Sequence analysis of a mammalian phospholipid-binding protein from testis and epididymis and its distribution between spermatozoa and extracellular secretions

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The cellular origin of a soluble phospholipid-binding protein (PBP) in rat testicular and epididymal secretions has been investigated genetically and immunologically. PBP is ubiquitous in tissue cytosols but is not present in blood serum, lymph or milk. The relatively large amounts present in cauda epididymal plasma (CEP) and rete testis fluid suggested therefore that it may be secreted specifically by these tissues. However, when PBP cDNAs from testis and epididymis were cloned and sequenced, they did not contain a signal peptide and only one size of transcript was obtained on Northern blots of RNAs from liver, brain, placenta, testis and epididymis. Moreover, PBP could not be detected in sperm-free CEP from castrated, androgen-stimulated animals or in medium from Sertoli cell cultures. Spermatozoa, on the other hand, contained significant amounts of PBP that could be solubilized by washing cells in dissociating reagents or high-salt solutions. These results indicate that, contrary to previous interpretations, PBP is not secreted by classical pathways in either the testis or epididymis but that its presence in CEP and rete testis fluid is attributable largely to release from spermatozoa. Thus, spermatozoa have a significant influence on the composition of CEP as well as on the secretory and absorptive activity of the epididymal epithelium. A possible role for PBP in membrane biogenesis and maintenance of antigen segregation in spermatozoa is discussed.

INTRODUCTION

The plasma membrane of spermatozoa, in common with that of other mammalian cells, contains a wide variety of proteins and glycoproteins, some of which are integral to the membrane whereas others are extrinsic or are only loosely attached (Voglmayer et al., 1980; Myles et al., 1981; Jones et al., 1983; Russell et al., 1983). In previous work on rat sperm plasma membranes we purified and characterized a major protein component with an M_r of 23000 on SDS/PAGE (Jones et al., 1983; Jones and Brown, 1987). This protein is also present in secretions collected from the testis and epididymis, prompting the interpretation that it can exist both as a soluble secreted form and as a membrane-bound form (Brooks, 1985; Jones and Brown, 1987). However, the protein is not present in other extracellular fluids (e.g. blood serum, milk and saliva) or erythrocyte membranes, although it is present in cytosols from a wide range of tissues taken from male and female rats (Jones and Brown, 1987). Subsequent N-terminal amino acid sequence analysis of CNBr-generated peptides revealed 85% sequence identity with a phosphatidylethanolamine (PE)-binding protein first isolated from bovine brain (Bernier et al., 1986). Further experiments established that the sperm M_r 23000 protein, now referred to as phospholipid-binding protein (PBP), also has preferential affinity for PE (Jones and Hall, 1991).

Notwithstanding this information, the presence of such a protein on spermatozoa and in secreted fluids from testis and epididymis remains an enigma. Two hypotheses have been suggested to explain its distribution. One states that the protein is secreted specifically in the testis and/or the epididymis and that it is adsorbed onto the sperm surface during spermiogenesis and epididymal maturation. If so, then one would expect that two classes of PBP transcript exist in these tissues: one possessing a region encoding a signal peptide (secreted form) and one without a leader sequence (cytoplasmic). The second hypothesis states that the protein is not secreted but during spermatogenesis is sequestered by different pathways into the sperm plasma membrane and cytoplasm and that its presence in testicular and epididymal secretions is attributable to release from spermatozoa.

In this paper we have investigated these possibilities from several different stand-points: by cloning and sequencing PBP cDNA from testis and epididymis to obtain genetic evidence for a secreted form of the protein; by examining the presence of PBP in epididymal secretions obtained from animals that had been castrated and stimulated with androgen, conditions which have been shown to induce the accumulation of secreted components in the lumen of the duct; and by investigating the release and cellular distribution of PBP during preparation of washed spermatozoa. The results obtained indicate that, contrary to previous assertions, PBP is not secreted by classical pathways in either the testis or epididymis and that spermatozoa are a major source of the soluble protein. That PBP is normally a major component of epididymal fluid suggests that substances emanating from spermatozoa have a significant influence on the composition of their surrounding milieu as well as the secretory and absorptive activity of the epididymal epithelium.

Abbreviations used: CEP, cauda epididymal plasma; FITC, fluorescein isothiocyanate; IIF, indirect immunofluorescence; NP-40, Nonidet-P40; PBP, phospholipid-binding protein; PE, phosphatidylethanolamine; RTF, rete testes fluid.

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The nucleotide sequence data reported appear in the EMBL Nucleotide Sequence Database under the accession numbers X71873 (rat PBP) and X73137 (monkey PBP).

MATERIALS AND METHODS

Materials

All restriction endonucleases were purchased from Pharmacia LKB. Hybond-N nylon membranes were obtained from Amersham International. [γ-^{32}P]ATP (> 3000 Ci/mmol), [γ-^{32}P]dCTP (> 3000 Ci/mmol) and random hexamer DNA-labelling kits were obtained from Du Pont-NEN. Nitrocellulose filters were supplied by Schleicher & Schuell. All other reagents were of AnalaR grade or the purest grade available.

Testes and epididymides were collected from neonatal and normal adult Wistar rats (*Rattus norvegicus*) following CO_2 asphyxiation and stored at −80 °C until required. Surgical procedures for castration, artificial cryptorchidism (transfer of testis and epididymis from scrotum to body cavity) and testosterone replacement have been described in detail previously (Walker et al., 1990; Perry et al., 1992). It has been shown that castration for 4 weeks followed by administration of 1.0 mg of testosterone per day subcutaneously for another 2 weeks produces an actively secreting epididymis devoid of spermatozoa (Jones et al., 1980). A similar result is achieved by 4 weeks of artificial cryptorchidism which destroys spermatogenesis (Setchell, 1978). Construction and storage of cDNA libraries from rat and monkey (*Macaca fascicularis*) have been reported (Luzio et al., 1990; Perry et al., 1992, 1993).

Purification of PBP and production of polyclonal antisera

PBP protein was purified to homogeneity from sperm-free cauda epididymal plasma (CEP) and polyclonal antisera raised in rabbits as described previously (Jones and Brown, 1987). The antibody was affinity purified against the original antigen coupled to CNBr-activated Sepharose-4B, dialysed against distilled water and freeze-dried.

