ISOLATION AND CHARACTERIZATION OF THE VITELLINE LAYER OF SEA URCHIN EGGS

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ABSTRACT

The vitelline layers (VLs) of unfertilized sea urchin eggs were isolated by homogenization in a hypotonic medium containing Triton X-100 and EDTA. The surface topography of the VL is not changed by isolation. The thickness of the isolated VLs (300-400 Å) is greater than that reported for VLs on intact eggs (100-200 Å). Sperm adhere to the isolated VLs. When both internal and external VL surfaces are accessible to sperm, the sperm attach only to the external surface, suggesting that the external surface may carry sperm receptor proteins not present on the internal surface. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis shows that isolated VLs are composed of numerous proteins ranging from >213,000 to 25,000 daltons. Lactoperoxidase-catalyzed 125I-iodination of unfertilized eggs labels two high molecular weight bands that stain faintly for carbohydrate. VLs are 90% protein and 3.5% carbohydrate. No predominance of a single amino acid or class of amino acids was found. Carbohydrate analysis yields fucose, mannose, galactose, glucose, xylose, glucosamine, galactosamine, and sialic acid. Controls for purity indicate that isolated VLs contain 2% protein of cytoplasmic origin and no more than 2.5% egg jelly.

KEY WORDS: fertilization, extracellular coat, sperm-egg recognition, vitelline layer, cell surface

The plasma membrane of the sea urchin egg is covered by a thin glycoprotein coat known as the vitelline layer (VL) (1, 11, 25) to which sperm attach during fertilization (20, 33, 34). The attached sperm must penetrate the VL to achieve contact and fusion with the plasma membrane of the egg. Within seconds after fusion, the egg cortical vesicles undergo exocytosis, releasing their contents under the VL. The VL elevates from the egg surface, combines with proteins from the cortical vesicles and transforms into the fertilization envelope (2, 25).

The mechanism by which sperm attach to the VL is not only of significance to fertilization research; it also represents an advantageous system for studying intercellular adhesion. Furthermore, sperm-to-VL adhesion is a species-specific phenomenon (31) which must involve recognition of macromolecules on gamete surfaces. We have begun a biochemical analysis of sperm-egg adhesion. In this paper, we report the isolation and characterization of the VL of unfertilized eggs.

MATERIALS AND METHODS

Isolation of VLs from Unfertilized Eggs

Gametes of Strongylocentrotus purpuratus were obtained by pouring 0.5 KCl into opened body cavities. Egg jelly coats were removed by a 2-min exposure to seawater, pH 5. Dejellied eggs were washed three times by settling in large volumes of fresh seawater, pH 8, and sedimented by gentle hand centrifugation in graduated
50-ml conical tubes. In most VL isolations reported here, the eggs were then treated for 5 min with 0.2 mM N-bromosuccinimide (NBS, pH 7.5) in seawater. Although omission of this step yields excellent fragmented VLs, we found that NBS pretreatment made the VLs resistant to fragmentation during homogenization, facilitating their recovery as intact ghosts. The VLs described below are from NBS-treated eggs, except where noted. After NBS treatment, the egg pellet was resuspended in 10 vol of isolation medium consisting of 20 mM EDTA, 50 mM sodium acetate, 0.4% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF) dissolved in acetone to 2% final concentration, and 0.1 mg/ml soybean trypsin inhibitor (SBTI). The pH was adjusted to 6.0 with HCl and NaOH and the medium stored at 0°C. All chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Egg lysis occurs immediately upon addition of isolation medium. The lysing eggs were placed in a Thomas smooth-glass, round-bottom tissue grinder (Arthur H. Thomas Co., Philadelphia) and 10 passes were made by hand with a loose-fitting Teflon pestle. This application of gentle shear was continued until no whole eggs or clumps of cytoplasm were visible by phase-contrast microscopy. The homogenate was centrifuged for 10 min at 1,000 g, the supernate removed by aspiration, and the white VL pellet resuspended in fresh isolation medium. The wash was repeated until the supernate was clear. The egg nuclei, which contaminate the VL preparation, form a small, coherent pellet at the tube bottom. The VLs can be separated from the nuclei by carefully pouring off the VLs while leaving the pellet of nuclei intact. The VL preparation was then washed twice in 2 M KCl and stored at 4°C in 50 mM sodium phosphate, pH 7.7, and postfixed for 30 min in 1% OsO4 at 0°C. The wash was repeated until the supernate was clear. The egg nuclei, which contaminate the VL preparation, can be removed at this stage, with slight shearing of the VLs.

