Phosphorylation of Nuclear and Flagellar Basal Apparatus Proteins during Flagellar Regeneration in *Chlamydomonas reinhardtii*

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Abstract. The antiphosphoprotein monoclonal antibody MPM-2 was used to investigate protein phosphorylation during flagellar regeneration in *Chlamydomonas reinhardtii*. MPM-2 recognizes a phosphorylated epitope and detects several *Chlamydomonas* proteins by Western immunoblot analysis. Two MPM-2 reactive proteins (34 and 90 kD) increase in Western immunoblot intensity after flagellar excision and decrease in intensity during flagellar regeneration. Immunofluorescence and immunogold labeling revealed MPM-2 staining within the nucleus, especially towards the nuclear periphery, the flagellar basal apparatus, and the nucleus-basal body connector after flagellar excision. Comparison of MPM-2 reactivity in wild-type cells and in the mutant *bald-2*, which lacks functional basal bodies, demonstrates that the 34-kD protein is localized in the nucleus and the 90-kD protein is localized in the flagellar basal region. MPM-2 reactivity is observed in cells competent for flagellar regeneration. However, when cells were treated with the kinase inhibitor, staurosporine, MPM-2 reactivity did not increase after flagellar excision and flagellar regeneration was impaired. These observations suggest that phosphorylation of the 34- and 90-kD proteins may be important for flagellar regrowth. Possible roles for phosphorylation in flagellar regeneration include transcriptional activation and transport of flagellar precursors to the base of the growing flagella.

PROTEIN phosphorylation is known to play an important regulatory role in numerous biological processes, such as enzyme activation, gene regulation, and mitosis (for review see Hunter, 1987). The possible roles of protein phosphorylation during flagellar biogenesis can be studied in *Chlamydomonas reinhardtii*, an organism which has proven to be a useful model system for understanding the genetics, molecular, and cell biology of flagella. When *Chlamydomonas* cells encounter environmental stresses, such as extremes of temperature or pH, noxious chemicals, or mechanical shear forces, their flagella become detached by an active centrin-based excision mechanism (Lewin and Lee, 1985; Sanders and Salisbury, 1989). On return of cells to more favorable conditions, flagella regrow within 2 h (Randall et al., 1967; Rosenbaum et al., 1969). Flagellar regeneration is a complex process which involves the coordinate synthesis and assembly of over 150 proteins (Dentler, 1987; Johnson and Rosenbaum, 1992; Lefebvre and Rosenbaum, 1986; Piperno et al., 1977; Schloss, 1984; Silflow and Rosenbaum, 1981). Piperno and coworkers (1976, 1981) first showed that phosphoproteins were present in *Chlamydomonas* flagella, and they identified over 80 phosphorylated flagellar components. Phosphorylation of some flagellar proteins occurs during flagellar regrowth and the same proteins are dephosphorylated during flagellar resorption (May, 1984). To date, however, there have been no reports on the phosphorylation of nuclear and basal apparatus proteins during flagellar regeneration.

Here, we describe the use of the antiphosphoprotein monoclonal antibody MPM-2 as a probe for the study of protein phosphorylation events during flagellar regeneration. MPM-2 was originally raised against mitotic HeLa cell extracts (Davis et al., 1983). It recognizes a conserved phosphorylated epitope, especially in, but not restricted to, mitotic cells (Vandré et al., 1984, 1986), including mitotic *Chlamydomonas* cells (Harper et al., 1990). MPM-2 antibodies have been used to identify mitosis-specific phosphoproteins associated with sites of microtubule nucleation, such as centrosomes and kinetochores (Centonze and Borisy, 1990; Vandré et al., 1984, 1986). In addition, MPM-2 reactive phosphoproteins have been reported to be part of a nucleoskeleton, associated with condensing chromosomes (Hirano and Mitchison, 1991). In several systems, MPM-2 recognizes phosphoproteins in nonmitotic cells, for example in association with microtubules in neuronal cells (Vandré et al., 1986), and in the flagellar basal apparatus of sea urchin sperm, *Physarum* flagellates, and *Paramecium* (Keryer et al., 1987; Vandré et al., 1986). In this study, we report that MPM-2 recognizes several phosphoproteins in *Chlamydomonas* cells which are located in the nucleus and flagellar

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basal apparatus, and which increase in MPM-2 reactivity after flagellar excision. The possible roles for these phosphoproteins during flagellar regeneration were investigated using mutants defective in either flagellar excision or flagellar basal apparatus structure, and through the use of inhibitors which block flagellar excision and/or the flagellar regeneration process.

Materials and Methods

Strains and Culture Conditions

The high mating efficiency *C. reinhardtii* strain CC 620+, derived from strain 137c (Harris, 1989), was used in the experiments described. Other strains used were the plus mating types of the flagellar autotomy mutant *fa-1* (Lewin and Burrascano, 1983), and the basal body-defective mutant *bald-2* (Goodenough and St. Clair, 1975). Cultures were obtained from the *Chlamydomonas* Genetics Center, Dr. E. Harris, Botany Department, Duke University, Durham, NC. Cells were grown in modified Sager and Granick medium I, MMI (Sager and Granick, 1953), with Hutner's trace element solution (Harris, 1989; Hutner et al., 1950). Cell stocks were maintained as agar slants or on plates by adding 1.5% washed Difco-bactoagar and 2 mM sodium acetate to MMI. To maintain plating efficiency of cells the agar was washed in several changes of deionized water to remove dissolved salts before use.

To avoid mitotic cells which also react with MPM-2 antibodies (Harper et al., 1990), gametes were employed for all flagellar excision experiments described in this work. In preliminary experiments both gametes and early G1 vegetative cells gave identical results. Gametes were produced by cell culture in aerated liquid MMI (300 ml) in 1 liter Erlenmeyer flasks under a 14:10 h light/dark cycle at 20°C, as described by Schmeisser et al. (1973). When cells reached a density of $1-2 \times 10^6$ cells per ml, they were taken at 6 h into the light phase, washed twice in MMI without nitrogen (MMI-N), and resuspended at $1-2 \times 10^6$ cells per ml in MMI-N under continuous light at 20°C for 24 h.

Flagellar Excision

In most of the experiments reported here, pH shock (Rosenbaum and Child, 1967) was used to induce flagellar excision. Cells were harvested by gentle centrifugation (400 *g*, 5 min) and resuspended in excision buffer (10 mM Tris HCl, 1 mM potassium phosphate buffer, 2 mM sodium acetate, plus 1/10 the volume of MMI-N, pH 7.0) at a density of 3×10^7 cells per ml. A previously determined volume of 0.1 N acetic acid was added to a gently stirred culture to bring the pH rapidly to 4.5. After 30 s the pH was returned to 7.0 by the addition of 0.1 N KOH. Samples were collected before shock (preshock) and at intervals during flagellar regrowth. Flagellar excision and regrowth were monitored as described below. Flagellar regeneration in >95% of cells was usually complete by 120 min. In some experiments, flagellar excision was also induced by mechanical shear forces generated by vortexing the cell suspension in a fluted tube. The G-protein agonist mastoparan (Sigma Chem. Co., St. Louis, MO) was added at 8 μM (Quarmby et al., 1992) to induce flagellar excision.

Cell Counts and Flagellar Measurement

Cells (0.5 ml) were fixed in an equal volume of 2% glutaraldehyde and counted on a hemacytometer slide. Flagellar lengths were measured using 100× phase optics with a calibrated ocular micrometer. At least 30 measurements were taken per sample.

