Evidence that Aggregation of Mouse Sperm Receptors by ZP3 Triggers the Acrosome Reaction

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Abstract. In the mouse, considerable evidence indicates that initial sperm binding to the zona pellucida (ZP) is mediated by ZP3. In addition, this same glycoprotein is also responsible for inducing the acrosome reaction (AR). Whereas the O-linked oligosaccharides of ZP3 appear to mediate sperm-ZP binding, the portion of ZP3 bearing AR activity has not been defined. To try to understand the bifunctional role of ZP3 (binding and AR inducing activities), we have examined the hypothesis that ZP3 aggregates sperm receptor molecules. By analogy with findings in a variety of other extracellular signal transducing systems, including receptors for growth factors and insulin, this aggregation event could initiate the cascade resulting in the AR. To test this hypothesis, we have generated monospecific polyclonal antibodies against ZP2 and against ZP3, and examined the effects of these probes on capacitated sperm incubated in the absence or presence of various ZP protein preparations. For some experiments, we have used proteolytic fragments of ZP3, a preparation known to retain specific binding, but not AR-inducing, activity. We show here that capacitated mouse sperm, incubated with ZP glycopeptides, displayed ARs when incubated subsequently with anti-ZP3 IgG; ARs did not occur when parallel sperm samples were incubated with anti-ZP2 IgG or with anti-ZP3 Fab fragments. When capacitated sperm were treated successively, with (a) ZP3 glycopeptides, (b) anti-ZP3 Fab fragments, and (c) goat anti-rabbit IgG, ARs occurred in the majority of sperm. An alternative approach to examine this hypothesis used ZP proteins obtained from tubal eggs treated previously with bioactive phorbol diester (12-O-tetradecanoyl phorbol-13-acetate [TPA]). This preparation arrests capacitated sperm in an intermediate state of the AR. We demonstrate here that these sperm can be induced to undergo a complete AR by subsequent treatment with anti-ZP3 IgG. Together, these findings are consistent with the hypothesis under examination, and suggest that the aggregation of sperm molecules recognized by ZP3 glycopeptides or by TPA-treated ZP is sufficient to trigger the events that occur during acrosomal exocytosis.

The mouse egg's zona pellucida (ZP) is an extracellular matrix composed of three glycoproteins termed ZP1, ZP2, and ZP3. These glycoproteins can be distinguished readily on the basis of size, with Mr of 200 kD for ZP1, 120 kD for ZP2, and 83 kD for ZP3 when electrophoresed using nondisulfide reducing conditions (2). Considerable evidence suggests that ZP3 serves as specific ligand for sperm binding to ZP during mouse fertilization (2). Furthermore, ZP3 also stimulates an exocytotic process, known as the acrosome reaction (AR), that is required by sperm for ZP penetration and fertilization (28).

Inquiry into the nature of ZP3 has revealed that ligand activity depends little upon the polypeptide backbone of ZP3, but resides in its O-linked oligosaccharides. Pronase digestion of ZP3 results in small glycopeptides (1.5-6 kD) that bind to sperm and are as effective as undigested ZP3 in competitively inhibiting sperm binding to intact ZP (9, 10). However, these small ZP3 glycopeptides do not induce the AR, suggesting that the polypeptide chain or intact glycoprotein plays a role in the latter activity. Recently, Endo et al. (6, 7) demonstrated that it is possible to dissociate both ZP3 activities (ZP binding and AR activities) without affecting the molecular mass of ZP3. Treating zona-intact mouse eggs with bioactive phorbol ester (12-O-tetradecanoyl phorbol-13-acetate [TPA]) before isolation of the zona proteins generates a form of ZP3 that is modified subtly; sperm incubated with this protein can initiate the AR, but not complete it. Since the polypeptide chain is present but nonfunctional in this case, it was suggested that this portion of ZP3 is not sufficient for AR inducing activity.

Receptor aggregation in response to biological stimuli has been described in other systems as an important mechanism to initiate signal transduction across the membrane (4, 13, 17, 21). In this context the polypeptide backbone of ZP3 might act to aggregate the sperm membrane proteins rec-
Materials and Methods

Collection of Sperm

Mouse sperm were obtained from cauda epididymides of mature CD-1 mice (Charles River Breeding Laboratories, Wilmington, MA) in CM (Krebs Ringer-bicarbonate medium supplemented with pyruvate, lactate, glucose, and BSA; 22). All gamete incubations were conducted under silicone oil (dimethylsiloxane 20 cc; Contour Chemical Co., North Reading, MA). For capacitation, sperm (~10^6 sperm/ml) were incubated for 60 min in 5% CO_2 in air at 37°C.

