The Transmembrane Signaling Pathway Involved in Directed Movements of *Chlamydomonas* Flagellar Membrane Glycoproteins Involves the Dephosphorylation of a 60-kD Phosphoprotein That Binds to the Major Flagellar Membrane Glycoprotein

Robert A. Bloodgood and Nancy L. Salomonsky

Department of Cell Biology and the Cancer Center, University of Virginia School of Medicine, Charlottesville, Virginia 22908

Abstract. Cross-linking of *Chlamydomonas reinhardtii* flagellar membrane glycoproteins results in the directed movements of these glycoproteins within the plane of the flagellar membrane. Three carbohydrate-binding reagents (FMG-1 monoclonal antibody, FMG-3 monoclonal antibody, concanavalin A) that induce flagellar membrane glycoprotein crosslinking and redistribution also induce the specific dephosphorylation of a 60-kD (pI 4.8–5.0) flagellar phosphoprotein (pp60) that is phosphorylated in vivo on serine. Ethanol treatment of live cells induces a similar specific dephosphorylation of pp60. Affinity adsorption of flagellar ^{32}P-labeled membrane-matrix extracts with the FMG-1 monoclonal antibody and concanavalin A demonstrates that pp60 binds to the 350-kD class of flagellar membrane glycoproteins recognized by the FMG-1 monoclonal antibody. In vitro, protein phosphatase 2B (calcineurin) removes 60% of the ^{32}P from pp60; this correlates well with previous observations that directed flagellar glycoprotein movements are dependent on micromolar calcium in the medium and are inhibited by calcium channel blockers and calmodulin antagonists. The data reported here are consistent with the dephosphorylation of pp60 being a step in the signaling pathway that couples flagellar membrane glycoprotein cross-linking to the directed movements of flagellar membrane glycoproteins.

THERE is an emerging view that cell surfaces serve as sensory receptors for motile cells; in particular, cells can detect physical and chemical cues in their environment and respond by activating cytoskeletal machinery that modulates cell adhesion, cell spreading and whole cell locomotion (Lackie, 1986; Singer and Kupfer, 1986; Bray and Hollenbeck, 1988; Stossel, 1989). Contact-induced information transfer across the plasma membrane is thought to be mediated by specific transmembrane glycoproteins (such as integrins) and second messenger pathways often involving protein phosphorylation and dephosphorylation (Damsky and Werb, 1992; Hynes, 1992; Schwartz, 1992; Juliano and Haskill, 1993; Williams et al., 1994).

The flagellar membrane of *Chlamydomonas* exhibits force transduction which is responsible for a form of whole cell locomotion referred to as gliding motility (Bloodgood, 1989, 1990). Vectorial labeling studies (iodination and biotinylation) have revealed that the major flagellar surface exposed proteins in *Chlamydomonas reinhardtii* are a class of high molecular mass concanavalin A binding glycoproteins referred to as the 350-kD flagellar membrane glycoproteins (Bloodgood, 1987). Use of a substrate immobilized iodination system has demonstrated that the 350-kD glycoproteins are the major flagellar surface components that interact with the substrate during gliding motility (Bloodgood and Workman, 1984). Cross-linking of the 350-kD glycoproteins with concanavalin A or specific anti-carbohydrate monoclonal antibodies induces a directed, energy-dependent redistribution of these glycoproteins within the plane of the flagellar membrane (Bloodgood et al., 1986) that is reminiscent of the "capping" of cell surface receptors observed on other systems (Schreiner and Unanue, 1976; Bourguignon and Bourguignon, 1984). Other *Chlamydomonas* flagellar membrane glycoproteins can also exhibit active, directed movements within the plane of flagellar membrane in response to antibody/lectin crosslinking (Homan et al., 1988; Kooijman et al., 1989) and/or flagella–flagella interactions during mating (Homan et al., 1987; Musgrave and van den Ende, 1987). These directed movements of flagellar membrane glycoproteins are dependent on the presence of micromolar concentrations of free calcium in the medium and are inhibited by calcium channel blockers and calmodulin antagonists (Bloodgood and Salomonsky, 1990). Use of a mutant cell line of *Chlamydomonas reinhardtii* with altered glycosylation of the 350-kD glycoproteins has suggested that whole cell gliding motility is dependent on the ability of the 350-kD glycopro-

Address all correspondence to Robert A. Bloodgood, Dept. of Cell Biology, Box 439, University of Virginia Health Sciences Center, Charlottesville, VA 22908.

teins to be translocated within the plane of the flagellar membrane (Bloodgood and Salomonsky, 1989).

Cross-linking of flagellar membrane glycoproteins with antibodies or lectins induces a signaling pathway that appears to activate the cytoskeletal machinery responsible for the directed movement of flagellar membrane glycoproteins within the plane of the flagellar membrane. The present study utilized in vivo phosphorylation and two-dimensional SDS-polyacrylamide gel electrophoresis to examine the role of protein phosphorylation in the signaling pathway that couples the sensory and motor functions of the *Chlamydomonas* flagellar surface.

Materials and Methods

Cell Strains, Antibodies, and Reagents

Chlamydomonas reinhardtii, strain pf18, cells were used for all experiments. Cells were grown vegetatively in a modified version of Medium I (Sager and Granick, 1953) at 21°C using a light/dark cycle of 14 h light and 10 h dark.

The FMG-1 (Bloodgood et al., 1986) and FMG-3 mouse monoclonal antibodies were obtained from ascites fluid collected from Balb/C mice injected with the appropriate hybridomas. The monoclonal antibodies were purified using protein A-Sepharose (Biorad Laboratories, Inc., Melville, NY) affinity chromatography.

