Cell Adhesion-dependent Inactivation of a Soluble Protein Kinase during Fertilization in *Chlamydomonas*

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Within seconds after the flagella of mt^+ and mt^- *Chlamydomonas* gametes adhere during fertilization, their flagellar adenylyl cyclase is activated several fold and preparation for cell fusion is initiated. Our previous studies indicated that early events in this pathway, including control of adenylyl cyclase, are regulated by phosphorylation and dephosphorylation. Here, we describe a soluble, flagellar protein kinase activity that is regulated by flagellar adhesion. A 48-kDa, soluble flagellar protein was consistently phosphorylated in an in vitro assay in flagella isolated from nonadhering mt^+ and mt^- gametes, but not in flagella isolated from mt^+ and mt^- gametes that had been adhering for 1 min. Although the 48-kDa protein was present in the flagella isolated from adhering gametes, we demonstrate that its protein kinase was inactivated by flagellar adhesion. Immunoblot analysis and inhibitor studies indicate that the 48-kDa protein in nonadhering gametes is phosphorylated by a protein tyrosine kinase. In vivo experiments showing that the protein tyrosine phosphatase inhibitor sodium orthovanadate inhibits fertilization suggest that protein dephosphorylation may be required for signal transduction. The 48-kDa protein and its protein kinase may be among the first elements of a novel signalling pathway that couples interaction of flagellar adhesion molecules to gamete activation.

INTRODUCTION

During fertilization in most sexual species, encounters between gametes take the form of a series of cell-cell interactions and signal transductions in an intimate cellular interplay that culminates in cell and nuclear fusion. When adhesion molecules on the sperm bind to their cognate egg surface molecules in a receptor/ligand-like interaction, signals are generated in the sperm (Garbers, 1989; Foltz and Lennarz, 1993; Myles, 1993). The sperm response to the signal includes recruitment and exposure of new adhesion/fusion molecules and exocytosis of enzymes that modify the extracellular surface of the egg (Myles, 1993; Snell, 1993). Apposition and adhesion of the plasma membranes of the two gametes is followed rapidly by membrane fusion and establishment of a common cytoplasm. In several organisms, both egg and sperm surface molecules have been identified

that play a role in these adhesion/signalling events. Although substantive evidence supports a role for G proteins (Ward and Kopf, 1993) and protein phosphorylation (Peaucellier *et al.*, 1988; Leyton and Salting, 1989; Leyton *et al.*, 1992; Ciapa and Epel, 1991; Jiang *et al.*, 1991; Duncan and Fraser, 1993; Kalab *et al.*, 1994) in sperm-egg signalling, the signalling pathways that couple interactions between sperm-egg adhesion molecules to cellular responses have not yet been elucidated.

Interactions between mt^+ and mt^- gametes during fertilization in the unicellular biflagellated alga, *Chlamydomonas reinhardtii*, comprise many of the events described above for animal systems (Goodenough, 1991; van den Ende, 1992; Snell, 1993). During the mating reaction in *Chlamydomonas*, interactions between adhesion molecules, agglutinins on the surfaces of the flagella of mt^+ and mt^- gametes activate a flagellar adenylyl cyclase that rapidly generates a 10-fold increase in intracellular cAMP (Pijst *et al.*, 1984; Pasquale and Goodenough, 1987;

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Kooijman *et al.*, 1990; Zhang and Snell, 1994). This rise of intracellular cAMP causes several cellular responses including the following: 1) secretion of a serine protease, which converts a prometalloprotease to an active enzyme that modifies and releases the extracellular matrix (Snell *et al.*, 1989); 2) movement of pre-existing agglutinins from the plasma membrane of the cell body onto the contiguous flagellar membrane (Hunnicut *et al.*, 1990); 3) erection on mt^+ gametes of an actin-filled mating structure or fusion organelle that displays adhesion/fusion proteins that interact with the mt^- fusion organelle (Friedmann *et al.*, 1968; Goodenough and Jurivich, 1978; Goodenough and Weiss, 1978; Detmers *et al.*, 1983); and 4) cell fusion to form a zygote.

In previous studies on the adenylyl cyclase in gametic flagella isolated from nonadhering cells, we and others have found no evidence that G proteins regulate the *Chlamydomonas* enzyme (Pasquale and Goodenough, 1987; Zhang *et al.*, 1991; Saito *et al.*, 1993; Zhang and Snell, 1993, 1994). Instead our recent evidence indicates that the adenylyl cyclase in gametic flagella is regulated by a novel, adhesion-dependent signalling pathway that includes a staurosporine-sensitive activity, probably a protein kinase (Zhang and Snell, 1994). In these experiments, when isolated gametic flagella of opposite mating types were mixed together *in vitro*, they adhered avidly, and the adenylyl cyclase activity was activated about threefold compared with nonmixed flagella. The protein kinase inhibitor staurosporine (50 nM) blocked about 70% of this adhesion-induced activation. Moreover, the same concentration of staurosporine also blocked flagellar adhesion-dependent signalling *in vivo*. Flagellar adhesion was unaffected by staurosporine, but cells agglutinating in the presence of this inhibitor failed to undergo the increase in intracellular cAMP shown by untreated gametes (Zhang and Snell, 1994).

To learn more directly about the role of protein phosphorylation in this signal transduction pathway during fertilization, we have examined the *in vitro* phosphorylation of proteins in flagella isolated from adhering and nonadhering gametes. Here we show that in an *in vitro* phosphorylation assay a 48-kDa soluble flagellar protein was consistently phosphorylated in flagella isolated from nonadhering mt^+ and mt^- gametes but not in flagella isolated from gametes that had been adhering for 1 min. The data indicate that the 48-kDa protein was present in the adhering flagella, but that the protein kinase that normally phosphorylated it was inactivated by flagellar adhesion. Taken together, our data indicate that protein phosphorylation plays an important role in this signal transduction pathway and may regulate coupling between flagellar adhesion and gamete activation.

