The Biologically Active Form of the Sea Urchin Egg Receptor for Sperm Is a Disulfide-bonded Homo-Multimer

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Abstract. Since many cell surface receptors exist in their active form as oligomeric complexes, we have investigated the subunit composition of the biologically active sperm receptor in egg plasma membranes from *Strongylocentrotus purpuratus*. Electrophoretic analysis of the receptor without prior reduction of disulfide bonds revealed that the surface receptor exists in the form of a disulfide-bonded multimer, estimated to be a tetramer. These findings are in excellent agreement with the fact that the NH_2-terminus of the extracellular domain of the sperm receptor is rich in cysteine residues. Studies with cross-linking agents of various length and hydrophobicity suggest that no other major protein is tightly associated with the receptor. Given the multimeric structure of the receptor, we investigated the effect of disulfide bond reduction on its biological activity. Because in quantitative bioassays fertilization was found to be inhibited by treatment of eggs with 5 mM dithiothreitol, we undertook more direct studies of the effect of reduction on properties of the receptor. First, we studied the effect of addition of isolated, pure receptor on fertilization. Whereas the non-reduced, native receptor complex inhibited fertilization in a dose-dependent manner, the reduced and alkylated receptor was inactive. Second, we tested the ability of the isolated receptor to mediate binding of acrosome-reacted sperm to polystyrene beads. Whereas beads coated with native receptor bound sperm, those containing reduced and alkylated receptor did not. Thus, these results demonstrate that the biologically active form of the sea urchin sperm receptor consists only of 350 kD subunits and that these must be linked as a multimer via disulfide bonds to produce a complex that is functional in sperm recognition and binding.

GAMETE interactions resulting in fertilization include the complex processes of cell recognition, membrane binding and fusion, and egg activation (Glabe et al., 1991; Myles, 1993; Wassarman, 1993). Sperm-egg contact results in immediate membrane depolarization (Jaffe, 1976) and triggers spatial and temporal Ca^{2+} waves (Miyazaki et al., 1993) that are mediated by both inositol triphosphate and ryanodine receptor channels (Galione et al., 1993). In sea urchins, the Ca^{2+}-induced release of Ca^{2+} is followed by exocytosis of cortical granules and the elevation of the fertilization envelope (Larabell and Chandler, 1991). The molecular characterization of echinoderm fertilization shows that these early developmental events originate from the successful interaction of complementary cell recognition molecules on the surface of the two gametes (Foltz and Lennarz, 1993; Lennarz, 1994). However, it is not yet known if sperm binding triggers egg activation via a receptor-mediated signal transduction event or by introduction of an activating factor into the egg cytoplasm. At present, the simplest model is that the sperm receptor on the egg surface, by interacting with the acrosomal sperm protein bindin (Vacquier and Moy, 1977), mediates the binding event. It remains to be determined if this binding directly or indirectly (via one or more additional gamete surface molecules) induces gamete membrane fusion and the subsequent egg activation.

In early studies a highly glycosylated receptor was postulated to exist on the sea urchin egg plasma membrane (Schmell et al., 1977; Rossignol et al., 1984; Ruiz-Bravo et al., 1986) and to participate in the binding of the acrosomal sperm protein bindin (Vacquier and Moy, 1977; Minor et al., 1989). More recently the isolation of a fragment of the receptor and subsequent molecular cloning and sequencing revealed that it was a transmembrane glycoprotein with an extracellular sperm-binding domain that exhibited sequence similarity to the heat shock protein 70 family (Foltz and Lennarz, 1990, 1992; Foltz et al., 1993). A recombinant protein representing the extracellular sperm-binding domain of the receptor was found to species-specifically bind to isolated bindin particles (Foltz et al., 1993), thus demonstrating that the receptor polypeptide backbone is directly involved in gamete adhesion. After the sequencing of the sperm receptor,

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purification of the 350-kD receptor from the crude egg surface complex was accomplished using lectin and ion exchange chromatography (Ohlendieck et al., 1993). The homogeneous receptor was found to consist of 70% carbohydrate. Lectin-binding studies, combined with compositional carbohydrate analysis, revealed that the oligosaccharide chains of the receptor are sulfated and that both N- and O-linked chains are present. Functional analysis showed that the sperm receptor retained its biological activity after purification, i.e., it bound species-specifically to acrosome-reacted sperm when adsorbed to polystyrene beads and it inhibited fertilization in a dose-dependent and species-specific manner (Ohlendieck et al., 1993).

The functional activity of many cell surface receptors depends on homodimerization or oligomerization (for review see Ullrich and Schlessinger, 1990). However, in the case of sea urchin fertilization, it is not known if the interaction between bindin on the acrosome-reacted sperm process and the sperm receptor directly results in the complex process of signal transduction and egg activation. Therefore, in analogy to studies on the oligomerization of other cell surface receptors, we investigated the subunit composition of the biologically active sperm receptor. Since the receptor exhibits a cysteine-rich domain at the extracellular NH_2 terminus (Foltz et al., 1993), we examined the possibility of formation of disulfide-bonds between receptor subunits using SDS-PAGE analysis under reducing and non-reducing conditions. In addition, to determine if other egg surface proteins interact with the 350-kD molecule, experiments were performed with a variety of cross-linking agents of various length and hydrophobicity using isolated egg surface ghost membranes.

The results of the present study revealed that the native sea urchin egg receptor for sperm exists as a homo-multimeric complex in the egg plasma membrane. No other major protein appears to be part of the egg surface membrane complex responsible for the recognition and binding of acrosome-reacted sperm. Most importantly, comparative fertilization bioassays using the isolated, native homo-multimer and the reduced receptor subunit showed that the ability of the isolated receptor to competitively inhibit fertilization depends on it being in oligomeric form. Furthermore, whereas acrosome-reacted sperm bind specifically to microspheres coated with the native, non-reduced receptor complex, they do not bind to beads coated with the reduced and alkylated receptor. Thus, effective adhesion to sperm cells requires the receptor to be a multimer, most likely a tetramer. These findings provide new insight into the structure of the receptor and also provide a molecular explanation for the inhibitory effect of DTT on fertilization reported over two decades ago (Epel et al., 1970).