Antibodies

The antiphosphoprotein monoclonal antibody MPM-2 (an ascites IgG; Davis et al., 1983) was a generous gift from Professor P. N. Rao, Department of Medical Oncology, The University of Texas, MD Anderson Cancer Center, Houston, TX. Production and characterization of anticentrin monoclonal antibody (20H5) will be described elsewhere (Baron, A. T., and J. L. Salisbury, unpublished observations).

Immunofluorescence Microscopy

Cells were fixed in freshly prepared 4% formaldehyde from a 16% EM grade stock solution (Electron Microscopy Sciences, Fort Washington, PA) in 10 mM Hepes buffer, pH 7.0, and allowed to settle for 30 min onto 8-welled microslides (Carlson Scientific, Peotone, IL), coated with 0.1% polyethylenimine (Sigma Chem. Co.). Excess fixative was removed by blotting the edge of the well with filter paper and slides were rinsed three times for 5 min each in PBS, pH 7.2 (10 mM phosphate buffer, 150 mM NaCl). Cells were permeabilized by immersion in −20°C methanol for 10 min, followed by −20°C acetone for 5 min. Slides were air dried, rehydrated, and rinsed in three changes of PBS and incubated in MPM-2 antibody at 1:800 dilution for 1 h at 37°C. After rinsing in PBS, cells were incubated in FITC-conjugated goat-anti-mouse antibody (Cappel, IgG; Oreganon Teknika Corp., West Chester, PA) at 1:400 dilution, for 1 h at 37°C. Slides were then washed in PBS (three times, 5 min each). Antibodies were diluted in PBS containing 5% goat serum (GIBCO BRL, Gaithersburg, MD), 1% glycerol, 0.1% BSA (fraction V; Sigma Chem. Co.) and 0.4% sodium azide. Slides were mounted in Gelvatol mountant (Rodriquez and Dienhardt, 1960) containing 2% *n*-propyl-gallate, pH 8.5 (Sigma Chem. Co.) and coverslips were sealed with nail polish to prevent evaporation. Preparations were viewed using a Nikon Microphot-FXA photomicroscope equipped with a 100 W mercury lamp and epifluorescence illumination, standard FITC and UV filter sets, and a Fluor 100 X, 1.30 NA, oil immersion objective. Photographs were recorded on Hypertech film at exposure times of between 30 and 45 s (Microfluor Ltd., Stony Brook, NY), and developed in Kodak D-19 developer for 6 min at 20°C. Controls employed secondary antibody alone or nonreactive primary monoclonal antibody.

Immunogold Labeling

Cells, before and 15 min after flagellar excision, were fixed in 4% formaldehyde with 0.5% glutaraldehyde, prepared from EM grade 16 and 25% stock solutions (Electron Microscopy Sciences), respectively, in 10 mM Hepes, pH 7.0, for 1 h at 4°C. Samples were treated with three changes, 5 min each, of 7 mg per ml sodium borohydride (Sigma Chem. Co.) to reduce free aldehyde groups and washed 3 times in 10 mM Hepes buffer, pH 7.0. Samples were rapidly dehydrated in a cold ethanol series and infiltrated with Lowicryl K4M resin (Pella Inc., Redding, PA) at −20°C. After resin polymerization by ultraviolet irradiation for 72 h at −20°C, sections were cut on an Ultracut E microtome (Reichert-Jung, Germany) and collected on formvar-coated nickel grids. For immunogold labeling, sections were hydrated with deionized water for 10 min, blocked in TBS (10 mM Tris buffer, 150 mM NaCl, pH 7.4) containing 5% normal goat serum and 0.1% BSA for 1 h, labeled with MPM-2 primary antibody at 1:1,000 dilution for 1 h at 20°C, washed in three changes of TBS, pH 7.4, containing 0.05% Tween-20 (TBST)[1] and incubated for 1 h in goat-anti-mouse antibody conjugated to 10 nm gold (Janssen Life Science Products, Piscataway, NJ). Sections were washed in TBST, as above, post stained in 1% OsO_4, 2% uranyl acetate, and Reynolds lead citrate and viewed with a JEOL 1200 electron microscope. Controls employed gold-conjugated secondary antibody only.

Polyacrylamide Gel Electrophoresis and Western Immunoblotting

Samples (3×10^7 cells per ml) were taken in parallel to those for immunofluorescence and pelleted by a low speed spin in 1.5-ml microfuge tubes. The cell pellet was then resuspended in 100 μl of ice-cold extraction buffer (20 mM Tris HCl, 150 mM NaCl, 5 mM EDTA, 1 mM DTT, 1 mM EGTA, 10 μM pepstatin A, 10 μM leupeptin, 1 mM sodium fluoride, 1 mM sodium pyrophosphate, 12 μM β glycerophosphate, 1 mM sodium orthovanadate, 2 mM PMSF, and 0.1% Tween-20, pH 7.4.), followed by 100 μl of hot 2 × SDS sample buffer (Laemmli, 1970). Cell lysis was completed by sonication with three 10-s bursts from an Ultrasonics processor (Sonics Inc., Danbury, CT). Samples were placed on ice between sonication bursts to avoid thermal degradation. Lysate was spun at 13,000 *g* for 30 s to pellet cell debris, rapidly frozen in liquid nitrogen, and stored at −70°C until run on a gel. For electrophoresis, samples were heated at 95°C for 3 min, and then 20-μl aliquots were loaded onto a 3-20% polyacrylamide gradient slab gel which was run overnight at 5 mA limiting current. Proteins were transferred onto PVDF membrane (Millipore Corp., Bedford, MA), using a modification of the method described by Towbin et al. (1979). The transfer buffer contained 0.01% SDS, and a two step transfer was employed (2 h at

1. *Abbreviations used in this paper*: APM, amiprophos methyl; CLA, calyculin-A; DIC, differential interference microscopy; OA, okadaic acid; TBST, Tris buffered saline containing 0.05% Tween-20.

60 V, followed by 1 h at 80 V) at 4°C. For identification of MPM-2 reactive proteins by Western immunoblot, the membranes were washed three times, 5 min each, in TBST, pH 7.4, blocked for 1 h with blocking buffer which consisted of TBST containing 5% normal goat serum (GIBCO BRL), and 2% BSA (fraction V, Sigma Chem. Co.), washed with TBST as above and incubated for 1 h at 20°C in MPM-2 antibody at 1:2,000 dilution in blocking buffer containing 0.02% sodium azide. After three washes (5 min each) in TBST, blots were incubated for 1 h at 20°C in horseradish peroxidase-conjugated goat-anti-mouse antibody (Cappel) at 1:1,000 dilution in azide free blocking buffer. Finally, blots were washed twice in TBST (5 min each), once in TBS (5 min), and the color reaction was produced with a freshly prepared solution containing 0.17 mg per ml 3-amino-9-ethylcarbazole, 0.47 mg per ml succinic acid, 2.38 mg per ml sodium acetate, and 0.03% hydrogen peroxide (Sigma Chem. Co.).

Acid Phosphatase Treatment

Western transfers of deflagellated *Chlamydomonas* whole cell extracts were treated with 33 U per ml, white potato acid-phosphatase (Sigma Chem. Co.) in 40 mM Pipes buffer, pH 6.0, for 8 h at 20°C, and then immunoblotted for MPM-2 reactivity as described above. Controls included an immunoblot incubated in Pipes buffer only, then reacted with MPM-2 antibody, and a phosphatase-treated blot subsequently reacted with anticentrin monoclonal antibody (20H5, 1:1,000).

