Identification and characterization of the 2D6 and M_r 23000 antigens on the plasma membrane of rat spermatozoa

Roy JONES and Colin R. BROWN

A.F.R.C. Institute of Animal Physiology, Animal Research Station, 307 Huntingdon Road, Cambridge CB3 0JQ, U.K.

Previous investigations [Jones, Brown, von Glos & Gaunt (1985) *Exp. Cell Res.* **156**, 31–44] have demonstrated the appearance of a new antigenic determinant (recognized by monoclonal antibody 2D6) on the plasma membrane of rat spermatozoa during post-testicular maturation in the epididymis. Identification of the 2D6 antigen on Western blots from one-dimensional SDS/polyacrylamide gels revealed that it co-migrated with a membrane protein (designated M_r 23000 antigen) present on testicular and immature germ cells, suggesting that one antigen might be a modified version of the other. In the present work, however, we demonstrate that, although they have similar M_r and are present in soluble and membrane-bound forms, the 2D6 and M_r 23000 antigens are biochemically and immunologically distinct molecules. The properties of the antigens are described and compared. The M_r 23000 antigen is present on both testicular and cauda epididymidal spermatozoa, has a pI of 6.1, contains no detectable carbohydrate, is not tissue-specific and is degraded by V8 protease. By contrast, the 2D6 antigen is glycosylated, has a broad pI from 4.5 to 6.1, is tissue- and species-specific and is resistant to digestion with V8 protease. Its role in sperm-egg recognition is discussed.

INTRODUCTION

It is now known that the plasma membrane of mammalian spermatozoa undergoes considerable remodelling during post-testicular development in the epididymis (reviewed by Eddy *et al.*, 1985). Freeze-fracture studies (Suzuki & Nagano, 1980), surface labelling experiments (Jones *et al.*, 1981, 1983; Voglmayr *et al.*, 1983), and the binding patterns of lectins and antibodies (Feuchter *et al.*, 1981; Koehler, 1981; Moore & Hartman, 1984; Parks & Hammerstedt, 1985) have revealed a variety of organizational and compositional differences in surface membrane glycoproteins and lipids between immature testicular spermatozoa on the one hand and mature cauda epididymidal spermatozoa on the other. In some instances specific alterations to the plasma membrane correlate closely with the acquisition of fertilizing capacity (Jones *et al.*, 1981, 1985), a finding that has heightened interest in identifying molecules on the sperm surface that may be involved in the recognition of the egg.

Recently, we described the appearance of a new antigen on the surface membrane of rat spermatozoa during maturation in the epididymis (Jones *et al.*, 1985). This antigen, recognized by monoclonal antibody 2D6, was not present on testicular spermatozoa but was distributed over the plasma membrane of cauda spermatozoa. Immunoblotting experiments revealed the antigen to have an M_r of 23000–24000 on SDS/polyacrylamide gels. However, an examination of proteins in sodium deoxycholate extracts of testicular and cauda spermatozoa showed a major Coomassie Blue-staining component in each cell type with an M_r of 23000–24000. Two explanations for this observation were proposed. Either the 2D6 monoclonal antibody was recognizing a new epitope produced on an existing membrane protein

by a post-translational modification process (e.g. glycosylation) or else it was detecting an entirely different antigen but with a coincident M_r . In the work described below we demonstrate that the latter interpretation is correct, namely that there are distinct antigens with M_r 23000–24000 on the surface membrane of cauda spermatozoa. One (referred to as 2D6 antigen) is present only on cauda spermatozoa, whereas the other (referred to as M_r 23000 antigen) is a major component of the plasma membrane of testicular as well as cauda spermatozoa. The antigens have been purified and their properties compared.

MATERIALS AND METHODS

Materials

All chemicals and enzymes were of the highest purity available commercially and were purchased from BDH, Sigma, Pharmacia or Miles as described previously (Jones *et al.*, 1985). Nitrocellulose membranes (0.45 μ m) were obtained from Schleicher & Schuell. 125 I-labelled Bolton and Hunter reagent and 14 C-labelled M_r marker proteins were supplied by Amersham International.

