

Breaching the Diffusion Barrier That Compartmentalizes the Transmembrane Glycoprotein CE9 to the Posterior-Tail Plasma Membrane Domain of the Rat Spermatozoon

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Abstract. CE9 is a posterior-tail domain-specific integral plasma membrane glycoprotein of the rat testicular spermatozoon. During epididymal maturation, CE9 undergoes endoproteolytic processing and then redistributes into the anterior-tail plasma membrane domain of the spermatozoon (Petruszak, J. A. M., C. L. Nehme, and J. R. Bartles. 1991. *J. Cell. Biol.* 114:917-927). We have determined the sequence of CE9 and found it to be a Type Ia transmembrane protein identical to the MRC OX-47 T-cell activation antigen, a member of the immunoglobulin superfamily predicted to have two immunoglobulin-related loops and three asparagine-linked glycans disposed extracellularly. Although encoded by a single gene and mRNA

in the rat, the majority of spermatozoal CE9 is of smaller apparent molecular mass than its hepatocytic counterpart due to the under-utilization of sites for asparagine-linked glycosylation. By fluorescence recovery after photobleaching, CE9 was determined to be mobile within the posterior-tail plasma membrane domain of the living rat testicular spermatozoon, thus implying the existence of a regional barrier to lateral diffusion that is presumed to operate at the level of the annulus. Through the development of an in vitro system, the modification of this diffusion barrier to allow for the subsequent redistribution of CE9 into the anterior-tail domain was found to be a time-, temperature-, and energy-dependent process.

CELLS with asymmetrical shapes compartmentalize their integral plasma membrane proteins to morphologically distinguishable surface domains. The mammalian spermatozoon has at least four compositionally distinct plasma membrane domains, corresponding to those covering the anterior and posterior segments of its head and tail (for example see Koehler, 1978; Friend, 1982; Primakoff and Myles, 1983; Holt, 1984). This highly asymmetrical cell compartmentalizes its plasma membrane proteins not only during spermiogenesis, but also through a series of processing and redistribution events that accompany posttesticular maturation in the epididymis, capacitation, and the acrosome reaction (Myles and Primakoff, 1984; Jones et al., 1990; Phelps et al., 1990; Cowan et al., 1991).

We recently identified a domain-specific plasma membrane protein of the rat spermatozoon called CE9 (Petruszak et al., 1991). Compartmentalized initially to the posterior-tail plasma membrane domain of the testicular spermatozoon, CE9 redistributes into the anterior-tail plasma membrane domain during maturation in the epididymis. This change in localization is preceded by the endoproteolytic

removal of a portion of the amino terminus of the protein.

In an effort to learn more about CE9 and its compartmentalization, we have determined the structure of rat CE9, measured its lateral diffusion within the posterior-tail plasma membrane domain of the living testicular spermatozoon and identified conditions that support the redistribution of CE9 on spermatozoa collected from the caput epididymidis in vitro.

Materials and Methods

Male Fischer F344 or Sprague-Dawley rats (250-350 g) were purchased from either Charles River Breeding Laboratories (Wilmington, MA) or Harlan Sprague-Dawley (Indianapolis, IN). Two rat liver cDNA libraries were used: a lambda gtl1 library obtained from Dr. James P. Hardwick (Division of Biological and Medical Research, Argonne National Laboratory, Argonne, IL) and a lambda ZAP II library purchased from Stratagene (La Jolla, CA). The following other materials were obtained from the designated sources: HRP-conjugated goat anti-rabbit IgG (Biorad Laboratories, Richmond, CA); pBluescriptSK+ plasmid (Stratagene); restriction endonucleases, Klenow enzyme and T4 ligase (Boehringer-Mannheim Biochemicals, Indianapolis, IN); E. coli DH5alpha (BRL, Gaithersburg, MD); [alpha-³²P]ATP and Na¹²⁵I (Amersham Corp., Arlington Heights, IL); GenescreenPlus (E. I. du Pont de Nemours & Co., Boston, MA); Immobilon (Millipore Corp., Bedford, MA); BSA (Fraction V, ICN Biochemicals, Cleveland, OH); Sequenase Version 2.0 kit (United States Biochem. Corp., Cleveland, OH); random primer pd(N)₆ (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ); N-Glycanase (Genzyme Corp., Cambridge, MA);

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rhodamine-labeled goat anti-rabbit IgG (either Southern Biotechnology Associates, Birmingham, AL, or Jackson ImmunoResearch Laboratories, Inc., West Grove, PA); and Fab fragment of goat anti-mouse IgG (Cappel, Malvern, PA).

All molecular biological procedures were carried out as described by Sambrook et al. (1989) or Ausubel et al. (1989) with minor modifications. cDNA libraries were screened with affinity-purified rabbit polyclonal anti-CE9. Inserts of positive clones were subjected to double-stranded sequencing in pBluescriptSK+ using the Sequenase Version 2.0 kit. Total RNA was isolated by guanidine thiocyanate extraction, CsCl centrifugation, and ethanol precipitation. 10- μ g samples of RNA were electrophoresed in formaldehyde-denaturing 1% agarose gels, transferred to nitrocellulose, and the blot probed with 32 P-CE9 cDNA prepared using [alpha- 32 P]ATP, Klenow enzyme and the pd(N)₆ random primer. Very high molecular-mass DNA was isolated from rat liver, and digested with restriction endonucleases. 40- μ g samples were electrophoresed in 0.7% agarose gels, transferred to GeneScreenPlus and probed with 32 P-CE9 cDNA. Sequence analyses and comparisons were made using the PC Gene program and GCG Sequence Analysis Software Package available through the Biotechnology Center at Northwestern University.

The following procedures have been described previously (Petruszak et al., 1991): collection of spermatozoa from the testis and different regions of the epididymis, preparation of tissue homogenates and gel samples, SDS-PAGE and Western blotting with affinity-purified rabbit polyclonal anti-CE9 antibodies and 125 I-Protein A, immunoprecipitation with monoclonal and polyclonal anti-CE9 antibody-Sepharose, immunoaffinity purification with polyclonal anti-CE9 antibody-Sepharose, gas-phase amino-terminal amino acid sequencing of immunoaffinity purified CE9 and immunofluorescent labeling of paraformaldehyde-fixed spermatozoa in the presence of 0.01% (wt/vol) of saponin in agarose squares (for quantification of distributions) or on poly-lysine-coated slides. Partial enzymatic deglycosylation of immunoprecipitated CE9 was conducted for 30–60 min at 37°C with 10 U/ml of N-Glycanase in 0.2 M NaPi, 10 mM *o*-phenanthroline, 10% (vol/vol) methanol, 1.25% (wt/vol) NP-40, 0.17% (wt/vol) SDS, pH 8.6.

