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

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Abstract. Originally identified as a basolateral domain-specific integral plasma membrane protein of the rat hepatocyte, CE9 mRNA and protein were also detected at high levels in the testis of the rat by Northern and Western blotting and immunoprecipitation. CE9 proved to be a domain-specific integral plasma membrane protein of the rat spermatozoon: on testicular spermatozoa, it was concentrated within the posterior tail domain of the plasma membrane, whereas on vas deferens spermatozoa, CE9 was concentrated within the anterior tail domain. This change in the localization of CE9 was observed to take place in a progressive fashion during the passage of the spermatozoa from the caput epididymidis to the cauda epididymidis and was preceded by the specific endoproteolytic cleavage of CE9 in the proximal portion of the caput epididymidis. Amino-terminal amino acid microsequencing of CE9 immunoaffinity purified from epididymis suggested that the cleavage occurred on the carboxy-terminal side of arginine-74 in the primary sequence of CE9, resulting in the loss of ~40% of the amino acids in the extracellular domain of this transmembrane glycoprotein.

PITHELIAL cells and other polarized cells, such as neurons and spermatozoa, compartmentalize their plasma membrane proteins to distinct domains, presumably to cope with an asymmetrical extracellular world (reviewed by Simons and Fuller, 1985). Previous studies have implicated tight junctions and/or the membrane skeleton in restricting the lateral diffusion of certain integral plasma membrane proteins on epithelial cells (reviewed by Rodriguez-Boulan and Nelson, 1989). However, considerably less is known about how this compartmentalization might be achieved by other types of polarized cells.

The life cycle of the mammalian spermatozoon affords a unique opportunity to examine the mechanisms by which a nonepithelial polarized cell establishes and remodels its plasma membrane domains. The spermatozoa of a variety of mammalian species have been observed to display distinct plasma membrane domains that differ in the distribution of intramembranous particles, lectin binding sites, lipids, and/or specific cell surface antigens (e.g., see Koehler, 1978; Friend, 1982; Primakoff and Myles, 1983; Holt, 1984). For the most part, these plasma membrane domains have been found to correlate with morphologically recognizable regions of the head and tail of the spermatozoon, e.g., the postacrosomal region of the head (posterior head) or the midpiece of the tail (anterior tail). As a consequence, it would be logical to assume that these domains were formed as the spermatids underwent morphological polarization during the process of spermiogenesis (Fawcett et al., 1971; Clermont, 1972; Hamilton, 1989). However, there is now mounting experimental evidence to support the notion that the plasma membrane domains of the mature spermatozoon are not constructed solely during spermiogenesis, but that they are also the result of extensive remodeling both during transit through the epididymis (Olson and Hamilton, 1978; Jones et al., 1983; Eddy et al., 1985; Holt, 1984; Jones, 1989; Phelps et al., 1990) and probably also during the processes of capacitation and the acrosome reaction (Friend et al., 1977; O'Rand, 1979; Myles and Primakoff, 1984).

Perhaps some of the more striking demonstrations of this concept of posttesticular remodeling at the level of specific plasma membrane proteins have come from recent analyses of the spermatozoa of the guinea pig: plasma membrane proteins distributed initially in a uniform fashion about the head (PH-30 and AH-50) or the head and tail (PH-20) of testicular spermatozoa become compartmentalized to specific regions of the head following epididymal maturation (Phelps et al., 1990). These changes in protein localization are thought to result from proteolysis at the surface of the guinea pig spermatozoon. Two of these proteins (PH-20 and PH-30) have been shown to undergo proteolytic cleavage upon transit of the spermatozoa through the epididymis, and a brief treatment of testicular spermatozoa with trypsin can simulate the

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effects of epididymal transit upon the localization of the plasma membrane proteins. Interestingly, one of these plasma membrane proteins (PH-20) exhibits a subsequent change in localization when spermatozoa obtained from the cauda epididymidis are made to undergo the acrosome reaction in vitro: under these conditions, PH-20 appears to migrate from the posterior to the anterior head of the spermatozoon, i.e., to the inner acrosomal membrane of the acrosome-reacted spermatozoon (Myles and Primakoff, 1984). Another example is the case of the cell surface antigen PT-1 which can be seen to migrate from the posterior to the anterior portion of the tail of the guinea pig spermatozoon when spermatozoa collected from the cauda epididymidis are placed in an artificial medium that supports their motility in vitro (Myles and Primakoff, 1984).

We have identified an integral plasma membrane glycoprotein of the rat spermatozoon called CE9. CE9 exhibits a novel combination of properties related to those enumerated above for the plasma membrane proteins of the guinea pig spermatozoon, but with many important differences. Originally identified as a basolateral-specific integral plasma membrane protein of rat hepatocytes (Hubbard et al., 1985; Bartles et al., 1985a,b), we have found that CE9 is compartmentalized to the posterior tail plasma membrane of testicular spermatozoa and then redistributes to the anterior tail plasma membrane upon transit of the spermatozoa through the epididymis. This redistribution is preceded by the endoproteolytic cleavage of CE9 in its extracellular domain.

Materials and Methods

Rats, Antibodies, and cDNA

Virus antibody-free male Fischer F344 (CDF) rats (225-250 g) were purchased from Charles River Breeding Laboratories (Wilmington, MA), maintained on a 14-h light/10-h dark cycle and given free access to rat chow and water for at least 1 wk before being used in experiments. Most experiments were conducted on rats weighing between 250 and 300 g. Mouse monoclonal and rabbit polyclonal antibodies directed against rat hepatocyte CE9 were prepared as described by Hubbard et al. (1985) and Bartles et al. (1985b), respectively. The polyclonal antibodies were affinity purified on columns of CE9-Sepharose as described by Bartles and Hubbard (1986). A cDNA encoding the entire rat hepatocyte CE9 protein was identified by using the affinity-purified polyclonal anti-CE9 antibodies to screen a rat liver lambda gt11 library. The details of the identification and characterization of this cDNA will be described in a separate communication (Nehme, C. L., J. A. M. Petruszak, and J. R. Bartles, manuscript in preparation).

Collection of Tissues and Spermatozoa

Rats were decapitated with a guillotine while maintained under ethyl ether anesthesia. Livers, testes, efferent ductules, epididymides, and vasa deferentia were removed surgically. The various segments of the epididymis, including the initial segment (Dym, 1983), were isolated by dissection with a scalpel blade. Spermatozoa were released from the testis and the segments of the epididymis by mincing in 2-3 vol (ml per g of moist tissue) of 4°C PBS (138 mM NaCl, 2 mM KCl, 38 mM NaP$_i$, pH 7.4) or the designated buffer with a scalpel blade followed by gentle gyratory shaking for 5-10 min in a small plastic Petri dish maintained in an ice-water bath. Residual tissue fragments were allowed to settle out for 1-2 min at unit gravity, and the supernate containing the spermatozoa was collected using a Pasteur pipet. In some experiments, testicular spermatozoa were separated from other testicular cells by subjecting 1.5-ml aliquots of such a minced preparation of testis made in 137 mM NaCl, 2.7 mM KCl, 8.1 mM NaP$_i$, 1.5 mM KP$_i$, pH 7.4, to centrifugation through a 10-ml gradient of 46% (vol/vol) Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) prepared in this same buffer for 10 min at 27,000 g in a rotor (JA 20; Beckman Instruments Inc., Palo Alto, CA) and J2-21 centrifuge at 10°C (Phelps et al., 1990). The testicular spermatozoa were collected in the lower one-third of the gradient above the

reddish layer containing erythrocytes, diluted threefold in PBS or the designated buffer and then washed twice by centrifugation for 20 min at 400 g in a rotor (AH-4; Beckman Instruments Inc.) and Accuspin centrifuge at 4°C. Spermatozoa were squeezed through a cut opening in the distal segment of the vas deferens by applying mild pressure with forceps on the outside surface of the vas deferens in the proximal to distal direction. These vas deferens spermatozoa were collected directly into 0.5-1 ml of 4°C PBS.

