Identification and Purification of a Sperm Surface Protein with a Potential Role in Sperm–Egg Membrane Fusion

Paul Primakoff, Hilary Hyatt, and Joanne Tredick-Kline

Department of Physiology, University of Connecticut Health Center, Farmington, Connecticut 06032

Abstract. Sperm–egg plasma membrane fusion during fertilization was studied using guinea pig gametes and mAbs to sperm surface antigens. The mAb, PH-30, strongly inhibited sperm–egg fusion in a concentration-dependent fashion. When zona-free eggs were inseminated with acrosome-reacted sperm preincubated in saturating (140 μg/ml) PH-30 mAb, the percent of eggs showing fusion was reduced 75%. The average number of sperm fused per egg was also reduced by 75%. In contrast a control mAb, PH-1, preincubated with sperm at 400 μg/ml, caused no inhibition. The PH-30 and PH-1 mAbs apparently recognize the same antigen but bind to two different determinants. Both mAbs immunoprecipitated the same two ^{125}I-labeled polypeptides with M_r 60,000 (60 kD) and M_r 44,000 (44 kD). Boiling a detergent extract of sperm severely reduced the binding of PH-30 but had essentially no effect on the binding of PH-1, indicating that the two mAbs recognize different epitopes.

Immunoelectron microscopy revealed that PH-30 mAb binding was restricted to the sperm posterior head surface and was absent from the equatorial region. The PH-30 and PH-1 mAbs did not bind to sperm from the testis, the caput, or the corpus epididymis. PH-30 mAb binding was first detectable on sperm from the proximal cauda epididymis, i.e., sperm at the developmental stage where fertilization competence appears.

After purification by mAb affinity chromatography, the PH-30 protein retained antigenic activity, binding both the PH-30 and PH-1 mAbs. The purified protein showed two polypeptide bands of 60 and 44 kD on reducing SDS PAGE. The two polypeptides migrated further (to ~49 kD and ~33 kD) on nonreducing SDS PAGE, showing that they do not contain interchain disulfide bonds, but probably have intrachain disulfides. 44 kD appears not to be a proteolytic fragment of 60 kD because V8 protease digestion patterns did not reveal related peptide patterns from the 44- and 60-kD bands. In the absence of detergent, the purified protein precipitates, suggesting that either 60 or 44 kD could be an integral membrane polypeptide.

MEMBRANE fusion is a universal process in eukaryotic cells. It occurs during membrane biosynthesis and membrane protein sorting, phagocytosis, endocytosis, exocytosis, and cell division. Cell–cell membrane fusion is also important in development. Examples include sperm–egg fusion in fertilization, and myoblast and osteoblast fusion in muscle and bone development.

Despite the importance of membrane fusion to cellular function and organismal development, little is known about how this process occurs. Considerable progress has been made in studying the mechanism of membrane fusion in only one case, the fusion of membrane-enveloped viruses with cellular membranes. With certain viruses, whose membrane protein composition is simple, two viral activities involved in fusion have been defined by liposome reconstitution studies. One activity binds the virus to the cellular membrane, while the second activity leads directly to fusion of the two membranes. The binding and fusion activities can be in the same protein, e.g., the HA protein of influenza virus, or in two distinct proteins, e.g., the HN protein for binding and the F protein for fusion, in Sendai virus. The studies with viruses have shown that "fusion protein"-induced membrane fusion will occur between plasma membranes of cells expressing a cloned gene for the fusion protein, between viral and intracellular membranes, or between viral and artificial membranes (White et al., 1983; Kondor-Koch et al., 1983; Florkiewicz and Rose, 1984; Paterson et al., 1985; Gething et al., 1986).

With the more complex membranes that fuse during cellular functions such as exocytosis, endocytosis, and fertilization, the molecular components involved in the fusion process are unknown. Antibodies can potentially be used to identify proteins involved in membrane fusion in these complex systems. The usefulness of the antibody approach is shown by recent studies of mAbs that recognize the fusion proteins of certain viruses. These studies collectively show that despite the relatively large size of antibody molecules and the requirement for close physical approach of two fusing membranes, only some mAbs to viral fusion proteins inhibit fusion, whereas others do not (Orvell and Grandien,

1982; Noble et al., 1983; Florkiewicz and Rose, 1984; Orvell, 1984).

The results in the current study identify the sperm-surface antigen PH-30 as a candidate for a protein with an essential function in sperm–egg fusion. We have found one mAb, PH-30, to the PH-30 protein that strongly inhibits guinea pig sperm–egg fusion, and a second mAb, PH-1, to the PH-30 protein that does not inhibit fusion, similar to the pattern found in the virus studies. Using affinity chromatography, we have purified the PH-30 protein and obtained it in a form where it is antigenically active in the dialyzable detergent octylglucoside. This should allow ready reconstitution of the PH-30 protein into liposomes and definition of its role in the membrane fusion process.

Materials and Methods

Preparation of Gametes

Sperm were obtained from the caudae epididymides of mature (>800 g) male Hartley guinea pigs and suspended at 1×10^8/ml in 0.9% NaCl. For sperm capacitation, 100 μl of this sperm suspension were added to 1.4 ml of modified Tyrode's medium (mT)[1] lacking $CaCl_2$ (mT-Ca^{2+}) and incubation of this 1.5-ml sperm suspension continued under mineral oil overnight (16–18 h) at 37°C (Fleming and Yanagimachi, 1981). The following morning 1.5 ml of mT containing 4 mM $CaCl_2$ (mT + $2 \times Ca^{2+}$) was added to induce the acrosome reaction of the sperm in the final volume, 3 ml of mT + 2 mM $CaCl_2$ (mT + Ca^{2+}) (Fleming and Yanagimachi, 1981).

Mature eggs were prepared by overnight culture of ovarian oocytes obtained from virgin female Hartley guinea pigs, 275–350 g (Yanagimachi, 1978). The ovaries were removed and placed in 5 ml oocyte medium (Table 2 in Yanagimachi, 1978). Extraneous tissue was dissected away and each ovary transferred to 3 ml fresh oocyte medium. Immature oocytes in a cumulus mass were extruded from the ovary by poking it with a sharp needle on a 1-cc syringe while gently holding the ovary with a forceps. Extruded oocytes in the cumulus mass were washed three times through 0.25 ml of oocyte medium under mineral oil at 37°C. Degenerated eggs were discarded. The remaining oocytes were cultured in oocyte medium 20–24 h at 37°C under 5% CO_2 and those that had extruded the first polar body were selected for the experiments. Yields of eggs varied considerably with each animal, but averaged about 15 eggs per animal. Shortly before they were mixed with sperm, the eggs' cumulus layers and zonae were enzymatically removed (Yanagimachi, 1972) and the eggs were washed in mT + Ca^{2+}.

Sperm–Egg Fusion Assay

A standard fusion assay was used in which the zona-free eggs are inseminated with acrosome-reacted sperm. About 10 min after Ca^{2+} addition to sperm to induce the acrosome reaction, 70 μl of purified mAb in mT + Ca^{2+} (test antibody) or 70 μl of mT + Ca^{2+} (medium control) was added to 30 μl of the acrosome-reacted sperm, diluted to achieve a final concentration of 5×10^4 sperm/ml in the 100-μl drop. After incubation of the sperm-mAb mixture at 37°C for 15–20 min, the zona-free guinea pig eggs were introduced into the 100-μl drop. After 1.5–2 h, eggs were fixed with acid–alcohol, stained with acetolacmoid, and scored for the presence of swollen sperm heads with associated tails in the cytoplasm to determine the number of sperm that had fused with each egg (Yanagimachi, 1972).