Isolation of ZP and Preparation of TPA-ZP

ZP from ovarian follicles of 2- to- old CD-1 mice were isolated as described (18). ZP (20-30 #g) were solubilized in CM containing no BSA and 0.4% polyvinylpyrrolidone by incubation at 60°C for 60 min. Treatment of tubal eggs with the phorbol ester, TPA, and subsequent acid solubilization were accomplished as described by Endo et al. (7).

ZP Glycopeptides Preparation

A preparation of ovarian ZP from ~35 mice was washed twice by centrifugation (12,000 g, 5 min) with 20 mM Tris/126 mM NaCl/1.7 mM CaC_2 buffer, pH 7.4, resuspended in 100 #l of the same buffer and solubilized by heating. Solubilized ZPs were incubated with insoluble pronase (pronase conjugated with carboxy methyl cellulose) following the procedure described by Florman et al. (10), except that the digestion was conducted for 12 h with readdition of fresh enzyme after 6 h. The digestion was assessed by SDS-PAGE (16) using a 15% polyacrylamide-bisacrylamide gel, 10 #l of the ZP protein solution (~1 #g of protein) was analyzed before and after protease digestion.

The ligand activity of the ZP glycopeptides was assayed using the binding competition assay described by Bleil and Wassarman (2). Four different dilutions of each glycopeptide preparation were tested and compared to the bioactivity of known concentrations of solubilized undigested whole ZP. The concentration of the glycopeptide preparations, in whole ZP equivalents/μl, was calibrated based upon biological activity of whole ZP.

The reactivity of the glycopeptides with anti-ZP pAbs was probed by dot blot (12) (Bio Dot microtitration apparatus, Bio-Rad Laboratories, Richmond, CA) using serial antibody dilutions. The antibodies were digested by SDS-PAGE (16) using a 15% polyacrylamide-bisacrylamide gel, 10 #l of the ZP protein solution (~1 #g of protein) was analyzed before and after protease digestion.

Anti-ZP Protein Polyclonal Antibodies

Polyclonal antibodies against isolated ZP proteins were raised in adult male New Zealand white rabbits. ZP, isolated from mouse ovaries, was boiled in sample buffer (62.5 mM Tris pH 6.8, 2% SDS, 10% glycerol) and the proteins were separated by electrophoresis under nonreducing conditions on a 7.5% polyacrylamide-bisacrylamide gel. After electrophoretic transfer of the proteins to nitrocellulose (26), ZP2 and ZP3 were identified according to their M_r. The area of the nitrocellulose sheet containing each protein was used to immunize rabbits as described by Knudsen (14). The animals received a total of four immunizations: day 1, ~1 μg in complete Freund's adjuvant; days 28, 56, and 100, ~1 μg, ~1 μg, and ~3 μg, respectively, all in incomplete Freund's adjuvant. All injections were administered subcutaneously. 15 d after each injection, sera were titrated by ELISA (20); bound Ig was detected with horseradish peroxidase-conjugated goat anti-rabbit IgG (Kierkegaard & Perry, Gaithersburg, MD) using 4-chloro-l-naphthol as substrate. Sera were also tested by immunoblot (Western blot) on whole ZP using 125I-labeled goat anti-rabbit IgG as the second antibody.

For purification, rabbit Ig was precipitated with 50% ammonium sulfate, dialyzed against PBS, and affinity-purified on protein-A Sepharose (11). Purified antibodies were titrated by dot blot, in which solubilized whole ZP (100 ng/well) was blotted onto nitrocellulose, followed by serial antibody dilutions. 125I-labeled goat anti-rabbit IgG, as described for dot blots with ZP glycopeptides, was used as the second antibody.

Preparation of Fab Fragments

Purified anti-ZP pAbs were digested using insoluble papain following the technique described by Goding (11). The IgG fraction (1 mg) was digested for 3 h at 37°C; the enzyme was removed by centrifugation. Digested material was applied to a protein-A Sepharose column to separate Fab fragments from Fc and undigested IgG molecules. Digestion and purification were followed by SDS-PAGE on a 10% gel, and the activity of the fragments was tested by Western blot on whole ZP. The protein concentration was determined by protein assay (Bio-Rad Laboratories) in 96-well microtiter plates; the results were quantified on an Enzyme Immuno Assay Reader (model 2250; Bio-Rad Laboratories).

