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Testicular biosynthesis and epididymal endoproteolytic processing of rat sperm surface antigen 2B1

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SUMMARY

Binding of mammalian spermatozoa to the zona pellucida of homologous eggs is mediated by specific molecules on their surface membranes. In the present investigation we describe the biogenesis, epididymal processing and cellular distribution of a plasma membrane antigen (2B1) on rat spermatozoa that has a potential role in mediating zona binding. 2B1 is expressed postmeiotically in the testis as a precursor glycoprotein (~60 kDa) that first appears on the plasma membrane of stage 6 to 8 round spermatids. Northern and western blot analyses show that there is a close correlation between the timing of transcription and expression of the glycoprotein on the cell surface. During spermatid elongation 2B1 is excluded from the head domain and is sequestered onto the sperm tail. As spermatozoa pass through the caput epididymidis 2B1 is endoproteolytically cleaved at a specific arginine residue (Arg 312) to produce a heterodimeric glycoprotein (~40 kDa and ~19 kDa) containing intramolecular disulphide bridges. Endoproteolysis at Arg 312 also takes place during culture of washed testicular or caput spermatozoa in vitro and can be prevented by serine proteinase inhibitors or enhanced by trypsinisation. However, neither processing in vivo or in vitro has any effect on the domain organisation of 2B1 antigen i.e. it remains localised to the tail. These results support the hypothesis that sperm antigens that are important for fertilization are synthesized as precursor molecules in the testis and are then 'activated' during epididymal maturation and capacitation, thereby ensuring that they only become fully functional at the site of fertilization.

Key words: Spermatogenesis, Fertilization, Sperm antigen processing

INTRODUCTION

Mammalian spermatozoa are highly polarised cells with well defined surface domains (Koehler, 1978; Primakoff and Myles, 1983). These domains are first established during spermiogenesis in the testis at the time when round spermatids begin to differentiate into fully formed spermatozoa. Many sperm-specific antigens are transcribed and translated postmeiotically and from recent studies on spermatogenesis in the mouse and guinea-pig it would seem that their expression is also temporally and spatially separated (Scully et al., 1987; Cowen and Myles, 1993). Superimposed on these early differentiation events is extensive remodelling of the plasma membrane during epididymal maturation, capacitation and to a lesser extent the acrosome reaction (Jones, 1989). These remodelling processes are not generalised phenomena but are carefully directed and highly selective for certain membrane components. The net result is that plasma membrane antigens are constantly being modified depending on the developmental status and environment of the spermatozoon, thereby leading to profound changes in activity and cellular location. Intriguingly, many of the antigens that are sequestered into specific surface domains in the testis and subsequently become targets for post-testicular processing are those found to be important for gamete interaction during fertilization. Particularly instructive in this respect is the behaviour of PH20 and PH30 antigens on guinea-pig spermatozoa. Initially, surface-bound PH20 antigen is found over the entire head region of testicular spermatozoa but during epididymal transit it undergoes endoproteolysis and becomes restricted to the postacrosomal region (Cowen et al., 1986; Lathrop et al., 1990; Phelps et al., 1990). PH20 appears to be a multifunctional protein as it shows hyaluronidase and zona binding activities (Lin et al., 1994). PH30 antigen (recently renamed 'fertilin') also localizes to the whole head of testicular spermatozoa, undergoes site-specific cleavage in the epididymis and as a result relocates to the postacrosomal domain on cauda spermatozoa (Primakoff et al., 1987; Blobel et al., 1990; Phelps et al., 1990). Fertilin has a putative role in sperm-oolema binding and fusion via integrin/disintegrin-like interactions (Myles et al., 1994; Perry et al., 1995). A third example of a sperm antigen that undergoes maturational processing is CE9 in which endoproteolysis near the N terminus precedes its redistribution from the posterior to anterior tail domains (Petruszak et al., 1991; Cesario and Bartles, 1994).

We report here on the biogenesis and post-testicular processing of a surface membrane glycoprotein (designated 2B1) on rat spermatozoa that has a presumptive role in mediating recognition and binding to the zona pellucida since: (a) a monoclonal antibody to it blocks fertilization in vitro in a dose dependent manner (Shalgi et al., 1990); (b) during capacitation 2B1 actively migrates from the tail to the acrosomal domain traversing at least one intramembranous barrier in the process (Jones et al., 1990); (c) only spermatozoa with 2B1 antigen on the acrosome have been observed to bind to the zona pellucida of homologous eggs (Shalgi et al., 1990). Although indirect, these findings are supported by the recent demonstration that 2B1 antigen is the rat orthologue of guinea-pig PH20 and that it has hyaluronidase activity (Hou et al., 1996). In the present investigation we show that 2B1 antigen is synthesised post-meiotically as a precursor glycoprotein in the testis. Initially it is distributed all over the plasma membrane of early round spermatids but during later stages of spermatid elongation it is excluded from the head domain and instead is sequestered specifically onto the plasma membrane overlying the sperm tail. As spermatozoa pass through the caput epididymidis 2B1 antigen is endoproteolytically cleaved at an arginine residue near the C terminus to produce the mature, heterodimeric form of the antigen. These results enforce the concept that important molecules on spermatozoa are first synthesised as precursors and only activated at the appropriate time and place for them to be fully functional, thereby allowing a degree of self-regulation by these transcriptionally inactive cells.

MATERIALS AND METHODS

Materials

All chemicals and enzymes were of the highest grade available commercially and unless stated otherwise were purchased from Sigma Chemical Co. (London, UK), BDH-Merck (Lutterworth, UK), Pharmacia-LKB (St Albans, UK), Boehringer Mannheim (Lewes, UK) or Pierce & Warriner (Chester, UK).

Antibodies

A mouse monoclonal antibody (mAb) to 2B1 glycoprotein was produced by fusion of splenic lymphocytes from immunized Balb/c female mice and an NS-1 myeloma cell line as described previously (Gaunt et al., 1983). The mAb was of the IgG_{2a} sub-class (Jones et al., 1990). Purified 2B1 IgG was prepared from ascites fluid produced in Pristane primed mice by affinity chromatography on Protein A-Sepharose 4B (Pharmacia-LKB) following the suppliers recommendations. A polyclonal antiserum to a synthetic l9-residue peptide derived from the C-terminal region of the 2B1 sequence (Hou et al., 1996) was produced by conjugating the peptide to a purified protein derivative of tuberculin followed by subcutaneous and intramuscular injection into BCG-sensitised Dutch rabbits. This peptide had the sequence EAGKYEVLGKPEVKDLEYF. Preimmune and immune antisera were collected by standard procedures, decomplemented at 56°C for 30 minutes and stored at −70°C. FITC-conjugated rabbit anti-mouse IgG (FITC-RAM), biotinylated rabbit anti-mouse IgG (biotin-RAM), biotinylated swine anti-rabbit IgG (biotin-SAR) and peroxidase conjugated avidin (Px-avidin) were purchased from Dako Laboratories (High Wycombe, UK) and used as supplied. Pancreatic trypsin was obtained from Boehringer and angiotensin converting enzyme (ACE) and tissue plasminogen activator (tPA) from Sigma.