Monoclonal Antibodies

Isolation of the hybridoma lines producing the mAbs PH-30 and PH-1 has been previously described (Myles et al., 1981; Primakoff and Myles, 1983). PH-30 and PH-1 recognize two different determinants on the same sperm surface protein (see Results). The PH-30 mAb is an IgG1 and PH-1 is an IgG2b as determined by Ouchterlony analysis (Kohler, 1980). PH-30 and PH-1 were purified from culture supernatant or ascites fluid by affinity chromatography on the Bio-Rad monoclonal antibody purification system, a protein A-agarose matrix, following the instructions of the supplier (Bio-Rad Laboratories, Richmond, CA). The isolated mAbs were >90% pure when examined by SDS PAGE and maintained approximately the same level of antigen binding activity found in the starting culture supernatant or ascites fluid.

1. *Abbreviations used in this paper*: mT, modified Tryode's medium; OG, octylglucoside; 44 kD and 60 kD, polypeptides of M_r ~44,000 and ~60,000, respectively.

Isolation of Sperm of Distinct Developmental Stages

Testicular sperm were obtained as previously described (Myles and Primakoff, 1983). To obtain selected segments of the epididymis, a testis with fat pad and epididymis intact were removed and submerged in Mg^{2+}-Hepes medium (Green, 1978). Fat cells were teased away and the epididymis was traced back to the caput and corpus regions. The caput, corpus, and proximal and distal cauda regions of the epididymis, as defined by Hoffer and Greenberg (1978), were dissected and the sperm were suspended in Mg^{2+}-Hepes, washed, and tested for mAb binding.

Indirect Immunofluorescence

Indirect immunofluorescence was performed as previously described (Myles et al., 1981; Primakoff and Myles, 1983). Live acrosome-reacted sperm were stained with PH-30 or PH-1 followed by rhodamine-conjugated goat anti-mouse IgG (CooperBiomedical Inc., Malvern, PA). After staining, cells were fixed with formaldehyde and photographed on a Zeiss Universal microscope equipped with epifluorescent optics using Kodak Tri-X film.

Immunoelectron Microscopy

Live acrosome-reacted and acrosome-intact sperm were stained with the PH-30 mAb followed by 10 nm colloidal gold-conjugated goat IgG anti-mouse IgG (Janssen Pharmaceutica, Beerse, Belgium). The cells were fixed with glutaraldehyde and osmium and processed by standard techniques for transmission electron microscopy.

Surface Labeling and Immunoprecipitation

Sperm, freshly removed from the cauda epididymis, were surface labeled with ^{125}I by the Iodogen method following procedures somewhat modified from our previous description (Primakoff and Myles, 1983). In particular, the Iodogen was stored desiccated at 4°C rather than without desiccation at room temperature, and the time between removing the sperm from the animal and completing the iodination was kept to an absolute minimum. Speed in preparing the sperm for the iodination increases the viability and motility observed when the iodination is complete and has resulted in improved immunoprecipitation with several mAbs in our collection. The steps in the surface labeling procedure were otherwise as described previously (Primakoff and Myles, 1983). The surface labeled sperm were lysed in 1% Triton X-100 and immunoprecipitation, SDS PAGE, and autoradiography were performed as previously described (Primakoff and Myles, 1983).

PH-30 Antigen Purification

The PH-30 antigen was purified by affinity chromatography on a PH-30 mAb-Sepharose column. The column was prepared by coupling purified PH-30 mAb to cyanogen-bromide-activated Sepharose 4B (Sigma Chemical Co., St. Louis, MO). PH-30 mAb in coupling buffer (Mescher et al., 1983) was incubated with washed, activated Sepharose beads at a ratio of 2 mg mAb to 1 ml swollen beads and subsequent steps in the coupling and washing of the beads were performed according to Mescher et al. (1983).

Caudae epididymides were removed from male guinea pigs and placed at 4°C in Mg^{2+}-Hepes medium containing a protease inhibitor cocktail that included 10 mM EDTA, 70 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, 2 μg/ml antipain, 10 μg/ml benzamidine, 1 μg/ml chymostatin, and 1 μg/ml pepstatin. Dissection of the caudae and all subsequent procedures were done at 4°C. The sperm were expressed from the caudae into the medium and washed in 200 ml of the medium. The sperm were resuspended at 1×10^8/ml in 60 ml of solubilization buffer, 0.01 M Tris, pH 8.0, 0.14 M NaCl, 30 mM octylglucoside (OG), and the protease inhibitor cocktail. The lysed sperm suspension was centrifuged at 100,000 *g* for 45 min to remove nuclei, axonemes, and particulate matter, and passed through a 45-μm Nalgene filter.

In initial experiments it was found that when the PH-30 mAb column was loaded to capacity (about 10 column volumes of centrifuged, filtered OG extract of sperm at 1×10^8 ml), almost all of the bound PH-30 antigen was lost during washing of the column. This finding of high losses during washing in mAb affinity chromatography typically indicates the utilized

mAb is of low or modest affinity (Johnson et al., 1985). In such cases, a useful purification strategy is to underload the column relative to its total antigen-binding capacity (Johnson et al., 1985). Underloading the column with antigen allows antigen that dissociates during the washing to rebind to unoccupied antibody sites.

Following this strategy, we used a 20-ml PH-30 mAb Sepharose column loaded with 60 ml of centrifuged, filtered OG extract of sperm at 1×10^8/ml. The extract was first applied to a 10-ml precolumn of Sepharose 4B, and the flow through from this column was applied to the 20-ml column of PH-30 mAb-Sepharose. The extract was loaded onto the column at a flow rate of 20 ml/h and the column was washed with 100 ml of solubilization buffer maintaining a flow rate of 20 ml/h. After the column was washed, the PH-30 antigen was eluted with 50 mM diethylamine, pH 11.5, containing 30 mM OG. After elution began, fractions were collected in 5-ml volumes, were immediately neutralized with 0.5 ml 2 M Tris-HCl, pH 7.6, and were concentrated by ultrafiltration before protein determination and SDS PAGE.

The starting lysate and other column fractions were applied to microtiter plates and assayed in the radioactive solid phase binding assay for their ability to bind the PH-30 and PH-1 mAbs. The fractions were also assayed for antigenic activity by measuring their ability to inhibit the solid-phase assay, since an inhibitory assay can give a quantitative measure of the amount of antigen present relative to the starting extract (Brown et al., 1981; Mescher et al., 1983; Johnson et al., 1985).

Radioactive Solid-Phase Binding Assay

This assay was performed essentially as previously described (Primakoff and Myles, 1983). Briefly, a 30-mM OG extract of 1×10^8 sperm/ml was prepared as previously described (Myles et al., 1984), diluted 1/10 in PBS so that it contained only 3 mM OG, and applied to a 96-well microtiter plate. The plate was washed, unbound sites blocked with 3% ovalbumin in PBS, and the mAb to be tested was incubated in the wells. After washing, bound mAb was detected with ^{125}I-rabbit anti-mouse IgG (New England Nuclear, Boston, MA).