Effect of Anti-ZP3 pAbs on the Acrosome Reaction-inducing Activity of ZP

Capacitated sperm (final concentration, 5 × 10^4 sperm/ml) were incubated in CM with solubilized whole ZP, TPA-ZP, or glycopeptides (each at 4 ZP equivalents/μl) for 30 min at 37°C in 5% CO_2/95% air. Separate samples of ZP glycopeptide-preincubated sperm received anti-ZP3 IgG, anti-ZP3 Fab or anti-ZP3 IgG for a second incubation period of 30 min using the same conditions. In other aliquots, sperm were incubated, sequentially, with ZP glycopeptide, followed by anti-ZP3 Fab, and finally with goat anti-rabbit IgG using the same conditions. Parallel experiments were conducted by replacing the ZP glycopeptide incubation for a similar incubation with TPA-ZP (4 ZP/μl) followed by further incubation with solubilized whole ZP, anti-ZP3 IgG, or anti-ZP2 IgG.

Controls for this entire series of experiments included sperm incubation with (a) anti-ZP protein pAbs in the absence of ZP preincubation, (b) glycopeptide followed by goat anti-rabbit IgG in the absence of anti-ZP3 Fab fragments, and (c) either ZP glycopeptides or TPA-ZP followed by preimmune rabbit sera. In all of these cases, no effect on acrosomal status was observed.

After the indicated incubations, acrosomal status was monitored by the CTC fluorescence assay that describes three different patterns: (a) intact sperm (B pattern); (b) sperm in an early intermediate state of AR (S pattern); and (c) acrosome reacted sperm (AR pattern) (22, 27). Control sperm, incubated in CM alone throughout the time course of each experiment, were examined using the chlortetracycline (CTC) assay to quantify the occurrence of spontaneous ARs; the proportions of B, S, and AR sperm did not differ significantly from beginning to end of each experiment. Repli cate experiments were conducted for each set of incubation conditions; at least 100 sperm were scored for each condition per experiment. The data presented represent the mean value obtained for all replicates of each experiment.

Unless indicated otherwise, all biochemical reagents were purchased from Sigma Chemical Co., St. Louis, MO.

Results

ZP Glycopeptides

Treatment of solubilized ZP with insoluble pronase gener-
ELISA TITRATION OF RABBIT SERA

Figure 1. ELISA assay to estimate the titer of preimmune and immune sera assayed on solubilized whole ZP. Whole ZP (100 ng/ml) was dried onto microtiter wells and probed with serial dilutions of preimmune and immune sera. Bound Ig was detected using horse-radish peroxidase-conjugated goat anti-rabbit IgG with 4-chloro-1-naphthol as substrate. Reactivity was followed by measuring OD at 405 nm. The titer, defined as 50% of maximal reactivity and shown by the broken line, was determined to be $1:2^{10}$ for anti-ZP2 serum and $1:2^{11}$ for anti-ZP3 serum.

Characterization of Anti-ZP-Antibodies

ELISA titration of rabbit sera collected after the fourth immunization indicated a titer of $1:2^{10}$ (or 1:1000) for anti-ZP2 pAbs and $1:2^{11}$ (or 1:2000) for anti-ZP3 pAbs, while the corresponding preimmune sera displayed low level, nonspecific responses (Fig. 1). By immunoblot analysis on electrophoretically separated whole ZP proteins, the antisera reacted specifically with the expected ZP protein (Fig. 2, lanes A and B). Digestion of anti-ZP3 IgG with papain and chromatographic separation on protein A-Sepharose yielded Fab fragments alone, uncontaminated by IgG, as judged by SDS-PAGE. When these Fab fragments were used to probe a nitrocellulose blot containing the separated ZP proteins, specific reactivity with ZP3 alone was observed (Fig. 2, lane C).

Dot blot analysis of the purified IgG fractions, probed on solubilized whole ZP similar to the method described above to estimate the titer of the rabbit antisera, was used to determine the IgG concentration that should be employed to examine specific physiological effects on sperm function. Anti-ZP2 IgG (dilution 1:2; i.e., 2 µg/ml) and anti-ZP3 IgG (dilution 1:2; i.e., 10 µg/ml) resulted in specific reactivity when compared with the preimmune IgG from the same animals (Fig. 3). These IgG concentrations were, therefore, chosen to initiate examination of effects on AR induction in capacitated sperm.