Materials and Methods

Materials

Cross-linking agents were purchased from Pierce (Rockford, IL). Ultrapure dithiothreitol, iodoacetamide, β-octylglucoside, acrylamide/bisacrylamide protein gel mix, and affinity-purified peroxidase-conjugated goat anti-rabbit IgG were obtained from Boehringer-Mannheim Corp. (Indianapolis, IN). Activated affinity support Affi-Gel 15 and the micro protein assay reagents were purchased from Bio-Rad Labs. (Richmond, CA). The enhanced chemiluminescence detection kit was from Amersham Corp. (Arlington Heights, IL), nitrocellulose membranes from Schleicher and Schuell, Inc. (Keene, NH), and prestained molecular weight standards were purchased from Bethesda Research Laboratories (Gaithersburg, MD). Polybead polystyrene microspheres were from Polysciences, Inc. (Warrington, PA) and DEAE-Sephacel from Pharmacia (Uppsala, Sweden). Calcium ionophore A23187, N-ethyl-maleimide, Protein A agarose and protease inhibitors, as well as all other analytical grade chemicals were purchased from Sigma Chem. Co. (St. Louis, MO).

Fertilization Bioassays

To evaluate the effect of reducing agents on sea urchin fertilization, dejellied, and washed eggs from *Strongylocentrotus purpuratus* (Marinus, Inc., Long Beach, CA) were incubated for 5 min with increasing amounts of dithiothreitol (DTT)[1] in filtered artificial sea water (FASW; Instant Ocean, Aquarium Systems, Mentor, OH). Collection and maintenance of gametes, as well as quantitative bioassays were performed using standard methods (Schmell et al., 1977; Rossignol et al., 1984; Ruiz-Bravo and Lennarz, 1986). For control purposes, appropriately diluted stock solutions of fresh sperm were treated in FASW with reducing agents by preincubation for 5 min at pH 8 or by adding DTT directly to the jelly coat mixture used for sperm activation. Comparative bioassays were performed with the native, non-reduced receptor complex and the egg receptor, that had been reduced using 10 mM DTT, and then alkylated with 40 mM iodoacetamide and extensively dialyzed against FASW. After DTT treatment or incubation with receptor preparations, eggs were tested for their ability to be activated by assessing elevation of the fertilization envelope by addition of 30 μM calcium ionophore A23187 dissolved in dimethyl sulfoxide.

Isolation of Homogeneous Whole Egg Ghost Membranes

To isolate whole egg ghost membrane vesicles, dejellied, and extensively washed eggs were resuspended in ice-cold Ca^{2+}-free sea water (0.5 M NaCl, 10 mM KCl, 25 mM $NaHCO_3$, 25 mM EGTA, 63 mM NaOH, pH 8; supplemented with 1 mM of each of phenylmethanesulfonyl fluoride, aprotinin, soybean trypsin inhibitor, antipain, leupeptin, and benzamidine) and homogenized using a hand operated glass homogenizer and Teflon pestle (Kinsey, 1986). After tenfold dilution, the ghost membrane vesicles were centrifuged for 2 min at 1,000 *g* and subsequently twice washed by pelleting. The preparation of ghost vesicles was evaluated by light microscopy and photography was performed with a Nikon Diaphot photomicroscope using Kodak Tmax-100 film. Isolated ghost membrane vesicles were used immediately for cross-linking experiments.

Cross-linking of Sperm Receptor

Cross-linking of sperm receptor was performed with homobifunctional N-hydroxysuccinimide cross-linkers according to Lomant and Fairbanks (1976). Egg ghost membrane vesicles were treated at room temperature for 30 min at pH 8 using 100 μg/ml of the following cross-linking reagents: Dithiobis(succinimidylpropionate) (DSP), dissolved in dimethyl sulfoxide; as well as bis(sulfosuccinimidyl)suberate (BS^3); or dithiobis(sulfosuccinimidyl propionate) (DTSSP); or disulfosuccinimidyl tartarate (S-DST), all dissolved in aqueous buffer. The reaction was stopped by the addition of 50 μl of 1 M ammonium acetate/ml reaction mixture. Cross-linked ghost membrane vesicles were then solubilized for 30 min on ice using 2% (wt/vol) β-octylglucoside and insoluble material removed by centrifugation at 105,000 *g* for 30 min. The egg receptor was chromatographically purified from the supernatant as previously described (Ohlendieck et al., 1993). Cross-linking was monitored by silver staining of SDS-PAGE gradient gels.

Gel Electrophoresis and Silver Staining

To compare the electrophoretic mobility of the egg receptor under non-reducing and reducing, as well as under cross-linking conditions, the SDS-PAGE analysis had to be modified to detect very large molecular weight protein complexes. Accordingly, electrophoresis was carried out with 3–10% or 3–15% SDS gradient gels (Laemmli, 1970) using SE-600 vertical slab gel units from HOEFER Scientific Instruments (San Francisco, CA) at a

1. *Abbreviations used in this paper*: BS^3, bis(sulfosuccinimidyl)suberate; DSP, dithiobis(succinimidylpropionate); DTSSP, dithiobis(sulfosuccinimidyl propionate); DTT, dithiothreitol; S-DST, disulfosuccinimidyl tartarate.

constant voltage of 200–400 V with continuous cooling and running times between 12–16 h. Protein samples were reduced by the addition of 10 mM DTT and neighboring non-reduced protein samples were protected from reduction by the addition of 10 mM N-ethyl-maleimide. To evaluate cross-linked protein complexes after reduction, cross-linked samples were electrophoretically separated under non-reducing conditions, and then gel slices containing the sperm receptor complex were excised and incubated with β-mercaptoethanol containing SDS-PAGE sample buffer (Laemmli, 1970). The receptor recovered after such treatment was subsequently re-electrophoresed on a second SDS-PAGE gradient gel under reducing conditions. Besides commercially available high molecular weight markers (Bethesda Research Laboratories), we also employed nebulin (600–800 kD) and titin (2,000–3,000 kD) (Eilertsen and Keller, 1992) for molecular weight comparison. SDS extracts from chicken pectoralis muscle containing titin and nebulin were a generous gift from Dr. Thomas C. S. Keller III (Florida State University, Tallahassee, FL).

