Isolation of bindin: The protein responsible for adhesion of sperm to sea urchin eggs

(fertilization/cell surface/intercellular adhesion/acrosome)

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Communicated by Daniel Mazia, March 17, 1977

ABSTRACT The insoluble granular material of the acrosome vesicle of sea urchin sperm has been isolated and shown to be a single 30,500 dalton protein for which the name "bindin" is proposed. The data presented are consistent with the hypothesis that bindin is the adhesive responsible for the attachment of sperm to the vitelline layer of the egg. Experimental results suggest that bindin may act by binding to carbohydrate receptors of vitelline layer glycoproteins. The speculation is made that sperm bindins may be the general mechanism by which animal sperm attach to eggs.

Sperm adhere to the surface of sea urchin eggs during fertilization. The adhesion is between the acrosome process of the sperm and the vitelline layer covering of the egg. Material derived from the sperm acrosome vesicle, which coats the acrosome process, is believed to be the adhesive substance responsible for gamete adhesion (1).

In sea urchins (2) and mammals (3, 4), sperm adhesion to the investing egg layer is a species-specific phenomenon that most probably involves recognition of specific macromolecules on gamete surfaces. Because of the large quantities of gametes available and the synchrony of gamete interaction (5), sea urchin sperm–egg adhesion is an advantageous model system for studying intercellular recognition mediated by cell surface macromolecules. Here we report the isolation of a protein from the sea urchin sperm acrosome vesicle that appears to be responsible for binding the sperm to the vitelline layer of the egg. The data presented support the hypothesis the sperm protein interacts with carbohydrate receptors on the vitelline layer. We propose that this protein is a member of a new class of sperm proteins that mediate gamete adhesion in many diverse species of the animal kingdom.

MATERIALS AND METHODS

Gametes. Gametes of Strongylocentrotus purpuratus were spawned into sea water from adult animals injected with 0.5 M KCl. Egg jelly coats were removed by exposure to pH 5 sea water for 2 min followed by extensive washing in sea water at pH 8.

Isolation of Insoluble Contents of Acrosome Vesicles. Sperm spawned into sea water was sedimented by centrifugation for 10 min in a Sorvall GSA 250-ml cup rotor at 2520 × g. Each 5 ml of packed sperm was gently resuspended in 100 ml of Ca-free sea water (6) containing 50 mM sodium acetate, 5 mM ethylene glycol bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid, 0.05% (wt/vol) sodium azide, and ovomucoid trypsin inhibitor (0.1 mg/ml). The pH was adjusted to 5.9. All chemicals were from Sigma Co. After complete resuspension of the cells, 100 ml of 75% (vol/vol) Ca-free sea water containing 2.5% (vol/vol) Triton X-100 was added, and the centrifuge bottle was tightly capped and shaken vigorously by hand for 1 min. This provided enough agitation to free the acrosome vesicle from the sperm. The mixture was centrifuged for 30 min in a swinging bucket rotor at 1000 × g, and the resultant supernatant was decanted into a clean beaker. Microscopic examination revealed contamination of the supernatant by whole sperm and sperm heads. The supernatant was then sieved through a glass-fiber column which allowed the passage of acrosome vesicles while trapping flagellae, whole sperm, and sperm heads. The column effluent was centrifuged for 50 min at 15,200 × g. The resulting pellets were washed with the isolation medium by repeated sedimentation and resuspension in Ca-free sea water at pH 5.8. The temperature was maintained at 0–4° throughout the procedure.

Glass fiber was prepared by blending spun glass (diameter, 8 μm) for 5 min at top speed in a Waring blender. The glass fibers, broken to an average length of 30 μm, were suspended in 2% Triton X-100 and poured into a flowing column until a 7-cm bed of fiber had settled; 2 cm of 2-mm glass beads was added to stabilize the top of the glass fiber bed. The column was washed with 4 liters of 2% Triton X-100 before use.