MATERIALS AND METHODS

Materials

4-(2-hydroxyethyl)-1-piperazineethanesulfonate (HEPES)¹ was obtained from Research Organics, Cleveland, OH; [γ -³²P]ATP was obtained from NEN, Boston, MA; N-[2-(methylamino)ethyl]-5-isoquinoline sulfonamide (H-8) was obtained from Seikagaku Kogyo, Tokyo, Japan; 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7) and microcystin were obtained from Calbiochem, La Jolla, CA; cAMP, ATP, papaverine, leupeptin, lima bean trypsin inhibitor, tosylphenylalanine chloromethylketone, N α -p-tosyl-L-lysine chloromethylketone, phenylmethanesulfonyl fluoride, polyethylenimine, and staurosporine were obtained from Sigma, St. Louis, MO; okadaic acid and the tyrosine kinase inhibitor sampler kit were obtained from Life Technologies, Gaithersburg, MD; and all other chemicals were reagent grade. The ECL Western blotting kit was from Amersham (Arlington Heights, IL).

Cells and Cell Culture

Chlamydomonas reinhardtii strains 21 gr (mt^+) and 6145C (mt^-) (available from the *Chlamydomonas* Genetics Center, Duke University, Durham, NC) were cultured at room temperature in Medium I or Medium II of Sager and Granick (Sager and Granick, 1954; Harris, 1989) on a 12-h light-dark cycle. Gametic cells were obtained as previously described (Snell, 1976) by transferring vegetative cells ($4\text{--}7 \times 10^6$ cells/ml) 6 h after the beginning of the light period into N-free Medium I containing 0.15 g/liter KH_2PO_4 and 0.3 g/liter K_2HPO_4 .

Isolation of Flagella

To isolate flagella from adhering gametes, mt^+ and mt^- gametes were mixed together for 1 min at room temperature, diluted about threefold with ice cold N-free Medium I, harvested by centrifugation, and flagella were separated from cell bodies by a modification of the pH shock method of Witman *et al.* (1972) as described earlier (Zhang *et al.*, 1991). Flagella were isolated from nonadhering gametes using the same procedure except the mt^+ and mt^- gametes were not mixed together before their flagella were isolated. In some experiments with nonadhering flagella, the cells were not diluted with ice cold N-free medium before being harvested for deflagellation, with no detectable effect on their *in vitro* phosphorylation pattern. The sedimented flagella were resuspended to a final concentration of 3–8 mg/ml flagellar protein in flagella buffer (20 mM Na-HEPES, pH 7.2, 4% sucrose, 1 mM EDTA, 2.5 mM $MgCl_2$). A mixture of protease inhibitors (7 μ M leupeptin, 3.2 μ g/ml trypsin inhibitor from lima bean, 60 μ M tosylphenylalanine chloromethylketone, 60 μ M N α -p-tosyl-L-lysine chloromethylketone, and 0.13 mM phenylmethanesulfonyl fluoride) was added to the suspension of flagella; the suspension was divided into small aliquots and immediately frozen and stored in liquid nitrogen until use. To prepare flagellar supernatants, flagellar samples were thawed rapidly and centrifuged at 10,000 rpm for 3 min at 4°C in an Eppendorf microfuge. (Similar results were obtained if the samples were centrifuged at 40,000 rpm for 30 min in a Beckman TL100 Tabletop Ultracentrifuge.)

Protein Determination

Protein was determined with the Coomassie blue protein assay reagent of Pierce Chemical (Rockford, IL) using crystalline bovine serum albumin as standard.

¹ Abbreviations used: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonate; HPLC, high performance liquid chromatography; H-8, N-[2-(methylamino)ethyl]-5-isoquinoline sulfonamide; H-7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine.

Assay for In Vitro Phosphorylation

Phosphorylation in vitro was assayed by addition of 4 μ l of frozen and thawed flagella to 21 μ l of assay buffer containing 20 mM HEPES, pH 7.2, 2.5 mM Mg, 1 mM EDTA, 4% sucrose, 50 μ M ATP (unless otherwise indicated in the figure legend) and 1 μ Ci [γ - 32 P]ATP at room temperature for 5 min. Samples were prepared for SDS-PAGE by addition of 8.5 μ l of 4 $\times$ SDS-PAGE sample buffer containing 0.2 M Tris, pH 6.8, 40% glycerol, 8% SDS, 8% bromophenol blue, and 40 mM dithiothreitol. After incubation at 45°C for 45 min, NEM was added to each sample to give a final concentration of 25 mM; the samples were incubated for an additional 30 min at 45°C and then immediately loaded onto the gels.

SDS-PAGE

Samples for SDS-PAGE were treated with SDS-PAGE sample buffer as described above and subjected to electrophoresis in 9% acrylamide mini-slab gels (Laemmli, 1974). After SDS-PAGE, the gels were fixed and silver stained (Merril *et al.*, 1980).

Autoradiography

The flagella samples that were phosphorylated in vitro with [γ - 32 P]ATP were analyzed by SDS-PAGE and autoradiography. After staining the gels were dried with a Stacked Gel Dryer SGD 300 (Savant) at 80°C for 30 min and exposed to x-ray film (Hyper Film-MP, Amersham) at -80°C for approximately 12–16 h before being developed.

Chromatography

A sample of mt^+ flagellar supernatant was diluted about 10-fold in Buffer A (20 mM HEPES, pH 7.2, 2.5 mM Mg, 1 mM EDTA, 3% sucrose) and injected into a high performance liquid chromatography (HPLC) DEAE 5PW column (Millipore, Milford, MA) equilibrated with Buffer A. The column was washed (flow rate of 1 ml/min), with Buffer A for 15 min and eluted with a linear gradient of 0–100% Buffer B (20 mM HEPES, pH 7.2, 2.5 mM Mg, 1 mM EDTA, 3% sucrose, 0.8 M NaCl). Fractions (1 ml) were collected in 1.5 ml microfuge tubes and assayed for in vitro phosphorylation as above and identical samples were separated by SDS-PAGE and silver stained.

Immunoblot Analysis

After SDS-PAGE, proteins were transferred to a polyvinylidene fluoride membrane (Immobilon P, Millipore, Bedford, MA) in buffer containing 25 mM Tris, pH 8.3, 192 mM glycine, 20% methanol at 100 mA overnight at 4°C. The membrane was blocked with 3% bovine serum albumin in TBS-T (20 mM Tris, pH 7.6, 137 mM NaCl, 0.05% Tween-20) for 2 h and then incubated with monoclonal anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology, Lake, Placid, NY) in blocking solution for 2 h. At the end of the incubations, the blots were washed with TBS-T and incubated for 1 h with horseradish peroxidase-conjugated goat anti-mouse antibodies in blocking solution. The membrane was then washed and developed with the ECL Western blotting reagents as described by the manufacturer (Amersham).