For fluorescence recovery after photobleaching, spermatozoa were allowed to diffuse out of decapsulated rat testes into 4°C modified Mg²⁺-Hepes buffer (140 mM NaCl, 4 mM KCl, 10 mM D-glucose, 2 mM MgCl₂, 4 mM Hepes, 0.4% (wt/vol) of BSA, pH 7.4; Green, 1978) after making a few slices through the mass of seminiferous tubules with a scalpel blade. The cells were then washed and labeled sequentially with mouse monoclonal anti-CE9 IgG (150 μ g/ml; 30 min) and rhodamine-labeled goat anti-(mouse IgG) Fab (Goding, 1976; 30 min) at 30–35°C in modified Mg²⁺-Hepes buffer, using centrifugation for 3 min at 1,500 g through a 0.5-ml step-gradient of 4% (wt/vol) of BSA in modified Mg²⁺-Hepes buffer after each step. A 5- μ l drop of the suspension of labeled cells was placed on a slide and sealed under a cover slip with nail polish for analysis. The optical and electronic systems used for the photobleaching measurements have been described elsewhere (for example see Koppel, 1979; Koppel et al., 1986). The argon-ion (514 nm) laser spot was defocused in one dimension with a cylindrical lens to produce a focused line perpendicular to the tail axis. Fluorescence intensity measurements were made at 31°C by scanning 12 points \sim 1.0 μ m apart in 1.92-s intervals before and after bleaching by a short pulse (0.05 s) with a high intensity beam. The data were fit by a multipurpose fitting function, which fits every point to a theoretical curve. The data were initially fit to a 3-variable, 5-parameter function (Koppel, 1979):

$$\frac{F_1(x,t)}{F(-)} = 1 - A \frac{\exp\{-x^2/[w^2(1 + t/T_D)]\}}{(1 + t/T_D)^{m/2}} - B \exp(-x^2/w^2) \quad (1)$$

where $F(x,t)$ is the postbleach fluorescence intensity at position x and time t , $F(-)$ is the prebleach fluorescence intensity, the term following the A is the mobile fraction (where m is the dimensionality of the beam), the term following the B is the immobile fraction, $A/(A + B)$ gives the recovery, w is the assumed $1/e$ radius of the Gaussian fluorescence scan, and $D = w^2/4T_D$ is the diffusion coefficient (Koppel, 1979). To take into account the small effects of imperfect bleaching, we then fit the data to a 3-variable, 7-parameter function:

$$[F_2(x,t)/F(-)] = \{[F_1(x,t)/F(-)] - C\} \exp(-\gamma t) \quad (2)$$

In this equation, the exponential term carries the effects of bleaching with the "monitoring" beam scan, and the constant term (C) signifies bleaching with a constant background added to the bleaching beam.

For incubation *in vitro*, spermatozoa were collected from dissected caput epididymidis (minus the initial segment; see Dym, 1983) of the rat by mincing with a scalpel blade in 1 ml of modified Hank's buffer (95 mM NaCl, 4.8 mM KCl, 1.2 mM MgSO₄, 25 mM NaHCO₃, 1.2 mM KH₂PO₄, 5.6

mM D-glucose, 21.6 mM lactic acid, 0.5 mM sodium pyruvate, 50 μ g/ml of streptomycin sulfate, 50 U/ml of sodium penicillin G, and 0.4% (wt/vol) of BSA, pH 7.4) that had been pre-equilibrated in a 35-mm petri dish with 5% CO₂ in a tissue culture incubator at 37°C. The dish was placed on a platform shaker and gyrated at 75 rpm for 5 min at room temperature. The spermatozoa were transferred to a 15-ml conical centrifuge tube, and remaining tissue fragments were allowed to settle out at 1 g for 2 min at room temperature. The supernate containing the spermatozoa was collected and placed back into a 35-mm petri dish and kept in the tissue culture incubator at the designated temperature in the presence of 5% CO₂. IgGs, metabolic inhibitors, and other chemicals were added to the incubation medium from concentrated stock solutions in modified Hank's buffer, with the exception of 2,4-dinitrophenol (DNP), which was added from a 4 M stock solution in DMSO; DMSO alone, at a final concentration of 0.075% (vol/vol) or less, had no effect either on the percent redistribution or viability. To assess viability, we determined the percentage of spermatozoa that excluded the chromosome-binding dye 4',6'-diamidino-2-phenylindole when applied at a final concentration of 0.2 μ g/ml and examined within 10 min at room temperature. At all times in control incubations, dye exclusion was found to be correlated in a one-to-one fashion with the motility of the circular swimming or twitching variety (Fray et al., 1972). At selected times, samples were removed and fixed for immunofluorescence. For the experiments shown in Fig. 9, the percent redistribution was calculated as the mean of the decrease in the percentage of spermatozoa showing the PT >> AT labeling pattern and the increase in the percentage of spermatozoa showing the PT = AT pattern after incubation for 20–24 h, with controls normalized to 100%.

Results

cDNA Sequence and Aberrant Glycosylation

The sequence of CE9 cDNA was deduced from four immunoreactive clones isolated from two rat liver cDNA libraries according to the strategy outlined in Fig. 1. The combined cDNA and predicted amino acid sequences are shown in Fig. 2, and the results are depicted schematically at the bottom of Fig. 1. The cDNA sequence was found to encode a 251-amino acid Type Ia transmembrane protein that proved identical to the MRC OX-47 T-cell activation antigen (Fossum et al., 1991) and to show significant sequence similarity to putative homologues in the mouse (84% identity to mouse gp42 or basigin; Altruda et al., 1989; Kanekura et al., 1991), human (58% identity to human basigin; Miyachi et al., 1991) and chicken (49% identity to the chicken HT-7 antigen or neurothelin; Seulberger et al., 1990; Schlosshauer and Herzog, 1990). The functions of these proteins are not currently known. CE9 is predicted to have a cleaved 21-amino acid signal peptide followed by a 187-amino acid extracellular tail that contains two immunoglobulin-related loops and three asparagine-linked glycans, a 23-amino acid membrane-spanning segment and a 41-amino acid cytoplasmic tail (Figs. 1 and 2).

