Liposome Fusion Induced by a M_r 18 000 Protein Localized to the Acrosomal Region of Acrosome-Reacted Abalone Spermatozoa†

Willie J. Swanson* and Victor D. Vacquier

Marine Biology Research Division and Center for Marine Biotechnology and Biomedicine, Scripps Institution of Oceanography, University of California, San Diego, La Jolla, California 92093-0202

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ABSTRACT: A M_r 18 000 protein is secreted by abalone spermatozoa during the acrosome reaction. Immunofluorescence of acrosome-reacted sperm localizes the protein as a coating on the spent acrosomal granule hull and on the surface of the acrosomal process. The membrane of the acrosomal process fuses with the egg plasma membrane at fertilization. The M_r 18 000 acrosomal protein aggregates negatively charged (but not neutral) large unilamellar liposomes and renders them permeable to internal probe. The M_r 18 000 proteins from two abalone species are potent inducers of intervesicular lipid mixing in the resonance energy transfer assay, suggesting that they mediate the fusion of lipid bilayers. Predicted secondary structures of these proteins show the presence of strongly amphipathic α-helices that may be active in the perturbation of phospholipid bilayers. The M_r 18 000 protein may mediate sperm−egg fusion during fertilization.

Study of the membrane fusion reactions of eukaryotic cells is an important field of cell research (Wilschut & Hoekstra, 1991; White, 1992). In metazoan animals, the nonpathogenic fusion of cells happens only twice in development: once at fertilization and again when myoblasts fuse to form the myotubes of skeletal muscle. The fusion of sperm and egg bridges successive generations; thus, its biological importance cannot be overstated. Sperm−egg fusion restores the diploid genome and metabolically activates the egg, setting the zygote on an irreversible pathway leading to mitosis. The identification of proteins mediating the fusion of sperm and egg is important to a deeper knowledge of fertilization.

Recent reports have identified cell surface proteins implicated in sexual fusion in slime molds (Aiba et al., 1993) and rat myoblasts (Lognonne & Wahrmann, 1988). Sperm proteins implicated in gamete fusion have been identified in three widely divergent animal species: the sea urchin, the abalone, and the guinea pig. Following the sea urchin sperm acrosome reaction, the protein bindin coats the sperm acrosomal process. Immunoperoxidase localization shows that bindin bridges the plasma membranes of sperm and egg (Moy & Vacquier, 1979). Ultrastructural data and measurements of membrane potential implicate bindin in sea urchin gamete fusion (Longo et al., 1994; Metz et al., 1995). Purified bindin was the first sperm protein shown to fuse liposomes (Glabe, 1985). Bindin fuses mixed-phase vesicles containing PC[1] and PS but does not fuse vesicles of dipalmitoylphosphatidylcholine. Deletion mutagenesis of bindin shows that the central domain of the protein, which has been highly conserved for at least 200 million years (Glabe & Clark, 1991; Vacquier et al., 1995), is the domain possessing affinity for biological membranes (Miraglia & Glabe, 1993).

A sperm membrane protein, PH-30, identified with a monoclonal antibody, has been proposed as the fusagen of sperm and egg in guinea pigs (Myles, 1993). PH-30 is a heterodimer composed of α- and β-subunits. Both subunits share sequence homology with metalloprotease and disintegrin domains of snake venom peptides (Wolfsberg et al., 1993). Synthetic peptides of the disintegrin domain of the β-subunit bind to eggs and block sperm−egg fusion (Myles et al., 1994). A synthetic peptide, representing the putative fusion domain of the α-subunit, fuses large unilamellar liposomes, supporting the hypothesis that PH-30 mediates sperm fusion with the egg (Muga et al., 1994).

Spermatozoa of abalones (genus *Haliotis*) possess a large acrosomal granule containing approximately equal quantities of two proteins of M_r 16 000 (hereafter termed "lysin") and M_r 18 000 (Lewis et al., 1982). They are the major proteins of abalone sperm. Upon fertilization, both proteins are released by exocytosis of the acrosomal granule onto the elevated vitelline envelope (VE) of the egg. Coincident with acrosomal exocytosis is the elongation of the acrosomal process from approximately 2 to 7 μm (Lewis et al., 1980). Lysin has been extensively studied; it creates a hole in the egg VE by a nonenzymatic mechanism (Vacquier & Lee, 1993; Shaw et al., 1993; Lee et al., 1995). Purified lysin renders neutral liposomes permeable and fuses negatively charged liposomes (Hong & Vacquier, 1986). Solution of the crystal structure of lysin, resolved at 1.9 Å, shows the presence of highly amphipathic α-helices and reveals a patch of solvent-exposed hydrophobic residues which could be active in membrane perturbation (Shaw et al., 1993).

Subsequently, the M_r 18 000 acrosomal protein was purified and crystallized (Swanson & Vacquier, 1995; W. J. Swanson, unpublished results). This protein has no effect

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* Corresponding author: Willie J. Swanson. Telephone: 619-534-2146. Fax: 619-534-7313. E-mail: jwswanson@ucsd.edu.

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[1] Abbreviations: ANTS, 1-aminonaphthalene-3,6,8-trisulfonic acid; DPX, *N,N′-p*-xylylenebis(pyridinium bromide); NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; PC, phosphatidylcholine (egg yolk); PE, phosphatidylethanolamine (egg); RET, resonance energy transfer; Rh-PE, *N*-(lissamine rhodamine B sulfonyl)-phosphatidylethanolamine; PS, phosphatidylserine (brain); VE, the vitelline envelope of abalone eggs.

