Binding of zona pellucida proteins to a boar sperm polypeptide of $M_r 53,000$ and identification of zona moieties involved

C. R. BROWN and R. JONES

AFRC Institute of Animal Physiology & Genetics Research, Animal Research Station, 307 Huntingdon Road, Cambridge CB3 0JQ, UK

Present address: AFRC Institute of Animal Physiology & Genetics Research, Department of Molecular Embryology, Babraham, Cambridge CB2 4AT, UK

Summary

Experiments have been carried out to identify proteins on boar spermatozoa that bind to components of the zona pellucida. Polypeptides in sodium deoxycholate extracts of boar spermatozoa and in whole seminal plasma have been separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis, transferred onto nitrocellulose sheet by electroblotting and probed with $^{125}$I-labelled heat-solubilized zona pellucida from pig oocytes or ovulated eggs. Zona proteins bound avidly and consistently to a polypeptide of $M_r 53,000$ on blots of capacitated and noncapacitated sperm and weakly to polypeptides of $M_r 67,000, 38,000$ and $18,000$. On blots of seminal plasma the $^{125}$I-labelled probes bound to two polypeptides of $M_r 65,000$ and $19–24,000$. Identification of the zona proteins that were binding to the aforementioned proteins on blots showed that all the major zona pellucida glycoproteins were involved, including those acquired from oviduct secretions. Binding of $^{125}$I-ovulated zona pellucida to the polypeptide of $M_r 53,000$ also occurred in extracts of testicular and epididymal boar spermatozoa. The results are discussed in relation to sperm–egg recognition in the pig.

Key words: porcine, zona pellucida, sperm, polypeptide, boar.

Introduction

A critical stage in mammalian fertilization is the recognition and binding of spermatozoa to the egg in the conducive environment of the oestrous oviduct. Once this propensity to unit has been acquired it seems logical to assume that the initial attachment of gametes is achieved via the interaction of adhesion molecules or receptors at their limiting surfaces. In the female gamete such molecules probably reside at the surface of the zona pellucida, the relatively thick glycoprotein matrix that encompasses the egg, and in sperm they are likely to be associated with the membranes overlying the head. The process of recognition and initial attachment of sperm to the egg is not entirely species specific unlike consolidated binding and sperm penetration through the zona pellucida (Gwatkin, 1977, review). While these broad concepts are widely accepted very little is known about the identity of such recognition molecules or their eventual interaction, although recently some progress has been made in this direction. For instance, in the mouse (Bleil & Wassarman, 1983) and pig (Sacco, Subramanian & Yurewicz, 1984) pre-exposure of spermatozoa to a component isolated from the zona pellucida has been shown to prevent sperm–egg recognition. There is also good evidence in the mouse that initial binding of spermatozoa to the zona pellucida is promoted by a sperm surface galactosyltransferase with a lectin-like affinity for $N$-acetylglucosamine residues on zona glycoproteins (Shur & Hall, 1982). In the guinea pig a role in sperm binding to the egg has been proposed for a mobile sperm surface antigen which transfers from its position over the posterior head of intact sperm to the inner acrosomal membrane of the acrosome-reacted sperm, presumably there to participate in consolidation of binding (Primakoff, Hyatt & Myles, 1985). Other putative sperm–zona pellucida binding molecules have been identified in sperm of various species by means of the novel approach of probing Western blots of sperm proteins with labelled zona proteins (Sullivan & Bleau, 1985; O’Rand, Matthews, Welch & Fisher, 1985). However, in some cases, e.g. in the pig, zonae
pellucidae invariably have been prepared on a large scale from ovarian oocytes and as such have not been exposed to the milieu of the oestrous oviduct. This may be significant in terms of identification of recognition molecules because it has recently been shown that the oestrous oviduct of the pig contains two glycoproteins which bind firmly to the zona pellucida of the freshly ovulated egg and which remain bound until at least the 2- to 4-cell stage (Brown & Cheng, 1986).

In view of the above points we have attempted to identify molecules on boar spermatozoa that have the ability to recognize zona pellucida moieties. In this paper we compare zonae pellucidae from pig oocytes with those from ovulated eggs in their respective ability to recognize and bind to boar sperm proteins on Western blots and we also identify the zona pellucida moieties involved in recognition.

