Sperm Exocytosis Increases the Amount of PH-20 Antigen on the Surface of Guinea Pig Sperm

Ann E. Cowan, Paul Primakoff, and Diana G. Myles

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

Abstract. Evidence has been presented that the PH-20 protein functions in sperm adhesion to the egg zona pellucida (Primakoff, P., H. Hyatt, and D. G. Myles, 1985, *J. Cell Biol.*, 101:2239–2244). The PH-20 protein migrates from its original surface domain to a new surface domain after the acrosome reaction (Myles, D. G., and P. Primakoff, 1984, *J. Cell Biol.*, 99:1634–1641). The acrosome reaction is an exocytotic event that results in insertion of a region of the secretory granule membrane, the inner acrosomal membrane (IAM), into the plasma membrane. After the acrosome reaction, PH-20 protein migrates to the IAM from its initial domain on the posterior head surface. We have now found a new dynamic feature of the regulation of PH-20 protein on the sperm surface; exocytosis increases the surface expression of PH-20 protein. After the acrosome reaction there is an approximately threefold increase in the number of PH-20 antigenic sites on the sperm surface. These new antigenic sites are revealed on the surface by insertion of the IAM into the plasma membrane. Our evidence indicates that before the acrosome reaction an intracellular population of PH-20 antigen is localized to the IAM. When migration of the surface population of the PH-20 protein is prevented, PH-20 protein can still be detected on the IAM of acrosome-reacted sperm. Also, PH-20 protein can be detected on the IAM of permeabilized acrosome-intact sperm by indirect immunofluorescence. Thus, the sperm cell regulates the amount of PH-20 protein on its surface by sequestering about two-thirds of the protein on an intracellular membrane and subsequently exposing this population on the cell surface by an exocytotic event. This may be a general mechanism for regulating cell surface composition where a rapid increase in the amount of a cell surface protein is required.

CELL surface function can be regulated by controlling the level of expression of surface molecules, their time of appearance, and their localization in the membrane. Our laboratory has been studying the function and localization of a guinea pig–sperm surface antigen, the PH-20 protein. We have obtained evidence that this protein has a role in sperm binding to the egg zona pellucida (Primakoff et al., 1985). Furthermore, we have found that the PH-20 protein is localized to the posterior head surface of acrosome-intact sperm, but after the acrosome reaction its pattern of localization is altered (Myles and Primakoff, 1984). The acrosome reaction is an exocytotic event that results in insertion of a region of the secretory granule membrane, the inner acrosomal membrane (IAM),[1] into the plasma membrane. After the acrosome reaction, the PH-20 antigen migrates rapidly and completely to the IAM. This is an example of a dynamic regulation of the localization of a cell surface molecule. The PH-20 migration is a specific process, since other antigens localized to the posterior head surface remain on the posterior head after the acrosome reaction.

1. *Abbreviations used in this paper*: FITC, fluorescein isothiocyanate; IAM, inner acrosomal membrane; MAb, monoclonal antibody; OAM, outer acrosomal membrane.

In this paper we report a second mechanism for the control of the surface expression of the PH-20 antigen that acts to regulate the amount of PH-20 on the cell surface. Initially, the PH-20 protein is found on both an intracellular membrane, the acrosomal membrane, and on the plasma membrane. We term the acrosomal membrane population PH-20_{AM} and the plasma membrane population PH-20_{PM}. When the acrosome reaction occurs, PH-20_{AM} is added to the cell surface and serves to increase surface expression of the PH-20 protein. Thus, sperm exocytosis, which is required for fertilization (Noda and Yanagimachi, 1976) results in both an "upregulation" of the PH-20 antigen and a dramatic alteration in its surface localization. We speculate that these changes in the surface expression of PH-20 protein may be important in regulating sperm–zona interaction.

Materials and Methods

Antibodies

The three monoclonal antibodies (MAbs) used in this study (PH-20, PH-21, and PH-22) are described in Primakoff and Myles (1983) and Primakoff et al. (1985). All three MAbs recognize the PH-20 protein and belong to the subclass IgG_1 (Primakoff et al., 1985). Competition experiments and tests for inhibition of sperm–zona binding show that at least two and probably

three different epitopes are recognized by the three MAbs (Primakoff et al., 1985).

The PH-22 MAb was purified from culture supernatants by protein A-affinity chromatography using the BioRad Monoclonal Antibody Purification System as described in the supplier's instructions (Bio-Rad Laboratories, Richmond, CA). The Fab' fragment of the PH-22 MAb was produced by pepsin digestion to produce a $F(ab')_2$ fragment that was subsequently reduced to produce the Fab' (Parham, 1983). Purified PH-22 IgG (5 mg) was incubated for 8 h at 37°C with 125 μl of pepsin-coated agarose beads (Bio-Rad Laboratories) in 0.1 M citrate buffer (pH 3.5) in a total volume of 0.5 ml to completely digest the IgG into $F(ab')_2$. The pH was neutralized by addition of 1/10th volume 0.3 M Tris (pH 8.6), and the pepsin-coated beads removed by centrifugation. The $F(ab')_2$ was reduced with 20 mM cysteine for 1 h at 37°C, and alkylated with 30 mM iodoacetamide for 15 min at room temperature. Fab' was purified from residual $F(ab')_2$ by Sephadex G-100 chromatography. The Fab' was completely free of contaminating material as determined by nonreducing SDS-polyacrylamide gels stained with Coomassie Blue.

The PH-22 Fab' was iodinated using the Bolton-Hunter reagent (New England Nuclear, Boston, MA), as detailed in the supplier's instructions. ^{125}I-Labeled Fab' was separated from hydrolysis products and unreacted reagent by Sephadex G-50 chromatography.

Rhodamine labeling of the purified PH-22 MAb was done using tetraethyl rhodamine isothiocyanate (RITC; Sigma Chemical Co., St. Louis, MO) in 0.1 M sodium carbonate buffer (pH 9.5) at a ratio of 25 μg RITC/mg protein (Goding, 1976). The rhodamine conjugate was separated from free dye by chromatography on a Sephadex G-25 column.

Second antibodies used were fluorescein isothiocyanate (FITC)- or RITC $F(ab)_2$ goat anti-mouse IgG and Fab goat-anti-mouse IgG (Cooper Biomedical, Malvern, PA). The latter antibody was conjugated with rhodamine as described above for purified PH-22 MAb.