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on the structural integrity of the egg VE, nor does it augment lysin-mediated dissolution of the VE (Swanson & Vacquier, 1995). Its cDNA and deduced amino acid sequences were determined for five species of California abalone. Sequence comparisons reveal that the M_r 18 000 protein has undergone extreme divergence which has been promoted by positive selection (Swanson & Vacquier, 1995). Homology between lysin and the M_r 18 000 protein shows that, although statistically unrelated in pairwise comparisons of primary structure, comparisons of the lysin crystal structure (Shaw et al., 1993) and predictions of the M_r 18 000 protein secondary structure (Rost & Sanders, 1994) and 3D profile analysis (Bowie et al., 1991) reveal a striking similarity between the two proteins, suggesting that they arose by gene duplication and subsequent divergence for functional specialization (Swanson & Vacquier, 1995). In this report, we show that the M_r 18 000 protein is a significantly more potent fusagenic protein than lysin.

EXPERIMENTAL PROCEDURES

Abalone sperm lysin was purified as described (Lewis et al., 1982; Vacquier & Lee, 1993). The M_r 18 000 acrosomal protein was purified from sperm of the abalone species *Haliotis rufescens* (Hr) and *Haliotis fulgens* (Hf) as previously described (Swanson & Vacquier, 1995). Both proteins were dialyzed into 100 mM NaCl/0.1 mM EDTA/5 mM HEPES/0.02% NaN_3 (pH 7.4) and stored at 4 °C. Antibody was commercially prepared in rabbits using the M_r 18 000 protein as the immunogen. The M_r 18 000 bands were excised from sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) gels that had been immersed in 2 M KCl for visualization of the M_r 18 000 band as a clear zone. The gel bands were ground with Freund's complete adjuvant and used for the initial inoculations. Booster inoculations were in incomplete Freund's adjuvant. The antisera gave a single reaction on a Western blot of whole spermatozoa, showing that it recognized only the M_r 18 000 protein (data not shown). Immunofluorescence using a rhodamine-conjugated goat antirabbit IgG as the secondary antibody was done by procedures previously used for sperm from marine invertebrates (Bookbinder et al., 1990).

Abalone sperm (species Hr) were induced to acrosome react by elevation of the pH of the seawater to 9.2 with NaOH. After 30 min (23 °C), the acrosome-reacted sperm were removed by centrifugation at 6000*g* for 30 min. The supernatant, containing the exocytosed acrosomal product, was concentrated by addition of 9 volumes of cold acetone (−20 °C, 30 min). The precipitate was collected by centrifugation at 10000*g* (30 min, 4 °C) and washed twice by resuspension in 70% acetone. The final precipitate was dissolved in 1% SDS, protein was determined by the BCA assay (Pierce), and samples were dissolved in electrophoresis sample buffer (Laemmli, 1970). SDS−PAGE of intact sperm, acrosome-reacted sperm, and the acrosomal exudate utilized 10% polyacrylamide gels (5−25 μg was loaded per lane). Gels were stained with Coomassie blue, destained, and dried between sheets of dialysis membrane (Hoefer, Inc.). The relative percentages of stain in lysin and the M_r 18 000 protein were determined by densitometry of the dried gels scanned at 550 nm. The relative amounts of the two acrosomal proteins were calculated by determining the area of the scanned peaks.

Large unilamellar phospholipid vesicles (liposomes) were prepared in 100 mM NaCl/0.1 mM EDTA/5 mM HEPES (pH 7.4) using PS:PC:PE (Avanti Polar Lipids) in a 1:1:1 molar ratio, by the reverse-phase evaporation technique (Szoka & Papahadjopoulos, 1978). The liposomes were then passed through a 0.2 μm pore diameter Nucleopore filter. For the liposome aggregation experiments, neutral vesicles were formed by the same procedure using only PC. For leakage experiments, liposomes (1:1:1 PS:PC:PE) were prepared in the above buffer with the inclusion of 45 mM DPX and 12.5 mM ANTS (Molecular Probes) and separated from the unencapsulated material on a BioGel P6 (BioRad Laboratories) column (30 × 1 cm) in the above buffer. The resonance energy transfer assay (RET) was used as described (Struck et al., 1981; Hoekstra, 1982) to assay lipid mixing as an indication of liposome fusion. Rh-PE and NBD-PE were incorporated into one batch of liposomes (1:1:1 PS:PC:PE, "labeled liposomes") at 1 mol % each relative to total lipid. The labeled liposomes were mixed in a ratio of 1:9 with unlabeled liposomes (1:1:1 PS:PE:PC); the base line fluorescence of the mixture represented 0% fusion (75 nmol of phospholipid in 1.5 mL in a 1 cm path length quartz cuvette). The fluorescence emission from 0.1 mol % labeled liposomes represented 100% fusion (complete intermixing of lipids results in a 10-fold dilution of labeled lipids). NBD fluorescence was continuously monitored with excitation at 455 nm and emission at 530 nm. Fusion (intermixing of labeled and unlabeled phospholipids) caused an increase in NBD-PE emission due to the loss of energy transfer from NBD-PE (donor) to Rh-PE (acceptor) which was the result of dilution of the labeled lipids. Where investigated in depth, the RET assay has proven to be a reliable indicator of interliposomal fusion (Rapaport et al., 1993; Murata et al., 1987; Wharton et al., 1988; Lear & DeGrado, 1987; Martin et al., 1992).