Northern and Western Blotting

Freshly isolated liver and testes were frozen and stored in liquid nitrogen. Total RNA was isolated by homogenization in guanidine thiocyanate, centrifugation in CsCl gradients, and precipitation with ethanol (Chirgwin et al., 1979). 10-20 μg samples of RNA (by A$_{260}$) were electrophoresed in formaldehyde-denaturing agarose gels, transferred to nitrocellulose by capillary action, and the blots were labeled with ^{32}P-CE9 cDNA prepared by random priming (Sambrook et al., 1989).

Spermatozoa or homogenates of freshly isolated liver, testis, or epididymis were prepared in the presence of a cocktail of protease inhibitors (1 mM PMSF, 1 μg/ml of antipain, 1 μg/ml of leupeptin, and 25 μg/ml of aprotinin) and resolved in reducing 7.5% polyacrylamide-SDS gels, transferred to nitrocellulose, and the blots were labeled with affinity-purified anti-CE9 antibody or preimmune IgG followed by ^{125}I-Protein A (Bartles et al., 1990). In some experiments, small segments of the epididymis were minced directly into hot SDS gel sample buffer and the resulting extracts analyzed by Western blotting. To distinguish soluble and peripheral membrane proteins from integral membrane proteins, homogenates of testis and epididymis were first treated with 50 mM sodium carbonate, pH 10.5, for 10 min at 4°C, centrifuged at 100,000 g for 60 min at 4°C and the supernate and pellet fractions analyzed by Western blotting (Fujiki et al., 1982).

Immunoprecipitation, Immunoaffinity Purification and NH$_2$-Terminal Amino Acid Microsequencing

CE9 was immunoprecipitated from Triton X-100/n-octyl-beta-D-glucoside extracts of homogenates prepared in the presence of protease inhibitors (see above) using monoclonal or polyclonal antibody-Sepharose, resolved in SDS gels and visualized by Western blotting (Bartles et al., 1985b). Immunoprecipitated CE9 was deglycosylated chemically using a combination of trifluoromethanesulfonic acid and anisole before being analyzed on Western blots (Bartles et al., 1985b). CE9 was purified on a larger scale in the presence of protease inhibitors (see above) from rat hepatocyte plasma membranes and epididymides by immunoaffinity chromatography on monoclonal and polyclonal antibody-Sepharose, respectively (Bartles et al., 1985b). 10-20 μg of immunoaffinity-purified CE9 was resolved in SDS gels, electrophoretically transferred to Immobilon membrane (Millipore Continental Water Systems, Bedford, MA), and subjected to gas-phase amino-terminal amino acid microsequencing by Dr. K.-L. Ngai using a protein sequencer (model 477A; Applied Biosystems, Foster City, CA) at the Northwestern University Biotechnology Facility (Evanston, IL).

Immunofluorescence

Spermatozoa were examined by immunofluorescence in agarose whole mounts. Freshly isolated preparations of spermatozoa were mixed with an equal volume of ice-cold 4% (wt/vol) paraformaldehyde in PBS and incubated for 5-10 min at 4°C. An equal volume of 60°C 3% (wt/vol) low-gelling temperature agarose (5510UA; Bethesda Research Laboratories, Gaithersburg, MD) was added and the mixture was allowed to gel into coverslip-thick layers between two microscope slides for 5 min at 4°C (Bartles et al., 1985a; deCamilli et al., 1983). Squares of the agarose whole mounts were quenched with 0.25% (wt/vol) NH$_4$Cl (2 × 5 min), incubated with affinity-purified polyclonal antibody or preimmune IgG (30 min), rinsed (3 × 5 min), incubated with rhodamine-labeled goat anti-rabbit IgG (15 min; Southern Biotechnology Associates, Birmingham, AL), and rinsed (3 × 5 min) in 0.15 M NaCl, 20 mM NaP$_i$, 3 mM NaN$_3$, pH 7.4, containing 0.5% (wt/vol) of BSA (ICN Biomedicals, Costa Mesa, CA), and 0.01% (wt/vol) of saponin (Sigma Chemical Co., St. Louis, MO) before being examined and photographed using a fluorescence microscope (E. Leitz, Inc., Rockleigh, NJ). In some experiments, spermatozoa isolated from the testis or caput epididymidis were treated with 0-20 μg/ml of trypsin (6502; Calbiochem-Behring Corp., La Jolla, CA) for 5 min at 22°C (Phelps et al., 1990) or spermatozoa obtained from the caput epididymidis were incubated in Mg^{2+}-MCM plus 1 mM KCl (102.3 mM NaCl, 25.1 mM NaHCO$_3$, 0.25 mM sodium pyruvate, 21.7 mM sodium lactate, 1 mM MgCl$_2$, 1 mM KCl, pH 7.4; Myles and Primakoff, 1984) or Mg^{2+}-Hepes

buffer (140 mM NaCl, 4 mM KCl, 4 mM Hepes-NaOH, 10 mM glucose, 2 mM MgCl$_2$, 100 μM EGTA, pH 7.4; Green, 1978) for 75 min at 37°C before fixation. In other experiments, preparations of spermatozoa in fixative were applied directly to poly-L-lysine–coated slides and allowed to dry at 22°C for 15 min before labeling.