Immunofluorescence

Immunofluorescence staining was carried out on live sperm or sperm fixed in 1.5% formaldehyde (made fresh from paraformaldehyde) as previously described (Myles et al., 1981). For immunofluorescence of permeabilized sperm, sperm were isolated from the cauda epididymis in Mg^{2+} Hepes medium (0.14 M NaCl, 4 mM KCl, 4 mM Hepes (pH 7.4) 10 mM glucose, and 2 mM $MgCl_2$; modified from Green, 1978) and a small drop of sperm placed on a polylysine-coated slide. A coverslip was applied and the slide immediately frozen on dry ice. While still frozen, the coverslip was removed and the slide submerged in 1.5% formaldehyde at room temperature and fixed for 20 min. The slides were washed with several changes of PBS. Subsequent incubations with antibody-containing solutions were carried out directly on the slide, and unbound antibody removed by washing in several changes of PBS.

Number of Antigenic Sites per Cell

The number of antigenic sites per cell was determined by measuring the amount of iodinated Fab' PH-22 bound to the cell at saturating Fab' concentrations, as described by Mason and Williams (1980). Sperm at 5×10^7/ml were first incubated with culture supernatant containing PH-22 MAb (control for nonspecific binding) or irrelevant MAb (PT-1; described in Myles and Primakoff, 1984) (experimental). Duplicate aliquots containing $\sim 5 \times 10^5$ sperm were then incubated for 30 min at room temperature with either 150 or 300 ng of ^{125}I-Fab' PH-22 (1.9×10^5 cpm/μg) in 100 μl PBS containing 1% BSA. Two concentrations of Fab' were used to ensure that the level of ^{125}I-Fab' was sufficient to saturate all available antigenic sites. The sperm were pelleted through 2 ml horse serum, resuspended in 200 μl PBS, and counted in a gamma counter. Background values were determined from the control sperm preincubated with culture supernatant containing PH-22 MAb. Background for acrosome-intact cells was 4,097 and 7,517 cpm/10^6 cells at 150 and 300 ng Fab', respectively; background for acrosome-reacted cells was 6,620 and 10,745 cpm/10^6 cells at 150 and 300 ng Fab', respectively. The number of antigenic sites per cell was determined from the cpms bound, the specific activity of the Fab', and the molecular weight of the PH-22 Fab' determined by SDS PAGE.

Acrosome Reaction

Two different methods were used to induce the acrosome reaction. In the first method, the acrosome reaction was induced by the addition of 1 μg/ml calcium ionophore A23187 (Calbiochem-Behring Corp., La Jolla, CA) to freshly obtained sperm at 1×10^7/ml in Ca^{2+} Hepes medium (0.14 M NaCl, 4 mM KCl, 4 mM Hepes (pH 7.4), 10 mM glucose, and 2 mM $CaCl_2$) at 37°C (Green, 1978). Previous results have shown that migration of PH-20 protein from the posterior head to the IAM occurs rapidly (within 5–10 min after A23187 addition) when sperm are acrosome-reacted by this method (Myles and Primakoff, 1984). Alternatively, sperm were incubated for 1 h in modified Tyrode's medium lacking calcium (109 mM NaCl, 2.8 mM KCl, 0.5 mM $MgCl_2$, 25 mM $NaHCO_3$, 5.6 mM glucose, 10 mM lactic acid, 1 mM Na-pyruvate, pH 7.6, and 0.3% BSA [fraction V, Calbiochem-Behring Corp.]) supplemented with 75 μg/ml lysophosphatidyl choline (Sigma Chemical Co.) at 37°C, and the acrosome reaction induced by the addition of an equal volume of modified Tyrode's medium containing 4 mM Ca^{2+} (Fleming and Yanagimachi, 1981). In contrast with sperm acrosome-reacted using A23187, sperm acrosome-reacted using lysophosphatidyl choline are hypermotile and capable of fertilizing eggs. This method for inducing the acrosome reaction resulted in a much slower rate of PH-20 migration (see Fig. 2). Sperm were scored as acrosome-intact or acrosome-reacted using phase-contrast optics.

Protease Treatment

Sperm at 5×10^7/ml in Mg^{2+} Hepes were digested with 2 mg/ml Pronase (Calbiochem-Behring Corp.) at 37°C for 30 min. The sperm were washed once, resuspended at 2×10^7/ml for Ca^{2+} Hepes medium at 37°C, and acrosome-reacted by the addition of 1 μg/ml A23187. The sperm were washed, fixed in formaldehyde, and stained for immunofluorescence.

Assay of Antigenic Activity

The amount of antigenic activity in cell fractions was measured by determining the ability of the fraction to inhibit an indirect solid-phase radioactive binding assay. The indirect binding assay has been described previously (Myles et al., 1981). Briefly, an octylglucoside extract of acrosome-intact sperm was bound to the wells of a 96-well plate. The plates were washed, unbound sites blocked with 3% ovalbumin in PBS, and MAb to be tested was incubated in the wells. After washing, MAb bound to the wells was detected using an ^{125}I-rabbit anti-mouse IgG (New England Nuclear). For the inhibition experiments, an appropriate dilution of MAb was chosen that was not saturating in the solid-phase assay. 100 μl of this dilution of MAb was incubated with 100 μl of serial twofold dilutions of the cell fraction to be tested for inhibition. Aliquots of the preincubated MAb–cell fraction mixture were used in the solid-phase binding assay to determine residual MAb binding. One unit of antigenic activity was defined as the amount of antigen needed to give 50% inhibition of the indirect radioactive-binding assay, as defined by Brown et al., (1981). Membrane-bound PH-20 was solubilized from acrosome-intact sperm and acrosomal vesicles with 30 mM octylglucoside. The detergent concentration was adjusted to 3 mM octylglucoside by dilution before incubation with MAb. Soluble PH-20 antigen that might be present in the acrosomal contents was also assayed for. Acrosomal contents were prepared as the 96,000 *g* supernatant after the acrosome reaction. The concentration of each cell fraction was adjusted so that within a series of six twofold dilutions, inhibition of antibody binding ranged from ~20 to 80%.

Purification of Membrane Vesicles Released by the Acrosome Reaction

The hybrid plasma-outer acrosomal membrane (OAM) vesicles released by

Table I. Quantitation of the Number of PH-22 MAb Binding Sites on Acrosome-intact and Acrosome-reacted Sperm

	Cpm bound/10^6 cells	Sites/cell
Acrosome-intact	19,763	$1.2 \pm 0.4 \times 10^6$
Acrosome-reacted	49,562	$3.1 \pm 0.1 \times 10^6$

Duplicate aliquots of acrosome-intact or acrosome-reacted sperm were labeled with PH-22 MAb or irrelevant MAb, then with ^{125}I-labeled Fab' fragment of the PH-22 MAb (sp act = 1.9×10^5 cpm/μg) under saturating conditions (300 and 600 ng/10^6 cells) of the Fab' fragment. The cells were washed to remove unbound antibody and counted in a gamma counter. The number of sites per cell was calculated from the cpm bound and the specific activity of the ^{125}I Fab'. Background values were obtained from the cells preincubated in the PH-22 MAb and have been subtracted from the data. Each value represents the average of three experiments +/− SD.