Other Procedures. For transmission electron microscopy, sperm and pellets of isolated acrosome vesicle material were fixed for 1 hr in 2% (vol/vol) glutaraldehyde in 80% Ca-free sea water and postfixed in 1% (wt/vol) osmium tetroxide also in Ca-free sea water. Dehydration was in ethanol followed with propylene oxide; embedding was in Epon. Sections were stained with uranyl acetate and lead citrate. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis (7) was performed on material dissolved in 2–10% sodium dodecyl sulfate/2% mercaptoethanol. For polyacrylamide gel electrophoresis in 6 M urea/5% (vol/vol) acetic acid (8), the acrosome vesicle material was dissolved in 8 M urea containing 0.1 M mercaptoethanol in 10% acetic acid. Glycopeptides of egg vitelline layers were prepared by digestion of a 10% (vol/vol) suspension of eggs with pancreatic trypsin ( Worthington) at 0.1 mg/ml. The supernatant was boiled for 30 min in a sealed tube to inactivate the trypsin. Glutaraldehyde-fixed eggs were prepared by 1-hr treatment with 1% glutaraldehyde in sea water followed by washing in sea water and storage in sea water containing 0.2% sodium azide. Sodium metaperiodate oxidation of egg surfaces was accomplished by treating fixed eggs for 3 hr with 50 mM NaIO₄ at pH 5.0 (9), followed by return to sea water.

For egg agglutination experiments, 2 ml of a 0.5% (vol/vol) egg suspension (2.91 × 10⁴ eggs) (5) was added to a 55-mm-diameter plastic culture plate (previously rinsed in 1% Photoflow) which was gently agitated on a rocker table. Ten to 100 μl of insoluble acrosome vesicle material, at concentrations ranging from 0.2–0.8 mg of protein per ml (10), was added. Agglutination of the eggs occurred within 2 min. Agglutination was qualitatively determined by visual inspection.
RESULTS

The intact acrosome vesicle

The membrane bound Golgi-produced acrosome vesicle (diameter, 0.3 μm) is located at the apex of the spermatozoan (Fig. 1). It is composed of a granular material and a proximally located morphologically distinct zone which we believe may be composed of membranes stacked upon each other (13).

Isolated acrosome vesicle material

Low-magnification electron micrographs of the final pellet from the isolation procedure showed it to be a relatively pure preparation of acrosome vesicles (Fig. 2 upper). Higher magnification (Fig. 2 lower) revealed that all membranous material of the intact acrosome vesicle was gone and only the insoluble granular material remained. Polyacrylamide gel electrophoresis of this material showed it to consist of a single component (Fig. 3 left) of apparent molecular weight 30,500. This component stained positively for protein (7) but was completely negative when stained for carbohydrate (14). Polyacrylamide gel electrophoresis of this material in 6 M urea/5% acetic acid also revealed one major component (Fig. 3 right). On a dry weight basis, with bovine serum albumin as a standard, this material dissolved in 1% sodium dodecyl sulfate was 105% protein. Because we believe that this insoluble protein is the substance which binds sperm to egg we refer to it by the name "bindin."

Involvement of bindin in sperm adhesion

Agglutination of Eggs by Bindin. If bindin is the adhesive that bonds the acrosome process to the egg vitelline layer, one would hypothesize bindin might agglutinate eggs. When we mixed particulate bindin (Fig. 2) in sea water with unfertilized, dejellied eggs, the eggs agglutinated within 2 min (Fig. 4 A and B). Because we believe the sperm–egg bond to be between bindin and the vitelline layer, we then isolated vitelline layers from unfertilized eggs and found that the addition of bindin caused their immediate agglutination (Fig. 4 C and D).

Vitelline Layer Glycopeptides Inhibit Bindin-Mediated Egg Agglutination. The vitelline layer contains glycoproteins (refs. 15 and 16; C. G. Glabe and V. D. Vacquier, unpublished data) that may be involved in sperm adhesion. Trypsinization of sea urchin eggs decreases their fertilizability, presumably by removing sperm receptor glycoproteins from the vitelline layer (15, 16). We reasoned that, if these receptors are glycoproteins, a glycopeptide digest of egg vitelline layers may inhibit the agglutination of eggs by bindin. We prepared a trypsin digest of unfertilized eggs, mixed a portion of it with isolated vesicles, and then added eggs. The eggs did not agglutinate (Fig. 4 E and F). Furthermore, these trypsinized eggs, when placed in fresh sea water, were not agglutinated by bindin (Fig. 4 G and H).