Liposome aggregation was measured as light scattering by recording the increase in absorption at 380 nm following addition of acrosomal proteins to 50 nmol of phospholipid in 1 mL of the above buffer (Hong & Vacquier, 1986). Leakage of liposome contents was determined by the ANTS/DPX assay (Ellens et al., 1985) with excitation at 360 nm and emission at 514 nm. The base line fluorescence emission of liposomes (75 nmol of phospholipid in 1.5 mL) represented 0% leakage of the probe, and a matching sample lysed in 0.1% Triton X-100 represented maximum leakage (Ellens et al., 1985).

Hydropathy profiles of the mature Hr and Hf M_r 18 000 proteins were made using a sliding window of seven residues (Kyte & Doolittle, 1982). The GenBank Accession Numbers for the two M_r 18 000 proteins are as follows: Hr, L36552; and Hf, L36589 (Swanson & Vacquier, 1995). Helical wheel diagrams of the predicted α-helices and hydrophobic moment calculations were made using the Staden program ANALYSEP (Staden, 1988) as implemented on the suite of programs, DNASYSTEM (Smith, 1988).

RESULTS

Immunofluorescence and Preferential Association of the M_r 18 000 Protein with Acrosome-Reacted Sperm. The two major proteins contained within the abalone sperm acrosomal granule, lysin and the M_r 18 000 protein, are encoded by different loci and are differentially packaged in the granule

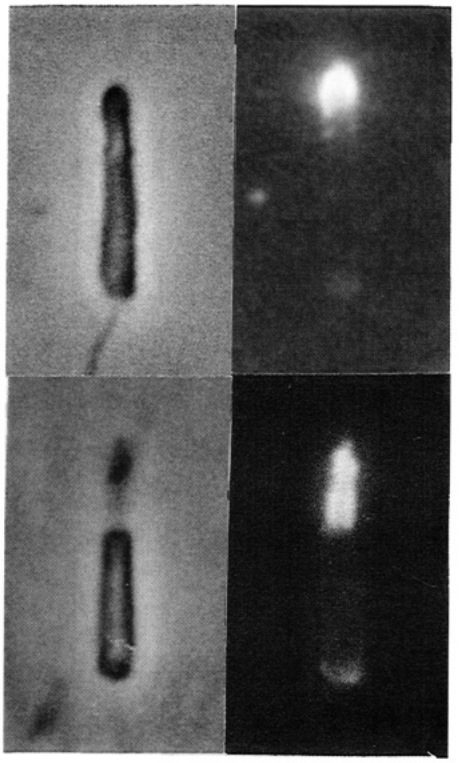


FIGURE 1: Immunofluorescence localization of the M_r 18 000 protein in the acrosomal region of Hr sperm. Left panels are phase contrast and right panels the corresponding immunofluorescence. Upper set shows a partially acrosome-reacted sperm and the lower set a completely acrosome-reacted sperm. Magnification is approximately 2500 times.

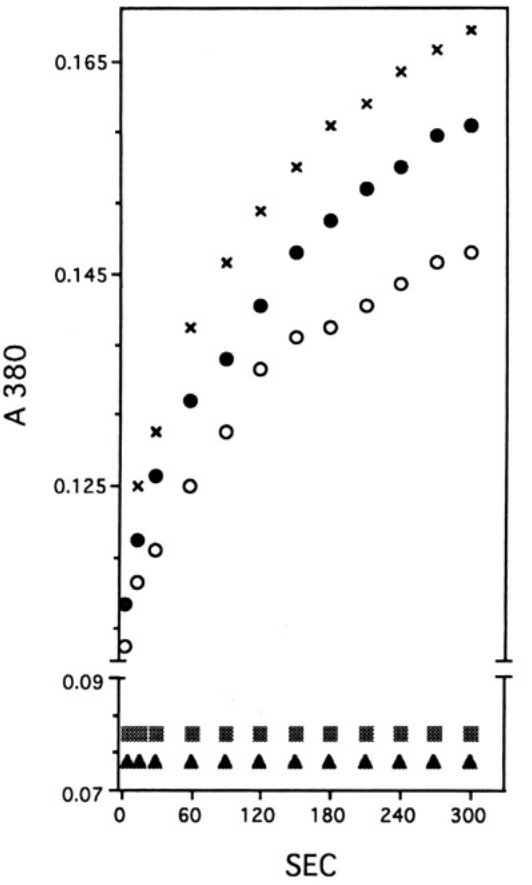


FIGURE 2: Time course of aggregation of PS:PC:PE (1:1:1) liposomes by Hf M_r 18 000 protein. Phospholipid (50 nmol) in 1 mL; 10 μg (×), 5 μg (●), and 2.5 μg (○) M_r 18 000 protein. Neutral liposomes made of only PC were not aggregated by 20 μg of Hf M_r 18 000 protein (■), nor were PS:PC:PE liposomes in buffer without protein or with 10 μg of lysozyme (▲ for both).

during spermiogenesis. Immunogold localization showed that lysin is stored posterior to the M_r 18 000 protein with a clearly visible separation between the two proteins (Haino-Fukushima & Usui, 1986). The M_r 18 000 protein is located at the most anterior part of the acrosome as shown by indirect immunofluorescence of sperm which have just begun to acrosome react (Figure 1, upper two panels; Lewis et al., 1980). Exocytosis of the acrosomal granule occurs at the anterior tip of the granule (Lewis et al., 1980; Usui, 1987). In completely acrosome-reacted sperm, the antibody localizes the M_r 18 000 protein to an area coating the hull of the spent acrosomal granule and the acrosomal process (Figure 1, lower two panels; Lewis et al., 1982, 1980). The greater intensity of the fluorescence from the granule hull obscures the fluorescence from the acrosomal process in photomicrographs. Preimmune serum does not react with sperm (data not shown).