Results

Detection of CE9 in the Testis and on Testicular Spermatozoa

In the course of our continuing studies on the expression and compartmentalization of integral plasma membrane proteins by the rat hepatocyte (Bartles et al., 1985a,b, 1987, 1990, 1991a,b), we encountered one such protein—CE9—whose mRNA (1.5 kb) could also be detected at high levels on Northern blots of RNA isolated from rat testis (Fig. 1; liver, lane a, testis, lane b). When the complementary comparison was carried out on Western blots of reducing SDS gels of testicular homogenate, we detected a multiplet of immunologically cross-reactive bands ranging from 40–48 kD (Fig. 1, lane d); most of the cross-reactive material was observed to migrate between 40 and 42 kD, i.e., slightly faster than the
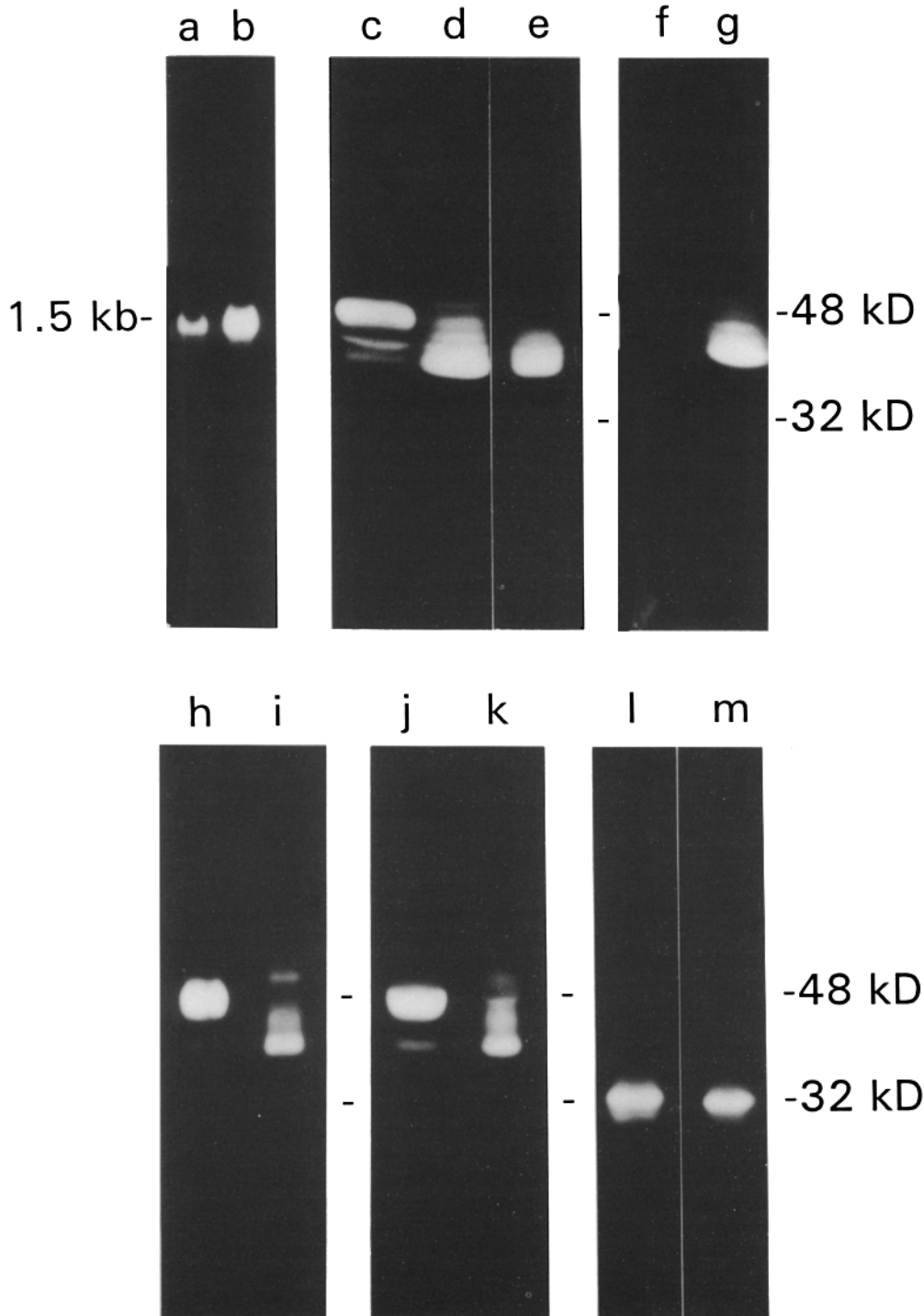
Figure 1. Biochemical comparison of hepatic and testicular CE9. (Lanes a and b) Northern blot of total RNA isolated from liver (lane a) and testis (lane b). (Lanes c-e) Western blot of homogenate of liver (lane c), homogenate of testis (lane d), and isolated testicular spermatozoa (lane e). (Lanes f and g) Western blot of the supernate (lane f) and pellet (lane g) resulting from the extraction of homogenate of testis with 50 mM sodium carbonate, pH 10.5. (Lanes h-k) Western blot of CE9 immunoprecipitated from detergent extract of liver (lanes h and j) or testis (lanes i and k) by monoclonal (lanes h and i) or polyclonal (lanes j and k) antibody-Sepharose. (Lanes l and m) Western blot of CE9 immunoprecipitated from detergent extract of liver (lane l) or testis (lane m) and deglycosylated with trifluoromethanesulfonic acid and anisole.

48-kD species which predominates in homogenate of liver (Fig. 1, lane c). A similar multiplet of bands (minus one or two of the more minor, slower-migrating bands) was also detected when samples of testicular spermatozoa, isolated from other testicular cells in gradients of Percoll, were analyzed on Western blots (Fig. 1, lane e). These crossreactive proteins appeared to represent bonafide CE9, because: (a) they were not detected on Western blots when preimmune IgG was substituted for the affinity-purified anti-CE9 antibody (data not shown); and (b) they could be immunoprecipitated from nonionic detergent extracts of testis with either monoclonal or polyclonal anti-CE9 IgG (Fig. 1, lanes i and k, respectively; cf., immunoprecipitates from liver, lanes h and j), but not unrelated IgGs (data not shown). The difference in electrophoretic mobility between the hepatic and testicular forms of CE9 appeared to reflect differential glycosylation of the same polypeptide, because both could be converted to the same apparent molecular mass (32 kD) by chemical deglycosylation with trifluoromethanesulfonic acid and anisole (Fig. 1; liver, lane l; testis, lane m). On the basis of its resistance to extraction from particulate fraction of testis in the presence of 50 mM sodium carbonate, pH 10.5 (Fig. 1; supernate, lane f; pellet, lane g) and its ready solubility in nonionic detergents (such as the combination of Triton X-100 and n-octyl-beta-D-glucopyranoside used in the immunoprecipitations) (Fig. 1, lanes i and k), the testicular form of CE9 appeared to be neither a peripheral membrane protein nor a soluble protein, but an integral membrane protein.

Localization of CE9 on Spermatozoa Collected from the Testis, Epididymis, and Vas Deferens

When we localized CE9 on testicular spermatozoa, we found it to be concentrated on the posterior portion of the tail of the spermatozoon, i.e., on the principal and end pieces situated behind the annulus, but not on the head of the spermatozoon or on the anterior portion of the tail (midpiece) (Fig. 2, a and b). This posterior tail localization was observed for 85-99% of the testicular spermatozoa, whether examined in minced preparations of testis or after isolation in gradients of Percoll (Table I). In contrast, when we localized CE9 on spermatozoa collected from the vas deferens, we observed the labeling to be concentrated over the anterior tail of 67-98% of the spermatozoa (Fig. 2, c and d; Table I). These localization patterns were observed under a variety of labeling conditions: e.g., with collection of spermatozoa at 4°C, room temperature, or 37°C; with or without prior fixation in 1-4% paraformaldehyde or 2% paraformaldehyde-lysine-periodate (McLean and Nakane, 1974); with or without 0.01% saponin as a membrane-permeabilizing agent; and with spermatozoa embedded in agarose or simply dried upon polylysine-coated slides. At the exposures shown, no labeling was apparent when preimmune IgG was substituted for the affinity-purified polyclonal anti-CE9 antibody (data not shown).

We also compared the localization of CE9 on spermatozoa collected from different segments of the epididymis (Dym, 1983). Although the frequency with which we observed a particular localization pattern at a given site within the epididymis varied somewhat from rat to rat, in each case we observed a progressive shift in the localization of CE9 from the posterior tail to the anterior tail coincident with transit of the spermatozoa through the epididymis (Fig. 3 and Table

I). Spermatozoa collected from the caput epididymidis typically displayed a posterior tail labeling pattern (Fig. 3 a and Table I) like that of the testicular spermatozoa (Fig. 2, a and b). But a small percentage of these spermatozoa also showed a significant amount of labeling over the anterior tail (Fig. 3 b and Table I). On spermatozoa collected from the cauda epididymidis, the labeling was concentrated over the anterior tail (Fig. 3, d and e and Table I), giving rise to a pattern like that shown above for spermatozoa collected from the vas deferens (Fig. 2, c and d). Spermatozoa collected from the corpus epididymidis reliably showed a labeling pattern that was intermediate between these two extremes, with the label being roughly equally distributed between the anterior and posterior tail domains (Fig 3 c and Table I).