Electron Microscopy

To check the purity and ultrastructure of isolated VL pellets, a portion of the pellet was fixed for 2 h in 2% glutaraldehyde, washed in 50 mM sodium phosphate, pH 7.7, and postfixed for 30 min in 1% OsO4 at 0°C. The pellet was dehydrated in ethanol and propylene oxide and embedded in Epon. Sections were stained with alcoholic uranyl acetate. For scanning electron microscopy (SEM), isolated VLs, with living sperm adhering, were fixed for 2 h in 5% formaldehyde in seawater, electrostatically bound to protamine-coated glass cover slips, and further prepared by a previously published method (37).

Analytical Procedures

Isolated VLs were prepared for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis according to the procedure of Laemmli (19). The protein concentration of the sample was adjusted to 2 mg/ml, using bovine serum albumin as a standard (21). Slab gel electrophoresis was done with the gel system of Laemmli (19), except that the ionic strength of the running gel buffer was increased to 560 mM. Gels were stained for protein with Coomassie Blue (41) or for carbohydrate by the PAS procedure (8). Molecular weight standards used were: skeletal muscle myosin, 200,000; phosphorylase a, 94,000; bovine serum albumin, 69,000; and lysozyme, 14,700.

Lactoperoxidase-catalyzed iodination of VLs before and after isolation was performed by the procedure of Johnson and Epel (15). Slab gels of 35S-labeled proteins were dried in a Hoefer slab gel drier (Hoefer Scientific Instruments, San Francisco, Calif.). Autoradiography was performed on dried gels with Kodak X-Omat R X-ray film. 20,000 g soluble egg cytoplasm from the isolation procedure was iodinated by the chloramine-T procedure (14). After iodination, egg cytoplasm was dialyzed against isolation medium until all dialyzable radioactivity was removed. The specific activity was 5.5 × 104 cpm/mg protein. Lipid was extracted from isolated VLs with chloroform-methanol 2:1. Phospholipid was analyzed by thin-layer chromatography (17). Sulfate content of isolated VLs was determined by the method of Terho (35).

For amino acid analysis, isolated VLs were hydrolyzed at 110°C in 5.7 N HCl for 18, 24, and 60 h before analysis with a Durrum D-500 AA analyzer (Durrum Instrument Corp., Sunnyvale, Calif.) (29). Amino sugars were determined after hydrolysis at 100°C for 6 h in 4 N HCl or 110°C for 18 h in 6 N HCl, with a Beckman 120C AA analyzer (Beckman Instruments, Inc., Fullerton, Calif.). The total content of neutral sugars was determined by the anthrone reaction (30) and the phenol sulfuric method (4), using an equal molar mixture of mannose and fucose as a standard. Quantitative determination of neutral sugars was done by gas chromatography of the alditol acetate derivatives, using a Varian model 1200 gas chromatograph (Varian Associates, Palo Alto, Calif.) (18). Samples were hydrolyzed at 100°C in 1 N HCl for 1, 4, 8, and 10 h. L-Arabinose was added before hydrolysis as an internal standard. Sialic acid was determined by the thioribarbituric acid assay after hydrolysis for 1 h in 0.1 N H2SO4 (30).

RESULTS

Morphology of Isolated VLs

After the final wash, isolated VLs appear in phase contrast as thin envelopes of uniform size (Fig. 1). Electron microscope examination of sections of VLs (Fig. 2) shows them to be fairly flat on one surface (arrows) with short, fuzzy, knoblike projections on the opposite surface. The knob-like projections of the VLs (Fig. 3) have an average length of 0.3 μm and are believed to be casts of the cytoplasmic microvilli of the egg. The thickness of the VLs averages ~300 Å in the narrowest, most distinct regions (arrows, Fig. 3).
**Figure 1** Phase-contrast photomicrograph of isolated VLs of NBS-treated *S. purpuratus* eggs. The VLs appear as thin, collapsed envelopes. × 275.