Finally, to ensure that the pAbs recognized the ZP glycopeptides after pronase digestion as well as they recognized undigested ZP, reactivity between the anti-ZP pAbs and ZP glycopeptides was tested in a dot blot assay. Both anti-ZP2 IgG and anti-ZP3 IgG bound to pronase-digested ZP glycopeptides specifically and to the same extent as that observed with the whole undigested ZP (Fig. 4).

Induction of AR

The CTC assay was used to monitor the sperm’s acrosomal status as a function of ZP and anti-ZP antibody exposure. At the start of the experiment (Fig. 5 A, T = 0), nearly 80% of capacitated sperm displayed the B pattern, indicating that they have intact acrosomes. Treatment of such a sperm population with ZP glycopeptide for 30 min (Fig. 5, A and B, Glycop) did not alter acrosomal status, despite the ability of the glycopeptide preparation to inhibit sperm-ZP binding competitively (see above). In contrast, when capacitated sperm were exposed to solubilized whole ZP (Fig. 5 A, WhZP), the majority of the population was observed in the...
autoradiographic analysis of the reactivity of anti-ZP2 IgG and anti-ZP3 IgG with whole ZP. Nitrocellulose wells were incubated with solubilized whole ZP (100 ng/well), followed by serially diluted IgG from preimmune (p-αZP2, p-αZP3) or immune (α-ZP2, α-ZP3) sera. After washing, the nitrocellulose sheet was probed with 125I-labeled goat anti-rabbit IgG. Starting IgG concentrations were adjusted to 1 mg/ml for p-ZP2 IgG and ZP2 IgG, and to 2.5 mg/ml for p-ZP3 IgG and ZP3 IgG.

AR pattern, indicating that the AR is completed. Treatment of capacitated sperm with glycopeptide for 30 min, followed by anti-ZP3 IgG (Fig. 5, Glycop → α-ZP3 Ab), produced the same pattern of acrosomal status in the population as whole ZP, i.e., ~60% of the population displayed the AR pattern. This effect appeared specific for the anti-ZP3 IgG preparation, since treatment of glycopeptide-preincubated sperm with anti-ZP2 IgG (Fig. 5 A, Glycop → α-ZP2 Ab) did not lead to ARs. Similarly, incubation of sperm with anti-ZP antibodies in the absence of ZP glycopeptide did not result in ARs (data not shown).

Since the protein concentration of the anti-ZP2 pAb that was selected for use in these experiments (on the basis of dot blot titration) was lower than that of the anti-ZP3 pAb (2 μg/ml versus 10 μg/ml, respectively), the effect of anti-ZP2 IgG at a protein concentration equivalent to that used for anti-ZP3 pAb on promotion of ARs was examined. Regardless of concentration, anti-ZP2 pAbs were never observed to effect ARs.

According to the hypothesis under examination, anti-ZP3 pAbs can aggregate the sperm’s ZP receptors in glycopeptide-treated sperm. If this is true, a corollary is that ZP glycopeptide-treated sperm would not undergo ARs in large numbers in the presence of univalent anti-ZP3 Fab fragments. This possibility was tested by treating capacitated sperm first with ZP glycopeptides, followed 30 min later with anti-ZP3 Fab fragments. 70% of such sperm were acrosome-intact, indicated by B pattern of the CTC assay (Fig. 5 B, Glycop → FaboZP3) Moreover, if these cells were incubated subsequently with goat anti-rabbit IgG, ARs were observed in the majority of the population (Fig. 5 B, Glycop → FaboZP3 → Gerabbit Ig). In the absence of anti-ZP3 Fab fragments, the goat anti-rabbit IgG had no effect on acrosomal status (data not shown).

Further support for a specific effect of anti-ZP3 pAbs on acrosomal status was demonstrated by the dose dependency of the response (Fig. 6). Populations of ZP glycopeptide-treated sperm displayed increasing proportions of AR pattern sperm as a function of increasing concentration of anti-ZP3 IgG. It is interesting to note that, with submaximal concentrations of anti-ZP3 IgG, the proportion of the population found in the S pattern of the CTC assay was substantially larger than that observed without anti-ZP3 IgG treatment.

It has been shown previously that incubation of capacitated sperm with TPA-ZP leads to an early stage of the AR (S pattern of the CTC assay) (7) and that the AR may be completed if whole ZP is then added (18). We now report that the same result may also be obtained if, instead of whole ZP, anti-ZP3 IgG (10 μg/ml) was added after pretreatment with TPA-ZP (Fig. 7). Neither anti-ZP2 IgG (2 μg/ml; Fig. 7) nor preimmune anti-ZP3 IgG (data not shown) promoted the same effect. None of the rabbit IgG preparations (either immune or preimmune) contained endogenous AR-promoting activity.