Assay of Antigenic Activity

The amount of antigenic activity in cell fractions (i.e., OG extracts) or column fractions was measured by determining the ability of the fraction to inhibit mAb binding in the radioactive solid-phase binding assay. For the inhibition experiments, the PH-30 or PH-1 mAb was diluted so that it showed binding 5–10-fold above background but was not saturating in the solid-phase assay, and the fractions were dialyzed against PBS containing 6 mM OG. 100 μl of diluted mAb was preincubated with 100 μl of serial twofold dilutions of the dialyzed fraction to be tested for inhibition. Aliquots of the preincubated mAb-fraction mixture were used in the solid-phase binding assay to determine residual mAb binding. A curve of percent inhibition versus dilution of fraction was plotted (for example, see Fig. 4). Following the definition of Brown et al. (1981), one unit of antigenic activity is defined as the amount of antigen needed to give 50% inhibition of the solid phase assay.

Protein Determination

The amounts of protein in the initial sperm extract and in the eluted, concentrated fractions from the affinity column were determined with the Pierce BCA protein assay reagent, according to the supplier's instructions (Pierce Chemical Co., Rockford, IL).

Results

Previously we have described the isolation of a collection of mAbs to surface antigens of guinea pig sperm (Primakoff and Myles, 1983). Many of these mAbs in culture supernatant, dialyzed against mT + Ca^{2+} medium, were surveyed for their ability to inhibit the fusion of guinea pig sperm with zona-free guinea pig eggs. One mAb, PH-30, strongly inhibited sperm–egg fusion. This led us to purify the mAb and retest its effect on the cell–cell fusion. In testing the purified PH-30 mAb, we compared it with purified PH-1 mAb, which binds to a different epitope on the same antigen (see below).

Purified PH-30 and PH-1 mAbs were examined in the same experiment for their ability to inhibit the sperm–egg fusion assay. Capacitated, acrosome-reacted sperm were preincubated with medium alone (medium control) or with one of the mAbs; 15–20 min later, zona-free eggs were introduced. The percent of eggs showing fusion (fertilization rate) was inhibited 65% by the PH-30 mAb; the average number of sperm fused per egg (fertilization index) was inhibited 76% by the PH-30 mAb (Table I). In contrast, the PH-1 mAb caused no inhibition (Table I).

Table II shows data compiled from 16 individual experiments, where inhibition of sperm–egg fusion was tested at different concentrations of the PH-30 mAb. A saturating inhibition of ~75% for the fertilization rate was observed at 140 μg/ml PH-30 mAb (Table II). The inhibition of the fertilization index by the PH-30 mAb paralleled the inhibition of fertilization rate; a saturating inhibition of ~75% was found at 140 μg/ml PH-30 mAb. The PH-1 mAb at 400 μg/ml did not inhibit (Table II).

Control experiments were done to check if the PH-30 mAb might be inhibiting sperm–egg fusion by an effect on sperm motility or by some effect on the eggs. Examining antibody preincubated sperm in the insemination dishes, we detected no effect of either the PH-30 or PH-1 mAbs on sperm motil-

Table I. Inhibition of Sperm–Egg Fusion by mAbs

	Eggs tested	Eggs fused	Fertilization rate* (% eggs fused)	Inhibition of fertilization rate	Total sperm fused	Fertilization index‡ (average number of sperm fused/egg)	Inhibition fertilization index
	n	*n*	%	%	*n*		%
PH-30	33	8	24	65	10	0.3	76
PH-1	34	24	71	0	52	1.5	0
Medium control	40	27	68	–	50	1.25	–

Inhibition of sperm–egg fusion by mAbs. Sperm were capacitated by overnight incubation in mT lacking Ca^{2+}. An equal volume of mT + 4 mM $CaCl_2$ was added to induce the acrosome reaction. Sperm were diluted in mT + Ca^{2+} and 30 μl of diluted sperm were added to 70 μl purified mAb in mT + Ca^{2+} or to 70 μl mT + Ca^{2+} alone (medium control). In the 100 μl, the final sperm concentration was 5×10^4/ml and the final mAb concentration was 400 μg/ml (PH-30, PH-1) or 0 μg/ml (medium control). After 15–20 min incubation, zona-free eggs were added to the 100-μl drop, and after an additional 1.5–2 h incubation, eggs were fixed, stained, and scored for the presence of swollen sperm heads with associated tails in the cytoplasm. Data from two individual experiments were added together to give the numbers of eggs tested, eggs fused, and sperm fused shown in the Table.

* Fertilization rate = $\frac{\text{(number of eggs fused)}}{\text{(number of eggs tested)}}$ expressed as a percentage.

‡ Fertilization index = $\frac{\text{(total number of sperm fused)}}{\text{(number of eggs tested)}}$ = average number of sperm fused per egg.

Table II. Inhibition of Sperm-Egg Fusion by mAbs

mAb	Inhibition of fertilization rate*	Inhibition of fertilization index‡	Number of experiments	Total eggs
μg/ml	%	%		*n*
PH-30 mAb				
40	32	9	1	28
100	32	39	2	45
140	74 ± 16	73 ± 19	7	160
400	77 ± 8	82 ± 8	6	154
PH-1 mAb				
400	0	0	3	48

Inhibition of sperm–egg fusion by the PH-30 and PH-1 mAbs. The protocol used in each experiment was the same as in Table I. The percent inhibition shown is the mean ± SD of the percent inhibition obtained in each individual experiment testing the mAb at the concentration shown.
* Fertilization rate, the percent of eggs that fuse, as defined in Table I.
‡ Fertilization index, the average number of sperm fused per egg, as defined in Table I.

ity. To test if the PH-30 mAb might be inhibiting fusion through some action on the egg, zona-free eggs were incubated in 140 μg/ml PH-30 mAb and then washed in mT + Ca^{2+} and inseminated. No inhibition was observed. In the sperm–egg fusion assay, fusion is detected by observing a later event, the swelling of the sperm nucleus in the egg cytoplasm. Thus the PH-30 mAb might conceivably block events subsequent to sperm–egg plasma membrane fusion and this possibility is considered further in the Discussion.

