High Density of Transmembrane Glycoproteins on the Flagellar Surface of Boar Sperm Cells

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ABSTRACT Membrane halves of boar sperm flagella were produced by freeze-fracture and labeled in situ with concanavalin A and wheat germ agglutinin; the lectins were visualized with protein-gold complexes. Concanavalin A and wheat germ agglutinin binding sites partition with both protoplasmic and exoplasmic halves of the membrane. A high density of lectin marking was found on protoplasmic membrane halves; we conclude that the label corresponds to transmembrane glycoproteins that, on freeze-fracture, are dragged across the outer (exoplasmic) half of the phospholipid bilayer. Our demonstration of numerous transmembrane proteins in sperm flagella offers the structural setting for previous models on flagellar surface motility that postulate accessibility of motile membrane components to the submembranous cytoskeleton.

Flagella of eucaryotic cells are specialized extensions of the cell body composed of similar basic components: a central axoneme, a thin layer of cytoplasm that may include mitochondria, accessory fibers or paraflagellar rods, and the limiting plasma membrane (1–4). They are responsible for two independent forms of whole-cell movement: swimming propelled by coordinated bending of the flagellum (4) and gliding on the surface of a solid substrate (5–7). Swimming is the result of sliding of axonemal microtubules, as characterized in a number of elegant studies (8–12). Gliding was first described in *Chlamydomonas* by Lewin (5) and appears to depend on the dynamics of the flagellar surface in association with cytoskeleton structures (7).

Flagellar surface motility is expressed by the saltatory movement of exogenous markers (polysterene microspheres or bacteria) (13). Bloodgood proposed that the markers bind to putative "motility-coupled cell-surface receptors" (7, 13). The density of these receptors slowly decreases upon incubation of flagella in inhibitors of protein synthesis (cycloheximide) and protein glycosylation (tunicamycin) (6, 14). Pronase digestion of a high-molecular-weight glycoprotein of the flagellar surface causes reversible loss of gliding ability (6, 15). Structural studies on flagella reported the presence of submembranous cytoskeleton-like elements that, in some cells, may connect the plasma membrane with the outer doublet microtubules of the axoneme (1–4, 16–18). ATPase activity was also found on the submembranous cytoplasm of flagella (19–25).

Current models on the coupling of surface receptors with the cytoskeleton postulate the existence of transmembrane glycoproteins on the flagellar membrane (3, 14). This topology of glycoproteins in the membrane would allow the same molecule to express (on its outer portion) the surface receptors and to be accessible (on its cytoplasmic portion) for interaction with the membrane skeleton or with axonemal microtubules. Here, we search for the topology of glycoconjugates on the flagellar surface of boar sperm cell using "fracture-label" techniques (26–28). The method allows direct labeling of membrane halves, obtained by freeze-fracture of cells, with specific cytochemical markers. We used lectin-protein-gold complexes to study the partition and density of glycoconjugates with sugar residues of mannose/glucose (concanavalin A [Con A] [29])[1] and sialic acid/*N*-acetyl-glucosamine (wheat germ agglutinin [WGA] [30, 31]) with protoplasmic and exoplasmic halves of the membrane. We show that protoplasmic halves of the flagellar plasma membrane have a high density of Con A and WGA receptors. We conclude that lectin labeling of protoplasmic membrane halves must correspond to transmembrane glycoproteins. Our finding is the first evidence for the transmembrane nature of numerous glycoproteins on a flagellar surface.

MATERIALS AND METHODS

Cells

Semen samples from mature boars, *Sus scrofa* (a generous gift of Dr. Larry Johnson, United States Department of Agriculture, Beltsville, MD), were

[1] *Abbreviations used in this paper:* Con A, concanavalin; WGA, wheat germ agglutinin.

centrifuged (500 *g*, 5 min), and the cell-rich pellets were washed by successive pelleting and resuspension (three times) in Hanks' balanced salt solution or in PBS. The spermatozoa were fixed in 1.5% glutaraldehyde in PBS, pH 7.4, at ice bath temperature for 1 h, and washed twice in PBS.