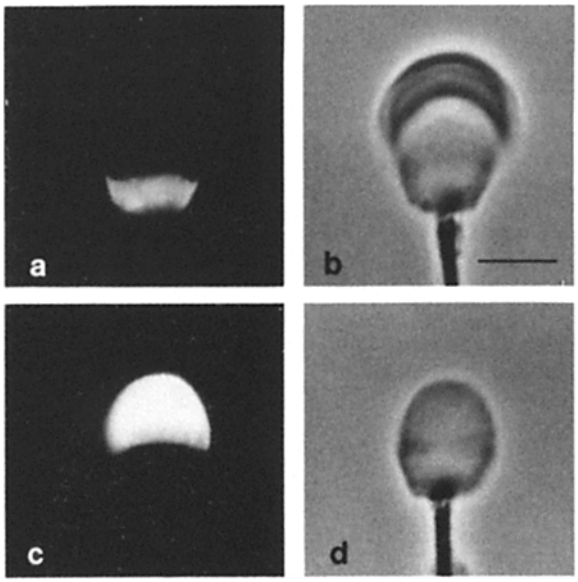

Figure 1. Indirect immunofluorescence surface-labeling of live sperm by the PH-22 MAb and a RITC-conjugated Fab second antibody. (*a* and *b*) A sperm stained before the acrosome reaction. (*c* and *d*) A sperm stained after the acrosome reaction. *a* and *c* are fluorescence images printed at identical exposures, *b* and *d* are phase images. Bar, 5 μm.

the acrosome reaction were purified on a sucrose gradient as described by Primakoff et al., (1980).

Fluorescence-activated Cell Sorter

Fixed and immunofluorescently stained sperm were adjusted to a concentration of 3–5 × 10^6/ml in PBS and analyzed by continuous flow fluorimetry using a FACS IV fluorescence-activated cell sorter (Becton Dickinson Labware, Paramus, NJ).

Immunoprecipitation

Immunoprecipitation, SDS PAGE, and autoradiography were carried out as previously described (Myles et al., 1981).

Results

Increased Binding of a MAb Directed against the PH-20 Protein Is Observed on the Sperm Surface after the Acrosome Reaction

We compared the number of antigenic sites on the surface of acrosome-intact and acrosome-reacted sperm, using one of the three MAbs that recognizes the PH-20 protein (see Materials and Methods). An ^{125}I-labeled Fab′ fragment of the PH-22 MAb was bound to the surface of live, acrosome-intact and acrosome-reacted sperm under saturating conditions of the ^{125}I-Fab. The number of antigenic sites was determined from the amount of ^{125}I-Fab′ bound and the specific activity of the Fab′ fragment (Table I). The results show that there are approximately three times as many antigenic sites on the surface of acrosome-reacted sperm (3.1 × 10^6 sites/cell) as there are on acrosome-intact sperm (1.2 × 10^6 sites/cell).

Some of the PH-20 Protein on the IAM of Acrosome-reacted Sperm Results from Migration of PH-20 Protein from the Posterior Head of Acrosome-intact Sperm

The surface localization of the PH-20 antigen is also different on acrosome-intact and acrosome-reacted sperm (Fig. 1 and Primakoff et al., 1985). On live sperm stained before the acrosome reaction, PH-20 protein is localized to the posterior head surface, whereas on live sperm stained after the acrosome reaction it is localized exclusively to the IAM. Because there is an apparent increase in the amount of PH-20 antigen on acrosome-reacted sperm, and all of this antigen is localized to the IAM, we sought to define the sources of the PH-20 antigen found on acrosome-reacted sperm. In previous studies we have presented evidence that the PH-20 protein originally present on the posterior head migrates to the IAM, and therefore must account for at least some of the PH-20 protein observed on the IAM of acrosome-reacted sperm (Myles and Primakoff, 1984). To confirm and extend the experiments that led to this conclusion, we performed further experiments in the present study.

Migration of PH-20 protein from the posterior head to the IAM was observed under conditions where the migration occurs more slowly than previously described, allowing a demonstration of the intermediate stages. Acrosome-intact sperm were prelabeled with PH-20 MAb and an RITC-conjugated Fab second antibody, then acrosome-reacted using lysophosphatidyl choline (see Materials and Methods). Fig. 2 presents intermediate stages, showing the progressive movement of labeled PH-20 protein from the posterior head to the IAM.

A bright ring of fluorescence was observed in the equatorial region, even 30 min after the acrosome reaction (Fig.

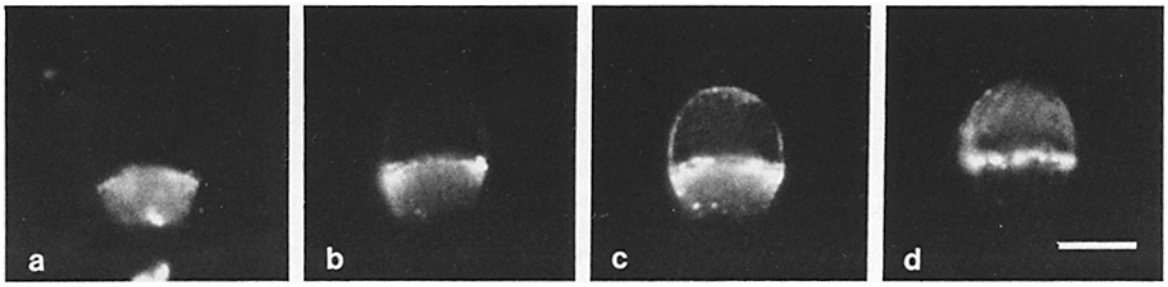

Figure 2. Migration of the PH-20 protein after the acrosome reaction. Acrosome-intact sperm were labeled with PH-20 MAb and an RITC-conjugated Fab second antibody, incubated in modified Tyrode's medium containing 75 μg/ml lysophosphatidyl choline, and induced to undergo the acrosome reaction by the addition of Ca^{2+}. Aliquots of sperm were fixed in formaldehyde at different times after the addition of Ca^{2+} and the migration of PH-20 protein assessed by fluorescence microscopy. *a*, 5 min; *b*, 8 min; *c*, 15 min; *d*, 30 min. The bright fluorescence on the sperm tail in *a* is an adherent piece of cellular debris. All micrographs are printed at identical exposures. Bar, 5 μm.