Materials and methods

Chemicals

Chemicals for electrophoresis and blotting procedures were obtained from Sigma Chemical Co. (London, UK) or from BDH (Poole, Dorset), and the reagent N-succinimidyl 3-(4-hydroxy, 5-[$^{125}$I]iodophenyl propionate and $^{14}$C-methylated molecular weight (M_{r}) markers from Amersham International, Amersham, Bucks, UK. Sodium deoxycholate (specially purified) was supplied by Fisons Scientific, Loughborough, Leics, UK, and pregnant mare's serum gonadotrophin (PMSG) and human chorionic gonadotrophin (hCG) by Intervet Laboratories Ltd, Cambridge, UK.

Collection of eggs and oocytes

Eggs were recovered surgically from 8- to 10-month-old crossbred (Large White and Landrace) gilts which had been injected on day 16 of the oestrus cycle with 1200 i.u. PMSG followed by 50 i.u. hCG on day 19-5. At 36 h post hCG oviducts were flushed with phosphate-buffered saline (PBS) containing 4 mg ml$^{-1}$ bovine serum albumin (BSA) (Brown & Cheng, 1986) and recovered eggs were washed thoroughly in PBS with polyvinyl alcohol (0-2 mg ml$^{-1}$) in place of BSA. Oocytes were aspirated from the follicles of ovaries obtained fresh from a slaughterhouse.

Iodination and solubilization of zonae pellucidae

The zona pellucida of eggs and oocytes was freed of corona radiata cells by gentle pipetting and the investment carefully removed with the aid of a Leitz micromanipulator. To groups of 25–50 isolated zonae, in 10 μl PBS, was added 2-5 μCi N-succinimidyl 3-(4-hydroxy, 5-[$^{125}$I]iodophenyl propionate in 20 μl 0-1 M-borate buffer pH 8-5 (Bolton & Hunter, 1973). After 15 min at 25°C the zonae were washed thorquely by centrifugation through five changes of 1 ml PBS (5 min at 1000 g). They were then solubilized by heating for 2 h at 70°C in 0-4 ml 0-03 M-sodium carbonate buffer pH 9-2 and stored frozen at −20°C.

Collection and extraction of spermatozoa

To obtain noncapacitated boar spermatozoa 5 ml of sperm-rich fraction of an ejaculate was centrifuged for 15 min at 650 g (25°C) and the loosely sedimented cells washed twice with 5 ml PBS. Sperm were capacitated by first washing in 0-9 % (w/v) NaCl containing 1 mg ml$^{-1}$ BSA followed by 4–6 h incubation at room temperature in supplemented Medium 199 (Cheng, 1985). Seminal plasma was cleared of residual spermatozoa and cellular debris by centrifugation at 10000 g for 10 min before use. Cauda epididymidal spermatozoa were obtained by retrograde flushing of the lumen of the cauda epididymidis with PBS and testicular spermatozoa by puncturing the efferent ductules. They were separated from epididymal and testicular fluids in the same way as ejaculated spermatozoa from seminal plasma. Spermatozoa were collected from the caput epididymidis by slicing the tissue in PBS. After incubating for 30 min supernatant fluids were drained, filtered through four layers of gauze and centrifuged for 30 s at 600 g. Supernatants contained spermatozoa with negligible contamination from tissue fragments. Sperm membrane proteins were solubilized by incubating the cells in 0-4 % w/v sodium deoxycholate (DOC) in PBS for 30 min at 4°C, and extracts recovered by centrifugation at 10000 g for 15 min.

Extraction of other tissues

Fresh tissue (0-5 g) from porcine liver, kidney, spleen, heart and lung was homogenized for 0-5 min in 4°C in 5 ml 0-4 % w/v DOC in 20 mm-Tris–HCl pH 8-4. Homogenates were incubated for 30 min at 4°C and extracts recovered by centrifugation at 10000 g for 30 min.