Densitometry of Western Immunoblots

Digital images of Western immunoblots were acquired using a Microtek color scanner (model 600ZS, Microtek Lab, Inc. Torrance, CA) in 256 gray-scale mode. Acquired images were analyzed, using the public domain program for the Macintosh, NIH Image (version 1.44), using the gel plotting macro. Regions of individual Western immunoblots to be analyzed were selected and measured after subtraction of the lane background. Mean values from at least three separate experiments for each treatment were determined for the 34- and the 90-kD MPM-2 reactive bands and were plotted as the density over the mean of their respective pretreatment control lanes.

Other Reagents Used

Neomycin sulfate (Sigma Chem. Co.) was added to cultures from a freshly prepared 1 mM aqueous stock solution to give 10 μM. Sodium pyrophosphate (Sigma Chem. Co.), which causes flagellar resorption over 3 h (Lefebvre et. al., 1978), was added at a final concentration of 10 mM. Aqueous solutions of cycloheximide and colchicine (Sigma Chem. Co.) were added to 10 μg per ml and 3 mg per ml, respectively (Rosenbaum et al., 1969; Lefebvre et al., 1978). The kinase inhibitor staurosporine (Sigma Chem. Co.) was dissolved in DMSO and added to cultures to give a final concentration of 20 μM. The phosphatase inhibitors okadaic acid (OA; GIBCO BRL) and calyculin-A (CLA; Calbiochem, San Diego, CA), were each added from 1-mM stock solutions to a final concentration of 1 μM. Stock solutions of OA and CLA were dissolved in DMSO and methanol, respectively. A 10-mM stock solution of amiprophos methyl (APM; Mobay Corp., St. Louis, MO) was dissolved in ethanol and added to a final concentration of 5 μM. The final concentrations of solvents (0.05% ethanol for APM and 0.1% for the other solvents) had no effect on flagellar regeneration or MPM-2 reactivity.

Results

MPM-2 Reactivity Increases in Deflagellated Cells

Chlamydomonas gametes have two apically positioned flagella, ~11-μm long, which are easily seen by phase or differential interference contrast (DIC) microscopy. Fig. 1 *A* shows a DIC image of three *Chlamydomonas* cells that have been processed for immunofluorescence with the antiphosphoprotein monoclonal antibody MPM-2 (Fig. 1 *B*). These cells gave little if any signal with MPM-2 when the antibody concentration was 1:800. Cells rapidly lose their flagella by an active excision mechanism after exposure to low pH, alcohol treatment, or mechanical shear (Sanders and Salisbury, 1989). After pH shock-induced flagellar excision (Fig. 1 *C*), cells stained brightly with MPM-2 (Fig. 1 *D*), demonstrating that there was an increase, either in phosphorylation or accessibility of MPM-2 reactive epitopes. The staining pattern was consistent with localization of MPM-2 reactive proteins in the nucleus, where the staining distribution appeared more intense toward the nuclear periphery, the flagellar basal apparatus region, and in the region of the centrin-based nucleus-basal body connector.

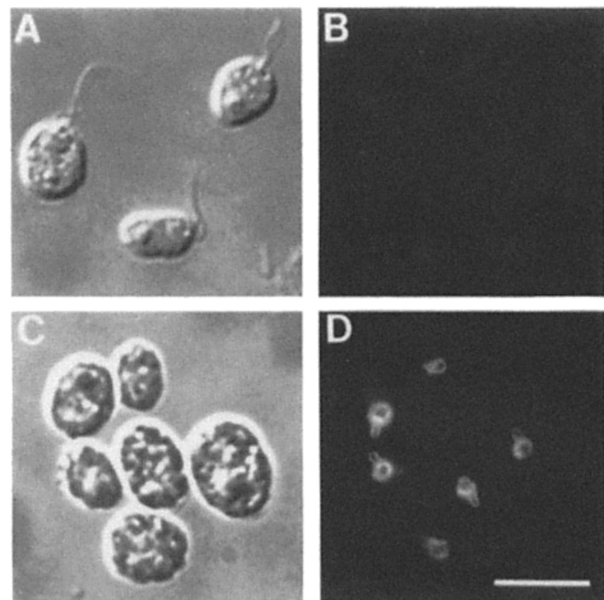

Figure 1. MPM-2 reactive structures appear after flagellar excision. DIC (*A* and *C*) and corresponding immunofluorescence (*B* and *D*) images of MPM-2 labeled *Chlamydomonas* cells. Cells with flagella (*A* and *B*) and 15 min after flagellar excision (*C* and *D*). Note that before flagellar excision (*A*), cells show little, if any, labeling with MPM-2 (*B*). After flagellar excision (*C* and *D*), MPM-2 reactive structures labeled strongly in the nucleus, basal apparatus, and nucleus-basal body connector (*D*). Bar, 10 μm.

MPM-2 Staining during Flagellar Regeneration

Under appropriate conditions, deflagellated *Chlamydomonas* cells regrow their flagella within 90–120 min (Rosenbaum et al., 1969). Fig. 2 illustrates the MPM-2 labeling pattern as seen by immunofluorescence in typical cells before flagellar excision (Fig. 2 *A*), and at times during flagellar regrowth (Fig. 2, *B*–F). Identical staining patterns and regeneration kinetics were produced if flagellar excision was induced by mechanical shear or pH shock. Before pH shock, cells showed weak nuclear MPM-2 immunofluorescence signal (Fig. 2 *A*). When cells were fixed 30 s after flagellar excision (Fig. 2 *B*), the nucleus and flagellar basal region were seen to stain brightly with MPM-2. The nuclear staining distribution appeared to be more intense toward the nuclear periphery. Note also that the nucleus lies closer to the basal apparatus than at later times (compare Fig. 2, *B*–*D*); this nuclear movement is caused by a contraction of the centrin-based nucleus-basal body connector, which occurs concomitant with flagellar excision (Salisbury et al., 1987). MPM-2 staining continued to be present in the nucleus and flagellar basal apparatus at the 15-min time point after flagellar excision (Fig. 2 *C*). In addition, the nucleus-basal body connector was seen to stain as it reextended and the nucleus began