For highly sensitive visualization of protein bands by silver staining, SDS polyacrylamide gels were incubated for 6 h with several changes of 0.1% acetic acid and 50% methanol (Foltz and Lennarz, 1990). After fixation and a 30-min incubation in 60 μM DTT, the gel was stained for 60 min in 0.1% silver nitrate in 0.22 μm-filtered distilled water, and then washed several times in distilled water. For development, gels were rinsed for 10 s and then stained in 0.02% formaldehyde and 0.28 M sodium carbonate. Staining was terminated by the use of 2 M citrate and gels were stored in 0.1 M citrate, 5% (vol/vol) glycerol before photography.

Antibody to Egg Receptor and Immunoprecipitation

The sea urchin egg receptor was purified to homogeneity using WGA lectin chromatography and DEAE-Sephacel ion exchange chromatography as previously described (Ohlendieck et al., 1993). Purification was monitored by SDS-PAGE analysis and immunoblot analysis using an antibody to a fusion protein which presents the extracellular sperm binding domain of the receptor (Foltz et al., 1993). Protein concentration was determined by the Bio-Rad micro protein assay with bovine mucin as a glycoprotein standard. Electrophoretic transfer of SDS-PAGE separated proteins to nitrocellulose was performed according to Towbin et al. (1979). Immunodetection was carried out with peroxidase-conjugated secondary antibodies using the enhanced chemiluminescence detection method as described (Ohlendieck et al., 1993). Immunization of a rabbit with the purified egg receptor was carried out by the Pocono Rabbit Farm and Laboratories (Canadensis, PA) as previously described in detail (Ohlendieck et al., 1993). The IgG fraction was isolated by ammonium sulfate precipitation followed by DEAE ion exchange chromatography according to Harlow and Lane (1988), and the purity of the antibodies was determined by Coomassie stained SDS polyacrylamide gels.

For the immobilization of purified IgG, antibodies were bound to Affigel-15 affinity matrix and to Protein A agarose by standard protocols (Harlow and Lane, 1988) and as described by the manufacturer. Binding of IgG to the support matrix was monitored by SDS-PAGE analysis. For immunoprecipitation, crude surface membrane complex (Kinsey, 1986) was solubilized for 60 min on ice with 2% (wt/vol) β-octylglucoside in 0.5 M NaCl, 50 mM Tris-Cl, pH 7.4. After centrifugation at 105,000 *g* for 30 min at 4°C, the supernatant was diluted fourfold with respect to detergent and incubated overnight with immobilized IgG on a shaker. Since treatment with low and high pH did not elute protein, bound egg receptor was eluted from the extensively washed IgG matrixes by incubation with 4 M potassium iodide. Alternatively, bound sperm receptor could be eluted using 1 M sodium thiocyanate or 4 M guanidine. After extensive dialysis, the eluted protein fractions were analyzed by silver staining of SDS polyacrylamide gels.

Sperm-binding Assay to Receptor-coated Microspheres

To evaluate the effect of reduction on the ability of the receptor to bind to acrosome-reacted sperm, binding assays were performed with microspheres coated with receptor which had been pretreated with 10 mM DTT or incubated with 10 mM DTT followed by treatment with 40 mM iodoacetamide. Untreated receptor-coated microspheres, as well as beads with reduced and alkylated receptor, were washed several times by pelleting with FASW, and then examined by electron microscopy as previously described (Foltz et al., 1993). The specificity of sperm binding was confirmed by control experiments with receptor-coated beads that were preincubated with antibodies to the extracellular sperm-binding domain (Foltz et al., 1993; Ohlendieck et al., 1993).

Results

Antibodies to Sperm Receptor Immunoprecipitate 350-kD Glycoprotein

After the initial isolation and partial characterization of the intact sperm receptor (Ohlendieck et al., 1993), we undertook the preparation of an antiserum to this glycoprotein. Purification of the sperm receptor using lectin and ion exchange chromatography was monitored by silver staining and immunoblot analysis with an antibody to a recombinant protein representing a portion of the extracellular domain of the receptor (not shown). Peak fractions eluted from the DEAE-Sephacel column were combined, concentrated, and used for rabbit immunization. Immunoprecipitation experiments were performed with the IgG fraction isolated from this new antiserum to the intact receptor protein and compared to findings using a previously characterized antibody prepared using the recombinant protein as immunogen (Ohlendieck et al., 1993). The comparison in Fig. 1 reveals that both antibodies precipitate a protein of similar electrophoretic mobility with an apparent molecular mass of 350 kD under reducing conditions. Immunoprecipitation experiments performed with anti-receptor IgG immobilized on Protein A agarose or on Affigel 15 matrix yielded similar results: the immunoprecipitated protein analyzed by SDS-PAGE followed by silver staining exhibited a 350-kD band with a characteristic light brown color, typical of highly glycosylated proteins.