RESULTS

Flagellar Adhesion and In Vitro Phosphorylation of a Soluble, 48-kDa Protein

To learn about changes in protein phosphorylation induced by flagellar adhesion, flagella isolated from nonadhering (ie., nonmixed) mt^+ and mt^- gametes and adhering (ie., mixed) mt^+ and mt^- gametes were

frozen and thawed, incubated with [γ - 32 P]ATP for 5 min, and phosphorylated proteins were examined by SDS-PAGE and autoradiography. Figure 1 shows that although the protein composition (Silver) and overall phosphorylation patterns (32 P) of nonadhering and adhering flagella were essentially identical, the phosphorylation of a 48-kDa protein (arrow) was reduced significantly in the adhering sample (Figure 1, compare + and - to $\pm$).

The frozen and thawed flagellar samples were fractionated by centrifugation to determine whether the 48-kDa protein kinase substrate and the protein kinase that phosphorylated it were soluble or sedimentable. Figure 2 shows the autoradiograms of unfractionated and fractionated mt^+ flagella that were incubated with [γ - 32 P]ATP before or after fractionation by centrifugation. The lane on the left (T) contains the total, unfractionated flagellar sample (the 32 P-labeled 48-kDa protein is indicated with an arrowhead); the middle set of lanes (Spin $\rightarrow$ 32 P) contains the supernatant (S) and pellet (P) of a sample that had been centrifuged before incubation for 5 min with [γ - 32 P]ATP; and the set of lanes on the right (32 P $\rightarrow$ Spin) contains the supernatant and pellet of a sample that was incubated with [γ - 32 P]ATP before centrifugation. As can be seen in Figure 2, although the proteins phosphorylated in the soluble and sedimentable compartments for the most part were unique to each compartment, the patterns of protein phosphorylation in the two compartments

Figure 1. In vitro phosphorylation of proteins in adhering and nonadhering gametic flagella. Samples (4 μ l) of frozen and thawed flagella isolated from nonadhering (+ and -) and adhering ($\pm$) gametes were added to 21 μ l of assay buffer containing 20 mM HEPES, pH 7.2, 2.5 mM Mg, 1 mM EDTA, 4% sucrose, 2 mM ATP, and 1 μ Ci [γ - 32 P]ATP and incubated at room temperature for 5 min. The assay was stopped by addition of SDS-PAGE sample buffer as described in MATERIALS AND METHODS. Samples were subjected to SDS-PAGE followed by autoradiography. The left panel shows the silver-stained gel and the right panel shows the autoradiogram of the dried gel. The arrow indicates the 48-kDa protein that was phosphorylated in nonadhering flagella (+ and -), but not in adhering ($\pm$) flagella.

Figure 2. Solubility of the 48-kDa protein and its protein kinase. Total (intact) frozen and thawed flagella (T) isolated from mt^+ gametes were incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ as described in Figure 1 and analyzed by SDS-PAGE and autoradiography. To determine if the 48-kDa protein was soluble, a portion of the sample was centrifuged at $10,000 \times g$ for 3 min at 4°C in an Eppendorf microfuge, the pellet was resuspended in flagella buffer to the original volume, and aliquots of the supernatant (S) and pellet (P) were analyzed by SDS-PAGE and autoradiography ($^{32}\text{P} \rightarrow \text{Spin}$). To determine if the protein kinase that phosphorylated the 48-kDa protein also was soluble, a sample of flagella was centrifuged first and the supernatant (S) and pellet (P) fractions were then incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and analyzed by SDS-PAGE and autoradiography ($\text{Spin} \rightarrow ^{32}\text{P}$). The arrowhead indicates the 48-kDa protein.

were similar in both sets of experiments. This suggested that the primary substrates for soluble protein kinases also were soluble and that the primary substrates for sedimentable protein kinase substrates were sedimentable. Moreover, the ^{32}P -labeled 48-kDa protein was present in the supernatant fraction, whether the samples were incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ before or after centrifugation, indicating that both the 48-kDa protein and its protein kinase were soluble.

To further study phosphorylation of the 48-kDa protein in flagellar supernatants, we determined the effects of varying the total ATP concentration and time of incubation. Figure 3A shows that in a 5-min incubation, the amount of incorporation of ^{32}P into flagellar proteins from nonadhering flagella varied with total ATP concentration. Some proteins (e.g., a protein of about 50 kDa) showed maximal incorporation at $0.1\text{--}2.0 \mu\text{M}$, while others (e.g., a protein of about 90 kDa) showed maximal incorporation at $50\text{--}150 \mu\text{M}$. For the 48-kDa protein, incorporation of ^{32}P was maximal around $10\text{--}50 \mu\text{M}$ ATP. When a comparable experiment was carried out with an adhering flagellar supernatant (Figure 3B), similar results were obtained except that there was no incorporation of ^{32}P into the 48-kDa protein at any concentration of ATP. A protein

of slightly lower M_r was phosphorylated when no unlabeled ATP was present in the assay (13 nM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$) in the adhering supernatant (*). But when samples of prelabeled, nonadhering, and adhering supernatants were mixed together and loaded onto a single lane of a gel (Figure 3C, middle lane), it was evident that the polypeptide that incorporated ^{32}P at low concentrations of ATP in the adhering supernatant (*) did not comigrate with the 48-kDa protein phosphorylated in the nonadhering supernatant (arrow). These results suggested that the inability of the assay to detect phosphorylation of the 48-kDa protein in the nonadhering samples was not due to an alteration in the affinity of the enzyme for ATP.

Figure 3D, which presents the results of varying the time of incubation in $50 \mu\text{M}$ ATP, shows that the 48-kDa protein had incorporated significant amounts of ^{32}P in both mt^+ (+) and mt^- (−) flagellar supernatants even after 1 min, whereas little if any incorporation into the 48-kDa protein was detectable in the adhering ($\pm$) supernatants in any of the samples. Taken together these results indicated that adhesion between mt^+ and mt^- gametic flagella induced a rapid and dramatic change in the regulation of phosphorylation of the 48-kDa protein.