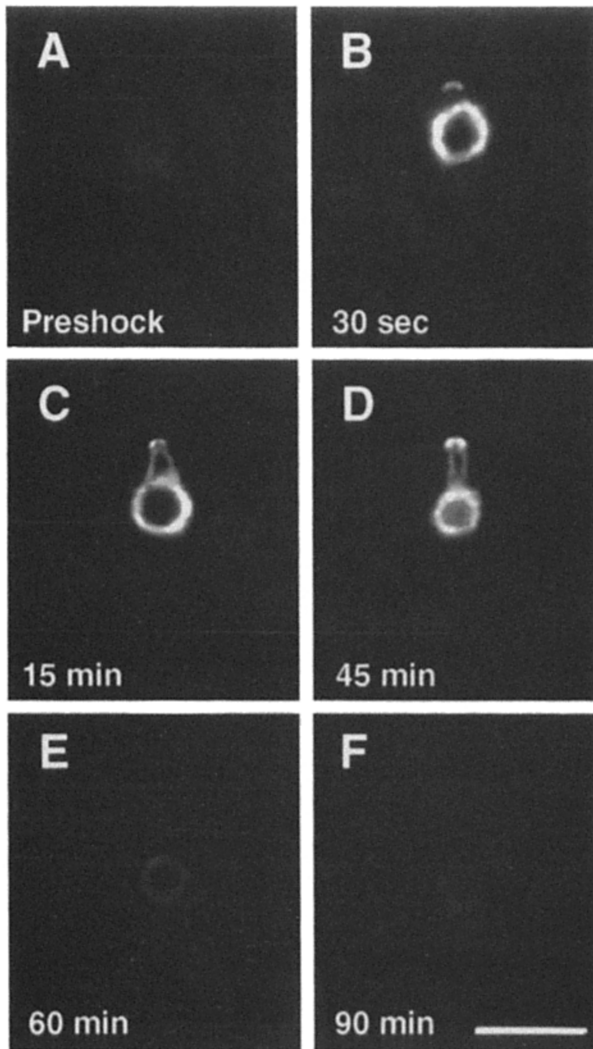

Figure 2. Immunofluorescence localization of MPM-2 reactive proteins in *Chlamydomonas* cells, before pH-induced flagellar excision (*A*) and during flagellar regeneration, after flagellar excision (*B–F*). Each cell is shown with flagellar basal apparatus toward the top of the photograph. Cells fixed 30 s after flagellar excision (*B*) stain brightly at the nuclear periphery and flagellar basal apparatus. Note that the nucleus has been displaced towards the cell anterior. At 15 min (*C*), the nucleus-basal body connector is labeled with MPM-2 and the nucleus has begun to move to a more central position. After 45 min (*D*) MPM-2 staining is similar to *C*. By 60 min (*E*), MPM-2 staining has diminished to a weak nuclear fluorescence, which by 90 min (*F*) has returned to preflagellar excision levels. Bar, 5 μm.

to return to a more central position within the cell (cf. Salisbury et al., 1987). By 45 min, after flagellar excision, flagella had regrown to approximately three quarters length and the MPM-2 staining pattern was similar to that at 15 min. By this time the nucleus-basal body connector had reextended further toward the cell center (Fig. 2 *D*). Between 60 and 120 min after flagellar excision, flagella regrew to their full length and MPM-2 staining had returned to low preflagellar excision levels (Fig. 2, *E–F*). These observations demonstrate that structures in the nucleus and flagellar basal apparatus become more reactive with MPM-2 antibodies after flagellar excision and that they remain MPM-2 reactive during the early period of flagellar regeneration. Furthermore, the nucleus-basal body connector region of the cell also increases in MPM-2 antibody reactivity during the period when the nucleus is moving from an extreme anterior to a more central position in the cell.

Immunogold Labeling in Cells before and after Flagellar Excision

Immunoelectron microscopy studies of control cells, which have full length flagella, show no MPM-2 immunogold labeling of the nucleus or flagellar basal apparatus (Fig. 3, *A–C*). Cells that were pH shocked to induce flagellar excision before fixation, however, showed substantial levels of MPM-2 immunogold labeling. In particular, a discrete nuclear zone near the nuclear periphery showed heavy MPM-2 labeling (Fig. 3 *D*, *open arrows*) and occasionally gold label was also seen at the nuclear envelope (Fig. 3 *D*, *closed arrows*). In addition, MPM-2 immunogold labeling was seen throughout the flagellar basal apparatus region including label on basal body associated-fibers (Fig. 3, *E–F*), the region of the outer doublets of the transition zone (Fig. 3 *G*), and the region of attachment of the basal bodies to the plasma membrane (Fig. 3 *H*).

MPM-2 Recognizes Several Phosphoproteins on Western Immunoblot Analysis, Two of Which (34 kD and 90 kD) Increase after Flagellar Excision

Fig. 4 shows a typical Western blot of MPM-2 reactive proteins from whole cell samples taken throughout the time course of flagellar regeneration. Before pH shock-induced flagellar excision (Fig. 4, *PS*), several MPM-2 reactive proteins were detected by Western immunoblot, including a band of 34 kD (*thin arrow*) and two higher molecular weight proteins of 130 and 210 kD. After flagellar excision, the 34-kD band increased in intensity and a 90-kD band, previously undetected with MPM-2, appeared (Fig. 4, *thick arrow*). The intensity of MPM-2 reactivity of the 34- and 90-kD proteins remained high in samples taken up to 15 min after flagellar excision, and then returned to basal levels by 120 min (Fig. 4). Analysis of MPM-2 Western immunoblots from over 20 separate experiments revealed that the 130- and 210-kD proteins did not change in MPM-2 reactivity after flagellar excision, however, the 34- and 90-kD proteins consistently increased in reactivity after flagellar excision and decreased again to preflagellar excision levels during the time course of flagellar regeneration. It should be noted that 34-kD protein reactivity was somewhat variable, since it increased slightly after sample manipulations (i.e., centrifugation, washing, resuspension in flagellar excision buffer). This variability could be reduced by preincubation in flagellar excision buffer for 60 min before pH shock. Nonetheless, the 34-kD protein did consistently increase in reactivity after flagellar excision, and reactivity of the 90-kD protein, which was not sensitive to preparative manipulations, appeared only during flagellar regrowth.

Fig. 5 illustrates a typical flagellar regeneration curve (Fig. 5 *A*), the corresponding areas of an MPM-2 Western immunoblot showing the 90-kD (Fig. 5 *B*) and 34-kD (Fig. 5 *C*) proteins, and densitometric analysis of band reactivity during flagellar regeneration. The densities of each of the two bands, averaged from three separate experiments, were plotted. Both proteins showed a marked increase in MPM-2

