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SksC, a Fertilization-Related Protein Kinase in *Chlamydomonas,* Is Expressed throughout the Cell Cycle and Gametogenesis, and a Phosphorylated Form Is Present in both Flagella and Cell Bodies

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Fertilization in the biflagellated eukaryote, *Chlamydomonas,* is initiated by flagellar adhesion between gametes of opposite mating types. An early event in the signal transduction pathway induced by these cell-cell interactions is the rapid inactivation of a flagellar protein kinase that phosphorylates a 48 kDa flagellar protein. Molecular cloning and characterization indicated that the 48 kDa substrate, termed SksC, itself is a novel protein kinase. Here, we have determined that its transcript levels were unchanged during prolonged flagellar adhesion. Moreover, resynthesis of new flagellar proteins following deflagellation was not accompanied by increases in transcript levels of SksC, suggesting that expression of this soluble protein kinase might not be restricted to flagella. Immunoblot analysis indicated that expression of SksC was ubiquitous: this soluble protein was found in both flagella and cell bodies and was expressed throughout the cell cycle and gametogenesis. Immunoprecipitation experiments indicated that SksC was phosphorylated in both flagella and cell bodies. Thus, in addition to its potential role in fertilization, this novel protein kinase may play a role in other signaling events in *Chlamydomonas.*

During fertilization in many species, gametes of opposite sexes recognize and communicate with their partners through interactions between cell surface molecules [1]. The gametic interactions at the cell surface generate signals that are translated into reversible, post-translational modifications on intracellular signaling proteins. While the initial interactions between gametes almost certainly involve species-specific adhesion molecules, downstream cellular events are likely to involve many signaling proteins some of which may transmit multiple extracellular signals. Protein phosphorylation has been recognized as one of the principal mechanisms through which cells relay signals generated at the cell surface to the cell interior [2,3]. In spite of the well-documented significance of protein phosphorylation in many cellular events, the role of protein kinases in fertilization is only beginning to be appreciated [4-9]. In higher plants, where pollen-pistil interactions restrict fertilization to compatible plant genotypes, a group of self-incompatible species contain a family of serine/threonine receptor kinases called S-locus receptor kinases (SRKs) [10,11]. While the precise roles of these molecules in pollen-pistil interactions and their downstream partners are not known, SRKs are presumed to initiate a signaling cascade that ultimately results in a decision to accept or reject prospective pollen [12]. In yeast, where the pheromone-induced mating pathway is well-understood, a battery of protein kinases generates a cascade of phosphorylation events that ultimately result in the transcriptional activation of mating-related genes [13,14]. It is becoming clear that the protein kinase module that operates in the pheromone-induced mating pathway in yeast may represent an example of evolutionarily-conserved signaling modules repeatedly utilized in several cellular and developmental signal transduction pathways [15,16].

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Fertilization in the unicellular eukaryotic alga, *Chlamydomonas* exhibits molecular and cellular similarities both to mating in yeast and to fertilization and zygote formation in multicellular organisms. Under appropriate environmental conditions, vegetatively growing mt^+ and mt^- *Chlamydomonas* cells differentiate into gametes [17]. When gametes of opposite mating types adhere to each other via their flagellar adhesion molecules, agglutinins, a signal transduction pathway is initiated that leads to a rapid increase in intracellular cAMP concentration [18,19] and subsequent downstream cellular events including cell wall loss and mating structure formation in preparation for cell fusion [20-22]. The increase in cAMP is due to the adhesion-induced activation of a gamete-specific adenylyl cyclase in the flagellar membrane [23]. We and others have previously shown that this gametic adenylyl cyclase is regulated not by G proteins, but by protein kinases [24,25]. One of these protein kinase activities is an ATP-dependent inhibitor of adenylyl cyclase that may be required to maintain the adenylyl cyclase at basal levels of activity in non-adhering gametes [23]. A second protein kinase activity is involved in the adhesion-dependent activation of flagellar adenylyl cyclase [26]. In *in vitro* phosphorylation experiments to identify fertilization-related protein kinases, we discovered that flagellar adhesion led to the inactivation of a soluble flagellar protein kinase [27]. Molecular cloning and characterization of the 48 kDa protein substrate for the adhesion-regulated protein kinase indicated that the 48 kDa substrate, termed SksC, also is a protein kinase, a novel member of the superfamily of eukaryotic protein kinases [28]. A bacterially expressed GST-fusion protein, GST-SksC is capable of autophosphorylation on serine and tyrosine, and phosphorylation of bovine β-casein on serine [28].