Flagellar Adhesion Leads to Inactivation of the Protein Kinase that Phosphorylates the 48-kDa Protein

To determine whether the reduction in ^{32}P incorporation into the 48-kDa protein of adhering flagellar samples was attributable to changes in activity of a protein phosphatase or a protein kinase, we used several approaches as described below. Inclusion in the *in vitro* assay of protein phosphatase inhibitors such as okadaic acid, microcystin, calyculin A, sodium fluoride, or sodium orthovanadate had no effect on the levels of phosphorylation of the 48-kDa protein in either the nonadhering or adhering samples (Figure 4). The 48-kDa protein was phosphorylated in all of the nonadhering (mt^+) samples (+) and was not phosphorylated in any of the adhering samples ($\pm$). These results suggested that the differences in phosphorylation of the 48-kDa protein in nonadhering and adhering flagellar samples were not due to changes in the activity of a protein phosphatase. This was further substantiated by two other experiments.

To assay directly for protein phosphatase activity, nonadhering samples (mt^+) were incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ for 5 min. After the 5-min labeling, one aliquot was prepared immediately for SDS-PAGE (Figure 4B, left lane), while another aliquot was supplemented with hexokinase and glucose to deplete the ATP, and the samples were incubated for a further 30 min (Figure 4B, right lane). Although there was some reduction in the amount of ^{32}P in the 48-kDa protein

Figure 3. Effect of ATP concentration and incubation time on phosphorylation of the 48-kDa protein. (A and B) Samples of nonadhering (mt^+ , A) and adhering flagellar supernatants (B) were incubated with a constant amount of [γ - ^{32}P]ATP (approximately 13 nM) under the conditions described in Figure 1, at the indicated concentrations of added nonradioactive ATP. The arrow in panel A indicates the 48-kDa phosphorylated protein in nonadhering flagella and the asterisk in panel B indicates a 46-kDa protein in adhering flagellar supernatants that was phosphorylated only at the lowest concentrations of ATP. It should be noted that in this and other experiments there was some variation from experiment to experiment in the relative levels of phosphorylation of proteins other than the 48-kDa protein. These variations were inconsistent and unlike the dramatic difference in phosphorylation of the 48-kDa protein between nonadhering and adhering flagella. (C) Supernatants from nonadhering (+) and adhering ($\pm$) flagella were assayed under the conditions described in Figure 1, with the following exceptions: in the reaction with nonadhering flagella (right lane, +) 50 μ M cold ATP was used to maximize the phosphorylation of 48-kDa protein (arrow). In the reaction with adhering flagella ($\pm$), no cold ATP was added to maximize the phosphorylation of the 46-kDa protein (asterisk). The middle lane ($\pm$, +) shows the profile of a mixture in which the two samples were phosphorylated, prepared for SDS-PAGE, and then mixed and loaded in the same well. (D) Supernatants from nonadhering (+ and -) and adhering ($\pm$) flagella were incubated with [γ - ^{32}P]ATP for the indicated times under the conditions described in Figure 1, except the concentration of cold ATP was 50 μ M. Just the region of the autoradiograph that includes the 48-kDa protein is shown.

after the 30-min incubation (Figure 4B, compare the left lane [^{32}P] to the right lane [$^{32}P \rightarrow \text{Hexo}$, 30']), phosphorylation of the 48-kDa protein was easily detectable after the 30-min incubation. These results indicated that nonadhering flagella did not contain a highly active protein phosphatase that dephosphorylated the 48-kDa protein. Thus, the dramatic difference in incorporation of ^{32}P into the 48-kDa protein in nonadhering and adhering flagella probably was not due to more rapid turnover of phosphate in the nonadhering sample. The absence of phosphorylation of any protein in the sample to which hexokinase and glucose were added before the 5-min incubation with [γ - ^{32}P]ATP (Figure 4B, middle lane), indicated that the

hexokinase/glucose treatment was effective in depleting the [γ - ^{32}P]ATP.

Figure 4C shows results of mixing experiments designed to determine whether the adhering supernatant contained activities that could block or reverse incorporation of ^{32}P into the 48-kDa protein. The first three lanes show the expected pattern of ^{32}P incorporation into control, nonadhering (+ and -), and adhering samples ($\pm$). Lanes 4 and 5 contain a mixture of nonadhering (mt^+) and adhering samples. Lane 4 contains both nonadhering and adhering supernatants that were combined and incubated together for 5 min before [γ - ^{32}P]ATP was added to initiate the reaction (Figure 4C, Mix $\rightarrow$ ^{32}P); lane 5 contains adhering su-

Figure 4. Protein phosphatase activity and phosphorylation of the 48-kDa protein. (A) Effects of protein phosphatase inhibitors. Supernatants from nonadhering (+) and adhering ($\pm$) flagella were incubated with [γ - 32 P]ATP under the conditions described in Figure 1 (except 50 μ M cold ATP was used) in the presence or absence of the following protein phosphatase inhibitors: Con, control (no addition); Oka, okadic acid (0.5 μ M); Mic, microcystin (1 μ M); Cal, calyculin A (50 nM); NaF, sodium fluoride (40 mM); and Van, sodium vanadate (100 μ M). (B) Direct assay for protein phosphatase activity. Supernatants from nonadhering flagella were incubated with [γ - 32 P]ATP under the conditions described in panel A. After a 5-min incubation, an aliquot was prepared for SDS-PAGE (left lane, 32 P). Glucose (1 mM) and 2.5 U of hexokinase were added to a second sample to deplete ATP from the reaction, and the incubation was continued for 30 min at 30°C before SDS-PAGE (right lane, 32 P $\rightarrow$ Hexo). As a control, a sample of the reaction buffer was pretreated with hexokinase/glucose for 3 min at room temperature to deplete the ATP before addition of the flagellar supernatant (center lane, Hexo $\rightarrow$ 32 P). (C) Effect of mixing of supernatants from adhering and nonadhering flagella on phosphorylation of 48-kDa protein. Supernatants from adhering ($\pm$) and nonadhering (+ and -) flagella were incubated with [γ - 32 P]ATP under the conditions described in Figure 4A. In one experiment both adhering and nonadhering flagellar supernatants were mixed together for 5 min at room temperature before addition of [γ - 32 P]ATP to initiate the reaction (Mix $\rightarrow$ 32 P). In another experiment both adhering and nonadhering flagella supernatants were incubated individually with [γ - 32 P]ATP first and then mixed together for 5 min at room temperature (32 P $\rightarrow$ Mix). The arrow indicates the 48-kDa protein.

pernatants that were incubated individually with [γ - 32 P]ATP for 5 min before being combined and incubated for a further 5 min (Figure 4C, 32 P $\rightarrow$ Mix). In both mixing experiments the levels of 32 P incorporation into the 48-kDa protein were equivalent and similar to the nonadhering sample incubated alone (Figure 4C, lane 1). These results were consistent with the idea that protein phosphatases did not play a significant role in the difference in phosphorylation of the 48 kDa in the nonadhering and adhering samples.