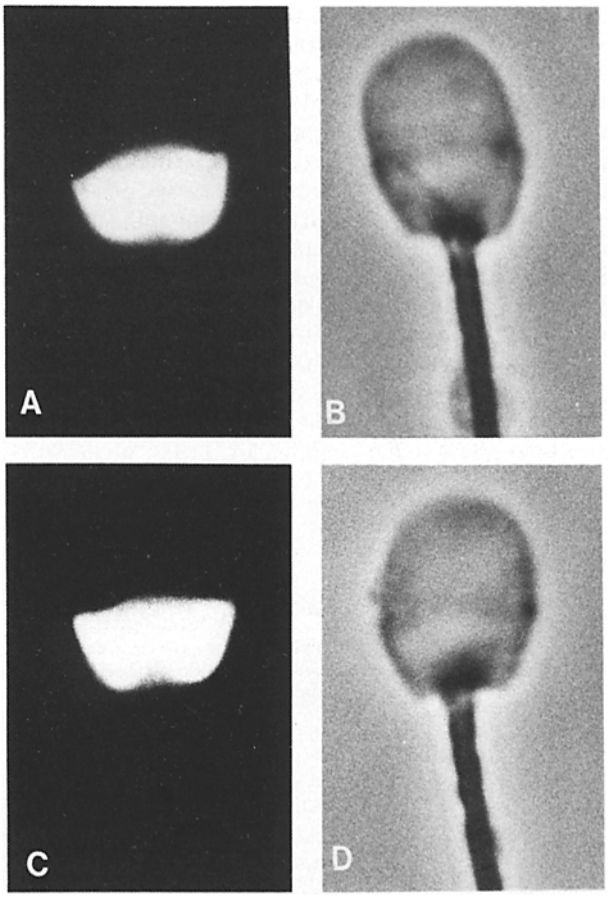

Figure 1. Indirect immunofluorescence of the PH-30 and PH-1 mAbs on acrosome-reacted sperm. Live acrosome-reacted sperm were stained with either the PH-30 or PH-1 mAb followed by rhodamine-conjugated goat anti-mouse IgG. (*A*) Fluorescence micrograph, PH-30. (*B*) Phase contrast micrograph of the same cell. (*C*) Fluorescence micrograph, PH-1. (*D*) Phase contrast micrograph of the same cell.

Surface Localization of the PH-30 Antigen

Previously the PH-30 and PH-1 mAbs have been shown to bind to the posterior head (post-acrosomal) region of acrosome-intact sperm (Primakoff and Myles, 1983). Because sperm that are capable of fusing with the egg are apparently acrosome-reacted (Yanagimachi, 1981), acrosome-reacted cells with bound PH-30 or PH-1 were observed by indirect immunofluorescence. mAb binding on these cells was also localized to the posterior head (Fig. 1).

Although it is clear the PH-30 mAb binds to the posterior head region, it is difficult to discern with immunofluorescence if PH-30 binds additionally to the equatorial region of the plasma membrane, which has been reported by some laboratories to be involved in sperm–egg fusion (for review, see Yanagimachi, 1981). The equatorial region of the plasma membrane is in the central region of the sperm head. It is the plasma membrane region that overlies the most posterior segment of the outer acrosomal membrane, a segment that does not undergo exocytotic membrane fusion during the acrosome reaction. To determine if the PH-30 mAb binds to the equatorial region of the plasma membrane, mAb binding was examined at the ultrastructural level. PH-30 mAb, bound to acrosome-reacted sperm, was visualized with an anti-mouse IgG second antibody conjugated to colloidal gold (Fig. 2). The gold particles were present on the sperm surface in the posterior head region and were absent from the equatorial region, whose limits are denoted by stars (Fig. 2). In the various sections examined from PH-30 mAb-stained acrosome-reacted and acrosome-intact sperm, gold particles were never observed in the equatorial region but were restricted to the head surface posterior to the equatorial region.

Developmental Appearance of the PH-30 Antigen

Because the sperm's ability to fertilize the egg requires sperm transit through the epididymis (Bedford, 1975; Hamilton, 1975; Orgebin-Crist, 1975), we determined at what stage of sperm development the antigenic determinants detected on the posterior head by the PH-30 and PH-1 mAbs first appear. It was found that both the PH-30 and PH-1 mAbs show no

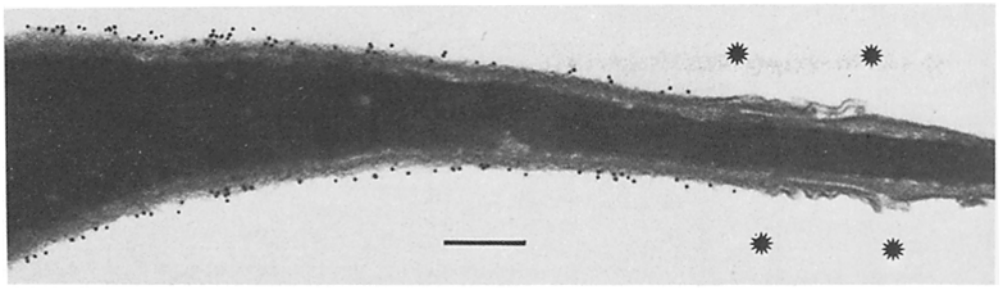

Figure 2. Immunoelectron micrograph of PH-30 mAb binding to acrosome-reacted sperm. Sperm were stained with the PH-30 mAb followed by colloidal gold-conjugated goat IgG anti-mouse IgG and processed for electron microscopy. The stars denote the limits of the equatorial segment. Bar, 0.1 μm.

binding to testicular, caput epididymal, or corpus epididymal sperm by indirect immunofluorescence. The cauda was the first region of the epididymis where mAb binding to sperm was observed (Table III). Division of the cauda epididymis into proximal and distal segments (Hoffer and Greenberg, 1978) revealed that PH-30 mAb binding can first be detected on sperm from the proximal segments of the cauda epididymis (Table III).

Identification of the PH-30 Antigen by Immunoprecipitation

In our previous studies using ^{125}I-labeled sperm, the PH-30 mAb failed to give a labeled immunoprecipitate and the PH-1 mAb gave an immunoprecipitate that showed low amounts of a labeled M_r ~60,000 polypeptide (Myles et al., 1981; Primakoff and Myles, 1983). With an improvement of the surface labeling procedure (Materials and Methods), we have obtained an immunoprecipitate with the PH-30 mAb; it is comprised of two ^{125}I-labeled polypeptides of M_r ~60,000 (60 kD) and M_r ~44,000 (44 kD) (Fig. 3, lane *1*). The 60-kD band was strongly labeled while the 44-kD band was only faintly labeled and required long exposures of the autoradiograph to be detected. With the improved labeling procedures, the PH-1 mAb also precipitated the strongly labeled 60-kD band and the faintly labeled 44-kD band (Fig. 3, lane *2*). These bands were absent in the control precipitate which used myeloma culture supernatant (Fig. 3, lane *3*).

To characterize the determinants recognized by the mAbs, we tested the heat-sensitivity of the PH-30 and PH-1 epitopes. Protein conformational determinants are heat-sensitive whereas sequential or carbohydrate determinants (on a glycoprotein) are generally heat-resistant (Stern et al., 1978). An OG extract of intact sperm, either untreated or boiled for 5 min, was tested for its ability to inhibit the solid-phase assay. The boiled extract showed a severe loss of its ability to inhibit PH-30 mAb binding but retained virtually all of its ability to inhibit PH-1 mAb binding (Fig. 4). This indicates that the epitopes recognized by the two mAbs are different and the PH-30 epitope may be a conformational determinant, whereas the PH-1 epitope is not conformational.

Table III. Binding of PH-30 and PH-1 mAbs to Sperm of Different Developmental Stages

	Immunofluorescence	
	PH-30	PH-1
Testicular	–	–
Caput epididymal	–	–
Corpus epididymal	–	–
Proximal cauda epididymal	+	ND
Distal cauda epididymal	+	+

Binding of the PH-30 and PH-1 mAbs to sperm of different developmental stages. Sperm were isolated from the testis or from various segments of the epididymis and stained with either the PH-30 or PH-1 mAb followed by a rhodamine-conjugated anti-mouse IgG second antibody. They were then examined for fluorescence. – denotes the absence of detectable fluorescence. + denotes fluorescence localized in the posterior head region, having roughly the intensity shown in Fig. 1.