Biochemical Changes in the CE9 Detected on Epididymal Spermatozoa

To determine whether there were any biochemical alterations in CE9 that might be correlated with the change in the protein's localization on the rat spermatozoon during epididymal maturation, we compared the electrophoretic mobility of the CE9 of spermatozoa collected from different sites along the male reproductive tract. We found that the CE9 of spermatozoa collected from the cauda epididymidis or the vas deferens migrated as a 23-33-kD multiplet on Western blots of reducing SDS gels (Fig. 4; cauda epididymidis, lane b; vas deferens, lane d), i.e., considerably faster than the 40-48-kD testicular form of CE9 (Fig. 4, lane a). At reduced exposures, it was apparent that two bands at the center of the multiplet (25 and 27 kD) were the major species. The spermatozoa collected from the caput epididymidis also expressed the 23-33-kD multiplet, but retained a small fraction of material that comigrated with the testicular form (Fig. 4, lane c). As was observed for testicular CE9, the 23-33-kD multiplet was not labeled on Western blots when preimmune IgG was substituted for affinity-purified polyclonal anti-CE9 antibody (data not shown), but it was specifically immunoprecipitated from nonionic detergent extracts of epididymal homogenate (pooled from all regions, caput through cauda) with polyclonal anti-CE9 IgG (Fig. 4, lane h; cf., immunoprecipitate from testis, lane g). However, in contrast to testicular CE9, that associated with the pooled epididymal spermatozoa could not be immunoprecipitated with Sepharose conjugates of the monoclonal anti-CE9 antibody (Fig. 4, lane f; cf., immunoprecipitate from testis, lane e), suggesting that the 23-33-kD form of CE9 had lost the epitope that reacted with the monoclonal anti-CE9 antibody in immunoprecipitation reactions.

To determine whether the decrease in the apparent molecular mass of CE9 observed upon entry of the spermatozoa into the epididymis was the result of proteolysis, we isolated CE9 from nonionic detergent extracts of rat epididymal homogenate by immunoaffinity chromatography using the polyclonal anti-CE9 antibody and then subjected the material migrating between 23 and 33 kD in SDS gels to gas-phase amino-terminal amino acid microsequencing. The results shown in Fig. 5 indicate that 23-33-kD form of the protein had a single amino-terminal sequence and that this sequence was different from that observed for the hepatic, and presumably also the testicular form of CE9, since both are encoded by a 1.5-kb mRNA species (Fig. 1, lanes a and b) and can be seen to comigrate in reducing SDS gels following chemi-
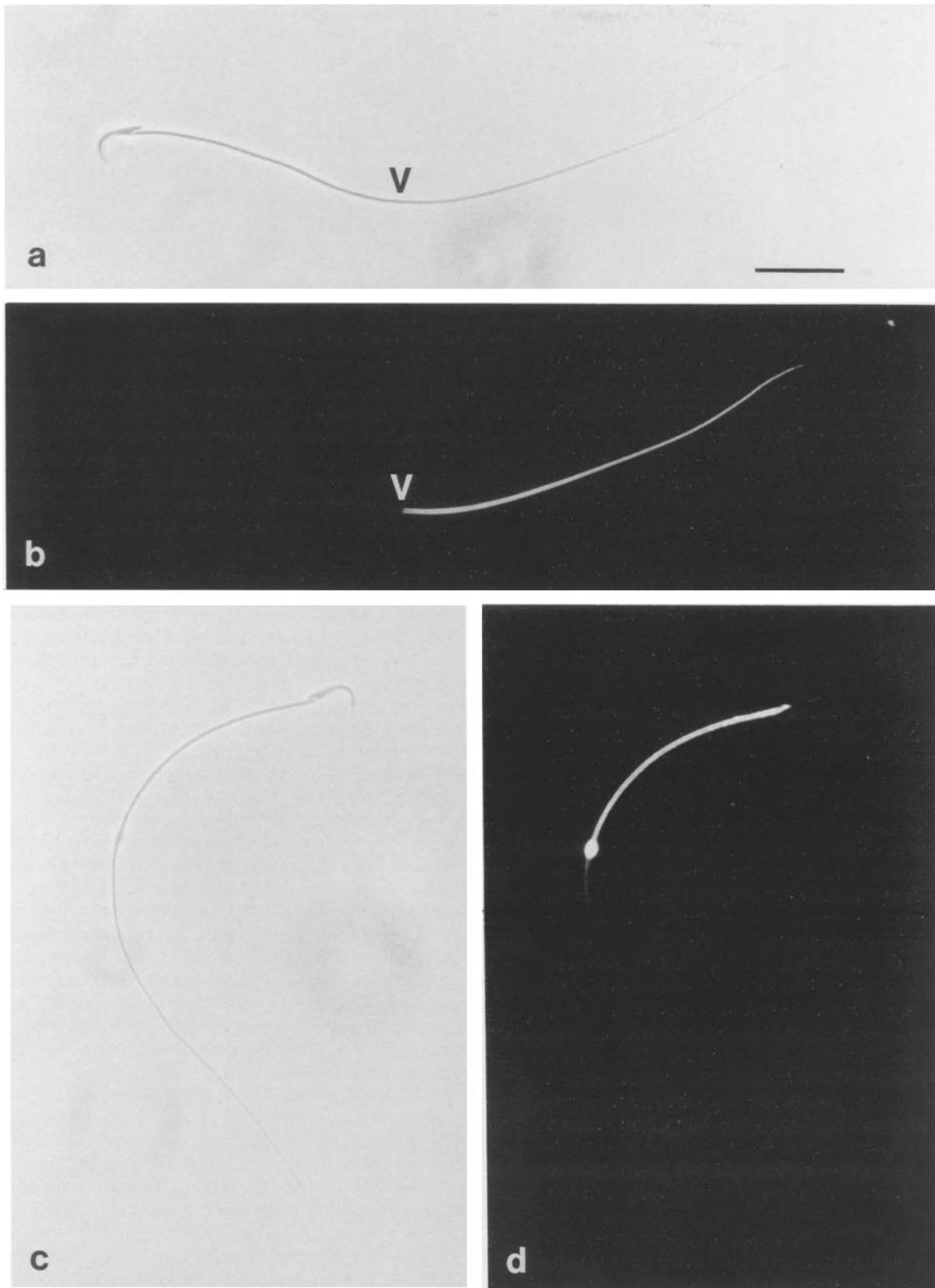
Figure 2. Immunofluorescence localization of CE9 on spermatozoa from testis (*a* and *b*) and vas deferens (*c* and *d*). *a* and *c*, phase contrast; *b* and *d*, fluorescence. Arrowhead in *a* and *b*, annulus that demarcates the posterior and anterior tail. Bar, 20 µm.