We next wanted to determine whether adhering supernatants contained a form of the 48-kDa protein that could be phosphorylated. To do this, we pre-incubated a nonadhering supernatant with nonradioactive ATP (2 mM) for 5 min to permit the phosphorylation sites on the 48-kDa protein to become occupied by nonradioactive phosphate groups. When [γ - 32 P]ATP subsequently was added to a portion of this pretreated sample, none of the proteins incorporated 32 P (left panel of Figure 5, lane 4, +). The first three lanes in the left panel of Figure 5, show that the expected phosphorylation pattern was observed when the [γ - 32 P]ATP and 2 mM ATP were mixed together before the flagellar samples were added. (The right panel of Figure 5 shows that the protein composition of all of the samples was indistinguishable). When the pretreated, nonadhering supernatant was mixed with a nonpretreated,

adhering supernatant and then [γ - 32 P]ATP was added, the 48-kDa protein was phosphorylated (Figure 5, left panel, lane 5). These experiments demonstrated that the 48-kDa protein was present in the adhering supernatant in a form that could be phosphorylated. Thus, the absence of phosphorylation of the 48-kDa protein in adhering samples was not attributable to depletion or modification of the protein. Rather the data were consistent with the idea that flagellar adhesion led to inactivation of the protein kinase that normally phosphorylates the 48-kDa protein.

The Protein Kinase that Phosphorylates the 48-kDa Protein Has the Properties of a Protein Tyrosine Kinase

To learn more about the properties of the adhesion-regulated protein kinase, we used anti-phosphotyrosine immunoblot analysis with partially purified 48-kDa protein, we tested the effects of several activators and inhibitors of protein kinases on *in vitro* phosphorylation of the 48-kDa protein, and we determined whether sodium orthovanadate, an inhibitor of protein tyrosine phosphatases, would inhibit fertilization when mt^+ and mt^- gametes were mixed together. For immunoblot analysis, an mt^+ flagellar supernatant was fractionated by HPLC. Analysis by SDS-PAGE

and autoradiography of the in vitro-phosphorylated fractions (Figure 6A, silver stain; Figure 6B, autoradiogram) showed that a protein of 48 kDa was enriched in fraction 30 and that the 48-kDa protein also was phosphorylated in this fraction. We next wanted to determine whether the 48-kDa protein reacted with anti-phosphotyrosine antibodies. To do this, a 48-kDa protein-enriched sample from an similar HPLC fractionation of mt^+ flagellar supernatant was incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, subjected to SDS-PAGE, and transferred to a polyvinylidene fluoride membrane. For detection of incorporation of ^{32}P , the membrane was exposed to x-ray film overnight (the corresponding autoradiogram is shown in Figure 6C, right lane). The same blot was then subjected to immunoblotting with an anti-phosphotyrosine antibody (Figure 6C, left lane). As Figure 6C shows, the ^{32}P -labeled 48-kDa protein also was stained by the anti-phosphotyrosine antibody. Staining was blocked if 100 μM O-phospho-L-tyrosine was included with the primary antibody,

Figure 5. Phosphorylation of the 48-kDa protein in adhering flagella by a protein kinase in nonadhering flagella. A nonadhering flagellar supernatant was incubated with 2 mM cold ATP at room temperature for 5 min to fill up available phosphorylation sites, and then a sample of nonpretreated adhering supernatant was mixed with the preincubated sample along with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and the incubation was continued for another 5 min (right lane, $\pm$, +). The phosphorylated 48-kDa protein is shown by the arrow. The second lane from the right (+) shows a sample of nonadhering supernatant that was incubated with 2 mM cold ATP for 5 min and then only $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (no adhering supernatant) was added and the incubation was continued for 5 min. Little if any protein phosphorylation was detected. The three lanes on the left show that if the $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was added to the 2 mM ATP before the supernatants were added, as expected, the 48-kDa protein was phosphorylated in the nonadhering samples (+ and $\pm$) and not in the adhering sample ($\pm$). The left panel (^{32}P) shows the autoradiogram and the right panel (Silver), the stained gel.

and staining was unaffected by 100 μM O-phospho-L-serine or 100 μM O-phospho-L-threonine (Figure 6D). These results suggested that the 48-kDa protein was phosphorylated by a protein tyrosine kinase.

Several activators and inhibitors of protein kinases were tested for their effects on phosphorylation of the 48-kDa protein. We found that neither the absence (Figure 7A, EGTA) nor the presence of Ca^{2+} (1 mM, Figure 7A, Ca^{2+}) in the assay had an effect on adhesion-regulated incorporation of ^{32}P into the 48-kDa protein. Similarly, addition of cAMP to the assay mix did not alter the pattern of incorporation of ^{32}P (Figure 7A, cAMP). In each case, as in the control sample (Figure 7A, Control), the 48-kDa protein was phosphorylated in the nonadhering supernatants and was not phosphorylated in the adhering supernatant. These results suggested that the adhesion-regulated protein kinase was neither a Ca^{2+} -calmodulin dependent protein kinase nor a cAMP-dependent protein kinase.

We also carried out phosphorylation experiments in the presence of several protein kinase inhibitors (Tamaoki *et al.*, 1986; Ruegg and Burgess, 1989; Levitzki, 1990). Figure 7 (panel B, silver stain; panel C, autoradiogram) shows the results of an experiment in which an mt^+ flagellar supernatant was incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (final concentration, 23 nM ATP) in the presence of the following protein kinase inhibitors: none, Con; staurosporine, ST; H-8, H-8; H-7, H-7; K252a, K252a; cinnamate, Cin; lavendustin A, Lav; tyrphostin 25 (3,4,5 trihydroxybenzylidene malononitrile), Tyr; aminobenzoic acid, Aba; genistein, Gen; or RCAM-lysozyme, RCA-L at the concentrations indicated in the figure legend. As shown in Figure 7C, several patterns of incorporation were observed: 50 nM staurosporine, K252a, cinnamate, lavendustin A, aminobenzoic acid, and RCAM-lysozyme had essentially no effect on incorporation of ^{32}P compared with the control, nontreated sample. H-7, genistein, and the higher concentration of staurosporine (1 μM), caused a reduction of incorporation of ^{32}P into most or all of the polypeptides. At 30 μM , H-8 caused an overall increase in incorporation of ^{32}P , whereas at 300 μM it led to a selective decrease in incorporation of ^{32}P into a polypeptide of about 52 kDa. Consistent with the results with the anti-phosphotyrosine antibodies, however, the protein tyrosine kinase inhibitor tyrphostin had a more discriminating effect on incorporation. This inhibitor had little or no effect on incorporation of ^{32}P into most of the polypeptides, but it caused a substantial and selective inhibition of incorporation of ^{32}P into the 48-kDa protein, again indicating that the 48-kDa protein was being phosphorylated by a protein tyrosine kinase.