In the present study, we have determined that transcript levels of SksC were unchanged in *Chlamydomonas* gametes undergoing prolonged adhesion and flagellar regeneration, suggesting that expression of SksC was not restricted to flagella. Immunological analysis using an affinity-purified polyclonal antibody raised against a bacterially expressed GST-SksC fusion protein indicated that SksC is expressed throughout vegetative growth and gametogenesis, and is present as a phosphoprotein in both flagella and cell bodies of *Chlamydomonas* gametes.

MATERIALS AND METHODS

Cells. Chlamydomonas reinhardtii strains 21gr (mt^+) and 6145C (mt^-) were cultured at 23 °C in 3.7 mM NH_4NO_3, 1.2 mM $MgSO_4$, 0.36 mM $CaCl_2$, 0.37 mM $FeCl_3$. 6 H_2O, 1.7 mM sodium citrate, 0.44 mM K_2HPO_4, 0.73 mM KH_2PO_4 plus trace elements on a 13:11 hour light-dark cycle [29]. Vegetative cells were induced to become gametes by resuspension in medium without NH_4NO_3 followed by culturing in continuous light at room temperature [30,31]. Activation of *Chlamydomonas* mt^+ gametes to undergo adhesion-mediated signal transduction was induced by incubation with flagella isolated from mt^- gametes [32,33], and deflagellation by pH-shock treatment was performed as previously described [34]. Mating competence was tested by microscopic evaluation of the ability of *Chlamydomonas* gametes to undergo agglutination and cell fusion [17].

Southern blot hybridizations. For Southern blots, genomic DNA from *Chlamydomonas* mt^+ cells (10 μg) digested with PvuII and XhoI was fractionated by electrophoresis on a 1% agarose gel, transferred to a nylon membrane and incubated with a probe derived from a 400 bp PCR-amplified fragment of SksC cDNA essentially as described earlier [28]. Hybridization was performed at 42 °C in 5× SSPE (1× SSPE = 10 mM sodium phosphate, pH 7.7, 0.18 M NaCl, 1 mM EDTA), 0.5% SDS, 200 μg/ml herring sperm DNA, 5× Denhardt's solution for 15-20 hours. Following hybridization, membranes were washed twice in 2× SSPE, 0.2% SDS at 42 °C for 30 min followed by two additional 30 min washes at 50 °C in 1× SSPE, 0.1% SDS and exposed to X-ray film at −80 °C.

Northern blot hybridizations. Total RNA was isolated from 200 ml (10^6 cells/ml) of mt^+ gametes or mt^+ gametes harvested ~10 min after deflagellation, using a modification of the procedures reported by Dobberstein *et al.* [35] and Lefebvre *et al.* [36] as described earlier [32]. Poly(A)$^+$ RNA was isolated essentially as described earlier [32]. For Northern blots, *Chlamydomonas* poly(A)$^+$ RNA (~1μg) was size-fractionated on a 1% denaturing formaldehyde agarose gel, transferred to a Nytran membrane (Schleicher & Schuell, Keene, NH) and incubated with a probe derived from a λZapII-derived clone containing most of the protein coding sequences and the 3′ untranslated region (Clone 4, Ref. 28). The probe was prepared using random primer labelling (Boeringer-Mannheim, Indianapolis, IN).

Production and characterization of antibodies. A plasmid derivative containing all but the first 12 amino acids of the coding sequence for SksC including the 3′ untranslated sequences was constructed in the prokaryotic expression vector, pGEX-KG as described earlier [28]. Bacterial clones harboring recombinant pGEX-KG plasmid were grown

to log phase in LB broth, induced by incubation with 1mM IPTG for ~1.5 hours, and the soluble GST-SksC fusion protein (~72 kDa) was extracted and purified as described previously [28]. The eluted protein was injected subcutaneously into 2 New Zealand white rabbits (~200 μg per rabbit) with TiterMax (Vaxcel, Inc.) as the adjuvant. Immune sera were collected at two week intervals after the initial injection. Following preliminary analysis to test for the presence of anti-SksC antibodies by immunoblots, antibodies against GST-SksC were affinity-purified. Affinity columns composed of Affigel 10 (Biorad, Richmond, CA) conjugated with GST-SksC or GST alone were prepared by incubation overnight at 4 °C of 1 ml of Affigel 10 resin with ~400 μg protein. Antibodies against GST-SksC and GST were selected by successive passages of the immune sera through GST- and GST-SksC-affinity columns, collected by elution in acid-glycine (0.3 N HCl-glycine, pH 2.4), and neutralized with Na_2PO_4, pH 10.4. Eluted antibody was stored at 4 °C in PBS (150 mM NaCl, 16 mM NaH_2PO_4, 4 mM Na_2HPO_4, pH 7.3) containing 0.05% sodium azide.

