An ATP-binding Cassette Transporter Is a Major Glycoprotein of Sea Urchin Sperm Membranes*

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Sperm are terminally differentiated cells that undergo several membrane-altering events before fusion with eggs. One event, the sea urchin sperm acrosome reaction (AR), is blocked by the lectin wheat germ agglutinin (WGA). In an effort to identify proteins involved in the AR induction, the peptide sequence was obtained from a 220-kDa WGA-binding protein. Degenerate PCR and library screening resulted in the full-length deduced amino acid sequence of an ATP-binding cassette transporter, suABCA. The protein of 1,764 residues has two transmembrane regions, two nucleotide-binding domains, and is most closely related to the human ABC subfamily A member 3 transporter (ABCA3). Sequence analysis suggests a large extracellular loop between transmembrane spanning segments 7 and 8, with five N-linked glycosylation sites. An antibody made to the loop region binds to non-permeabilized cells, supporting that this region is extracellular. suABCA is found in sperm membrane vesicles, it can be solubilized with nonionic detergents, and it shifts from 220 to 200 kDa upon protein N-glycanase F digestion. suABCA localizes to the entire surface of sperm in a punctate pattern, but is not detected in lipid rafts. Based on its relationship to subfamily A, suABCA is most likely involved in phospholipid or cholesterol transport. This is the first investigation of an ABC transporter in animal sperm.

As spermatocytes mature, their shape is altered from round, undifferentiated spermatocytes to long, highly compartmentalized, terminally differentiated cells. Most sperm consist of a head containing an acrosomal vesicle and a nucleus, a midpiece containing mitochondria, and a long flagellum. Upon release into seawater, sea urchin sperm activate motility and, before fusing with eggs, undergo the exocytotic acrosome reaction (AR).1 In mammals, this process is further complicated by the need to undergo capacitation before the AR can be triggered. Capacitation is a poorly understood process involving cholesterol efflux, changes in intracellular ion concentrations, and tyrosine phosphorylation (1). All of these processes involve changes in membrane structure and composition (2), and the molecules involved in these changes are only now being discovered.

One of the best-studied membrane-altering events in fertilization is the sea urchin sperm AR. When sperm contact a fucose sulfate polymer contained in the jelly layer surrounding the egg, multiple fusions occur between the acrosomal vesicle and the plasma membrane of sperm resulting in exocytosis of the vesicle (3). A newly exposed membrane, covering the actin-containing acrosomal process, then fuses with the egg plasma membrane (4). Wheat germ agglutinin (WGA) blocks the egg jelly-induced AR of sea urchin sperm (5). One WGA-binding protein, suREJ3, has been demonstrated to be involved in triggering the AR (6), and another, suREJ3, has been implicated in this process (7). This paper identifies another major WGA-binding protein of sea urchin sperm as an ATP-binding cassette (ABC) transporter, most closely related to human ABCA3.

ABC transporters make up one of the largest families of transmembrane proteins and have been identified in every organism. These proteins use ATP to drive the transport of a wide variety of substances across the membrane including phospholipids, amino acids, peptides, toxins, metals, and antibiotics. The functional unit contains two distinct transmembrane regions, each with five to eight transmembrane spanning segments (TMSs), and two distinct nucleotide-binding domains (NBDs). In bacteria, each domain is a separate polypeptide, whereas in eukaryotes a single polypeptide chain can contain a half-transporter (one transmembrane region and one NBD) or a full transporter with two transmembrane regions and two NBDs (8, 9).

Forty-eight human ABC transporters are divided into seven distinct families based on sequence similarity. Human subfamily A contains 12 members, which can be further subdivided into two distinct groups (10). The first group in subfamily A has seven members, all of which are implicated in phospholipid and cholesterol transport. Here we identify a sea urchin ABC transporter belonging to the subfamily A. This is the first description of an ABC transporter in animal sperm.