Two lines of evidence suggested that spermatozoal and hepatocytic CE9 were encoded by a single mRNA and gene in the rat. First, Northern blotting indicated the presence of a single 1.5-kb mRNA in several rat tissues, including testis (Fig. 3, left panel). Second, single-labeled bands were detected on Southern blots of rat DNA that had been digested with restriction endonucleases that did not cut within the CE9 cDNA (Fig. 3, right panel).

We have postulated that tissue-specific differences in glycosylation of the same \sim 32-kD polypeptide backbone account for why spermatozoal is of smaller apparent molecular mass than its hepatocytic counterpart (Petruszak et al., 1991). When the \sim 48-kD liver CE9 was subjected to partial enzymatic deglycosylation using N-Glycanase, we observed the expected ladder of three products, ranging from \sim 43–32

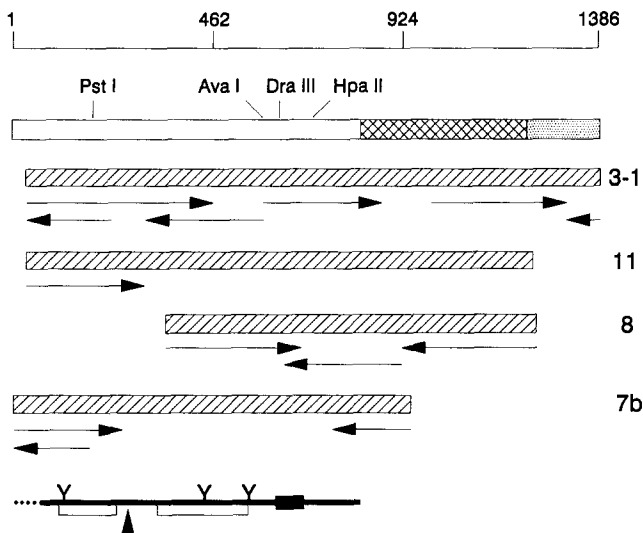


Figure 1. Schematic depiction of sequencing strategy and predicted outcome. Upper diagram, ~1,386 nucleotide (nt) CE9 cDNA with selected restriction sites indicated within the 828-nt coding sequence (□), 408-nt 3'-untranslated sequence (▨), and ~150-nt poly (A) tail (▩). Middle four diagrams (▨), four CE9 cDNA clones sequenced as indicated by underlying arrows. Lower diagram, the 251-amino acid mature CE9 protein (solid line) with its 21-amino acid amino-terminal signal peptide (dotted line), 23-amino acid membrane-spanning segment (thickened section in line), consensus sites for asparagine-linked glycosylation (Y) and the two pairs of disulfide-bonded cysteines that define the postulated immunoglobulin-related loops (thin connecting lines). The site of endoproteolytic cleavage observed during spermatozoal maturation in the epididymis (Petruszak et al., 1991) is indicated by the arrowhead.

kD, each separated by ~5 kD (Fig. 4, see lanes L- and L+). In contrast, the majority of the testicular CE9 migrated nearest the form of partially deglycosylated hepatocytic CE9 that was missing two of its three N-linked glycans (Fig. 4, lane T-) and yielded only a single ~32-kD product upon short-term digestion with N-Glycanase (Fig. 4, lane T+). These data suggested that, unlike hepatocytic CE9, the bulk of the spermatozoal CE9 had only one asparagine-linked glycan. This glycan would most likely need to be present on Asn-132 or Asn-168, since the truncated form of CE9 detected on epididymal spermatozoa is missing Asn-23, yet was also found to contain only a single asparagine-linked glycan by this criterion (Fig. 4, lanes E- and E+).

Measurements of Diffusion

Fluorescence recovery after photobleaching was selected to identify the basis of the compartmentalization of CE9 to the posterior-tail plasma membrane domain. Since the rabbit polyclonal anti-CE9 IgG and its Fab showed considerably lower levels of specific binding to unfixed rat spermatozoa (data not shown), we resorted to the use of the intact mouse monoclonal anti-CE9 IgG followed by rhodamine-labeled Fab (Myles et al., 1984). Unfortunately, this limited our analysis to testicular spermatozoa, because the mAb does not react well with the endoproteolytically processed form of CE9 found on epididymal spermatozoa (Petruszak et al., 1991; and our own unpublished results).

When we labeled freshly isolated rat testicular spermatozoa, virtually all the spermatozoa were brightly and