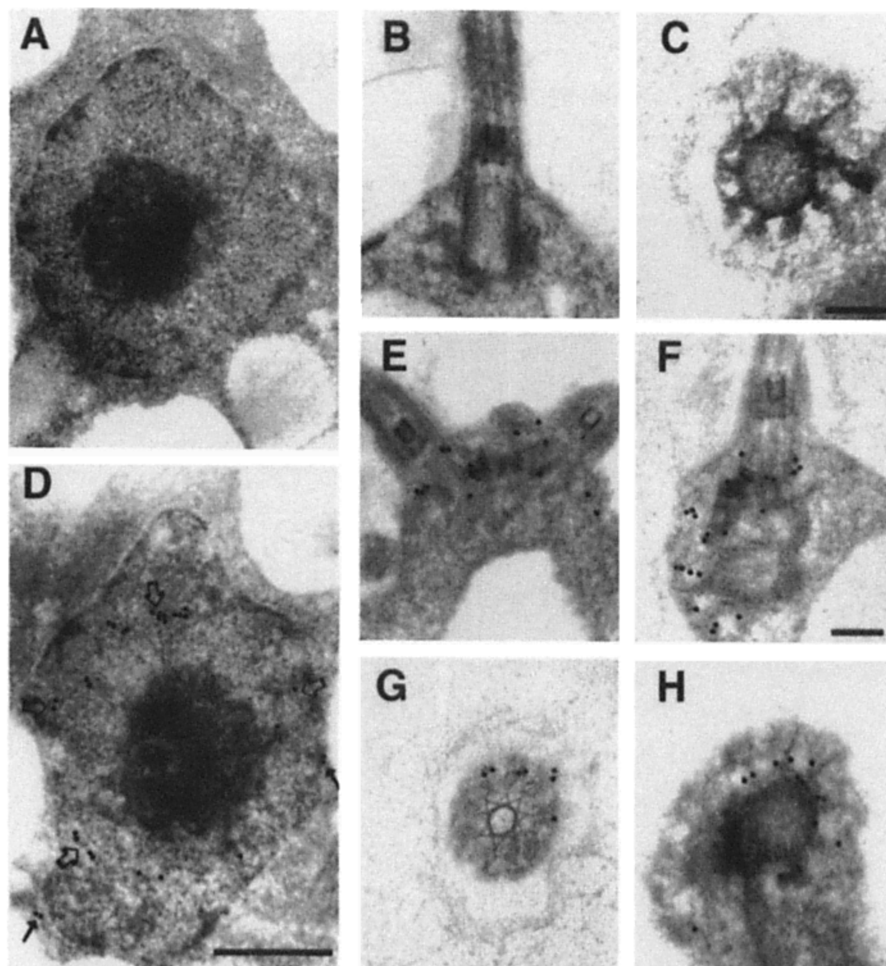

Figure 3. Immunogold labeling with MPM-2 antibodies before flagellar excision (*A–C*) and 15 min after flagellar excision (*D–H*). Note that no labeling is present in the nucleus (*A*) or basal apparatus (*B* and *C*) before flagellar excision. After flagellar excision cells show substantial levels of label in the nuclear periphery (*D, open arrows*) and occasionally on the nuclear envelope (*D, closed arrows*). Labeling is also observed throughout the flagellar basal apparatus region, including basal associated fibers (*E–F*), the region of the outer doublets of the transition zone (*G*), and on anchoring fibers (*H*). Bars: $D = 1\ \mu m$ (*A* and *D*); $C = 0.2\ \mu m$ (*C* and *G*); $F = 0.2\ \mu m$ (*B, E, F* and *H*).

reactivity after flagellar excision, which subsequently decreased, usually to below preflagellar excision levels, as flagellar regeneration progressed.

To verify that the MPM-2 antibody recognizes phosphoproteins in deflagellated *Chlamydomonas* cells, we treated Western transferred whole cell extracts with acid phosphatase. This treatment prevented the subsequent binding of MPM-2 antibody to the 34-kD and 90-kD proteins (Fig. 6 *B*). Two types of controls were employed. First, incubation of immunoblots in buffer only for the same period of time as the acid phosphatase treatment, resulted in no loss of reactivity with MPM-2 antibody (Fig. 6 *A*). Second, an acid phosphatase-treated blot was subsequently reacted with anticentrin monoclonal antibody (20H5), and then further processed with secondary antibody in parallel to the samples treated for MPM-2 reactivity. This control gave a strong centrin band (Fig. 6 *C*) indicating that the phosphatase treatment had no effect on the subsequent reactivity of an antibody that does not recognize phosphorylated epitopes.

Comparison of Wild-type and Mutant Cells

Table I summarizes MPM-2 reactivity in wild-type cells and two mutant strains after flagellar excision. In wild-type cells, as demonstrated above, flagella were excised upon pH shock and the cells stained brightly with MPM-2 antibody in the flagellar basal region, nucleus-basal body connector, and in the nuclear periphery. In addition, there was an increase in reactivity by Western immunoblot analysis of the 34- and 90-kD proteins. Mechanical shear treatment (i.e., rapid vortexing) also resulted in an increase in MPM-2 immunofluorescence and reactive proteins (Table I). Cells of the flagellar autotomy mutant *fa-1* (Lewin and Burrascano, 1983), do not excise their flagella, due to a defect in the excision mechanism (Sanders and Salisbury, 1989). After pH shock, *fa-1* cells show immunofluorescence labeling with MPM-2 antibody at the flagellar basal region and nucleus, however, in contrast to wild-type control cells, the nucleus-basal body connector did not stain at all at any time after pH shock (Fig. 7 *A*). We also note that as reported in earlier studies (Salisbury et al., 1987), *fa-1* cell nuclei did not move to an anterior position after pH shock. After pH shock of *fa-1* cells, Western immunoblots revealed an increase in MPM-2 reactivity for both the 90- and 34-kD proteins (Fig. 8).

Bald-2 mutants do not have functional basal bodies, and therefore do not grow flagella (Goodenough and St. Clair, 1975). After pH shock of *bald-2* cells, an increase in nuclear staining was observed, however for ~99% of the cells no staining was seen in the region corresponding to the flagellar basal apparatus or nucleus-basal body connector of wild-type cells (Fig. 7 *B*). In a few rare instances, individual cells did label near the cell border which may represent the location of a defective flagellar basal apparatus. Corresponding Western immunoblots of *bald-2* cells after pH shock revealed 34-kD band reactivity and a variable and weak 90-kD

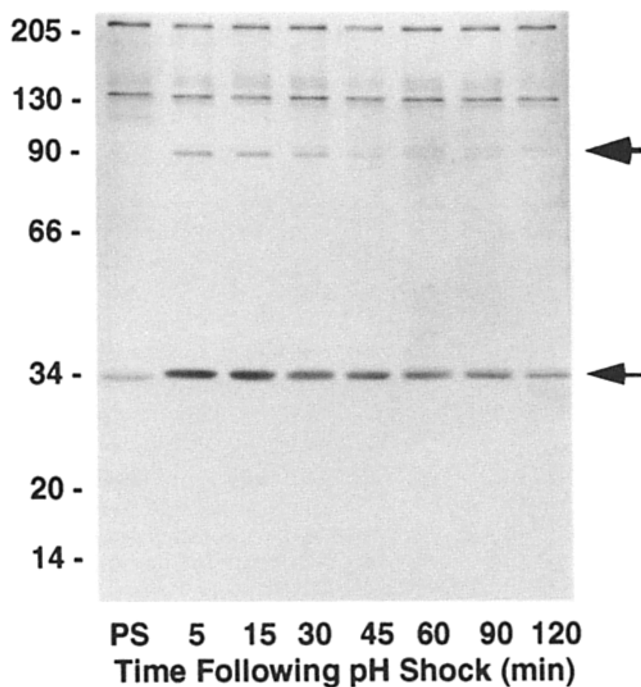

Figure 4. Western immunoblot of whole cell extracts of *Chlamydomonas* showing MPM-2 reactivity before pH shock-induced excision (*PS*) and during flagellar regeneration, after flagellar excision (5–120 min). Molecular weight markers in kD are presented along the left hand side of the blot. Note that before flagellar excision (*PS*), MPM-2 recognized several proteins of ~210, 130, and 34 kD. After flagellar excision (5 min), the reactivity of the 34-kD protein (*thin arrow*) increased and a 90-kD protein appeared (*thick arrow*). MPM-2 reactivity of the 34- and 90-kD proteins then decreased gradually throughout the time course of flagellar regeneration.