Protein A-Sepharose CL-4B (17-0780-01), concanavalin A Sepharose 4B (17-0440-01) and the Ampholine carrier ampholites used for isoelectric focusing were purchased from Pharmacia Biotech, Inc. (Piscataway, NJ); the affinity-purified goat anti-mouse IgG secondary antibodies were purchased from Hyclone Laboratories, Inc. (Logan, UT); avidin D (A-2004) was purchased from Vector Laboratories, Inc. (Burlingame, CA), NHS-LC-Biotin (21335-G) was purchased from Pierce Chemical Company (Rockford, IL); Microcystin-LR was purchased from Sigma Chemical Company (St. Louis, MO) and Calbiochem (La Jolla, CA); Okadaic Acid (31665A) was purchased from GIBCO BRL (Gaithersburg, MD); protein grade Nonidet P-40 (10% solution) was purchased from Calbiochem; PDA[1] (piperazine diacrylamide) and acrylamide were purchased from Bio Rad Laboratories, Inc. (Melville, NY). All other reagents were obtained from Sigma Chemical Company.

Biotinylation of Live Cells

Vectorial labeling of surface exposed proteins on live cells was accomplished by biotinylation of amine groups with the NHS-LC-biotin reagent (catalog no. 21335; Pierce Chemical Company, Rockford, IL). For labeling of nonradioactive cells, 200 mls of 6×10^7 cells/ml were incubated with NHS-LC-biotin at a final concentration of 1 mg/ml for 15 min in medium I of Sager and Granick (1953). The cells were pelleted, resuspended in fresh growth medium, mixed with 300 ml of unlabeled cells at the same concentration and the flagella purified by the pH shock method (Witman et al., 1972).

For certain experiments, cells were both labeled with ^{32}P-orthophosphoric acid and surface biotinylated. In this case, cells were incubated with ^{32}P-orthophosphoric acid (as described below) for 45 min at which time 10 ml of a 10 mg/ml freshly dissolved solution of NHS-LC-biotin in the ^{32}P-labeling medium were added to each 100 ml of cell solution and incubation continued for an additional 15 min. Flagella isolation was performed using the dibucaine method described below for ^{32}P-labeled cells.

In Vivo Phosphorylation and Cell Fractionation

Chlamydomonas were grown in Medium I of Sager and Granick (1953) containing 10% of the normal level of potassium phosphate plus 10 mM Hepes buffer, pH 7.2. Cells were harvested and resuspended at a concentration of 4.5×10^7 cells per ml in Medium I in which all of the potassium phosphate was replaced by 20 mM Hepes buffer, pH 7.2. 100–200 mls of cell suspension were incubated at room temperature with a final concentration of 25 μCi/ml of ^{32}P-orthophosphoric acid (NEX-053; New England Nuclear, Boston, MA) for 60 min (unless otherwise indicated). Flagella were isolated and purified by the dibucaine method of Witman (1986); immediately after deflagellation, two volumes of ice-cold STOP solution (20 mM Hepes, pH 7.2, 20 mM EGTA, 200 mM sodium fluoride, 4% sucrose, 200 units/ml Trasylol [aprotinin], 1 μM Microcystein-LR, 0.1 μM Okadaic Acid) were added to the preparation. All subsequent solutions contained 1 μM Microcystin-LR. The flagella were washed twice in Immunoprecipitation Buffer A (190 mM sodium chloride, 50 mM Tris-HCl, pH 8.3, 5 mM EGTA, 1 mM $MgCl_2$, 200 units/ml Trasylol [aprotinin], 1 μM microcystin-LR) or in 20 mM Hepes and 5 mM EGTA, pH 7.2. The flagella were extracted with 0.05% Nonidet P-40 in 20 mM Hepes, pH 7.2 (for two-dimensional gel electrophoresis) or Immunoprecipitation Buffer A (for immunoprecipitation) for 15 min on ice and then centrifuged for 20 min at 89,000 *g* in the Beckman TLX tabletop centrifuge using the TLA-120.2 rotor. The resulting supernate (referred to as the membrane-matrix fraction) was used for one-dimensional or two-dimensional polyacrylamide gel electrophoresis or was utilized for immunoprecipitation experiments.

Immunoprecipitation

The phosphorylated membrane-matrix extract was incubated overnight at 4°C with the appropriate antibody (50 μg total in most cases). In some cases, the ^{32}P-labeled flagellar membrane-matrix extract was divided in half and one aliquot was treated with 0.5–1% SDS at 100°C for 2 min; Nonidet P-40 (equal volume of a 5% solution) was then added to both aliquots in order to sequester the SDS into mixed micelles so that it did not interfere with subsequent antibody-antigen binding. The protein A-Sepharose CL-4B was prepared by washing 4× with Immunoprecipitation Buffer B (150 mM NaCl, 10 mM Tris-HCl, pH 8.3, 5 mM EGTA, 1 mM $MgCl_2$, 0.05% Nonidet P-40, 100 units/ml Trasylol [aprotinin], and 1 μM Microcystin-LR), and incubated overnight at 4°C with affinity-purified rabbit anti-mouse IgG H & L antibody (Catalog no. EA2060; Hyclone Laboratories). This bridge antibody approach was necessary because the mouse monoclonal antibodies FMG-1 and FMG-3 used in this study are of the IgG_1 subclass and do not have a high binding affinity for protein A-Sepharose beads. The next morning, the beads were washed 4× with Immunoprecipitation Buffer B and incubated for 2 h at room temperature with the mixture of flagellar extract and mouse monoclonal antibody. At the end of the incubation, the beads were washed 10× using 10 ml of Immunoprecipitation Buffer B for each wash. Bound protein was eluted from the beads with Laemmli sample buffer (Laemmli, 1970), if it was to be used for one-dimensional acrylamide gels, or with a non-ionic detergent-urea buffer (9 M urea, 8% Nonidet P-40, 2% Ampholines [3 parts pH 5-7, 3 parts pH 6–8, 4 parts pH 3.5–10], 5% β-mercaptoethanol), if it was to be used for two-dimensional gels.

Polyacrylamide Gel Electrophoresis and Western Blot Procedures

One-dimensional SDS-polyacrylamide slab gel electrophoresis was performed using the Jarvik and Rosenbaum (1980) modification of the Laemmli (1970) procedure except that PDA was substituted for bis-acrylamide. All acrylamide slab gels used for one-dimensional and two-dimensional acrylamide gel electrophoresis contained a 4–16% gradient of acrylamide and a 3–6 M gradient of urea and were stained for protein using either Coomassie Brilliant Blue R-250 or the silver stain procedure of Oakley et al. (1980), dried, and exposed to Kodak XAR x-ray film at −70°C with a Cronex enhancing screen (Du Pont Co., Wilmington, DE).