EXPERIMENTAL PROCEDURES

Protein Preparation and Peptide Sequencing—All procedures were on ice or at 4 °C. Sea urchins, Strongylocentrotus purpuratus, were spawned by injection with 0.5 M KCl and the undiluted sperm collected with a Pasteur pipette. Sperm were resuspended in 0.45-μm filtered seawater (FSW), coelomocytes were removed by three (5 min) centrifugations at 200 × g and sperm cells sedimented at 5,000 × g (15 min). Sperm membranes were solubilized by suspending sperm pellets in 0.15 M NaCl, 10 mM HEPES, pH 7.4, and 1% Nonidet P-40. Solubilized protein was obtained from the supernatant after centrifugation at...
100,000 × g for 1 h. This supernatant was applied to a WGA-agarose column (EY Laboratories). The column was washed with 50 column volumes of wash buffer (0.15 M NaCl, 10 mM HEPES, 0.1% Nonidet P-40, pH 7.4) and the protein eluted in wash buffer containing 100 mM N-acetyl-D-glucosamine. SDS-PAGE was performed (11) and the gel stained with Coomassie Brilliant Blue. The 220-kDa band was cut excised from the gel, destained, and sent to the Stanford University FACility for trypan digestion and peptide sequencing.

**DNA Sequencing and Sequence Analysis—** Degenerate primers were designed according to peptides 1 and 3 and used to amplify a message from cDNA from an entire sea urchin. Specific primers were made to this message and 3′ rapid amplification of cDNA ends (RACE) with testis mRNA was performed to obtain most of the 3′ end of the sequence. This product was used to screen a testis λ ZAP II library (Stratagene). Overlapping clones were obtained and sequenced.

**Antibody Production—** A portion of suABCA DNA corresponding to Ala961–Pro1158 was ligated into the pET15b vector (Novagen), which contains N-terminal His tag, and bacterial expression was performed according to a previously published protocol (7). The resulting purified recombinant protein, ABCe, was separated by SDS-PAGE and negatively stained with cunic chloride (20). The protein was excised, destained, and used to make commercially raised anti-ABCe rabbit antibodies (Strategic BioSolutions). The antibodies were subsequently affinity purified on an ABCe-conjugated Aff-Gel 15 column (Bio-Rad).

**Other Methods—** Sperm membranes vesicles (SMVs) were made according to the pH 9 method (21, 33). For protein-N-glycosidase F (PNGase F) treatment of sperm protein, 40 µg of WGA eluate containing 0.5% SDS and 50 mM β-mercaptoethanol was boiled 5 min. Nonidet P-40 was added to 7.5%, followed by 2.4 µl of PNGase F and 27.6 µl of distilled water. The sample was incubated overnight at 37 °C. Following deglycosylation, the sample was separated on SDS-PAGE, transferred to PVDF, and Western blots performed using ABCe antibody. Lipid rafts were made following a previously described protocol (22). Briefly, 200 µl of dry sperm were solubilized with 1 ml of 1% Triton X-100 in solubilization buffer (SB; 10 mM Tris-HCl, pH 7.5, 0.15 mM NaCl, 5 mM EDTA), incubated on ice for 20 min, and homogenized with 10 strokes of a Dounce homogenizer. The cell debris was removed by a 5 min × 1,300 g centrifugation. The supernatant was mixed with equal volume of 55% (v/v) sucrose in SB. Two milliliters of the resulting milliliters of the resulting supernatant were layered successively with 6 ml 30% sucrose in SB and 3.5 ml 5% sucrose in SB. The lipid rafts were obtained by ultracentrifugation at 200,000 × g at 4 °C for 18 h, and 1-ml fractions were collected. Protein was separated by SDS-PAGE, and gels were stained with silver (23), Coomassie Brilliant Blue, or transferred to PVDF for immunoblotting. Transfer to PVDF was in 20% methanol/80% water with 192 mM glycine and 25 mM Tris-OH at pH 8.3. Blots were blocked for 1 h with a 1:20,000 dilution of ABCe monoclonal antibody, had absolutely no fluorescence.