Collection of spermatozoa and secretions from testis and epididymis

Testicular fluid was collected from the rete testis 18–20 h after ligation of the efferent ductules (Jones and Brown, 1987). Rete testicular fluid (RTF) was centrifuged at 1000 *g*, the supernatant was removed, and detergent-soluble proteins were extracted from the sperm pellet with 1.0% (v/v) Nonidet-P40 (NP-40) in PBS, pH 7.4, at 4 °C for 45 min. Extracts were clarified by centrifugation at 12000 *g* for 10 min. Luminal contents from the cauda epididymidis of normal rats were either collected without dilution into capillary tubes and subsequently centrifuged at 12000 *g* for 120 min [the resultant supernatant is referred to as 'neat' cauda epididymal plasma (CEP)] or collected and diluted into 20 vol. of PBS after which the spermatozoa were pelleted by centrifugation at 1000 *g* for 15 min. Supernatants were removed and spermatozoa extracted with 1% (v/v) NP-40 as described above.

Sperm-free CEP was collected from castrated androgen-stimulated epididymides by flushing the duct via the vas deferens with mineral oil and drawing the fluid extruded from the cauda region into capillary tubes. In experiments to investigate release of PBP from spermatozoa, cells were washed 4 times by centrifugation in PBS. Successive wash fluids were collected, re-centrifuged at 100000 *g* for 60 min at 4 °C and stored frozen until analysis. Alternatively, after the first wash, sperm pellets were resuspended in 0.5 M KCl, pH 7.4, or 0.1 M Na_2CO_3, pH 11.0, or 0.2% (w/v) trypsin in PBS or 1% (v/v) NP-40 in PBS or PBS. Suspensions were incubated for 30 min at 20 °C, centrifuged at 1000 *g* for 10 min and the supernatants were removed. Sperm pellets were re-extracted with 1% (v/v) NP-40 as described above. Supernatants were then clarified by re-centrifugation at 100000 *g* for 60 min at 4 °C and dialysed against 6.25 mM Tris/HCl, pH 7.4, containing 0.2% (w/v) SDS before analysis by SDS/PAGE.

Electrophoresis and Western blotting

Proteins (60 μg/track) were separated by reducing SDS/PAGE on 8.5–15% (w/v) polyacrylamide gels and either stained directly with 0.5% (w/v) Coomassie Blue R-250 in methanol/acetic acid/water (40:7:53, by vol.) or electroblotted on to polyvinylidene difluoride (Immobilon) membranes (Millipore). Blots were probed with an anti-PBP polyclonal antibody (20 μg/ml) followed by peroxidase-conjugated goat anti-(rabbit IgG) (1:400, v/v; Dako Ltd.) as described previously (Jones and Brown, 1987). 4-Chloronaphthol was used as the dye reagent.

Indirect immunofluorescence

Spermatozoa from the cauda epididymidis were prepared for visualization by indirect immunofluorescence (IIF) microscopy by two different procedures. In both cases, spermatozoa were washed once in PBS by centrifugation, resuspended in 0.1% (w/v) BSA in PBS, and either mixed directly with affinity-purified anti-PBP IgG (100 μg/ml) or fixed in 4% *p*-formaldehyde in PBS for 30 min at 4 °C. Fixed sperm were then washed twice in PBS/BSA and resuspended in antibody solution as described above. Suspensions were incubated for 40 min at 4 °C, washed twice in PBS/BSA and mixed with a 1:50 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-(rabbit IgG). After incubation for 40 min at 4 °C and two PBS/BSA washes, spermatozoa were resuspended in CITIFLUOR antifade medium (City University, London) and viewed with u.v. light at ×400 magnification using a Zeiss Axiophot photomicroscope.

Frozen sections of testis tissue were cut on a cryostat at −20 °C, air-dried on to glass slides and blocked with 5% (w/v) BSA/5% (w/v) normal goat serum in PBS for 1 h at room temperature. Sections were then probed with affinity-purified anti-PBP IgG (100 μg/ml) followed by 1:50-diluted FITC-conjugated goat anti-(rabbit IgG) and viewed as described above.

RNA isolation and Northern blot analysis

Isolation of RNA from tissues was carried out as previously described (Girotti et al., 1992). Total RNA was subjected to Northern blot analysis following fractionation by electrophoresis through a formaldehyde-containing, 1.1% (w/v) agarose gel. Following capillary transfer to a Hybond-N nylon membrane, samples were prehybridized and hybridized as described previously (Walker et al., 1990) using a cDNA insert labelled with ^{32}P to high specific radioactivity by a random priming protocol (Feinberg and Vogelstein, 1984). Equivalent track loadings were verified by reprobing filters with mouse α-actin cDNA (Humphries et al., 1981).

Isolation and sequence analysis of rat and monkey PBP cDNA clones

Epididymal or testicular cDNA clones ($\sim 5 \times 10^4$) were transferred to nitrocellulose filters and probed for clones possessing PBP cDNA inserts. Hybridization with oligonucleotide probes (see the Results section) was performed for 40 h under conditions

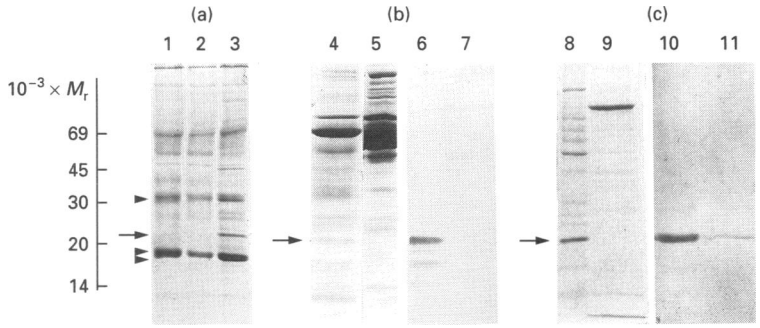

Figure 1 Identification of PBP in (a) epididymal secretions, (b) testicular secretions and (c) Sertoli cell culture medium by SDS/PAGE and Western blotting