Collection of spermatozoa and secretions from testis and epididymis

Testicular and cauda epididymidal spermatozoa, rete testis fluid (RTF) and luminal plasma from the cauda epididymidis (CEP) were collected as described previously (Jones *et al.*, 1983). Secretions were centrifuged at 10000 *g* for 10 min and stored frozen at -20°C . Spermatozoa were washed twice in PBS, pH 7.4, by centrifugation at 300 *g* for 8 min in round-bottom test-tubes. Detergent-soluble proteins from spermatozoa were prepared by resuspending the sperm pellet in 0.4%

(w/v) sodium deoxycholate in 0.264 M-sucrose/10 mM-Tris/HCl, pH 8.5, at 4 °C for 45 min and centrifuging at 10000 *g* for 10 min. Surface membranes from spermatozoa were prepared as described by Jones (1986), and solubilized in O'Farrell (1974) lysis buffer containing 5% (v/v) 2-mercaptoethanol.

Column chromatography

Soluble proteins in CEP were separated on a column (20 cm × 1.8 cm²) of DEAE-A25 equilibrated in 50 mM-Tris/HCl buffer, pH 8.2, exactly as described previously (Jones *et al.*, 1980). Bound proteins were eluted with a linear gradient of 0–0.5 M-NaCl in buffer. Samples from selected fractions containing equal amounts of proteins were dialysed against distilled water, freeze-dried and analysed on SDS/polyacrylamide gels.

Electrophoresis and Western blotting

Proteins were separated on non-denaturing 7% polyacrylamide gels (Davis, 1964) or on reducing SDS/polyacrylamide gels (Laemmli, 1970) using a gradient of 8–15% (w/v) polyacrylamide. Flat-bed isoelectric focusing of membrane proteins was carried out under dissociating conditions as described by O'Farrell (1974) using ampholytes pH 3–10 (Pharmacia). Gel strips were equilibrated in 62.5 mM-Tris/HCl (pH 7.4)/2% (w/v) SDS/5% (v/v) 2-mercaptoethanol for 30 min at 20 °C before electrophoresis in the second dimension on SDS gels. Proteins were stained with 0.5% (w/v) Coomassie Blue R-250 in methanol/acetic acid/water (40:7:53, by vol.) and destained in the same solvent without dye. Glycoproteins were detected by the PAS method as modified by Dubray & Bezard (1982). Alternatively, electrophoresed proteins were electroblotted onto nitrocellulose membranes (Towbin *et al.*, 1979) and probed with antibodies as described previously (Jones *et al.*, 1985).

Production of antibodies

Monoclonal antibodies to cauda epididymal spermatozoa were prepared as described previously (Gaunt *et al.*, 1983). One monoclonal antibody, designated 2D6, that recognizes a surface membrane antigen on cauda epididymal spermatozoa has been described in detail (Jones *et al.*, 1985). A polyclonal antibody to a secreted epididymal protein with *M_r* 23000 (Jones *et al.*, 1980) was prepared by injecting 500 µg of immunogen in Freund's complete adjuvant into rabbits. It was found that the purified *M_r* 23000 secreted protein elicited a poor immunological response, but when injected in a semi-purified form (the principal contaminants being the *M_r* 18500 and *M_r* 19000 androgen-dependent epididymal proteins; Jones *et al.*, 1980) a good antibody titre was obtained. When this antiserum was tested on Western blots of proteins in CEP it recognized antigens with *M_r* 23000, 19000 and 18500. Western blots of proteins in RTF on the other hand gave a reaction only over the *M_r* 23000 antigen. Therefore, an affinity-purified antibody to the *M_r* 23000 antigen was prepared by coupling proteins in RTF to CNBr-activated Sepharose 4B (Pharmacia recommendations) and passing a 25% (w/v) (NH₄)₂SO₄ fraction from whole serum [dialysed against 50 mM-Tris/HCl (pH 7.4)/0.1 M-NaCl] through the column equilibrated in the same buffer. Bound antibody was eluted with 50 mM-diethylamine, pH 11.0, adjusted to pH 7.0 with 1.0 M-HCl, dialysed against distilled water and freeze-dried. The antibody was dissolved in PBS to give 0.1 mg/ml and used at a final dilution of 1:100. This antibody recognized a single antigen with an *M_r* of 23000 on Western blots of proteins in CEP and RTF and was used in all subsequent investigations. An immunoglobulin fraction from pre-immune rabbit serum was prepared by precipitation with 25% (NH₄)₂SO₄, dialysis against distilled water and freeze-drying. Proteins were dissolved in PBS to give 0.1 mg/ml and used as described above.