Two-dimensional polyacrylamide gel electrophoresis (2D PAGE) was modified from the method of O'Farrell (1975). The isoelectric focusing (IEF) gel solution, containing 9 M urea, 3.7% acrylamide-PDA, 2% Nonidet P40, 2% ampholines (3 parts pH 5-7 ampholines, 3 parts pH 6-8 ampholines, and 4 parts pH 3.5-10 ampholines), was poured to a height of 12 cm in glass tubes (14 cm long × 2.5 mm ID), overlayed with 8 M urea, allowed to polymerize for 2–4 h and used the same day. After prefocusing the gels, samples were loaded and run for a total of 8400 volt-hours. The tube gels were equilibrated in sample equilibration buffer (2.3% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.0625M Tris-HCl, pH 6.8) and placed on a SDS-PAGE gradient slab gel as described above. All two-dimensional gels are oriented such that the acidic end of the gel is to the left and the basic end of the gel is to the right. For quantification of the relative amounts of ^{32}P label in the 60 kD spot on dried two-dimensional gels, a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) was utilized. A series

1. *Abbreviations used in this paper*: IEF, isoelectric focusing; PDA, piperazine diacrylamide.

of four 2-D gels from the same calcineurin experiment were exposed together using the same screen for the same period of time. ImageQuant software (Molecular Dynamics) was used to integrate the amount of signal in an identical area outlining the same spot on all four gels. Identical areas of background were integrated from each gel and used for background subtraction.

Electroblot transfer of one- and two-dimensional polyacrylamide slab gels to 0.45 μm nitrocellulose paper (BAS-85; Schleicher and Schuell, Keene, NH) was performed according to the method of Towbin et al. (1979) for 1,200 mAmp-hours in a blotting buffer containing 0.19 M glycine and 0.05 M Tris-base, pH 8.7. Proteins were visualized by staining the nitrocellulose with 0.1% Amido Black in 50% methanol-10% acetic acid, destained and blocked overnight at 4°C in the appropriate blocking buffer (0.1% Tween-20 in PBS, pH 7.2, for primary and secondary antibody labeling and 10 mM Hepes, pH 7.2, 0.1% Tween-20 and 0.005% thimerisol for HRP-labeled avidin D labeling of biotinylated proteins). These same buffers were used for all washes (3×, 10 min each) and for diluting the antibodies and avidin-D. After incubation of blots with affinity purified HRP-labeled goat anti-mouse IgG antibody or HRP-labeled Avidin-D (Vector Labs), color development was achieved using 1.0 mM diaminobenzidine and 0.03% hydrogen peroxide in PBS, pH 7.2.

Phosphoamino Acid Analysis

Phosphoamino acid analysis was performed by the method of Hemmings et al. (1984).

Results

Incubation of *Chlamydomonas reinhardtii*, strain pf18, with ^{32}P-orthophosphoric acid results in the in vivo labeling of many flagellar proteins, presumably due to the coupled activity of flagellar protein kinases and flagellar protein phosphatases. Isolated flagella were fractionated into membrane-matrix and axonemal compartments by extraction with 0.05% Nonidet P-40 and analyzed by one- and two-dimensional acrylamide gel electrophoresis and autoradiography. The flagellar membrane-matrix compartment exhibited a large number of silver-stained protein spots (Fig. 1 *A*) over 30 of which were phosphorylated (Fig. 1 *B*). The phosphoproteins tend to cluster around the pI range of 4.5–6.0 (Figs. 1 *B* and 3 *A*). Phosphoamino acid analysis of the entire membrane-matrix fraction and of a dozen randomly selected phosphoprotein spots taken from a two-dimensional gel revealed that most of the phosphoproteins in the flagellar membrane-matrix fraction are phosphorylated on serine (data not shown). In vivo labeling times from 10–70 min revealed a similar two-dimensional polyacrylamide gel pattern of radioactive phosphoprotein spots in the flagellar membrane-matrix fraction (for instance, compare Fig. 1 with Fig. 3).

The major proteins of the *Chlamydomonas reinhardtii* flagellar membrane are a class of high molecular mass concanavalin A-binding glycoproteins, referred to as the 350-kD glycoproteins (Bloodgood, 1987, 1990), that are recognized by the FMG-1 mouse monoclonal antibody (Bloodgood et al., 1986). These glycoproteins are exposed at the flagellar surface and are the major flagellar proteins that are labeled by surface biotinylation (Fig. 2 *A*) or surface iodination (Bloodgood and Workman, 1984). Another anti-carbohydrate monoclonal antibody, FMG-3, recognizes a group of four minor flagellar membrane glycoproteins (Fig. 2 *C*), all of which are surface exposed as judged by their ability to be biotinylated in vivo (Fig. 2 *D*). One of the four surface-exposed flagellar membrane glycoproteins recognized by the FMG-3 monoclonal antibody (the FM-3C glycoprotein) is phosphorylated in vivo (Fig. 2 *E*).