Densitometry of Coomassie blue-stained SDS−PAGE gels of sperm before and after the acrosome reaction, and of the seawater soluble exocytotic product of acrosomal granules, showed that, relative to lysin, a disproportionately greater amount of the M_r 18 000 protein remains associated with sperm after acrosomal granule exocytosis. In relative amounts of Coomassie blue staining protein, in unreacted Hr sperm, the relative quantities of the two acrosomal proteins are 60% lysin and 40% M_r 18 000 protein. However, in acrosome-reacted sperm, this relative percentage changes to 38% lysin and 62% M_r 18 000 protein (a relative increase of 22% in the M_r 18 000 protein). In the seawater soluble acrosomal exudate, the relative amount of lysin is 84% compared to 16% for the M_r 18 000 protein (a relative decrease of 24% in the M_r 18 000 protein). Overnight incubation (4 °C) of acrosome-reacted sperm and soluble exocytotic product before addition of SDS electrophoresis sample buffer showed no differences in the relative amounts of the two acrosomal proteins. Also, the amount of low-molecular weight material migrating at the gel front did not increase with increasing time of incubation at 23 °C before addition of electrophoresis sample buffer. These two observations suggest that proteolytic degradation was not a cause of the disproportionately greater localization of the M_r 18 000 protein, relative to lysin, with acrosome-reacted sperm.

The M_r 18 000 Protein Induces Aggregation, Leakage, and Fusion of Liposomes. The Hf M_r 18 000 protein causes negatively charged liposomes to aggregate as assayed by increased absorption at 380 nm. However, neutral liposomes are not aggregated (Figure 2). Lysozyme, a protein of similar size and pI, does not aggregate these large, unilammellar, negatively charged vesicles (Figure 2). A time course of trypsin digestion of the M_r 18 000 protein, monitored by SDS−PAGE, demonstrated that after partial digestion both the rate and extent of liposome aggregation decrease (data not shown). This suggests that the structure of the M_r 18 000 protein is important for aggregation. The Hf M_r 18 000 protein renders negatively charged liposomes permeable to entrapped probe (Figure 3), showing that the protein associates closely with the lipid bilayer.

Both Hr and Hf M_r 18 000 acrosomal proteins induce the concentration dependent lipid bilayer mixing of negatively

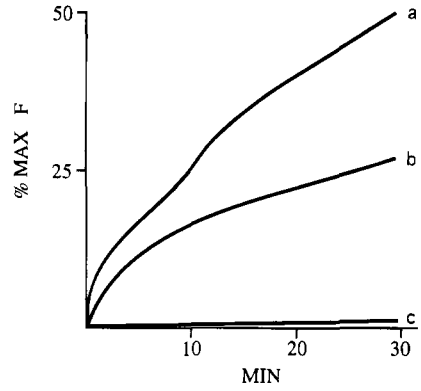


FIGURE 3: Time course of Hf M_r 18 000 protein-induced leakage of liposome contents after addition of 10 μg (a) and 5 μg (b) of protein. Buffer alone (c) did not cause leakage (75 nmol of phospholipid, 1.5 mL). % MAX F refers to the percent maximum fluorescence when the liposomes are lysed with 0.1% Triton X-100.

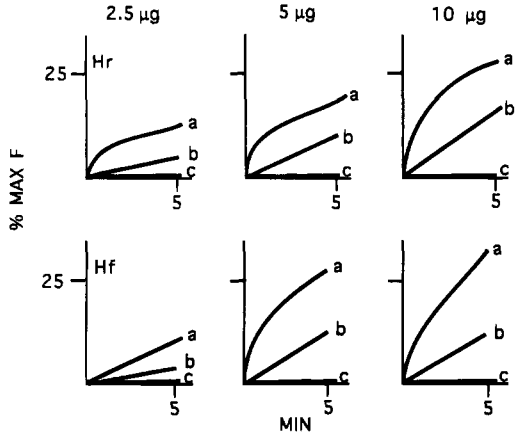
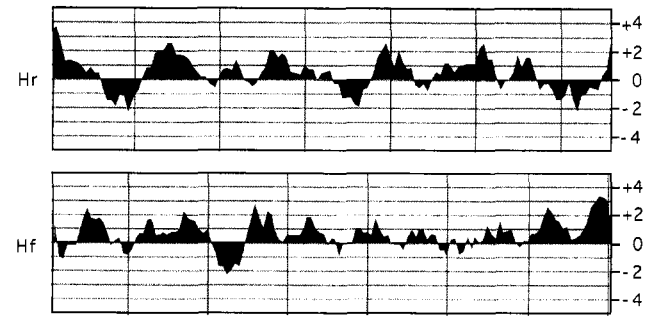


