D. W. (1974). Membrane differentiations in freeze-fractured mammalian sperm. *J. Cell Biol.* **63**, 641-664.

Friend, D. S. (1989). Sperm maturation: membrane domain boundaries. *Ann. NY Acad. Sci.* **567**, 208-221.

Holt, W. V. (1984). Membrane heterogeneity in the mammalian spermatozoon. *Int. Rev. Cytol.* **87**, 159-194.

Hunnicutt, G. R., Kosfiszer, M. G. and Snell, W. J. (1990). Cell body and flagellar agglutinins in Chlamydomonas reinhardtii: the cell body plasma membrane is a reservoir for agglutinins whose migration to the flagella is regulated by a functional barrier. *J. Cell Biol.* **111**, 1605-1616.

Jones, R. (1987). Membrane remodelling during sperm maturation in the epididymis. In *Oxford Reviews of Reproductive Biology*, vol. 11 (ed. S. R. Milligan), pp. 285-337. Oxford University Press, Oxford.

Jones, R., Shalgi, R., Hoyland, J. and Phillips, D. M. (1990). Topographical rearrangement of a plasma membrane antigen during capacitation of rat spermatozoa in vitro. *Dev. Biol.* **139**, 349-362.

Kan, F. W. K. and Pinto da Silva, P. (1987). Molecular demarcation of surface domains as established by label-fracture cytochemistry of boar spermatozoa. *J. Histochem. Cytochem.* **35**, 1069-1078.

Kasinrerk, W., Fiebiger, E., Stefanova, I., Baumruker, T., Knapp, W. and Stockinger, H. (1992). Human leukocyte activation antigen M6, a member of the Ig superfamily, is the species homologue of rat OX-47, mouse basigin, and chicken HT7 molecule. *J. Immunol.* **149**, 847-854.

Koehler, J. K. (1978). The mammalian sperm surface: studies with specific labeling techniques. *Int. Rev. Cytol.* **54**, 73-108.

Koehler, J. K. (1983). Structural heterogeneity of the mammalian sperm flagellar membrane. *J. Submicrosc. Cytol.* **15**, 247-253.

Koppel, D. E., Primakoff, P. and Myles, D. G. (1986). Fluorescence photobleaching analysis of cell surface regionalization. In *Application of Fluorescence in the Biomedical Sciences* (ed. D. L. Taylor), pp. 477-497. Alan R. Liss, Inc., New York.

Miyauchi, T., Kanekura, T., Yamaoka, A., Ozawa, M., Miyazawa, S. and Muramatsu, T. (1990). Basigin, a new, broadly distributed member of the immunoglobulin superfamily, has strong homology with both the immunoglobulin V domain and the α-chain of major histocompatibility complex class II antigen. *J. Biochem.* **107**, 316-323.

Miyauchi, T., Masuzawa, Y. and Muramatsu, T. (1991). The basigin group of the immunoglobulin superfamily: complete conservation of a segment in and around the transmembrane domains of human and mouse basigin and chicken HT7 antigen. *J. Biochem.* **110**, 770-774.

Musgrave, A., de Wildt, P., van Etten, I., Pijst, H., Schloma, C., Kooyman, R., Homan, W. and van den Ende, H. (1986). Evidence for a functional membrane barrier in the transition zone between the flagellum and cell body of Chlamydomonas eugametos gametes. *Planta* **167**, 544-553.

Myles, D. G. and Primakoff, P. (1984). Localized surface antigens of guinea pig sperm migrate to new regions prior to fertilization. *J. Cell Biol.* **99**, 1634-1641.

Myles, D. G., Primakoff, P. and Koppel, D. E. (1984). A localized surface protein of guinea pig sperm exhibits free diffusion in its domain. *J. Cell Biol.* **98**, 1905-1909.