Table I. Localization of CE9 on Spermatozoa Obtained from Different Segments of the Male Reproductive Tract

Source of spermatozoa	No. of experiments	Localization			
		PT	PT > AT	PT = AT	PT < AT
		%			
Testis	2	85–92	3–6	5–9	0
Testis (Percoll gradient)	2	95–99	1–5	0	0
Initial segment of caput epididymidis	2	72–84	6–11	10–19	0
Caput epididymidis	9	83 ± 9	9 ± 6	8 ± 6	0
Corpus epididymidis	3	1 ± 1	3 ± 6	96 ± 6	0
Cauda epididymidis	3	0	0	16 ± 10	84 ± 10
Vas deferens	2	0	0	2–33	67–98

Spermatozoa were collected from the designated sources, fixed, and embedded in agarose. The localization of CE9 was determined by immunofluorescence. A total of 100 spermatozoa obtained from the designated source were analyzed in each experiment. The data are reported either as ranges for two independent experiments or as mean ± SD for numbers of independent experiments greater than two. *PT,* posterior tail; *AT,* anterior tail. *PT,* patterns similar to those shown in Figs. 2, *a* and *b* or 3 *a*; *PT > AT,* patterns intermediate to those shown in Fig. 3, *a* and *b*; *PT = AT,* patterns intermediate to those shown in Fig. 3, *b* and *c*; *PT < AT,* patterns similar to those shown in Figs. 2, *c* and *d* or 3, *d* and *e*.

cal deglycosylation (Fig. 1, lanes *l* and *m*). Repeated attempts to isolate testicular CE9 for comparative microsequencing have failed because of an inability to resolve it sufficiently from a contaminant which is most likely actin (data not shown). However, we have recently isolated and sequenced a cDNA corresponding to the entire coding sequence of CE9 (Nehme, C. L., J. A. M. Petruszak, and J. R. Bartles, manuscript in preparation). As a consequence, we have been able to pinpoint the 10 amino-terminal residues of the 23–33-kD epididymal CE9 to an internal stretch of amino acids ranging from glycine-75 through arginine-84 of the full-length CE9 polypeptide. This places the putative proteolytic cleavage site on the carboxy-terminal side of arginine-74, and, based upon the disposition predicted for CE9 in the plasma membrane bilayer (Nehme, C. L., J. A. M. Petruszak, and J. R. Bartles, manuscript in preparation), this cleavage would be expected to result in the loss of 74 of the 186 amino acids in the extracellular domain of this transmembrane protein (Fig. 5). One prediction from these findings is that 23–33-kD form of CE9 should remain an integral plasma membrane protein and not become a peripheral membrane protein or a soluble protein of the epididymal fluid. Consistent with this expectation, the 23–33-kD form of CE9 was found to resist extraction from the particulate fraction of epididymal homogenate with 50 mM sodium carbonate, pH 10.5 (Fig. 4; supernate, lane *i*; pellet, lane *j*), yet it was readily extracted by nonionic detergents, e.g., in preparation for immunoprecipitation (Fig. 4, lane *h*).

To attempt to better pinpoint the site in the male reproductive tract at which the proteolytic cleavage of spermatozoal CE9 took place, we analyzed SDS gel sample buffer extracts of successive 2–3-mm segments of the caput epididymidis by Western blotting. We observed a progressive conversion of CE9 to a faster migrating form between the first and third such segment (Fig. 4, lanes *k–m*). Although the mobilities of the CE9 in these samples prepared by direct extraction into hot SDS gel sample buffer were slightly different, the higher molecular mass form of CE9 detected in the first segment was observed to comigrate with the testicular and efferent ductular forms of CE9 when processed and analyzed in parallel (data not shown), and the lower molecular mass form detected from the second 2–3-mm segment of the caput epididymidis on was observed to comigrate with the CE9 of

spermatozoa collected from the cauda epididymidis (Fig. 5, lane *n*). In no instance did we detect the accumulation of significant amounts of intermediates with mobilities between the testicular and epididymal forms.

The Effects of Trypsinization and Incubation In Vitro on the Localization of Spermatozoal CE9

We wanted to establish whether the CE9 actually underwent a redistribution on the tail of the spermatozoon or whether new CE9 epitopes were somehow availed in the anterior tail upon movement of the spermatozoa through the epididymis. A partial answer to this question may be obtained simply by reviewing the immunofluorescence micrographs shown in Fig. 3. These spermatozoa were collected from the different segments of the epididymis of a single rat and were fixed, embedded, immunolabeled, and photographed in parallel under identical conditions. Note that the overall labeling intensity did not appear to undergo a marked change during epididymal transit. This observation was consistent with the idea that the change in the localization of CE9 was the result of the redistribution of existing CE9 molecules along the tail of the spermatozoon rather than an unmasking of a large number of new CE9 epitopes located in the anterior tail.

Since trypsinization had recently been shown to alter the compartmentalization of certain plasma membrane proteins on the guinea pig spermatozoon (Phelps et al., 1990), we also wanted to examine the effect of such a trypsin treatment on the localization of CE9. Of course, for such a mechanism to be responsible for the observed change in the localization of CE9, we would have to hypothesize that trypsinization would either elicit a redistribution of CE9 epitopes from the posterior to the anterior tail or somehow avail new CE9 epitopes on the anterior tail while simultaneously destroying those present originally on the posterior tail. We subjected spermatozoa isolated from the testis and caput epididymidis to digestion with 5, 10, or 20 µg/ml of trypsin for 5 min at room temperature according to the procedure of Phelps et al. (1990), and, in no case, did we observe a significant change in the localization of CE9 from posterior tail to anterior tail (Table II). Furthermore, trypsinization under these conditions did not result in the proteolysis of the 40–48-kD testicular form of CE9 (Fig. 5; control, lane *o*; treated with 20 µg/ml of trypsin, lane *p*), nor did it cause a perceptible
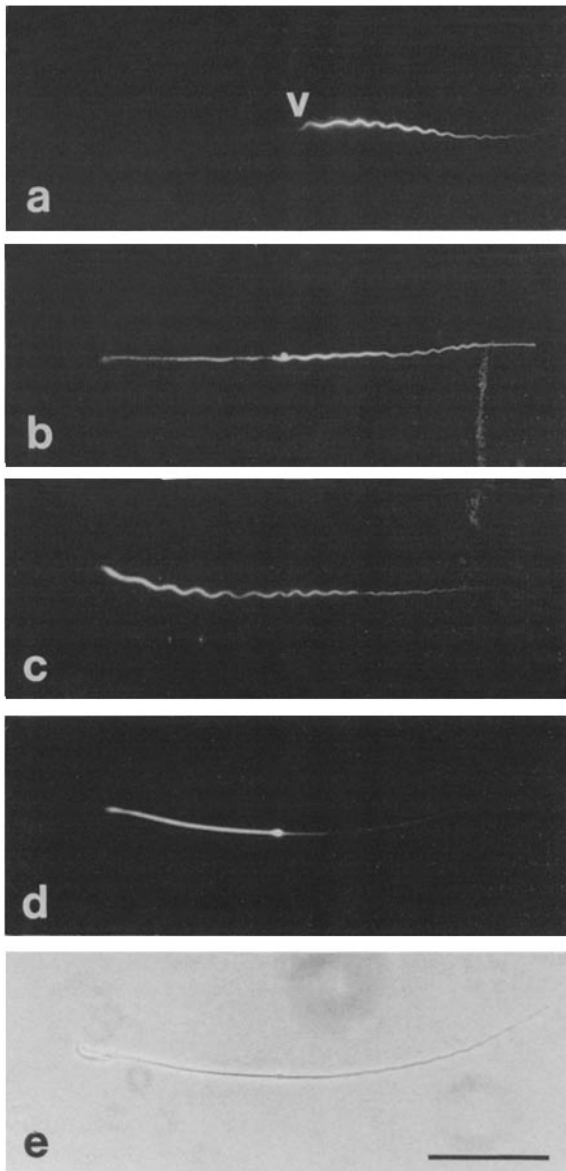
Figure 3. Immunofluorescence localization of CE9 on spermatozoa collected from the caput epididymidis (*a* and *b*), corpus epididymidis (*c*), and cauda epididymidis (*d* and *e*) of a single rat. *a–d*, fluorescence; *e*, phase contrast image of *d*. The spermatozoa were fixed, embedded, labeled, and photographed in parallel under identical conditions and are coaligned with their heads at the left. Arrowhead in *a*, annulus. Bar, 40 μm.