Finally, because our results were consistent with the idea that protein dephosphorylation was required for signalling during fertilization, we wanted to deter-

Figure 6. Fractionation of the 48-kDa protein on HPLC and immunoblotting with an anti-phosphotyrosine antibody. (A and B) HPLC fractionation. A sample (300 μ l) of a supernatant of mt^+ flagella was diluted about 10-fold in Buffer A and fractionated on a DEAE 5 PW column. After fractionation, aliquots (10 μ l) of the indicated fractions were diluted (the final assay volume was 50 μ l) and assayed for *in vitro* phosphorylation under the conditions described in Figure 4A. The samples were then subjected to SDS-PAGE and stained with silver (A) and analyzed by autoradiography (B). (C and D) Immunoblot analysis with an anti-phosphotyrosine antibody and specific blocking of the antibody staining by *O*-phospho-L-tyrosine. A supernatant from mt^+ flagella was fractionated as above and a sample enriched in the 48-kDa protein was incubated with [γ - 32 P]ATP under the conditions described in Figure 4A, subjected to SDS-PAGE, and transferred to a PVDF membrane. To detect the 32 P-labeled protein the membrane was exposed overnight for autoradiography (right lane of C). (Some variation from experiment to experiment was noted in the extent of phosphorylation of other proteins in the HPLC fraction enriched in the 48-kDa protein; this, along with the variabilities involved with electrophoretic transfer to PVDF membranes may account for the dominance of the 48-kDa protein in this autoradiogram compared with the autoradiogram in Figure 6B). To detect phosphotyrosine, the PVDF membrane then was subjected to immunoblotting with an anti-phosphotyrosine antibody as described in MATERIALS AND METHODS (left lane of C). The double-headed arrow indicates the 48-kDa protein. In the experiment shown in panel D, an mt^+ flagellar supernatant was fractionated on an SDS-PAGE curtain gel, transferred to a PVDF membrane, and strips of the membrane containing the transferred proteins were incubated with anti-phosphotyrosine antibody without (C) or with 100 μ M *O*-phospho-L-tyrosine (pY), *O*-phospho-L-serine (pS), or *O*-phospho-L-threonine (pT).

mine whether sodium orthovanadate, a protein tyrosine phosphatase inhibitor (Gordon, 1991; Liao and Lane, 1995; Lin and Grinnell, 1995), would inhibit fertilization. To do this, mt^+ and mt^- gametes were pretreated separately with 1 mM sodium orthovanadate for 15 min and then mixed together in the continued presence of the inhibitor. After 5–7 min, portions of the samples were fixed in glutaraldehyde for determination of the extent of cell fusion. The results from three experiments are shown in Table 1. As indicated by the 33–45% reduction in the number of cells that fused to form zygotes compared with control gametes, sodium orthovanadate inhibited fertilization. Inhibition of fertilization by vanadate was not due to a general toxic effect on the cells as assessed by several criteria. First, examination by phase contrast microscopy of the cells before mixing indicated that motility of the control and vanadate-pretreated cells was indistinguishable. Second, in both control and vanadate-pretreated suspensions, greater than 90% of the cells swam to the bottom of their incubation tubes within 1 min after agitation was interrupted, indicating that vanadate had no detectable effect on the cells' ability to undergo phototaxis. Third, as assessed by phase contrast microscopy, the extent of aggregation after mixing of the cells in vanadate was indistinguishable from the aggregation of control gametes. In both control and pretreated samples, greater than 90–95% of the cells adhered to each other, forming large clumps of aggregating gametes. Finally, Table 1 shows that the effects of vanadate pretreatment were reversible: if vanadate pretreated gametes were washed out of vanadate 15 min before mixing (Experiments 1 and 2), there was little if any inhibition of fertilization.

DISCUSSION

Although much is known about signal transduction induced by binding of soluble ligands such as peptide hormones or growth factors to their cellular receptors, the molecular mechanisms that couple cell-matrix and cell-cell interactions to generation of intracellular biochemical signals are just beginning to be elucidated (Dustin and Springer, 1991; Hynes, 1992; Hart *et al.*, 1993; Ward and Kopf, 1993; Schaller and Parsons, 1994; Schlaepfer, *et al.*, 1994). The working model for understanding cell-cell signalling is that cell surface adhesion proteins act both as adhesion/recognition molecules and as ligands and/or receptors. For example, during fertilization in many species, binding between sperm and egg surface molecules activates guanylyl cyclase (Garbers, 1989) or adenylyl cyclase (Leclerc and Kopf, 1995) and regulates protein phosphorylation (Bentley *et al.*, 1987; Peaucellier *et al.*, 1988; Vacquier *et al.*, 1989; Leyton *et al.*, 1992, 1995; Duncan and Fraser, 1993; Kalab *et al.*, 1994). With only a few notable exceptions (Chinkers and Garbers, 1991; Ley-

ton *et al.*, 1992; Leyton and Saling, 1989; Foltz *et al.*, 1993; Ward and Kopf, 1993), however, the proteins responsible for these events have not been identified or characterized.

During *Chlamydomonas* fertilization, interactions between flagellar adhesion molecules on mt^+ and mt^- gametes initiate a signal transduction cascade that ultimately leads to cell fusion. Our recent studies of *Chlamydomonas* fertilization have demonstrated that the early events in this pathway can be inhibited, both in vivo and in vitro, by low concentrations of the protein kinase inhibitor staurosporine (Zhang and Snell, 1994), suggesting that flagellar protein kinase activity is regulated by flagellar adhesion. To learn more about flagellar adhesion-dependent changes in protein kinase activity we carried out in vitro phosphorylation studies on flagella that were isolated from mt^+ and mt^- gametes that had been mixed together for 1 min to initiate sexual signalling. We found that gametic flagella contain a soluble, 48-kDa protein whose level of in vitro phosphorylation is regulated by flagellar adhesion. This 48-kDa protein may be the first bona fide, soluble protein kinase substrate identified in the *Chlamydomonas* flagellum, and the protein that phosphorylates it, the first soluble flagellar protein kinase activity reported in this organism.