Discussion
Receptor-mediated cellular responses may be triggered by receptor aggregation in a variety of systems (4, 13). In some cases, it has been reported that ligand fragments can bind to their receptors but cannot induce a response unless a cross-linking molecule is present (3, 23, 24). In this manuscript, we examined receptor-effector coupling as a consequence of receptor aggregation in a novel system, that of mammalian gamete interaction leading to fertilization. In the mouse, receptors in the sperm plasma membrane bind to the ZP, specifically to ZP3; ZP3 then stimulates the occurrence of the AR, an exocytotic event that permits sperm penetration of the zona matrix and fusion with the egg plasma membrane (28). We have tested the hypothesis that acrosomal exocytosis is initiated by aggregation of the sperm receptors recognized by the antibodies. The Journal of Cell Biology, Volume 108, 1989 2166
by ZP3; the results of this study are consistent with such an hypothesis.

Small glycopeptides of ZP3 can bind to the sperm surface but fail to induce the AR (10). In this paper, we demonstrated that it was possible to reconstitute this activity by binding the glycopeptides with pAbs raised against ZP3. The antibody by itself was unable to produce this effect. Furthermore, if, instead of the polyvalent anti-ZP3 pAb, the corresponding Fab fragment was used, no AR was induced. However, AR activity could again be reconstituted by addition of another reagent (goat anti-rabbit IgG) that cross-linked the Fab fragments. These observations lead to the suggestion that sperm receptor aggregation may play an important role in AR induction. A further test of this model will be possible when the identity of the sperm's receptor(s) for ZP3 are known. Polyclonal, but not univalent, antibodies against the receptors should promote the AR in the absence of ZP. In this connection it is interesting to note that a mAb that identifies a subset of sea urchin sperm membrane proteins is able, by itself, to induce ARs in that system (25).

Recently, Endo and colleagues (6, 7) found that exposure of zona-intact mouse eggs to TPA results in modified bioactivity of ZP3 without alteration of the glycoprotein's M, suggesting that the polypeptide portion of ZP3 is present. Treatment of capacitated mouse sperm with TPA-ZP arrests the cells at an early stage of the AR, visualized as the S pattern in the CTC assay. Such TPA-ZP-treated sperm can be induced to undergo ARs if they are incubated subsequently with either (a) whole solubilized ZP (18), or (b) anti-ZP3 pAbs (Fig. 7). In the case of TPA-modified ZP3, perhaps the patching ability of the protein is restricted, such that receptor aggregation occurs to a subthreshold extent despite normal ligand activity. The results obtained with TPA-ZP are reminiscent of those observed when different anti-ZP3 IgG concentrations were used; when the antibody concentration is decreased, a large proportion of sperm remained in S pattern, and did not complete the AR (Fig. 6). It will be interesting to determine whether related mechanisms are operating in these two cases to produce such similar sperm populations.

For the insulin receptor, it has been reported that receptor subunit aggregation can activate a tyrosine kinase (21), and it has been proposed that the main function of this activation is to induce a conformational change in the receptor that is transmitted to one or more regulatory proteins (8). A large number of cell surface receptors exert their actions through specific GTP-binding regulatory proteins (G-proteins), which couple the receptor to appropriate effector systems (19). Recent evidence suggests that G-proteins are present in mam-
malian sperm (1, 15), and may be involved in ZP-induced ARs in mouse sperm since inactivation of a 41-kD α-like subunit of a G-protein heterotrimer by pertussis toxin inhibits physiological ARs (5). The results presented here suggest that ZP3 mediated aggregation of receptors in the sperm's plasma membrane may represent the initial event in this G-protein–coupled cascade that leads to acrosomal exocytosis. Work is directed presently toward identifying the sperm's receptor(s) for ZP3 and determining whether other mechanisms found in model systems where receptor aggregation plays a key role in extracellular signal transmission also operate during gamete interaction.

We are grateful to D. Bunch and C. Carron for constructive criticism of the work and thoughtful review of the manuscript, and to Dr. K. Burridge for donation of 125I-labeled goat anti-rabbit IgG as well as helpful review of the manuscript. We would also like to thank Ms. A. Robinson for production of the mouse ZP preparations.

The work was funded by grants from the National Institutes of Health and the Andrew W. Mellon Foundation. L. Leyton acknowledges generous support from the Rotary Foundation of Rotary International.

Received for publication 6 December 1988 and in revised form 9 February 1989.

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