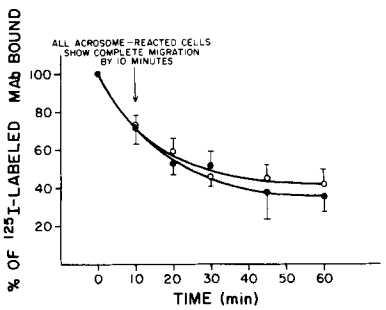

Figure 3. Dissociation rate of PH-22 MAb from acrosome-reacting (○) and acrosome-intact sperm (●). Acrosome-intact sperm were labeled with intact ^{125}I-PH-22 MAb (2×10^4 cpm and 20 ng IgG/10^6 sperm) and washed to remove unbound IgG. Half the sperm were acrosome-reacted in Ca^{2+} Hepes medium containing 1 μg/ml A23187 and 100 μg/ml unlabeled PH-20 MAb at 37°C. When sperm are acrosome-reacted by this method, migration of PH-20 protein to the IAM is completed in 5–10 min. The other half was resuspended in Mg^{2+} Hepes medium containing 100 μg/ml unlabeled PH-20 MAb at 37°C. At different times, duplicate aliquots of sperm were pelleted and the bound cpm as percentage of the value at time zero (~1,700 cpm/10^6 sperm) determined. Background values were determined from sperm preincubated with unlabeled PH-20 MAb before labeling with ^{125}I-PH-20 MAb, and have been subtracted from the data. Each point represents the average of three experiments. Error bars indicate 1 SD.

2 *d*). Bright fluorescence in this region was not previously observed in the final stages of migration after A23187-induced acrosome reaction (Myles and Primakoff, 1984). It is not clear which of these conditions more closely reflects the in vivo situation with respect to PH-20 migration, as the in vivo stimulus for the acrosome reaction in guinea pig sperm is unknown. The bright ring of equatorial region fluorescence seen after the lysophosphatidyl choline-induced acrosome reaction could result from incomplete migration and may reflect the presence of labeled PH-20 protein on the three overlapping layers of surface membrane (plasma membrane, OAM, and IAM, see Fig. 7) that are present in this region. Alternatively, bright fluorescence could result from unique properties of the equatorial region membrane.

The observed movement of fluorescently labeled PH-20 MAb to the IAM cannot be accounted for by dissociation of the labeled MAb from sites on the posterior head region and reassociation to new sites revealed on the IAM (these sites are described in the next section). Previously, we tested for dissociation and reassociation by staining acrosome-intact sperm with PH-20 MAb and FITC-Fab second antibody, and inducing the acrosome reaction in the presence of excess unlabeled PH-20 MAb (Myles and Primakoff, 1984). To avoid the complication of the indirect label, acrosome-intact sperm were prelabeled with RITC-PH-22 MAb, and acrosome-reacted in the presence of a 100-fold excess of unlabeled PH-22 Mab. Fluorescence was still observed on the IAM of >95% of the acrosome-reacted cells. Under these conditions, unlabeled PH-22 Mab will compete with any rebinding of the labeled PH-22 Mab to IAM, so the appearance of fluorescence on the IAM results from the movement of the antibody–antigen complex. Further, we have used ^{125}I-labeled PH-22 MAb to demonstrate quantitatively that a high percentage of the PH-22 MAb remains bound to the sperm during the change in localization. Fig. 3 shows the dissociation of bound ^{125}I-PH-22 MAb from sperm undergoing the acrosome reaction in the presence of a 50-fold excess of unlabeled MAb. The results of this experiment show that in sperm acrosome-reacted using A23187 at 37°C, <30% of the antibody dissociates from the sperm head (Fig. 3) during the 10 min in which all of the PH-20 protein migrates to the IAM (Myles and Primakoff, 1984). Therefore, >70% of the antibody remains bound to the sperm surface during the migration. In addition, the dissociation rate was identical for acrosome-intact sperm, in which no migration occurs, and acrosome-reacting sperm, in which PH-20 protein is migrat-

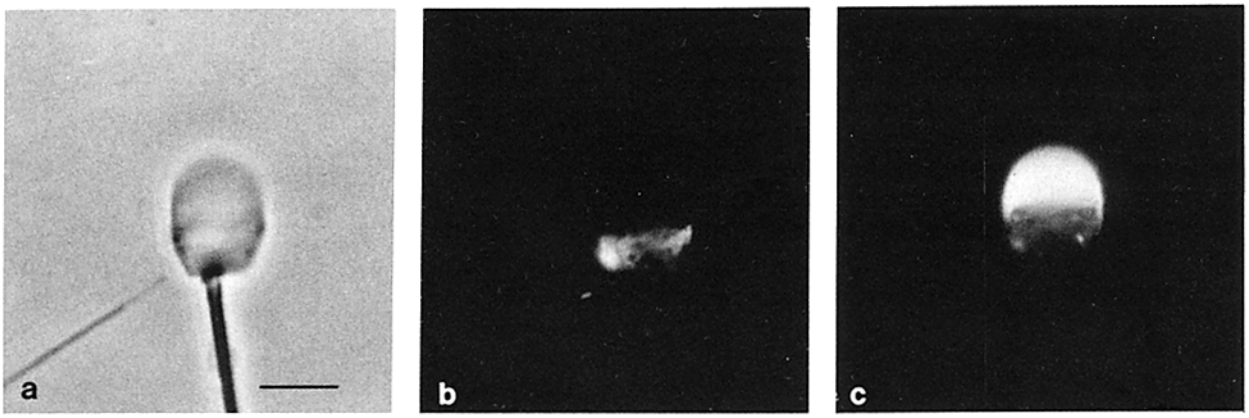

Figure 4. Presence of PH-20 protein on the IAM of acrosome-reacted sperm in which PH-20 migration from the posterior head has been blocked. Acrosome-intact sperm were labeled with the PH-20 MAb and an FITC-conjugated $F(ab')_2$ second antibody. The sperm were acrosome-reacted, fixed, and restained with the PH-20-MAb followed by an RITC-conjugated second antibody. (*a*) Phase image of acrosome-reacted sperm. The fluorescein fluorescence (*b*) is localized to the posterior head, indicating that PH-20 migration from the posterior head to the IAM has been prevented. The fluorescein image appears patchy due to crosslinking by the divalent second antibody. The rhodamine fluorescence (*c*) reveals PH-20 on the entire surface of the IAM, even though migration is blocked, as *b* shows. Identical results were obtained regardless of whether the sperm were fixed before restaining with the PH-20 MAb or were stained live. Some rhodamine fluorescence is observed on the posterior head, probably as a result of some additional binding of the rhodamine second antibody to the fluorescein second antibody–PH-20 MAb complexes. Bar, 5 μm.

ing to the IAM. This result shows that the acrosome reaction does not significantly alter the dissociation of the antibody from the posterior head antigen.

PH-20 Protein Can Be Observed on the IAM of Acrosome-reacted Sperm in the Absence of PH-20 Migration from the Posterior Head

The observed increase in the number of PH-20 antibody binding sites on the surface of acrosome-reacted sperm suggests that there is a source of PH-20 protein in addition to the migrating PH-20 protein on the plasma membrane. (For clarity, the population of migrating PH-20 protein on the posterior head plasma membrane has been termed PH-20_{PM}). One way to test this hypothesis is to determine if PH-20 protein can still be detected on the IAM surface of live acrosome-reacted sperm in the absence of PH-20_{PM} migration. Two different experimental approaches were used to eliminate the contribution of PH-20_{PM} on the IAM of acrosome-reacted cells.