Procedure for probing proteins from sperm and seminal plasma on Western blots with $^{125}$I-zona pellucidae glycoproteins

Polypeptides in seminal plasma and in DOC extracts of spermatozoa were separated on nonreducing sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) employing a 7-7-14 % w/v gradient of polyacrylamide gel. Sperm membrane proteins were solubilized by incubating either with Coomassie Brilliant Blue R250 (0-05 % w/v) or stained for carbohydrate with the periodic acid Schiff reaction (PAS) (Fairbanks, Steck & Wallach, 1971). Alternatively, proteins were transferred onto nitrocellulose paper (0-45 μm; Schleicher & Schull Ltd) using the electroblotting method of Towbin (1979). Blots were washed three times in PBS (10 min with shaking) blocked for 1 h at 25°C with 5 % w/v BSA in PBS and then probed with $^{125}$I-zona pellucidae (approx. 1–2×10$^{6}$ cts min$^{-1}$ in 0-4 ml) for 3 h at 25°C. The blots were washed three times in PBS which, in some experiments, included Nonidet P-40 (0-5 % w/v), and dried. Labelled proteins were detected by autoradiography using prefloashed Kodak X-Omat S film.

Several technical problems arose during initial experiments with the above protocol. One was the random appearance on autoradiographs of 'hot' spots, e.g. see Fig. 2. These small areas of intense radioactivity were caused by the adherence of tiny particles of incompletely solubilized $^{125}$I-labelled zona to the nitrocellulose. This problem could be circumvented by centrifuging the melted
Zone proteins at 10,000 g for 5 min before probing the blot. Preliminary experiments showed that despite incomplete dissolution zona proteins do not behave preferentially in their solubility properties during heating at 70°C (results not shown). A second difficulty was the requirement for a 'preblot' with the freshly solubilized 125I-labelled zona proteins. It was found that the first time the probe was used background radioactivity on blots was unacceptably high, despite effective blocking of the nitrocellulose with 5% w/v BSA. O'Rand et al. (1985) have reported the same phenomenon and also employed a preblot. However, the second and third times the probe was used background levels became weak and it was possible to detect specific binding. Again preliminary experiments showed that the preblot did not remove preferentially any particular class of zona proteins (results not shown).

**Extraction and electrophoresis of 125I-zonae pellucidae recognized on blots**

It is known that depending upon whether or not the egg has been ovulated the pig zona pellucida may consist of up to three major glycoproteins (Brown & Cheng, 1986) and in order to examine if one or all of these glycoproteins are involved in binding to sperm and seminal plasma polypeptides, relevant areas of the probed Western blot were cut out and extracted for 5 min at 100°C in 50 μl 62.5 mm-Tris–HCl pH 7.4 containing 2% w/v SDS. SDS–PAGE was carried out as described above.

**Results**

**Electrophoretic analysis of solubilized zona pellucidae**

In preliminary experiments we investigated if the solubilization process had any effect on the macromolecular constituents of the investment. A comparison was made of the profile of 125I-labelled polypeptides, separated on nonreducing SDS–PAGE, of zonae removed manually from freshly ovulated eggs and either dissolved directly in SDS or solubilized by heating as described in Materials and methods. There was no difference between the two procedures as each profile of proteins was identical to that shown in Fig. 1j. Similarly, solubilizing zonae in acid Tyrode's solution pH 2.9 or PBS pH 2.5 had no significant effect on the profile of polypeptides on SDS–PAGE compared to those dissolved directly in SDS.

**Identification of molecules in sperm and seminal plasma that recognize 125I-zonae pellucidae glycoproteins**

The range of polypeptides present in sodium deoxycholate extracts of ejaculated sperm and in seminal plasma after staining with Coomassie blue is shown in Fig. 1a,b. Sperm extracts separated into a relatively complex profile of proteins with Mr between 14,000 to 100,000 whereas seminal plasma contained fewer proteins but with a major group between Mr 14,000 and 25,000. Those proteins that stain with the PAS procedure to detect carbohydrate are indicated by an asterisk; in some cases the reaction was quite strong although, as can be seen from Fig. 1, the Coomassie blue stain appeared weak.

When Western blots of parallel gels of those shown in Fig. 1a,b were probed with 125I-zonae from follicular oocytes (125I-FZP) consistent binding was observed to a sperm component of Mr 53,000 (Fig. 1c). Weaker affinity binding was found over a polypeptide Mr 18,000 while two others, Mr 67,000 and 38,000, varied in the amount of probe they bound, the maximum being seen in track c. In seminal plasma (track d) there was strong binding of 125I-FZP over an area ranging from Mr 19,000 to 24,000 and weak binding towards a polypeptide of Mr 65,000.