Proteins in lanes 1–5, 8 and 9 were stained with Coomassie Blue. Lanes 6, 7, 10 and 11 represent Western blots probed with anti-PBP antibody followed by detection with peroxidase-conjugated goat anti-(rabbit IgG) and 4-chloronaphthol dye reagent. **(a)** CEP: lane 1, sperm-free CEP 4 weeks after castration and after 2 weeks of testosterone stimulation; lane 2, sperm-free CEP from a 4-week cryptorchid epididymis; lane 3, CEP collected from normal epididymis; spermatozoa were separated from CEP by centrifugation without dilution, i.e. to yield 'neat' CEP. **(b)** RTF: lane 4, RTF collected from normal testis; lane 5, sperm-free RTF collected from 4-week cryptorchid testis; lanes 6 and 7, Western blot from parallel gel to lanes 4 and 5. **(c)** Lane 8, proteins solubilized from cauda spermatozoa with 1% (v/v) NP-40; lane 9, Sertoli cell culture medium; lanes 10 and 11, Western blot from parallel gel to lanes 8 and 9. On all gels, the arrow indicates the position of PBP. Arrowheads in lane 1 indicate the position of three epididymal-specific, androgen-dependent secretory proteins (Brooks et al., 1986a; Walker et al., 1990). Values of M_r were calculated from the mobility of proteins of known size (Pharmacia low M_r kit).

of moderate stringency [6 × SSC at 45 °C (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate)]. Following two rounds of clone purification by re-screening under identical conditions, plasmid DNA from persistently hybridizing clones were isolated and characterized. Screening of libraries with cloned cDNA probes was performed for 40 h at a higher stringency (6 × SSC, 65 °C).

Where appropriate, cDNA inserts were sequenced on both DNA strands using a custom primer walking strategy and employing a Du Pont Genesis 2000 automated sequencer. All PBP cDNA sequence data obtained in this way were compiled and aligned using the LASERGENE suite of programs (DNASTAR Ltd.).

Other procedures

Total protein was measured by dye binding (Bradford, 1976) using BSA as standard. Serum-free Sertoli cell culture medium collected from 4-day-old cultures was a generous gift of Dr. Yan Cheng (Population Council, New York, NY, U.S.A.). The Sertoli cell culture medium, which had a protein concentration of 90 μg/ml, was concentrated 10-fold on Centricon 10 (Amicon) membranes and then was analysed by SDS/PAGE and Western blotting. Cytosolic fractions from neonatal testes were prepared as described previously (Jones and Brown, 1987).

RESULTS

Is PBP actively secreted by the testis and epididymis?

In initial experiments designed to test the hypothesis that PBP is a secreted protein, we endeavoured to produce sperm-free fluids from testis and epididymis while maintaining the secretory activity of both organs as close to normal as possible, thereby eliminating spermatozoa as a source of soluble PBP. A sperm-free epididymis can be produced by manipulating the androgen status of the animal. Approximately 4 weeks after castration, all spermatozoa are lost from the epididymis and the gland atrophies. Testosterone administration (1 mg/day subcutaneous) for 2 weeks subsequently restores epididymal secretory activity (Walker et al., 1990). SDS/PAGE analysis of proteins in this sperm-free CEP and in normal CEP, in which spermatozoa had been present, revealed the presence of PBP in the normal but not the sperm-free secretions (Figure 1a). Confirmation that the epididymis had responded to exogenous androgen treatment is shown by the re-induction and secretion of three testosterone-dependent secretory proteins in the sperm-free CEP (Figure 1a) (Jones et al., 1980; Brooks and Higgins 1980; Brooks et al., 1986a,b; Walker et al., 1990). These three proteins were also found to be present in sperm-free CEP from the cryptorchid epididymis, but again PBP was absent (Figure 1).

RTF, containing 10^6 spermatozoa per ml, was collected under acute conditions following ligation of the efferent ductules for 18–20 h (which causes fluid to accumulate within the seminiferous tubules). After collecting this fluid by puncturing the extra-testicular rete testis and removing spermatozoa by centrifugation, the resultant supernatant was analysed by SDS/PAGE and Western blotting. As shown in Figure 1(b), PBP was consistently found to be present in this fluid, albeit as a minor component, and gave a strong reaction upon Western blotting with anti-PBP antibody. To investigate whether Sertoli cells were the source of this PBP, rats were artificially cryptorchidized for 4 weeks to disrupt spermatogenesis, leaving only spermatogonia and Sertoli cells present in the seminiferous tubules. In cryptorchid testes, Sertoli cells continue to secrete fluid at near normal rates (Setchell, 1978). However, the sperm-free RTF collected from these animals did not contain any detectable PBP when analysed by SDS/PAGE and Western blotting (Figure 1), suggesting that PBP is not a secretory product of Sertoli cells.

This conclusion is further supported by an analysis of the medium from Sertoli cells cultured *in vitro* under conditions where they have been shown to secrete proteins such as transferrin, clusterin, androgen-binding protein, etc. (Cheng et al., 1986). SDS/PAGE of proteins in this Sertoli cell culture medium showed a major component at $M_r \sim 75000$ when stained with Coomassie Blue but a protein could not be detected that was coincident with the mobility of PBP (Figure 1c). Although a Western blot of Sertoli cell culture medium revealed a very weak signal at M_r 23000 when probed with anti-PBP antibody (Figure 1c), this could represent a small amount of cell lysis during culture and collection of medium, bearing in mind that immunodetection systems offer ~ 100 times the sensitivity of Coomassie Blue.

Evidence that PBP is present in Sertoli cells and germ cells in

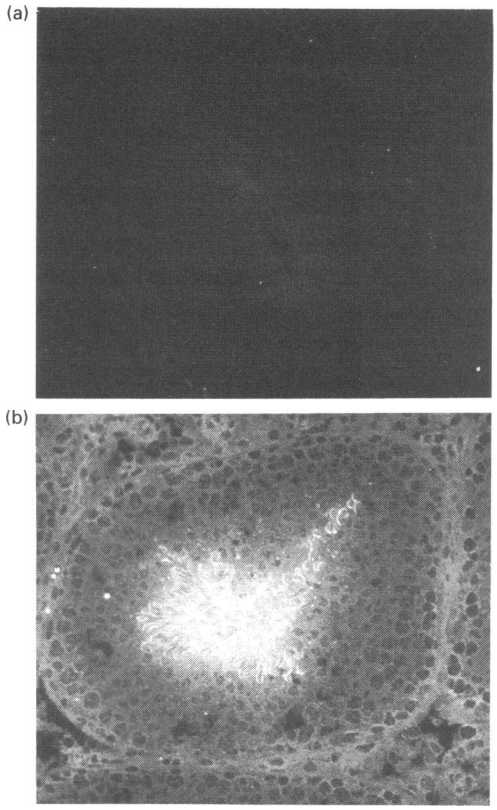

Figure 2 Immunocytochemical localization of PBP in rat testis

Frozen sections of adult testis were probed with anti-PBP antibody followed by FITC–goat anti-(rabbit IgG) and viewed with u.v. light. (**a**) Control section incubated with second layer antibody only. (**b**) Test section probed with anti-PBP antibody. Note that fluorescence is present in all cell-types throughout the seminiferous epithelium as well as on spermatozoa in the lumen of the duct. Magnification × 520.