The three carbohydrate-binding reagents described above (FMG-1 monoclonal antibody, FMG-3 monoclonal antibody, and concanavalin A) all bind to the flagellar surface and, in live cells, induce the energy-dependent redistribution

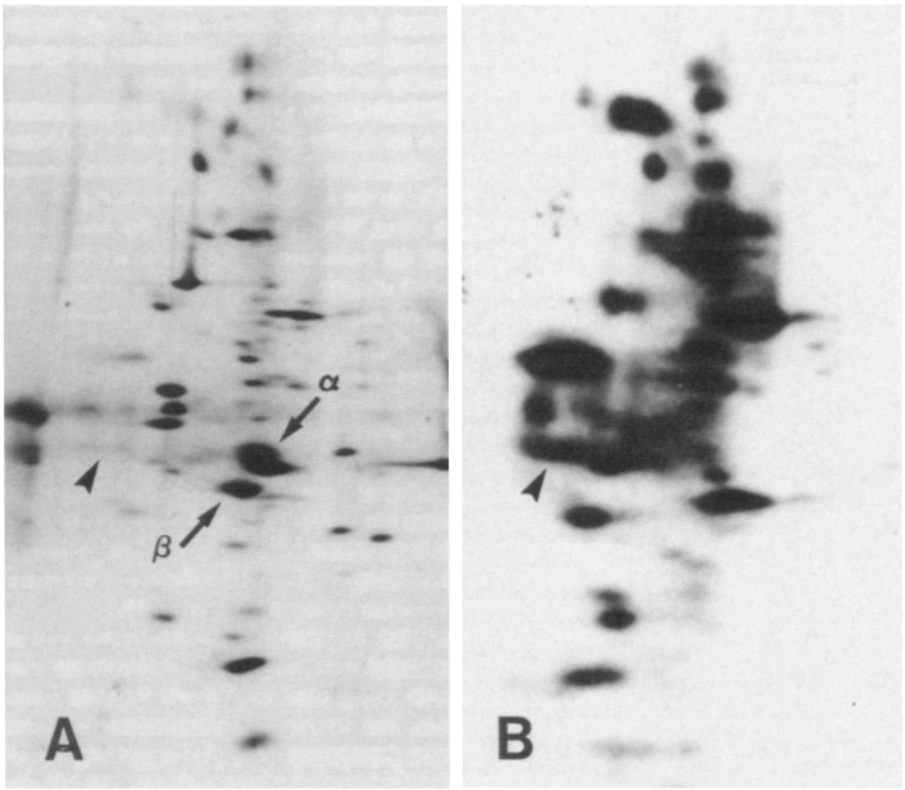

Figure 1. Identification of phosphoproteins in the membrane-matrix fraction of *Chlamydomonas* flagella. Live cells were labeled with ^{32}P-orthophosphoric acid for 10 min. Flagella were isolated and extracted with 0.05% Nonidet P-40; the solubilized proteins were separated on a two-dimensional acrylamide gel, stained with silver and exposed to X-ray film to identify proteins that were phosphorylated in vivo. (*A*) Silver stained pattern of protein spots in the gel over the pI range of 4.0 to 6.0. (*b*) Corresponding autoradiogram. α-tubulin and β-tubulin are indicated in *A* with arrows. The 60-kD phosphoprotein is indicated by arrowheads in *A* and *B*. Acid end of the pH gradient to the left.

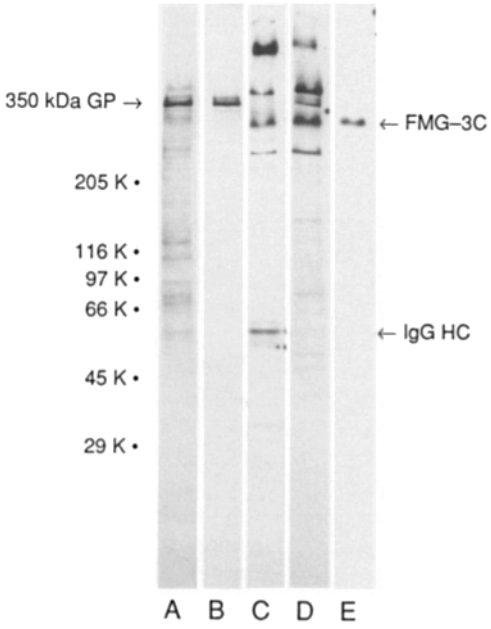

Figure 2. This figure illustrates the flagellar membrane glycoproteins recognized by the FMG-1 and FMG-3 anti-carbohydrate mouse monoclonal antibodies. Live cells were incubated with ^{32}P-orthophosphoric acid to label phosphoproteins and NHS-LC-biotin to biotinylate surface-exposed proteins. Flagella were isolated and extracted with 0.05% Nonidet P-40 to obtain the membrane-matrix fraction. Aliquots of the phosphorylated and biotinylated extract were immunoprecipitated with either the FMG-1 or FMG-3 monoclonal antibodies. Samples were run on a 4–16% SDS-polyacrylamide gradient slab gel and blotted to nitrocellulose, all samples shown come from the same gel and the same blot. (*A*) Entire Nonidet P-40 flagellar extract stained with HRP-avidin to identify biotinylated (and hence flagellar surface-exposed) proteins. (*B*) Western blot of FMG-1 immunoprecipitate stained with the FMG-1 monoclonal antibody. (*C*) Western blot of FMG-3 immunoprecipitate stained with the FMG-3 monoclonal antibody. This anti-carbohydrate monoclonal antibody recognizes four glycoproteins which are referred to as FMG3A-3D, A being the slowest migrating glycoprotein and D the fastest migrating glycoprotein. (*D*) FMG-3 immunoprecipitate stained with HRP-avidin to identify biotinylated (and hence flagellar surface-exposed) proteins. (*E*) Autoradiogram of the same blot strip shown in lane D demonstrating that one of the four surface exposed proteins recognized by the FMG-3 antibody (designated FMG-3C) is phosphorylated in vivo.