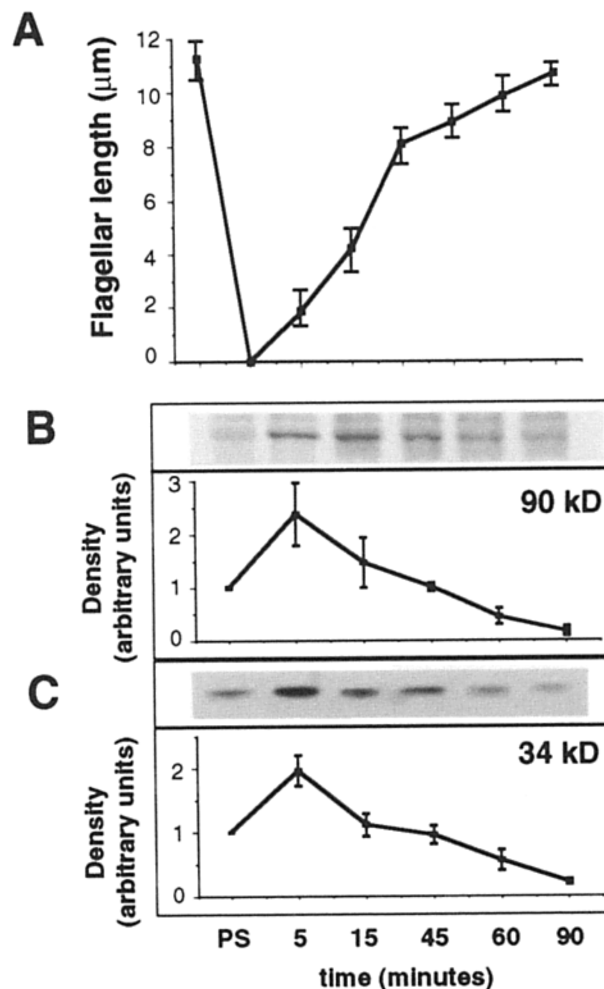

Figure 5. Flagellar regeneration kinetics (*A*) and representative areas of Western immunoblot along with densitometry measurements of the 90-kD (*B*) and 34-kD (*C*) MPM-2 reactive proteins. Density measurements were averaged from three independent experiments and show that after flagellar excision MPM-2 reactivity initially increased, and then subsequently decreased to below preflagellar excision levels over the time course of flagellar regeneration. Standard error bars are shown.

band reactivity (Fig. 8); in some experiments, 90-kD protein reactivity was undetected. Therefore, MPM-2 antibody reactivity of the 34-kD protein always corresponded to nuclear periphery staining. The 90-kD band reactivity was greatly diminished in *bald*-2 cells which also failed to stain in the flagellar basal apparatus region. It is likely, therefore that the 34-kD MPM-2 reactive protein is nuclear associated and the 90-kD MPM-2 reactive protein is located in the flagellar basal apparatus region.

Inhibitor Studies

We have employed a number of inhibitors in our studies (summarized in Table I), some of which have been shown by other workers to affect flagellar excision or regeneration. The aminoglycoside antibiotic, neomycin, blocks the inositol phosphate signaling pathway by binding to phosphatidylinositol 4,5-bisphosphate preventing its hydrolysis by phospholipase C (Gabev et al., 1989). Quarmby et al. (1992) demonstrate that neomycin treatment of *Chlamydomonas* cells results in inhibition of inositol phosphate metabolism and prevents flagellar excision after pH shock. We confirmed that neomycin treatment prevents pH shock induced flagellar excision. In these cells, MPM-2 staining was not detected by immunofluorescence in either the nucleus or flagellar basal apparatus region (Fig. 7 *C*). While the 34- and 90-kD proteins were detectable by immunoblot, they were less intense than parallel controls (Fig. 8). When neomycin was added to cells immediately after pH shock, cells excised their flagella, however, flagellar regeneration kinetics were slower than normal. Western immunoblots reveal that MPM-2 reactivity of the 34- and 90-kD proteins increased in cells treated with neomycin immediately after pH shock (data not shown). These results suggest that signaling through the inositol phosphate pathway is a rapid event and confirm the observation that phosphorylation of MPM-2 reactive proteins is an early step in the flagellar regeneration process.

The G-protein agonist mastoparan (final concentration of 8 μM) causes flagellar excision in *Chlamydomonas* cells (Quarmby et al., 1992). In this study mastoparan also induced excision and MPM-2 staining was observed in the nucleus, flagellar basal apparatus, and the nucleus-basal body connector (Fig. 7 *D*). On Western immunoblot, MPM-2 reactivity of the 34- and 90-kD proteins increased after flagellar excision but was weaker than in cells excised by pH shock. This was reflected by the fact that flagellar regenera-

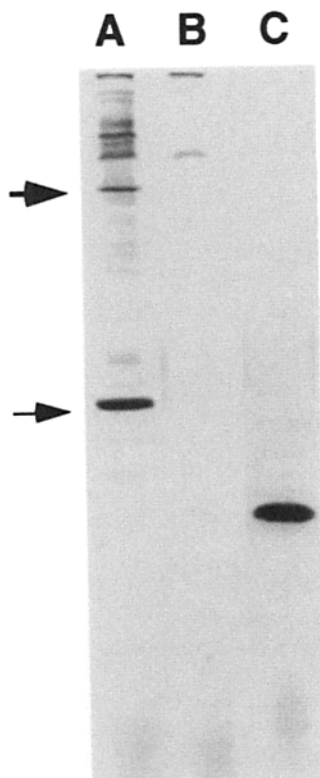

Figure 6. Acid phosphatase treatment of whole cell extracts from deflagellated *Chlamydomonas* cells prevents subsequent reactivity of MPM-2 antibody. MPM-2 reactive proteins were observed after incubation in phosphatase buffer only (*A*). Note the 90-kD (*thick arrow*) and 34-kD (*thin arrow*) proteins. Incubation of Western immunoblots in 33 U per ml acid phosphatase for 8 h resulted in the loss of MPM-2 reactivity for the 90-kD and 34-kD proteins. A control acid phosphatase-treated blot (*C*) stained strongly with anticentrin monoclonal antibody.

tion was variable in mastoparan-treated cells, where typically only about 70% of the cells regrew their flagella.

The phosphoric amide APM has been shown to block tubulin mRNA induction and tubulin synthesis in *Chlamydomonas* (Collis and Weeks, 1978; Quader and Filner, 1980). APM has also been shown to inhibit plant microtubule polymerization (Morejohn and Forsket, 1984). In the presence of APM, *Chlamydomonas* cells deflagellated after exposure to low pH, in a mechanically stirred suspension, but these cells failed to regrow flagella (Table I). These cells showed an increase in nuclear, nucleus-basal body connector, and flagellar basal apparatus staining with MPM-2 by immunofluorescence (Fig. 7 *E*). However, in contrast to control cells, the nucleus of APM-treated cells did not move to the extreme anterior region after pH shock. Western immunoblot analysis, of APM-treated cells, revealed that the 34- and 90-kD proteins initially increased in MPM-2 reactivity (Fig. 8), but they rapidly decrease to background levels within 30 min. Remarkably, when APM was washed out after 60 min of treatment, the nucleus rapidly moved to the extreme cell anterior and stained brightly as did the flagellar basal region, with MPM-2 by immunofluorescence (Fig. 7 *F*). The 34- and 90-kD MPM-2 reactive proteins appeared again to increase in intensity on Western immunoblot analysis (Fig. 8) and flagella regrew with essentially normal kinetics. These results suggest that APM acts to inhibit flagellar regeneration at a point distal to phosphorylation of MPM-2 reactive proteins. We have also found that APM may act at a second site as well, since living cells and detergent extracted cell models treated with APM failed to excise their flagella after pH shock or elevated calcium treatment when there was no mechanical shear load on the system (i.e., when cells were not stirred, Sanders, M. A., and J. L. Salisbury, unpublished data). Thus, APM may also act in a more direct, MPM-2 phosphoprotein independent fashion on the flagellar excision mechanism itself.