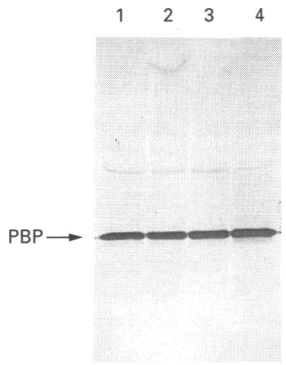

Figure 3 Neonatal development of testicular PBP

Testicular cytosols prepared from 10-day-old (lane 1), 20-day-old (lane 2), 30-day-old (lane 3) and adult (lane 4) animals were analysed by reducing SDS/PAGE, blotted and probed with anti-PBP antibody as described in the legend to Figure 1. The arrow indicates the position of PBP.

the testis is shown in Figures 2 and 3. Frozen sections of adult testis probed with anti-PBP antibody gave a diffuse reaction in the cytoplasm of all cell-types in the interstitial tissue and in the seminiferous epithelium, most noticeably in spermatogonia, spermatocytes, spermatids and spermatozoa in the lumen (Figure 2). Control sections were negative. The antigen, however, is not restricted to post-meiotic germ cells, as testicular cytosols from 10-day-old neonates (at this age only Sertoli cells and spermatogonia are present in the seminiferous tubules) contained similar amounts of PBP as did cytosols from adult testes (Figure 3). This confirms our earlier observations that PBP is ubiquitous in its cellular distribution (Jones and Brown, 1987).

Taken together, these data suggest that PBP is not secreted by the testis or epididymis. Therefore, genetic evidence for a secreted form of PBP was sought by cloning and sequencing PBP cDNA from testicular and epididymal libraries.

Cloning and sequence analysis of rat and monkey epididymal PBP cDNA

Rat epididymal cDNA clones ($\sim 5 \times 10^4$) were screened for those possessing cDNA inserts that hybridized to a 96-fold redundant oligonucleotide (5′-CARGCBGARTGGGAYGAYTAYGT-3′) corresponding to amino acid residues 169–176 (DQAEWDDYV) of bovine brain PBP (Glatz and Veerkamp, 1983). Following clone purification, plasmid DNAs from eight persistently hybridizing clones were isolated and the sizes of their cDNA inserts were determined. Those clones with the three largest inserts were sequenced. The same rat epididymal cDNA library was subsequently rescreened with a non-redundant 27-base oligonucleotide complementary to nucleotides 146–172 of the compiled rat PBP cDNA sequence (Figure 4), and a further 13 clones were identified and subjected to DNA sequence analysis.

Overall, the 16 sequenced independent rat epididymal cDNA clones were of similar size (900–1037 bp) and were completely superimposable except for a single sequence discrepancy: a C/T polymorphism at nucleotide position 766 in the compiled sequence (Figure 4).

The nucleotide sequence of rat epididymal PBP cDNA possesses an open reading frame that encodes a translational product of 187 amino acid residues (predicted M_r 20803), and which commences with the 5′-proximal ATG codon (nucleotides 29–31; Figure 4). The partial amino acid sequences of two peptides produced by CNBr cleavage of rat CEP PBP showed good alignment with the sequence deduced from the cloned cDNA (Jones and Hall, 1991).

Grandy et al. (1990) have reported the sequence of a single rat brain PBP cDNA clone which, apart from five single base differences, is identical to the epididymal sequence shown in Figure 4. These minor differences, all of which occur in non-coding regions, may represent genuine polymorphisms, rat strain differences (Sprague–Dawley versus Wistar) or sequencing artefacts (four of the five differences in the rat brain cDNA occur in regions sequenced on only one DNA strand).

In addition to isolating rat epididymal PBP clones, a cynomolgus monkey epididymal cDNA library was screened with the complete cDNA insert of a rat PBP clone, and 11 independent, strongly hybridizing clones were isolated and purified. Six of these were subsequently sequenced and although none was derived from sibling clones, each contained the complete coding sequence of monkey PBP (Figure 5).

Alignment of rat and monkey epididymal PBP predicted amino acid sequences with each other and with that of the bovine brain protein revealed a very high degree of sequence conservation (Figure 6). However, the two epididymal PBP sequences appeared to lack a signal peptide (characteristic of proteins destined for secretion) on the basis of N-terminal sequence alignment with the cytosolic brain homologue (Schoentgen et al., 1987). To confirm this finding in a single species, rat liver