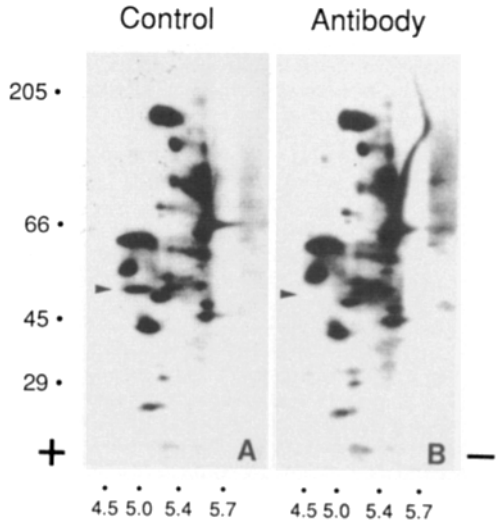

Figure 3. Treatment of ^{32}P-labeled cells with the FMG-1 mouse monoclonal antibody results in specific dephosphorylation of the 60-kD phosphoprotein (*arrowheads*) found in the flagellar membrane-matrix fraction. These figures are autoradiograms of two-dimensional polyacrylamide gels of the Nonidet P-40 soluble flagellar proteins from control cells or cells that had been treated with the FMG-1 mouse monoclonal antibody (100 μg/ml) to crosslink the 350-kD population of flagellar membrane glycoproteins. Cells were labeled with ^{32}P-orthophosphoric acid for 70 min before addition of the antibody for 10 min. + and − indicates the acidic and basic ends of the gel. Molecular mass standards are indicated on the left of the figures (in kD).

of the populations of membrane glycoproteins they recognize (Bloodgood et al., 1986). In order to identify changes in the level of phosphorylation of flagellar proteins that occur coincident with flagellar membrane glycoprotein redistribution, cells were labeled with ^{32}P-orthophosphoric acid and then treated with each of these reagents at a concentration of 100 μg/ml for 10 min to induce flagellar glycoprotein redistribution. The most dramatic and consistent change in the pattern of protein phosphorylation resulting from cross-linking of flagellar membrane glycoproteins by all three of these reagents (FMG-1 monoclonal antibody, FMG-3 monoclonal antibody, concanavalin A) was the virtually complete dephosphorylation of a spot with apparent molecular mass of 57–60 kD and pI in the range of 4.8–5.0 (Fig. 3). This protein (henceforth referred to as the 60-kD phosphoprotein), exhibits clear pH and molecular weight heterogeneity in some of our two-dimensional gels, separating into at least three spots (see Figs. 5 and 6). Phosphoamino acid analysis of the 60-kD spot confirmed that this spot(s) was a phosphoprotein, and demonstrated that all of the radioactive phosphate was attached to serine residues (data not shown).

Dephosphorylation of the 60-kD flagellar phosphoprotein was also induced by treatment of live cells with 8% ethanol (Fig. 4). Ethanol induces deflagellation of *Chlamydomonas* (Lewin et al., 1982). In the experiment shown in Fig. 4, 8% ethanol resulted in about 50% deflagellation; the data shown in Fig. 4 represent the membrane-matrix extract from the flagella that remained attached to the cells. In a separate experiment, the flagella that were released by ethanol were compared with the flagella that remained attached to the flagella after ethanol treatment; in both populations of flagella, the ethanol treatment induced complete dephosphorylation of the 60-kD phosphoprotein. Neomycin sulfate (at 40 and 80 μM) did not prevent the ethanol-induced or the FMG-1 antibody-induced dephosphorylation of the 60-kD phosphoprotein (data not shown) although it did prevent the ethanol induced deflagellation, implying that the neomycin was effective in preventing a rise in IP_3 which is a prerequisite for flagellar excision (Quarmby et al., 1992).

When the ^{32}P-labeled flagellar membrane-matrix extract was immunoprecipitated with the FMG-1 monoclonal antibody that specifically recognized the 350-kD flagellar membrane glycoproteins, the 60-kD phosphoprotein was also found in the immunoprecipitate (Fig. 5) suggesting an association of the 60-kD phosphoprotein with the 350-kD gly-

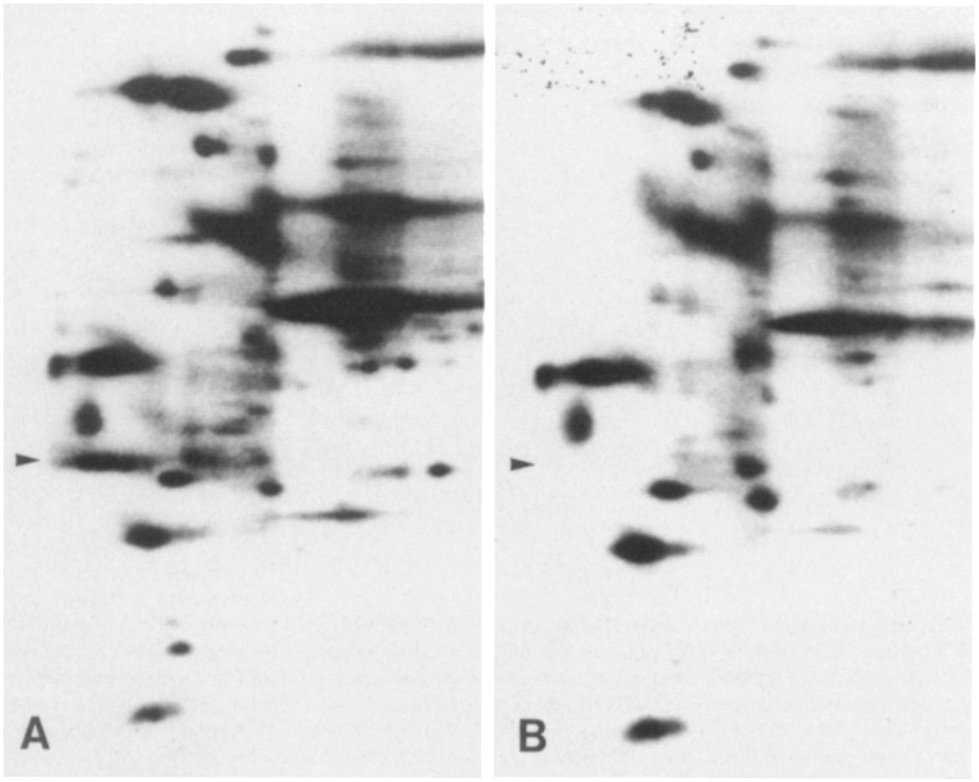

Figure 4. These autoradiograms of two-dimensional acrylamide gels of flagellar membrane-matrix proteins demonstrate that treatment of live cells with 8% ethanol, after in vivo labeling with ^{32}P-orthophosphoric acid for 60 min, induced specific dephosphorylation of the 60-kD flagellar phosphoprotein (arrowheads). (*A*) Control cells. (*B*) Cells treated with 8% ethanol for 10 min.