The first method made use of the observation that migration of PH-20_{PM} protein from the posterior head to the IAM can be inhibited by crosslinking the protein using the PH-20 MAb and an FITC-labeled divalent second antibody (Myles and Primakoff, 1984). When sperm labeled in this manner were acrosome-reacted, none of the FITC-labeled MAb was observed to migrate to the IAM (Fig. 4 *b*), thus demonstrating that migration of PH-20 from the posterior head region was prevented. If the sperm were then relabeled with PH-20, PH-21, or PH-22 MAb and an RITC-conjugated second antibody, the RITC label was observed on the entire surface of the IAM in >95% of the acrosome-reacted sperm (Fig. 4 *c*). No RITC label was observed on the IAM when the acrosome-reacted sperm were relabeled with a control Mab, PH-30, or myeloma supernatant.

The second method of eliminating the contribution of PH-20_{PM} to the IAM fluorescence on acrosome-reacted cells entailed proteolytically removing PH-20 protein from the posterior head surface. Treatment of live, acrosome-intact sperm with pronase abolished immunofluorescence labeling of the PH-20 protein on the posterior head surface. When pronase-treated sperm were acrosome-reacted and then immunofluorescently labeled with the PH-22 MAb, staining was nevertheless observed on the entire surface of the IAM of >95% of the cells. No IAM staining was observed using the control MAb PH-30 or myeloma supernatant. Thus, the PH-20 protein observed on the surface of the IAM of acrosome-reacted sperm must arise from an additional source besides the migration of PH-20_{PM} from the posterior head.

PH-20 Protein Pre-exists on the IAM of Acrosome-intact Sperm

We found that when acrosome-intact sperm were extensively permeabilized, PH-20 antigen could be detected in the region of the IAM. If acrosome-intact sperm were permeabilized by freezing before formaldehyde fixation, PH-22 MAb staining was detected on the posterior head region and in the region of the IAM (Fig. 5, *a* and *b*). All three of the MAbs that recognize the PH-20 protein exhibited this pattern of staining, which appeared on >90% of the cells in the preparation. We have called the intracellular population of PH-20

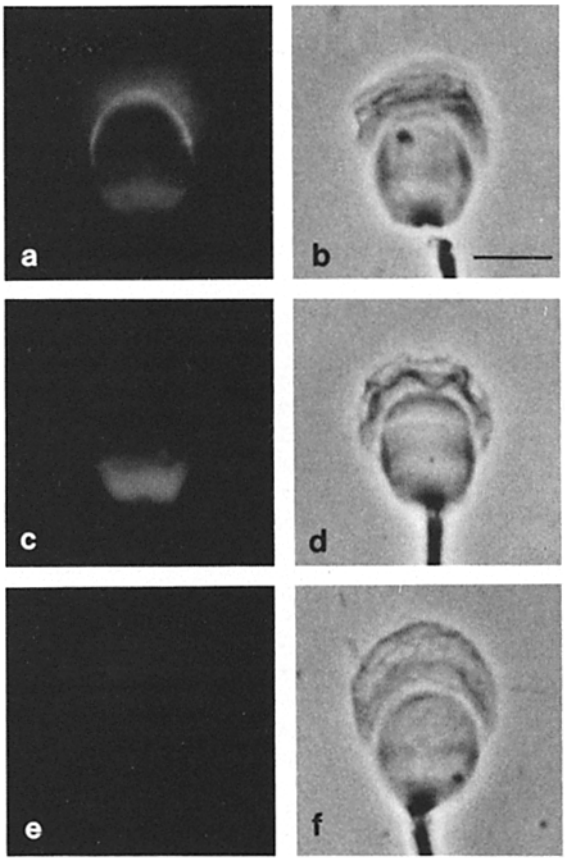

Figure 5. Indirect immunofluorescence staining of permeabilized, acrosome-intact sperm by the PH-22 MAb. Acrosome-intact sperm were permeabilized, fixed, and stained with either the PH-22 MAb (*a* and *b*), PH-30 MAb, (*c* and *d*), or myeloma culture supernatant (*e* and *f*). The PH-22 MAb stains the posterior head region and a narrow band on the anterior tip of the IAM. In controls, neither the PH-30 MAb, which recognizes a different antigen localized to the posterior head domain, or myeloma supernatant stain the IAM. *a*, *c*, and *e*, fluorescence optics; *b*, *d*, and *f*, phase optics. Fluorescence micrographs are printed at identical exposures. Bar, 5 μm.

antigen that pre-exists on the IAM of acrosome-intact sperm PH-20_{AM}, to distinguish it from the population originally on the posterior head surface, PH-20_{PM}.

Two controls were performed to demonstrate the specificity of PH-20 antibody binding to the IAM in these preparations. First, another MAb, PH-30, that recognizes a different antigen localized to the posterior head domain (Primakoff and Myles, 1983), did not stain the IAM under these conditions (Fig. 5, *c* and *d*). In addition, no staining was observed by the second antibody alone (Fig. 5, *e* and *f*).

Two features of the immunofluorescence staining pattern observed on permeabilized acrosome-intact sperm (Fig. 5, *a* and *b*) warranted further investigation because they bear on the precise location of the newly identified intracellular PH-20 antigen. First, while bright specific staining occurred only in the immediate area of the IAM, a halo of staining was observed in the rest of the acrosomal region (Fig. 5, *a* and *b*). This halo may simply be due to a higher background staining by the PH-22 MAb relative to control MAb (Fig. 5, *c* and *d*), or could indicate the presence of PH-20 antigen in the acrosomal contents or in the OAM. Second, only a thin

band, or horseshoe, in the region of the IAM that overlies the anterior tip of the nucleus was stained in permeabilized acrosome-intact cells. However, 15 min after the acrosome reaction, PH-20_{AM}-specific staining was observed on the entire surface of the IAM (see Fig. 4 *c*). We have addressed these issues in the following sections.