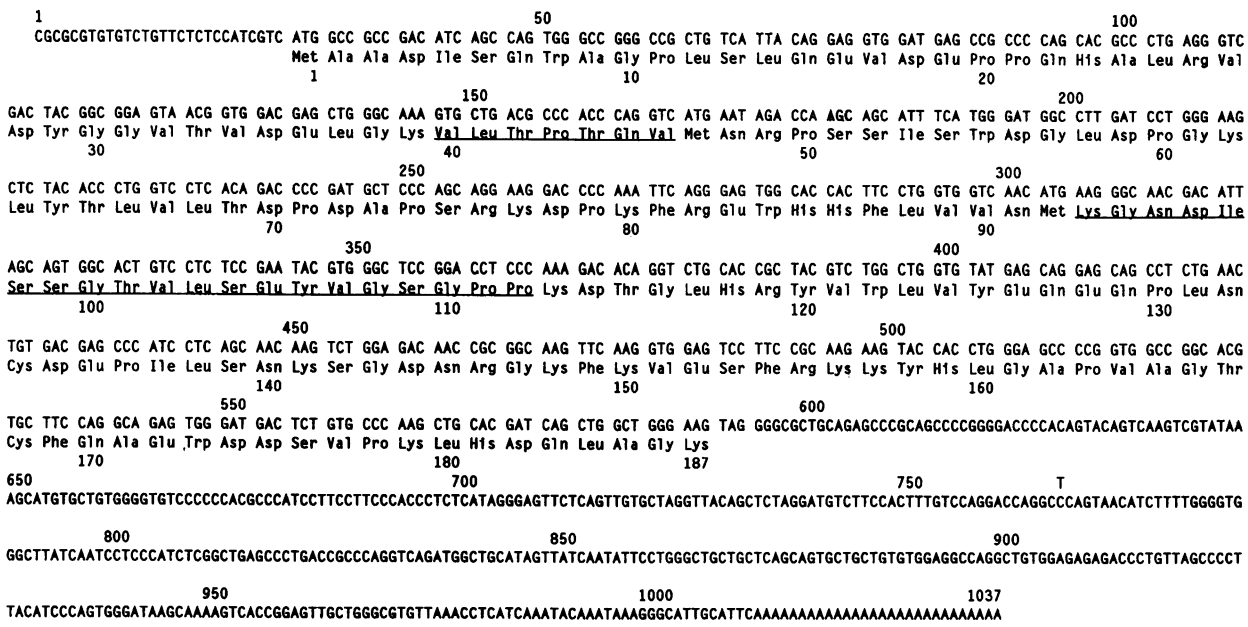

Figure 4 Complete nucleotide and deduced amino acid sequence of cloned rat epididymal cDNA

This sequence was compiled from 16 independent rat epididymal cDNA clones. The partial amino acid sequences of two peptides produced by CNBr cleavage of rat epididymal PBP which show good alignment with the deduced amino acid sequence of cloned PBP cDNA are underlined. A C/T polymorphism at nucleotide position 766 is indicated.

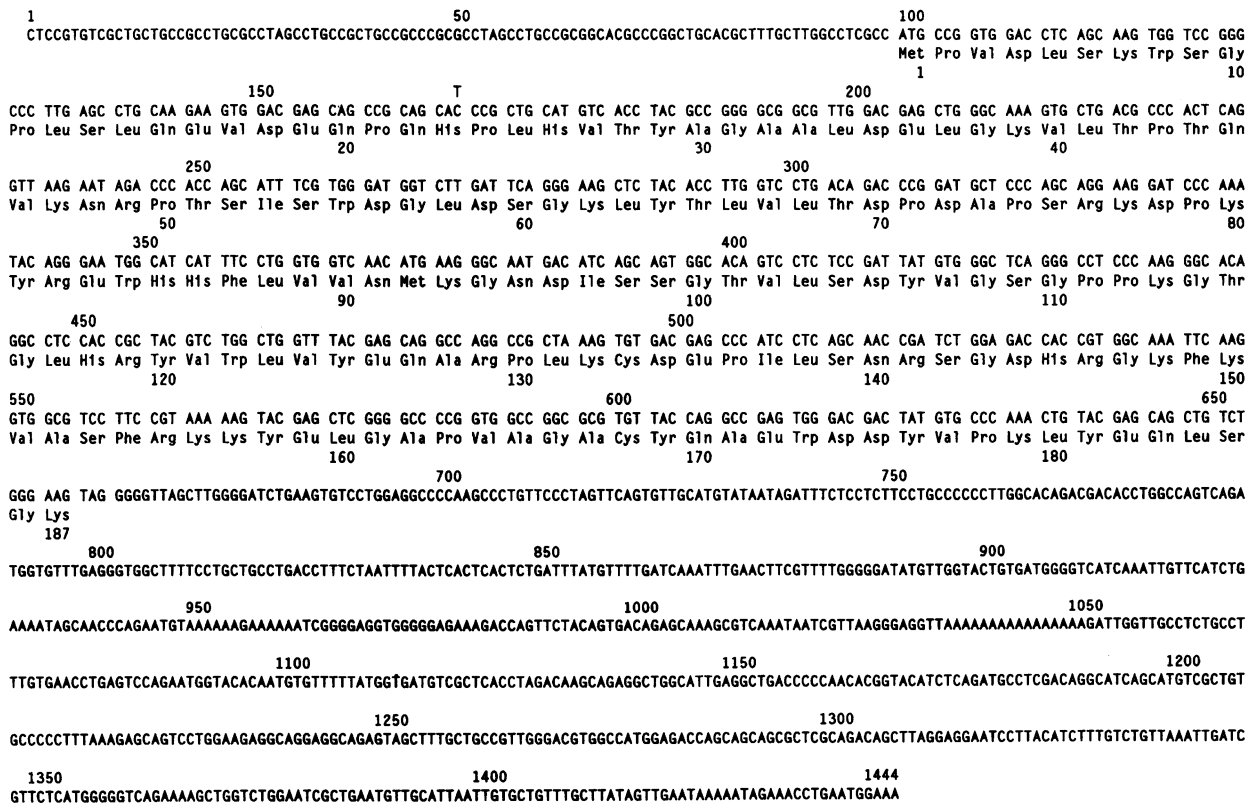

Figure 5 Complete nucleotide and deduced amino acid sequence of monkey epididymal cDNA

This sequence was compiled from six independent monkey epididymal cDNA clones and five independent monkey testicular cDNA clones. A C/T polymorphism at nucleotide position 166 is indicated.

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                    1       10        20        30        40        50        60        70        80
Rat epididymal PBP  MAADISQWAGPLSLQEVDEPPQHALRVDYGGVTVDELGKVLTPTQVMNRPSSISWDGLDPGKLYTLVLTDPDAPSRKDPK
                    | | | | |||||||||| ||| | | | | |||||||||||| ||| |||||||| ||||||||||||||||||||
Monkey epididymal PBP MPVDLSKWSGPLSLQEVDEQPQHPLHVTYAGAALDELGKVLTPTQVKNRPTSISWDGLDSGKLYTLVLTDPDAPSRKDPK
                     |||||||||||||||||| ||||| | | || |||||||||||||||||||||| ||||| ||||||||||||||||||||
Bovine brain PBP    PVDLSKWSGPLSLQEVDERPQHPLQVKYGGAEVDELGKVLTPTQVKNRPTSITWDGLDPGKLYTLVLTDPDAPSRKDPK

                    90       100       110       120       130       140       150       160       170       180   187
                    FREWHHFLVVNMKGNDISSGTVLSEYVGSGPPKDTGLHRYVWLVYEQEQPLNCDEPILSNKSGDNRGKFKVESFRKKYHLGAPVAGTCFQAEWDDSVPKLHDQLAGK
                     |||||||||||||||||||||||| |||||||| |||||||||||||| || |||||||| ||| |||||| |||||| ||||||| | |||||| |||| || ||
                    YREWHHFLVVNMKGNDISSGTVLSDYVGSGPPKGTGLHRYVWLVYEQARPLKCDEPILSNRSGDHRGKFKVASFRKKYELGAPVAGACYQAEWDDYVPKLYEQLSGK
                    ||||||||||||||| |||||||||||||||||||||||||||||||| ||||| ||||||||||||||||||||||||||||||||| ||||||||||||||||||||
                    YREWHHFLVVNMKGNNISSGTVLSDYVGSGPPKGTGLHRYVWLVYEQEGPLKCDGPILSNRSGDHRGKFKVASFRKKYELGAPVAGTCYQAEWDDYVPKLYEQLSGK
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Figure 6 Alignment of the deduced amino acid sequences of rat and monkey epididymal PBP with that of bovine brain PBP

The sequence of bovine brain PBP is taken from Schoentgen et al. (1987).