Purification of the PH-30 Antigen

The PH-30 antigen was purified from OG extracts of sperm by affinity chromatography on PH-30 mAb-Sepharose. In the peak eluted fraction we recovered 45% of the PH-30 antigen present in the starting OG extract of 6×10^9 sperm. This is 120 μg of protein or ~0.1% of the total protein in the initial extract (Table IV). (The remainder, ~55% of the PH-30 antigen, was lost on the Sepharose 4B precolumn and in the wash of the PH-30 mAb column). The isolated PH-30 protein thus retained antigenic activity as measured by the assay for antigenic activity (Table IV) and by the direct solid-phase assay (Table V).

On SDS PAGE, the purified PH-30 antigen shows two bands of 60 kD and 44 kD (Fig. 5, lanes *2–5*), corresponding to the two bands observed in the ^{125}I-labeled immunoprecipitate (Fig. 4). The intensity of the two bands is different in

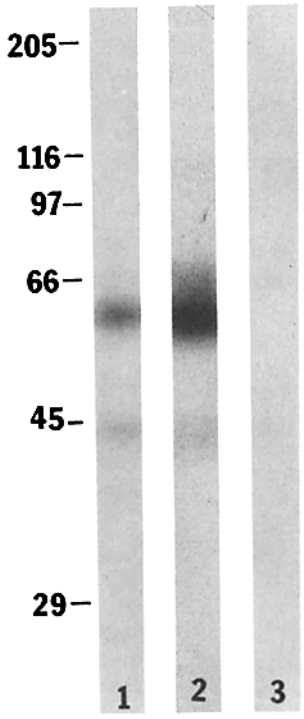

Figure 3. Autoradiograph of ^{125}I-labeled immunoprecipitates electrophoresed on SDS PAGE. The ^{125}I surface-labeled sperm extract was precipitated with an mAb, the precipitates were electrophoresed on a 10% SDS polyacrylamide gel, the gel was dried and autoradiographed. Lane *1*, PH-30 mAb. Lane *2*, PH-1 mAb. Lane *3*, Control, myeloma supernatant used in place of mAb. M_r standards are as follows: myosin, M_r 205,000; β-galactosidase, M_r 116,000; phosphorylase B, M_r 97,000; BSA, M_r 66,000; ovalbumin, M_r 45,000; and carbonic anhydrase, M_r 29,000.

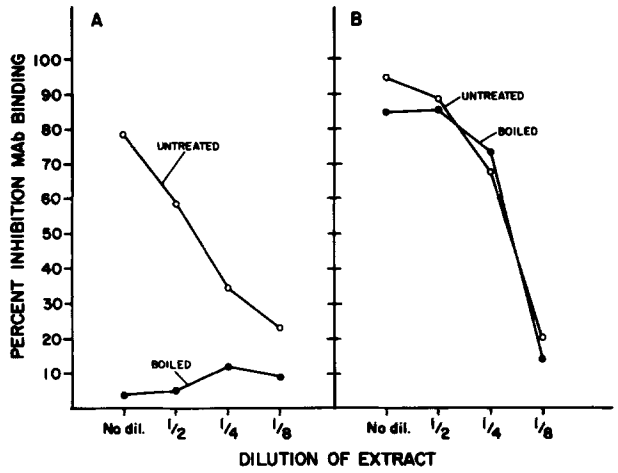

Figure 4. Heat sensitivity of the PH-30 and PH-1 antigenic determinants. An OG extract of 1×10^8 sperm/ml was split in half, one half was boiled for 5 min, and the other remained at room temperature for 5 min (untreated). The boiled and untreated extracts were tested for their ability to inhibit PH-30 or PH-1 mAb binding in the solid-phase binding assay using the protocol described in the assay for antigenic activity (Materials and Methods). The data points are the averages of duplicate determinations each made in two experiments. (*A*) PH-30 mAb binding. (*B*) PH-1 mAb binding.

the Coomassie Blue–stained, purified antigen (Fig. 5 *A*, lane *2*) compared with the ^{125}I-labeled, immunoprecipitated antigen. In the purified antigen the 44-kD band is the more intensely stained of the two polypeptides, whereas in the ^{125}I-labeled antigen the 44-kD band is very faint. This may reflect relatively poor ^{125}I labeling of the 44-kD polypeptide. However, Coomassie Blue staining of 60 kD as well as 44 kD may be aberrant as they stain poorly or diffusely compared with the molecular mass standards on the same gel (compare Fig. 5 *A*, lane *2*, 14 μg of purified PH-30 antigen to Fig. 5 *A*, lane *1*, 1 μg of each molecular mass standard).

With an overloaded, Coomassie Blue–stained sample (14 μg) of the purified PH-30 protein, no bands other than 60 kD and 44 kD are seen (Fig. 5 *A*, lane *2*). If increasing loads (1, 5, 10 μg) of purified PH-30 protein are silver-stained (Fig. 5 *A*, lanes *3–5*), only 60 kD and 44 kD are detected in the 1-μg load, but additional bands are detected in the 5- and 10-μg loads. These additional bands are between 60 and 44 kD or below 44 kD (with one exception, 160 kD, Fig. 5 *A*, lane *5*). Thus, the additional bands may be proteolytic fragments of the PH-30 polypeptides or they may be contaminants. We

Table IV. Purification of the PH-30 Antigen

	Protein	Antigenic activity	Relative specific activity	Yield of antigenic activity
	mg	*units* × 10^{-3}		%
Starting OG extract (6×10^9 cells)	126	5.6	1	(100)
Peak eluted fraction from PH-30 column	0.12	2.5	469	45

Yield of purified PH-30 protein. The amount of protein in the fractions was determined with the Pierce BCA protein assay reagent. The amount of antigenic activity in the fractions was determined using the assay for antigenic activity.

Table V. Binding of PH-30 and PH-1 mAbs to Fractions from a PH-30 mAb-Sepharose Column

	cpm bound in solid-phase assay	
Fraction	PH-30	PH-1
OG extract	1,503	2,869
Column flow through	180	162
Peak eluted fraction	1,610	2,446

Binding of mAbs to various fractions obtained in the purification of the PH-30 protein. 50 μl of each of the indicated fractions was assayed in duplicate in the radioactive solid-phase binding assay for ability to bind the PH-30 and PH-1 mAbs.

have found that in our hands the silver-staining procedure detects the molecular mass standards down to 5 ng, suggesting the rough estimate that silver-stainable proteolytic fragments or contaminants of the purified PH-30 protein are present at a level of <5 ng in 1 μg, i.e., at <1 part in 200.

The 60- and 44-kD bands are not linked to each other by disulfide bonds, since the purified PH-30 protein examined under nonreducing conditions gives two broad bands whose central regions migrate at ~49 and ~33 kD (Fig. 5 *B*, lane *2*). Compared with the reduced PH-30 protein, run on the opposite side of the same gel (Fig. 5 *B*, lane *1*), the two nonreduced polypeptides stain better with silver and migrate further. They also stain more diffusely, showing what appear to be multiple sub-bands in the broad 49- and 33-kD regions (Fig. 5 *B*, lane *2*), possibly because the nonreduced polypeptides have more than one conformation when denatured by SDS.