Production of Homogeneous Whole Egg Surface Complex Ghosts

An important prerequisite for studying the subunit composition of the sperm receptor by cross-linking experiments was the reproducible preparation of large egg surface vesicles without any proteolysis. Conventional mechanical homogenization of sea urchin eggs produces small vesicular structures (not shown). Since resealing of small vesicles and the

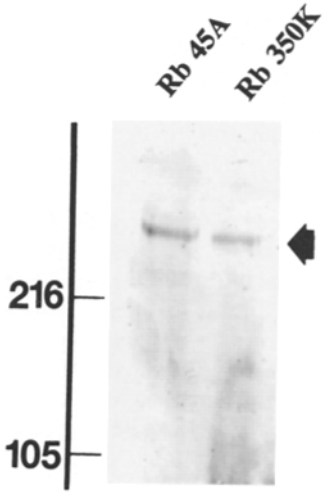

Figure 1. Immunoprecipitation of sperm receptor. After incubation of solubilized egg surface membranes with anti-receptor IgG (immobilized on Protein A agarose), eluted proteins were subjected to 3–10% gradient SDS-PAGE under reducing conditions, and then analyzed by silver staining. Both anti-receptor IgG fractions precipitated a protein band of apparent 350 kD (*arrow*). Rb-45A is directed against a recombinant protein representing the extracellular sperm-binding domain of the receptor and Rb-350 K is directed against the intact, homogenous receptor molecule. Molecular weight standards ($M_r \times 10^{-3}$) are indicated on the left.

production of heterogeneous inside-out and right-side-out vesicles can not be controlled in these preparations, we produced instead homogeneous whole ghost vesicles using a hand operated glass homogenizer with a Teflon pestle. After careful rupturing of dejellied eggs, the surface complex containing plasma membrane, vitelline layer, and cortical granules was isolated in the presence of protease inhibitors. In Fig. 2 *b* is shown a preparation of the resulting whole egg surface ghosts as compared to intact eggs (Fig. 2 *a*); Fig. 2 *c* indicates the homogeneity of a larger field of this preparation. Since these large ghosts are not sealed vesicles, cross-linking agents can potentially reach both the inside and outside of the egg surface complex. Initial attempts to directly analyze the cross-linked receptor in ghost preparations failed because the receptor concentration was too low to be detectable by immunological analysis. Therefore, after cross-linking the ghosts were solubilized by β-octylglucoside treatment, and the egg receptor purified chromatographically before SDS-PAGE and Western blot analysis as previously described (Ohlendieck et al., 1993).

Native Sperm Receptor Exists as a Disulfide-bonded Homo-Multimer

Comparative SDS-PAGE analysis using 3–10% gradient gels run at high voltage for a long duration revealed a large reduction in electrophoretic mobility of the sperm receptor from 350 kD under reducing conditions to ~1,500 kD under non-reducing conditions (Figs. 3 *a* and 4 *b*). Values for the molecular weight of the native receptor, nebulin and titin were corrected by a factor (0.81) to account for the difference observed in the estimated molecular weight of the reduced receptor using gradient SDS gels of differing polyacrylamide concentration (Fig. 4, *a* and *b*). This finding suggested that the sperm receptor exists in the egg surface complex as a disulfide-bonded oligomer most likely consisting of four identical 350 kD subunits. Interestingly, the deduced primary sequence of the receptor predicts a cluster of cysteine residues near the NH_2 terminus of the receptor (Foltz et al., 1993) which agrees with the presence of a disulfide-bonded oligomeric complex. After initial experiments to establish the optimum incubation conditions and cross-linker concentration, the oligomeric complex was treated with the homo-bifunctional N-hydroxysuccinimide agent DSP. Such treatment resulted in an additional reduction in electrophoretic mobility (Fig. 3 *a*). Incubation at different temperatures, with different buffers and for different time periods resulted in the same change of electrophoretic mobility (not shown). Molecular weight comparison with nebulin and titin from chicken pectoral muscle (not shown) indicated that upon cross-linking the molecular weight of the 1,500-kD receptor complex doubled to ~3,100 kD. This implies the formation of a cross-linked receptor octamer consisting of two identical homo-tetramers. However, the linear relationship between the electrophoretic mobility and the logarithm of the relative molecular weight is not highly reliable with very large protein complexes. Nevertheless, studies by Eilertsen and Keller (1992) showed the usefulness of titin and nebulin for the comparative determination of high molecular weights in SDS-PAGE analysis. Furthermore, the long electrophoresis running times using constant high voltage resulted in reproducible SDS-PAGE profiles; these findings were confirmed by the reduction of cross-linked complexes (see Fig. 5).

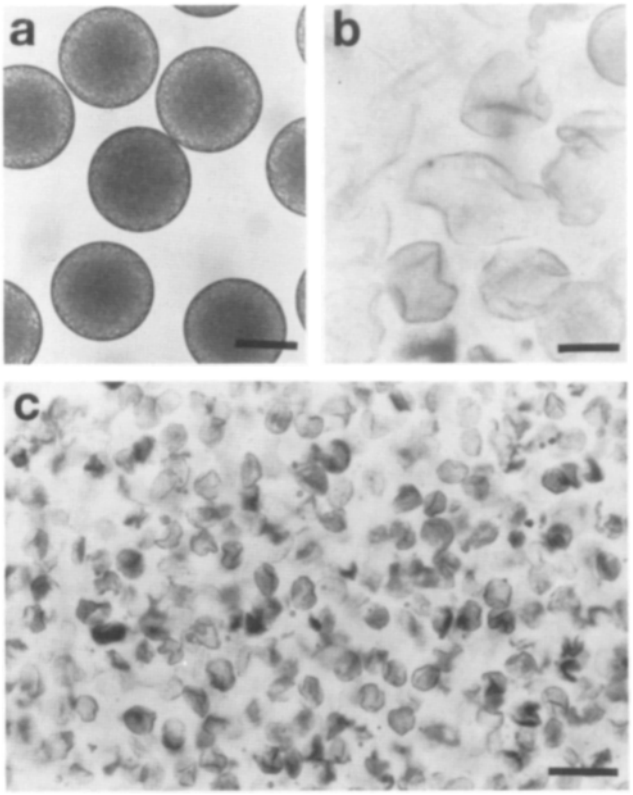

Figure 2. Egg surface membrane ghosts from *S. purpuratus*. Shown is a micrograph of intact dejellied sea urchin eggs (*a*) and ghosts prepared as described in the text (*b*) (Bar, 40 μm). A representative field of the ghosts used in the cross-linking studies is shown in *c* (Bar, 16 μm).