coproteins. The specificity of this interaction is suggested by the fact that only the 60-kD phosphoprotein, out of a large assortment of phosphoproteins in the flagellar extract (Fig. 1 *B*), was found associated with the 350-kD glycoproteins. Control experiments demonstrated that the 60-kD phosphoproteins did not interact with the FMG-1 monoclonal antibody, the rabbit anti-mouse bridge antibody or the protein A–Sepharose beads. In particular, two observations suggest that the 60-kD phosphoprotein is immunoprecipitated by the FMG-1 antibody because of its association with the 350-kD flagellar membrane glycoproteins and not because it is directly recognized by the FMG-1 antibody. When a portion of the ^{32}P-labeled membrane-matrix extract is boiled in 1% SDS and the SDS is then sequestered by addition of an excess of Nonidet P-40, subsequent immunoprecipitation with the FMG-1 antibody no longer brings along the 60-kD phosphoprotein (compare Fig. 6, *a* and *b*) even though the antibody is still able to bind to the 350-kD membrane glycoproteins (Fig. 6 *c*). Secondly, when the ^{32}P-labeled FMG-1 immunoprecipitate was run on a two-dimensional polyacrylamide gel, blotted to nitrocellulose, probed with the FMG-1 monoclonal antibody and then autoradiographed, it was observed that the 60-kD spot does not bind the FMG-1 antibody (data not shown).

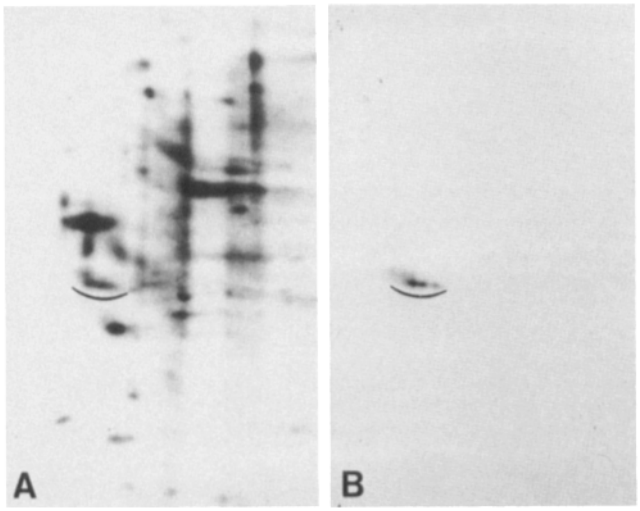

Figure 5. Autoradiograms of two-dimensional acrylamide gels demonstrating that the FMG-1 monoclonal antibody immunoprecipitates the 60-kD flagellar phosphoprotein. (*A*) Complete ^{32}P-labeled Nonidet P-40 flagellar extract illustrating the large number of flagellar membrane-matrix phosphoproteins present. (*B*) FMG-1 immunoprecipitate obtained with the ^{32}P-labeled extract shown in *A*. The position of the 60-kD phosphoprotein isoforms in each panel is indicated by the brackets.

Incubation of the ^{32}P-radiolabeled membrane-matrix extract with concanavalin A beads also results in binding the 60-kD flagellar phosphoprotein to the beads (data not shown), presumably due to the association of the 60-kD phosphoprotein with the 350-kD glycoproteins, which represent the major concanavalin A-binding glycoproteins of the flagellum (Monk et al., 1983; Bloodgood et al., 1986). Pretreatment of the ^{32}P-labeled flagellar extract with SDS results in a loss of the 60-kD phosphoprotein (data not shown), again arguing that its original binding was due to interaction with a concanavalin A-binding glycoprotein and not because of direct interaction with concanavalin A.

Taken together, the immuno-affinity and lectin-affinity data demonstrate that at least some of the 60-kD phosphoprotein is associated with the flagellar membrane through