Nehme, C. L., Cesario, M. M., Myles, D. G., Koppel, D. E. and Bartles, J. R. (1993). Breaching the diffusion barrier that compartmentalizes the transmembrane glycoprotein CE9 to the posterior-tail plasma membrane domain of the rat spermatozoon. *J. Cell Biol.* **120**, 687-694.

Olson, G. E., Lifsics, M., Fawcett, D. W. and Hamilton, D. W. (1977). Structural specializations in the flagellar plasma membrane of opossum spermatozoa. *J. Ultrastruct. Res.* **59**, 207-221.

Orgebin-Crist, M.-C., Danzo, B. J. and Davies, J. (1975). Endocrine control of the development and maintenance of sperm fertilizing ability in the epididymis. In *Handbook of Physiology*, section 7. Endocrinology, vol. 5 (ed. D. W. Hamilton and R. O. Greep), pp. 319-338. American Physiological Society, Bethesda.

Peters, A., Palay, S. and Webster, H. (1991). *The Fine Structure of the Nervous System.* Oxford University Press, New York.

Peterson, R. N. and Russel, L. D. (1985). The mammalian spermatozoon: a model for the study of regional specificity in plasma membrane organization and function. *Tissue & Cell* **17**, 769-791.

Petruszak, J. A. M., Nehme, C. L. and Bartles, J. R. (1991). Endoproteolytic cleavage in the extracellular domain of the integral plasma membrane protein CE9 precedes its redistribution from the posterior to the anterior tail of the rat spermatozoon during epididymal maturation. *J. Cell Biol.* **114**, 917-927.

Phelps, B. M., Koppel, D. E., Primakoff, P. and Myles, D. G. (1990). Evidence that proteolysis of the surface is an initial step in the mechanism of formation of sperm cell surface domains. *J. Cell Biol.* **111**, 1839-1847.

Primakoff, P. and Myles, D. G. (1983). A map of the guinea pig sperm surface constructed with monoclonal antibodies. *Dev. Biol.* **98**, 417-428.

Primakoff, P., Hyatt, H. and Myles, D. G. (1985). A role for the migrating sperm surface antigen PH-20 in guinea pig sperm binding to the egg zona pellucida. *J. Cell Biol.* **101**, 2239-2244.

Primakoff, P., Hyatt, H. and Tredick-Kline, J. (1987). Identification and purification of a sperm surface protein with a potential role in sperm-egg membrane fusion. *J. Cell Biol.* **104**, 141-149.

Rodriguez-Boulan, E. and Powell, S. K. (1992). Polarity of epithelial and neuronal cells. *Annu. Rev. Cell Biol.* **8**, 395-427.

Sarma, V. R., Silverton, E. W., Davies, D. R. and Terry, W. D. (1971). The three-dimensional structure at 6 Å resolution of a human γG1 immunoglobulin molecule. *J. Biol. Chem.* **246**, 3753-3759.

Schlosshauer, B. and Herzog, K.-K. (1990). Neurothelin: an inducible cell

surface glycoprotein of blood-brain barrier-specific endothelial cells and distinct neurons. *J. Cell Biol.* **110**, 1261-1274.

Schneeberger, E. E. and Lynch, R. D. (1992). Structure, function and regulation of cellular tight junctions. *Amer. J. Physiol.* **262**, L647-L661.

Seulberger, H., Lottspeich, F. and Risau, W. (1990). The inducible blood-brain barrier specific molecule HT7 is a novel immunoglobulin-like cell surface glycoprotein. *EMBO J.* **9**, 2151-2158.

Stackpole, C. W. and Devorkin, D. (1974). Membrane organization in mouse spermatozoa revealed by freeze-etching. *J. Ultrastruct. Res.* **49**, 167-187.

Weiss, R. L., Goodenough, D. A. and Goodenough, U. W. (1977). Membrane particle arrays associated with the basal body and with contractile vacuole secretion in Chlamydomonas. *J. Cell Biol.* **72**, 133-143.

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