Collection and immunofluorescence staining of spermatogenic cells and spermatozoa

Testis tissue (~ 0.5 g) from adult rats (Wistar strain) was minced in 5 ml PBS (138 mM NaCl, 2 mM KCl, 10.3 mM NaH_2PO_4, 8.5 mM Na_2HPO_4, pH 7.2; 290 mOsmol/kg) containing a cocktail of protease inhibitors (1.0 mM AEBSF, 5.0 mM *p*-aminobenzamidine, 2.0 μM leupeptin, 1.3 μM pepstatin) and large fragments of tissue allowed to settle for 5 minutes at room temperature. Cells remaining in the supernatant were stained by indirect immunofluorescence (IIF) microscopy with 2B1 mAb/FITC-RAM as described (Jones et al., 1990). Clumps of cells or obviously damaged cells were not included in the analysis. Spermatogenic cell-types were identified and classified according to the method of Russell et al. (1990).

To study the postnatal development of 2B1 antigen by western blotting, testes from 10-, 20-, 40- and 60-day-old rats were minced as described above and cells dispersed by homogenising with 3 strokes of a loose-fitting glass-teflon Potter homogeniser. Suspensions were centrifuged at 600 ***g*** for 15 minutes and pellets extracted with 1% NP-40 detergent in PBS for 45 minutes at 4°C. Cell-free supernatants were collected by centrifugation at 10,000 ***g*** for 10 minutes and stored frozen until analysis.

Testicular spermatozoa were collected by puncturing the extratesticular rete testis of anaesthetised rats 18-20 hours after ligation of the efferent ductules. Typically, 100-150 μl of testicular fluid, free of contamination from blood or lymph and containing ~10^6 spermatozoa/ml, could be obtained from each rat. Spermatozoa were collected from the caput, corpus and cauda epididymidis by mincing the tissue in PBS/5 mM glucose + protease inhibitors (see above) and purified by washing through a Percoll step gradient (20%:30%:40% in PBS) by centrifugation at 250 ***g*** for 10 minutes. Recovered spermatozoa were either used for in vitro culture experiments (see later) or membrane proteins solubilised by incubation in 4 vols of 0.4% sodium deoxycholate (DOC) in PBS/10 mM Tris-HCl, pH 8.5, for 45 minutes at 4°C. Detergent extracts were collected by centrifugation at 10,000 ***g*** for 10 minutes and stored frozen at −20°C until analysis.

Immunohistochemistry

Pieces of testis were snap frozen on dry ice at −80°C and 10 μm thick sections cut on a cryostat. Sections were dried onto pre-cleaned glass slides, blocked with 5% BSA/5% normal rabbit serum in PBS for 1 hour and then probed with 2B1 mAb supernatant/FITC-RAM as described (Jones et al., 1985). HAT medium was used as control. Sections were covered in antifadant solution (Citifluor, UKC, Canterbury, UK) in glycerol/PBS (1:1) and viewed by epifluorescence microscopy on a Zeiss Axiophot photomicroscope.

Electrophoresis and western blotting

Proteins in detergent extracts were separated by 1-dimensional SDS-PAGE (Laemmli, 1970) under non-reducing or reducing (5% 2-mercaptoethanol for 4 minutes at 100°C) conditions and gels either stained with 5% Coomassie Blue R-250 in fixative (40% methanol, 7% acetic acid, 53% water) or electroblotted on to PVDF nylon membranes (Immobilon; Millipore (UK) Ltd, Watford) using a semi-dry blotting system (Schleicher and Schuell Ltd, Germany). Blots were probed with 2B1 mAb/biotin-RAM/Px-avidin or 2B1 peptide antiserum/biotin-SAR/Px-avidin essentially as described previously (Jones et al., 1990) and antigen-antibody complexes visualised with 4-chloronaphthol dye. Molecular masses were estimated from relative mobilities of known protein standards (Rainbow markers, Pharmacia-LKB).

For 2-dimensional SDS-PAGE, a plasma membrane enriched fraction was prepared from testicular and cauda spermatozoa by vortex-mixing (Jones, 1986), the pellet dissolved in O'Farrell lysis buffer (O'Farrell, 1974) and proteins separated by iso-electric focusing in the first dimension with ampholyte pH 3-10 and reducing SDS-PAGE in the second dimension on a Millipore electrophoresis system according to the manufacturer's instructions. This system utilizes very large second dimension gels (23 cm × 26 cm) to achieve high resolution of separated proteins. Gels were either silver-stained (Giulian et al., 1983) or electroblotted onto Immobilon and probed with 2B1 mAb as described above.

Protein sequencing

Western blots from 1- or 2-dimensional SDS-PAGE gels were stained lightly with Coomassie Blue R-250 as described above and the position of 2B1 antigen located by reference to parallel blots stained with 2B1 mAb/biotin-RAM/Px-Avidin. Further identification of 2B1 antigen on 2-D blots was aided by its highly characteristic appearance (see later). Relevant protein bands were excised, washed thoroughly in distilled water and subjected to N-terminal amino acid sequence analysis on an Applied Biosystems gas phase sequencer (Model 470A) equipped with an on-line phenylthiohydrontoin analyser (Model 120A). The sequencing programme was run as recommended by the manufacturer.

Culture of spermatozoa in vitro

Percoll-purified spermatozoa from the testis, proximal caput epididymidis and cauda epididymidis were suspended in a modified RFM medium (Shalgi et al., l990) containing 107 mM NaCl, 1.2 mM $MgSO_4$, 4.3 mM KCl, 1.2 mM KH_2PO_4, 4.5 mM $CaCl_2$, 5.5 mM D-glucose, 24.1 mM sodium lactate, 0.5 mM sodium pyruvate, 24.0 mM $NaHCO_3$, 0.4% BSA, 0.5 μg/ml streptomycin sulphate and 0.6 μg/ml sodium penicillin/gentamicin. The pH was adjusted to 6.8 with 32 mM Pipes which gave an osmolality of 290-310 mOsmol/kg. This pH was chosen to resemble that of epididymal fluid in vivo (~7.0; Jones, 1978); it rose by 0.2 units during incubation. Sperm suspensions (~10^6 sperm/ml) were incubated under water-saturated mineral oil at 37°C in an atmosphere of air/5% CO_2 in the absence (control) or presence of 10.0 mM sodium fluoride + 10.0 mM 2,4-dinitrophenol or 2.0 mM AEBSF or 1.8 μM aprotinin or 12.0 μM leupeptin or 4.1 μM pepstatin or 1 mM benzamidine or 270.0 μM TLCK or RFM minus $CaCl_2$ + 1 mM EGTA. Samples were withdrawn after 20 hours, centrifuged at 10,000 ***g*** for 3 minutes and pelleted spermatozoa extracted with 0.4% DOC as described above. Solubilized proteins were analysed by SDS-PAGE/western blotting. Alternatively, caput spermatozoa were incubated for 3 hours in the presence of trypsin (25 μg/ml) or ACE (0.1 unit/ml) or tPA (10 μg/ml) followed by extraction and analysis of proteins as described above.