Fig. 1. Identification of *M_r* 23000 and 2D6 antigens on testicular and cauda epididymal spermatozoa

Deoxycholate-soluble proteins from testicular (T) and cauda (Cd) spermatozoa were separated on SDS/polyacrylamide gels and visualized with Coomassie Blue R-250 (a), or stained for carbohydrate by the PAS procedure (e). Parallel gels were electroblotted and probed with the anti-*M_r* 23000 antibody (b), 2D6 monoclonal antibody (c), or HAT medium (d). Blots probed with immunoglobulins from pre-immune rabbit serum were similar to that shown for HAT medium. The second-layer antibody was peroxidase-conjugated goat anti-rabbit (for the anti-*M_r* 23000 antibody) and peroxidase-conjugated rabbit anti-mouse (for 2D6 antibody). 4-Chloronaphthol was the final chromogen (Jones *et al.*, 1985).

Iodination of proteins and peptide mapping

Slices from SDS gels containing either 2D6 antigen or the M_r 23000 protein were equilibrated in several changes of 5 ml of 0.1 M-borate buffer, pH 8.5, until the pH remained stable. The slices were then transferred to 200 μ l of fresh 0.1 M-borate buffer, pH 8.5, containing 10 μ Ci of 125 I-labelled Bolton and Hunter reagent and left to stand at 20 °C for 30 min with occasional shaking. Slices were washed three times in 5 ml of borate buffer (5 min each wash) and transferred to 2 ml of 62.5 mM-Tris/HCl (pH 7.4)/2% (w/v) SDS/5% (v/v) 2-mercaptoethanol for 30 min at 20 °C. The iodinated proteins in gel slices were applied to separate tracks on top of an SDS gel and digested with 25 μ g of *Staphylococcus aureus* V8 protease for 60 min at room temperature as described by Cleveland *et al.* (1977). Labelled peptides were detected by autoradiography of the dried gel using pre-flashed Kodak X-Omat S film and intensifying screens.

Other procedures

Cytosols were prepared by homogenizing tissues in 5 vol. of PBS containing 10 mM-iodoacetamide/1 mM-phenylmethanesulphonyl fluoride in a Polytron Ultra-turrax for 30 s and centrifuging at 40000 rev./min in a Beckman Ti rotor at 5 °C for 60 min. Total protein was measured by the method of Bradford (1976) or by measurement of A_{280} (assuming $A_{280}^{1\text{ mg/ml}} = 1.0$). Milk was extruded manually from the mammary glands of lactating rats.

RESULTS

Identification of M_r 23000 and 2D6 antigens on testicular and cauda epididymal spermatozoa

An analysis of proteins in deoxycholate extracts of testicular and cauda epididymal spermatozoa on a one-dimensional SDS gel revealed the presence of a major Coomassie Blue-staining component in each cell type with an M_r of 23000–24000 (Fig. 1a). A Western blot taken from a parallel gel and probed with the rabbit anti- M_r 23000 antibody produced a strong reaction over this antigen on both testicular and cauda spermatozoa (Fig. 1b), but when similar blots were probed with 2D6 monoclonal antibody a reaction was obtained only in extracts of cauda spermatozoa (Fig. 1c). The antigen recognized by 2D6 monoclonal antibody had an M_r of 23000–24000.

Since the epitope recognized by the 2D6 monoclonal antibody is not known, the above results cannot be interpreted as evidence that it is recognizing an entirely different antigen to the anti- M_r 23000 antibody. The first indication that the anti- M_r 23000 and 2D6 antibodies were detecting separate antigens arose from a two-dimensional electrophoretic analysis of membrane proteins from testicular and cauda spermatozoa. Coomassie Blue staining of gels of membrane proteins from testicular spermatozoa revealed a major protein component with M_r 23000 and a pI of 6.1 (Fig. 2a). This component was recognized by the anti- M_r 23000 antibody on a Western blot from a parallel gel (Fig. 2b). Some heterogeneity was present, as one major and three minor proteins were always detected. An essentially similar picture was obtained on blots of cauda sperm membranes probed with the anti- M_r 23000 antibody

(Fig. 2f). However, Coomassie Blue-stained gels of cauda sperm membranes showed that, in addition to the protein component at M_r 23000, another intensely staining band was present with an M_r that extended from 23000 to 24000 and which focused as a 'streak' between pI 4.5 and 6.0 (Fig. 2e). It was this component that was recognized by the 2D6 monoclonal antibody on Western blots (Fig. 2g), whereas no reaction was observed on blots of testicular sperm membrane proteins (Fig. 2c). Control blots probed with pre-immune rabbit immunoglobulins or HAT medium were negative (Figs. 2d and 2h).