Flagellar Adhesion Regulates the Activity of a Flagellar Protein Kinase

Although several proteins were phosphorylated in vitro in flagella both from nonadhering and adhering gametes, the 48-kDa protein was the only one whose phosphorylation was inhibited substantially and consistently in the adhering flagella (Figure 1). Several types of experiments indicated that protein phosphatase activity did not play a significant role in the difference in incorporation of ^{32}P into the 48-kDa protein in the supernatants from nonadhering and adhering samples. First, protein phosphatase inhibitors included during the incubation with $[\gamma\text{-}^{32}P]\text{ATP}$ had no effect on the phosphorylation pattern (Figure 4A); second, we could find no evidence for a highly active protein phosphatase activity in the nonadhering supernatants (Figure 4B). Furthermore, experiments in which supernatants from nonadhering and adhering flagella were mixed together either before or after the $[\gamma\text{-}^{32}P]\text{ATP}$ was added (Figure 4C) indicated that adhering supernatants did not contain activities that could block or reduce the phosphorylation of the 48-kDa protein in nonadhering flagellar supernatants. In this regard, Bloodgood and Salomonsky (1994) have shown that the detergent soluble fraction of flagella

(Figure 7 cont). ($\pm$) flagella were incubated with $[\gamma\text{-}^{32}P]\text{ATP}$ under the conditions described in Figure 4A in the absence (Control) or presence of 1 mM EGTA (EGTA), 1 mM Ca^{2+} (Ca^{2+}), or 100 μM cAMP (cAMP). The arrow indicates the 48-kDa protein. (B and C) Effect of protein kinase inhibitors on phosphorylation of 48-kDa protein in nonadhering flagella. Supernatants from nonadhering mt^+ flagella were incubated with $[\gamma\text{-}^{32}P]\text{ATP}$ under the conditions described in Figure 4A (except the concentration of cold ATP in this experiment was 10 nM) in the presence of the following protein kinase inhibitors (from Life Technologies). Control (No addition), Con; staurosporine (50 nM or 1 μM), ST; cinnamate (2 μM), Cin; Lavendustin A (50 nM), Lav; tyrphostin-25 (40 μM), Tyr; aminobenzoic acid (100 nM), Aba; genistein (100 μM), Gen; RCAM-lysozyme (40 μM), RCA-L. Panel B shows the silver stained gel and panel C shows the autoradiogram of the same gel. The arrow in panel C indicates the phosphorylated 48-kDa protein.

Figure 7. Characterization of the protein kinase that phosphorylates the 48-kDa protein. (A) Effect of EGTA, Ca^{2+} , and cAMP. Supernatants from nonadhering (+ and -) and adhering

Table 1. Inhibition of fertilization by sodium orthovanadate

	Control ^a			1 mM Vanadate, wash ^b				1 mM Vanadate ^c			
	# Single	# Fused	% Zygotes	# Single	# Fused	% Zygotes	Inhibition	# Single	# Fused	% Zygotes	Inhibition
Exp. 1	136	130	49	137	132	49	0%	171	62	27	45%
Exp. 2	114	172	60	126	152	55	8%	155	94	38	37%
Exp. 3	114	206	64	...	...	...	...	147	110	43	33%

The extent of fertilization, indicated as the percent of single mt^+ and mt^- gametes that fused to form quadriflagellated zygotes, was determined in a phase contrast microscope from counts of the number of cells with two flagella (# Single) and the number of cells with four flagella (# Fused) after they were mixed together for the indicated time.

^{a,c} Mt^+ and mt^- gametes (2 ml each, $\sim 2 \times 10^7$ cells/ml) were incubated separately in N-free medium 1 without^a or with^c 1 mM sodium orthovanadate for 15 min at room temperature, mixed together in the continued presence of the inhibitor for 7 min (Exp. 1), 6 min (Exp. 2), or 5 min (Exp. 3), and then 50- μ l portions were fixed for determination of the extent of cell fusion by mixing with 50 μ l of 1% glutaraldehyde.

^b Pretreated cells were washed out of the vanadate 15 min before mixing.

contains highly active phosphatases. It may be that a membrane-bound phosphatase plays an important role in regulation of the phosphorylation state of the 48-kDa protein, but the experiments in Figure 4 were carried out with the soluble fraction of flagella, and any membrane-associated phosphatase activity would not have been detected.

Other mixing experiments (Figure 5) demonstrated that even though no incorporation of ^{32}P into the 48-kDa protein could be detected in the supernatants from adhering flagella, the 48-kDa protein indeed was present in these samples in a form that could be phosphorylated by a protein kinase in the nonadhering supernatant. Taken together, the simplest explanation for these results is that the flagellar adhesion-dependent changes in the level of ^{32}P incorporation into the 48-kDa protein in the *in vitro* assay were a consequence of the adhesion-regulated inactivation of a protein kinase.

Initial experiments to characterize the protein kinase activity that phosphorylated the 48-kDa protein also provided clues about the position of the protein kinase in the overall cellular response to flagellar adhesion. It was possible that the adhesion-dependent inactivation of phosphorylation was a downstream response to increases in cAMP signalling (Saito *et al.*, 1993; Wu *et al.*, 1993). But the result that inclusion of cAMP in the incubation had no effect on the phosphorylation of the 48-kDa protein in nonadhering samples and did not promote phosphorylation in the adhering samples, indicated that the protein kinase was not a cAMP-dependent protein kinase. This suggested that the protein kinase was not regulated by events downstream of activation of adenylyl cyclase, and was consistent with the idea that the protein kinase acts early in the signalling pathway. In addition, the lack of effect of Ca^{2+} or EGTA on the phosphorylation of the 48-kDa protein indicated it was not being phosphorylated by

a Ca^{2+} /calmodulin-dependent protein kinase. This suggested that the mechanism for regulation also may be upstream of or at least independent of the adhesion-dependent change in intracellular Ca^{2+} reported by Goodenough and colleagues (Goodenough *et al.*, 1993).