After experiments with DSP, other cross-linkers of different length and solubility were tested for their ability to cross-link egg surface membranes. Independent of their hydrophobicity or length (ranging from 6.4 to 12 × 10^{-10} m), the cross-linking agents DSP, DTSSP, BS^3, and S-DST produced the same increase in mass (Fig. 3 *b*). In the case of DTSSP, BS^3, and S-DST a second protein band of lower molecular weight was detected by silver-staining. This was found to be a cross-linked egg surface protein unrelated to the sperm receptor that sometimes copurified with receptor in lectin chromatography (Fig. 3 *b*). The presence of silver-stained proteins at the top of the gel using the cross-linkers DSP, DTSSP, and BS^3 is probably due to very high molecular weight complexes which can not be resolved by SDS-PAGE.

To investigate whether the receptor tetramer cross-links exclusively to itself or also to other cellular components, the receptor complex was cross-linked under non-reducing conditions, recovered from a gel, reduced, and then analyzed by SDS-PAGE. The results in Fig. 5 demonstrate that the reduced complex contained only the characteristic 350-kD protein band of the sperm receptor subunit; no additional proteins could be detected, although staining was performed with a sensitive silver staining procedure. To exclude the possibility that low molecular weight proteins were cross-

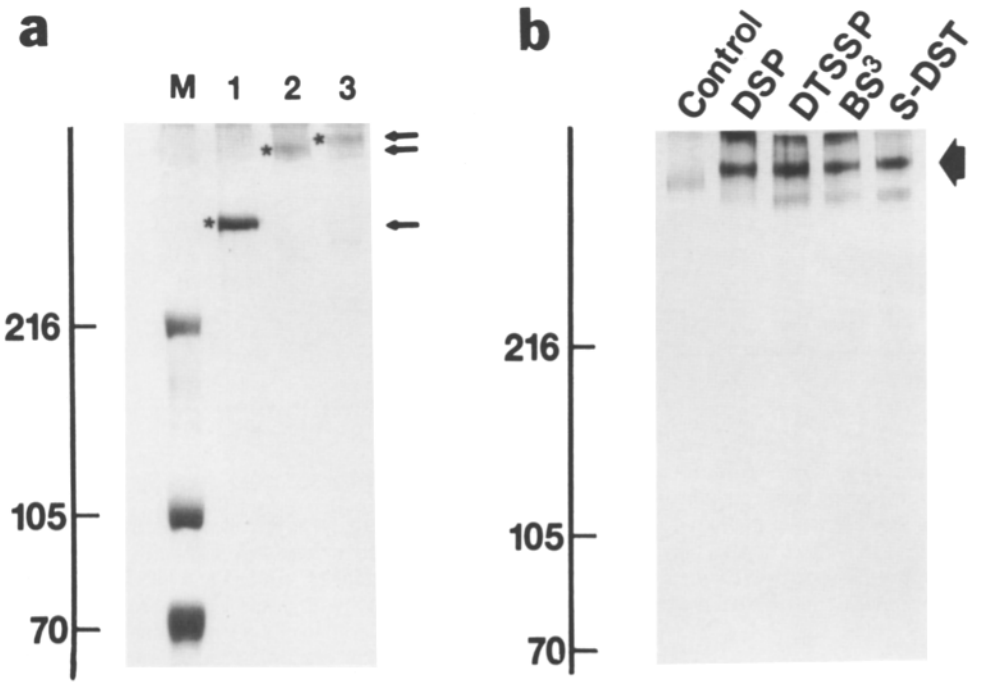

Figure 3. Cross-linking of sperm receptor oligomeric complex. Shown are silver-stained 3–10% gradient gels of the sperm receptor complex under reducing, non-reducing, and cross-linked conditions. (*a*) Reduced receptor, treated with 10 mM DTT (lane *1*); Non-reduced receptor (lane 2); and DSP cross-linked receptor (lane *3*), both treated with 10 mM N-ethyl-maleimide. The sperm receptor is indicated by arrows. (*b*) Non-reduced receptor, and receptor cross-linked using DSP, DTSSP, BS^3, and S-DST. A characteristic decrease in electrophoretic mobility of the cross-linked receptor as compared to the native receptor, is indicated by the arrow. Molecular weight standards ($M_r \times 10^{-3}$) (lane *M*) are indicated on the left.

linked to the receptor complex, a reducing gradient gel of higher polyacrylamide concentration was run, but again no subunits in addition to that of 350 kD were detected (not shown).

Inhibition of Fertilization by DTT Is Not Due to Removal of the Vitelline Layer

Since comparative SDS-PAGE analysis under reducing and non-reducing conditions (Fig. 3 *a*) demonstrated that the native sperm receptor existed as a disulfide-bonded homomultimer, experiments were performed to evaluate the effect of reduction on the biological activity of the receptor complex. Preincubation of washed and dejellied eggs with increasing amounts of DTT (1–20 mM) resulted in a dramatic reduction in their ability to be fertilized (Fig. 6). In contrast, a control experiment in which sperm was exposed for seconds to DTT, under the conditions of the bioassay, revealed that there was little or no effect of reducing agent on sperm (Fig. 6).