A notable feature of the 2D6 antigen was the considerable charge heterogeneity displayed after isoelectric focusing under dissociating conditions (Fig. 2e). It is likely that this microheterogeneity is caused by the presence of significant amounts of carbohydrate, as a strong PAS-positive reaction was obtained on SDS gels at M_r 23000–24000 in deoxycholate extracts of cauda spermatozoa but not testicular spermatozoa (Fig. 1e). This result was confirmed by PAS staining of two-dimensional gels of cauda sperm membrane proteins. A faint but positive reaction was present over the protein which focused as a broad band between pI 4.5 and 6.1 at M_r 23000–24000 (results not shown). By contrast, no reaction was obtained over the M_r 23000 antigen on two-dimensional gels of testicular sperm membrane proteins.

Identification of M_r 23000 and 2D6 antigens in RTF and CEP

Further evidence for dissimilarity between M_r 23000 and 2D6 antigens was obtained from an analysis of proteins in RTF and CEP. Electrophoresis on polyacrylamide gels at pH 8.9 (Davis, 1964) in the first dimension followed by analysis on SDS gels in the second dimension revealed a major Coomassie Blue-staining component with an M_r of 23000 in both RTF and CEP (Figs. 3a and 3e). This protein was recognized specifically by the rabbit anti- M_r 23000 antibody on Western blots (Figs. 3b and 3f). To obtain accurate identification of antigens on blots from two-dimensional gels, some blots were overdeveloped in the dye reagent to allow background to increase above normally acceptable levels. This enabled the position of other non-reactive proteins to be located and from this the M_r 23000 protein could be pinpointed. However, the 2D6 monoclonal antibody did not react with this antigen in either RTF or CEP but instead detected an antigen in CEP at M_r 23000–24000 that migrated as a streak in the cathodic half of the first dimension gel (small arrowheads in Fig. 3g). As reported previously (Jones *et al.*, 1985), 2D6 monoclonal antibody also recognizes several other proteins in CEP with M_r of 32000, 28000 and 21000, although the latter antigens are not detected to a significant extent on blots of proteins from cauda spermatozoa. Blots of RTF probed with 2D6 monoclonal antibody produced no reaction (Fig. 3c). Similarly, blots probed with pre-immune rabbit immunoglobulins or HAT medium were negative (Figs. 3d and 3h).

Purification of M_r 23000 and 2D6 antigens

2D6 monoclonal antibody is an IgM (Jones *et al.*, 1985) and loses its affinity after coupling to CNBr-activated Sepharose 4B. Consequently, we were not able to use immunoaffinity columns to purify the membrane-bound

Fig. 2. Two-dimensional polyacrylamide-gel electrophoresis of membrane proteins from testicular and cauda epididymidal spermatozoa

Membrane proteins from testicular (a-d) and cauda (e-h) spermatozoa were separated in the first dimension by isoelectric focusing on polyacrylamide gels and in the second dimension on gels containing SDS. Proteins were stained with Coomassie Blue R-250 (a and e) or blotted onto nitrocellulose paper and probed with anti-M_r 23000 antibody (b and f), 2D6 monoclonal antibody (c and g) or HAT medium (d and h). Blots probed with pre-immune rabbit immunoglobulins gave a similar result to that shown in (d) and (h). Large arrow indicates M_r 23000 antigen; small arrow-heads indicate 2D6 antigen.

