Binding of zona pellucida proteins to a boar sperm polypeptide of M_r 53000 and identification of zona moieties involved

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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 53000 on blots of capacitated and noncapacitated sperm and weakly to polypeptides of M_r 67000, 38000 and 18000. On blots of seminal plasma the ^{125}I-

labelled probes bound to two polypeptides of M_r 65000 and 19–24000. 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 53000 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 oestrus 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 oestrus oviduct. This may be significant in terms of identification of recognition molecules because it has recently been shown that the oestrus 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 thoroughly 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 at 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-zonae 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 (Laemmli, 1970). Separated proteins were visualized 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-zonae 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 preflashed 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 zonae to the nitrocellulose. This problem could be circumvented by centrifuging the melted

zonae at 10 000 g for 5 min before probing the blot. Prelimi-
nary experiments showed that despite incomplete dissol-
ution 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 ^{125}I-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 phenom-
enon 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 ^{125}I-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 polypep-
tides, 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 zonae pellucidae

In preliminary experiments we investigated if the
solubilization process had any effect on the macro-
molecular constituents of the investment. A com-
parison was made of the profile of ^{125}I-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 ^{125}I-zonae pellucidae
glycoproteins*

The range of polypeptides present in sodium deoxy-
cholate 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 M_r between 14 000 to
100 000 whereas seminal plasma contained fewer

proteins but with a major group between M_r 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 ^{125}I-zonae from fol-
licular oocytes (^{125}I-FZP) consistent binding was
observed to a sperm component of M_r 53 000
(Fig. 1c). Weaker affinity binding was found over a
polypeptide M_r 18 000 while two others, M_r 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 ^{125}I-FZP over an
area ranging from M_r 19 000 to 24 000 and weak
binding towards a polypeptide of M_r 65 000.

Probing Western blots of sperm proteins with
zonae from ovulated eggs (^{125}I-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 M_r 53 000.
We detected no difference in binding either qualitat-
ively or quantitatively between capacitated and non-
capacitated sperm (tracks e and f).