Even a 5-min preincubation of sperm cells with 5 mM DTT, which completely inhibits eggs, caused only ~50% inhibition of fertilization (not shown), probably due to reduced sperm mobility. This observation suggested that reduction of one or more proteins of the sea urchin egg surface membrane has an inhibitory effect on fertilization, in confirmation of earlier studies of Epel et al. (1970). These authors concluded that DTT treatment inhibited fertilization due to the removal or disruption of the vitelline layer. To test this hypothesis, we

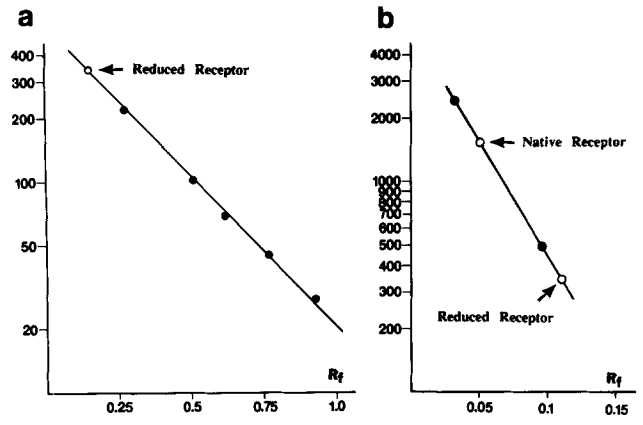

Figure 4. Estimation of molecular weight of native sperm receptor. Shown are plots of the logarithm of the relative molecular weight ($M_r \times 10^{-3}$) of standard proteins (●) versus their relative migration during gradient 3–15% (*a*) or 3–10% (*b*) SDS-PAGE. The relative molecular weight ($M_r \times 10^{-3}$) of the sperm receptor (○) under reducing or native, non-reducing conditions is indicated by arrows. Molecular markers used were myosin H-chain, phosphorylase B, BSA, ovalbumin, and carbonic anhydrase in *a* and titin and nebulin in *b*. Values for the molecular weight of the native receptor, nebulin, and titin were corrected by a factor (0.81) to account for the difference observed in the estimated molecular weight of the reduced receptor using the two different gradients.

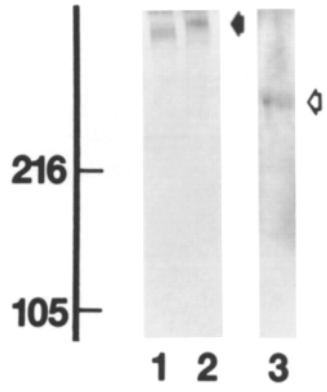

Figure 5. Analysis of cross-linked sperm receptor complex. Shown are silver-stained 3–10% gradient gels of non-reduced receptor (lane *1*), DSP cross-linked (lanes *2–3*) receptor before (lane *2*) and after reduction (lane *3*). SDS-PAGE analysis failed to reveal that the multimeric receptor is cross-linked to any protein other than itself. Molecular weight standards ($M_r \times 10^{-3}$) are indicated on the left.

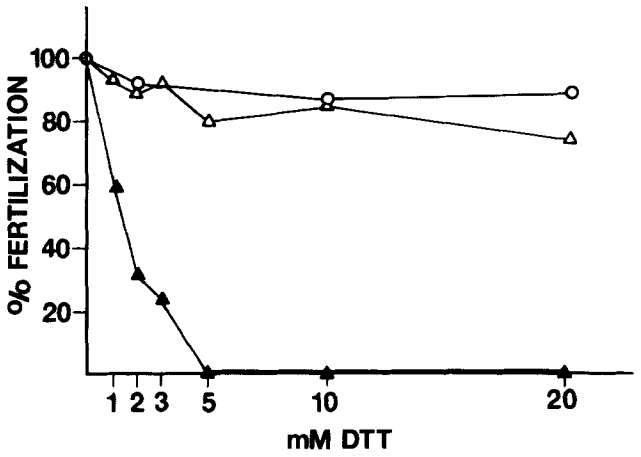

Figure 6. Dithiothreitol inhibits fertilization. Eggs from *S. purpuratus* were preincubated for 5 min at pH 8 with control buffer containing no DTT (○) or increasing amounts of DTT dissolved in filtered artificial sea water (▲). To exclude the possible effect of DTT on the sperm rather than the egg, sperm were treated with DTT that had been added to the jelly coat mixture (△) used for initiation of the acrosome reaction.

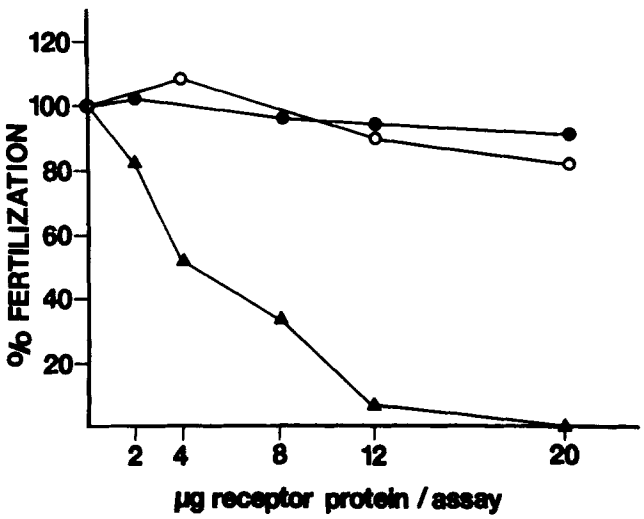

Figure 7. Exogenous sperm receptor inhibits fertilization. Dejellied and washed eggs from *S. purpuratus* were incubated with increasing amounts (2–20 µg protein) of reduced and alkylated receptor (○), native receptor (▲) or buffer control (●). After addition of acrosome-reacted sperm, elevation of the fertilization envelope was determined microscopically by examining 250 eggs and normalized to untreated controls.

treated eggs with the calcium ionophore A23187 at a final concentration of 30 µM after treatment with 5 mM DTT to inhibit fertilization (not shown). We found that over 70% of the DTT-treated eggs were able to elevate their fertilization envelope when the process was initiated by ionophore. In addition, we found that DTT treatment did not exhibit short term effects on the morphology. Membrane budding and general morphological dissociation of egg membranes appeared only after longer incubation times (>45 min) with DTT. Thus, DTT treatment does not remove components of the vitelline layer that are functional in fertilization envelope formation.