Fracture-Label

THIN SECTIONS: Fixed and washed boar spermatozoa were mixed in 30% (wt/vol) BSA in PBS, and centrifuged (2,000 *g*, 10 min). The pellet of BSA cells was gelled by the addition of glutaraldehyde to a final concentration of 1%. In some samples, glutaraldehyde-fixed human erythrocytes were mixed with the sperm cells as an internal control of the freeze-fracture process: patterns of lectin labeling of freeze-fractured erythrocytes were previously established in this laboratory (42); erythrocytes are a reliable marker for surfaces exposed in gels by slicing at room temperature because, in these instances, the red blood cells are always separated in toto (never cross-fractured) with gel surfaces. The gels were sliced, impregnated in 30% glycerol in PBS (3 h), frozen in partially solidified Freon 22, and fractured in liquid nitrogen with a glass pestle (27, 29, 33). Fractured gel fragments were thawed, deglycerinated, and labeled with Con A-peroxidase-gold or WGA-ovomucoid-gold, as described below (see Cytochemistry). The samples were then processed for thin-section electron microscopy.

Other samples of fixed and washed sperm cells were precoated with cationized ferritin in PBS, pH 7.2 (1 mg/ml, Miles Biochemicals, Elkhart, IN) before fracture-label. Three successive incubations of 15 min were performed to assure saturation of all anionic sites available at the sperm surface. Coated spermatozoa were washed in PBS, and mixed in 30% BSA in PBS. BSA cell gels were obtained by glutaraldehyde cross-linking, and processed for freeze-fracture and WGA-ovomucoid-colloidal-gold labeling (see Cytochemistry).

PLATINUM-CARBON REPLICAS: Fixed and washed boar spermatozoa were impregnated in 30% glycerol in PBS, and mixed in a solution of 30% BSA in 30% glycerol (in PBS). The specimens were freeze-fractured with a scalpel, as described before (27), or "sandwiched" between two glass coverslips (28). For the "sandwich" method, small droplets of the mixture were dispersed on precleaned glass coverslips that were superimposed on other coverslips paved with glutaraldehyde (3% in 30% glycerol/PBS). Both types of preparations were frozen in the liquid phase of partially solidified Freon 22, and transferred to liquid nitrogen. Freeze-fracture of "sandwiched" BSA cell gels was performed by mechanical separation of complementary coverslips immersed in liquid nitrogen. The specimens were thawed, deglycerinated, and labeled with Con A-peroxidase-gold or WGA-ovomucoid-gold, as described below (see Cytochemistry). Washed specimens were fixed in 1% osmium tetroxide in veronal buffer for 30 min, dehydrated in ethanol, and critical-point-dried in ethanol/carbon dioxide (27). The fractured gels were shadowed with platinum/carbon in a Balzers 301 apparatus. Replicas of the fracture-faces were isolated by digestion of the gel in sodium hypochloride, and mounted on formvar-coated grids for electron-microscopic observation.

Cytochemistry

Samples of isolated cells or BSA–sperm gels were incubated in solutions of Con A (0.25 mg/ml) or WGA (0.25 mg/ml) in Sorensen's phosphate buffer, 4% polyvinylpyrrolidone, pH 7.4, for 1 h at room temperature (Con A) or 37°C (WGA). Controls were preincubated in lectin-competitive sugars—0.4 M methyl-α-D-mannopyranoside (Con A experiments) or 0.4 M *N*-acetyl-D-glucosamine (WGA experiments)—for 15 min, followed by incubation in the lectin and the sugar as described above. After washing in Sorensen's buffer, the samples were labeled in a colloidal gold/horseradish peroxidase complex (33–35) (Con A experiments) or in ovomucoid-coated colloidal gold (34–36) (WGA experiments), for 3 h at 4°C. All samples were washed in Sorensen's buffer and processed either for platinum/carbon replication or for thin-section electron microscopy.

Processing for Thin-Section Electron Microscopy

Gel fragments were postfixed in a solution of 2% osmium tetroxide and 1% potassium ferricyanide (37–39) in veronal buffer, stained en bloc with uranyl acetate (5 mg/ml) in the same buffer, pH 5.8, dehydrated in acetone, and embedded in Epon 812. Thin sections were observed by transmission electron microscopy after counterstaining with lead citrate only.