**Figure 2** Longitudinal section of isolated NBS-treated VLs stained with alcoholic uranyl acetate. The regularly spaced microvillus projections of the egg surface are preserved in the isolated VL. The stacks of VLs appear homogeneous and free from visible cytoplasmic contamination. Arrows point to flat (internal) surface of VL. × 17,325.
These VL projections examined in transverse section (Fig. 4) have a center-to-center spacing of 0.26 μm and an inside diameter of 0.13 μm.

Controls for Purity

ARE THESE ISOLATED ENVELOPES REALLY VLS? Could these envelopes be fertilization envelopes, hyaline layers, or cortices of the cells? On the basis of the spacing and morphology of the knob-like VL projections (Figs. 2-4, 6), compared to the morphology of the fertilization envelope of this species (33, 34, 40), the possibility that these envelopes are fertilization envelopes can be excluded. Also, if unfertilized eggs are treated with dithiothreitol (6) or trypsin (5) which disrupt the VL, the structures shown in Figs. 1-6 are not present in the egg homogenate. The structures we call VLs are not egg hyaline layers because the hyaline layer does not form, and if formed, is not preserved in the absence of divalent cations and the presence of Ca²⁺ chelators (16, 36). The isolated VLs are not the "cortex" of the egg because they do not isolate in medium which preserves the cortex (26). For these reasons, we believe that the envelopes we isolate from unfertilized eggs are vitelline layers.

CYTOPLASMIC CONTAMINATION OF ISOLATED VLS: The following experiment was performed to determine the amount of cytoplasmic contamination of isolated VLs. 5 ml of eggs (400 mg protein) were suspended in 35 ml of isolation medium containing 400 mg of ¹²⁵I-labeled cytoplasmic protein (5.5 × 10⁴ cpm/mg). Homogenization and isolation of the VLs were performed as described, and the radioactivity contaminating the final VL pellet was determined. The data (Table I) show that ~2% of the total protein of isolated VLs is protein of cytoplasmic origin which is associated with the isolated VLs and is not removed by 2 M KCl. Analysis by thin-layer chromatography showed the VLs to be <0.1% phospholipid by weight.

CONTAMINATION BY EGG JELLY: The sea urchin egg is surrounded by a viscous jelly layer which is solubilized by pH 5 seawater. The possibility of contamination of isolated VLs by egg jelly was assessed by indirect immunofluorescence using rabbit antibody to soluble egg jelly (furnished by Dr. B. Brandriff of this laboratory). Rabbit anti-egg jelly immunoglobulin reacts with isolated VLs while appropriate controls do not (data not shown). Egg jelly is a sulfated carbohydrate-protein complex containing 17% sulfate by dry weight (Dr. B. Brandriff, unpublished observations). Assuming that the sulfate content of isolated VLs is due solely to contaminating jelly, the maximum amount of contamination can be estimated. Isolated VLs contain 0.43% sulfate by weight, giving an estimate of 2.5% for the maximum possible contamination by egg jelly.

Adhesion of Sperm to Isolated VLs

Isolated VLs were suspended in seawater containing 1–10 μg/ml soluble egg jelly to initiate the acrosome reaction. Fresh sperm were added, and after 60 s an equal volume of 5% formaldehyde in seawater was added. The suspension was centrifuged for 5 min at 500 g, the pellet was resuspended in fresh formaldehyde-seawater, and centrifugation was repeated until the supernate was clear. The VLs were prepared as described and viewed in the SEM. Sperm bind by their acrosome processes to both NBS-treated and untreated isolated VLs (Figs. 5 and 6). SEM observations show that the internal surface of the VL (Fig. 6, inset, the surface in contact with the plasma membrane) is flat and does not possess the knob-like projections of the outer surface (Figs. 2, 3 and 6). The internal and external surfaces of isolated VLs can therefore be distinguished. When sperm are mixed with isolated VLs, they adhere only to the external surface (Figs. 5 and 6). In a random-selection observation of 22,730 μm² of the external surface, we found 108 adhering sperm. In a similar tally of 4,810 μm² of internal VL surface, only one adhering sperm was found.