Detection of glycosylated forms of 2B1 antigen

The presence of carbohydrate on 2B1 antigen was investigated in 2 ways: (1) periodic acid Schiff (PAS) reaction for total carbohydrate. Detergent solubilized proteins from testicular and cauda spermatozoa were separated by SDS-PAGE and gels fixed and stained with PAS reagent (Dubray and Bezard, 1982). Parallel tracks from the same gel were either stained with Coomassie Blue R-250 or western blotted and probed with 2B1 mAb as described above. (2) Binding of peroxidase-conjugated lectins. Western blots containing sperm proteins separated by SDS-PAGE were probed with peroxidase-conjugated lectins from: *Cancavalia ensiformis* (specificity for α-mannose and α-glucose), *Triticum vulgaris* (specificity for N-acetylglucosamine and N-acetyl-neuraminic acid), *Pisum sativum* (specificity for α-mannose), *Arachis hypoglycia* (specificity for α-galactose and N-acetyl-1,3-galactosamine) and *Ulex europeus* (UEA I, specificity for α-L-fucose). Bound lectin was detected with 4-chloronaphthol dye reagent as described above.

Isolation of total RNA and northern blot analysis

Testes were removed from adult and neonatal rats (20, 30, and 50 days post partum), snap frozen in liquid nitrogen and stored frozen at −70°C until required. Total RNA was extracted using a TRIzol™ (Gibco-BRL)-based method. Samples (15 μg) were then subjected to northern blot analysis as described (Hou et al., 1996). The integrity of the RNA samples and equivalence of lane loadings was confirmed by reprobing the blot with α-actin cloned cDNA (Humphries et al., 1981).

RESULTS

Expression of 2B1 antigen during spermatogenesis

We have reported previously that 2B1 mAb binds strongly to spermatozoa collected from the extratesticular rete testis (Gaunt et al., 1983) indicating that the antigen is expressed during spermatogenesis and is not an epididymal maturation antigen. To investigate if its synthesis was cell- and stage-specific, frozen sections of adult testis were probed with 2B1 mAb/FITC-RAM. Results showed that there was no reaction in the interstitial cells of Leydig, blood or lymphatic vessels and that fluorescence was confined to the seminiferous tubule (Fig. 1). Within the seminiferous epithelium, Sertoli cells and spermatogonia were negative and staining was confined to post-meiotic germ cells and fully formed spermatozoa in the lumen of the duct. This stage-specific expression was confirmed by staining freshly isolated cells released from testis minces. 2B1 antigen could not be detected on spermatogonia, primary spermatocytes or stage 1-4 round spermatids but it was present all over the plasma membrane of stage 8-10 spermatids (Fig. 2). During later stages (stages 10-12) the antigen was excluded from the elongating sperm head so that on stage 18-19 spermatids it was found exclusively on the plasma membrane overlying the residual body and tail domain (Fig. 2). Restriction of 2B1 to the tail domain was also characteristic of fully formed spermatozoa released into the lumen of the seminiferous tubule (Fig. 2).

Further confirmation of the post-meiotic expression of 2B1 antigen was provided by documenting its appearance in the testis during post-natal development. In the rat, pachytene spermatocytes first appear at day 15 and round spermatids between days 25 and 30 (Yang et al., 1990). No reaction was present on frozen sections of 20-day-old testis when viewed by IIF and it was not until 30 days that a positive reaction was obtained in the seminiferous tubules (results not shown). Similarly, western blots of 1% NP-40 solubilized proteins from whole cell suspensions showed that 2B1 antigen was not present in 20-day-old testis but became detectable after 30 and 60 days (Fig. 3A). The apparent molecular mass of 2B1 antigen at 30

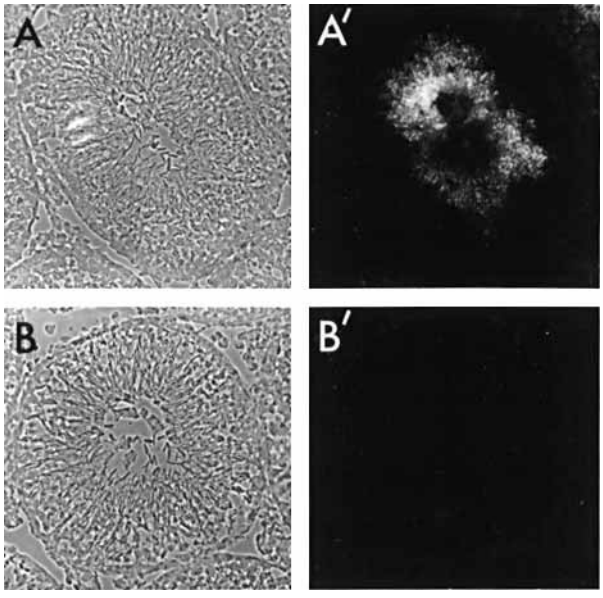

Fig. 1. Indirect immunofluorescence of frozen sections of adult rat testis probed with 2B1 mAb/FITC-RAM (A,A′) or HAT medium/FITC-RAM (B,B′). A and B, phase contrast; A′ and B′, fluorescence. ×45.