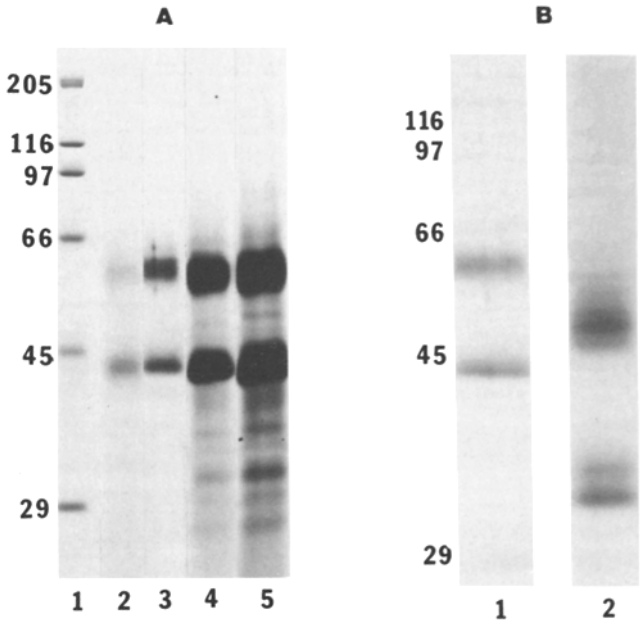

Figure 5. SDS PAGE analysis of affinity-purified PH-30 protein. (*A*) Lane *1*, same molecular weight standards used in Fig. 3, each present at 1 μg. Lane *2*, 14 μg purified PH-30 protein. After electrophoresis lanes *1* and *2* were stained with Coomassie Blue. Lane *3*, 1 μg PH-30 protein; lane *4*, 5 μg PH-30 protein; lane *5*, 10 μg PH-30 protein. Lanes *3–5* were silver stained. (*B*) The purified PH-30 protein was run reduced and nonreduced on opposite sides of the same gel to allow direct comparison of polypeptide migrations under the two conditions (Allore and Barber, 1983; Samelson, 1985). Lane *1*, PH-30 protein, reduced sample. Lane *2*, PH-30 protein, nonreduced sample. The gel was silver stained.

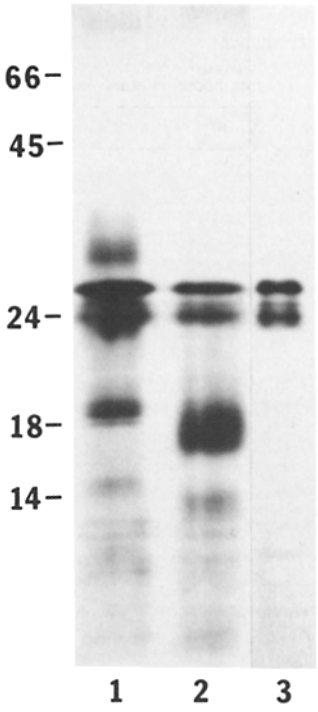

Figure 6. Cleveland digests of the 60- and 44-kD polypeptides. The 60- and 44-kD bands were excised from a 10% SDS-polyacrylamide gel, placed in lanes of a second 15% SDS-polyacrylamide gel with an overlay of 500 ng V8 protease (Sigma Chemical Co.), and electrophoresed into the second gel according to the protocol of Cleveland et al. (1977). Lane *1*, digest of 60 kD; lane *2*, digest of 45 kD; lane *3*, 500 ng V8 protease alone. The gel was silver stained. The M_r standards (Sigma Chemical Co.) are: M_r 66,000, BSA; M_r 45,000, ovalbumin; M_r 24,000, trypsinogen; M_r 18,000, β-lactoglobulin; M_r 14,000, lysozyme.

The PH-30 and PH-1 mAbs might bind to both 60 kD and 44 kD because these are two distinct, structurally related proteins or because 44 kD is a proteolytic fragment of 60 kD. The Cleveland digest method using *Staphylococcus aureus* V8 protease was used to see if a structural relationship between the two polypeptides could be detected (Cleveland et al., 1977). After digestion with the V8 protease, 60 kD gave rise to four major peptides at 33, 24, 19, and 14.5 kD (Fig. 6, lane *1*). The major peptide at 24 kD co-migrates with and is partially obscured by the V8 protease itself, which runs as a triplet in the 24–27-kD range and a minor band at 11 kD (Fig. 6, lane *3*). The digest from 44 kD showed none of the four major peptides from 60 kD, but gave rise to different major peptides at 17 kD, 13 kD, and a doublet near the front at <10 kD (Fig. 6, lane *2*). In addition to the differences in molecular masses of the major peptides derived from 60 kD and 44 kD, they also stained different colors on the original silver-stained gel.

Discussion

To analyze fusion between complex membranes with many externally disposed proteins, one must have a lead to investigate, i.e., an indication of which protein(s) may be fusion-related. One must also be able to purify the protein(s) so that their possible fusion-related activities can be tested. The present results meet both of these criteria: the PH-30 antigen has been identified as a candidate for a protein with a role in sperm–egg membrane fusion and it has been purified from detergent lysates of sperm.

We found that the PH-30 mAb strongly inhibited the standard fusion assay that has been used in most investigations of mammalian sperm–egg membrane fusion. This assay detects the occurrence of fusion by observation of a subsequently occurring event, the swelling of the sperm nucleus in the egg cytoplasm. It is conceivable that the PH-30 mAb actually allows sperm–egg membrane fusion to occur and blocks some other subsequent event prerequisite to sperm head swelling. This possibility seems unlikely since the PH-30 mAb binds to the sperm plasma membrane, but ruling it out would require development of an assay that measures the plasma membrane fusion event directly.

Although the PH-30 mAb inhibited the sperm–egg fusion assay, the PH-1 mAb did not inhibit. In comparing other features of the PH-30 and PH-1 mAbs, no major difference was noted other than their binding to distinct epitopes. The pH-30 mAb is an IgG1; PH-1 is an IgG2b (Materials and Methods). They showed fluorescence of roughly equal intensity when binding to live, acrosome-reacted sperm (Fig. 1) and PH-1 bound at a somewhat higher level to sperm extracts and to purified PH-30 antigen in the solid-phase assay (Table V). Compared with PH-30, the PH-1 mAb generally immunoprecipitated more antigen from Triton extracts of sperm (Fig. 3) and its binding in the solid-phase assay was inhibited to a higher level by preincubation with OG extracts of sperm (Fig. 4), suggesting that the PH-1 mAb may be of somewhat higher affinity than the PH-30 mAb. The finding that the PH-1 mAb gives no inhibition of sperm–egg fusion indicates that the PH-30 mAb inhibition does not arise from PH-30 mAb simply coating the sperm and preventing close approach of the sperm and egg. Rather, the results suggest that the PH-30 mAb may specifically affect an active site on the PH-30 protein essential in sperm–egg fusion events, while the PH-1 mAb does not affect this site.

Developmental Appearance and Localization of the PH-30 Antigen

Our results on developmental appearance and surface localization of the PH-30 antigen bear on two long standing questions in fertilization research: (*a*) what essential changes occur in the sperm cell during epididymal maturation that make the sperm competent for fertilization? and (*b*) which regions of the sperm plasma membrane are involved in fusion with the egg plasma membrane?