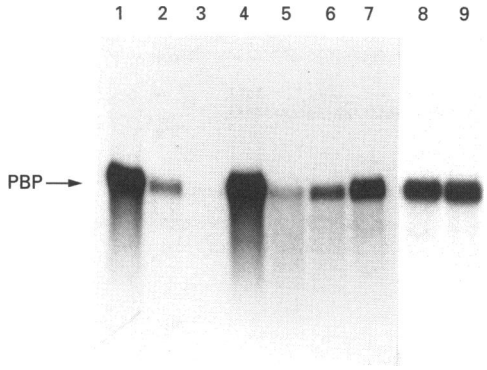

Figure 7 Northern blot analysis of the tissue distribution of rat PBP transcripts

Total RNA (10 μg/lane) was isolated from frozen rat tissues, electrophoresed on a denaturing agarose gel, blotted and probed with a rat epididymal PBP cDNA insert (see the Materials and methods section). The sources of the RNA samples were as follows: lane 1, liver; lane 2, brain; lane 3, placenta; lane 4, testis; lane 5, epididymis from a 14-day castrated animal; lane 6, epididymis from a 14-day castrated animal subsequently treated with testosterone for 14 days; lane 7, normal epididymis; lane 8, cauda epididymidis; and lane 9, caput epididymidis.

cytosolic PBP was cloned from a rat liver cDNA library and was found to have an identical sequence to its epididymal counterpart throughout the entire coding and non-coding regions (results not shown). Such sequence identity, together with the lack of a signal peptide sequence in the many clones examined here, argues against the secretion of PBP by the epididymis via a classic secretory pathway. Although it is possible that PBP is secreted by an unusual pathway that does not involve an N-terminal signal peptide (Muesch et al., 1990), such a possibility would imply that the non-classical secretory mechanism would be epididymis-specific, because PBP with an identical sequence is apparently cytosolic in other tissues. Alternatively, PBP found in epididymal fluid might be derived from the testis despite the evidence to the contrary shown in Figure 1. If so, significant amounts of PBP mRNA would be expected in the testis. A preliminary analysis was therefore sought of the tissue distribution and abundance of rat PBP transcripts.

Tissue distribution of rat PBP transcripts

Northern blot analysis of total RNA isolated from rat liver, brain, placenta, testis and epididymis revealed the testis to be a major site of PBP expression (Figure 7), with a higher steady-state PBP transcript level than that found in the epididymis or the brain. In all tissues examined, PBP mRNAs migrated as a single band, although this does not preclude the possibility that secreted and non-secreted forms of PBP are encoded by similarly sized transcripts. Figure 7 also demonstrates that castration resulted in a marked reduction in epididymal PBP transcript levels which was only partially reversed by subsequent testosterone treatment. This latter finding is consistent with earlier work on protein synthesis in the epididymis, assayed by [^{35}S]methionine incorporation (Jones et al., 1980). Analysis of RNA isolated from the caput and cauda regions of the epididymis (Figure 7) indicates that PBP is expressed throughout the epididymis, unlike many epididymis-specific transcripts which are often much more highly expressed in one region (Girotti et al., 1992; Perry et al., 1992, 1993).

Cloning and sequence analysis of testis PBP cDNA

The availability of a cynomolgus monkey testis cDNA library enabled cloned monkey testicular PBP transcripts to be isolated and characterized using a non-redundant oligonucleotide probe under moderately stringent hybridization conditions. This probe sequence, which is highly conserved in rat and monkey epididymal PBP cDNA, corresponded to an internal peptide sequence (Figure 4, amino acid residues 40–48) identified in PBP isolated from rat CEP (Jones and Hall, 1991). In total, five monkey testicular PBP clones were isolated and their cDNA inserts sequenced. In each case, the DNA sequence was identical to that of monkey epididymal PBP, and although the clones were completely or almost full-length, there was no indication of a signal peptide sequence (see Figure 5).

Evidence that PBP is derived from spermatozoa

Previous work has shown that PBP constitutes a major component of the total detergent-solubilized protein from testicular and cauda epididymal spermatozoa (Jones et al., 1983). In addition, an enriched plasma membrane fraction prepared from caudal spermatozoa contains PBP as a major component when analysed by SDS/PAGE (Jones and Brown, 1987). Spermatozoa, therefore, are a potential source of PBP, and as the foregoing results indicated that PBP is not actively secreted by either the testis or epididymis, the possibility was investigated that PBP found in normal RTF and CEP is derived from spermatozoa themselves. To this end, once-washed caudal spermatozoa were resuspended in PBS (control) or 0.5 M KCl, or 0.1 M Na_2CO_3, pH 11.0, or 0.2 mg/ml trypsin in PBS or 1% NP-40 in PBS.

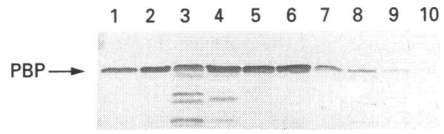

Figure 8 Extraction of PBP from rat spermatozoa with detergents and dissociating reagents

Western blot analysis shows the relative amounts of PBP solubilized from cauda spermatozoa with various reagents (lanes 1–5) together with PBP remaining in the treated sperm pellets (lanes 6–10). The second extraction was carried out with 1% (v/v) NP-40. Reagents: lanes 1 and 6, PBS; lanes 2 and 7, 0.5 M KCl; lanes 3 and 8, 0.2% (w/v) trypsin; lanes 4 and 9, 0.1 M Na_2CO_3, pH 11.0; lanes 5 and 10, 1% (v/v) NP-40.