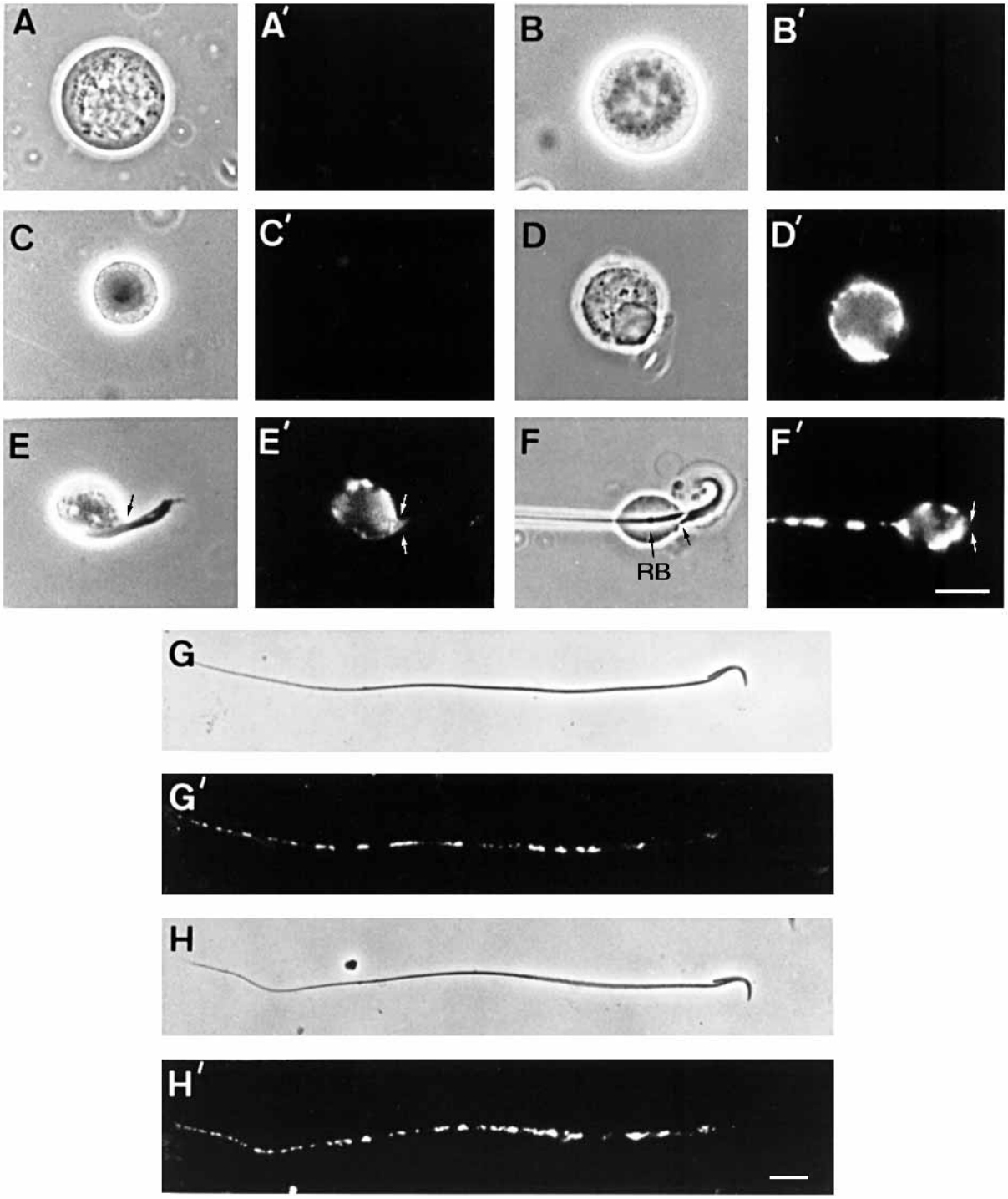

Fig. 2. Paired phase contrast (A to H) and fluorescence (A′ to H′) photographs of spermatogenic cells from minces of adult rat testis stained with 2B1 mAb/FITC-RAM. (A,A′) Spermatogonium. (B,B′) Pachytene spermatocyte. (C,C′) Stage 1-4 round spermatid. (D,D′) Stage 6-8 round spermatid. (E,E′) Stage 10-12 elongating spermatid (at this stage the tails are fragile and break off easily). (F,F′) Stage 16-18 elongated spermatid. (G,G′) Testicular spermatozoon. (H,H′) Cauda spermatozoon. RB, residual body. Arrows in E,E′ and F,F′ indicate the neck region. Bar, 5 μm.

days was the same as that on fully formed spermatozoa collected from the rete testis, suggesting that this form (~60 kDa on reducing SDS-PAGE) represents the precursor version of the antigen.

Since temporal expression of many proteins in the testis is controlled at the translational level (Hecht, 1986), we next investigated if 2B1 mRNA transcripts could be detected in pre-meiotic as well as post-meiotic germ cells. Northern blot analysis of RNA from neonatal testes revealed that 2B1 transcripts (2.2 kb) were not present at 20 days but became

detectable after 30 days (Fig. 3B) suggesting that the timing of transcription and expression of 2B1 glycoprotein on the cell surface are closely linked.

Processing of 2B1 antigen during epididymal maturation in vivo

As described above, 2B1 antigen is distributed uniformly over the tail domain of 95% of fully formed immature testicular spermatozoa, only occasionally extending anteriorly towards the postacrosomal or equatorial segments. This localization does not change during epididymal maturation (Fig. 2) and cauda spermatozoa always show strong patchy fluorescence on the tail when viewed by IIF microscopy. In previous work we have shown that patching is a clustering reaction induced by the polyvalent second layer antibody and that it is a reflection of the ability of 2B1 antigen to move laterally within the plane of the plasma membrane (Gaunt et al., 1983). Restriction of 2B1 antigen to the tail domain is also consistently observed under a variety of labelling conditions, e.g. spermatozoa air dried on glass slides or pre-fixed in 4% paraformaldehyde or after 2 cycles of freeze-thawing to expose intracellular antigens. However, incubation of spermatozoa in membrane solubilizing reagents, such as 0.4% DOC, abolishes labelling completely. This confirms our previous results using immunogold electron microscopy that 2B1 antigen is found exclusively on the plasma membrane and is not associated with intracellular organelles (Jones et al., 1990).

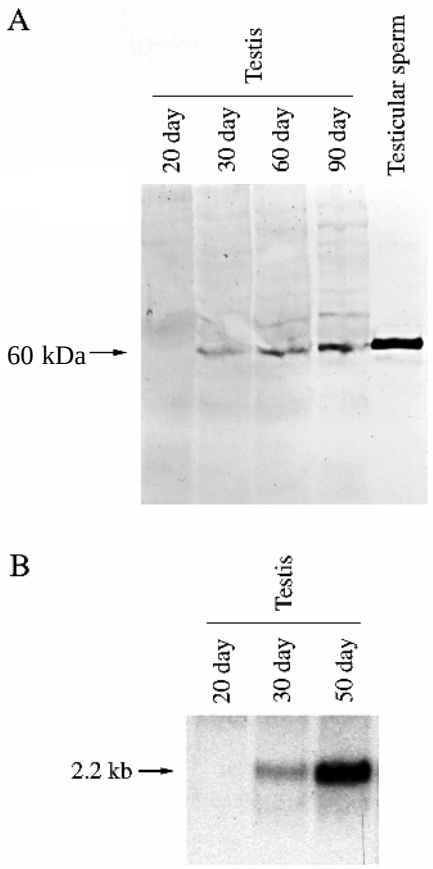

Fig. 3. (A) Western blot of detergent-soluble proteins extracted from neonatal testis (20, 30, 60 and 90 days old) and testicular spermatozoa probed with 2B1 mAb/biotin-RAM/Px-avidin. Proteins were separated by reducing SDS-PAGE. Relative molecular masses are indicated. (B) Northern blot analysis of 2B1 transcripts in total RNA isolated from testes of 20-, 30-, and 50-day-old rats. The relative size of the transcript is indicated.