Probing Western blots of sperm proteins with zonae from ovulated eggs (125I-OZP) gave essentially the same profile of polypeptide recognition as did probing with follicular zonae (Fig. 1e) although in general there was an increased avidity of the probe, particularly for the sperm polypeptide of Mr 53,000. We detected no difference in binding either qualitatively or quantitatively between capacitated and non-capacitated sperm (tracks e and f).

On blots of seminal plasma proteins the 125I-OZP probe bound to a polypeptide doublet Mr 65,000 and an area of polypeptides Mr 19,000–24,000 (tracks d and g).

Washing blots probed with either 125I-FZP or 125I-OZP in PBS containing 0.5% w/v NP-40 reduced considerably background labelling as well as the amount of radiolabel associated with the Mr 67,000, 38,000 and 18,000 polypeptides on spermatozoa. However, the amount of label associated with the sperm Mr 53,000 polypeptide remained undiminished after washing four times with PBS/NP-40.

**Identification of zona moieties recognizing sperm and seminal plasma polypeptides**

When those areas of the blots occupied by the polypeptides of Mr 18,000, 19,000–24,000, 53,000 and 65,000 were cut out and the bound 125I-zona proteins extracted as described in the Materials and methods section it was possible to identify which of the zona moieties were involved in recognizing the sperm proteins. As shown in Fig. 1 there was a marked difference in the polypeptide profiles depending upon which 125I-zona probe was used. In all cases where 125I-FZP was employed a single polypeptide of Mr 79,000 was resolved with a broader area of label spread directly below it, whereas when 125I-OZP was used each extract separated into three polypeptides of Mr 250,000, 90,000 and 79,000. Again there was a broader area of label below with a lower molecular
weight limit of approximately 55,000. This difference is shown in Fig. 1 together with polypeptide profiles of the zona from an oocyte and an ovulated egg and it can be seen that the former matches extracts of polypeptides probed with follicular zonae and the latter those probed with ovulated zonae.

Recognition of 125I-zona pellucida by polypeptides of immature and mature spermatozoa

The Mr 53,000 polypeptide was present in DOC extracts of testicular, epididymal and ejaculated sperm as revealed by staining with Coomassie blue and in each case it was recognized by 125I-OZP glycoproteins after blotting (Fig. 2). It was noticeable that more 125I-OZP bound to the Mr 53,000 polypeptide from cauda epididymal and ejaculated sperm than from testicular and proximal caput epididymal sperm. There was also weak recognition of a polypeptide of approximate Mr 67-68,000 in testicular and proximal caput sperm extracts and of an approximate Mr 38,000 polypeptide in epididymal and ejaculated, but not testicular, sperm extracts.

Tissue specificity of the Mr 53,000 polypeptide

When blots of DOC extracts of porcine liver, kidney, spleen, heart and lung polypeptides separated on
SDS–PAGE were probed with $^{125}\text{I}-\text{OZP}$ there was no binding of label to an area of the blot corresponding to $M_r 53000$ (Fig. 3).

Fig. 2. SDS–PAGE of sodium deoxycholate extracts of testicular (a), caput (b) and cauda (c) epididymal and ejaculated (d) spermatozoa blotted and probed with $^{125}\text{I}-\text{OZP}$. Each track received approximately 150 µg protein.

Fig. 3. SDS–PAGE of sodium deoxycholate extracts of porcine spermatozoa (a), liver (b), kidney (c), spleen (d), heart (e) and lung (f) blotted and probed with $^{125}\text{I}-\text{OZP}$. Each track received approximately 150 µg protein.

Discussion

A major conclusion from the work described in this paper is that glycoproteins from the zona pellucida of pig eggs have a strong avidity for a protein of $M_r 53000$ present in DOC extracts of boar spermatozoa. This protein is recognized by zona glycoproteins from follicular as well as ovulated eggs. It is also present in detergent extracts of spermatozoa from the testis and various levels of the epididymis so it is therefore unlikely that the protein is acquired during sperm maturation in the epididymis. Another aspect of these results is the specificity and strong avidity of the zona glycoproteins for the sperm $M_r 53000$ component which, due to its absence from several porcine tissues, is apparently not a ubiquitous protein.