RESULTS

Ultrastructure of Boar Sperm Flagella

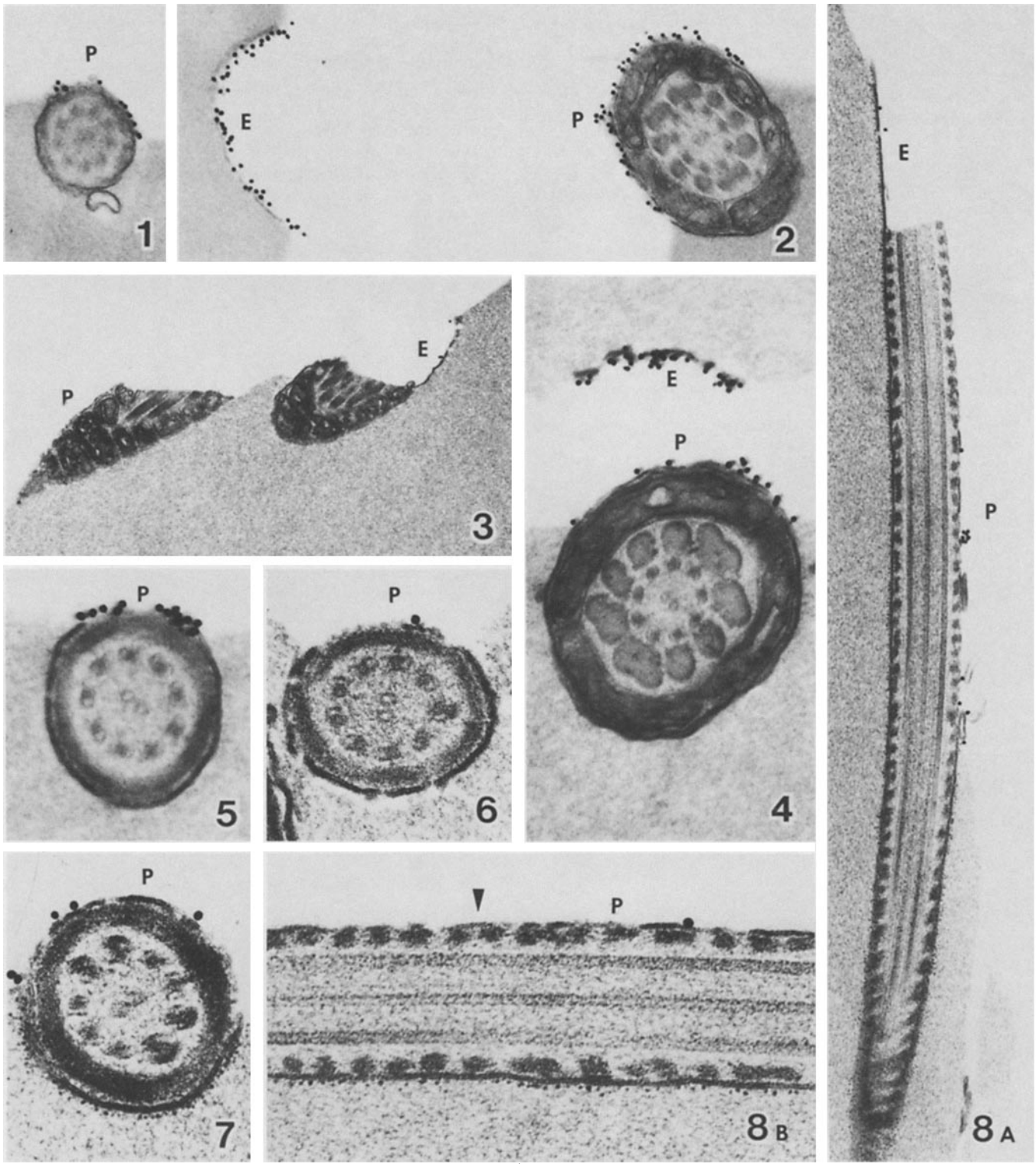
Interpretation of our results requires a brief review of the general morphology of the boar sperm flagellum. The central component of the flagellum of boar spermatozoa is a 9 + 2 axoneme. Flagella of mammalian sperm are differentiated into two major segments: the midpiece and the principal piece (for review see reference 41). At the midpiece, the axoneme is surrounded by nine dense fibers that are, on the outside, circumferentially covered by mitochondria (illustrated here in Figs. 2–4). At its proximal end, the dense fibers of the midpiece fuse to form the connecting piece between sperm head and sperm tail. At the principal piece, the longest segment of the sperm flagellum, the axoneme is surrounded only by transversal rings of fibrous material (illustrated here in Figs. 5–8) that terminate short of the distal end of the axoneme (illustrated here in Fig. 1).