PH-20 Protein Is Absent or Present at a Low Level in the Acrosomal Contents or OAM

We have investigated whether the intracellular population of the PH-20 antigen is restricted to the IAM, or whether the antigen also exists in the OAM or acrosomal contents, by assaying for PH-20, PH-21, and PH-22 antibody binding activity in the material released by the acrosome reaction. Sperm were acrosome-reacted, pelleted, and the supernatant containing the released acrosomal contents and hybrid plasma–OAM vesicles centrifuged at high speed to separate the soluble acrosomal contents from the membrane vesicles (Primakoff et al., 1980). The soluble acrosomal contents were then assayed for the ability to compete for antibody binding in an indirect solid-phase radioimmune assay. As Table II shows, the acrosomal contents contained on average only 9% of the binding activity for the three anti–PH-20 MAbs found in detergent extracts of acrosome-intact sperm. Similar results were obtained when hybrid plasma–OAM vesicles were purified from the supernatant (Primakoff et al., 1980), and PH-20, PH-21, and PH-22 MAb binding activity in detergent extracts of these vesicles determined. The amount of PH-20, PH-21, and PH-22 MAb binding activity observed in the extracts was only 9% of that observed in detergent extracts of whole sperm. To determine background values, the extracts were also tested for binding activity to the PT-1 MAb (Myles and Primakoff, 1984), which recognizes an antigen localized to the sperm tail and thus is not specifically released by the acrosome reaction. The amount of anti–PH-20 antibody binding activity (9%) was only slightly greater than the background value obtained for PT-1 binding activity (7%). These results suggest that PH-20 antigen is absent or present only in low amounts in either the acrosomal contents or the OAM.

Table II. Amount of PH-20 Antigen Released by the Acrosome Reaction

	Units of antigenic activity/10^6 cells*		
	Detergent extract of acrosome-intact sperm	Acrosomal contents	Detergent extract of purified vesicles
PH-20 MAb	22.5	0.8 (4%)	1.8 (8%)
PH-21 MAb	27	2.2 (8%)	2.1 (8%)
PH-22 MAb	20.5	2.9 (14%)	2.2 (11%)
PT-1 MAb‡	9.9	ND	0.7 (7%)

ND, not determined.
* One unit of antigenic activity is defined as the amount of antigen needed to give 50% inhibition of an indirect radioactive binding assay, as defined by Brown et al. (1981). Numbers in parentheses give the amount of antigenic activity as a percentage of that found in detergent extracts of acrosome-intact sperm. The results are the average of two experiments.
‡ The PT-1 MAb recognizes a protein localized to the sperm tail, and thus serves as a control for antigen not specifically released by the acrosome reaction.

Some of the PH-20_{AM} May Not Be Accessible to MAb Binding in Acrosome-intact Sperm

The "horseshoe" pattern of staining observed on the IAM of permeabilized cells (Fig. 5 *a*) is also observed on acrosome-reacted cells at early times (1–3 min) after induction of the acrosome reaction (not shown) under conditions where PH-20_{PM} migration is blocked (using the protocol in Fig. 4). At later times (>5 min), the entire IAM is stained on these acrosome-reacted cells (see e.g., Fig. 4 *c*). A possible explanation for the different staining patterns is that the PH-20_{AM} is not accessible to antibody in specific regions of the IAM either on permeabilized cells or during the earliest stages of the acrosome reaction. This hypothesis predicts an increase in the amount of anti–PH-20 antibody bound to PH-20_{AM} as the acrosomal contents are released. We therefore used a fluorescence-activated cell sorter to quantitate the amount of fluorescent antibody bound to individual sperm stained specifically for PH-20_{AM} (see legend to Fig. 4), and compared the staining intensity on cells exhibiting either the "horseshoe" pattern or complete staining of the IAM. The peak in the cell number vs. fluorescence intensity plot obtained from sperm exhibiting complete IAM staining of the PH-20_{AM} (5 min after inducing the acrosome reaction) was found to be increased ~1.6 arbitrary fluorescence units compared with sperm exhibiting the horseshoe pattern of PH-20_{AM} staining (1 min after the acrosome reaction). Thus, the different patterns of PH-20_{AM} staining correspond to different amounts of antibody bound. This result suggests that PH-20_{AM} is present on the entire region of the IAM on acrosome-intact sperm, but is masked from antibody binding in some regions. As the acrosome reaction proceeds, new PH-20 MAb binding sites are exposed on the IAM.

Comparison of PH-20_{AM} and PH-20_{PM} Antigens by SDS-polyacrylamide Gels

The fact that all three MAbs directed against the PH-20 antigen immunofluorescently stained both the PH-20_{PM} and PH-20_{AM} antigens suggests that these two antigens are the same or closely related proteins. However, it is also possible that there are in fact two distinct antigens that share antigenic determinants. To examine this question we have compared the immunoprecipitates obtained from detergent extracts of sperm surface-labeled with ^{125}I before the acrosome reaction, where only the PH-20_{PM} antigen can be labeled, and after the acrosome reaction, where both the PH-20_{PM} and PH-20_{AM} antigens can be labeled. A diffuse band of ~M_r 62,000 was obtained from both acrosome-intact and acrosome-reacted surface-labeled cells, and no additional bands were detected from the extract of acrosome-reacted sperm (Fig. 6). Furthermore, there was an increase in the amount of the M_r 62,000 band obtained from acrosome-reacted sperm, as would be expected if the PH-20_{AM} antigen is closely related structurally to the PH-20_{PM} antigen. Further studies are in progress to determine if relatively subtle structural differences exist between PH-20_{PM} and PH-20_{AM}.

Discussion

In the experiments presented here we have discovered that exocytosis in guinea pig sperm results in a change in the amount of PH-20 on the sperm cell surface. The increase in

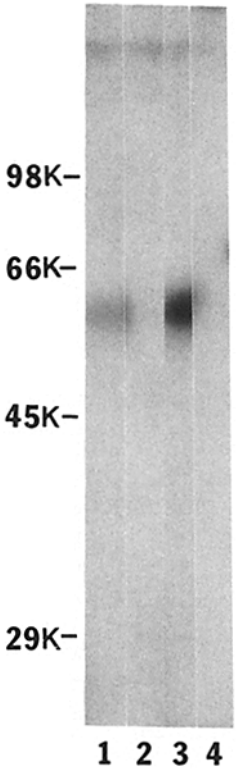

Figure 6. Comparison of the immunoprecipitates of ^{125}I-surface-labeled PH-20 protein from acrosome-intact or acrosome-reacted sperm. 3×10^7 acrosome-intact or acrosome-reacted sperm were each surface-labeled with 0.33 mCi ^{125}I, washed, extracted with Triton X-100, cleared by ultracentrifugation, and dialyzed. Each dialyzed extract contained 1×10^7 cpm/400 μl of extract from 3×10^7 sperm. 40 μl of each extract was used for immunoprecipitation, and the entire immunoprecipitate loaded on the gel. Immunoprecipitates obtained from acrosome-reacted sperm using any of the three MAbs directed against PH-20 protein consistently contained 2–5-fold higher cpms compared with immunoprecipitates obtained from equal numbers of acrosome-reacted sperm. Lanes *1* and *2* show the immunoprecipitates obtained from acrosome-intact sperm using PH-21 MAb (lane *1*) or myeloma culture supernatant (lane *2*); lanes *3* and *4* show the immunoprecipitate from acrosome-reacted sperm using PH-21 MAb (lane *3*) or myeloma supernatant (lane *4*).