SDS Gel Electrophoresis of VLs

We experienced a problem of artifact when first determining the number of protein molecular weight classes comprising the VL. We believe that it is necessary to report this artifact because other workers may repeat this experiment without recognizing the problem. Isolated VLs were dissolved in 2% SDS, 5% mercaptoethanol, and polyacrylamide gel electrophoresis was performed by the procedure of Maurer (see reference 22, system no. 1). 10% acrylamide running gels were prepared containing 375 mM Tris HCl, pH 8.8; the 3% stacker gel containing 6 mM Tris and 32 mM phosphate, pH 6.8. The electrode buffer was 5 mM Tris-base, 38 mM glycine, pH 8.8, and 0.1% SDS throughout the system. The gels were stained with either Coomassie Blue (41) or PAS (8). Solubilized VLs electrophoresed in this system migrate as a single heavy band of apparent
Figure 3 High magnification longitudinal section of isolated VLs showing details of the microvillus projections. On the basis of the shape and orientation of the microvillus projections, it is possible to distinguish the internal and external VL surfaces. The VL appears to consist of a unit structure ~300 Å in width (arrows) consisting of two parallel electron-dense lines separated by a less electron-dense space. Amorphous fibrous material is associated with this unit structure. × 63,200.

Figure 4 High magnification transverse section through microvillus projections of isolated VLs. The center-to-center spacing of the VL projections is 0.27 μm. × 70,600.
Figure 5  Scanning electron micrograph (SEM) of sperm adhering to an isolated VL. Living sperm and VLS were mixed; after 1 min, the preparation was fixed in formaldehyde and prepared for SEM. When both internal and external VL surfaces were accessible, sperm adhered preferentially to the outer surface showing that the two sides must differ in composition. It is possible that sperm receptor molecules may reside on the outer VL surface (28). × 2,600.

Figure 6  High magnification SEM of sperm attached to the external VL surface by their acrosome processes (arrows). Inset: SEM of internal VL surface. × 13,000.
TABLE I
Cytoplasmic Contamination of Isolated VLs

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<tr>
<th>Protein</th>
<th>²¹²²I/mg</th>
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<tr>
<td>mg</td>
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<tr>
<td>Total homogenate</td>
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<td>Isolated VLs</td>
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<td>6.25 × 10²</td>
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% contamination = VL cpm after isolation / specific activity homogenate cytoplasm mg VL protein isolated

mol wt 160,000 daltons that stains for both protein and carbohydrate. If, however, the gel system of Laemmli is employed and the ionic strength of the running gel increased by 50% as given in Materials and Methods, the VL material separates into many components (Fig. 7a).

In each pair of gels in Fig. 7, the left gel is stained for protein and the right gel is its corresponding autoradiograph showing the location of ¹²⁵I-labeled proteins. In one series of experiments, eggs were radioiodinated before VL isolation but not treated with NBS. VLs from these eggs resolve into ~40 protein bands with one major component of apparent mol wt 54,000 (Fig. 7a). Autoradiographs of VLs from eggs labeled before isolation of the VL reveal only two high molecular weight bands, one of which does not enter the running gel and the other which migrates ~2 mm into the running gel (Fig. 7a). Both of these bands stain faintly for carbohydrate (Fig. 7a and b, arrows on right). This pattern of labeling indicates that these two components are accessible to lactoperoxidase-catalyzed iodination before isolation of the VLs and suggests that they are components of the external surface of the VL.

In another experiment, intact eggs were radioiodinated then treated with NBS and the VLs were isolated. The protein components of the NBS-treated VLs (Fig. 7b) were very similar to those of untreated eggs (Fig. 7a). The autoradiograph of the NBS VLs shows, however, that the labeled material is present in a higher molecular weight form that does not enter the stacking gel (Fig. 7b). This suggests that NBS crosslinks the VL surface labeled material (Fig. 7a) causing aggregates which do not break down in 2% SDS, 5% mercaptoethanol at 80°C.