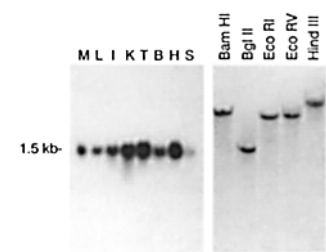
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1 GGACGAGGCGACATGGCGGGCGCTGCTGCTGGCGCTGGCCCTCACGTTCTCGAGTGGC
  METAlaAlaAlaLeuLeuLeuAlaLeuAlaPheThrPheLeuSerGly
61 CAAGGGCGCTCGCGGGCGGGCCACCATCGTAACTCTGTCCAGGAAGTGTACTCCAAG
  -5 GlnGlyAlaCysAlaAlaAlaGlyThrIleValThrSerValGlnGluValAspSerLys
121 ACACAGCTTACCTGCTTTTTGAACAGCAGTGGCATGGATCGTGGCCACCGCTGGATG
  16 ThrGlnLeuThrCysPheLeuAsnSerSerGlyIleAspIleValGlyHisArgTrpMet
181 AGAGTGGCAAGGTACTGCAGGAAGACACGCTGCCGATCTACAGATGAAGTACACGGTG
  36 ArgGlyGlyLysValLeuGlnGluAspThrLeuProAspLeuGlnMetLysTyrThrVal
241 GATCGAGTACCGCTCTGGAGAATATCTCTGCATCTCTCTCGAGCTGTGGGCGAGA
  56 AspAlaAspAspArgSerSerGlyGluTyrSerCysIlePheLeuProGluProValGlyArg
301 GGCAACATCAATGTGGAGGGGCCACCCAGGATCAAGTGGGAAAGAAATCGGAACACGGC
  76 GlyAsnIleAsnValGluGlyProProArgIleLysValGlyLysLysSerGluHisAla
361 AGTGAGGGAGAGTTTGTGAAGCTGATCTGCAAGTCTGAGGCGTCCCACCTCTGTGGAT
  96 SerGluGlyGluPheValLysLeuIleCysLysSerGluAlaSerHisProProValAsp
  Pro
421 GAGTGGGTCTGGTTAAGACCTCTGACACTGGGACGACACTCTCCACGGCAGTGTAG
  116 GluTrpValTrpPheLysThrSerAspThrGlyAspGlnThrIleSerAsnGlyThrGlu
481 GCCAATAGCAAGTACGTCATTATATCCACGCTGAGCTGCAGAGCTGATCATCAGCGAC
  136 AlaAsnSerLysTyrValIleIleSerThrProGluLeuSerGluLeuIleIleSerAsp
541 CTGGACATGAAAGTGGACCTGGCACCTACGTGTGCAATGCCACCAACTCCAGGGCAGT
  156 LeuAspMetAsnValAspProGlyThrTyrValCysAsnAlaThrAsnSerGluGlySer
601 GCTCGGAGACCATCTCACTGCTGTCGCGCAGCGCCCTGGCAGCCCTCTGGCCCTTCTGT
  176 AlaArgGluThrIleSerLeuArgValArgSerArgLeuAlaAlaLeuTrpProPheLeu
661 GGCATTGTGGCGAGGTCCTGGTGTGGTCACCATCATCTTCTACTCAGAGAGAGGGCGG
  196 GlyIleValAlaGluValLeuValLeuValThrIleIlePheIleTyrGluLysArgArg
721 AAGCCGGACAGCCCTGGACAGGATGATCTGGCGGGCCCGCCACTGAAGGCCAGCCGG
  216 LysProAspGlnThrLeuAspGluAspAspProGlyAlaAlaProLeuLysGlySerGly
781 TCTCACCTGAATGACAAAGGACAAAGTGTGGCCAGAGGAACGCCACTGAGCGGGGGG
  236 SerHisLeuAsnAspLysAspLysAsnValArgGlnArgAsnAlaThr
841 CAGCGGGGAGGGGAGTGGCCAGGGCACTGACCCAGCCAGCGTCTGCCTCCACTCCT
  901 GTGTCCATCTCTGCCGACCTGAGCCCTGCCAACCCCACTCTTATCCAGCCCAAGTGT
  961 AAGACAGAGCCTTACCTTACAGAAAACCCATCTGGGAGAAAGCAGGCCACTTGCAGTACC
  1021 CTGTTCTAGTTAAACTGAATGAGGTTTCTATGACAGCAATCCATTCTTATGGGCTACG
  1081 TTTTATTTTCTTCCCTCTGAAGTATGCTACTACAGCCCTGGGAGTGGGGAGTGGG
  1141 GTCATGAGGAAAGCCAGGGCACACATCTCTACTTACCTTACCTGTTGGAGGGGGCTGG
  1201 CTGCTGGGACCCCAATAAAGACCTACACCCAC
  
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Figure 2. Combined cDNA and amino acid sequence predicted for CE9. Single underline, the first 19 amino acids of the mature hepatocytic CE9 as determined by gas-phase amino-terminal amino acid sequencing. (The initial pair of alanines was not resolved in our earlier attempt at amino-terminal amino acid sequencing [Petruszak et al., 1991], thus causing the tentative numbering scheme in that earlier article to be off by one amino acid.) Double underline, the membrane-spanning segment. (*), Consensus sites for asparagine-linked glycosylation; (") cysteines. nt 394 was found to be a C in clone 3-1, but a T in two other clones, one from each cDNA library. These sequence data are available from EMBL/GenBank/DBJ under accession number X67215.

uniformly labeled in their posterior-tail domains (Fig. 5, a and b), thus providing support for a cell-surface localization for that CE9 detected previously by immunofluorescence on fixed testicular spermatozoa embedded in agarose blocks or immobilized on poly-lysine-coated slides (Petruszak et al., 1991). We next measured the recovery and diffusion coefficient for the specifically bound fluorescent Fab-mAb com-

Figure 3. (Left panel) Northern blot of total RNA isolated from rat skeletal muscle (M), liver (L), small intestine (I), kidney (K), testis (T), brain (B), heart (H), and spleen (S). (Right panel) Southern blot of fragments of very high molecular-mass rat liver DNA generated by digestion with the designated restriction endonuclease. Arrowhead, position of the undigested DNA.



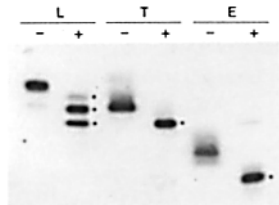


Figure 4. Western blot of polyclonal antibody immunoprecipitates from rat liver (L), testis (T), and cauda epididymidis (E), either without (-) or with (+) limited enzymatic deglycosylation with N-Glycanase. Dots, deglycosylation products.

plex on unfixed testicular spermatozoa (Koppel, 1979; Koppel et al., 1986). For these experiments, we deliberately selected spermatozoa that exhibited periodic twitching of the distal tail extremity as a sign of cell viability. The data collected from a representative spermatozoon are depicted in Fig. 6, where decay of fluorescence depletion $\{1-[F(x,t)/F(-)]\}$ has been plotted as a function of time measured in 0.16-s intervals (n). The decay of fluorescence depletion was found to be both relatively complete and rapid for a transmembrane protein in a biological membrane (reviewed by Jacobson et al., 1987). The curves in Fig. 6 show a theoretical fit using Eqs. 1 and 2 in which the recovery and diffusion coefficient were assigned values of 93% and 9.2×10^{-10} cm²/s, respectively. When the data from four different spermatozoa were analyzed in this fashion, the average recovery and diffusion coefficient were computed to be $105 \pm 12\%$ and $(1.1 \pm 0.3) \times 10^{-9}$ cm²/s, respectively. Since the divalent monoclonal primary antibody could elicit some cross-linking, this calculated value for the diffusion coefficient should be viewed as defining a lower limit. The high degree of mobility of spermatozoal CE9 within the posterior-tail plasma membrane domain suggested that CE9 was compartmentalized by a regional diffusion barrier whose effects were realized beginning at the junction between anterior- and posterior-tail plasma membrane domains, i.e., at the level of the annulus.