FIGURE 4: Fusion of PS:PC:PE (1:1:1) liposomes induced by the M_r 18 000 protein (a) and lysin (b) at the three concentrations indicated. Buffer alone (c) did not induce fusion. Upper panels are Hr acrosomal proteins, and lower three panels are Hf proteins (75 nmol of phospholipid in 1.5 mL). % MAX F refers to percent maximum fluorescence when total mixing occurred between labeled and unlabeled liposomes.

charged liposomes in the RET assay (Figure 4). Over longer time periods (60 min, 10 μg of protein), these proteins induce 100% mixing of phospholipids. A control experiment using only labeled vesicles showed that no increase in fluorescence occurred upon addition of the M_r 18 000 protein. This indicates that the increased fluorescence of the probe in Figure 4 resulted from its dilution into the bilayer of the unlabeled liposomes (Wilschut & Hoekstra, 1991). Lysin from species Hr had previously been shown to be fusagenic in this assay (Hong & Vacquier, 1986). Thus, it was of interest to compare the fusagenic abilities of lysin and the M_r 18 000 protein under identical conditions. Fusion experiments with negatively charged liposomes show that, for both species, the M_r 18 000 protein is a more potent fusagen than is lysin (Figure 4). The greatest differences in rates of fusion were observed immediately after addition of the proteins to the liposomes. At the three protein concentrations tested, the fusagenic ability of the M_r 18 000 protein was always greater than that of lysin. After 10 min of assay, the extent of fusion in the M_r 18 000 samples was significantly greater (10 replicas, $p < 0.01$) than in the lysin samples. Comparison of the two proteins on the basis of micrograms is valid. The molecular weights of the two proteins from these two species are approximately equal, Hr lysin 16 269 and Hf lysin

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Hr  DKKSTVSKENAAAMKVAMIKFLDSRTDRFKKRIEKIGYPITPPQVTTLLYYNRERLMDWCHNYVEVSKKII
Hf  FDDVV..RQEQSYVQRG.VN...EEMHKLV..FRDMRWNLGPGFVFL.KKV....M.RY.MD.ARY....L

Hr  LLGGNKLNKKNFARMGRIIGWKNQWILKRRQWHM----VRVMRRYKASAIAKKIVAMKVADLPCN
Hf  Q.KHLPV...TLTK...FV.YR.-YGVI.ELYADVFRD.QGF.GP.MT.AMR.YSSKDPGTF..KNEKRRG
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FIGURE 5: Hydropathy plots of the two mature M_r 18 000 proteins (sliding window of seven residues; Kyte & Doolittle, 1982). Vertical lines denote segments of 20 amino acids. Hydrophilic is positive and hydrophobic negative. The Hr (132 residues) and Hf (141 residues) amino acid sequences are shown below. Dots in the Hf sequence denote portions identical to the Hr sequence, and dashes are inserted for alignment (Swanson & Vacquier, 1995).

16 317, compared to the M_r 18 000 proteins of Hr 15 663 and Hf 16 921 (Vacquier & Lee, 1993; Swanson & Vacquier, 1995). The denotation of "M_r 18 000" comes from the M_r of the proteins on SDS–polyacrylamide gels relative to the migration of standard proteins. The migration of the M_r 18 000 protein may be retarded due to its high net positive charge.

Hydropathy Profiles of M_r 18 000 Proteins. The amino acid sequences of the mature Hr and Hf M_r 18 000 proteins are reproduced in Figure 5 along with their hydropathy profiles. The sequences are only 34% identical, indicating that extreme divergence has occurred between these two proteins (Swanson & Vacquier, 1995). The hydropathy profiles of the two proteins show no hydrophobic domains of more than 10 amino acids. The profiles are quite dissimilar, with only four hydrophobic domains of 6–10 residues in the Hr protein and only one such domain in the Hf protein (Figure 5). The plots show that the M_r 18 000 proteins of these two species are predominately hydrophilic.

The predicted α-helical domains of these two proteins (Swanson & Vacquier, 1995) are presented as helical wheels in Figure 6. Each protein has five predicted α-helices in comparable positions (Swanson & Vacquier, 1995; Rost & Sanders, 1994). All 10 predicted α-helices are visually amphipathic, in that charged residues are localized on one side of the helix, whereas hydrophobic residues occupy the opposite side (Figure 6). The hydrophobic moments of these helices (μ_H), a quantitative measure of helical amphipathicity (Eisenberg et al., 1984a), are presented in Table 1. All values of μ_H for the M_r 18 000 proteins are relatively high. For helices 1–4 of both M_r 18 000 proteins, μ_H is greater than that of melittin, a peptide known to be a potent disrupter of membranes (Fujii et al., 1993). Despite the extensive sequence divergence of the two M_r 18 000 proteins, the values of μ_H of corresponding helices are strikingly similar. Furthermore, the charged residues of the corresponding helices of the two proteins are in similar locations in the wheel diagrams (Figure 6). When displayed as a hydrophobic moment plot, the predicted helices group on the boundary of surface active and globular proteins (Eisenberg et al., 1984a). Values of μ_H for the M_r 18 000 proteins are generally higher than those of the homologous helices of