We were surprised to find that lactoperoxidase-catalyzed radioiodination of intact eggs before VL isolation labeled only two regions of the gel (Fig. 7a). We thought that the isolation procedure might solubilize other labeled components which would then be lost during the successive washings of the VLs. To answer this question, intact eggs were ¹²⁵I-labeled as described and instantly solubilized by addition of a solution containing 2% SDS, 5% mercaptoethanol, and the protease inhibitors paraaminobenzamidine (50 mM) and PMSF (2 mM) at 80°C. The protein components and corresponding autoradiograph of the whole egg preparation (Fig. 7c) show that only two bands appear on the autoradiograph corresponding to the location of the two labeled components of the isolated VLs (Fig. 7a). This suggests that no surface labeled components are lost or degraded during the isolation procedure. If VLs are ¹²⁵I-labeled after isolation, essentially all protein bands appear labeled (Fig. 7d). It is possible that the unlabeled bands (Fig. 7a, b, and c) are not exposed at the external surface of the VLs of intact eggs and therefore are not accessible to lactoperoxidase labeling.

Figure 7 SDS gel electrophoresis of isolated vitelline layers. In each set of gels, the gel on the left displays the protein components. The corresponding autoradiograph of the gel is shown on the right. (a) Eggs were surface labeled with ¹²⁵I by the lactoperoxidase method before isolation of VLs. The eggs were not treated with NBS. The VLs resolve into ~40 protein staining bands. There is one major component of apparent mol wt of 54,000 daltons. The autoradiograph of this gel shows only two regions of label, one at the top of the running gel and the other about 2 mm into the running gel. (b) Eggs ¹²⁵I-labeled and treated with NBS before isolation of VLs. No apparent differences exist in the protein composition, but the surface labeled material does not enter the stacking gel. We believe that this is so because NBS cross-links this material. PAS arrows indicate the position of carbohydrate-containing bands. (c) Eggs were ¹²⁵I-labeled and dissolved in a solution containing 2% SDS, 5% mercaptoethanol, 50 mM paraaminobenzamidine, and 2 mM PMSF at 80°C. The protein composition of the whole eggs is shown on the left. The autoradiograph shows the same two zones of label that appear in Fig. 7a. This shows that no surface labeled material is lost or degraded during isolation of VLs. (d) When VLs were ¹²⁵I-labeled after isolation, all the protein staining bands appear to be labeled.
Biochemical Analysis

Isolated VLs are 90% protein, 3.5% carbohydrate, and 0.43% sulfate. Their amino acid composition is presented in Table II. There are no unusual percentages of amino acids present. The carbohydrate composition of VLs is presented in Table III. A jelly contamination of 2.5% by weight would account for only approx. one-third of the total fucose content of isolated VLs. The other sugars found in VLs do not occur in appreciable concentration in soluble jelly (Dr. B. Brandriff, unpublished data). Pretreatment with NBS before isolation of VLs does not alter the carbohydrate composition.

DISCUSSION

In this paper we report the isolation and analysis of VLs from unfertilized sea urchin eggs. Before some isolations, eggs were treated with NBS, the exact action of which on this material is unknown. The differences noted between NBS-treated and untreated VLs are the increased resistance to mechanical shear and the increase in apparent size of the surface radioiodinated, PAS-positive VL components (Fig. 7a and b). NBS may form covalent cross-links between surface proteins by the oxidative formation of dityrosine linkages between adjacent proteins (10). This reagent can oxidize sulfhydryl groups to disulfides (23), and it is known that disulfide bonds are necessary for the stability of the intact VL (6). We feel, however, that NBS-mediated disulfide formation is not a likely explanation for the difference in the autoradiographs of the surface iodinated components (Fig. 7a and b) because the formation of the high molecular weight material by NBS (Fig. 7b) is not reversed by agents such as mercaptoethanol and dithiothreitol.