Sperm from all mammalian species tested are incompetent to fertilize until they have undergone a maturation process in the epididymis (Bedford, 1975; Hamilton, 1975; Orgebin-Crist, 1975). The first direct evidence for functional maturation in the epididymis was obtained in the guinea pig by Young (1931). One essential change sperm undergo during epididymal maturation is the acquisition of motility, which is required for fertilization. Two lines of evidence indicate that, in addition to motility acquisition, other changes must occur in the epididymis for the sperm to achieve fertilization competence. Hypophysectomy blocks acquisition of fertilization competence in the epididymis while leaving motility unaffected (Dyson and Orgebin-Crist, 1973), and polyclonal antisera, raised to mixtures of epididymal fluid proteins, block in vitro fertilization in the rat without affecting sperm motility (Cuasnicu et al., 1984). The appearance during epididymal maturation of the PH-30 antigen, or certain determinants on it, could be an essential change required for fertilization competence. PH-30 mAb binding was not detected on caput or corpus epididymal sperm, but was found on sperm from the proximal cauda epididymis. Sperm from the proximal cauda in the guinea pig have a significant, but not a maximal, level of fertilization competence (Young, 1931). The proximal cauda may be the first stage where a

significant level of fertilization competent sperm exist in the guinea pig, if guinea pig epididymal maturation is analogous to that in two other rodents, rat and hamster, where the proximal cauda has been shown to be the site where fertilization competence first appears (Dyson and Orgebin-Crist, 1973; Horan and Bedford, 1972).

Which region of the sperm plasma membrane is involved in fusion with the egg plasma membrane has been a subject of extended controversy. A variety of electron microscopic studies have suggested that the region of the sperm plasma membrane that fuses with the egg plasma membrane is either the posterior head or the equatorial region, or both of these regions (reviewed in Yanagimachi, 1981). We have found that binding of the PH-30 mAb is restricted to the posterior head surface and is absent in the equatorial region. This result implicates plasma membrane molecules present in the posterior head and not present in the equatorial region as playing a required role in the fusion. Our findings of course leave open the possibility that surface molecules in the equatorial region also participate in membrane fusion events.

Purification and Characterization of the PH-30 Protein

We purified the PH-30 protein by affinity chromatography on PH-30 mAb-Sepharose. The purified PH-30 antigen shows two bands on reducing SDS PAGE, one at 60 kD and one at 44 kD. The two polypeptides migrate further (to 49 and 33 kD) on a nonreducing gel. This shows they do not have interchain disulfide bonds, but probably contain intrachain disulfides. Since nonreduced polypeptides with intrachain disulfides can not assume a fully extended conformation in SDS, they generally migrate further (to apparent lower molecular masses) than their reduced counterparts (Allore and Barber, 1983; Samelson, 1985). Finding a lower apparent molecular mass with a non-reduced polypeptide compared with a reduced polypeptide is diagnostic of the presence of intrachain disulfide bonds and has been used to show their presence in newly discovered proteins, for example, the T cell receptor for antigen (Samelson, 1985).

Cleveland digests did not indicate that the 44- and 60-kD bands are two structurally related proteins or that 44 kD is a proteolytic fragment of 60 kD. Further analysis will be required to determine the relationship of 44 kD and 60 kD. We have observed that the purified PH-30 protein precipitates when OG is removed by dialysis, which suggests either 44 kD or 60 kD could be an integral membrane polypeptide. It can be speculated that the PH-30 protein may be a complex of 44 kD and 60 kD. This complex might be formed in the epididymis, since the PH-30 protein is first detectable on sperm in the proximal cauda epididymis. On proximal cauda sperm, PH-30 is localized to the posterior head. One PH-30 polypeptide might be derived from the epididymal fluid and bind to the other, acting as a "receptor," an integral membrane polypeptide localized to the sperm posterior head surface prior to the sperm's arrival in the cauda epididymis.

Recent investigations have pointed to two other proteins that may have a role in the sperm–egg fusion process. Saling et al. (1985) have shown that the IgM monoclonal antibody M-29 inhibits mouse sperm fusion with zona-free mouse eggs. The M-29 mAb does not bind to live acrosome-intact sperm, but will bind to the equatorial region of fixed acrosome-intact sperm or live acrosome-reacted sperm (Saling et al., 1985). This indicates that in contrast to the PH-30 antigen, the M-29 antigen is not a plasma membrane protein but is located on the outer and/or inner acrosomal membrane in the equatorial region. Such a location suggests that the M-29 mAb may affect sperm–egg plasma membrane fusion indirectly or that the M-29 antigen may redistribute to the equatorial region plasma membrane after the acrosome reaction.

In sea urchins the suggestion has been made that the protein bindin, which is known to be involved in binding of sperm to the egg's vitelline layer, may also be involved in fusion of the sea urchin sperm with the egg plasma membrane (Glabe, 1985*a*, *b*). Experiments have shown that isolated bindin can lead to aggregation or fusion of phospholipid vesicles with certain lipid compositions. Experiments have not yet been done with gametes indicating that bindin has a role in the process of sperm–egg fusion.

The availability of purified PH-30 antigen will allow us to test possible fusion-related activities of this membrane protein. An important test will be to see if the purified protein in liposomes will allow the liposomes to bind to or fuse with zona-free eggs. Since we have isolated the PH-30 protein in the dialyzable detergent OG, it can be readily freed of detergent and reconstituted into liposomes. The purified PH-30 protein is antigenically active, retaining activity for binding the PH-30 and PH-1 mAbs. If the PH-30 protein, isolated by our methods, retains functional activity, it should be possible to use the purified antigen in liposomes to observe fusion-related activities inhibitable by the PH-30 mAb and not by the PH-1 mAb.

We gratefully acknowledge Dr. Diana Myles for discussions and encouragement and Bonnie Phelps for testing antibody binding to sperm in early stages of epididymal maturation. Dori Bearce provided technical assistance and Christine Pearson did the immunoelectron microscopy. We also thank Drs. Laurinda Jaffe and Diana Myles for critical reading of the manuscript, and Joan Jannace for its careful preparation.

This work was supported by a grant from the Rockefeller Foundation, a Grant-in-Aid from the American Heart Association, and National Institutes of Health grant HD-16580. P. Primakoff held an Established Investigatorship Award from the American Heart Association during the course of this work.

Received for publication 16 May 1986, and in revised form 23 September 1986.

References

Allore, R. J., and B. H. Barber. 1983. Inter- and intramolecular disulfide bonding among lymphocyte plasma membrane proteins and glycoproteins. *Mol. Immunol.* 20:383–395.

Bedford, J. M. 1975. Maturation, transport and fate of spermatozoa in the epididymis. *In* Handbook of Physiology. Section 7, Endocrinology. Vol. 5, Male Reproductive System. D. W. Hamilton and R. O. Greep, editors. American Physiological Society, Bethesda. 303–317.

Brown, W. R. A., A. N. Barclay, C. A. Sunderland, and A. F. Williams. 1981. Identification of a glycophorin-like molecule at the cell surface of rat thymocytes. *Nature (Lond.).* 289:456–460.