Immunoblots. For SDS-PAGE and immunoblotting, samples were boiled for 4 min at 100°C in a a modified Laemmli sample buffer (62.5 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 100 mM DTT and 0.001% bromophenol blue), and separated by electrophoresis on 9% gels [37]. Proteins were electrophoretically transferred to PVDF membranes as described earlier [26]. After transfer, membranes were blocked in 20 mM Tris, pH 7.6, 137 mM NaCl, 0.05% Tween-20 (TBS-T) with 5% dry milk (Carnation; Nestle Food Co., Glendale, CA) for 60 min, followed by incubation with affinity-purified antibody diluted 1:500 in TBS-T containing 3% dry milk for 1 hour. After a brief wash with TBS-T, the membranes were incubated with goat anti-rabbit antibody against whole rabbit IgG conjugated to horse radish peroxidase (Coppell) in TBS-T containing 3% dry milk for 30 min. Following incubation with the secondary antibody, the membranes were washed extensively with TBS-T, and treated with Enhanced Chemiluminescence (ECL) reagents as recommended by the supplier (Amersham Corp., Arlington Heights, IL), and exposed to X-ray film.

Immunoprecipitation. Flagella and cell body extracts prepared as described below were incubated with affinity-purified anti-GST-SksC antibodies for one to several hours on ice. The antigen-antibody complexes were recovered by incubation with protein A agarose beads for two hours with occasional stirring, the beads were washed twice with PBS containing 1% Triton X-100, followed by two additional washes with PBS. The beads were resuspended in sample buffer, boiled for 4 min at 100 °C and the proteins were analyzed by SDS-PAGE. For *in vitro* phosphorylation of SksC, supernatants from frozen and thawed flagella were incubated in an assay buffer containing 20 mM Hepes, pH 7.2, 2.5 mM $MgCl_2$, 1 mM EDTA, 4% sucrose and 50 μM [γ-^{32}P]ATP (1.8×10^3 dpm/pmol) as described earlier [27].

In vivo phosphorylation of SksC. $\sim 1 \times 10^8$ (100 ml) synchronously growing vegetative cells (mt^+) were collected after 8 hours in the light period and induced to become gametes by resuspension in low phosphate, nitrogen-free medium (1.2 mM $MgSO_4$, 0.36 mM $CaCl_2$, 0.37 mM $FeCl_3$. 6 H_2O, 1.7 mM sodium citrate, 0.04 mM K_2HPO_4, 0.07 mM KH_2PO_4, 10 mM Hepes, pH 7.2 plus trace elements) and growth in continuous light with aeration for 20 hrs at 23 °C. Cells were collected by centrifugation, resuspended in the above medium without phosphates supplemented with 200 μCi [^{32}P]H_3PO_4, and incubated for 3 hours in continuous light at room temperature under constant aeration. Flagella and cell bodies were harvested essentially as described earlier [38]. Briefly, cells were collected by centrifugation, washed once in the above medium, resuspended in 1.8 ml of 7% sucrose, 10 mM Tris, pH 7.6, and deflagellated by pH-shock [23,34]. Samples of the flagella and cell bodies were underlaid with 400 μl of 25% sucrose, 10 mM Tris, pH 7.6, and cell bodies were sedimented by centrifugation at 3000 RPM for 5 min at 4°C in a microfuge. Supernatants, containing flagella, were transferred to fresh tubes and the flagella were collected by centrifugation at 14000 RPM for 10 min at 4°C. Cell bodies and flagella were resuspended in 10 mM Tris, pH 7.6, 20 mM NaCl buffer containing protease and phosphatase inhibitors (1mM phenylmethyllsulfonyl fluoride, 10 μM leupeptin, 1 μM pepstatin, 1 mM ortho-phenanthroline, 5 μM microcystin, 0.1 μM okadaic acid, 0.5 μM sodium vanadate, 10 nM calyculin) and stored under liquid N_2 until use.

Cell fractionation. Cell bodies and flagella were thawed at 37 °C, and subjected to three additional freeze-thaw cycles. The frozen and thawed samples were centrifuged at 100,000 RPM for 20 min at 4 °C in a table top ultracentrifuge, the supernatants were removed, and sedimented material was washed twice and resuspended in 10 mM Tris, pH 7.6, 20 mM NaCl with protease and phosphatase inhibitors (10 μM leupeptin, 1 μM pepstatin, 1 mM ortho-phenanthroline, 1 mM sodium vanadate, 50 mM sodium fluoride, 10 mM ammonium molybdate).