Sea urchin eggs contain trypsin-like protease activity (3, 9) that alters the VL surface making it incapable of binding sperm (3, 38). For this reason, we included 1 mM PMSF and 0.1 mg/ml SBTI in the isolation medium, both of which are potent inhibitors of the egg protease (3, 7, 9, 38). If protease inhibitors are omitted, the isolated VLs will not bind sperm and the high molecular weight protein bands (Fig. 7a, b, and d) decrease in number with the concomitant appearance of low molecular weight material that migrates with the tracking dye.

Figs. 2-6 reveal that the major morphological features of the isolated VLs are the regularly spaced, knob-like projections. Tegner and Epel (33, 34) have described the morphology of the VL on the intact egg of this species. They find the VL to exhibit a very dense and uniform array of knob-like casts of the cytoplasmic microvilli underlying the VL. They report that the VL projections have a center-to-center spacing of 0.25 μm, a length of 0.27 μm and a diameter of 0.16 μm (33). The projections of isolated VLs shown in Figs. 2-4 have a center-to-center spacing of 0.26 μm (Fig. 4), an inside diameter of 0.13 μm (Fig. 4) and an average length of 0.3 μm (Figs. 2 and 3). These measurements are important because the center-to-center spacing of the projections changes from

## Table II

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>mol %</th>
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<td>Lys</td>
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<td>Arg</td>
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</tr>
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</tr>
<tr>
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n.d. = not determined

## Table III

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<td>Gal</td>
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<tr>
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<tr>
<td>Sulfate</td>
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Amino acid analysis of sea urchin VL (Table II) is similar to that found for the VL of Xenopus (42). Our analysis of intact VLs differs significantly from that reported for the VL material solubilized from the sea urchin egg surface by 1 M urea (1). Our analysis does not show a predominance of one amino acid or a class of amino acids as is seen in VLs from the keyhole limpet Megathura, which are 61 mol % threonine (13). Similarly, very high percentages of threonine and serine were found for the VLs of the snail Tegula (12). Carbohydrate analysis of VLs (Table III) does not show a predominance of a single sugar. Sialic acid is a component of the isolated VL although it was not found to occur in VL material dissolved from eggs of another species of sea urchin by urea treatment (1).

We are unaware of any other report of the isolation of the intact VL from sea urchin eggs. Aketa (1) reported the isolation of soluble VL glycoproteins by treating eggs with 1 M urea during the first minute after insemination when the VL is transforming into the fertilization envelope. This extraction not only solubilizes the elevating VL but it must also solubilize cortical granule proteins and possibly proteins from the plasma membrane. Osanai (24) has isolated the VL from the palolo worm Tylorrhynchus (polychaeta) by homogenizing eggs in hypotonic seawater. As is true of isolated sea urchin VLs (Fig. 5), sperm will attach to palolo VLs. The VL of the mollusc Tegula has been isolated by homogenization in 0.5% SDS (12). Analysis shows it to be 33% protein and 50% carbohydrate. The VL of the keyhole limpet Megathura has been isolated and found to be 37% protein and 63% carbohydrate (13). Xenopus VLs have most recently been isolated and found to be 85% protein and 15% carbohydrate (42). Electrophoresis of SDS-solubilized Xenopus VLs shows them to be composed of two high molecular weight glycoproteins, two major proteins comprising two-thirds of the VL mass and at least seven other protein components (42). Our analysis of sea urchin VLs shows them to be composed of numerous proteins ranging from apparent mol wt >213,000 to 25,000 daltons, only two of which stain faintly for carbohydrate.

The VL is the site of species-specific sperm adhesion (1, 31). This special example of intercellular adhesion may serve to maximize gamete contact, thus facilitating sperm penetration of the VL and fusion with the egg plasma membrane. After the sperm acrosome reaction has occurred, a protein derived from the acrosomal vesicle which we call bindin (39) coats the outer surface of the sperm acrosome process (32). We have isolated bindin and shown by several criteria that it is the species-specific sperm attachment substance (footnote 1 and reference 39). One would assume that

molecular recognition must occur between bindin and a receptor on the VL surface. Evidence has recently been presented that such species-specific glycoprotein sperm receptors may exist on the surfaces of sea urchin eggs (27).

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