Sodium Metaperiodate Oxidation Renders Eggs Non-agglutinable by Bindin. When returned to sea water, aldehyde-fixed eggs bind sperm at the same rate and in approximately the same numbers (5) as do living eggs. Apparently the bindin-receptor system of the vitelline layer is not damaged by either formaldehyde or glutaraldehyde treatment. If the receptor for bindin is composed of sugar residues, sperm binding and bindin-mediated agglutination of eggs should be destroyed by metaperiodate oxidation of the surfaces of fixed eggs. We found the ability of glutaraldehyde-fixed eggs to bind sperm (5) decreased in proportion to the duration of metaperiodate exposure. In addition, metaperiodate-treated eggs did not agglutinate upon addition of bindin (Fig. 4 I and J). On the basis of these results, we tentatively conclude that bindin rec-
Fig. 3. (Left) Sodium dodecyl sulfate/12.5% polyacrylamide gel electrophoresis of 50 μg of acrosome vesicle pellet. The membraneless vesicles consist of a single protein of molecular weight 30,500 which we call “bindin.” Arrow points to tracking dye. (Right) On 7.5% polyacrylamide gel electrophoresis in 6 M urea/5% acetic acid of 5 μg of insoluble acrosome vesicle material, only one major band (bindin) appears.

Fig. 4. (A and B) Bindin agglutinates unfertilized eggs. (A) Egg suspension (2 ml) was gently agitated on a rocker table and 0.2 ml of a suspension of bindin (0.12 mg of protein) was added. The eggs agglutinated within 2 min. (B) Eggs without bindin are shown as a control. (C and D) Bindin agglutinates isolated vitelline layers. (C) Isolated vitelline layers (2 ml; 5 mg of protein) in sea water were rocked in a dish and 0.2 ml of a suspension of bindin (0.12 mg of protein) was added. The isolated vitelline layers agglutinated. (D) Vitelline layers without acrosome vesicles did not agglutinate. (E and F) Glycopeptides from vitelline layers inhibit the agglutination of eggs by bindin. (E) Bindin suspension (0.2 ml; 0.12 mg of protein) was mixed with 0.4 ml of glycopeptide preparation (1.1 mg of protein) for 5 min before addition of 2 ml of egg suspension. The glycopeptides blocked agglutination of eggs by bindin. (F) Agglutinated control eggs. (G and H) Trypsinized eggs are not agglutinated by bindin. Eggs were trypsinized and then washed by settling and resuspension in sea water containing soybean trypsin inhibitor at 0.1 mg/ml. Control eggs were also washed in soybean trypsin inhibitor. Bindin (0.2 ml; 0.12 mg of protein) was added to 2 ml of egg suspension. (G) Trypsinized eggs did not agglutinate. (H) Control eggs agglutinated. (I) Glutaraldehyde-fixed eggs are rendered unagglutinable by bindin after metaperiodate oxidation. (J) Glutaraldehyde-fixed eggs not treated with metaperiodate show strong agglutination in response to bindin.

FIG. 4. (Legend appears at bottom of the left-hand column.)
ognizes a carbohydrate receptor on the vitelline layer which may be the glycopeptide portion of a glycoprotein.

**DISCUSSION**

For biochemical studies of the molecular mechanism of intercellular recognition and adhesion, an ideal experimental system would exhibit the following characteristics: the cell populations should be homogeneous and be obtainable in large quantity; the intercellular interaction should be easy to quantitate and occur with great synchrony; it should be possible to reconstruct the specific interaction from isolated subcellular fractions; and lastly, the intercellular adhesion should be one for which the biological significance has been well established. The study of adhesion of sperm to sea urchin eggs possesses these attributes.