Cleveland, D. W., S. G. Fischer, M. W. Kirschner, and U. K. Laemmli. 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. *J. Biol. Chem.* 252:1102–1106.

Cuasnicu, P. S., F. G. Echeverria, A. D. Piazza, M. S. Cameo, and J. A. Blaquier. 1984. Antibodies against epididymal glycoproteins block fertilizing ability in rat. *J. Reprod. Fertil.* 72:467–471.

Dyson, A. L. M. B., and M.-C. Orgebin-Crist. 1973. Effect of hypophysectomy, castration and androgen replacement upon the fertilizing ability of rat epididymal spermatozoa. *Endocrinology.* 93:391–402.

Fleming, A. F., and R. Yanagimachi. 1981. Effects of various lipids on the acrosome reaction and fertilizing capacity of guinea pig spermatozoa with special reference to the possible involvement of lysophospholipids in the acrosome reaction. *Gamete Res.* 4:253–273.

Florkiewicz, R. Z., and J. K. Rose. 1984. A cell line expressing vesicular

stomatitis virus glycoprotein fuses at low pH. *Science (Wash. DC).* 225:721–722.

Gething, M.-J., R. W. Doms, D. York, and J. White. 1986. Studies on the mechanism of membrane fusion: site-specific mutagenesis of the hemagglutinin of influenza virus. *J. Cell Biol.* 102:11–23.

Glabe, C. G. 1985*a*. Interaction of the sperm adhesive protein, bindin, with phospholipid vesicles. I. Specific association of bindin with gel-phase phospholipid vesicles. *J. Cell Biol.* 100:794–799.

Glabe, C. G. 1985b. Interaction of the sperm adhesive protein, bindin, with phospholipid vesicles. II. Bindin induces the fusion of mixed-phase vesicles that contain phosphatidylcholine and phosphatidylserine in vitro. *J. Cell Biol.* 100:800–806.

Green, D. P. L. 1978. The induction of the acrosome reaction in guinea pig spermatozoa by the divalent metal cation ionophore A23187. *J. Cell Sci.* 32:137–151.

Hamilton, D. W. 1975. Structure and function of the epithelium lining the ductuli efferentes, ductus epididymidis and ductus deferens in the rat. *In* Handbook of Physiology. Section 7, Endocrinology. Vol. 5, Male Reproductive System. D. W. Hamilton and R. O. Greep, editors. American Physiological Society, Bethesda. 259–301.

Hoffer, A. P., and J. Greenberg. 1978. The structure of the epididymis, efferent ductules and ductus deferens of the guinea pig: a light microscope study. *Anat. Rec.* 190:659–678.

Horan, A. H., and J. M. Bedford. 1972. Development of the fertilizing ability of spermatozoa in the epididymis of the Syrian hamster. *J. Reprod. Fertil.* 30:417–423.

Johnson, P., A. F. Williams, and G. R. Woollett. 1985. Purification of membrane glycoproteins with monoclonal antibody affinity columns. *In* Hybridoma Technology in the Biosciences and Medicine. T. A. Springer, editor. Plenum Publishing Corp., New York. 163–176.

Kohler, G. 1980. Hybridoma Techniques. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. 65 pp.

Kondor-Koch, C., B. Burke, and H. Garoff. 1983. Expression of Semliki Forest virus proteins from cloned complementary DNA. I. The fusion activity of the spike glycoprotein. *J. Cell Biol.* 97:644–651.

Mescher, M. F., K. C. Stallcup, C. P. Sullivan, A. P. Turkewitz, and S. H. Herrmann. 1983. Purification of murine MHC antigens by monoclonal antibody affinity chromatography. *Methods Enzymol.* 92:86–109.

Myles, D. G., and P. Primakoff. 1983. Establishment of sperm surface topography during development analyzed with monoclonal antibodies. *In* The Sperm Cell. J. Andre, editor. Martinus Hijhoff, The Hague. 127–130.

Myles, D. G., P. Primakoff, and A. R. Bellve. 1981. Surface domains of the guinea pig sperm defined with monoclonal antibodies. *Cell.* 23:433–439.

Myles, D. G., P. Primakoff, and D. E. Koppel. 1984. A localized surface protein of guinea pig sperm exhibits free diffusion in its domain. *J. Cell Biol.* 98:1905–1909.

Noble, A. G., G. T.-Y. Lee, R. Sprague, M. L. Parish, and P. G. Spear. 1983. Anti-gD monoclonal antibodies inhibit cell fusion induced by herpes simplex virus type 1. *Virology.* 129:218–224.

Orgebin-Crist, M.-C., B. J. Danzo, and J. Davies. 1975. Endocrine control of the development and maintenance of sperm fertilizing ability in the epididymis. *In* Handbook of Physiology. Section 7, Endocrinology. Vol. 5, Male Reproductive System. D. W. Hamilton and R. O. Greep, editors. American Physiological Society, Bethesda. 319–338.

Orvell, C. 1984. The reactions of monoclonal antibodies with structural proteins of mumps virus. *J. Immunol.* 132:2622–2629.

Orvell, C., and M. Grandien. 1982. The effects of monoclonal antibodies on biological activities of structural proteins of Sendai virus. *J. Immunol.* 129:2779–2787.

Paterson, R. G., S. W. Hiebert, and R. A. Lamb. 1985. Expression at the cell surface of biologically active fusion and hemagglutinin/neuraminidase proteins of the paramyxovirus simian virus 5 from cloned cDNA. *Proc. Natl. Acad. Sci. USA.* 82:7520–7524.

Primakoff, P., and D. G. Myles. 1983. A map of the guinea pig sperm surface constructed with monoclonal antibodies. *Dev. Biol.* 98:417–428.

Saling, P. M., G. Irons, and R. Waibel. 1985. Mouse sperm antigens that participate in fertilization. I. Inhibition of sperm fusion with the egg plasma membrane using monoclonal antibodies. *Biol. Reprod.* 33:515–526.

Samelson, L. E. 1985. An analysis of the structure of the antigen receptor on a pigeon cytochrome c-specific T cell hybrid. *J. Immunol.* 134:2529–2535.

Stern, P. L., K. R. Willison, E. Lennox, G. Galfre, C. Milstein, D. Secher, A. Ziegler, and T. Springer. 1978. Monoclonal antibodies as probes for differentiation and tumor-associated antigens: a Fforssman specificity on teratocarcinoma stem cells. *Cell.* 14:775–783.

White, J., M. Kielian, and A. Helenius. 1983. Membrane fusion proteins of enveloped animal viruses. *Q. Rev. Biophys.* 16:151–195.

Yanagimachi, R. 1972. Fertilization of guinea pig eggs in vitro. *Anat. Rec.* 174:9–20.

Yanagimachi, R. 1978. Calcium requirement for sperm-egg fusion in mammals. *Biol. Reprod.* 19:949–958.

Yanagimachi, R. 1981. Mechanism of fertilization in mammals. *In* Fertilization and Embryonic Development In Vitro. L. Mastorianni, Jr. and J. D. Biggers, editors. Plenum Publishing Corp., New York. 81–182.

Young, W. C. 1931. Study of the function of the epididymis. III. Functional changes undergone by spermatozoa during their passage through the epididymis and vas deferens in the guinea pig. *J. Exp. Biol.* 8:151–162.