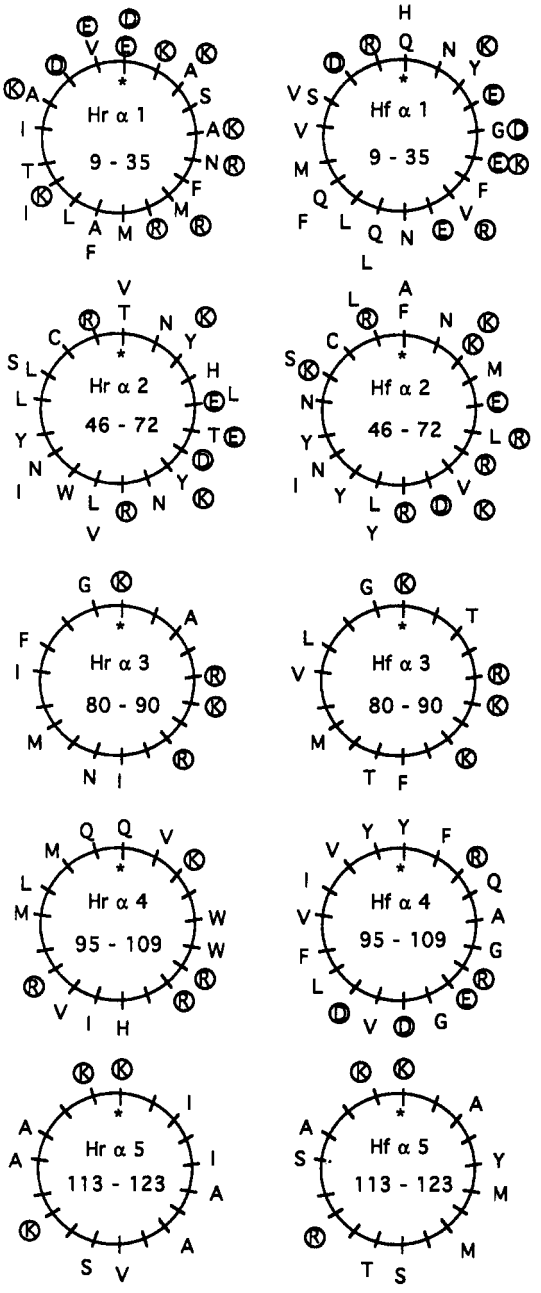

FIGURE 6: Helical wheels of the five predicted α-helices of the Hr and Hf M_r 18 000 proteins. Charged residues are circled, and amino acid positions comprising each helix are given inside each wheel. The NH_2-terminus of each helix is marked with an asterisk. Each perpendicular line is 20° of arc, contiguous residues are 100° apart, and progression from the NH_2-terminus is clockwise. Numbering refers to the Hr sequence (Swanson & Vacquier, 1995).

lysin (Swanson & Vacquier, 1995; Table 1) which could explain why the M_r 18 000 protein is a more potent fusagen than lysin (Figure 4).

DISCUSSION

Myoblast fusion and fertilization are the only normal developmental processes involving cell−cell fusion. The spermatozoa of abalones are advantageous for such studies since they have large acrosome granules from which hundreds of milligrams of "gamete recognition" proteins can be isolated (Lewis et al., 1982; Vacquier & Lee, 1993). The M_r 18 000 abalone acrosomal protein localizes to the proper place to be the mediator of sperm−egg fusion (Mozingo et al., 1995). The protein's location within the acrosomal granule suggests that it coats the acrosomal process as the acrosomal actin polymerizes. Elongation of the acrosomal process from 2 to 7 μm during the acrosome reaction causes it to pass through the bulk of the stored M_r 18 000 protein (Lewis et al., 1980; Haino-Fukushima & Usui, 1986; Usui, 1987). The potent membrane-perturbing character of this protein may be the reason it is contained within a paracrystalline shell (hull) made of 13 nm diameter filaments that lines the inner surface of the acrosomal granule membrane (Lewis et al., 1982; Haino-Fukushima & Usui, 1986; Usui, 1987). The cell may use this packaging strategy to encapsulate the M_r 18 000 protein to protect its cellular membranes during and after spermiogenesis.

Table 1: Hydrophobic Moments of Predicted Amphipathic Helices of the Acrosomal Proteins

	M_r 18 000		lysin	
helix	Hr	Hf	Hr	Hf
1	0.76	0.72	0.78	0.68
2	0.58	0.79	0.45	0.48
3	0.74	0.70	0.24	0.24
4	0.59	0.64	0.38	0.15
5	0.40	0.39	0.43	0.09
mean	0.61	0.65	0.46	0.33

Comparing acrosome-intact to acrosome-reacted sperm, a disproportionately greater amount of the M_r 18 000 protein, compared to lysin, remains associated with acrosome-reacted sperm. The purified, seawater soluble M_r 18 000 protein has no effect on the egg VE nor on the surface of the oocyte (Swanson & Vacquier, 1995). Unlike the acrosomal protein from the marine worm *Urechis caupo* (Gould & Stephano, 1991), the M_r 18 000 protein alone, or mixed with lysin, does not activate abalone oocytes (as judged by the absence of polar body formation).