Developmental expression of SksC. To assess possible changes in levels of SksC during the life cycle of *Chlamydomonas*, approximately 2 ml samples ($\sim 2 \times 10^6$) of cells were collected at various times during the vegetative cell cycle (V6 through V24) and gametogenesis (G0 through G18). The numbers after 'V' represent the number of hours during the light period at which cells were harvested. The numbers after 'G' represent the number of hours after resuspension in gamete-induction medium at which cells were harvested. Cells were collected by centrifugation in a microfuge, and resuspended in ice cold 0.5% SDS, 10 mM Tris, pH 7.6, 20 mM NaCl with protease and phosphatase inhibitors (1mM phenylmethyllsulfonyl fluoride, 10 μM leupeptin, 1 μM pepstatin, 1 mM ortho-phenanthroline, 5 μM microcystin, 0.1 μM okadaic acid, 0.5 μM sodium vanadate, 10 nM calyculin) and kept on ice. After all samples were collected, they were centrifuged at 15000 $\times$ g for 10 min at 4°C and the supernatants were collected. Protein amounts were determined using Bradford assay [39], and equal amounts of protein from each sample (~10 μg) were analyzed by SDS-PAGE and immunoblotting.

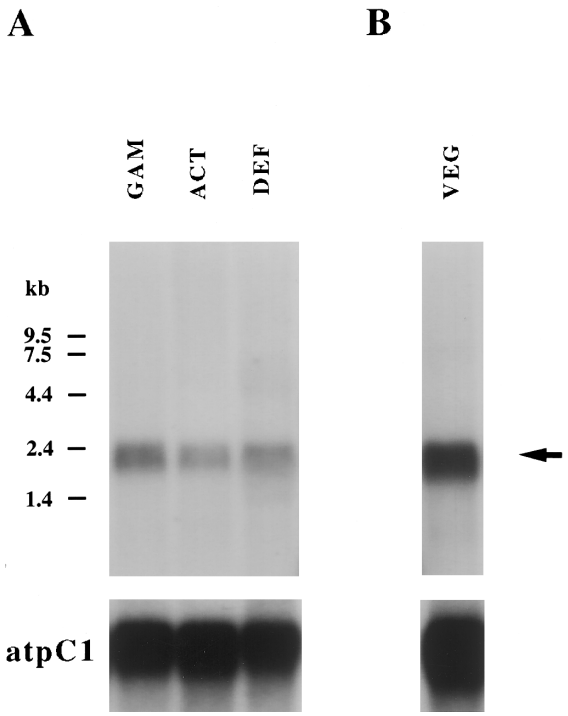

FIG. 1. Northern hybridization. (A) Samples of poly(A)$^+$ RNA isolated from mt$^+$ gametes (GAM), mt$^+$ gametes activated by the addition of flagella isolated from mt$^-$ gametes (ACT), and mt$^+$ gametes undergoing flagellar regeneration (DEF) were fractionated on 1% denaturing formaldehyde agarose gels, transferred to a Nytran membrane and incubated with a nucleotide probe derived from SksC cDNA [28](upper panel). The position of the 2.3 kb transcript identified by the SksC cDNA probe is indicated by an arrow. RNA size standards are shown on the left. As a loading control, the blot was stripped by incubation in 0.1× SSPE, 0.1% SDS for 2× 15 min at 100°C, and rehybridized to a cDNA probe for ATP synthase subunit C (lower panel)[50]. (B) Northern blot hybridization of poly(A)$^+$ RNA isolated from vegetatively growing *Chlamydomonas* cells.

RESULTS

SksC Transcript Levels Were Unchanged during Prolonged Adhesion and after Deflagellation

SksC originally was identified as a soluble flagellar protein whose phosphorylation was regulated by adhesion of *Chlamydomonas* gametes [27,28]. To determine if SksC was transcriptionally up-regulated by prolonged adhesion and signal transduction, we experimentally induced *Chlamydomonas* mt$^+$ gametes to undergo adhesion-mediated signal transduction without allowing them to undergo cell fusion. Under these conditions, synthesis of gamete-specific signaling molecules such as agglutinins is known to be induced [40,41]. When poly(A)$^+$ RNA from resting and activated gametes was analyzed by Northern blot hybridization, transcript levels of SksC in activated gametes did not appear to be significantly higher (Fig. 1, lanes GAM and ACT), suggesting that regulation of SksC during the early stages of fertilization occurred, not by new synthesis, but by post-translational modifications.