We propose, as have others (1, 11), that, after the acrosome reaction, molecules of bindin coat the surface of the acrosome process and act as an adhesive to bind the sperm to the vitelline layer of the egg. The data presented here are suggestive evidence that bindin may interact with a carbohydrate receptor on the vitelline layer. Working on this hypothesis we attempted to inhibit the agglutination of eggs by bindin by the addition of various mono- and disaccharides. Although we have not as yet found a saccharide that will inhibit bindin as an egg agglutinin, we have not excluded the possibility that bindin may have affinity for specific terminal saccharides of vitelline layer glycoproteins. We have recently isolated native vitelline layers from unfertilized eggs; when sperm are mixed with these isolated layers, the sperm attach only to the outer surfaces of the layers (unpublished data). This finding supports the idea of sperm receptor molecules existing on the outer vitelline surface.

As previously mentioned, sperm adhesion to sea urchin eggs is a species-specific phenomenon (2). We have found that bindin will only agglutinate eggs of its own species. Although the agglutination of eggs by bindin is species specific, the attachment of sperm to various types of surfaces can also be nonspecific. For example, sperm attach to glass microscope slides by the acrosome process. Bindin from two species of sperm will bond the cells together by their acrosome processes and the acrosome process can attach to the midpiece of another sperm cell (17).

Evidence for species-specific carbohydrate sperm receptors on the sea urchin vitelline layer has recently been provided by the work of Schmell et al. (16). These workers demonstrated the quantitative inhibition of fertilization by the addition of vitelline layer fragments prepared from eggs of two species of sea urchins. This inhibition is species specific and presumably results from competition for sperm between the sperm receptors on vitelline fragments and the sperm receptors on intact egg surfaces. The sperm receptor activity can be removed by trypsin without destruction, and the freed receptor binds to concanavalin A-Sepharose. Addition of concanavalin A to sperm/egg mixtures also inhibits fertilization (16). In S. purpuratus, we have found no effect of concanavalin A on bindin-mediated agglutination of eggs.

The hypothesis that the interaction of animal gametes is based on a protein–carbohydrate binding mechanism is not unreasonable, given the numerous examples of this mechanism in other intercellular adhesions. For example, the bacterium *Escherichia coli* adheres to human mucosal cells by the presence of a lectin on its surface that recognizes mannose receptors on the human cells (9). In yeast, a glycoprotein can be isolated from one mating type that agglutinates cells of the opposite mating type (18, 19). In chlamydomonads, mating between + and − gamete types depends on a cell surface glycoprotein. The surface reception of this glycoprotein can be blocked by treating the cells with protease, concanavalin A, or specific glycosidases (20). In higher plants the reception of pollen by the surface of the stigma may also depend on a protein–carbohydrate interaction (21). Several lectins, isolated from tissues, appear to mediate intercellular adhesion of their own cell type. For example, the cell aggregation factor from certain sponges is specifically inhibited by uronic acid (22), and in this case a putative receptor molecule has been identified (23). In cellular slime molds, proteins that bind specific carbohydrates appear to mediate the adhesion of aggregating amebae (24, 25). Lectins have also been demonstrated to be present in plasma membranes of bovine liver cells (26) and in chick embryonic muscle (27).

The adhesion of sperm to the surfaces of eggs occurs in many diverse animal groups. In addition to sea urchins, other examples include the Palolo worm *Tylorrhynchus* (Polycheata) (28), the horseshoe crab *Limulus* (subphylum Chelicerata) (29), the hemichordate *Saccoglossus* (30), the amphibian *Xenopus* (31), the mouse (32), and the hamster (3, 4). These diverse species may all possess specific bindins that mediate sperm–egg adhesion. The presence of a granular, electron-dense, acrosome vesicle material similar to that of sea urchin sperm bindin has been observed in the acrosome vesicles of many species of invertebrate sperm (12, 30).

Mammalian sperm also exhibit species-specific attachment to the zona pellucida of the egg (3, 4). It would be of interest to attempt to isolate bindin from mammalian sperm. If mammalian bindin exists, it might be a perfect antigen to use in attempts to induce mammalian immunosterility without risking the development of autoimmune complications.

We are indebted to Ms. Silvia Brice for technical assistance, to Ms. Karen Audittore for the urea gels, and to Dr. B. Brandriff for a critical reading of the manuscript. We also thank Mr. Charles Glabe for many helpful discussions and suggestions. Work supported by National Institutes of Health Grant 1-R01-HD 08645.

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