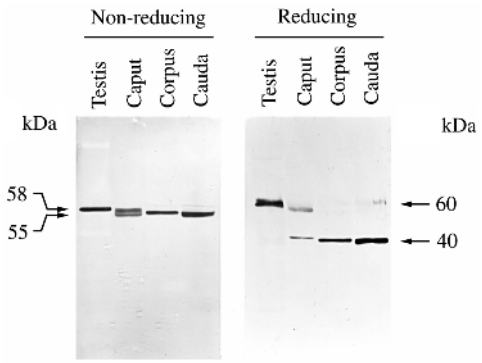

Fig. 4. Processing of 2B1 antigen during epididymal maturation in vivo. Western blots containing reduced or non-reduced detergent-soluble proteins from testicular, proximal caput, corpus and cauda epididymidal spermatozoa were probed with 2B1 mAb/biotin-RAM/Px-avidin.

To investigate if there were any biochemical changes to 2B1 antigen during epididymal maturation, 0.4% DOC-solubilized proteins from testicular, proximal caput, corpus and cauda spermatozoa were analysed by SDS-PAGE/western blotting. Under non-reducing conditions testicular 2B1 has a molecular mass of ~58 kDa but on proximal caput spermatozoa a doublet appears at ~56 kDa to ~57 kDa (Fig. 4). In extracts of corpus spermatozoa a single band predominates at ~56 kDa and on cauda spermatozoa there is a further decrease to ~55 kDa. These differences in electrophoretic mobility of 2B1 are enhanced following reduction of the protein with 2-mercaptoethanol. Testicular 2B1 now has a mass of ~60 kDa, proximal caput 2B1 a doublet at ~58 kDa and ~42 kDa and corpus and cauda 2B1 ~40 kDa (Fig. 4). The fact that a cocktail of protease inhibitors was present during solubilization of membrane proteins and that similar results could be obtained using 1% SDS (a potent inhibitor of most proteases) instead of 0.4% DOC, argues against the observed differences being artifacts of the extraction protocol. No reaction was observed on western blots probed with HAT medium/PxRAM or non-immune mouse IgG/Px-RAM (results not shown).

Although 2B1 antigen is readily detectable on western blots with the mAb, we have not been able to immunoprecipitate it from detergent extracts of any type of spermatozoa using Protein A-Sepharose 4B or 2B1 IgG conjugated to Sepharose 4B beads. This suggests that in solution the precipitating epitope on 2B1 antigen is either blocked or modified by the detergent or is simply not available to the mAb because of conformational changes. A scan of different detergents (Triton X-100, Nonidet P-40, Chaps, Tween-20) did not improve the situation. However, our evidence indicates that 2B1 mAb recognizes a protein- rather than a carbohydrate-based epitope since: (i) treatment of cauda spermatozoa with a cocktail of exoglycosidases has no effect on binding of 2B1 mAb (results not shown); (ii) a bacterial expressed fusion protein (which is not glycosylated) containing the N-terminal 150 residues of 2B1 sequence, is recognised strongly by 2B1 mAb (Hou et al., 1996).

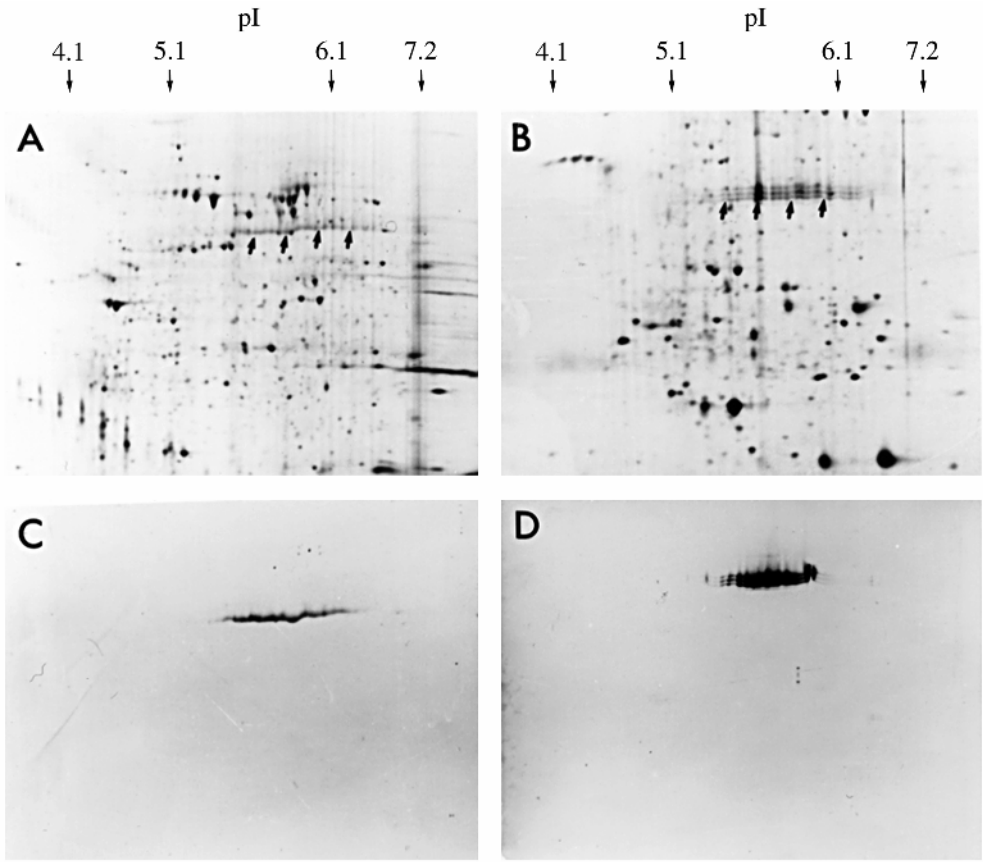

Fig. 5. Membrane proteins from testicular (A and C) and cauda epididymidal (B and D) spermatozoa were separated by 2-D SDS-PAGE and either visualised by silver staining (A and B) or transferred to western blots and probed with 2B1 mAb/biotin-RAM/Px-avidin (C and D). The position of 2B1 antigen in A and B is indicated by arrows. Parallel blots to C and D were stained with Coomassie Blue and areas containing 2B1 antigen excised and subjected to N-terminal sequence analysis.