Inhibition of Fertilization Bioassays by Sperm Receptor Depends on Oligomeric Structure

To further investigate if the effect of DTT on fertilization was due to reduction of disulfide bonds of the sperm receptor or other more non-specific phenomena, we performed a competition bioassay of fertilization with the native and the reduced receptor (Fig. 7). The results in Fig. 7 demonstrate that the reduced and alkylated receptor does not inhibit fertilization, while the native, non-reduced receptor does so in a dose-dependent manner. After inhibition of fertilization by the native receptor complex, addition of the calcium ionophore A23187 resulted in elevation of the fertilization envelope in 88% of the inhibited eggs (not shown). This shows that exogenous receptor does not inhibit fertilization by interacting with eggs and somehow poisoning them. Rather, it strongly implies that exogenous receptor competes for sperm binding with the endogenous receptor on the egg surface.

Gamete Binding to Sperm Receptor Depends on Multimeric Structure

To determine if the observed inhibition of fertilization after reduction was actually due to a lack of sperm binding, rather than a possible secondary effect such as reduced signal transduction by the non-oligomeric receptor, we carried out previously described microsphere/sperm binding assays (Foltz et al., 1993; Ohlendieck et al., 1993) to test the adhesive properties of the reduced receptor. Beads with adsorbed homogeneous sperm receptor were treated with DTT or DTT followed by alkylation with iodoacetamide (Table I). Sperm binding to washed microspheres containing the reduced or reduced and alkylated receptor was compared to sperm binding to beads which had been adsorbed with the native, non-reduced receptor complex. Reduction of the receptor was found to result in a 70% decrease in sperm binding, while reduction followed by alkylation abolished all sperm binding (Table I). The additional reduction in sperm binding upon alkylation of reduced receptor suggests that some of the non-alkylated, reduced receptor might have been reoxidized during washing with filtered artificial sea water.

Discussion

The objective of the present study was to determine the subunit composition of the sea urchin egg receptor for sperm in order to understand the structural features essential for its biological activity. Before this compositional analysis, it was important to clearly establish that the sperm receptor previously identified by a cloning and sequencing strategy (Foltz et al., 1993), and the receptor molecule recently isolated by lectin and ion exchange chromatography (Ohlendieck et al., 1993) were in fact identical. The results of comparative immunoprecipitation experiments conclusively show that the 350-kD egg surface glycoprotein is the sea urchin sperm receptor. Based on this finding we proceeded to carry out an analysis of the subunit composition of the biologically active receptor. The results of several types of experiments revealed that recognition and binding of acrosome-reacted sperm to the receptor depends on its oligomeric structure. First, SDS-PAGE analysis under reducing and non-reducing conditions

Table I. Sperm Binding to Receptor-coated Beads

Receptor-coated beads	Sperm observed	Sperm bound to beads
	(*n*)	(*n*)
Native receptor (*non-reduced*)	189	102 (54%)
Native receptor + *anti-70kD*	103	3 (3%)
Reduced receptor (*10 mM DTT*)	151	23 (15%)
Reduced receptor + *anti-70 kD*	120	4 (3%)
Reduced and alkylated receptor (*10 mM DTT, 40 mM iodoacetamide*)	116	3 (3%)
Reduced and alkylated receptor + *anti-70 kD*	101	2 (2%)

Sperm binding assays were performed with microspheres coated with receptor which had been pretreated with 10 mM DTT or incubated with 10 mM DTT followed by treatment with 40 mM iodoacetamide. For control purposes, antibodies to the extracellular sperm-binding domain were employed to confirm the specificity of the binding assay.

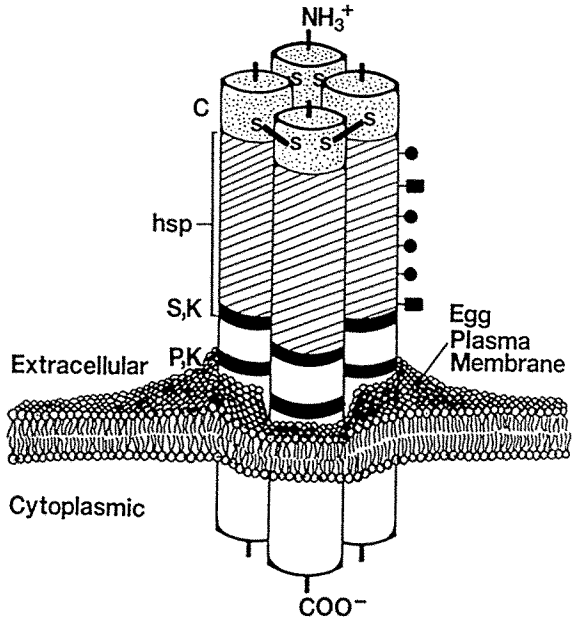

Figure 8. Model of the structure of the sea urchin egg receptor for sperm. Shown is the oligomeric complex consisting of identical 350 kD subunits as deduced from the results of the present study. It is estimated that the receptor is composed of four subunits. The subunits are proposed to be disulfide-bonded in the cysteine-rich, NH_2-terminal domain (Foltz et al., 1993). For simplicity, a partial overlap between the cysteine-rich domain and the hsp-like domain is not shown. Charged sequences (*S,K* and *P,K*), a domain with homology to the heat shock 70 family of proteins (*hsp*), as well as putative O-linked (●) and N-linked (■) glycosylation sites are denoted.

combined with cross-linking studies revealed that the native sperm receptor exists as a disulfide-bonded multimer, estimated to be a homo-tetramer. Second, since competition fertilization bioassays showed that the native receptor complex, but not the reduced receptor subunit inhibited fertilization, it seemed likely that initiation of the egg activation cascade depended on sperm binding to the native multimer. Third, direct proof for the effect on binding was obtained in experiments showing that sperm did not bind to polystyrene beads coated with reduced and alkylated receptor, but did bind to native receptor. Thus, the multimer configuration of the sperm receptor appears to be indispensable for sea urchin gamete adhesion. Further support for this idea is provided by the recent observation that glutathione inhibits bindin-mediated egg agglutination (Stears, R. L., and W. J. Lennarz, unpublished results), a finding that suggests that the reduced receptor on the egg cell surface does not functionally interact with bindin.