Suspensions were incubated at room temperature for 30 min, centrifuged at 12000 *g* and supernatants removed. These supernatants (first extracts) were re-centrifuged at 100000 *g* for 60 min at 4 °C. Sperm pellets were then re-extracted in 1% (v/v) NP-40 at 4 °C for 45 min, centrifuged at 12000 *g* for 10 min and the supernatants (second extracts) were stored frozen. Western blot analysis (Figure 8) revealed the presence of PBP in all of the first extracts, the 0.5 M KCl and 0.1 M Na_2CO_3, pH 11.0, extracts being especially rich in the protein. Densitometric analysis of the proportion of PBP from spermatozoa remaining in the second extracts indicated that 0.5 M KCl solubilized 68% of PBP, trypsin 74%, 0.1 M Na_2CO_3, pH 11.0, 87% and 1% (v/v) NP-40 98%.

Attempts to localize PBP on spermatozoa by IIF proved difficult and results were equivocal irrespective of the fixing and staining procedure. When unfixed spermatozoa in suspension were incubated at 4 °C, < 10% of cells showed any fluorescence. Those spermatozoa that were positive had patchy fluorescence along the tail and were probably damaged, as areas of plasma membrane appeared to be detached from the surface. Cytoplasmic droplets were also fluorescent on these spermatozoa. Fixation in 4% (v/v) paraformaldehyde in PBS for 30 min followed by incubation with antibodies at 4 °C did not increase the incidence, or alter the appearance, of labelled spermatozoa. Similar results were obtained when incubations were carried out at room temperature.

DISCUSSION

PBP is one of five or six major proteins present in normal rat CEP and detergent extracts of cauda epididymal spermatozoa (Jones et al., 1983; Brooks, 1985; Klinefelter and Hamilton, 1985). It is also synthesized by epididymal epithelial cells as shown by [^{35}S]methionine incorporation (Brooks and Higgins, 1980; Jones et al., 1980). Because the epididymis is known to secrete a wide variety of proteins and glycoproteins into the luminal fluid (Cooper, 1986), many of which are regulated specifically by androgens and integrate into or 'coat' the plasma membrane of spermatozoa, it was logical to presume that PBP was one such protein and that it would be secreted by classic pathways. However, the evidence obtained in the present work points to a different interpretation; namely, that PBP is not actively secreted by the epididymis but is derived principally from spermatozoa. The reasons for arriving at this conclusion are as follows. Firstly, the genetic evidence indicates that PBP is a non-secreted protein. PBP does not contain a classic N-terminal signal peptide and is identical throughout its entire sequence to cytosolic PBP from liver. Also, similar-sized mRNA transcripts are present on Northern blots of all tissues examined, suggesting that there is only one form of the protein. Although the absence of an obvious signal peptide cannot, in itself, be regarded as definitive evidence that a protein is not exported from a cell, as there is a growing list of proteins that are secreted by non-classic pathways (e.g. lipocortin and interleukin 1; Muesch et al., 1990), there is no indication from the present work, or elsewhere, that PBP belongs to this category.

Secondly, PBP cannot be detected in CEP from an epididymis rendered sperm-free by castration/androgen replacement or by artificial cryptorchidism. Under these circumstances, CEP does not contain any sperm components and can be regarded as representing 'pure' epididymal secretions. Support for this view comes from the presence in sperm-free CEP of three epididymal-specific androgen-dependent glycoproteins previously referred to as B, C and D/E (Brooks and Higgins, 1980; Jones et al., 1980; Walker et al., 1990). All three proteins are accepted as being secreted because: (a) they are not present in the testis or testicular spermatozoa; (b) they are consistently found in sperm-free CEP from castrated androgen-stimulated animals; and (c) their cDNAs have been sequenced and found to contain classic signal peptides. PBP does not meet any of these criteria.

Thirdly, the fact that PBP can be solubilized from spermatozoa with trypsin and dissociating reagents strongly suggests that germ cells are the primary source of the protein in CEP and RTF. Luminal contents collected directly from the cauda epididymidis are highly viscous (comparable with 10 M glycerol) and are usually diluted 20–30-fold in PBS before removal of spermatozoa by centrifugation. From the results shown in Figure 8 this treatment would dissociate significant amounts of PBP from spermatozoa causing it to appear in the dilute CEP. However, the fact that PBP is also present in CEP collected without dilution ('neat' CEP) suggests that it is normally released from spermatozoa during their maturation process. If so, this represents some of the first evidence that spermatozoa have a direct influence on the composition of CEP in addition to the epididymal epithelium.

PBP is also present in testicular spermatozoa (Jones and Brown, 1987) and can be solubilized from them in the same way as described above for cauda spermatozoa (R. Jones, unpublished work). As PBP could not be detected in sperm-free RTF from cryptorchid testes and the small amounts detected by Western blotting in Sertoli cell culture medium are not consistent with it being a major secretory product of Sertoli cells, all the evidence suggests once again that PBP is derived from germ cells. PBP in 'normal' RTF may, in fact, be an artifact of the collection technique. Testicular spermatozoa are usually allowed to accumulate in the testis over a period of 18–20 h following ligation of the efferent ducts and it is possible that during the time they reside in the rete area some degenerate and release PBP, especially as RTF contains ~ 120 mM NaCl.

The cellular localization of PBP on spermatozoa remains an enigma. The fact that it is present in preparations of plasma membranes (Jones and Brown, 1987) and has affinity for PE suggests that it is associated (however loosely) with the lipid bilayer on either the external or internal face. However, it could not be visualized by IIF irrespective of the labelling protocol, and we are forced to conclude either that PBP is inaccessible to the antibody or that it is leached off the membrane during the washing steps that are necessary to remove unbound antibodies. Accessibility is undoubtedly a problem given that the protein is cytoplasmic in other tissues. Moore et al. (1987) reported similar difficulties with immunodetection of a M_r 24000 antigen on rat spermatozoa with monoclonal antibody B109. This antibody localized to cytoplasmic droplets and along the inner face of their surrounding plasma membrane but only faint staining could be

observed along the tail of a small proportion of fixed spermatozoa. Fresh, unwashed spermatozoa did not stain. It is not clear whether PBP and B109 antigen are identical, but clearly the properties of some proteins make them difficult to detect immunologically.