surface expression of the PH-20 protein coincides with a change in its localization. As summarized in the model diagrammed in Fig. 7, these changes are brought about by both the insertion of a secretory granule membrane containing PH-20 protein into the surface and a directed movement of PH-20 protein from one surface domain to another. On acrosome-intact sperm, there are two distinct populations of the PH-20 protein. One population, PH-20_{PM}, is localized to the posterior head surface. The second population, PH-20_{AM}, is localized to an intracellular membrane, the IAM. The acrosome reaction, which is the Ca^{2+}-dependent exocytosis of a single large secretory granule, results in joining of the IAM and plasma membrane via the equatorial segment. This event exposes PH-20_{AM} on the sperm surface, resulting in an increase in the amount of PH-20 protein on the sperm surface. The continuity of the plasma membrane and IAM also allows migration of the PH-20_{PM} to the IAM. As a result, PH-20 protein from two different sources is concentrated on the surface of the anterior half of the acrosome-reacted sperm.

Two lines of evidence suggest that the PH-20_{AM} protein is closely related structurally to the PH-20_{PM} protein. First, immunoprecipitates of detergent extracts from both acrosome-intact and acrosome-reacted sperm contain the same surface-labeled band of 62 kD. No labeled polypeptides in addition to the 62-kD band are immunoprecipitated using surface-labeled acrosome-reacted sperm. Rather, there is an increase in the amount of the 62-kD polypeptide, consistent with the increased number of sites observed on acrosome-reacted sperm. Second, the three antibodies directed against the PH-20 protein give the same immunoprecipitate and apparently recognize three different epitopes (Primakoff et al., 1985). All three antibodies label both the posterior head and IAM populations of PH-20 protein by indirect immunofluorescence, which also suggests a substantial degree of homology between the two populations of PH-20 antigen.

The evidence we have presented shows that one mechanism for regulating the amount of surface proteins on a cell is the localization of the protein to a secretory granule membrane and subsequent exocytosis. Traditionally the function of Ca^{2+}-regulated exocytosis has been viewed as the release of soluble components into the extracellular medium. Our results indicate a second function for sperm exocytosis—regulating the level of expression of a surface protein. A similar mechanism for regulating cell surface composition has been proposed in several other systems. Borregaard et al. (1983) have provided convincing evidence that the b-cytochrome component of the human neutrophil microbial oxidase is associated with intracellular vesicle membranes of unstimulated neutrophils and becomes transported to the surface when the neutrophils are stimulated to undergo exocytosis. In addition, the increase on stimulated neutrophils in the f-met-leu-phe receptor (Fletcher and Gallin, 1980, 1983; Fletcher et al., 1982) and possibly the receptor for the C3b complement factor (Fearon and Collins, 1983) may be regulated by exocytosis. Rapid changes in the rate of cellular

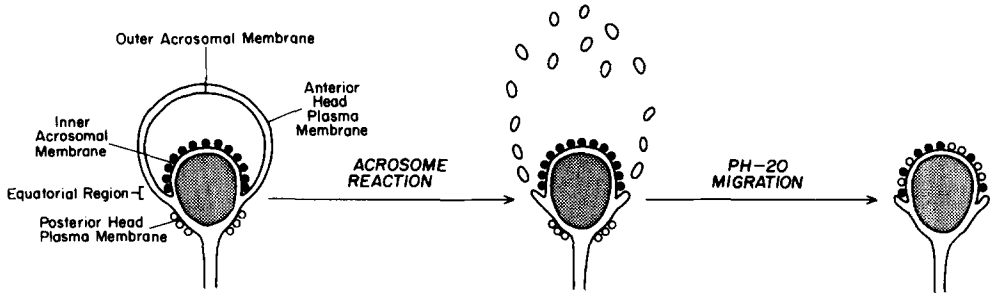

Figure 7. Diagram of PH-20 localization on acrosome-intact and acrosome-reacted sperm. Two populations of PH-20 protein are detected on acrosome-intact sperm. PH-20_{PM} is localized to the surface of the posterior head region, whereas PH-20_{AM} is intracellular, and localized to the IAM. After the acrosome reaction, the posterior head plasma membrane becomes continuous with the IAM, allowing the migration of the PH-20_{PM} to the IAM. On acrosome-reacted sperm, all the PH-20 protein is concentrated on the IAM.

transport functions upon hormonal stimulation in a variety of cell types has also been proposed to occur by exocytosis of vesicles containing transporter proteins (reviewed in Leinhard, 1983). The variety of these systems implies that exocytosis may be a general mechanism for regulating cell surface composition. Such a mechanism is particularly attractive in cases where a rapid increase in the amount of cell surface protein is required, where increases in protein synthesis and processing rates would be insufficient to meet the cell's needs.

The presence of PH-20 protein on both an external and internal membrane in acrosome-intact sperm raises questions about how this protein is targeted to two different membranes. The acrosome is derived from vesicles of the Golgi complex in the developing sperm (Burgos and Fawcett, 1955). In other systems, sorting of most membrane proteins occurs in the Golgi apparatus and is thought to require specific receptors that recognize an "address" signal contained either in the amino acid sequence or in the pattern of glycosylation of a membrane protein (Sabatini et al., 1982; Garoff, 1985; Farquhar, 1985). If this is true, there are at least two hypotheses that could account for the targeting of the PH-20 protein to two different membranes. One hypothesis would predict the existence of two isoforms of the PH-20 protein that differ in the address sequence. A second hypothesis would predict a single form of the PH-20 protein containing an address sequence that would be recognized by two different receptors.

The restricted localizations of both PH-20_{AM} and PH-20_{PM} protein and the migration of PH-20_{PM} dramatically illustrate the complexities involved in the regulation of membrane topography. Both populations of the PH-20 protein on acrosome-intact sperm are restricted to a limited region of a continuous membrane system. PH-20_{PM} is localized to a restricted region of the plasma membrane, while PH-20_{AM} is restricted to a discrete region of an organelle membrane, the acrosomal membrane. The acrosomal membrane has previously been hypothesized to contain specific membrane domains, based on the different functional roles of the OAM and IAM (Holt, 1984; Olson and Winfrey, 1985). In addition, these two regions of the acrosomal membrane show distinct differences in stability; the OAM is easily disrupted by changes in ionic strength and sonication, whereas the IAM remains intact under a variety of conditions (Wooding, 1973; McRorie and Williams, 1974; Dudkiewicz et al., 1979). Morphological differences between the OAM and IAM have been observed by freeze-fracture electron microscopy (Olson and Winfrey, 1985). Furthermore, an antiserum raised against purified OAM does not stain the IAM (Hinrichsen et al., 1985). The localization of specific proteins to a discrete region of a continuous intracellular membrane system has been observed in other systems. In chloroplasts, components of Photosystem II are restricted to the grana partitions and components of Photosystem I are excluded from this region (Staehelin 1976; Anderson and Andersson, 1980, 1982). Lateral segregation of intracellular membrane proteins is also necessary for protein sorting in the endoplasmic reticulum and Golgi apparatus.