Concomitant with these results that neither cAMP nor Ca^{2+} affected the phosphorylation of the 48-kDa protein, other experiments indicated that the 48-kDa protein may have been phosphorylated by a protein tyrosine kinase. The immunoblotting experiments showed that the 48-kDa protein was stained by an anti-phosphotyrosine antibody (Figure 6), and only the protein tyrosine kinase inhibitor tyrphostin selectively blocked incorporation of ^{32}P into the 48-kDa protein (Figure 7, B and C). These results, which suggest that flagella contain protein tyrosine kinase activity, are consistent with an earlier report by Segal and Luck (1985), who showed that flagellar axonemes contained proteins that were tyrosine phosphorylated.

Sexual Signalling, the 48-kDa Protein, and Regulation of Phosphorylation

One model consistent with our data is that this newly discovered, adhesion-regulated protein kinase and its 48-kDa substrate are the immediate, downstream molecules whose activity is regulated by agglutinin interactions. For example, much like receptor aggregation in other systems (Kornberg *et al.*, 1991; Hynes, 1992; Johnson and Vaillancourt, 1994; Weiss and Littman, 1994), interactions between mt^+ and mt^- agglutinins might induce agglutinin aggregation in the plane of the membrane (Goodenough and Jurivich, 1978; Kooijman *et al.*, 1989; Bloodgood, 1992b) thereby leading to inhibition of the adhesion-regulated protein kinase. Based

on the results presented here our working model is that a nonphosphorylated form of the 48-kDa protein is required in the signalling pathway that couples agglutinin interactions to activation of adenylyl cyclase. Normally the 48-kDa protein is phosphorylated, and thereby maintained in an inactive form, through the action of the adhesion-regulated protein kinase, which may be a protein tyrosine kinase. Inactivation of this protein kinase during flagellar adhesion along with the continued activity of a putative, constitutively active protein phosphatase, possibly a protein tyrosine phosphatase, leads to the net dephosphorylation of the 48-kDa protein. Our results that the protein tyrosine phosphatase inhibitor sodium orthovanadate inhibited fertilization (Table 1) supported this model and also were consistent with in vitro experiments (Figure 6) that indicated that the adhesion-regulated protein kinase is a protein tyrosine kinase. Recent studies in a higher plant system also have implicated a protein tyrosine kinase in signal transduction. Suzuki and Shinshi (1995) reported that a 47-kDa protein serine/threonine kinase in tobacco cells is phosphorylated on tyrosine during signalling induced by a fungal elicitor. It will be interesting to determine whether the 47-kDa protein described by these investigators is related to the 48-kDa protein described here.

The model we currently favor for flagellar adhesion-induced signalling events during fertilization is that both inactivation of the adhesion-regulated, 50 nM staurosporine-insensitive (Figure 7) protein kinase described here, as well as activation of a 50 nM staurosporine-sensitive protein kinase (Zhang and Snell, 1994) are elements of the mechanism, possibly a protein kinase cascade, that couples agglutinin interactions to activation of adenylyl cyclase. Recently, Segall *et al.* (1995) showed that a mitogen-activated protein kinase is required for activation of adenylyl cyclase during morphogenesis in *Dictyostelium*. One difference between this *Chlamydomonas* system, however, and other protein kinase cascades is that protein phosphorylation often activates the next protein kinase in the pathway (reviewed in Davis, 1993 and Johnson and Vaillancourt, 1994), but for integration of multiple signals, negative regulation by phosphorylation also is important (Wu *et al.*, 1993; Rossomando *et al.*, 1994; Ueki *et al.*, 1994).

Other workers have reported that aggregation of molecules in the flagellar membrane may involve phosphorylation of flagellar proteins (Bloodgood and Salomonsky, 1991; Bloodgood, 1992a,b). Recently the Bloodgood laboratory (Bloodgood and Salomonsky, 1994) discovered that antibody-induced glycoprotein redistribution in *Chlamydomonas* vegetative cells was accompanied by the specific de-phosphorylation of a 60-kDa flagellar protein.

These workers have proposed that dephosphorylation of the 60-kDa protein may be a step in the signalling pathway that couples glycoprotein cross-linking to directed movements of flagellar membrane glycoproteins. The 48-kDa protein was not one of the phosphorylated proteins previously noted (Bloodgood, 1992a; Bloodgood and Salomonsky, 1994) but future experiments should elucidate possible relationships between agglutinin interactions, flagellar glycoprotein redistribution, and these newly described mechanisms of regulation of protein phosphorylation.

In our previous studies (Zhang *et al.*, 1991; Zhang and Snell, 1993) we reported that flagella isolated from nonadhering gametes contain a membrane-associated, ATP-dependent, heat-labile inhibitor of flagellar adenylyl cyclase. We proposed (Zhang and Snell, 1994) that this inhibitory activity might be required to maintain the low levels of cAMP required for flagellar motility (Hasegawa *et al.*, 1987). Because gametes use cAMP as a primary signalling molecule for fertilization, however, they must be able to activate the adenylyl cyclase rapidly upon flagellar adhesion. It may be that the adhesion-regulated protein kinase described in the present report, which is inactivated by adhesion, is related to the previously described ATP-dependent activity that inhibits the adenylyl cyclase in nonadhering flagella.

We anticipate that, like signal transduction mediated by protein kinases in other systems, sexual signalling in *Chlamydomonas* will involve a complex interplay of protein kinases and protein phosphatases. We believe that we have identified a novel, adhesion-regulated event in *Chlamydomonas* sexual signalling. A clearer understanding of the role of the adhesion-regulated protein kinase and its 48-kDa substrate in this *Chlamydomonas* signalling pathway, as well as information about the similarities of the *Chlamydomonas* protein kinases to protein kinases in other signalling pathways, will require further biochemical as well as molecular characterization of the *Chlamydomonas* molecules.

Note added in proof: Since the submission of this manuscript Kurvari *et al.* [Kurvari, V., Zhang, Y., Luo Y., and Snell, W.J. (1996). Molecular cloning of a protein kinase whose phosphorylation is regulated by gametic adhesion during *Chlamydomonas* fertilization. *Proc. Natl. Acad. Sci. USA* 93, 39–43.] have reported the molecular cloning of a cDNA for the 48kDa protein, called Sks-C. The studies indicate that the deduced amino acid sequence of Sks-C has homology to protein kinases and that a Sks-C fusion protein autophosphorylates on serine and tyrosine.

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