A more precise comparison can be made with a mouse epididymal protein (MEP9) that, from peptide sequence data, appears to be the homologue of PBP (Vierula et al., 1992). MEP9 was detected immunocytochemically on tissue sections in elongating spermatids, principal epithelial cells in the distal caput, corpus and cauda epididymidis and sperm cytoplasmic droplets but not in spermatogonia, spermatocytes, Sertoli cells, the proximal caput epididymidis or sperm plasma membranes. The testicular distribution of MEP9 contrasts with our own observations on PBP in the rat. PBP is present in testicular cytosols from neonates as early as 5 days, an age when only Sertoli cells and spermatogonia are present in the seminiferous tubules. Thus, it is not hormonally induced and is present in somatic cells in the testis as well as in germ cells. The explanation for these discrepancies between rat PBP and mouse MEP9 is not clear but may reside in the nature of the antibodies.

Loss of PBP during antibody labelling is also a legitimate explanation. Antibody-induced shedding of surface antigens has been reported in the parasitic worm *Toxocara canis*, where it is thought to function as a defence mechanism against immune attack by the host (Page et al., 1992). One such antigen, the TES-32 glycoprotein, can be detected on the cuticle by IIF with monoclonal antibodies if incubations are performed at 4 °C but at 37 °C the antigen–antibody complexes are shed into the medium. TES-32 glycoprotein is especially interesting as it shows significant sequence similarity (30 %) with PBP. It differs from PBP, however, in having an N-terminal extension that contains a signal peptide (D. Gems and R. M. Maizels, personal communication). The existence of a secreted homologue of PBP may be an important precedent and raises the possibility that soluble PBP in the male reproductive tract could have an extracellular role.

The widespread tissue distribution of PBP suggests that it has a role as a lipid carrier protein during membrane biogenesis in the cytoplasm (Schoentgen et al., 1987). Such a protein would clearly be important in spermatogenesis when different autoantigenic determinants, characteristic of mature spermatozoa, are directed towards separate surface domains. How these domains are formed and maintained is currently one of the fundamental questions in sperm biology. Fluorescent recovery after photobleaching (FRAP) measurements have indicated that many membrane lipids are segregated into fluid and gel phases and that these phases are established early on during spermiogenesis (Wolf et al., 1990). If so, then PBP could be involved in directing lipids towards different surface compartments. Further work is required to establish whether other PBPs are present in spermatozoa and whether they have an extracellular role during maturation in the epididymis.

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REFERENCES

Bernier, I., Tresca, J.-P. and Jollès, P. (1986) Biochim. Biophys. Acta **871**, 19–23
Bradford, M. M. (1976) Anal. Biolchem. **72**, 248–254
Brooks, D. E. (1985) Biochim. Biophys. Acta **841**, 59–70
Brooks, D. E. and Higgins, S. J. (1980) J. Reprod. Fert. **59**, 363–375
Brooks, D. E., Means, A. R., Wright, E. J., Singh, S. P. and Tiver, K. K. (1986a) Eur. J. Biochem. **161**, 13–18
Brooks, D. E., Means, A. R., Wright, E. J., Singh, S. P. and Tiner, K. K. (1986b) J. Biol. Chem. **26**, 4956–4961
Cheng, C. Y., Mather, J. P., Byer, A. L. and Bardin, C. W. (1986) Endocrinology (Baltimore), **118**, 480–488
Cooper, T. G. (1986) The Epididymis, Sperm Maturation and Fertilization, Springer-Verlag, Berlin
Feinberg, A. P. and Vogelstein, B. (1984) Anal. Biochem. **137**, 266–267
Girotti, M., Jones, R., Emery, D. C., Chia, W. and Hall, L. (1992) Biochem. J. **281**, 203–210
Glatz, J. F. C. and Veerkamp, J. H. (1983) Anal. Biochem. **132**, 89–95
Grandy, D. K., Hanneman, E., Bunzow, J., Shih, M., Machida, C. A., Bidlack, J. M. and Civelli, O. (1990) Mol. Endocrinol. **4**, 1379–1376
Humphries, S. E., Whittal, R., Minty, A., Buckingham, M. and Williamson, R. (1981) Nucleic Acids Res. **9**, 4895–4908
Jones, R. and Brown, C. R. (1987) Biochem. J. **241**, 353–360
Jones, R. and Hall, L. (1991) Biochim. Biophys. Acta. **1080**, 78–82
Jones, R., Brown, C. R., von Glos, K. I. and Parker, M. G. (1980) Biochem. J. **188**, 667–676
Jones, R., von Glos, K. I. and Brown, C. R. (1983) J. Reprod. Fert. **67**, 299–306
Klinefelter, G. R. and Hamilton, D. W. (1985) Biol. Reprod. **33**, 1017–1027
Luzio, J. P., Brake, B., Banting, G., Howell, K. E., Braghetta, P. and Stanley, K. K. (1990) Biochem. J. **270**, 97–102
Moore, A., Enstrud, K., Baker, J., Wenstrom, J. and Hamilton, D. W. (1987) Ann. N.Y. Acad. Sci., **513**, 204–214
Muesch, A., Hartmann, E., Rohde, K., Rubartelli, A., Sita, R. and Rapoport, T. A. (1990) Trends Biochem. Sci. **15**, 86–88
Myles, D. G., Primakoff, P. and Bellve, A. K. (1981) Cell **23**, 433–439
Page, A. P., Rudin, W., Fluri, E., Blaxter, M. L. and Maizels, R. M. (1992) Exp. Parasitol. **75**, 72–86
Perry, A. C. F., Jones, R., Barker, P. J. and Hall, L. (1992) Biochem. J. **286**, 671–675
Perry, A. C. F., Jones, R. and Hall, L. (1993) Biochem. J. **293**, 21–25
Russell, L. D., Peterson, R. N., Russell, T. A. and Hunt, W. (1983) Biol. Reprod. **28**, 393–413
Schoentgen, F., Saccoccio, F., Jollès, J., Bernier, I. and Jollès, P. (1987) Eur. J. Biochem. **166**, 333–338
Setchell, B. P. (1978) in The Mammalian Testis, pp. 360–366, Paul Elek, London
Vierula, M. E., Araki, Y., Rankin, T. L., Tulsiani, D. R. P. and Orgebin-Crist, M.-C. (1992) Biol. Reprod. **47**, 844–856
Voglmayr, K. J., Fairbanks, G., Jackowitz, M. A. and Colella, J. (1980) Biol. Reprod. **22**, 655–667
Walker, J. E., Jones, R., Moore, A., Hamilton, D. W. and Hall, L. (1990) Mol. Cell Endocrinol. **74**, 61–68
Wolf, D., Maynard, V. M., McKinnon, C. A. and Melchior, D. L. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 6893–6896