The kinase inhibitor, staurosporine has been reported to block flagellar tubulin mRNA increase in deflagellated cells (Cheshire, J., and L. Keller, personal communication). Cells treated with staurosporine excise their flagella normally after pH shock or mechanical shear, however the extent of flagellar regrowth is sensitive to staurosporine concentration. Cells deflagellated in 3 μg/ml staurosporine regrow half-length flagella, while 10 μg/ml staurosporine results in a delay in initiation of flagellar regrowth and cells regrow less than half-length flagella (Cheshire, J., and L. Keller, personal communication). Here, we observed a similar effect of staurosporine on flagellar regrowth. Immunofluorescent MPM-2 staining was variable and weak, ranging from no

Table I. Summary of MPM-2 Reactivity of Chlamydomonas after Various Treatments

Cell-type	Treatment	MPM-2 Localization			MPM-2 Western blot		Flagellar excision competence	Flagellar regrowth
		BBA	NBBC	NP	34 kD	90 kD		
Wild-type	pH shock	+++	+++	+++	+++	+++	99–100%	>95%
Wild-type	Mechanical shear	+++	+++	+++	+++	+++	95–100%	>95%
fa-1	pH shock	+++	−−−	++−	++−	+++	0%*	*
bald-2	pH shock	−−−	−−−	++−	++−	+−−	0%‡	‡
Wild-type	Neomycin¶	−−−	−−−	+−−	+−−	+−−	∿5%	0%
Wild-type	Mastoparan‖	++−	++−	++−	++−	++−	>95%	∿70%
Wild-type	APM¶	++−	++−	++−	++−	++−	>95%§	0%
	APM washout	+++	+++	+++	+++	+++		>90%
Wild-type	Staurosporine¶	−−−	−−−	+−−	+−−	−−−	>95%	impaired
Wild-type	Okadaic acid¶	+++	+++	+++	+++	+++	>95%	>95%
Wild-type	Calyculin-A¶	+++	+++	+++	+++	+++	>95%	>95%

*, *Fa-1* cells fail to excise their flagella under any conditions.
‡, *Bald-2* cells do not have flagella or functional basal bodies.
§, APM-treated cells excise their flagella only when samples are mechanically sheared (i.e., stirred).
‖, These treatments caused flagellar excision without the requirement of pH shock or excess mechanical shear.
¶, Inhibitor treatment preceded pH shock induced flagellar excision.
BBA, basal bodies and flagellar apparatus;
NBBC, nucleus-basal body connector;
NP, nuclear periphery.

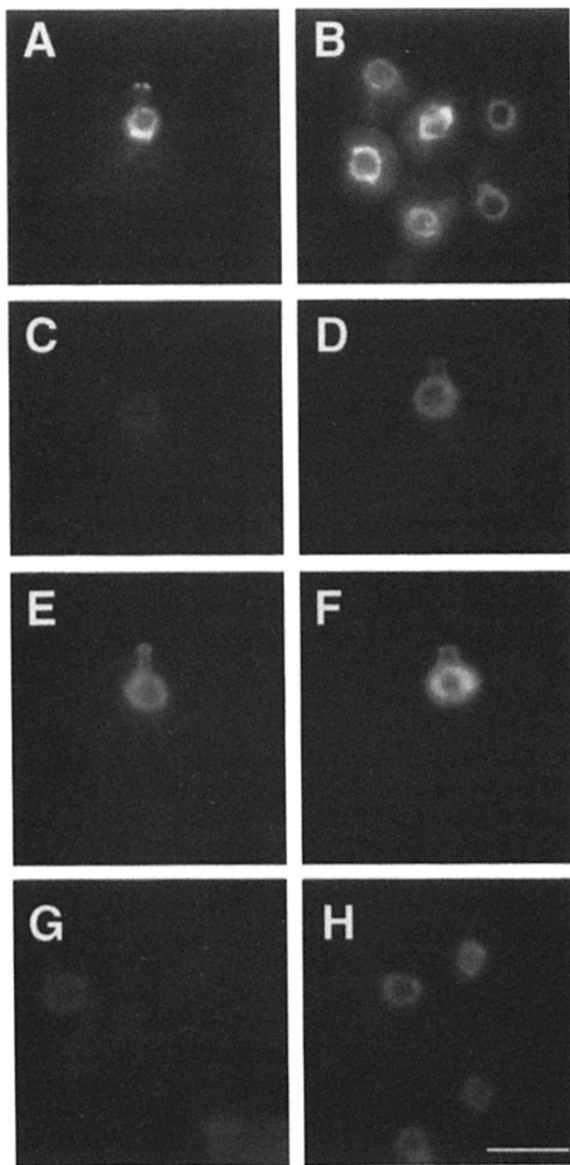

Figure 7. MPM-2 immunofluorescence localization in mutants and after treatments summarized in Table I. (*A*) *Fa-1* mutant after pH shock, reveals staining of the nucleus and flagellar basal apparatus. The nucleus-basal body connector does not stain at any time. (*B*) In *bald-2* mutants after pH shock, only nuclear staining is observed. (*C*) Neomycin treatment prevents subsequent pH shock-induced excision and results in only very weak nuclear fluorescence with MPM-2. (*D*) The G-protein agonist mastoparan causes flagellar excision and results in MPM-2 labeling identical to that seen in cells deflagellated by pH shock. (*E*) APM added to cells after flagellar excision, results in no flagellar regrowth or anterior nuclear movement, until the inhibitor was washed out (*F*). (*G*) Treatment with the kinase inhibitor staurosporine did not prevent pH-induced flagellar excision, but impaired flagellar regeneration and greatly diminished MPM-2 labeling. (*H*) Labeling flagellated cells with MPM-2 at a higher concentration (1:400) reveals nuclear staining, but basal apparatus or nucleus-basal body connector staining was not seen. Bar, 5 μm.

staining to very weak nuclear flagellar basal apparatus and nucleus-basal body connector staining (Fig. 7 *G*). MPM-2 reactivity of the 34- and 90-kD proteins after flagellar excision was also greatly reduced as compared to controls; at early time points after flagellar excision the 34-kD band was greatly diminished, but surprisingly its reactivity increased slightly after 45 min (Fig. 8). In staurosporine-treated cells, the 90-kD protein reactivity with MPM-2 antibody was typically below detection limits (Fig. 8). We also investigated the effects of two potent phosphatase inhibitors, OA (Fig. 8) and CLA, which had no inhibitory affect on flagellar regrowth at the concentrations used (Table I). MPM-2 reactive proteins and immunofluorescence in these treatments were similar to that seen in controls except that the reactivity of the 34-kD protein was somewhat delayed (Fig. 8) with maximum 34-kD reactivity occurring at 45 min after pH shock-induced excision. These results are consistent with the phosphorylation of MPM-2 reactive proteins after flagellar excision.

Observations Using Higher MPM-2 Antibody Concentrations

We have found that in wild-type cells with flagella, nuclei stained when the MPM-2 antibody was used at higher concentration (i.e., 1:400; Fig. 7 *H*). This staining corresponded to 34-kD MPM-2 reactivity by Western immunoblot analysis also detectable at higher antibody concentrations. The flagellar basal apparatus or nucleus-basal body connector do not stain under these conditions nor is 90-kD protein reactivity detected with MPM-2 antibodies in these cells. Regardless of antibody concentration, an increase of both the nuclear fluorescence and the 34-kD MPM-2 reactivity by Western immunoblot analysis consistently occurred upon flagellar excision.