Peptide Sequencing and Sequence Analysis—Several high molecular weight proteins are enriched in WGA eluates of solubilized sperm protein when compared with the starting material, including suREJ1 and suREJ3 (6, 7). The 220-kDa band is enriched in the eluate and can also be detected in the starting material (Fig. 1). To purify this protein, a WGA-eluate was separated by SDS-PAGE and the gel stained with Coomassie Brilliant Blue. The 220-kDa protein was excised and trypan digestion and peptide sequencing performed. Three peptide sequences were obtained (Fig. 2). Peptides 1 and 3 were used to design degenerate primers and the primers used to amplify a partial sequence from cDNA. The initial PCR product was then used to obtain the full-length sequence by a combination of screening a testis cDNA library and 3′ rapid amplification of cDNA ends with testis cDNA. Using BLAST to search GenBank identified this protein as a new member of the ABC transporter superfamily, named suABCA.

suABCA is a 1,764-amino acid full ABC transporter, containing two NBDs and two transmembrane regions of six TMS each (Fig. 2). SignalP predicts a signal sequence from Met4 to Arg14. All three peptides were found in the deduced suABCA amino acid sequence. The NBDs of suABCA contain the subfamily A signature sequences, including the Walker A motif, the #50 sequence, the "hot spot," the ATS/C region, the Walker B motif, and the Switch #162 sequence (Fig. 3A; Refs. 24 and 25). Within all of these regions, the two homologous domains of suABCA vary by only three amino acids. Also, suABCA is conserved in the additional ABCA regions 100–130 amino acids downstream of the Walker B motif, varying by only two amino acids.
A Kyte-Doolittle plot (Fig. 3B) indicates that suABCA has 12 predicted TMS, with the first TMS located within the putative signal sequence. The TMS are also predicted by TMHMM (15). Between TMS 1 – 2 and TMS 7 – 8 are large, putative extracellular loops of 158 and 242 residues, respectively. The first loop contains three potential N-linked glycosylation sites, and five are predicted in the second loop (Fig. 3D). Two of the peptide sequences are found in the second loop and each includes an N-linked glycosylation site. In both cases, the asparagine could not be determined by Edman degradation, most likely due to possession of oligosaccharide chains.

The human ABC transporter subfamily A is divided into two groups based on phylogeny (10). Human ABCA1, -2, -3, -4, -7, and -12 fall into one group. Multiple sequence alignments of suABCA to this group show the sea urchin transporter to be most similar to member 3 (Fig. 3C). The sea urchin transporter is also similar in size to human ABCA3 (1,764 compared with 1,704 amino acids) and, like human ABCA3, contains a shorter extracellular loop between TMS 1 and 2 in comparison to the other members of this group. In summary, suABCA has a large glycosylated extracellular loop in the first transmembrane region and a second larger glycosylated loop in the second transmembrane region. Six TMS are predicted in each transmembrane region; however, TMS1 may be cleaved after the signal sequence (Fig. 2).

Immunoblots—Anti-ABCe antibody reacts with a band at the expected size of 220 kDa in an Nonidet P-40 extract of whole sperm (Fig. 4A). The antigen is enriched in sperm membrane vesicles and is not present in the supernatant of the vesicle preparation. A second band, appearing in the SMV sample, is most likely a breakdown product. suABCA is highly enriched in the WGA eluate. Lanes N, V, and S contain 10/100 g of protein, while lane W contains 0.5/100 g of protein. The density of the band in lane W is similar to that of the SMV preparation, indicating that the WGA eluate provides approximately a 20-fold enrichment of the suABCA transporter. Based on amino acid se-
sequence alone, suABCA should be ~195 kDa. Treatment of WGA-binding proteins with PNGase F, followed by anti-ABCe immunoblotting, demonstrates that suABCA shifts in relative mass from 220 to 200 kDa (Fig. 4B), confirming N-linked glycosylation.

Localization of suABCA—Immunofluorescence using the anti-ABCe antibody localizes the antigen to the entire surface of sperm in a punctate pattern (Fig. 5A). This pattern and level of fluorescence was seen in both nonpermeabilized and permeabilized cells and was not altered by time of fixation. Also, decreasing the concentration of antibody resulted in the same punctate pattern with lower levels of fluorescence. Cells reacted with only the secondary antibody had absolutely no fluorescence. Because of the punctate pattern and possible role of suABCA in phospholipid or cholesterol transport, lipid rafts were isolated and tested for the presence of suABCA (Fig. 5B). A sucrose cushion was floated on top of the detergent-extracted supernatant and the sample ultracentrifuged. This resulted in a lipid-dense band in fraction 4, which has been characterized previously as the glycosphingolipid-rich raft fraction (22). Fractions taken from top to bottom show that suABCA is mostly solubilized by detergent and is not detected in the lipid raft fraction.