Both testicular and cauda forms of 2B1 antigen are glycosylated to similar extents as judged by the intensity of the PAS reaction on SDS-polyacrylamide gels and the fact that they bind peroxidase-conjugated *Cancavalia ensiformis* and *Pisum savitum* lectins with equal intensity (results not shown). The only evidence for processing of carbohydrate moieties during epididymal maturation was shown by significantly stronger binding of *Triticum vulgaris* agglutinin to cauda 2B1 relative to testicular 2B1, suggesting addition of sialic acid residues to the termini of carbohydrate chains (results not shown).

Identification of endoproteolytic cleavage site(s) in 2B1 glycoprotein during epididymal maturation

One explanation for the large difference in size between testicular and cauda forms of 2B1 antigen following analysis by reducing SDS-PAGE is that the glycoprotein has undergone endoproteolytic cleavage with the smaller fragment(s) attached to the larger portion of the protein by one or more disulphide bridges. To assess if the cleavage site(s) were located towards the N or C terminus, enriched preparations of plasma membranes from testicular and cauda spermatozoa were solubilized in O'Farrell lysis buffer and proteins separated by 2-dimensional SDS-PAGE under reducing conditions. The position of 2B1 antigen was identified by a combination of silver staining and probing western blots from parallel gels with 2B1 mAb. Both testicular and cauda forms of 2B1 antigen focused with a broad pI between pH 5.3 and 6.3 (Fig. 5). Testicular 2B1 gave a single band whereas cauda 2B1 migrated as 3 closely spaced parallel bands indicating greater heterogeneity in size. N-terminal microsequencing of each form excised from 2-dimensional western blots stained with Coomassie Blue R-250 gave the amino acids VDYRAT... for cauda 2B1 and VXYRAT... for testicular 2B1 (X represents an unidentified residue). This close correspondence strongly suggests that cleavage has not released an N-terminal fragment. To ascertain the exact position of the internal cleavage site, western blots of reduced cauda 2B1 antigen separated by SDS-PAGE were probed with a polyclonal antibody raised to a synthetic peptide representing residues 376 to 394 (2B1 antigen is 477 residues long; Hou et al., 1996). A single band was obtained at 19 kDa (Fig. 6). Direct microsequencing of this fragment gave SAGCPILRQY... which represents residues 313 to 321 of the full sequence. This suggests that there is a single cleavage site on the C-terminal side of Arg 312. As calculated from the amino acid composition, this would release a fragment of ~18.4 kDa which is close to the size of that observed on the western blot with the anti-peptide antibody (Fig. 6).

Processing of 2B1 antigen during culture of immature spermatozoa in vitro

Spermatozoa normally pass through the initial segment and proximal caput epididymidis in 1 to 2 days. During this time >90% of testicular fluid is re-absorbed and remodelling of the plasma membrane of spermatozoa begins. However, it is not clear how much of this remodelling is intrinsic to spermatozoa and how much is induced by epididymal secretions. To elucidate the mechanisms underlying the processing of 2B1 antigen observed in vivo, we attempted to reproduce the phenomenon in vitro under defined conditions. For this purpose, spermatozoa from the testis and proximal caput epididymidis

were collected under sterile conditions, washed through Percoll and resuspended in RFM to approximately 10^6/ml. Spermatozoa were incubated at 37°C in 95% air/5% CO_2 and samples withdrawn at 0, 5 and 20 hours for analysis by reducing SDS-PAGE/western blotting with 2B1 mAb. Results showed that after incubation of testicular spermatozoa for 5 hours, a weakly staining band appeared at 45 kDa that increased in intensity after 20 hours (Fig. 7). Concomitantly, there was a noticeable decrease in staining of the 60 kDa antigen. This conversion from a higher to lower molecular mass form was much more pronounced during incubation of proximal caput spermatozoa. With these cells the intensity of the 58 kDa component decreased substantially in amount during incubation whilst the 45 kDa form increased in a parallel fashion suggesting a precursor-product relationship. In contrast to the above, the mature 40 kDa form of 2B1 antigen did not change in size or relative amounts during incubation of cauda spermatozoa in vitro (Fig. 7).

These results suggest that conversion of testicular sperm 2B1 antigen to a cauda-like form takes place spontaneously in the absence of epididymal secretions during incubation in vitro over a time-scale similar to that for passage of spermatozoa through the proximal caput epididymidis in vivo. To determine the mechanisms regulating this transformation, proximal caput spermatozoa were incubated for 20 hours in RFM in the presence of different combinations of metabolic poisons and protease inhibitors. These were 10.0 mM sodium fluoride + 10.0 mM 2,4-dinitrophenol, or 1 mM EGTA or 270 μM TLCK or 2 mM AEBSF or 1.8 μM aprotinin or 12.0 μM leupeptin or 4.1 μM pepstatin or 1.0 mM benzamidine-HCl. It was found that the presence of metabolic poisons or absence of Ca^{2+} ions had no effect on the conversion of the caput form of 2B1 antigen to the cauda-like form but several protease inhibitors

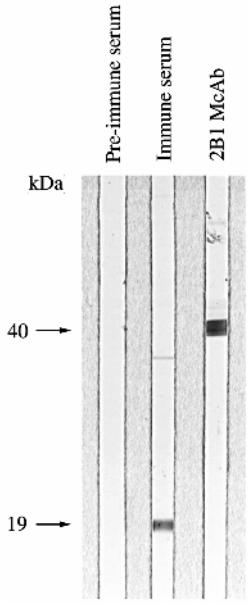

Fig. 6. Western blot of detergent-extracted proteins from cauda epididymidal spermatozoa separated by reducing SDS-PAGE and probed with 2B1 mAb/biotin-RAM/Px-avidin or a rabbit polyclonal antiserum to a C-terminal peptide derived from the 2B1 sequence (Hou et al., 1996).

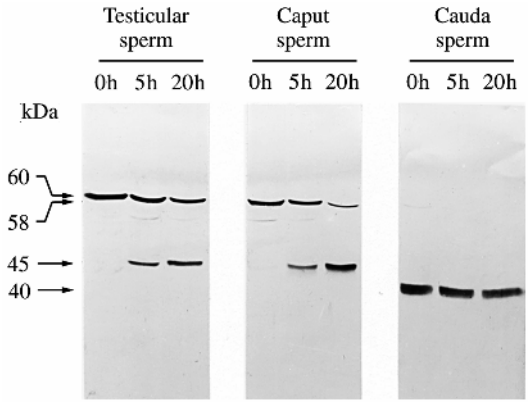

Fig. 7. Testicular, proximal caput and cauda epididymidal spermatozoa were incubated in vitro for 0, 5 and 20 hours and detergent-extracted proteins analysed by reducing SDS-PAGE/western blotting using 2B1 mAb. With testicular and proximal caput spermatozoa there was a progressive increase in the amounts of a 45 kDa component concomitant with a decrease in the 60/58 kDa antigen. By contrast, the 40 kDa cauda antigen remained unaffected.

prevented it completely (Fig. 8A). The most effective were AEBSF and leupeptin, both known to inhibit serine and cysteine proteases. Aprotinin, pepstatin, benzamidine-HCl and TLCK were not inhibitory at the concentrations tested. Transformation of caput 2B1 antigen into the cauda-like form was also temperature sensitive as it was retarded by 50% to 60% if proximal caput spermatozoa were incubated at 5°C rather than 37°C for 20 hours (results not shown).