Because SksC was identified in gametic flagella, we tested if transcript levels of SksC, like several other flagellar proteins [36,42], would be altered in response to deflagellation of gametes. As shown in Fig. 1 (lanes GAM and DEF), SksC transcript levels remained relatively unaffected in cells undergoing flagellar regeneration (DEF). These results suggesting that the majority of cellular SksC may not be in the flagella led us to determine if SksC was expressed

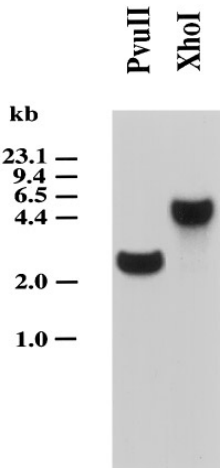

FIG. 2. Southern hybridization. *Chlamydomonas* genomic DNA digested with PvuII or XhoI was fractionated by electrophoresis on a 1% agarose gel, transferred to a nylon membrane and incubated with a probe derived from a 400 bp PCR-amplified fragment from SksC cDNA [28]. DNA size standards are indicated on the left.

in vegetatively growing *Chlamydomonas* cells. To do this, poly(A)$^+$ RNA from vegetative cells was analyzed by Northern blot hybridization. As shown in Fig. 1B, the 2.3 kb SksC transcript also was detected in vegetative cells, further suggesting that this protein kinase might be involved in multiple cellular processes.

To determine the genomic copy number and organization of SksC, *Chlamydomonas* DNA was analyzed by Southern blot hybridization using a probe derived from SksC cDNA sequences. As shown in Fig. 2, a single fragment was evident in *Chlamydomonas* genomic DNA digested with PvuII or XhoI, indicating that SksC is encoded by a single copy gene.

Subcellular Distribution and Developmental Expression of SksC

To determine the cellular localization of SksC, we utilized immunoblot analysis with polyclonal antibodies raised against a bacterially expressed GST-fusion protein. Fig. 3A shows the results of extraction and purification of the bacterially expressed, recombinant GST-SksC. The 72 kDa recombinant protein was detectable in IPTG-induced *E.coli* cells and most of the protein was soluble in detergent (TX-100 S/N). The fusion protein was extracted with glutathione agarose beads, followed by elution with reduced glutathione. SDS-PAGE analysis showed that the eluted GST-SksC was largely free of proteolytic fragments and contaminating bacterial proteins (Fig. 3A, GST-SksC), and immunoblot analysis indicated that the affinity-purified polyclonal antibodies raised against the bacterially expressed fusion protein specifically reacted with GST-SksC (Fig. 3B, GST-SksC), and with endogenous SksC (Flagella).

To determine the location of SksC in *Chlamydomonas*, mt$^+$ gametes were deflagellated and the flagella and cell bodies were analyzed by immnublotting with the affinity-purified α-SksC antibodies. As shown in Fig. 4, SksC was present in both flagella and cell bodies. Moreover, fractionation by centrifugation after freezing and thawing indicated that this protein kinase was soluble in both cellular compartments of *Chlamydomonas* gametes (Fig. 4, Fl.Soluble and CB.Soluble).

To study the expression of SksC during development, we collected *Chlamydomonas* cells at various times during vegetative growth and during gametogenesis, and analyzed extracts by immunoblotting. As shown in Fig. 5, SksC was detected at all stages of vegetative growth and levels did not change during gametogenesis, suggesting that constitutive expression of