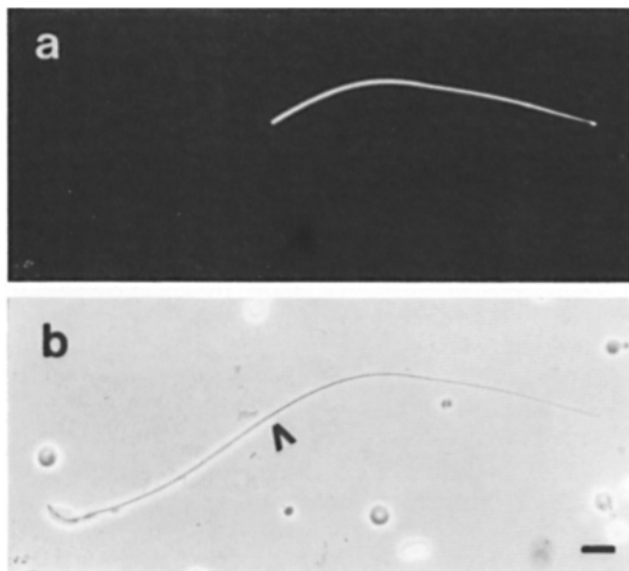


Figure 5. Immunofluorescent labeling of unfixed rat testicular spermatozoon with mAb and rhodamine-labeled Fab. (a) Fluorescence; (b) Phase contrast. Arrowhead, annulus. Bar, 15 μ m.

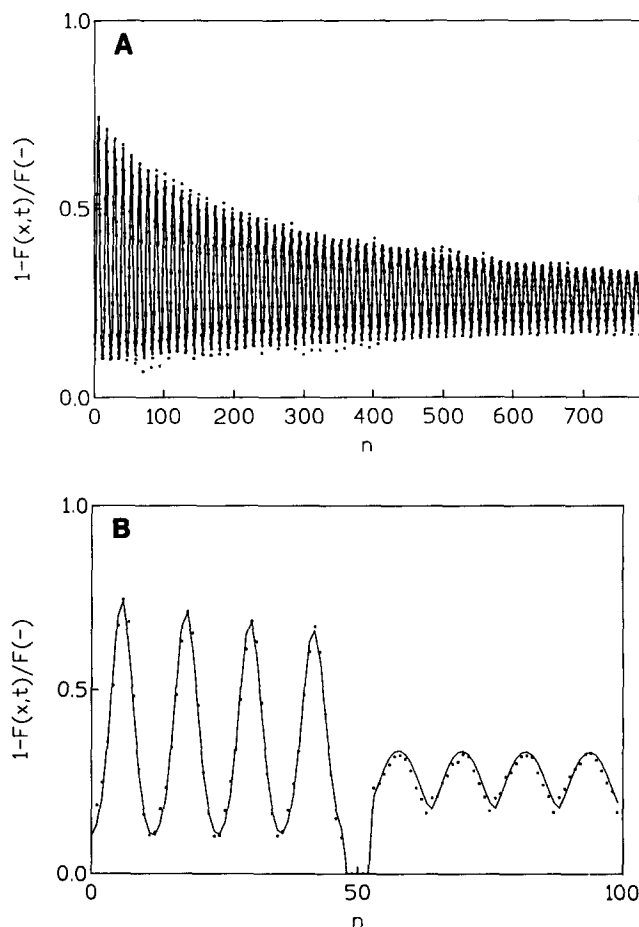


Figure 6. Fluorescence recovery after photobleaching of a living rat testicular spermatozoon at 31°C. Plots of decay of fluorescence depletion $\{1-[F(x,t)/F(-)]\}$ versus time in 0.16-s intervals (n) for all of the scans (A) and the first and last four scans shown on an expanded scale (B). The curves were fit using Eqs. 1 and 2 with the recovery and diffusion coefficient assigned values of 93% and 9.2×10^{-10} cm²/s, respectively.

Redistribution In Vitro

The regional diffusion barrier must presumably be breached to account for the redistribution of CE9 during maturation in the epididymis (Petruszak et al., 1991). We wondered whether it would be possible to identify conditions that would support a similar redistribution of CE9 on rat spermatozoa maintained in vitro. We used spermatozoa from the caput epididymidis for these experiments, because they have the endoproteolytically processed form of CE9 and would presumably represent the relevant precursors in vivo. After comparing a number of different conditions, we selected incubation at 37°C in a modified Hank's buffer for further study because it supported significant redistribution with a minimal loss in cell viability.

As expected, the majority of the caput spermatozoa displayed CE9 in a posterior-tail location immediately after isolation (Fig. 7, a and b; Fig. 8 A, $PT \gg AT$). Typically 80% of the freshly isolated spermatozoa were judged to be viable on the basis of their ability to exclude the chromosome-binding dye 4',6-diamidino-2-phenylindole (Fig. 8 A, *DYE-EXCL.*). With incubation at 37°C, there was a change in the localization of CE9 such that an increasing percentage of the