In Fig. 8 is shown a schematic diagram of the receptor that includes the earlier deductions about its primary sequence, as well as the new findings about the composition of its biologically active form. It is postulated that the cysteine rich domain is the site of cross-linking of the subunits. Efforts to detect protein subunits other than the 350-kD glycoprotein failed. Thus, it appears that the disulfide-linked multimer alone is sufficient for recognition and binding of acrosome-reacted sperm. Based on the yield of receptor isolated from egg surface complex it was previously calculated that each egg contains ~1.25×10^6 receptor subunits on its surface (Ohlendieck et al., 1993). Since an average of 1,744 sperm were experimentally determined to bind to an *S. purpuratus* egg (Vaquier and Payne, 1973), an average maximum valency of 170 receptor homo-tetramers per bound sperm can be calculated. Interestingly, even under theoretical conditions of saturating sperm binding (see Ohlendieck et al., 1993), up to 13 receptor complexes would be available to bind to each sperm cell. These values are in the range calculated as being sufficient to overcome the physical force exerted by a sperm with an actively moving flagellum (Lennarz, 1994; McLauglin, S., and W. J. Lennarz, unpublished results).

The finding of a homo-multimer structure for the sperm receptor is interesting in analogy to other known ligand/receptor interactions. It is well established that many cell surface receptors exist in their active form as oligomeric complexes (Ullrich and Schlessinger, 1990). For example, the endogenous functional insulin receptor exists as a disulfide-linked $\alpha_2\beta_2$ heterotetrameric complex (Treadway et al., 1990). In contrast, the heterodimeric $\alpha\beta$ insulin receptor displays markedly decreased binding affinity for insulin (Swanson and Pessin, 1989) and lacks insulin-stimulated kinase activity (Boni-Schnetzler et al., 1986). Thus, oligomerization via disulfide-bridges is an essential prerequisite both for insulin binding and signal transduction. The findings of the current study clearly parallel this behavior, since oligomerization is required for binding.

Since the functional binding of gametes is a crucial first step in fertilization (Foltz and Lennarz, 1993), the new findings on the subunit composition of the egg surface receptor responsible for sperm binding have potentially important implications for the steps that follow binding, namely, signal transduction and egg activation. Recent studies by Galione et al. (1993) demonstrate that spatial and temporal Ca^{2+} waves in activated sea urchin eggs are contributed by both inositol triphosphate and ryanodine receptor channels. Lee et al. (1993) found that in addition to inositol triphosphate, cyclic adenosine diphosphate-ribose participates in mobilizing Ca^{2+} in the sea urchin egg. Since blockage of either of these Ca^{2+} signaling systems alone was not sufficient to prevent sperm-induced Ca^{2+} transients (Lee et al., 1993),

redundant mechanisms of Ca^{2+} release appear to underlie Ca^{2+} waves in sea urchin fertilization. Interestingly, the measurement of the inositol triphosphate content in sea urchin sperm shows that the amount present in one sperm is high enough to trigger Ca^{2+}-release and egg activation when it is introduced into eggs (Iwasawa et al., 1990). The oligomeric sperm receptor complex might in some way participate in the introduction of such an activating factor into the egg cytosol. Alternatively, since sperm bound via the sperm receptor to the egg plasma membrane have an actively moving flagellum, local membrane disturbance could trigger egg activation via the opening of stretch-activated Ca^{2+}-channels (Guharay and Sachs, 1984). In such a case, the sperm receptor complex would not directly mediate egg activation but would function primarily as a species-specific anchor to bind sperm tightly to the egg surface. Interestingly, preliminary electrophysiological experiments indicate that sperm fusion to artificial black lipid bilayers is significantly increased by the addition of homogeneous sperm receptor (Ohlendieck, K., W. J. Lennarz, A. Lievano, and A. Darszon, unpublished results). This would suggest that the sperm receptor exhibits fusiogenic properties.

The cross-linking studies presented in this investigation do not exclude the possibility of the association of non-stoichiometric or transiently bound proteins to the receptor complex. Such low abundance receptor components might not have been detected by the cross-linking approach using SDS-PAGE analysis and silver staining for detection. Besides G-proteins and cytoskeletal components such as peripheral actin, possible candidates which might interact with the egg receptor are kinases, since a rapid change in phosphorylation on tyrosine residues is known to accompany sea urchin fertilization (Ciapa and Epel, 1991). Interestingly, a protein tyrosine kinase was recently described in the sea urchin cortex which might play a role in the egg activation process (Peaucellier et al., 1993). Furthermore, binding of sperm or soluble bindin to eggs has been shown to stimulate tyrosine phosphorylation of the sperm receptor (K. F. Foltz. 1993. *Mol. Biol. Cell.* 4:231*a*). However, the fact remains that only the 350-kD subunits have been detected and these subunits, in the form of a multimer, are biologically active. Native, non-reduced receptor preparations inhibit fertilization in a dose-dependent manner and mediate adhesion of sperm to coated microspheres. Clearly, these processes do not require other egg cell surface proteins. Whether or not other sperm proteins, in addition to bindin, are required for gamete binding and subsequent egg activation is an open question.

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