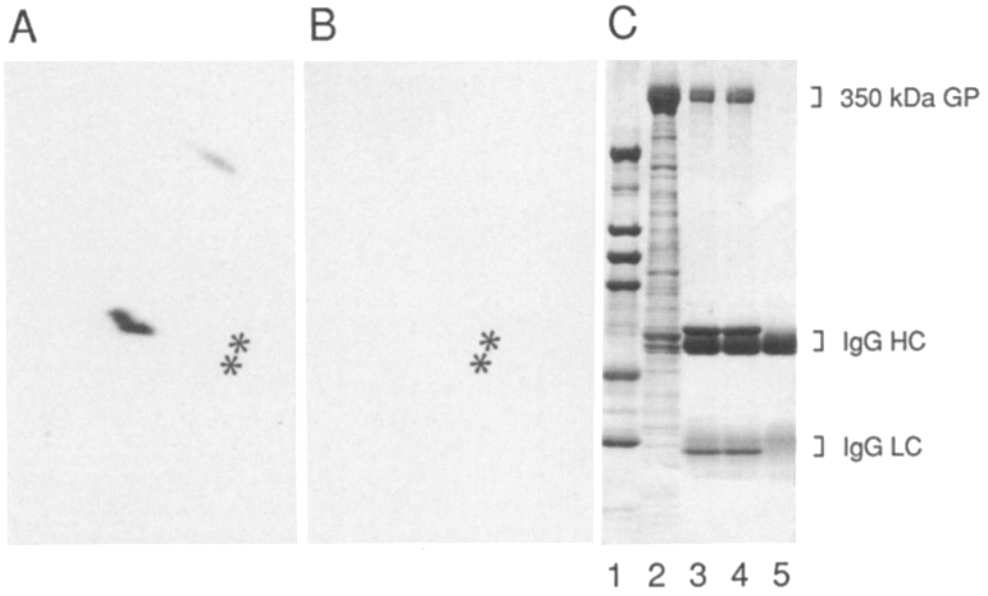

Figure 6. Effect of SDS pretreatment of the ^{32}P-labeled Nonidet P-40 flagellar extract on immunoprecipitation of the 60-kD flagellar phosphoprotein by the FMG-1 monoclonal antibody. (*A*) Autoradiogram of a portion of a two-dimensional gel of an FMG-1 immunoprecipitate. (*B*) Autoradiogram of a portion of a two-dimensional gel of an FNG-1 immunoprecipitate obtained using an aliquot of the same ^{32}P-labeled Nonidet P-40 extract that had been pretreated with 1% boiling SDS (followed by addition of excess Nonidet P-40) in order to disrupt all non-covalent protein-protein interactions. The asterisks indicate the positions where α-tubulin and β-tubulin would migrate (*C*) Coomassie Blue stained one-dimensional SDS-acrylamide gradient slab gel of the same FMG-1 immunoprecipitation samples shown in *A* and *B* demonstrating that the SDS pretreatment of the ^{32}P-labeled flagellar Nonidet P-40 extract did not inhibit the ability of the FMG-1 monoclonal antibody to recognize and immunoprecipitate the 350-kD major flagellar membrane glycoproteins (labeled as 350 kD GP). Lane *1*, molecular mass markers: 205, 116, 97, 66, 45, and 29 kD. Lane *2*, complete Nonidet P-40 flagellar extract (flagellar membrane and matrix proteins). Lane *3*, FMG-1 immunoprecipitate (same sample shown in *A*). Lane *4*, FMG-1 immunoprecipitate of Nonidet P-40 extract that was pretreated with 1% SDS, followed by addition of excess Nonidet P-40 (same sample shown in *B*). Lane *5*, Control immunoprecipitate of Nonidet P-40 extract in which the FMG-1 primary antibody was omitted; the heavy (IgG HC) and light (IgG LC) chains of the rabbit anti-mouse IgG bridge antibody were still present.

an association with the 350-kD flagellar membrane glycoproteins. Unlike the FMG-1 antibody and concanavalin A, the FMG-3 antibody did not immunoprecipitate the 60-kD phosphoprotein. This is a somewhat surprising result because all three reagents, when used on live cells, stimulate the dephosphorylation of the 60-kD phosphoprotein.

The dephosphorylation of the 60-kD flagellar phosphoprotein that accompanies transmembrane signaling could result from the inhibition of a protein kinase, the activation of a protein phosphatase or some combination of the two. Because of the highly specific nature of the dephosphorylation of the 60-kD phosphoprotein and because flagellar membrane glycoprotein movements require calcium in the medium and are inhibited by calcium channel blockers and calmodulin antagonists (Bloodgood and Salomonsky, 1990), the most likely phosphatase to be involved in dephosphorylation of the 60-kD phosphoprotein is protein phosphatase 2B (calcineurin), which is a calcium and calmodulin dependent enzyme (Klee et al., 1988).

In order to determine if the 60-kD phosphoprotein is a substrate for calcineurin (PP2B), FMG-1 antibody immunoprecipitates (containing the 350-kD membrane glycoproteins and the 60-kD phosphoprotein) from ^{32}P-labeled membrane-matrix extracts were treated with various combinations of calcium, calmodulin and bovine brain calcineurin from two different sources (Sigma Chemical Company and Dr. Angus C. Nairn at Rockefeller University, NY). As shown in Fig. 7, each of the two sources of brain calcineurin used removed ~60% of the ^{32}P-phosphate (that has been incorporated in vivo) from the 60-kD phosphoprotein in a calcium and calmodulin-dependent manner. At the same time, the heterogeneity of the 60-kD autoradiographic spot was decreased with the higher molecular mass and more acidic components disappearing. This experiment also demonstrates that calcineurin was able to partially dephosphorylate

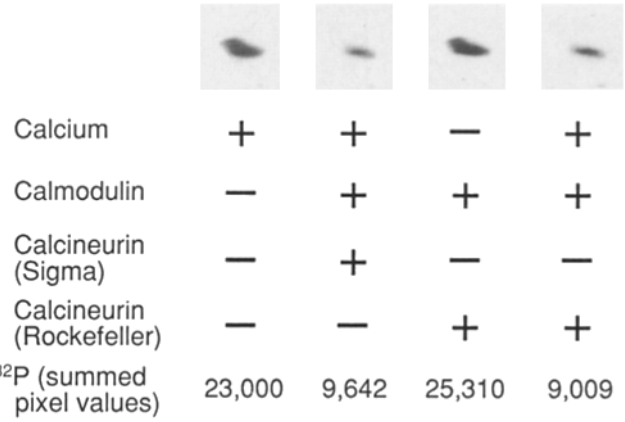

Figure 7. Effect of protein phosphatase 2B (calcineurin) treatment of FMG-1 antibody immunoprecipitates on the ^{32}P-content of the 60-kD flagellar phosphoprotein. The photographs are of the 60-kD phosphoprotein spot from autoradiograms of two dimensional gels of FMG-1 immunoprecipitates treated as indicated in the table. The amount of ^{32}P associated with the 60-kD phosphoprotein spot was quantitated using a PhosphorImager (Molecular Dynamics) and the ImageQuant software.

the 60-kD phosphoprotein while it was associated with the 350-kD glycoprotein.

Discussion

Cross-linking of a population of flagellar membrane glycoproteins by antibodies or lectins induces their movement within the plane of the flagellar membrane (Bloodgood et al., 1986; Homan et al., 1988; Kooijman et al., 1989). Micromolar concentrations of free calcium ions are required in the extracellular medium for the directed movements of flagellar glycoproteins (Bloodgood and Salomonsky, 1990). Since calcium channel blockers inhibit antibody-induced flagellar glycoprotein redistribution at permissive levels of free calcium in the medium (Bloodgood and Salomonsky, 1990), it is assumed that the initial event in the signal transduction pathway initiated by the flagellar glycoprotein cross-linking is an influx of calcium which increases the intracellular free calcium concentration, either globally or locally. The present study was designed to explore whether the next step in the signal transduction pathway involves a change in the level of phosphorylation of one or more flagellar proteins.