Because there are no quantitative, controllable assays to assess the fusagenic potential of an isolated protein in the sperm−egg fusion reaction, assays employing liposomes have been used to investigate the fusagenic properties of sperm proteins. Abalone sperm lysin was previously shown to fuse negatively charged liposomes (Hong & Vacquier, 1986). Here, it is shown that the M_r 18 000 protein is a more potent inducer of interliposomal bilayer mixing than lysin, especially immediately after addition of protein (Figure 4). This is consistent with the general observation that sperm−egg fusion in animals occurs within seconds after the acrosome reaction. We previously demonstrated that lysin and the M_r 18 000 protein are duplicated genes by comparison of lysin's crystal structure and the predicted secondary structure of the M_r 18 000 protein and confirmed this observation with 3D compatibility analysis (Swanson & Vacquier, 1995; Bowie et al., 1991). Hypothetically, the ancestral acrosomal protein possessed the ability both to dissolve the egg VE and to mediate the fusion of gamete membranes. Currently, the M_r 18 000 protein is ineffective at dissolving the egg VE but fuses liposomes at a faster rate than lysin. Lysin dissolves the VE and still retains some membrane-fusing activity.

The high net positive charge cannot be the sole reason for the M_r 18 000 protein being a more potent fusagen of liposomes than lysin (Figure 4). For the Hf comparison, lysin is more positively charged than the M_r 18 000 protein. The

net positive charges on these two acrosomal proteins from these two abalone species are Hr lysin +13 (pI 10.0) and Hf lysin +21 (pI 11.3), compared to M_r 18 000 Hr +22 (pI 10.6) and Hf +18 (pI 10.4). It has previously been shown that polylysine does not induce intervesicular lipid mixing of large negatively charged liposomes in the RET assay, although it does aggregate them (Fujii et al., 1992). Lysozyme (MW 14 300, pI 10.0) and partially proteolysed M_r 18 000 protein do not aggregate negatively charged liposomes, indicating that aggregation is not solely due to small charged molecules. However, the greater average amphipathicity of the α-helices (Table 1) of the M_r 18 000 proteins, compared to the lysins, correlates with their greater fusagenic potency (Figure 4). Although the two M_r 18 000 proteins in this study are only 34% identical in amino acid sequence, prediction of secondary structure, placement of charged residues as observed on a helical wheel (Figure 6), and hydrophobic moment calculations of the amphipathic helices (Table 1) are strikingly similar for the two proteins.

As recently discussed (Rapaport et al., 1993), the three attributes of most fusagenic proteins are high positive charge, high α-helical content, and aggregation of the protein within the lipid bilayer. The M_r 18 000 proteins of abalone sperm fulfill the first two criteria; however, nothing is yet known concerning aggregation of the protein within the bilayer. The virus fusion proteins have well-defined hydrophobic regions of approximately 20 amino acids located in the amino-terminal or central portion of their sequence which are thought to insert into the bilayer (Gething et al., 1986; Lear & DeGrado, 1987). The M_r 18 000 proteins do not possess such "fusion domains." Given the background information on proteins which are capable of fusing lipid bilayers, the most important attribute of the M_r 18 000 proteins is the possession of multiple, strongly amphipathic α-helices. By strongly amphipathic, we mean that these helices have hydrophobic moments greater than that of mellitin, one of the most potent membrane disruptive natural peptides known (Morgan et al., 1984; Murata et al., 1984). The induction of lipid mixing in the PS:PC:PE vesicles most probably occurs by neutralization of the protein's positive charge by PS (Yoshimura et al., 1992; Fujii et al., 1992), followed by insertion of the protein into the lipid bilayer (Rapaport et al., 1993; Fujii et al., 1992). An important general characteristic of fusagenic proteins may be their ability to form a three-dimensional amphipathic structure (Fuji et al., 1993). The cationic β-structured defensin peptides are amphipathic and induce fusion of liposomes (Fujii et al., 1992). The potential fusion domain of the guinea pig sperm protein PH-30 forms β-structure when bound to membranes and mediates liposome fusion by the RET assay (Muga et al., 1994). The hydrophobic moment (calculated on the basis of β-structure, $\delta = 160°$; Eisenberg et al., 1984b; Fujii et al., 1992) of the fusagenic peptide from PH-30 (μ_H 0.50) is quite high and is similar to that of the fusagenic defensins (μ_H 0.49−0.60; Fujii et al., 1992). The guinea pig sperm protein PH-30 and the abalone sperm M_r 18 000 protein may share a similar mechanism of membrane fusion despite the extreme differences in structure.

Location of the M_r 18 000 protein on sperm, predicted secondary structure, calculated hydrophobic moments, and interaction with liposomes are consistent with the hypothesis that M_r 18 000 abalone sperm proteins play a role in sperm−egg fusion. The extreme divergence of this protein, driven by natural selection (Swanson & Vacquier, 1995), suggests that it may mediate the fusion process in a species-selective manner. For this to be the case, the fusion process mediated by the M_r 18 000 sperm protein would have to involve a complementary receptor on the egg plasma membrane.