Preliminary evidence indicates that the position of the internal cleavage site is the same in vitro as in vivo. First, the C-terminal subunit produced in vitro has a molecular mass of 19.5 kDa as detected on a western blot probed with the anti-peptide polyclonal antibody (results not shown). Second, microsequencing of this 19.5 kDa subunit gave SXGX-PILXQX which represents 6 out of 10 residues that correspond in position to the amino acids between Ser 313 and Try 321 (this sequence is SAGCPILRQY as determined by cDNA cloning; Hou et al., 1996; X indicates an unidentified residue). We tentatively conclude, therefore, that during incubation of immature spermatozoa in vitro a single cleavage still occurs at Arg 312 but that the N-terminal subunit so produced has a higher molecular mass than that found in vivo where further modifications take place that are not reproduced in vitro. This does not appear to be the case with the C-terminal subunit which has a similar size in vivo and in vitro.

Processing of 2B1 antigen by exogenous proteinases

To investigate if exogenously added proteinases could mimic under acute culture conditions the processing observed in vivo or in vitro, proximal caput spermatozoa were incubated at 37°C for 3 hours in the presence of pancreatic trypsin (2.5 μg/ml) or tPA (10 μg/ml) or ACE (0.1 unit/ml), washed and DOC extracted-proteins analysed by SDS-PAGE/western blotting with 2B1 mAb1. tPA and ACE were chosen because they have been detected in testicular and epididymal secretions (Vanha-Perttula et al., 1985; Huarte et al., 1987). Trypsin caused a rapid breakdown of caput 2B1 antigen to a 45 kDa component

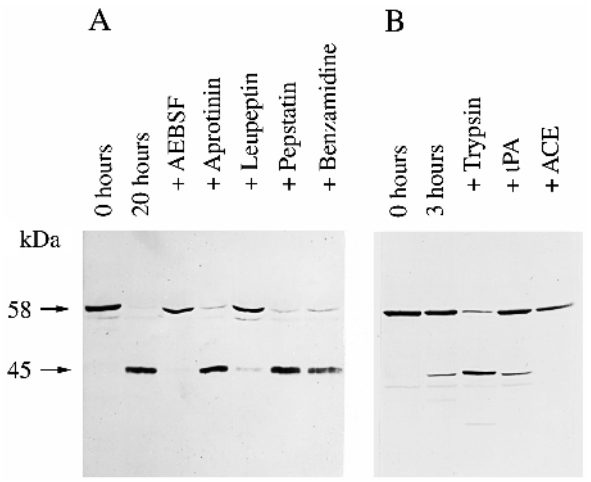

Fig. 8. Effects of co-incubation with protease inhibitors (A) and exogenous proteinases (B) on processing of 2B1 antigen on caput spermatozoa in vitro. Spermatozoa were incubated under conditions described in detail in Materials and Methods. ACE, angiotensin converting enzyme; tPA, tissue plasminogen.

that co-migrated with the cauda-like form produced after 20 hours of culture in vitro (Fig. 8B). tPA had no apparent effect whereas ACE appeared to be inhibitory relative to incubated controls.

Effects of endoproteolysis on domain organisation of 2B1 antigen

The domain distribution of 2B1 antigen does not change during maturation of spermatozoa in vivo in the epididymis (Fig. 2) indicating that endoproteolysis per se does not induce the antigen to migrate in a manner similar to that observed after capacitation (Jones et al., 1990). To investigate if the processed 45 kDa form of 2B1 antigen that is produced during prolonged incubation in vitro, or after trypsinisation, changes its domain organisation, proximal caput spermatozoa were incubated as described above and stained with 2B1 mAb/FITC-RAM. As a positive control, spermatozoa were incubated in the presence of 2 mM AEBSF. Results showed that in all samples 2B1 antigen remained on the tail and did not migrate to the acrosome (results not shown).

DISCUSSION

This work has shown that the rat sperm antigen 2B1 is synthesised postmeiotically in the testis as a precursor glycoprotein of ~60 kDa and although initially it is found all over the plasma membrane of early round spermatids, during later stages of differentiation it is excluded from the head domain and instead is confined to the residual body and growing flagellum. As spermatozoa pass through the caput epididymidis 2B1 antigen is cleaved endoproteolytically at a single site (Arg 312) to produce a heterodimeric glycoprotein (N-terminal of ~40 kDa and C-terminal subunit of ~19 kDa) that is capable of actively migrating from the tail to the acrosomal domain during capacitation. In vitro incubation of washed caput spermatozoa, or brief digestion with trypsin, induces the formation of a 'cauda-like' form of 2B1 that is internally cleaved at the same site but has a larger N-terminal subunit (~45 kDa) suggesting that other processing events must take place in addition to endoproteolysis.

Biosynthesis of sperm surface membrane domains during spermiogenesis

A multiplicity of mechanisms could account for the differential localisation of surface membrane antigens during spermiogenesis. The first requires directional migration of pre-existing antigens to specific sites on the differentiating spermatid. This has been described for mouse galactosyltransferase which is distributed all over the plasma membrane of spermatogonia and spermocytes but during spermiogenesis coaleses onto the acrosomal cap domain with a 77-fold increase in surface density (Scully et al., 1987). The mechanisms that induce sperm antigens to move against such large concentration gradients are not clear (see later) but Scully et al. (1987) have suggested that filamentous actin, which co-localises with GalTase on spermatids and mature spermatozoa, plays a role. 2B1 is also a migrating antigen with a transmembrane domain (Hou et al., 1996) and its exclusion from the head region at stages 10-12 of spermiogenesis could be due to active transport in a posterior direction, particularly since it is about this time that a potential diffusion barrier, the posterior ring, is established in the neck region. If such a barrier could act like an impermeable filter to 2B1, it would account for its selective removal from the head domain.

A second mechanism that could cause disappearance of 2B1 from the sperm head would be specific internalisation and degradation rather than migration. Although a theoretical possibility, this has not been documented convincingly for any sperm surface antigen and we have not observed 2B1 intracellularly on sperm heads either by fluorescence or immunogold electron microscopy (Jones et al., 1990).