Thin-Section Fracture-Label

Similar to what we observed in sperm heads (28), a notable preservation of the fine structure of boar sperm flagella was found in cells submitted to thin-section fracture-label. Moreover, due to BSA embedding of sperm cells after fixation, the flagellar membrane did not show the usual wrinkling artifact observed when spermatozoa are routinely processed without the support of a cross-linked protein matrix (40). The membrane halves of flagella are seen, in thin sections, as interrupted trilaminar profiles (see Fig. 8*B*), similar to what was described in other fracture-labeled cells and tissues (26, 28, 32, 41–43). This appearance of membrane halves results from reorganization of membrane apolar groups into bimolecular structures upon exposure of freeze-fracture-induced monolayers to an aqueous environment, on thawing of the preparations (see references 26 and 32 for evidence and detailed discussion of the process).

Both protoplasmic and exoplasmic membrane halves of the boar sperm flagellum show high density of Con A and WGA binding sites visualized by gold/protein complexes (Figs. 1, 2, 4, and 5). This is best seen in gel "cracks" because they provide complementary views of freeze-fractured membranes (Fig. 2). Labeling was virtually absent on membrane halves of flagella incubated in the lectins in the presence of their competitive sugars (Figs. 3 and 6). Drastic reduction (70% or more) of WGA labeling was also found on membrane halves of sperm flagella coated with cationized ferritin prior to freeze-fracture (Figs. 7 and 8).

We observed also thin sections of freeze-fractured gels containing glutaraldehyde-fixed human erythrocytes, in addition to sperm cells. The presence of erythrocytes in sperm–BSA gels was used to identify faces induced by slicing of the gel at room temperature rather than by freeze-fracture: in previous studies (26, 32), we demonstrated that in genuine freeze-fracture edges erythrocytes are frequently cross-fractured; in contrast, gel faces due to slicing at room temperature never display cross-cut erythrocytes as they partition in toto with either side. In addition, the fractured edges of the gels were inspected for frayed edges, an additional signal for a gel "cut" induced at room temperature. In these preparations, labeling patterns of spermatozoa as well as the ultrastructure of their fractured membranes were similar to those reported above. It should be noted that because the gels are crushed into very small fragments, the possibility of observing a "cut" face is far smaller than that of observing a freeze-fracture face since the total surface of freeze-fracture generated during crushing of gels, under liquid nitrogen, far exceeds the original surface of the ("cut") gel piece.