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

Washing blots probed with either ^{125}I-FZP or [125]I-
OZP in PBS containing 0·5 % w/v NP-40 reduced
considerably background labelling as well as the
amount of radiolabel associated with the M_r 67 000,
38 000 and 18 000 polypeptides on spermatozoa. How-
ever, the amount of label associated with the sperm
M_r 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 M_r 18 000, 19 000–24 000, 53 000 and
65 000 were cut out and the bound ^{125}I-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 ^{125}I-zona probe was used. In all cases
where ^{125}I-FZP was employed a single polypeptide of
M_r 79 000 was resolved with a broader area of label
spread directly below it, whereas when ^{125}I-OZP was
used each extract separated into three polypeptides of
M_r 250 000, 90 000 and 79 000. Again there was a
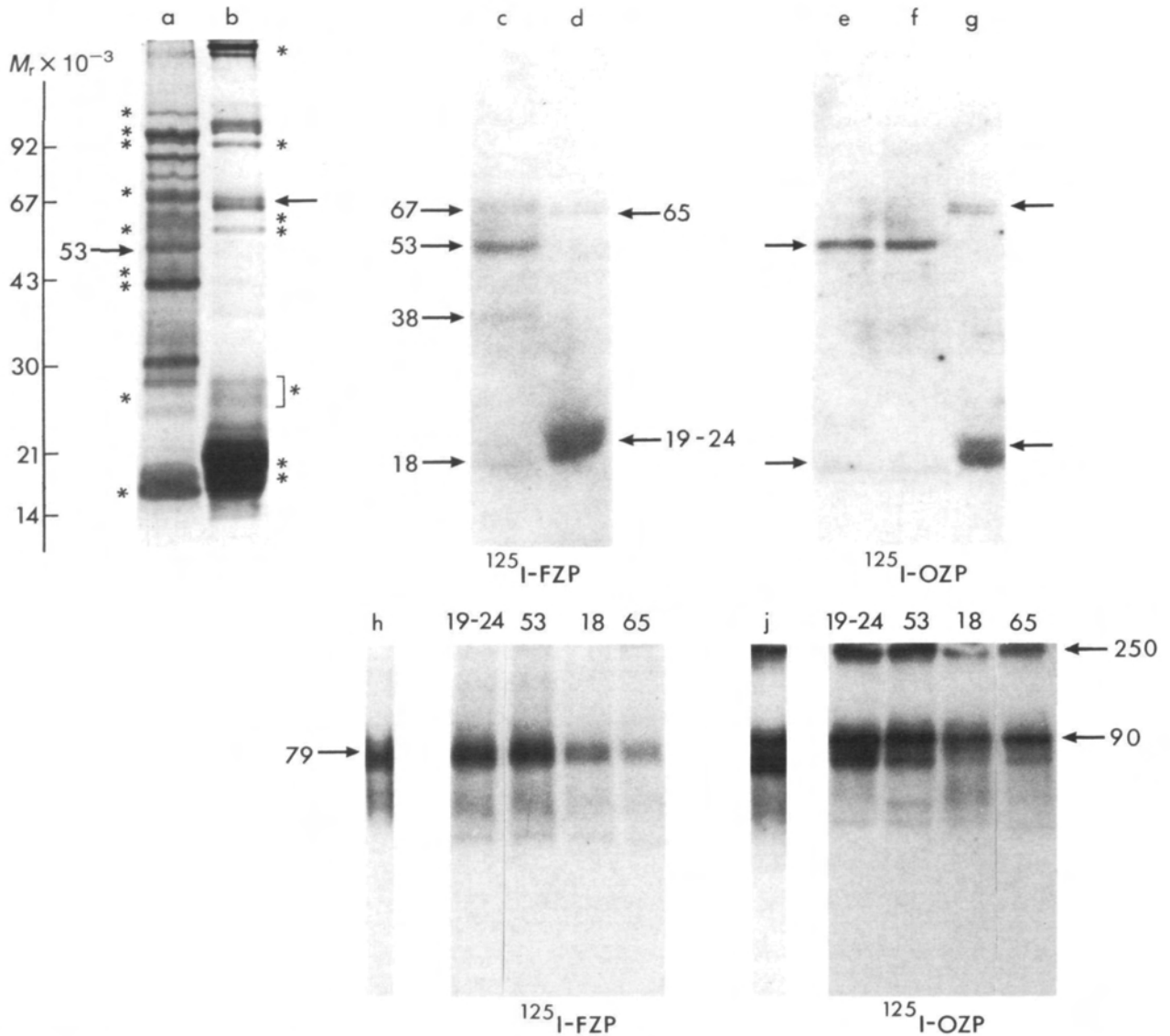broader area of label below with a lower molecular

Fig. 1. SDS–PAGE of sodium deoxycholate extracts of ejaculated sperm (a) and of seminal plasma (b) stained with Coomassie brilliant blue R250; tracks c and d are similar separations transferred to nitrocellulose and probed with ^{125}I-FZP and f and g represent the same but probed with ^{125}I-OZP. For track e sperm were capacitated before extraction. The electrophoretograms in the lower half of the figure are nonreduced SDS–PAGE separations of zona components extracted from areas of the blot as indicated by their M_r; tracks h and j represent an individual zona from an oocyte and from an ovulated egg, respectively.

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 ^{125}I-zona pellucida by polypeptides of immature and mature spermatozoa

The M_r 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 ^{125}I-OZP glycoproteins

after blotting (Fig. 2). It was noticeable that more ^{125}I-OZP bound to the M_r 53 000 polypeptide from cauda epididymidal and ejaculated sperm than from testicular and proximal caput epididymidal sperm. There was also weak recognition of a polypeptide of approximate M_r 67–68 000 in testicular and proximal caput sperm extracts and of an approximate M_r 38 000 polypeptide in epididymal and ejaculated, but not testicular, sperm extracts.