The functional consequences of these complex patterns of localization and redistribution are unknown. In view of the evidence that the PH-20 antigen has a role in sperm binding to the egg zona pellucida (Primakoff et al., 1985), it can be speculated that the change in amount and location of PH-20 protein on the sperm surface might serve to control sperm–zone interaction. The acrosome reaction results in a change in the amount of the PH-20 protein on the sperm surface, in the environment of the protein, and in the positioning of the PH-20 protein on the sperm surface. These changes may result in an altered strength of sperm–zona binding, or could be important in creating the proper orientation of sperm binding to facilitate subsequent sperm penetration through the zona.

We thank Bonnie Phelps for help in performing some of the radioimmune binding assays, Jerry Murphy for technical assistance in using the fluorescence-activated cell sorter, and Denise Gagnon for preparing the manuscript.

A. E. Cowan was supported by a Damon Runyon-Walter Winchell Cancer Fund Fellowship grant, DRG-804. This work was supported by the National Institutes of Health grant HD 16580 to D. G. Myles and American Heart Association (AHA) Grant-in-aid 82-1120 with funds contributed in part by the AHA Connecticut Affiliate. During this work, D. G. Myles held a Faculty Research Award from the American Cancer Society, and P. Primakoff held an Established Investigatorship Award from the AHA.

Received for publication 24 February 1986, and in revised form 9 June 1986.

References

Andersson, B., and J. M. Anderson. 1980. Lateral heterogeneity in the distribution of chlorophyll-protein complexes of the thylakoid membranes of spinach chloroplasts. *Biochim. Biophys. Acta.* 593:427–440.

Anderson, J. M., and B. Andersson. 1982. The architecture of photosynthetic membranes: lateral and transverse organization. *Trend Biochem. Sci.* 6:288–292.

Burgos, M. H., and D. W. Fawcett. 1955. Studies on the fine structure of the mammalian testis I. Differentiation of the spermatids in the cat (Felis Domestica). *J. Biophys. Biochem. Cytol.* 1:287–300.

Borregaard, N., J. M. Heiple, E. R. Simons, and R. A. Clark. 1983. Subcellular localization of the *b*-cytochrome component of the human neutrophil microbial oxidase: translocation during activation. *J. Cell Biol.* 97:52–61.

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.

Dudkiewicz, A. B., P. N. Srivastava, C. H. Yang, and W. L. Williams. 1979. Extraction of human and rabbit acrosomes: a comparison of sequential and sonication methods. *Andrologia.* 11:355–366.

Farquhar, M. G. 1985. Progress in unraveling pathways of Golgi traffic. *Annu. Rev. Cell Biol.* 1:447–488.

Fearon, D. T., and L. A. Collins. 1983. Increased expression of C3b receptors on polymorphonuclear leukocytes induced by chemotactic factors and by purification procedures. *J. Immunol.* 130:370–375.

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 lysopholipids in the acrosome reaction. *Gamete Res.* 4:253–273.

Fletcher, M. P., and J. I. Gallin. 1980. Degranulating stimuli increase the availability of receptors on human neutrophils for the chemoattractant f-met-leu-phe. *J. Immunol.* 124:1585–1588.

Fletcher, M. P., and J. I. Gallin. 1983. Human neutrophils contain an intracellular pool of putative receptors for the chemoattractant n-formyl-methionyl-leucyl-phenylalanine. *Blood.* 62:792–799.

Fletcher, M. P., B. E. Seligmann, and J. I. Gallin. 1982. Correlation of human neutrophil secretion, chemoattractant receptor mobilization, and enhanced functional capacity. *J. Immunol.* 128:941–948.

Garoff, H. 1985. Using recombinant DNA techniques to study protein targeting in the eukaryotic cell. *Annu. Rev. Cell Biol.* 1:403–445.

Goding, J. W. 1976. Conjugation of antibodies with fluorochromes: modifications to the standard methods. *J. Immunol. Methods.* 13:215–226.

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

Hinrichsen, A. C., E. Topfer-Petersen, T. Dietl, C. Schmoeckel, W.-B. Schill. 1985. Immunological approach to the characterization of the outer acrosomal membrane of boar spermatozoa. *Gamete Res.* 11:143–155.

Holt, W. V. 1984. Membrane heterogeneity in the mammalian spermatozoon. *Int. Rev. Cytol.* 87:159–193.

Leinhard, G. E. 1983. Regulation of cellular membrane transport by the exocytotic insertion and endocytotic retrieval of transporters. *Trends Biochem. Sci.* 8:125–127.

Mason, D. W., and A. F. Williams. 1980. The kinetics of antibody binding to membrane antigens in solution and at the cell surface. *Biochem. J.* 187:1–20.

McRorie, R. A., and W. L. Williams. 1974. Biochemistry of mammalian fertilization. *Annu. Rev. Biochem.* 43:777–803.

Myles, D. G., and P. Primakoff. 1984. Localized surface antigens of guinea pig sperm migrate to new regions prior to fertilization. *J. Cell Biol.* 99:1634–1641.

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.

Noda, Y. D., and R. Yanagimachi. 1976. Electron microscopic observations of guinea pig spermatozoa penetrating eggs in vitro. *Dev. Growth & Differ.* 18:15–23.

Olson, G. E., and V. P. Winfrey. 1985. Structure of membrane domains and matrix components of the bovine acrosome. *J. Ultrastruct. Res.* 90:9–25.

Parham, P. 1983. On the fragmentation of monoclonal IgG1 IgG2a, and IgG2b from Balb/c mice. *J. Immunol.* 131:2895–2902.

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

Primakoff, P., D. G. Myles, and A. R. Bellve. 1980. Biochemical analysis of the released products of the mammalian acrosome reaction. *Dev. Biol.* 80:324–331.

Primakoff, P., H. Hyatt, and D. G. Myles. 1985. A role for the migrating sperm surface antigen PH-20 in guinea pig sperm binding to the egg zona pellucida. *J. Cell Biol.* 101:2239–2244.

Sabatini, D. D., G. Kreibich, T. Morimoto, and M. Adesnik. 1982. Mechanisms for the incorporation of proteins in membranes and organelles. *J. Cell Biol.* 92:1–22.

Staehelin, L. A. 1976. Reversible particle movements associated with unstacking and restacking of chloroplast membranes in vitro. *J. Cell Biol.* 71:136–158.

Wooding, F. B. P. 1973. The effect of Triton X-100 on the ultrastructure of ejaculated bovine sperm. *J. Ultrastruct. Res.* 42:502–516.