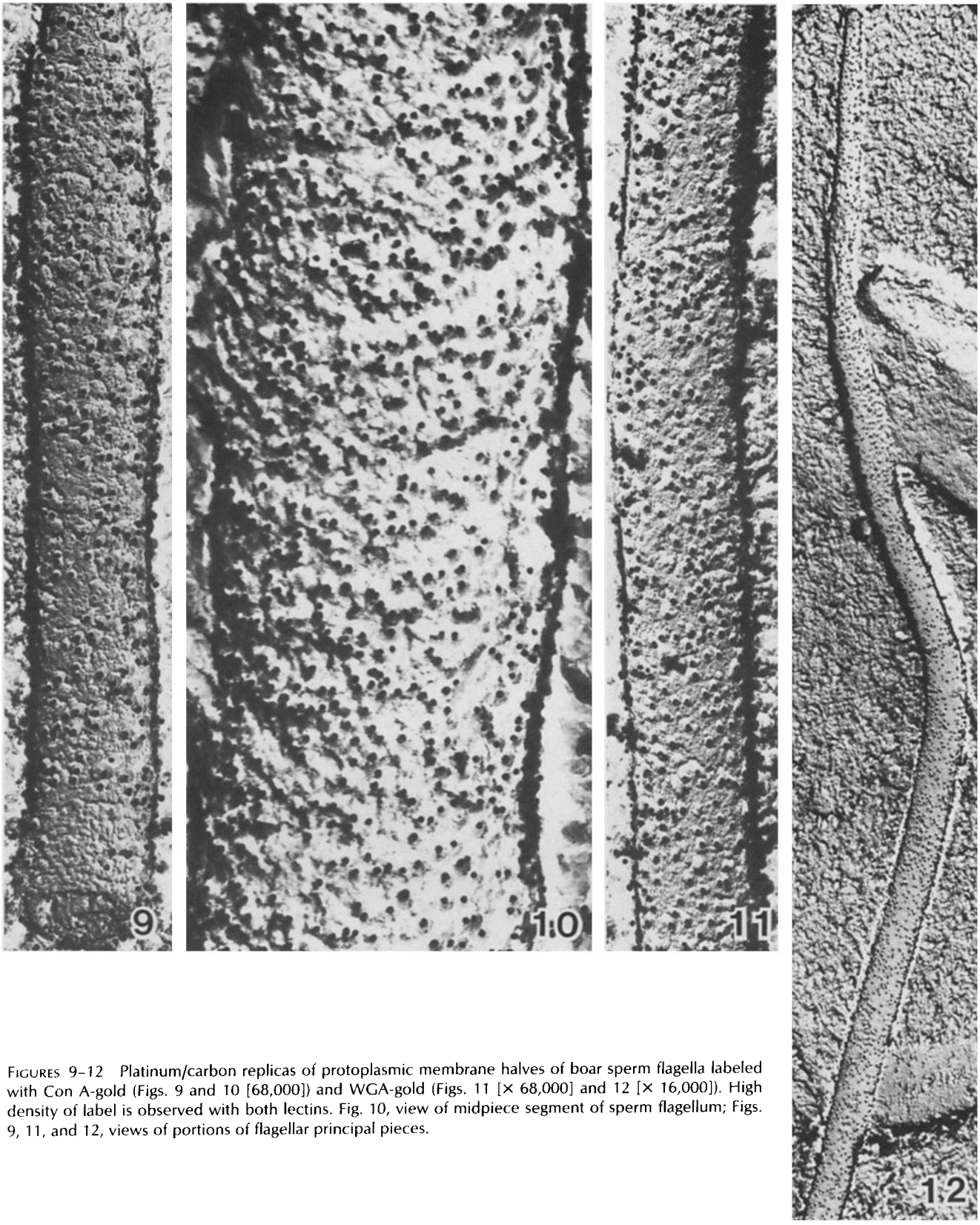
FIGURES 1–8 Thin sections of boar sperm flagella embedded in BSA, freeze-fractured, and labeled with complexes of Con A-peroxidase-gold (Figs. 1–3) or WGA-ovomucoid-gold (Figs. 4–8). Protoplasmic membrane halves (*P*) of the principal piece (Fig. 1 [× 48,000] and Fig. 5 [× 115,000]) and of the midpiece (Fig. 2 [× 50,000] and Fig. 4 [× 61,000]) of flagella are well marked by both Con A (Figs. 1 and 2) and WGA (Figs. 4 and 5). Complementary membrane halves (*P* and *E*) are shown in Figs. 2 and 4. Decrease in lectin-gold labeling is observed in control experiments (Con A incubation in presence of methyl-α-D-mannopyranoside, Fig. 3 [× 30,000], WGA incubation in presence of *N*-acetyl-D-glucosamine, Fig. 6 [× 115,000]). Precoating of flagella with cationized ferritin results in decrease of WGA labeling on membrane halves (Fig. 7 [× 130,000 and 8*A* [× 44,000]). Membrane halves appear as interrupted trilaminar profiles (Fig. 8*B*, arrowhead [× 101,000]).

Critical-Point Drying (CPD) Fracture-Label

In these preparations, face views of cleaved flagellar membranes are obtained. They contrast with the images seen in thin-sections where prolonged longitudinal views of membrane halves (as shown in Fig. 8*A*) are rarely obtained (see reference 28 for explanation). In critical-point drying specimens, reorganization of freeze-fracture membrane halves re-

sults in a rugose structure (Fig. 9) where membrane-intercalated particles are not identifiable (27, 28, 41–43).