DISCUSSION

WGA blocks the egg jelly-induced AR of sea urchin sperm (5), and there are ~10 major WGA-binding proteins visible on a silver-stained gel. One or more of these proteins must play a role in AR induction. suREJ1 was the first WGA-binding protein shown to be a receptor for the egg jelly fucose sulfate polymer, a known AR inducer (3, 6). Also, the NH2- and COOH-terminal halves of suREJ3 bind WGA and are implicated in the AR, due to their location on the plasma membrane covering the acrosomal vesicle (7).

The 220-kDa (previously reported as 190 kDa; Refs. 21 and 33) WGA-binding protein was shown to be an ABC transporter belonging to the human subfamily A transporters. Twelve members of this subfamily have been identified from human, which are all full transporters with two transmembrane regions and two NBDs (25). Available data show that the members of subfamily A are involved in cholesterol or phospholipid transport (10). Members of subfamily A that cluster with suABCA include ABCA1, -2, -3, -4, -7, and -12. suABCA is most similar to human ABCA3 (Fig. 3C). ABCA3 is found associated with the lamellar bodies of lung alveolar type II cells and is thought to play a role in phospholipid transport during surfactant production (26, 27). The best studied member of this subfamily is ABCA1, mutations in which cause the autosomal recessive disorder Tangier disease. Patients with Tangier disease have virtually no plasma high density lipoprotein (HDL), they accumulate cholesterol in macrophages, and they have a high incidence of atherosclerosis (28, 29). Accumulating evi-
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A.

![Image of filipin-cholesterol complexes](image)

**Fig. 5.** suABCA is present in a punctate pattern all over sperm but is not detected in lipid rafts. A, phase contrast image of sperm on the left and the same sperm on the right showing immunofluorescence of anti-ABCe. In the absence of primary antibody there was absolutely no fluorescence associated with the cells. B, anti-ABCe immunoblot of fractions taken from the sucrose gradient of the lipid raft experiment. The first panel is a silver-stained gel, and the second panel is the immunoblot. Fraction 4 contains the lipid rafts; fractions 6, 8, and 10 are part of the sucrose gradient; and fraction 12 is the solubilized sperm protein.

Transmission electron micrographs of filipin-cholesterol complexes show that during capacitation, cholesterol increases over the guinea pig acrosomal vesicle (2). During capacitation, mammalian sperm lose cholesterol from their membranes. Bovine serum albumin, cyclodextrins, and HDL are all capable of binding cholesterol and increasing the rate of capacitation (1). In mammalian sperm, no mechanism has been described for cholesterol removal from membranes. An ABCA transporter would be an ideal protein to perform such a task. However, sea urchin sperm no not appear to have a capacitation-like process and are capable of acrosome reacting immediately upon release into seawater.

Anti-ABCe was made against the region between TMS 7 and 8. This region is thought to be a regulatory region in ABCA1 (25), and several models have been proposed describing the topology of this region in subfamily A members (24, 30). With ABCA1, data support a model for an intracellular location with the hydrophobic region (TMS 7) sticking into the membrane but not passing through it (24). In ABCA4, evidence indicates that the region corresponding to TMS 7 passes through the membrane so that the region between TMS 7 and 8 is extra-cellular. Like ABCA4, our data support an extracellular location of this region. Twenty kDa of the relative molecular mass of suABCA can be accounted for by N-linked glycosylation, and five N-linked sites are predicted in this region. Two sites are present in the sequenced peptides, and asparagine residues could not be identified via Edman degradation, indicating that they are glycosylated. Also the anti-ABCe antibody made against this putative extracellular region binds non-permeabilized cells, and the level of binding does not change after permeabilization. Because this region may be a regulatory site in some ABCA members (25), anti-ABCe was dialyzed into FSW and applied to live sperm. No differences could be detected in swimming behavior, cell agglutination, or acrosome reactions (data not shown).

suABCA has a distinct punctate pattern of immunofluorescence. To test the possibility that suABCA was present in specific membrane microdomains, anti-ABCe immunoblots were performed on isolated lipid rafts. Rafts have previously been isolated from sea urchin sperm and shown to be enriched in glycosphingolipids but not cholesterol (22). Several sea urchin membrane proteins such as the speract receptor, suREJ1, adenylate cyclase, and guanylate cyclase have been detected in the raft fraction but suABCA is not (31). Likewise, human ABCA1 does not associate with sphingomyelin/cholesterol-rich lipid rafts (32).