Other sperm polypeptides recognized by the zona glycoproteins were those of $M_r 67000$, $38000$ and $18000$ although binding to these was more variable and with lower affinity. O'Rand et al. (1985), using the same species and experimental approach, reported major binding of zona proteins to sperm polypeptides of $M_r 16000$ and $18000$ with less binding to other species of $M_r$ between $63000$ and $88000$, and $45000$ and $53000$. Our results differ mainly in that the polypeptide $M_r 53000$ is consistently the major species recognized in our experiments although we did detect a small amount of binding at $M_r 18000$. The only major area of recognition that we saw corresponding to an $M_r$ value of 18–19 000 was in seminal plasma. Possible reasons why the results of O’Rand et al. are different from those reported here could be that they adopted a different labelling protocol (solubilized zonae were labelled with $^{125}\text{I}$-iodogen) or that their proteins were prepared by extracting spermatozoa directly with SDS. It is possible that the polypeptide $M_r 53000$ is a glycoprotein because of its ability to bind $^{125}\text{I}$-concanavalin A. However, we were unable to detect carbohydrate on the $M_r 53000$ polypeptide whereas several others in sperm and seminal plasma were quite strongly PAS-positive.

It is interesting that zona glycoproteins bind to polypeptides in seminal plasma as well as in spermatozoa. Both zona probes ($^{125}\text{I}-\text{FZP}$ and $^{125}\text{I}-\text{OZP}$) recognized several polypeptides with an $M_r$ value ranging from 19 000 to 24 000 and also, though again to a variable extent, a polypeptide doublet of $M_r 45000$.
raised the possibility that boar seminal plasma proteins derived from the seminal vesicle secretion (Hunt, Russell, Peterson & Strack, 1983) can bind to the porcine zona pellucida. Hunt et al. have shown that relatively high levels of seminal plasma proteins bind readily to boar spermatozoa and that a group of $M_r$ 16–19000 is represented in plasma membrane preparations. These are present in seminal vesicle secretion and thus could be responsible for the binding of the zona proteins to blots of seminal plasma components, particularly the lower molecular weight group. Since there is likely to be less of these proteins on sperm after washing it is possible that the sperm polypeptide of $M_r$ 18000 originates from the seminal plasma.

An alternative approach used to identify molecules involved in sperm–egg recognition utilizes purified or enriched components of either the zona pellucida or sperm membrane to block sperm–zona binding in vitro. For instance in the pig a zona component of $M_r$ 85000 is effective in this respect (Sacco et al. 1984) as are enriched sperm membrane preparations (Peterson, Henry, Hunt, Saxena & Russell, 1985). Although the experimental approach that we have used is arguably less physiological than the above it has the advantage of testing the complete profile of zona glycoproteins against a wide range of sperm polypeptides, thus increasing the chance of identifying potentially interesting molecules. Also in the pig a similarly comprehensive approach has been adopted by Peterson et al. (1985). They incubated solubilized sperm plasma membrane preparations with isolated zonae and like us found that several proteins with molecular weights ranging from approximately 35000 to 100000 showed affinity for the zona.

The mouse zona pellucida consists of three major glycoproteins and one of these, $M_r$ 83000 (ZP3), has sperm-binding properties (Bleil & Wassarman, 1980). Similarly in the pig sperm receptor activity has been attributed to a glycoprotein of $M_r$ 58000 (Sacco et al. 1984). However, in our experiments recognition of sperm polypeptides was not restricted to a particular zona component. Analysis of $^{125}$I-zona probe that bound to sperm polypeptides clearly showed that all zona moieties were involved. Although at present we are unable to elaborate on this common binding property of zona glycoproteins the results do suggest that a significant structural homology between them exists. The zona pellucida of the oocyte is heavily glycosylated (Dunbar, Wardrip & Hedrick, 1980) and both oviduct glycoproteins react strongly with PAS (Brown & Cheng, 1986) so it is possible that the sperm polypeptide $M_r$ 53000 is recognizing common sugar sequences. This would not be surprising for evidence is accruing that carbohydrates are critically involved in the initial events of gamete fusion (Lopez, Bayna, Litoff, Shaper, Shaper & Shur, 1985; Florman & Wassarman, 1985). Further experiments are in progress to investigate the nature of the binding we have observed and its possible significance in relation to sperm–egg recognition processes.

We are grateful to Dr S. J. Gaunt for helpful discussions, Mr K. I. von Gloß for technical assistance and Mr K. T. Elsome for supplying pig eggs.

References