Observation of replicas of boar sperm flagella illustrated clearly the high density of both Con A and WGA receptors along protoplasmic membrane halves of the different segments of the sperm tail (Figs. 9–12). In critical-point drying preparations, the possibility of confusing surfaces exposed during gel slicing at room temperature with genuine faces produced by freeze-fracture is remote: cut faces are excluded during orientation of the gel fragments to platinum/carbon



FIGURES 9–12 Platinum/carbon replicas of protoplasmic membrane halves of boar sperm flagella labeled with Con A-gold (Figs. 9 and 10 [68,000]) and WGA-gold (Figs. 11 [× 68,000] and 12 [× 16,000]). High density of label is observed with both lectins. Fig. 10, view of midpiece segment of sperm flagellum; Figs. 9, 11, and 12, views of portions of flagellar principal pieces.

shadowing, according to their different texture as observed with the dissecting microscope (27, 41). In frozen "sandwiched" preparations, only fracture faces can be revealed (28). The patterns of labeling were similar in both types of critical-point drying specimens and were consistent with the observations made by thin-section "fracture-label."

DISCUSSION

We characterize here the topology and density of Con A and WGA binding glycoconjugates on a flagellar surface through the analysis of the partition and planar distribution of lectin-gold markers on membrane halves, as induced by freeze-fracture of glutaraldehyde-fixed boar sperm cells. We show that Con A and WGA receptors partition with both protoplasmic and exoplasmic halves of the membrane.

Lectins interact with sugar residues of oligosaccharides of biological membranes (29). Carbohydrates of eucaryotic membranes are exclusively expressed at the exoplasmic surface of the bilayer (28, 44, 45). Therefore, lectin labeling observed either on protoplasmic or on exoplasmic membrane halves must originate from the carbohydrate moieties of glycoconjugates that, in intact cells, are exposed at the outer surface of the plasma membrane. As freeze-fracture splits biological membranes through its hydrophobic plane (46, 47), we conclude that labeling on protoplasmic halves results from dragging, during fracture, of oligosaccharides of transmembrane glycoproteins across the outer (exoplasmic) half of the membrane. Previous comparisons on the partition of the major integral proteins of human erythrocytes with freeze-fracture membrane halves suggested the operation of a stochastic process that reflects the relative expression of each species of transmembrane protein at each side of the membrane as well as its interaction with components of the membrane skeleton (26, 42). We showed that glycophorin, preferentially expressed at the outer surface and without linkages to the membrane skeleton (48, 49), partitions preferentially with the outer half of the erythrocyte membrane while band 3, which has a large hydrophilic segment exposed at the inner surface and known associations to the membrane skeleton (50, 51), partitions preferentially with the inner half of the membrane (26, 42). Should this interpretation prove correct, lectin labeling of protoplasmic membrane halves suggests that the transmembrane glycoproteins of the sperm flagella are preferentially expressed at the inner surface and/or linked to submembranous components. When the balance of associations of integral proteins is changed in favor of the outer side of the membrane, obtained here through binding of cationized ferritin to the flagellar surface, labeling of protoplasmic membrane halves suffers drastic reduction (Figs. 7 and 8). Lectin labeling of exoplasmic membrane halves is more difficult to ascribe. It may correspond to glycolipids, peripheral and integral glycoproteins; evidence for this interpretation is retrieved on our previous "fracture-label" studies on the partition of lectins with plasma membrane halves of *Acanthamoeba castellanii* (52), and of human erthrocytes (26, 42).

Comprehensive models on the molecular basis of flagellar surface motility proposed by Dentler (3) and Bloodgood (14) postulate the central role of transmembrane membrane proteins on the force transduction from the membrane cytoskeleton or axonemal microtubules to the motile surface receptors. These surface receptors belong to a high-molecular-weight glycoprotein (6, 15); their motility is reversibly inhibited by cytoskeleton disruptors (15). Flagellar surface motility has been studied using *Chlamydomonas* as the major experimental model because they are easy to grow in the laboratory and immobile mutants are available (3, 7). Surface motility similar to that observed on flagellar membranes has also been found in fibroblasts (53–56), sea urchin blastula cells (57), and neuronal axons (58). Our results are referred to the flagellar segment of boar sperm cells. Structural and cytochemical studies have documented the similarity of sperm tail with flagella from other cells: submembranous cytoskeleton elements, as well as membrane-microtubule "bridges," were described on mammalian sperm tail (2); ATPase activity, detected cytochemically by lead phosphate deposition, was also reported on the submembranous cytoplasm of the sperm tail (20, 24). Our "fracture-label" results provide the first experimental evidence for the transmembrane topology of numerous glycoproteins on a flagellar membrane; they are consistent with models (3, 14) that envisage glycoproteins of flagella as transmembrane couplers of surface receptors with submembranous elements.

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