Two other features of spermiogenesis that provide potential control points for vectoral transport of proteins are postmeiotic gene expression and translational regulation of stored mRNAs. Together, these processes have a major influence on the fate of sperm antigens (Hecht et al., 1986; Eddy et al., 1993). Direct evidence that temporal control of protein translation can affect its destination has been provided by Braun et al. (1989) who found in transgenic mice containing a human growth hormone (hGH) gene under the control of a protamine promoter that translation of hGH mRNA in early round spermatids resulted in acrosomal localisation whereas if translation was delayed to the elongating spermatid stage (the timing of translation depended on the length of the 3′ untranslated region of the transgene transcript) hGH was distributed intracellularly, but excluded from the acrosome. Given that the same protein was involved in each instance, this suggests that beyond a certain time-point it can only be routed to specific locations. From their studies on temporal expression of antigens during spermatogenesis in guinea-pigs, Cowen and Myles (1993) have proposed a model in which localisation patterns would always be produced if newly synthesised proteins were inserted into anterior-most domains and diffusion between domains was prevented. Thus, tail-specific antigens would be expressed at the pachytene or early spermatid stage whereas whole head or acrosomal antigens would be produced later at the elongation stages and prevented from diffusing posteriorly by intramembraneous barriers. Although this scheme can explain the

pattern of localisation of a number of antigens detected on the guinea-pig sperm surface with monoclonal antibodies, others are less easily accounted for, e.g. galactosyltransferase mentioned above. Another enigma is guinea-pig PH20 which contains a GPI anchor but during spermiogenesis is directed to the inner acrosomal membrane as well as the surface membrane (Phelps et al., 1990). The mechanisms responsible for this dichotomy in routing are difficult to explain by the above model, particularly since a GPI anchor is the classic signal for sorting proteins to the apical plasma membrane (Lisanti and Rodriguez-Boulan, 1989). In addition, given the high degree of sequence identity between species homologues of PH20/2B1 (Hou et al., 1996), it is not clear how their different surface locations (posterior head in guinea pig, tail in rat, acrosome in mouse and human) are achieved without invoking other systems.

A fifth mechanism for directional targeting of antigens is storage in the ER and Golgi pathways prior to their expression on the surface as described recently for CE9 (Cesario et al., 1995) and pre-fertilin (Carroll et al., 1995). Both CE9 (rat) and pre-fertilin (guinea-pig) are synthesised in primary spermatocytes and can be detected intracellularly well before their appearance on the surface membrane of spermatids at stage 11. Thereafter, CE9 locates to the posterior tail domain and pre-fertilin to the posterior head concomitant with a decrease in their amounts in the Golgi cisternae. This implies that within the same spermatid different proteins can be stored in the ER and then transported to various parts of the cell when the correct developmental stage has been reached. However, we have no evidence that 2B1 glycoprotein or its mRNA transcripts are stored intracellularly since during neonatal development there is co-incidence between the timing of its transcription and expression on the surface membrane as shown by northern and western blotting experiments.

Overall, therefore, a number of mechanisms exist within the developing spermatid for sorting and targeting of surface membrane antigens. They appear to operate concurrently and the pathways probably interconnect making it difficult to decipher which system relates to which antigen at any particular time.

Processing of sperm membrane antigens during epididymal maturation

It is known that extensive remodelling of the plasma membrane of spermatozoa takes place during epididymal maturation coincident with development of their fertilizing capacity (Bedford, 1975; Jones, 1989). The majority of these changes are caused by interaction with epididymal secretory proteins, either directly by adsorption onto the plasma membrane, or indirectly by modification of existing membrane proteins. The present results clearly demonstrate that the latter, in the form of endoproteolysis, is an important aspect of post-testicular sperm development in the rat. In vivo, testicular 2B1 antigen is cleaved at the C-terminal side of Arg 312 as spermatozoa pass through the proximal caput epididymidis. Since there is only a single cleavage site, despite the presence of numerous arginine and lysine residues throughout the sequence (Hou et al., 1996), it suggests that the conformation of the extracellular domain of the protein is important in determining accessibility to the proteinase. The 2B1 dimer so produced is cross-linked by one or more intramolecular disulphide bridges (the C-terminal fragment contains 9 cysteine residues and the larger N-terminal fragment 5 cysteines; Hou et al., 1996) which explains its behaviour on reducing versus non-reducing SDS-PAGE. The difference in molecular mass between the larger N-terminal subunit (40 kDa) of authentic cauda 2B1 and that produced after trypsinization or culture of caput spermatozoa in vitro (45 kDa) probably reflects other processing events we have not detected, e.g. to the carbohydrate moiety. Another tail specific antigen on rat spermatozoa, CE9, also undergoes endoproteolysis in the proximal caput epididymidis except that in this case the cleavage site is located towards the N terminus at Arg 74 and the smaller fragment is lost (Petruszak et al., 1991). The source and specificity of the extracellular proteinase(s) involved are not known but at least some must be serine proteinases on the basis of a consistent arginine residue in the cleavage site and the ability of type-specific inhibitors to prevent processing in vitro. Extracellular proteinases, such as ACE and tPA, have been detected in epididymal secretions (Vanha-Perttula et al., 1985; Huarte et al., 1987) as have serine protease inhibitors (Perry et al., 1993). Together, they could conceivably mediate and regulate these transformations in vivo. However, exogenous ACE and tPA have no effect on 2B1 in vitro and since spermatozoa were Percoll-washed to remove epididymal secretions, it follows that the proteinases must be firmly bound to the surface membrane. Alternatively, they may leak out of prematurely acrosome reacted spermatozoa during prolonged incubation. This would obviously not be the case in vivo, however, as spermatozoa reach the cauda epididymidis in a fully fertile and intact state. Additional studies are required to determine the source of these proteinases and their effects on spermatozoa.

It is of interest that the hyaluronidase activity of rat 2B1 and its guinea pig counterpart, PH20, is present in both precursor and processed forms (Lin et al., 1994; Hou et al., 1996), indicating that cleavage at Arg 312 is not obligatory for formation of the enzyme's active site. Of equal interest is the presence of several hyaluronan binding motifs, $B(X_7)B$, within the primary sequences of 2B1 and PH20 (Hou et al., 1996). This motif is common to a wide range of cell surface glycoproteins that mediate binding to extracellular matrices. Well known examples are CD44, RHAMM and link protein (Yang et al., 1994). 2B1 and PH20, therefore, appear to be multifunctional proteins with roles in facilitating penetration of spermatozoa through the cumulus oophorus as well as in attachment of spermatozoa to the zona pellucida (which contains hyaluronic acid). Internal cleavage of Agr 312 may be necessary to confer the latter property on 2B1 by inducing conformational changes that expose the appropriate motif. This is supported by the fact that testicular spermatozoa (which carry precursor 2B1) cannot bind to the zona pellucida whereas cauda spermatozoa (which carry processed 2B1) are able to do so.

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