Immunofluorescence of testis homogenates shows that the protein is present in a similar pattern on all immature sperm-matogenic cells (data not shown). A potential role for suABCA could be involved in the early stages of membrane structuring to create the terminally differentiated mature spermatozoa.

Although many proteins and carbohydrates have been identified as important players in sperm-egg interactions (4), the membrane alterations during fertilization are poorly understood. One interesting question raised by this work is why suABCA is such an abundant membrane protein present on the entire surface of the sperm. Sperm might alter their membranes during spermogenesis, motility, the acrosome reaction, and ultimately sperm-egg fusion.

This research has implications beyond sperm physiology. Membrane vesicles can be isolated from sperm as right-side-out vesicles by the pH 9 method (33) or as a mixture of right-side-out and inside-out vesicles by nitrogen cavitation (34). suABCA remains tightly associated with SMVs. SMV preparations can be combined with methods such as WGA chromatography or affinity chromatography to enrich for suABCA-containing lipid vesicles. The abundance of suABCA may offer an excellent opportunity to study the biochemical properties of these transporters in their native environment.

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**REFERENCES**

1. Visconti, P. E., Westbrook, V. A., Chertihin, O., Demarco, I., Sleight, S., and Dieckman, A. B. (2002) *J. Reprod. Immunol.* 53, 133–150
2. Bearer, E. L., and Friend, D. S. (1990) *J. Electron Microsc. Technol.* 16, 281–297
3. Vaequier, V. D., and Moy, G. W. (1997) *Dev. Biol.* 192, 125–135
4. Podell, S. B., and Vacquier, V. D. (1984) *J. Cell Biol.* 99, 1588–1604
5. Moy, G. W., Mendoza, L. M., Schulz, J. B., Swanson, W. J., Glabe, C. G., and Vacquier, V. D. (1996) *J. Cell Biol.* 133, 809–817
6. Cropp, J. M. (1998) *Methods Enzymol.* 292, 101–116
7. Klein, I., Sarkadi, B., and Varadi, A. (1999) *Biochim. Biophys. Acta* 1461, 257–262
8. Dean, M., Hamon, Y., and Chintini, G. (2001) *J. Lipid Res.* 42, 1007–1017
9. Laemmli, U. K. (1970) *Nature* 227, 680–685
10. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) *J. Mol. Biol.* 215, 403–410
11. Nielsen, H., Engelbrecht, J., Brunak, S., and von Heijne, G. (1997) *Protein
14. Kyte, J., and Doolittle, R. F. (1982) J. Mol. Biol. 157, 105–132
15. Krogh, A., Larsson, B., von Heijne, G., and Sonnhammer, E. L. L. (2001) J. Mol. Biol. 305, 567–580
16. Higgins, D. G., Thompson, J. D., and Gibson, T. J. (1996) Methods Enzymol. 266, 383–402
17. Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. (1997) Nucleic Acids Res. 25, 4876–4882
18. Nicholas, K. B., Nicholas, H. B., Jr., and Deerfield, D. W., II (1997) EMBNew News 4, 14
19. Page, B. D. (1996) Comput. Appl. Biosci. 12, 357–358
20. Lee, C., Levin, A., and Branton, D. (1987) Anal. Biochem. 166, 308–312
21. Vacquier, V. D. (1986) Methods Cell Biol. 27, 15–40
22. Ohta, K., Sato, C., Matsuda, T., Toriyama, M., Lennarz, W. J., and Kitajima, K. (1999) Biochem. Biophys. Res. Commun. 258, 616–623
23. Bungert, S., Molday, L. L., and Molday, R. S. (2001) J. Biol. Chem. 276, 23539–23546
24. Podell, S. B., Moy, G. W., and Vacquier, V. D. (1984) Biochem. Biophys. Acta 778, 25–37