Fig. 3. Two-dimensional polyacrylamide-gel electrophoresis of soluble proteins in RTF and CEP

Proteins in RTF and CEP were separated by electrophoresis on 7% polyacrylamide gels in the first dimension and on SDS gels in the second dimension. Proteins were stained with Coomassie Blue R-250 (*a* and *e*) or blotted onto nitrocellulose membranes and probed with anti- M_r 23000 antibody (*b* and *f*), 2D6 monoclonal antibody (*c* and *g*) or HAT medium (*d* and *h*). Pre-immune rabbit immunoglobulins gave a similar result to HAT medium. Large arrow indicates M_r 23000 antigen and small arrowheads the 2D6 antigen. The area bounded in (*a*) and (*e*) is that shown in the blots.

form of the 2D6 antigen. However, CEP proved to be a convenient source for the purification of the soluble forms of both 2D6 and M_r 23000 antigens.

Ion-exchange chromatography of proteins in CEP on DEAE-Sephadex A-25 at pH 8.2 was carried out as

described in the Materials and methods section. Elution of bound proteins with a gradient of 0–0.5 M-NaCl produced three major peaks (Fig. 4*a*). Analysis of proteins in selected fractions on SDS gels showed that the M_r 23000 antigen was eluted in the first peak between

approx. 40 mM- and 90 mM-NaCl (Fig. 4b) followed by the M_r 18 500 and 19 000 androgen-dependent proteins at approx. 100–160 mM-NaCl and lastly by the M_r 32 000 androgen-dependent protein at approx. 250–350 mM-NaCl (Jones *et al.*, 1980).

Western blots of parallel gels that had been probed with the anti- M_r 23 000 antibody confirmed the results obtained with Coomassie Blue staining, namely that the first protein peak was rich in the M_r 23 000 antigen (Fig. 4c). The immunoblot in Fig. 4(c) also demonstrates another property of the M_r 23 000 antigen. Following dialysis against distilled water the antigen breaks down to at least two lower M_r forms. These breakdown products constitute < 10% of the parent molecule, as they are not revealed by Coomassie Blue staining but are disclosed by the greater sensitivity of immunodetection systems.

Western blots probed with 2D6 monoclonal antibody, on the other hand (Fig. 4d), showed that an antigen with an M_r of 23 000–24 000 was eluted between 175 mM- and 300 mM-NaCl (fractions 80–120). The amount of 2D6 antigen in these fractions, however, was insufficient to stain with Coomassie Blue.

Peptide mapping of purified M_r 23 000 and 2D6 antigens

Because of the small amounts of 2D6 antigen recovered from the DEAE-A25 column, proteins were iodinated with Bolton and Hunter reagent before peptide mapping with V8 protease. For comparison, the M_r 23 000 antigen was purified in a similar manner. Fractions 30–40 (Fig. 4a) were pooled, a sample electrophoresed on a one-dimensional SDS gel and the area corresponding to M_r 23 000 excised. This gel slice contained pure M_r 23 000 antigen as shown by: (a) a single band was observed when re-analysed on SDS gels, (b) the antigen contained in the gel slice gave a positive reaction on Western blots probed with anti- M_r 23 000 antibody but gave a negative reaction when probed with 2D6 monoclonal antibody (results not shown). A similar protocol was used to obtain pure 2D6 antigen from pooled fractions 80–120. Following iodination of the respective proteins in the gel slice and digestion with V8 protease the M_r 23 000 antigen was degraded to several lower M_r forms, chiefly polypeptides of 18 000 and 15 000 (Fig. 5a). However, the 2D6 antigen remained resistant to digestion under similar conditions (Fig. 5b).

Specificity of M_r 23 000 and 2D6 antigens

Western blots were prepared of cytosolic proteins from epididymis, testis, ventral prostate, liver, kidney, spleen, brain muscle and mammary gland (lactating) along with milk and serum proteins and probed with anti- M_r 23 000 and 2D6 antibodies. The M_r 23 000 antigen was detected in cytosols from all tissues investigated (Fig. 6a) but the 2D6 antigen at M_r 23 000–24 000 was specific to epididymis (Fig. 6b). However, the M_r 23 000 antigen was not detectable in whole milk (Fig. 6) or blood serum (results not shown). The 2D6 antigen was also species-specific as no reaction was obtained over Western blots of deoxycholate extracts of sperm from mouse, hamster, bull, ram and boar (results not shown). A weak reaction for the M_r 23 000 antigen was detected on mouse spermatozoa but not on the other species tested.