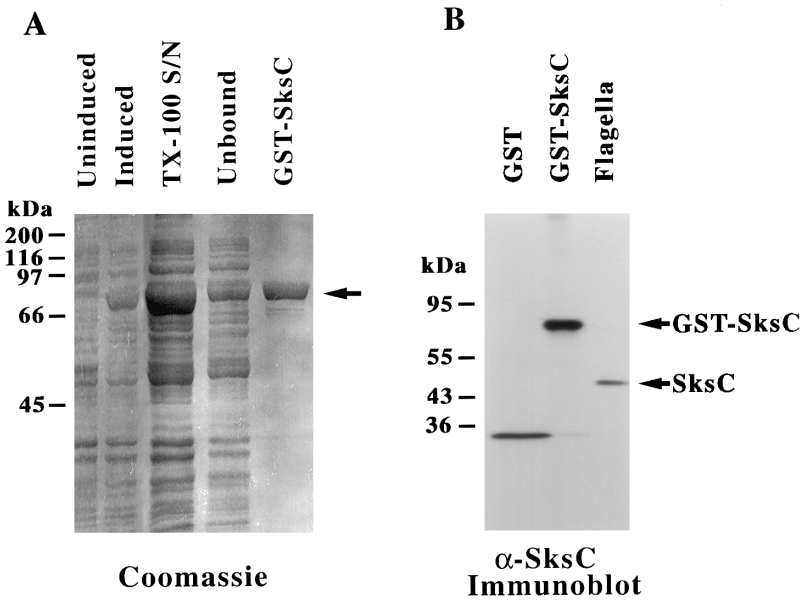

FIG. 3. Characterization of α-SksC antibodies. (A) Extraction and purification of GST-SksC. SksC cDNA was subcloned into the pGEX-KG vector in frame with the coding sequence for glutathione S-transferase as described earlier [28]. Late log phase *E.coli* DH5α cells harboring the recombinant vector (Uninduced) were induced by incubation with 1 mM IPTG (Induced), extracted by sonication in PBS containing 1% Triton X-100 (TX-100 S/N), and purified using glutathione agarose beads (GST-SksC). Equivalent portions of each sample were electrophoretically resolved by 9% SDS-PAGE, and stained with Coomassie blue. The position of the 72 kDa GST-SksC is indicated by an arrow. Molecular weight standards are indicated on the left. (B) Affinity-purified polyclonal antibodies raised against GST-SksC reacted with the endogenous SksC in *Chlamydomonas* flagella. A rabbit polyclonal antibody was affinity-purified using GST-SksC-conjugated matrix as described in Materials and Methods. $\sim$1.0 μg each of purified GST (GST), GST-SksC (GST-SksC) and $\sim$30 μg of soluble flagella supernatant from mt$^+$ *Chlamydomonas* gametes (Flagella) were fractionated by 10% SDS-PAGE, transferred to a PVDF membrane, and analyzed by immunoblotting using α-SksC antibodies. Positions of GST-SksC fusion protein and endogenous SksC are indicated by arrows. Although the anti-SksC antibody was affinity purified on affinity matrices conjugated with GST and GST-SksC, some cross-reactivity to GST was observed (left lane). Molecular weight standards are shown on the left.

SksC may be important throughout *Chlamydomonas* development. Taken together, these results indicated that SksC is ubiquitously expressed during *Chlamydomonas* development, and that it is present as a soluble protein in both cell bodies and flagella.

SksC Is Present in a Phosphorylated Form in Flagella and Cell Bodies, and a Protein Kinase That Phosphorylates SksC Is Detectable in Flagella

Previous experiments on the phosphorylation of SksC in gametic flagella were done *in vitro* [27]. To determine if SksC was phosphorylated *in vivo*, and if both flagellar and cell body forms were phosphorylated, we employed immunoprecipitation methods on extracts of metabolically labeled *Chlamydomonas* gametes. To do this, gametes were incubated with [^{32}P]H_3PO_4 for 3 hours in gamete-induction medium lacking other phosphorus sources, and flagella and cell bodies were harvested as described in Materials and Methods. Soluble fractions from *in vivo* phosphorylated flagella and cell bodies were incubated with anti-SksC antibodies, followed by incubation with protein A agarose, and the immune complexes were analyzed by SDS-PAGE and autoradiography. As shown in Fig. 6A, SksC was present as a phosphoprotein in both flagella and cell bodies of *Chlamydomonas* gametes. Moreover, consistent with our previous studies, a protein kinase activity that phosphorylates SksC could be detected using

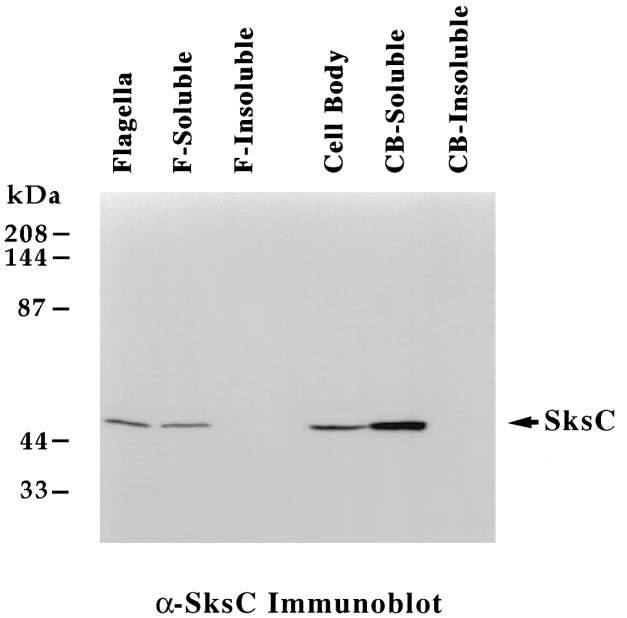

FIG. 4. Subcellular localization of SksC protein. ~5 × 10^7 Flagella (Flagella) and ~1 × 10^7 cell bodies (Cell Body) from mt^+ *Chlamydomonas* gametes were fractionated into soluble (Fl.Soluble; CB.Soluble) and insoluble (Fl.Insoluble; CB.Insoluble) fractions as described in Materials and Methods, and analyzed by 10% SDS-PAGE and immunoblotting using α-SksC antibody. Position of SksC is indicated by an arrow. Molecular weight standards are shown on the left.