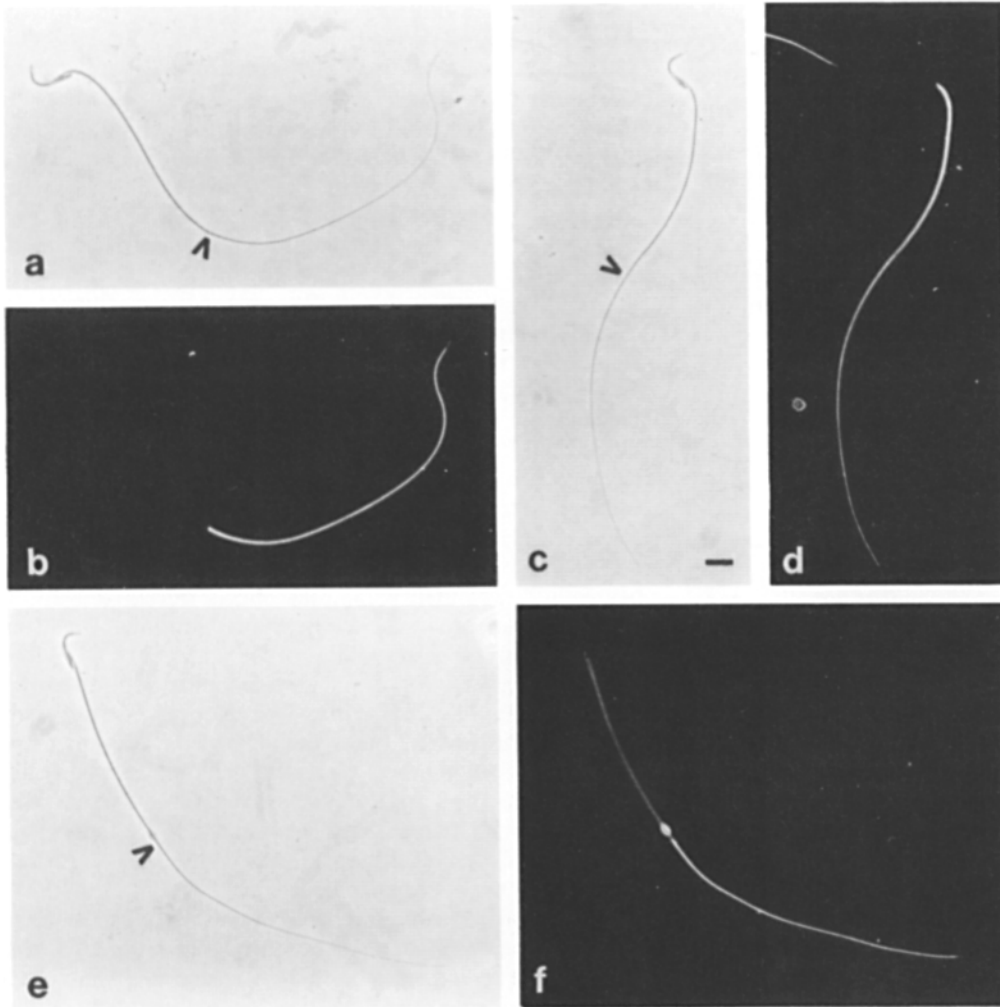


Figure 7. Immunofluorescent labeling of rat spermatozoa, either immediately after isolation from the caput epididymidis (*a* and *b*) or after incubation for either 22 (*c* and *d*) or 8 (*e* and *f*) h in vitro. (*a*, *c*, and *e*) Phase contrast. Arrowheads, annulus. Bar, 9 μm .

spermatozoa showed a more equal labeling of anterior- and posterior-tail plasma membrane domains (Fig. 7, *c* and *d*; Fig. 8 *A*, $PT = AT$). The rate of appearance of spermatozoa exhibiting this $PT = AT$ localization pattern was relatively slow during the first 5 h of the incubation, increased nearly ninefold between 7 and 10 h and then leveled off after ~ 12 h. After incubation for 20–24 h, an average of 66% of the spermatozoa showed a change in the localization of CE9 from the $PT \gg AT$ category to the $PT = AT$ category, while during the same interval there was a decrease of only 16% in the number of spermatozoa that excluded the 4',6-diamidino-2-phenylindole. The concentration of spermatozoa did not change during the course of the incubation (data not shown).

Given the temporal relationship between the disappearance of the spermatozoa exhibiting the $PT \gg AT$ pattern and the appearance of those with the $PT = AT$ pattern, the change in localization most likely represented the breaching of the diffusion barrier by CE9 and its subsequent lateral diffusion into the anterior-tail plasma membrane domain. The behavior exhibited by spermatozoal CE9 in this in-vitro assay was different from that observed during spermatozoal maturation in the epididymis in that there was no evidence of a marked concentration of CE9 within the anterior-tail plasma membrane (Petruszak et al., 1991). At all times, it was possible to identify significant numbers of spermatozoa

with labeling patterns intermediate between the two extremes of $PT \gg AT$ and $PT = AT$ (Fig. 8 *A*; $PT > AT$). These spermatozoa typically had relatively low, yet readily discernible levels of specific labeling distributed throughout their anterior-tail domains (e.g., see Fig. 7, *e* and *f*). The percentage of these presumptive intermediates was seen to increase transiently just before and during the period of most rapid redistribution, yet at no time examined did their numbers exceed 17%.

To try to learn more about the requirements for breaching the diffusion barrier, we examined the effects of various treatments on the redistribution of CE9 in the in-vitro assay (Fig. 9). None of the treatments shown in Fig. 9 was found to adversely affect the ability of the spermatozoa to exclude 4',6-diamidino-2-phenylindole when compared with controls (data not shown). Consistent with a mechanism that involved the lateral diffusion of cell surface CE9, the redistribution was reduced to 2% of controls by polyclonal rabbit anti-CE9 IgG, presumably due to the cross-linking of CE9 within the posterior-tail plasma membrane domain. The effect was judged to be specific, because preimmune IgG at the same concentration caused no such inhibition. The breaching of the diffusion barrier was most likely not the result of the action of proteases released during tissue disruption or the subsequent incubation, because the inclusion of a vast excess of

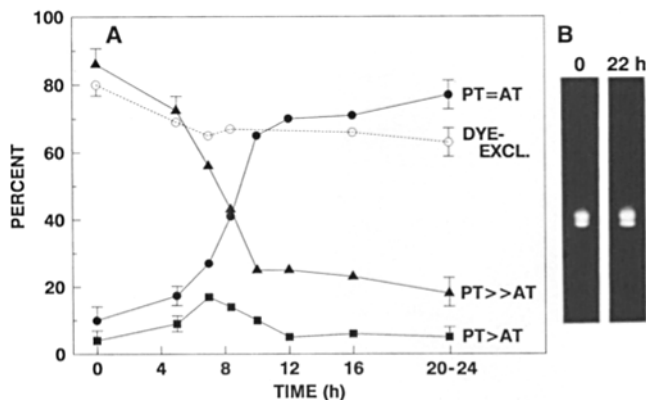


Figure 8. Redistribution of CE9 on spermatozoa collected from the caput epididymidis during incubation in vitro. (A) Changes in the immunofluorescent labeling pattern (closed symbols with specifications of relative labeling intensities of posterior-tail [PT] and anterior-tail [AT] domains shown at right) and ability to exclude 4',6-diamidino-2-phenylindole (\circ , DYE-EXCL.) as a function of time. Localization patterns: PT >> AT (\blacktriangle , Fig. 7, a and b), PT = AT (\bullet , Fig. 7, c and d) and PT > AT (\circ , anything in between these two extremes, e.g., Fig. 7, e and f). Each time point represents an independent experiment; the 0-, 5-, and 20–24-h time points are plotted as mean \pm SD for six independent experiments. 100–120 spermatozoa were analyzed in each experiment. (B) Western blot of the spermatozoa either before (0 h) or after (22 h) incubation for 22 h in vitro.

nontoxic protease inhibitors with a broad spectrum of coverage for both serine and thiol proteases—antipain (100 μ M), leupeptin (100 μ M) and aprotinin (50 μ M)—inhibited the redistribution by only 18%. When analyzed on Western blots, neither the concentration nor the electrophoretic mo-