Fig. 4. Purification of soluble forms of M_r 23 000 and 2D6 antigens

Proteins in CEP were chromatographed on DEAE-A25 (a) as described in the Materials and methods section and selected fractions were analysed on SDS gels (b). Elution of the M_r 23 000 antigen and 2D6 antigen was monitored on Western blots of parallel gels (c and d respectively). M_r markers in (b) were (from the top) phosphorylase b (M_r 94 000), bovine serum albumin (M_r 67 000), ovalbumin (M_r 43 000), carbonic anhydrase (M_r 30 000), soybean trypsin inhibitor (M_r 20 100) and α -lactalbumin (M_r 14 400). Large arrow in (b) and (c) indicates M_r 23 000 antigen, and arrowheads in (b) and (d) the position of the 2D6 antigen. Numbers above tracks in (b) refer to fractions tested from column in (a).

DISCUSSION

This work has established that there are two protein components on the surface membrane of rat cauda epididymidal spermatozoa that have similar M_r on

Fig. 5. Peptide maps of purified M_r 23000 and 2D6 antigens

Purified M_r 23000 and 2D6 antigens were iodinated with ^{125}I -labelled Bolton and Hunter reagent, digested with V8 protease (Cleveland *et al.*, 1977) and labelled peptides separated on an SDS gel followed by autoradiography. (a) M_r 23000 antigen, (b) 2D6 antigen.

Fig. 6. Tissue specificity of M_r 23000 and 2D6 antigens

Cytosolic proteins from (i) epididymis, (ii) testis, (iii) ventral postate, (iv) liver, (v) kidney, (vi) spleen, (vii) brain, (viii) muscle, (ix) mammary gland and (x) milk were separated on SDS gels, blotted onto nitrocellulose paper and probed with anti- M_r 23000 antibody (a) or 2D6 monoclonal antibody (b).

SDS/polyacrylamide gels but can be distinguished from each other biochemically and immunologically. One of these components (the M_r 23000 antigen) is already present on germ cells leaving the testis whereas the other (the 2D6 antigen) arises specifically during passage of spermatozoa through the epididymal duct.

Although we do not know the exact nature of the antigenic site recognized by 2D6 monoclonal antibody, the possibility that the 2D6 antigen is a modified version of the M_r 23000 antigen is unlikely for several reasons. First, the anti- M_r 23000 antibody is polyclonal and would, therefore, be expected to cross-react with variant forms. That it clearly does not do this suggests that the M_r 23000 and 2D6 antigens were unrelated. Second, an analysis of plasma membrane proteins by two-dimensional gel electrophoresis shows that the 2D6 antigen is a major component on cauda epididymal spermatozoa but is completely absent from testicular spermatozoa. By contrast, the M_r 23000 antigen is present on both testicular and cauda sperm membranes. Third, the 2D6 antigen is resistant to digestion with V8 protease whereas the M_r 23000 antigen is degraded to a variety of lower

M_r forms. Therefore, other than a similarity in M_r there does not appear to be any biochemical or immunological relationship between the M_r 23000 and 2D6 antigens.

It also seems unlikely that the 2D6 antigen is a modified form of any other membrane protein on testicular spermatozoa. It is too major a component to be a derivative of one of the relatively minor proteins that migrate with an M_r at or below 23000–24000 on 'maps' of testicular sperm membranes (Fig. 2a). In addition, modification of these proteins (e.g. by glycosylation) would require a considerable alteration in charge (from very basic pI to very acidic pI) to assume the behaviour of the 2D6 antigen. Together with the fact that the 2D6 antigen (or its precursor) can be detected in the epithelial cells lining the epididymal duct and in CEP (Jones *et al.*, 1985), the evidence favours the concept that it is a 'coating' antigen and that it does not arise on the sperm surface by re-orientation or modification of existing membrane components (Eddy *et al.*, 1985).

It may be significant that 2D6 antigen is a major PAS-positive glycoprotein on the plasma membrane of mature spermatozoa. A comparison of the relative intensity of the PAS reaction at M_r 23000–24000 in CEP and in a detergent extract of cauda spermatozoa indicates that there is at least 5 times more 2D6 antigen on the sperm membrane than in the secretions. Recent evidence suggests an important role for carbohydrate moieties during sperm-egg recognition in both vertebrates and invertebrates. In sea urchin spermatozoa a lectin-like protein termed 'bindin' has been shown to recognize fucose residues on the vitelline layer of eggs (Glabe *et al.*, 1982), and in the hamster acetylated amino sugars have been reported to block sperm-egg attachment *in vitro* (Ahuja, 1982). In the rat, fucoidan has been found to be a potent inhibitor of fertilization (Shalgi *et al.*, 1986). Furthermore, Wassarman and co-workers (Florman & Wassarman, 1985) have demonstrated in the mouse that O-linked carbohydrate on zona pellucida proteins is crucial for sperm-egg recognition. The nature of the carbohydrate on the 2D6 antigen remains to be elucidated, but the demonstration that the antigen is tissue- and species-specific and that its appearance on the plasma membrane of spermatozoa in the epididymis coincides with development of fertilizing capacity is suggestive of a selective function.