an *in vitro* phosphorylation assay. To do this, soluble flagellar proteins from mt^+ gametes were incubated with [γ-^{32}P]ATP as described earlier [27] and immunoprecipitated using anti-SksC antibodies. As shown in Fig. 6B, a phosphorylated form of SksC was immunoprecipitated (α-SksC), whereas affinity-purified anti-GST antibodies did not immunoprecipitate any phosphorylated *Chlamydomonas* proteins under these conditions (α-GST). Taken together, these results indicated that *Chlamydomonas* flagella contain an active protein kinase activity that phosphory-

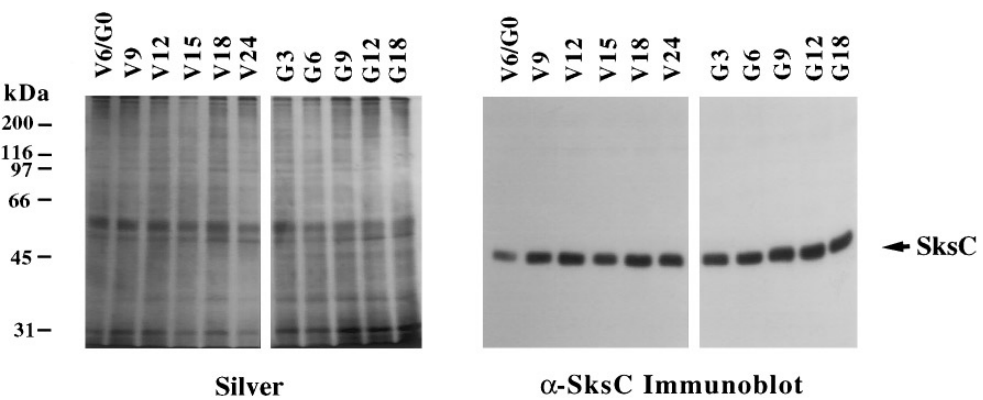

FIG. 5. Expression of SksC during *Chlamydomonas* development. Samples were collected at various times during *Chlamydomonas* vegetative growth (V6 through V24) and during gametogenesis (G0 through G18), and ~10 μg each sample was analyzed by immunoblotting with α-SksC antibody as described in Materials and Methods. Positions of SksC and the molecular weight standards are indicated. Left panel, silver stain; right panel, α-SksC immunoblot.

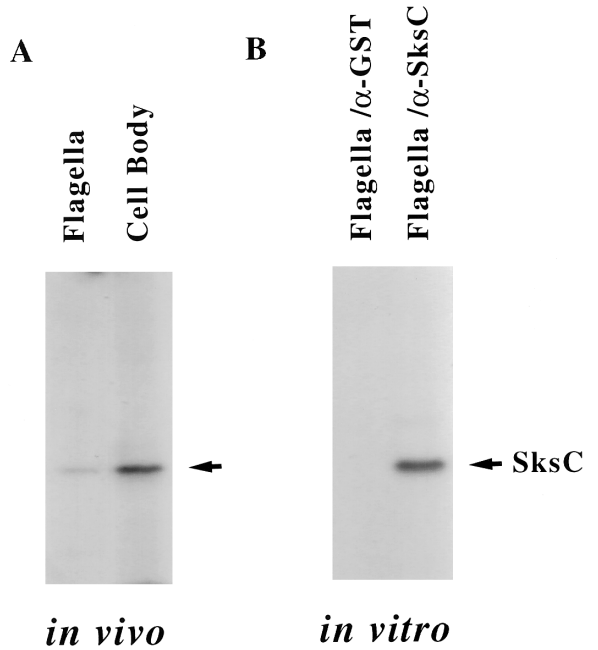

FIG. 6. Immunoprecipitation of phosphorylated SksC. (A) Freeze-thaw supernatants from flagella and cell bodies of metabolically-labeled *Chlamydomonas* gametes were immunoprecipitated using α-SksC antibodies as described in Materials and Methods and analyzed by SDS-PAGE and autoradiography. (B) Freeze-thaw supernatants from *Chlamydomonas* gametic flagella were phosphorylated *in vitro* as described in Materials and Methods, immunoprecipitated with α-SksC antibody, and analyzed by SDS-PAGE and autoradiography (right lane). As a control, a flagella supernatant was incubated with α-GST antibody (left lane). Arrow indicates the position of SksC.

lates SksC *in vitro*, and that phosphorylated SksC is present in both flagella and cell bodies of *Chlamydomonas* gametes.