decrease in fluorescent labeling intensity (data not shown). But what we did observe was the loss of the heads from a significant fraction of those spermatozoa treated with the higher concentrations of trypsin (Table II, numbers in parentheses). The inability to unmask any additional CE9 epitopes in the anterior tail domain with exogenous trypsin lent additional support to the notion that the pre-existing CE9 epitopes actually redistributed along the tail of the spermatozoon during epididymal transit.

The PT-1 antigen of the guinea pig spermatozoon remains compartmentalized to the posterior tail of spermatozoa collected from the cauda epididymidis, but it can be made to migrate from the posterior to the anterior tail when these spermatozoa are placed into an artificial medium (Myles and Primakoff, 1984; Myles et al., 1984). To determine whether we might be able to elicit a similar redistribution of CE9 in vitro, we incubated spermatozoa obtained from the caput epididymidis under those conditions that resulted in maximim redistribution of PT-1 on guinea pig spermatozoa (namely, up to 75 min at 37°C in Mg^{2+}–MCM buffer plus 1 mM KCl; Myles and Primakoff, 1984) or in another buffer which seemed to be effective at supporting the viability of the spermatozoa, Mg^{2+}-Hepes buffer (Green, 1978). In neither case did any significant redistribution of CE9 occur (Table II).

Discussion

CE9 as Domain-specific Integral Plasma Membrane Protein of Hepatocytes and Spermatozoa

We have identified CE9 as an integral plasma membrane protein that exhibits a complex pattern of expression and modification during the life cycle of the rat spermatozoon. To our knowledge, CE9 represents the first clear example of an integral plasma membrane protein that is compartmentalized to specific plasma membrane domains by both epithelial cells (hepatocytes; see Hubbard et al., 1985; Bartles et al., 1985*a,b*, 1990) and spermatozoa. The only possible precedent of which we are aware is a galactose-binding protein immunologically crossreactive with the rat hepatic lectin-2/3 subunit of the asialoglycoprotein receptor of rat hepatocytes that has been found to be concentrated on the surfaces of certain rat spermatogenic cells and over the dorsal head region of rat epididymal spermatozoa (Abdullah and Kierszenbaum, 1989).

Following spermiogenesis, CE9 is compartmentalized to the posterior tail plasma membrane domain of testicular spermatozoa. Does this mean that we should somehow equate the posterior tail domain of the spermatozoon with the basolateral plasma membrane domain of the hepatocyte? For that matter, when does CE9 perform its biological function for the spermatozoon, before or after its proteolytic cleavage and appearance in the anterior tail plasma membrane domain? In the absence of any conclusive information regarding the biological function of CE9, it is difficult to even venture a guess. Nevertheless, we are actively exploiting CE9 as a means to compare and contrast the mechanisms by which diverse polarized cell types establish and maintain their plasma membrane domains. For example, will CE9 prove (by direct analogy to the case of the hepatocyte; Bartles et al., 1987) to be targeted directly to the posterior tail plasma membrane domain of an already polarized late spermatid or spermatozoon? Or will there prove to be a more random initial insertion of this transmembrane glycoprotein into the plasma membrane of a spermatogonium, spermatocyte, or early spermatid followed by its specific removal and/or redistribution during spermiogenesis? In preliminary immunofluorescence experiments examining the localization of CE9 on semithin frozen sections of rat testis, we have observed the most intense labeling to be that associated with the elongated spermatids and spermatozoa near to or within the lumina of the seminiferous tubules (unpublished results). Additional experiments, including attempts to localize CE9
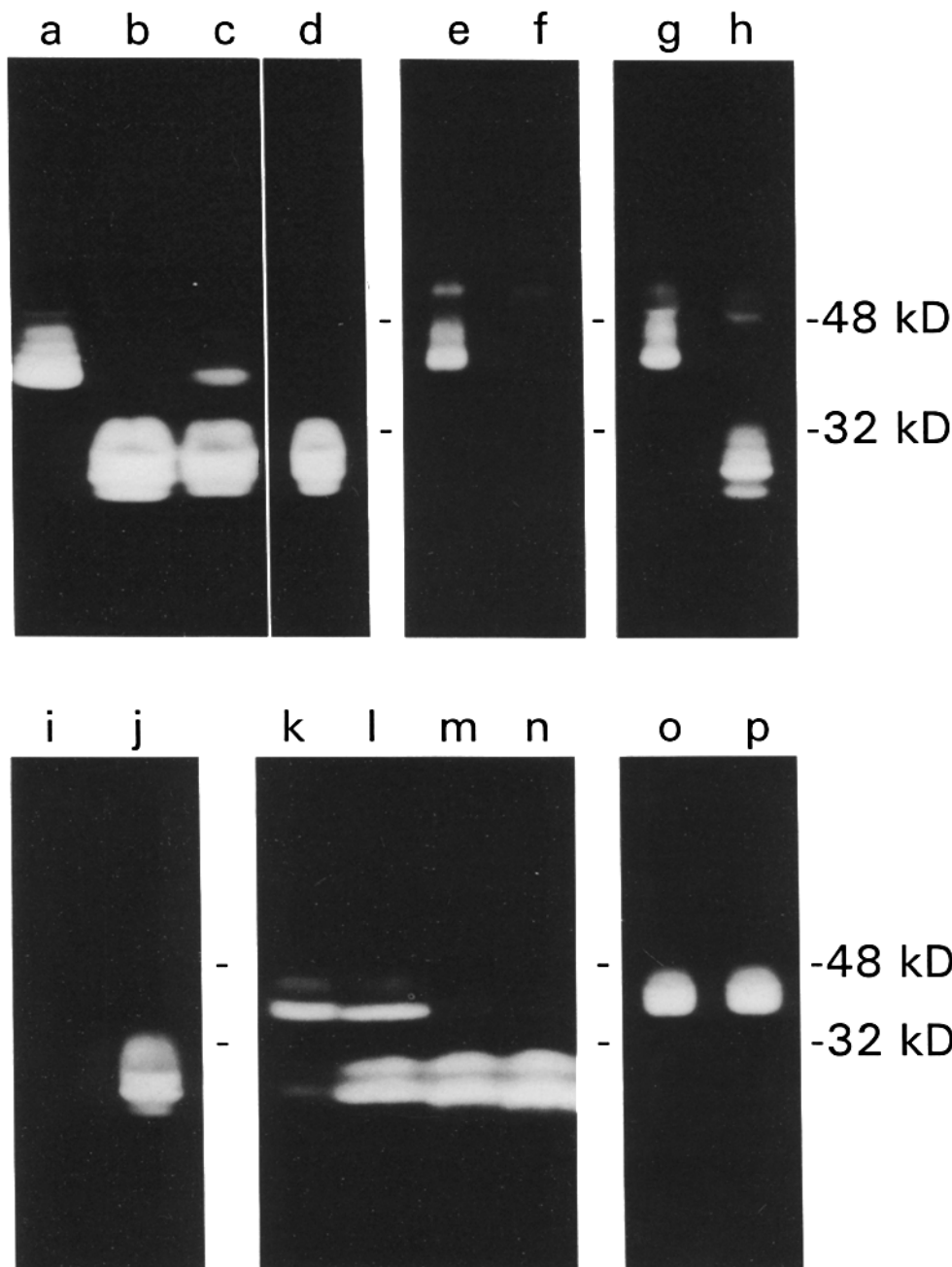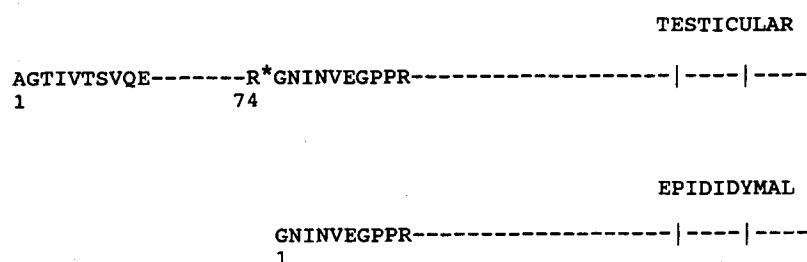
Figure 4. Biochemical comparison of testicular and epididymal CE9. (Lanes *a–d*) Western blot of homogenate of testis (lane *a*), caput epididymidis (lane *c*), and cauda epididymidis (lane *b*) and isolated vas deferens spermatozoa (lane *d*). (Lanes *e–h*) Western blot of CE9 immunoprecipitated from detergent extract of testis (lanes *e* and *g*) or total epididymis (lanes *f* and *h*) by monoclonal (lanes *e* and *f*) or polyclonal (lanes *g* and *h*) antibody-Sepharose. (Lanes *i* and *j*) Western blot of the supernate (lane *i*) and pellet (lane *j*) resulting from the extraction of homogenate of epididymis with 50 mM sodium carbonate, pH 10.5. (Lanes *k–n*) Western blots of gel sample buffer extracts of the first three 2–3-mm-thick segments of the caput epididymidis (proximal-to-distal) (lanes *k–m*) and the cauda epididymidis (lane *n*). (Lanes *o* and *p*) Western blot of isolated testicular spermatozoa without (lane *o*) and with (lane *p*) treatment with 20 μg/ml of trypsin for 5 min at 22°C.