The M_r 23000 antigen on the other hand is a ubiquitous protein as it is present in cytosols from a wide variety of tissues. Its apparent absence from milk and blood serum indicates that it is not a universally secreted protein. The exception to this is clearly in the epididymis, where it constitutes a major component of CEP. Previously, we have shown that an M_r 23000 protein is synthesized by principal cells in the epididymis although it is not androgen-dependent (Jones *et al.*, 1980). The M_r 23000 antigen is also a major component of sperm membranes, although it is not detectable in red cell ghosts or on hepatocyte plasma membranes (R. Jones, unpublished work). It is not clear at present why this protein should be secreted by some tissues but remain cytoplasmic in others, and why it should be a component of sperm membranes but not erythrocytes or hepatocyte membranes. So far, we have not detected significant differences in the electrophoretic behaviour of the secreted versus non-secreted antigen. The molecular mechanisms that direct protein transport within cells are still poorly understood, but according to current concepts

most of the information necessary to determine the fate of the protein is contained in its primary structure (Sabatini *et al.*, 1982). Membrane proteins contain stop-transfer sequences that terminate transmembrane transport, whereas secreted proteins presumably have a signal sequence but no stop-transfer sequence. The difference in the ability of various tissues to secrete the M_r 23000 antigen, therefore, may reside in very subtle alterations in the sequence of few amino acids manifested during translation. Unusually for a secreted protein, the epididymal M_r 23000 antigen does not appear to be glycosylated yet it displays microheterogeneity after isoelectric focusing under dissociating conditions.

The properties of the M_r 23000 antigen described here are very similar to those of an M_r 22000 protein reported recently by Brooks (1985). This protein was found on rat sperm membranes, in tissue cytosols but not in extracellular secretions other than those from testis and epididymis. Our observations are in good general agreement with these results, but also differ in several respects. Brooks (1985) observed a tendency for the M_r 22000 protein to aggregate during purification, whereas we found that the M_r 23000 antigen had a propensity to degrade to several lower- M_r forms. Brooks (1985) also reported by using indirect immunofluorescence techniques that the M_r 22000 protein was restricted to the membrane domain overlying the sperm flagellum, and because it had a patchy distribution suggested that it was mobile within the plane of the membrane. The patching was not prevented by fixation with glutaraldehyde, which seems unusual since cross-linking membrane proteins is well known to inhibit their mobility and prevent antibody-induced patching (Gaunt *et al.*, 1983). For reasons as yet unclear, we have not yet obtained satisfactory fluorescence on spermatozoa with our affinity-purified antibody even though it recognizes the antigen strongly on Western blots. Nonetheless, it seems likely that the M_r 22000 protein (Brooks, 1985) and the M_r 23000 antigen described here are identical.

Although there are clearly many differences between the 2D6 and M_r 23000 antigens, one feature in common is that they are present in soluble and membrane-bound forms. A number of precedents for this phenomenon are known, e.g. fibronectin (Yamada & Olden, 1978), chromogranin (Settleman *et al.*, 1985), and VSG protein of *Trypanosoma* parasites (Turner, 1984). In the case of fibronectin and several other cell adhesion molecules, it has been shown that a discrete internal domain, the Arg-Gly-Asp tripeptide, is crucial for interaction with a cell-surface receptor (Ruoslahti & Pierschbacher, 1986). However, it is not known if such a tripeptide is present in either the M_r 23000 or 2D6 antigens. It has also been shown that some glycoproteins, e.g. VSG and *Leishmania* p63 antigen (Bouvier *et al.*, 1985) can exist in hydrophilic and amphiphilic forms despite the absence of any substantial sequence of hydrophobic amino acids in the C-terminal region of the molecule. The membrane anchoring domains of these glycoproteins have been shown to utilize covalently attached phosphatidylinositol as their hydrophobic 'tails' (reviewed by Low *et al.*, 1986). It will be important in future work to com-

pare the hydrophilic and amphiphilic forms of the 2D6 and M_r 23000 antigens and their disposition within the sperm plasma membrane.