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REFERENCES

Aiba, K., Yanagisawa, K., & Urushihara, H. (1993) *J. Gen. Microbiol. 139*, 279−285.
Bookbinder, L. H., Moy, G. W., & Vacquier, V. D. (1990) *J. Cell Biol. 111*, 1859−1866.
Bowie, J. U., Luthy, R., & Eisenberg, D. (1991) *Science 253*, 164−170.
Eisenberg, D., Schwarz, E., Komaromy, M., & Wall, R. (1984a) *J. Mol. Biol. 179*, 125−142.
Eisenberg, D.,Weiss, R. M., & Terwilliger, T. C. (1984b) *Proc. Natl. Acad. Sci. U.S.A. 81*, 140−144.
Ellens, H., Bentz, J., & Szoka, F. C. (1985) *Biochemistry 24*, 3099−3106.
Fujii, G., Horvath, S., Woodward, S., Eiserling, F., & Eisenberg, D. (1992) *Protein Sci. 1*, 1454−1464.
Fujii, G., Selsted, M. E., & Eisenberg, D. (1993) *Protein Sci. 2*, 1301−1312.
Gething, M.-J., Doms, R. W., York, D., & White, J. (1986) *J. Cell Biol. 102*, 11−23.
Glabe, C. G. (1985) *J. Cell Biol. 200*, 800−806.
Glabe, C. G., & Clark, D. (1991) *Dev. Biol. 143*, 282−288.
Gould, M., & Stephano, J. L. (1991) *Dev. Biol. 146*, 509−518.
Haino-Fukushima, K., & Usui, N. (1986) *Dev. Biol. 115*, 27−34.
Hoekstra, D. (1982) *Biochim. Biophys. Acta 692*, 171−175.
Hong, K., & Vacquier, V. D. (1986) *Biochemistry 25*, 543−549.
Kyte, J., & Doolittle, R. F. (1982) *J. Mol. Biol. 157*, 105−132.
Laemmli, U. K. (1970) *Nature 227*, 680−685.
Lear, J. D., & DeGrado, W. F. (1987) *J. Biol. Chem. 262*, 6500−6505.
Lee, Y.-H., Ota, T., & Vacquier, V. D. (1995) *Mol. Biol. Evol. 12*, 231−238.
Lewis, C. A., Leighton, D. L., & Vacquier, V. D. (1980) *J. Ultrastruct. Res. 72*, 39−46.
Lewis, C. A., Talbot, C. F., & Vacquier, V. D. (1982) *Dev. Biol. 92*, 227−239.
Lognonne, J. L., & Wahrmann, J. P. (1988) *Cell Differ. 22*, 245−258.
Longo, F. J., Cook, S., McCulloh, D. H., Ivonnet, P. I., & Chambers, E. L. (1994) *Zygote 2*, 317−331.
Martin, I., Defrise-Quertain, F., Nielson, N. M., Saermark, T., Burny, A., Brasseur, R., Vandenbranden, M., & Ruysschaert, J. M. (1992) in *Membrane Interactions of HIV: Implications for Pathogenesis and Therapy of AIDS* (Aloia, R. C., & Curtain, C. C., Eds.) pp 365−376, Wiley-Liss, New York.
Metz, E. C., Kane, R. E., Yanagimachi, H., & Palumbi, S. R. (1994) *Biol. Bull. 187*, 23−34.
Miraglia, S. J., & Glabe, C. G. (1993) *Biochim. Biophys. Acta 1145*, 191−198.
Morgan, D. G., Williamson, H., Fuller, S., & Hudson, B. (1984) *Biochem. Biophys. Acta 732*, 668−674.
Moy, G. W., & Vacquier, V. D. (1979) *Curr. Top. Dev. Biol. 13*, 31−44.
Mozingo, N. M., Vacquier, V. D., & Chandler, D. E. (1995) *Mol. Reprod. Dev. 41*, 493−502.
Muga, A., Neugebauer, W., Hirama, T., & Surewicz W. K. (1994) *Biochemistry 33*, 4444−4448.
Murata, M., Sugahara, Y., Takahashi, S., & Ohnishi, S. (1984) *J. Biochem. 102*, 957−962.
Murata, M., Nagayama, K., & Ohnishi, S. (1987) *Biochemistry 26*, 4056−4062.
Myles, D. G. (1993) *Dev. Biol. 158*, 35−45.

Myles, D. G., Kimmel, L. H., Blobel, C. P., White, J. M., & Primakoff, P. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 4195−4198.
Rapaport, D., Hague, R., Pouny, Y., & Shai, Y. (1993) *Biochemistry 32*, 3291−3297.
Rost, B., & Sanders, C. (1994) *Proteins 19*, 55−72.
Shaw, A., McRee, D. E., Vacquier, V. D., & Stout, C. D. (1993) *Science 262*, 1864−1867.
Smith, D. W. (1988) *Comput. Appl. Biosci. 4*, 212.
Staden, R. (1988) *Comput. Appl. Biosci. 4*, 53−60.
Struck, D., Hoekstra, D., & Pagano, R. E. (1981) *Biochemistry 20*, 4093−4099.
Swanson, W. J., & Vacquier, V. D. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 4957−4961.
Szoka, F. C., & Papahadjopoulos, D. (1978) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4194−4198.
Usui, N. (1987) *Gamete Res. 16*, 37−45.
Vacquier, V. D., & Lee, Y.-H. (1993) *Zygote 1*, 181−196.
Vacquier, V. D., Swanson, W. J., & Hellberg, M. E. (1995) *Dev., Growth Differ. 37*, 1−10.
Wharton, S. A., Martin, S. R., Ruigrok, R. W. H., Skehel, J. J., & Wiley, D. C. (1988) *J. Gen. Virol. 69*, 1847−1857.
White, J. M. (1992) *Science 258*, 917−924.
Wilschut, J., & Hoekstra, D., Eds. (1991) *Membrane Fusion*, p 902, Marcel Dekker Inc., New York.
Wolfsberg, T. G., Bazan, F., Blobel, C. P., Myles, D. G., Primakoff, P., & White, J. M. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 10783−10787.
Yoshimura, T., Goto, Y., & Aimoto, S. (1992) *Biochemistry 31*, 6119−6126.

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