on testicular cells obtained by mechanical and/or enzymatic dissociation, will be required to establish whether the bulk of testicular CE9 is indeed expressed only relatively late in spermiogenesis or whether there are certain limitations on antibody accessibility that interfere with the labeling of other testicular cells in the semithin sections.

Differences between CE9 and the PT-1 Antigen of Guinea Pig Spermatozoa

To our knowledge, there is only a single precedent for a posterior tail–specific plasma membrane protein on mammalian spermatozoa: the PT-1 antigen detected on the sper-

TESTICULAR

```
AGTIVTSVQE-------R*GNINVEGPPR-------------------|----|----
1              74
```

EPIDIDYMAL

```
GNINVEGPPR-------------------|----|----
1
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Figure 5. Schematic depiction of the partial primary structure and disposition predicted for the CE9 of testicular (*upper*) and epididymal (*lower*) spermatozoa on the basis of amino-terminal amino acid microsequencing and cDNA sequencing. The extracellular domain lies to the left of the two vertical bars which signify the site of insertion into the plasma membrane. The putative site of endoproteolytic cleavage is after arginine-74 of testicular CE9 (*asterisk*).

Table II. The Effects of Various Treatments on the Localization of CE9 on Spermatozoa

Source of spermatozoa	Treatment	Posterior tail localization
		%
Testis (Percoll gradient)	None	99(0)
"	5 min, 22°C, 5 µg/ml trypsin	94(12)
"	5 min, 22°C, 10 µg/ml trypsin	95(25)
"	5 min, 22°C, 20 µg/ml trypsin	95(58)
Caput epididymidis	None	83(0)
"	5 min, 22°C, 5 µg/ml trypsin	81(28)
"	5 min, 22°C, 10 µg/ml trypsin	78(32)
"	5 min, 22°C, 20 µg/ml trypsin	81(46)
Caput epididymidis	None	82
"	75 min, 37°C, PBS	82
"	75 min, 37°C, Mg^{2+}-MCM + 1 mM KCl	76
"	75 min, 37°C, Mg^{2+}-Hepes	84

Spermatozoa were collected from the designated sources and, after the indicated treatment (see Materials and Methods for details), were fixed and embedded in agarose. The localization of CE9 was determined by immunofluorescence. A total of 100 spermatozoa were analyzed for each condition. The failure of either type of treatment to elicit a significant change in the localization of CE9 was confirmed qualitatively in at least two independent experiments. Numbers in parentheses represent the percentages of spermatozoa in the trypsinization experiments which no longer retained their heads at the time of analysis of immunofluorescence.

matozoa of the guinea pig (Myles and Primakoff, 1984; Myles et al., 1984). And although there are some intriguing similarities between PT-1 and CE9, there are many more striking differences. For example, PT-1 remains compartmentalized to the posterior tail of guinea pig spermatozoa even after they have passed through the epididymis and has been observed to migrate to the anterior tail only in vitro when spermatozoa collected from the cauda epididymidis are placed into an artificial medium (Myles and Primakoff, 1984). In contrast, CE9 appears to redistribute from the posterior to the anterior tail of the rat spermatozoon in vivo during epididymal transit, but does not exhibit a similar change in localization when spermatozoa collected from the caput epididymidis are incubated in vitro. Relatively little is known about PT-1 in a biochemical sense, because the available anti-PT-1 antibodies do not react on Western blots or in immunoprecipitations. However, Myles et al. (1984) have shown that PT-1 exhibits a sedimentation coefficient of 6.8 S when n-octyl-beta-D-glucopyranoside extracts of guinea pig spermatozoa are analyzed by isopycnic centrifugation in sucrose density gradients. When we performed the exact same analysis on the testicular and epididymal spermatozoa of the rat, we found that CE9 exhibited a sedimentation coefficient in the range of 2.5–3 S (unpublished results). Furthermore, by solid-phase antibody–binding assay, PT-1 was not detected in liver, kidney, or brain (Myles et al., 1981), three particularly rich sources of CE9 (Nehme, C. L., J. A. M. Petruszak, and J. R. Bartles, manuscript in preparation). On the basis of these criteria, we think it unlikely that CE9 represents the rat equivalent of guinea pig PT-1.

Redistribution of CE9 Versus the Appearance of New Epitopes

In the case of the PT-1 antigen, it has been possible to monitor the fate of prebound monoclonal Fab fragments to demonstrate that PT-1 molecules pre-existing on the surface of the guinea pig spermatozoon actually redistribute laterally from the posterior tail to the anterior tail under certain con-

ditions in vitro (Myles and Primakoff, 1984). Comparably definitive experiments to establish the occurrence of any such redistribution of rat spermatozoal CE9 are not possible at the present time, since we have not yet identified conditions that support a change in the localization of this transmembrane protein in vitro. Nevertheless, from our data, we can conclude that the change in the localization of CE9 is not accompanied by a drastic increase in the level of its expression on the surface of the rat spermatozoon (Fig. 3). It is very unlikely that any additional CE9 might be contributed by the secretions of the epididymis (cf., Olson and Hamilton, 1978; Jones et al., 1983; Holt, 1984; Eddy et al., 1985; Jones, 1989); on the basis of amino acid sequence, CE9 is predicted to be a transmembrane protein (Fig. 5; and Nehme, C. L., J. A. M. Petruszak, and J. R. Bartles, manuscript in preparation), and it displays the solubility characteristics of an integral membrane protein rather than those of a soluble protein present in the epididymal fluid (Fig. 1, lanes *f, g, i,* and *k*; Fig. 4, lanes *h, i,* and *j*). Furthermore, the CE9 molecules expressed on the anterior tail and the posterior tail of rat epididymal spermatozoa share a rather distinctive posttranslational modification: the proteolytic removal of a large segment of extracellular domain (Figs. 4 and 5). One favored alternative explanation would involve the coordinated masking and unmasking of pre-existing CE9 epitopes in the posterior tail and anterior tail plasma membrane domains, respectively. However, we have thus far been unable to unmask any such cryptic CE9 epitopes on or within the anterior tail, even when spermatozoa collected from the caput epididymidis are subjected to extensive washing, trypsinization, and/or permeabilization with detergents (Fig. 3, Table II, and unpublished results). To our knowledge, other possible explanations requiring either active CE9 biosynthesis and turnover by the spermatozoon or the equivalent of CE9 transcytosis (from posterior tail to anterior tail domains) are without precedent for spermatozoal plasma membrane proteins. Therefore, at this time, we are most inclined to ascribe the change in the localization of CE9 during epididymal maturation to the lateral redistribution