Although it has long been known that the flagellar axoneme possesses many phosphoproteins, including radial spoke proteins, central pair associated proteins and dynein polypeptides (Piperno and Luck, 1976; Adams et al., 1981; Huang et al., 1981; Piperno et al., 1981; Segal and Luck, 1985; Hasegawa et al., 1987; Tash, 1989), the present study is the first published characterization of phosphoproteins associated with the membrane and matrix compartments of the *Chlamydomonas* flagellum utilizing in vivo phosphorylation. Gregory S. May's published thesis research in Joel Rosenbaum's laboratory (May, 1984) involved the first description of phosphoproteins in the membrane-matrix compartment of the *Chlamydomonas reinhardtii* flagellum. Bloodgood (1992) described the in vitro phosphorylation and dephosphorylation of proteins in the membrane-matrix fraction of the *Chlamydomonas reinhardtii* flagellum. There have been a few reports of phosphoprotein association with ciliary membranes of *Paramecium* (Lewis and Nelson, 1981; Eistetter et al., 1983; Klumpp and Schultz, 1991) and sheep trachea (Salathe et al., 1993). The present study demonstrates that the *Chlamydomonas* flagellum has a surprisingly large number of phosphoproteins in the membrane-matrix compartment (operationally defined as those proteins that can be solubilized in 0.05% Nonidet P-40). These phosphoproteins tend to cluster around a fairly narrow pI range (4.6–5.8) and are primarily phosphorylated on serine.

The major finding of the present study is that three different carbohydrate binding reagents (FMG-1 antibody, FMG-3 antibody, and concanavalin A), all of which are known to bind to and cross-link the external domains of flagellar membrane glycoproteins and to induce redistribution of the populations of glycoproteins they recognize (Bloodgood et al., 1986), also induce the selective dephosphorylation of a 60-kD phosphoprotein that binds to the major flagellar membrane glycoprotein (referred to as the 350-kD glycoproteins). The observation that three reagents that induce glycoprotein redistribution also induce dephosphorylation of the 60-kD phosphoprotein suggests, albeit does not prove, that the dephosphorylation of the 60-kD phosphoprotein is one of the steps in the signaling pathway that couples flagellar membrane glycoprotein crosslinking to the activation of intraflagellar machinery responsible for directed movements of these glycoproteins within the plane of the flagellar membrane.

The present study has utilized protein A–Sepharose and concanavalin A–Sepharose affinity binding to demonstrate that the 60-kD phosphoprotein is associated with one or more of the membrane glycoproteins recognized by the FMG-1 monoclonal antibody and by concanavalin A. Since the FMG-1 monoclonal antibody recognizes primarily the 350-kD glycoprotein doublet (Figs. 2 and 6 *c*; see also Bloodgood et al., 1986), which is also known to bind concanavalin A (Monk et al., 1983; Bloodgood et al., 1986), it is likely that the association of the 60-kD phosphoprotein with the flagellar membrane is through its binding to the 350-kD flagellar membrane glycoproteins.

The dephosphorylation of the 60-kD phosphoprotein could be due to activation of a protein phosphatase or inhibition of a protein kinase. The observed dephosphorylation of the 60-kD phosphoprotein is highly specific (in the sense that only one of the large array of phosphoproteins in the membrane-matrix fraction is affected), involves the removal of phosphate associated with serine, and occurs in response to an event (glycoprotein cross-linking) which is thought to be associated with calcium influx (Bloodgood and Salomonsky, 1990). These observations are consistent with the involvement of protein phosphatase 2B (calcineurin), which is a calcium- and calmodulin-activated, serine-threonine–specific protein phosphatase with a very limited substrate specificity (Klee et al., 1983, 1988). The present study shows that 60% of the ^{32}P-labeled phosphates that are put on serine residues of the 60-kD phosphoprotein in vivo are removed by in vitro treatment with brain calcineurin from two different sources. Another flagellar membrane associated phosphoprotein (FMG-3C glycoprotein recognized by the FMG-3 antibody), which fails to be dephosphorylated in vivo in response to flagellar membrane crosslinking, is not a substrate for calcineurin in vitro (data not shown). The calcineurin results reported in this paper correlate well with the previous observations that the signaling pathway associated with flagellar membrane glycoprotein movements is dependent upon micromolar calcium in the medium and is inhibited by calcium channel blockers and calmodulin antagonists (Bloodgood and Salomonsky, 1990).

Another finding of the present study is that treatment of *Chlamydomonas* with 8% ethanol mimics the effect of antibody or concanavalin A cross-linking by stimulating virtually complete dephosphorylation of the 60-kD flagellar phosphoprotein. Ethanol increases the intracellular concentrations of free calcium and IP_3 in rat liver hepatocytes (Hoek et al., 1987). In *Chlamydomonas eugametos*, ethanol activates mating structures, induces deflagellation and increases the intracellular IP_3 level (Musgrave et al., 1992). In *Chlamydomonas reinhardtii*, ethanol induces deflagellation but it has not been determined if ethanol increases intracellular IP_3 or calcium. Neomycin is an inhibitor of phosphoinositide hydrolysis (Gavev et al., 1989) and prevents both deflagellation and the deflagellation-associated increase in IP_3 in *Chlamydomonas reinhardtii* (Quarmby et al., 1992). Although neomycin inhibited the ethanol induced deflagellation, it did not inhibit either the ethanol or the antibody-induced dephosphorylation of the 60-kD flagellar

phosphoprotein observed in the present study, suggesting that the ethanol and antibody induced dephosphorylation of the 60-kD flagellar phosphoprotein are not mediated through IP_3 but, instead, may involve an IP_3-independent rise in intracellular free calcium concentration.

The present study has demonstrated that cross-linking of flagellar transmembrane glycoproteins induces the dephosphorylation of a 60-kD membrane-associated phosphoprotein. Since all of the cross-linking agents that induced phosphorylation of the 60-kD phosphoprotein also induce an activation of the intraflagellar machinery responsible for the directed movements of glycoproteins within the plane of the flagellar membrane, it is reasonable to conclude that the dephosphorylation of the 60-kD phosphoprotein is an intermediate step in the transmembrane signaling pathway that couples the sensory and motor functions of the *Chlamydomonas* flagellar surface.

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