DISCUSSION

We have demonstrated previously that SksC is a novel protein kinase whose phosphorylation is regulated by adhesion between *Chlamydomonas* gametes of opposite mating types. Its phosphorylated state in non-adhering gametes was proposed to be maintained by a flagellar protein kinase activity, which is in turn regulated by cell-cell adhesion [27]. Consistent with this notion, the predicted amino acid sequence of SksC indicated the presence of putative phosphorylation sites for a tyrosine kinase, protein kinase C and ERK/MAP kinases [28]. Here we report further characterization of this novel protein kinase.

Northern blot hybridization indicated that SksC is encoded by a single transcript of 2.3 kb in *Chlamydomonas* gametes and vegetative cells (Fig. 1). Moreover, when *Chlamydomonas* cells were subjected to prolonged adhesion with the isolated flagella of mt^- gametes, the transcript levels of SksC were unchanged. Furthermore, deflagellation of *Chlamydomonas* gametes also did not affect the levels of SksC transcript (Fig. 2). These results were somewhat surprising since SksC was characterized as a flagellar protein and, like many flagellar proteins, was expected to be transcriptionally up-regulated by deflagellation. For example, removal of flagella induces transcriptional activation of synthesis of flagellar proteins such as tubulins [36]. Prolonged activation of mt^+ gametes by mixing with flagella isolated from mt^- gametes also induces a dramatic up-regulation of transcripts for a gamete-specific molecule with a probable role in fertilization-related signal transduction events (V.K. and W.J.S., in prepara-

tion). The results presented here suggest that regulation of SksC is accomplished, not at the transcriptional level, but perhaps by other means such as post-translational modifications, consistent with available evidence that fertilization in *Chlamydomonas* is not mediated by transcriptional activation of new genes [20, 43]. Our earlier observation that phosphorylation of SksC was inhibited during adhesion-induced signaling events also is consistent with this notion. Although the expression and biochemical functions of many protein kinases are regulated at the transcriptional and translational levels [44-46], biochemical functions of many protein kinases are regulated by post-translational modifications such as phosphorylation [47]. In other experiments to determine the molecular complexity behind SksC expression, we analyzed the genomic organization of SksC using Southern blot hybridization. As shown in Fig.1, unlike many protein kinases from higher organisms whose genes are organized into complex multigene families [3], SksC is encoded by a single copy gene. This is perhaps not surprising given the small genome size for *Chlamydomonas* (~70,000 kb).

Because our evidence indicated that expression of SksC might not be restricted to gametic flagella, we raised polyclonal anti-SksC antibodies using a bacterially-expressed GST-fusion protein, and purified the antibodies on an affinity matrix coupled to GST-SksC. The affinity-purified anti-SksC antibodies reacted specifically with endogenous SksC in immunoblots, and immunoprecipitated it from *Chlamydomonas* extracts (Figs. 3 and 4). Using these antibodies, we determined the subcellular location of SksC. Our evidence indicated that SksC was a soluble protein present in about equal concentrations in flagella and cell bodies of *Chlamydomonas* (Fig. 4A). In addition, SksC was expressed in both flagella and cell bodies of *Chlamydomonas* vegetative cells (data not shown), and at all stages of the vegetative cell cycle and gametogenesis during *Chlamydomonas* life cycle (Fig. 5).

Immunoprecipitation experiments indicated that phosphorylated SksC was present both in flagella and cell bodies of *Chlamydomonas* gametes (Fig. 6A). Recently, many signaling proteins have been reported to participate in multiple, often seemingly unrelated biological processes in a single cell type. Well-known examples of protein kinases with multiple functions are the components of the MAP kinase cascade in *Saccharomyces cerevisiae* (Ste20, Ste11, Ste7 and a Fus3-like MAP kinase), which are involved in two distinct developmental programs in a single cell type: the pheromone response pathway during mating, and filamentous growth [48]. The behavior of PC12 cells in response to epidermal growth factor (EGF) and nerve growth factor (NGF) is another example where transient activation of ERK/MAP kinase by EGF leads to cell proliferation whereas prolonged activation of the ERK/MAP kinase by NGF induces these cells to arrest growth and proliferation and differentiate into neurons [49]. Similarly, the ubiquitous distribution of SksC in *Chlamydomonas* gametes and vegetative cells suggests that, in addition to its involvement in adhesion-induced signaling events during *Chlamydomonas* fertilization, SksC may be involved in multiple developmental pathways.

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