Discussion

We report the use of the antiphosphoprotein antibody MPM-2, to investigate the phosphorylation of proteins during flagellar regeneration. MPM-2 antibodies localized to the nuclear periphery, the flagellar basal apparatus, and the nucleus-basal body connector during flagellar regeneration. The flagellar basal apparatus and nucleus labeled immediately after flagellar excision, while the nucleus-basal body connector label appeared coincident with its reextension and the return of the nucleus to a more central position within the cell (Salisbury et al., 1987). We also show that MPM-2 reactivity of a 34- and a 90-kD phosphoprotein rapidly increased after flagellar excision, and that this reactivity returned to basal levels as flagella regrew. The increased reactivity of the 34- and 90-kD proteins with MPM-2 antibody after flagellar excision was not dependent on new protein synthesis, since pretreatment of cells with the protein synthesis inhibitor cycloheximide had no effect on the rapid appearance of the MPM-2 reactive labeling by immunofluorescence or by Western blot analysis (not shown). It is well known that gene upregulation occurs almost normally in the absence of new protein synthesis (Baker et al., 1984). The rapid increase in MPM-2 reactivity that we observed, therefore, probably represents the phosphorylation of preexisting pools of these phosphorylation substrates. Our studies suggest that the 34-kD MPM-2 reactive protein is associated with the nuclear periphery, and that the 90-kD MPM-2 reactive protein is associated with the flagellar basal apparatus. This conclusion is based on immunofluorescence and immunogold

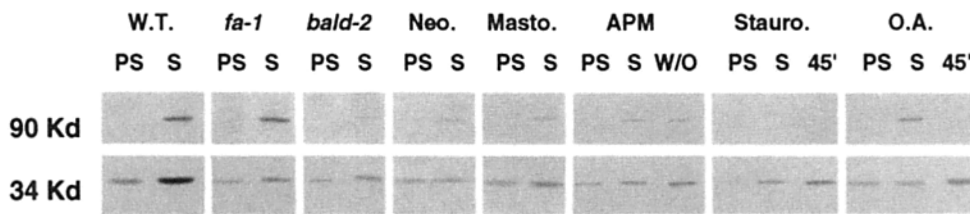

Figure 8. Panels from Western immunoblots showing MPM-2 reactivity of the 34-kD and 90-kD proteins in mutants and after treatments summarized in Table I. *WT*, wild-type cells; *fa-1*, flagellar autotomy mutant; *bald-2*, mutant defective in basal body formation; *Neo*, neomycin sulfate added to cells before pH shock; *Masto*, mastoparan induces flagellar excision; *APM*, amiprophos methyl; *Stauro*, staurosporine; *OA*, okadaic acid; *PS*, before pH shock or treatment; *S*, after pH shock or treatment; *45'*, 45 min after pH-induced flagellar excision; *W/O*, inhibitor wash out.

labeling, and observations using *Chlamydomonas* mutants (see below).

We examined the localization of the MPM-2 reactive phosphoproteins in a mutant lacking functional basal bodies (*bald-2*), and a mutant defective in the excision process (*fa-1*). After pH shock, the *bald-2* mutant shows little, if any, reactivity of the 90-kD protein by Western blot analysis. *Bald-2* cells also fail to localize MPM-2 reactive structures outside the nucleus. Nonetheless, these cells show nuclear staining and reactivity of the 34-kD protein with MPM-2 antibodies. This data supports the conclusion that the 34-kD protein is in the nucleus and the 90-kD is in the flagellar basal apparatus. In *fa-1* after pH shock the flagellar basal region and nuclear staining increased, as in wild-type cells. However, the nucleus-basal body connector never stained with MPM-2. In contrast to wild-type cells (Salisbury et al., 1987), the nucleus of the *fa-1* mutant fails to undergo anterior displacement after pH shock. These observations further suggest that nucleus-basal body connector reextension and nuclear movement occur coincident with phosphorylation of nucleus-basal body components.

We also examined the role of phosphorylation of MPM-2 reactive proteins through the use of kinase and phosphatase inhibitors. The kinase inhibitor staurosporine, suppressed the increase in MPM-2 reactive proteins after flagellar excision and resulted in impaired flagellar regrowth. This observation suggests that staurosporine sensitive protein kinase activity and phosphorylation of MPM-2 reactive proteins may be related and in addition may play an important role in flagellar regeneration. In this regard, staurosporine has been shown to inhibit tubulin mRNA increase in deflagellated *Chlamydomonas* cells, resulting in impaired flagellar regrowth (Cheshire, J., and L. Keller, personal communication). The phosphatase inhibitor studies, however, suggest that such a model may be oversimplified. Treatment with the phosphatase inhibitors, OA and CLA, had no effect on the appearance of the 90-kD MPM-2 reactive proteins or flagellar basal apparatus and nucleus-basal body fluorescence. While these compounds did delay an increase in the 34-kD MPM-2 reactivity and nuclear fluorescence, it is possible that under normal conditions dephosphorylation events precede the phosphorylation cascade resulting in the increase in 34-kD reactivity.

The increase in nuclear, nucleus-basal body connector, and basal apparatus localization of MPM-2 reactive proteins after flagellar excision suggests that they may play a role in flagellar gene induction and/or flagellar precursor transport. This possibility is also supported by the temporal phosphorylation of the 34- and 90-kD MPM-2 reactive proteins after flagellar excision and their decrease in reactivity concomitant with flagellar regrowth. Cells induced to resorb their flagella by incubation in pyrophosphate (Lefevre et al., 1978) give immunofluorescence staining with MPM-2, identical to deflagellated cells, when the pyrophosphate is washed out (data not shown). This is evidence to support the notion that it is not the flagellar excision event itself which is causing an increase in MPM-2 reactivity, but that the MPM-2 reactive phosphoproteins are perhaps part of a signal cascade involved in flagellar regrowth. Drugs that block flagellar regrowth may uncouple the phosphorylation process from regrowth since colchicine-treated cells fail to regrow flagella, yet show a rapid increase in MPM-2 reactivity in both the nucleus and flagellar apparatus similar to control cells (data not shown). In addition, cells treated with APM do not regrow flagella and show only a modest increase in MPM-2 reactivity over a shorter duration (30 min). Interestingly, after APM washout, MPM-2 reactivity appears more intense than before and flagella rapidly regrow to full length. Finally, the correlation of increased MPM-2 reactivity and flagellar biogenesis is consistent with this hypothesis since we have not observed flagellar regrowth in treatments that do not result in an increased reactivity with MPM-2 antibodies.

Localization of the MPM-2 reactive phosphoproteins in the nucleus and basal apparatus, during flagellar regeneration, leads us to speculate on the possible roles they may be playing. In immunoprecipitation experiments, the 34-kD protein proved to be a fairly insoluble protein in detergent extracted cells (data not shown), suggesting that it might be an integral part of the nucleoskeleton (see Verheijen et al., 1988). Phosphorylation of the 34-kD protein might play a role in transcriptional regulation of flagellar precursor protein genes by acting on the transcription apparatus, preparing it for a rapid increase in activity (see Hunter and Karin, 1992). As noted above, the 34-kD protein was also sensitive to changes in the external milieu (cf. Martindale and Salisbury, 1990) which suggests that the 34-kD protein may play a more general role in nuclear signal transduction. Phosphorylation of the 34-kD nuclear protein may be an important signaling event responsive to a wide range of cell stimuli. In this study, the increase of the 34-kD protein was consistently greater after flagellar excision and during the early stages of flagellar regrowth. The 90-kD MPM-2 reactive protein appears to be intimately associated with the flagellar basal apparatus, since its localization is undetected in *bald-2* mutants which lack normal basal bodies and its reactivity by Western blot analysis is greatly diminished in these cells. The localization of the 90-kD protein in the tran-

sition region, which is believed to play a role in the control of flagellar assembly (Dentler, 1987), suggests that it may function in flagellar precursor packaging or transport into the growing axoneme. The observations presented here suggest that the phosphoproteins recognized by MPM-2 may play important roles in the flagellar regeneration process.

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