of pre-existing, proteolytically processed CE9 molecules within the plasma membrane bilayer of the tail of the spermatozoon.

Possible Mechanisms Involved in the Compartmentalization and Redistribution of CE9

What might keep plasma membrane proteins like CE9 and PT-1 in place on the tail of the spermatozoon? In the case of the guinea pig PT-1 antigen, experiments using the technique of fluorescence-recovery-after-photobleaching have suggested that the protein is relatively unhindered in its ability to diffuse laterally within the plane of the bilayer of the posterior tail plasma membrane (Myles et al., 1984). In such a case, it is relatively easy to envision a barrier to unrestricted lateral diffusion, perhaps somewhat analogous to a tight junction, that would operate at the level of the annulus. The idea of the annulus participating in such a barrier is not incompatible with its unique ultrastructure and its kinetics of appearance during spermiogenesis (Fawcett et al., 1971; Fawcett, 1975; Friend, 1989; Phillips, 1989). Other related barriers might similarly limit lateral diffusion between head and tail domains (the posterior striated ring), between the anterior and posterior domains of the head (the equatorial segment), and between the inner acrosomal membrane and posterior head domain on acrosome-reacted spermatozoa (Fawcett, 1975; Friend, 1989; Cowan et al., 1987). It is presently unclear whether such a mechanism would also extend to the case of CE9 on the rat spermatozoon. But, because of the specific proteolytic processing step observed for CE9, we are also considering the possibility that CE9 is kept in place within the posterior tail domain in part by interacting with other molecules locally at the cell surface or in the surrounding extracellular matrix. Wier and Edidin (1988) have presented evidence to support the notion that transmembrane proteins may experience reductions in lateral diffusibility via interactions involving their extracellular domains. Likewise, the PH-20 protein is compartmentalized to the posterior head plasma membrane domain of the guinea pig epididymal spermatozoon (Phelps et al., 1990) despite the protein's apparent attachment to only the outer leaflet of the plasma membrane bilayer via a phosphatidylinositol-glycan anchor (Phelps et al., 1988). Upon the proteolytic removal of that specific portion of the extracellular domain of spermatozoal CE9, these interactions would presumably be interrupted and allow CE9 to either diffuse or be moved laterally into the anterior tail domain coincidently with the breakdown of the putative annular diffusion barrier. Since CE9 appears to eventually become so concentrated within the anterior tail plasma membrane domain (Figs. 2 d and 3 d), it is tempting to speculate either that the redistribution is an active process or that the proteolytically processed CE9 might simply diffuse but then somehow become trapped within the anterior tail plasma membrane domain. Perhaps the putative annular diffusion barrier is itself converted into a selective one-way gate for CE9 during epididymal maturation.

Role of Proteolysis in the Posttesticular Processing of CE9

From the foregoing discussion, it should be apparent that proteolysis could prove to be an important aspect of the post-testicular processing of CE9 on the rat spermatozoon. While the exact relationship of the proteolysis to the function and the localization of CE9 remain unclear, the proteolytic removal of the amino-terminal 74 amino acids of CE9's 186-amino acid extracellular domain definitely precedes the molecule's apparent redistribution into the anterior tail plasma membrane domain during epididymal transit. It should be pointed out that epididymal transit takes time, 8-12 d in most species, and is associated with a number of changes in the physiology of the spermatozoon, perhaps most notably the acquisition of the capacities for progressive motility and fertilization (Bedford, 1975; Jones, 1989). Therefore, the proteolysis and redistribution of CE9 likely represent just two of a whole spectrum of changes normally occurring at the surface of the spermatozoon during its journey through the epididymis (Jones, 1989). Proteolysis has also been implicated in the posttesticular processing of the plasma membrane proteins and domains of the guinea pig spermatozoon. For example, a brief treatment of guinea pig testicular spermatozoa with exogenous trypsin has been found to simulate the effects of epididymal transit, both by promoting the compartmentalization of certain plasma membrane proteins and by causing other plasma membrane proteins that are already domain specific to change their localizations (Phelps et al., 1990). In both rats and guinea pigs, and sites of action of the relevant proteases have been traced to the proximal portion of the epididymis (Fig. 4, lanes k-m; and see Phelps et al., 1990). It remains unclear whether these proteases actually belong to the spermatozoon, are present in the fluid of the male reproductive tract, or are a surface component of the epithelial cells which line the tract. However, the inability to detect forms of CE9 intermediate between the 40-48-kD testicular form and the 23-33-kD epididymal form (Fig. 4, lanes k-m) and the homogeneity of the NH$_2$-terminal amino acid sequence of the 23-33-kD form (Fig. 5) both suggest that the cleavage of CE9 is endoproteolytic in nature. On the basis of the identification of arginine as the amino acid on the carboxy-terminal side of the proposed cleavage site in CE9, one might anticipate that the relevant enzyme would have a trypsin-like specificity. However, the failure of even relatively large amounts of exogenous trypsin to cleave the CE9 on isolated testicular spermatozoa (Fig. 4, cf., lanes o and p) argues strongly against this possibility and suggests instead that the protease that cleaves CE9 has an altogether different specificity. In this context, it may also be pertinent to note that computer-based comparisons (unpublished results) have revealed no instances of significant amino acid sequence similarity around the cleavage site identified for CE9 and that recently postulated for the PH-20 plasma membrane protein of the guinea pig spermatozoon (Lathrop et al., 1990).

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