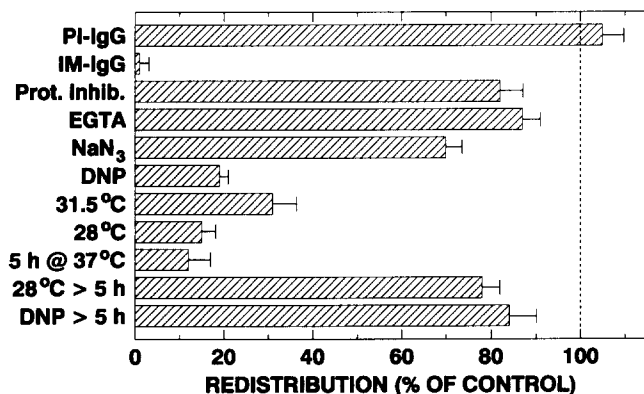


Figure 9. The effects of treatments on the redistribution of CE9 on caput spermatozoa incubated in vitro for 20–24 h. PI-IgG, 0.5 mg/ml of preimmune IgG; IM-IgG, 0.5 mg/ml of rabbit polyclonal anti-CE9 IgG; Prot. Inhib., protease inhibitors (100 μ M leupeptin, 100 μ M antipain, and 50 μ M aprotinin); EGTA, 100 μ M EGTA; NaN₃, 6 mM NaN₃; DNP, 3 mM DNP; 31.5°C, 31.5°C instead of 37°C; 28°C, 28°C instead of 37°C; 5 h @ 37°C, assayed after 5 h of incubation instead of 20–24 h; 28°C > 5 h, shifted to 28°C for 16 h after 5 h of incubation at 37°C; and DNP > 5 h, shifted to 3 mM DNP for 16 h after 5 h incubation under control conditions. The data are reported either as mean \pm SD for three independent determinations or as mean \pm (range/2) for two independent determinations. 100–120 spermatozoa were analyzed in each experiment.

bility of spermatozoal CE9 was found to change significantly during the control incubation for 22 h at 37°C (Fig. 8 B). Unlike the redistributions of some other spermatozoal plasma membrane proteins during capacitation or the acrosome reaction in vitro (Myles and Primakoff, 1984; Jones et al., 1990), the redistribution of CE9 did not require exogenous calcium ion and was inhibited by only 14% by the inclusion of the calcium ion chelator EGTA (100 μ M) (Fig. 9). The process was, however, judged to be energy dependent in that it was inhibited 81% by DNP (3 mM), an uncoupler of oxidative phosphorylation. By comparison, sodium azide (6 mM), which blocks only one of the three proton-pumping sites in the mitochondrial respiratory chain, caused only a 30% inhibition. Both metabolic inhibitors did, however, block the circular swimming or twitching type of motility exhibited by these spermatozoa (Fray et al., 1972; data not shown). The redistribution in vitro proved to be remarkably sensitive to temperature, being inhibited by 69 and 85% simply by lowering the incubation temperature from 37 to 31.5 and 28°C, respectively. Reduced temperature and DNP appeared to exert their inhibitory effects during the initial phase(s) of the process, because the redistribution was still quite efficient if a shift to 28°C or the addition of DNP was carried out after a 5-h preincubation under control conditions (Fig. 9). The 78% redistribution observed after such a shift to 28°C was 2.9 times greater than the maximum expected as the sum of the 12% redistribution observed at the time of shift plus the 15% redistribution expected with prolonged incubation at 28°C. Likewise, the 84% redistribution observed after a shift to 3 mM DNP at 5 h was 2.7 times greater than the maximum expected as the sum of the 12% redistribution observed at the time of shift plus the 19% redistribution observed after prolonged incubation in 3 mM DNP. These data suggested that 28°C and DNP inhibited some critical step(s) involved in the modification of the diffusion barrier and not the ensuing lateral redistribution into the anterior-tail domain.

Discussion

The mechanisms used to compartmentalize, endoproteolytically process, and redistribute spermatozoal CE9 must ultimately reflect the structural features of the protein. On the basis of cDNA sequence reinforced with biochemical evidence, the CE9 on a rat testicular spermatozoon is predicted to be a 251-amino acid Type Ia transmembrane glycoprotein with a 41-amino acid cytoplasmic tail, a 23-amino acid membrane-spanning segment, and a 187-amino acid extracellular tail that contains two immunoglobulin-related loops and predominantly only one of three possible asparagine-linked glycans. During posttesticular maturation in the rat epididymidis, the amino-terminal 75 amino acids of the extracellular tail of CE9 (including the more distal of two predicted immunoglobulin-related loops) are removed by endoproteolytic cleavage as a prelude to the redistribution of the remaining, now truncated transmembrane glycoprotein from the posterior-tail into the anterior-tail plasma membrane domain (Petruszak et al., 1991). CE9 proved to be identical in sequence to the MRC OX-47 T-cell activation antigen (Fossum et al., 1991) and to show significant sequence similarity to putative homologues identified previously in mice, humans, and chickens (Kanekura et al., 1991; Miya-

uchi et al., 1991; Seulberger, 1990). The functions postulated for this subset of immunoglobulin superfamily members presently run the gamut from a cell adhesion molecule to a lymphokine receptor to a receptor or transporter at the blood-brain barrier (Altruda et al., 1989; Seulberger et al. 1990; Schlossauer and Herzog, 1990; Schlosshauer, 1991; Kanekura et al., 1991; Miyauchi et al., 1991; Fossum et al., 1991).

Compartmentalization by a Regional Diffusion Barrier

Our measurements of lateral diffusion suggest that CE9 is compartmentalized to the posterior-tail plasma membrane domain by a regional diffusion barrier, rather than by local interactions with components of the membrane skeleton, extracellular matrix, or other membrane proteins (Nelson and Hammerton, 1989; Phelps et al., 1988; Wier and Edidin, 1988). In some respects, this barrier may be thought of as the functional equivalent of the tight junction that restricts lateral diffusion between apical and basolateral plasma membrane domains on epithelial cells (Stevenson et al., 1988; Rodriguez-Boulan and Nelson, 1989; Gumbiner, 1990). The only other example of a posterior-tail-specific spermatozoal surface antigen, the PT-1 antigen of the guinea pig spermatozoon, was also found to be highly mobile within its domain (Myles et al., 1984). Unfortunately, it has not been possible to characterize the PT-1 antigen, because its mAb fails to react by immunoprecipitation or by Western blotting. On the basis of differences in sedimentation coefficient, tissue distribution, and timing of redistribution into the anterior-tail plasma membrane domain, we have argued that CE9 is not the rat equivalent of the guinea pig PT-1 antigen (Petruszak et al., 1991). So, for the moment, it looks as though there may be at least two examples of mobile plasma membrane proteins that are compartmentalized to the posterior-tail plasma membrane domain of the spermatozoal tail by a regional diffusion barrier. On the basis of position, we would predict that the regional diffusion barrier that compartmentalizes CE9 might involve the annulus. Long appreciated in transmission electron micrographs, the annulus is composed of an electron-dense ring, circular or triangular in cross section (depending upon the species), positioned subjacent to the plasma membrane at the boundary between the anterior-tail and posterior-tail domains (Fawcett et al., 1971; Fawcett, 1975; Friend, 1989). Other ultrastructural barriers have been postulated to exist at the posterior striated ring and equatorial segment to limit lateral diffusion between posterior-head and anterior-tail plasma membrane domains and anterior-head and posterior-head plasma membrane domains, respectively (Fawcett, 1975; Friend, 1989; Cowan et al., 1987).