In summary, therefore, we have demonstrated that there are two distinct antigens on the surface membrane of rat cauda epididymal spermatozoa with an M_r of 23000–24000. Although both antigens are present in soluble and membrane-bound forms they have different origins and different biochemical properties.

We thank Mr K. I. von Glos for able technical assistance.

REFERENCES

- Ahuja, K. K. (1982) *Exp. Cell Res.* **140**, 353–362
- Bouvier, J., Etages, R. J. & Bordier, C. (1985) *J. Biol. Chem.* **260**, 15504–15509
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Brooks, D. E. (1985) *Biochim. Biophys. Acta* **841**, 59–70
- Cleveland, D. W., Fischer, S. G., Kirschner, M. W. & Laemmli, U. K. (1977) *J. Biol. Chem.* **252**, 1102–1106
- Davis, B. J. (1964) *Am. N.Y. Acad. Sci.* **121**, 404–427
- Dubray, G. & Bezard, G. (1982) *Anal. Biochem.* **119**, 325–329
- Eddy, E. M., Vernon, R. B., Muller, C. H., Hahnel, A. C. & Fenderson, B. A. (1985) *Am. J. Anat.* **175**, 225–237
- Feuchter, F. A., Vernon, R. B. & Eddy, E. M. (1981) *Biol. Reprod.* **24**, 1099–1110
- Florman, H. M. & Wassarman, P. M. (1985) *Cell* **41**, 313–324
- Gaunt, S. J., Brown, C. R. & Jones, R. (1983) *Exp. Cell Res.* **144**, 275–284
- Glabe, C. G., Grabel, L. B., Vacquier, V. D. & Rosen, S. D. (1982) *J. Cell Biol.* **94**, 123–128
- Jones, R. (1986) *J. Reprod. Fertil.* **77**, 435–449
- Jones, R., Brown, C. R., von Glos, K. I. & Parker, M. G. (1980) *Biochem. J.* **188**, 667–676
- Jones, R., Pholpramool, C., Setchell, B. P. & Brown, C. R. (1981) *Biochem. J.* **200**, 457–460
- Jones, R., von Glos, K. I. & Brown, C. R. (1983) *J. Reprod. Fertil.* **67**, 299–306
- Jones, R., Brown, C. R., von Glos, K. I. & Gaunt, S. J. (1985) *Exp. Cell Res.* **156**, 31–44
- Koehler, J. K. (1981) *Arch. Androl.* **6**, 197–217
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Low, M. G., Ferguson, A. J., Futerman, A. H. & Silman, I. (1986) *Trends Biochem. Sci.* **11**, 212–215
- Moore, H. D. M. & Hartman, T. D. (1984) *J. Reprod. Fertil.* **70**, 175–183
- O'Farrell, P. H. (1974) *J. Biol. Chem.* **250**, 4007–4021
- Parks, J. E. & Hammerstedt, R. H. (1985) *Biol. Reprod.* **32**, 653–668
- Ruoslahti, E. & Pierschbacher, M. D. (1986) *Cell* **44**, 517–518
- Sabatini, D. D., Kriebach, G., Morimoto, R. & Adesnik, M. (1982) *J. Cell Biol.* **92**, 1–22
- Settleman, J., Nolan, J. & Angeletti, R. H. (1985) *J. Biol. Chem.* **260**, 1641–1644
- Shalgi, R., Matityahu, A. & Nebel, L. (1986) *Biol. Reprod.* **34**, 446–452
- Suzuki, F. & Nagano, T. (1980) *Biol. Reprod.* **22**, 1219–1231
- Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354
- Turner, M. J. (1984) *Philos. Trans. R. Soc. London Ser. B* **307**, 27–40
- Voglmayr, J. K., Fairbanks, G. & Lewis, R. G. (1983) *Biol. Reprod.* **29**, 767–775
- Yamada, K. M. & Olden, K. (1978) *Nature (London)* **275**, 179–184