Tissue specificity of the M_r 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}I-OZP there was no binding of label to an area of the blot corresponding to M_r 53 000 (Fig. 3).
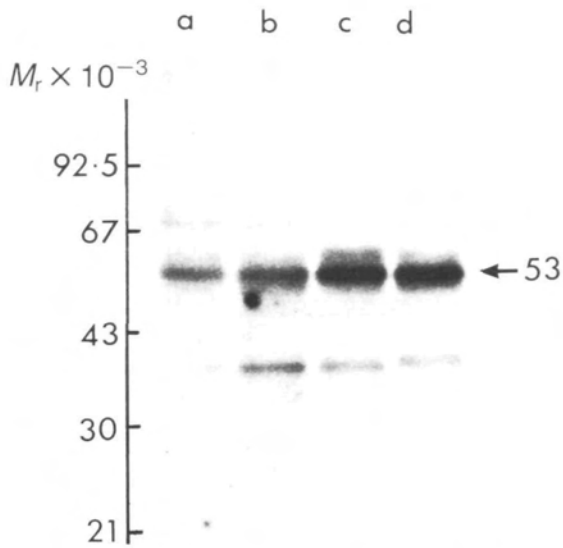

Fig. 2. SDS–PAGE of sodium deoxycholate extracts of testicular (a), caput (b) and cauda (c) epididymal and ejaculated (d) spermatozoa blotted and probed with ^{125}I-OZP.
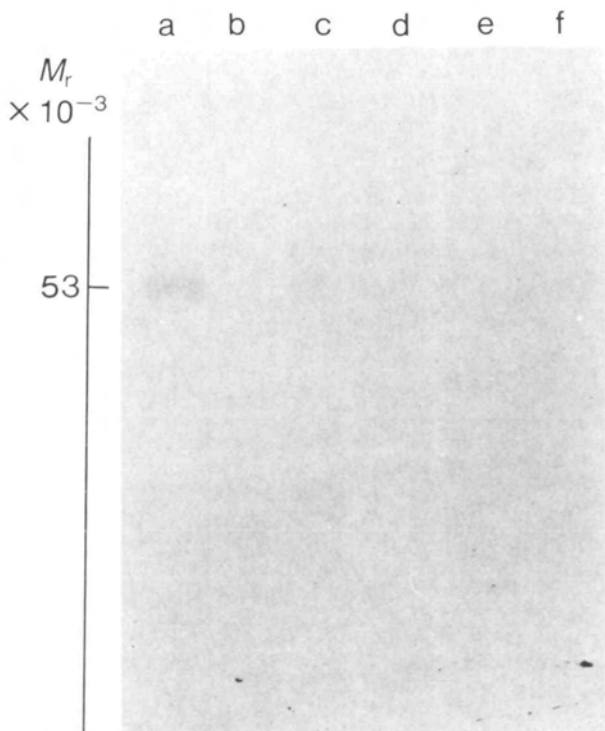

Fig. 3. SDS–PAGE of sodium deoxycholate extracts of porcine spermatozoa (a), liver (b), kidney (c), spleen (d), heart (e) and lung (f) blotted and probed with ^{125}I-OZP. Each track received approximately 150 μg protein.

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 53 000 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 53 000 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 67 000, 38 000 and 18 000 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 16 000 and 18 000 with less binding to other species of M_r between 63 000 and 88 000, and 45 000 and 53 000. Our results differ mainly in that the polypeptide M_r 53 000 is consistently the major species recognized in our experiments although we did detect a small amount of binding at M_r 18 000. 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}I-iodogen) or that their proteins were prepared by extracting spermatozoa directly with SDS. It is possible that the polypeptide M_r 67 000 represents one of the minor bands occasionally seen by O'Rand *et al.* and it is interesting that we also found this to be a spasmodic and in our case low-affinity recognition. Using the same approach in the hamster, Sullivan & Bleau (1985) identified a sperm plasma membrane polypeptide of M_r 26 400. This is thought to be a glycoprotein because of its ability to bind ^{125}I-concanavalin A. However, we were unable to detect carbohydrate on the M_r 53 000 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}I-FZP and ^{125}I-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 65 000. Peterson, Russell & Hunt (1984) have

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 16–19 000 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 18 000 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 85 000 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 35 000 to 100 000 showed affinity for the zona.

The mouse zona pellucida consists of three major glycoproteins and one of these, M_r 83 000 (ZP3), has sperm-binding properties (Bleil & Wassarman, 1980). Similarly in the pig sperm receptor activity has been attributed to a glycoprotein of M_r 58 000 (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 53 000 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.

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