Breaching the Diffusion Barrier

Whatever the nature of the diffusion barrier, clearly it must be breached to allow for the redistribution of spermatozoal CE9 from the posterior-tail to the anterior-tail plasma membrane domain during epididymal maturation (Petruszak et al., 1991). Up until now, it has been difficult to study this phenomenon and to dissociate the breaching from what is most likely a diffusion-trapping or active translocation process (Cowan et al., 1991) that concentrates CE9 within the anterior-tail plasma membrane domain by the time the sper-

matozoa have reached the cauda epididymidis (Petruszak et al., 1991). Here we have succeeded in developing and characterizing a simplified *in vitro* system that allows for observation of the relatively rapid and synchronous breaching of the diffusion barrier by CE9 on rat spermatozoa collected from the caput epididymidis.

The redistribution of spermatozoal CE9 is nearly complete after only 12 h *in vitro*. The result is CE9 distributed in a more or less uniform fashion throughout the plasma membrane of the entire tail. Thus, the process most closely resembles the redistribution observed *in vivo* during passage between the caput and corpus epididymidis (Petruszak et al., 1991). Considering that epididymal transit takes 8–12 d in most mammals, including rats (Bedford, 1976; Jones, 1989), the journey from the caput to the corpus epididymidis may be estimated to take at least a few days. That the redistribution can be blocked specifically by polyclonal rabbit anti-CE9 IgG is consistent with a mechanism involving the lateral diffusion of pre-existing cell surface CE9. Even though this polyclonal antibody is inferior to the anti-CE9 mAb at labeling unfixed spermatozoa (see above), it is presumably still able to bind to these unfixed cells in sufficient amounts to bring about the cross-linking of CE9 within the posterior-tail plasma membrane domain. A major factor contributing to the relative rapidity of the redistribution in the *in vitro* assay appears to be the use of 37°C, since the process is slowed considerably even by lowering the temperature to 31.5 or 28°C. The redistribution is also inhibited by DNP, an uncoupler of oxidative phosphorylation, thus implying a need for metabolic energy in the form of ATP.

As assayed by immunofluorescence, the redistribution of spermatozoal CE9 *in vitro* can be divided operationally into at least two phases: a 5-h lag period, during which little redistribution is evident, followed by a 7-h period of lateral redistribution into the anterior-tail plasma membrane domain. In theory, the process could actually be more continuous than it appears, with earlier redistribution falling below the threshold for detection by immunofluorescence. But the fact that we can readily detect presumptive redistribution intermediates, many with considerably lower levels of specific labeling over their anterior-tail domains (e.g., Fig. 7, *e* and *f*), reduces the likelihood that the lag period is an artifact of analysis. It argues, instead, that the concept of a lag period is likely to apply, at least to the redistribution of a substantial proportion of the spermatozoal CE9. The ability to experimentally dissect the redistribution into successive periods of greater and lesser sensitivity to the inhibitors DNP and 28°C further reinforces the notion that we are observing a multistep process. The existence of the lag period is suggestive of a preparatory or priming phase during which the regional diffusion barrier is somehow modified to allow for the subsequent passage of CE9. Since DNP and 28°C are ineffective as inhibitors of redistribution when introduced near the end of the lag period (i.e., at 5 h), we infer that there are steps involved in the modification of the diffusion barrier that require ATP and a temperature above 28°C.

Proteolysis at the cell surface is thought to provide the impetus for the redistribution of the PH-20, PH-30, and AH-50 plasma membrane proteins on the guinea pig spermatozoon during epididymal maturation (Phelps et al., 1990). If proteases are involved in the modification of the regional diffusion barrier on the rat spermatozoon in our *in vitro* as-

say, then these proteases: (a) are not sensitive to inhibition by relatively high concentrations of the nontoxic serine and thiol protease inhibitors aprotinin, antipain, and leupeptin; (b) can act in the presence of a vast excess of BSA; and (c) do not proteolyze CE9 directly. We have already established that the same brief treatment with trypsin that can elicit the redistribution of the PH-20, PH-30, and AH-50 plasma membrane proteins on guinea pig testicular spermatozoa in vitro (Phelps et al., 1990) brings about neither the proteolysis nor the redistribution of CE9 on rat spermatozoa collected from the testis or the caput epididymidis (Petruszak et al., 1991). All of this is not to say that the endoproteolytic removal of the amino-terminal 75 amino acids of spermatozoal CE9 that takes place in the proximal caput epididymidis (Petruszak et al., 1991) is not a prerequisite for breaching the diffusion barrier.

In terms of its sensitivity to reduced temperature and uncouplers of oxidative phosphorylation, the breaching of the diffusion barrier by rat spermatozoal CE9 appears more similar to the redistribution of the PT-1 antigen from the posterior-tail to anterior-tail plasma membrane domain during the capacitation of guinea pig spermatozoa in vitro (Myles and Primakoff, 1984). The major differences are that the redistribution of the PT-1 antigen is much faster, being virtually complete within 1 h at 37°C with a lag period of <15 min, and that the redistribution of the PT-1 antigen takes place on spermatozoa from a much later stage of maturation, those from the cauda epididymidis instead of those from the caput epididymidis. Taken at face value, we think that the data currently available for CE9 and the PT-1 antigen hint at the intriguing possibility that the regional diffusion barrier that demarcates the posterior- and anterior-tail plasma membrane domains of the mammalian spermatozoon may be breached by specific proteins at different rates and times during posttesticular maturation.

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