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Biochemistry

Molecular cloning of a protein kinase whose phosphorylation is regulated by gametic adhesion during *Chlamydomonas* fertilization

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ABSTRACT **Fertilization in *Chlamydomonas* is initiated by adhesive interactions between gametes of opposite mating types through flagellar glycoproteins called agglutinins. Interactions between these cell adhesion molecules signal for the activation of adenylyl cyclase through an interplay of protein kinases and ultimately result in formation of a diploid zygote. One of the early events during adhesion-induced signal transduction is the rapid inactivation of a flagellar protein kinase that phosphorylates a 48-kDa protein in the flagella. We report the biochemical and molecular characterization of the 48-kDa protein. Experiments using a bacterially expressed fusion protein show that the 48-kDa protein is capable of autophosphorylation on serine and tyrosine and phosphorylation of bovine β-casein on serine, confirming that the 48-kDa protein itself has protein kinase activity. This protein kinase exhibits limited homology to members of the eukaryotic protein kinase superfamily and may be an important element in a signaling pathway in fertilization.**

Many biological processes are regulated by protein phosphorylation/dephosphorylation after receptor–ligand interactions at the cell surface. While there is evidence to suggest that cell–cell adhesion between gametes is translated into phosphorylation reactions inside the cell, with the exception of those involved in the yeast pheromone response and mating (1–3), no protein kinase with a biochemical role in fertilization has been identified. In the unicellular eukaryotic alga *Chlamydomonas*, adhesive interactions between receptor-like glycoproteins on gametic flagella of opposite mating types lead to the activation of a flagellar adenylyl cyclase (4–6) through a complex interplay of several protein kinase activities (6–8). One of these protein kinase activities, an ATP-dependent inhibitor of adenylyl cyclase, is proposed to maintain the adenylyl cyclase at basal levels of activity in nonadhering gametes (6–8). A second protein kinase activity is required for the adhesion-dependent activation of flagellar adenylyl cyclase (6). The activation of adenylyl cyclase in turn results in a rapid increase in intracellular cAMP levels, which trigger downstream cellular events including cell wall loss and mating structure formation in preparation for cell fusion (9).

In experiments to identify adhesion-dependent changes in flagellar protein kinase activity, we found (Y.Z., Y.L., and W.J.S., unpublished data) that flagellar adhesion between gametes of opposite mating types during fertilization rapidly leads to inhibition of a flagellar protein kinase activity that phosphorylates a 48-kDa flagellar protein. In *in vitro* phosphorylation assays, the 48-kDa protein was phosphorylated in flagella isolated from nonadhering gametes, but its phosphorylation could not be detected in flagella isolated from gametes that had been adhering for 1 min. Available evidence indicates that the inactivation of this protein kinase may be an event upstream of the activation of adenylyl cyclase: the inhibition of the soluble protein kinase is a rapid event, inhibition is not reversed by the addition of cAMP, and the activity of the protein kinase in nonadhering gametes is unaffected by addition of protein kinase inhibitors that block downstream cAMP-induced signaling events (Y.Z., Y.L., and W.J.S., unpublished data). To further define the function of the 48-kDa flagellar protein in *Chlamydomonas* fertilization and to determine its molecular properties, we have identified and characterized the cDNA‡ encoding this protein.

MATERIALS AND METHODS

Isolation of Flagella. *Chlamydomonas reinhardtii* 21gr (mt^+) and 6145C (mt^-) were cultured (10) and gametogenesis was induced as described (11). Flagella were isolated by a modification of the pH shock method of Witman *et al.* (12) as described (7). The sedimented flagella were resuspended to a final concentration of 3–8 mg of flagellar protein per ml in flagella buffer [20 mM Hepes, pH 7.2/4% (wt/vol) sucrose/1 mM EDTA/2.5 mM $MgCl_2$] and a mixture of protease inhibitors was added (7).

Protein Kinase Renaturation Assay. The protein kinase renaturation assay was performed essentially by the method of Ferrell and Martin (13) with ≈25 μg of gametic flagella. Renaturable protein kinase activity was detected by renaturing the flagellar proteins immobilized on poly(vinylidene difluoride) membrane (Immobilon-P, Millipore), followed by incubation in a kinase reaction buffer containing 30 mM Tris·HCl (pH 7.4), 10 mM $MgCl_2$, 2 mM $MnCl_2$, and 16 nM [γ-^{32}P]ATP (6.6×10^6 dpm/pmol; Amersham) for 30 min at room temperature.

Protein Purification. Supernatant (200 μl) from frozen and thawed flagella that had been centrifuged at 15,000 × *g* for 10 min at 4°C was diluted ≈1:10 in buffer A (20 mM Hepes, pH 7.2/2.5 mM $MgCl_2$/1 mM EDTA/3% sucrose). For *in vitro* phosphorylation, the sample was incubated for 5 min at room temperature with 50 μM [γ-^{32}P]ATP (1.8×10^3 dpm/pmol). Unreacted ATP was removed by use of a prepacked PD-10 desalting column (Pharmacia) equilibrated with buffer A. The radiolabeled sample was injected into an HPLC DEAE 5PW column (Millipore) equilibrated with buffer A. The column was washed (flow rate of 1 ml/min) with buffer A for 15 min and material was eluted with a linear gradient of 0–100% buffer B (buffer A with 0.8 M NaCl) for 30 min. Fractions (1 ml) were collected in 1.5-ml microfuge tubes. Amino acid sequences were obtained through an on-campus facility from

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Abbreviations: Sks-C, sexual signaling kinase substrate in *Chlamydomonas*; GST, glutathione *S*-transferase; DTT, dithiothreitol.

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‡The sequence reported in this paper has been deposited in the GenBank data base (accession no. U36196).

six distinct peaks after CNBr or tryptic digestions and HPLC separation of resulting peptides.

cDNA Cloning and Sequencing. Two degenerate oligonucleotide primers, GTGAATTCGARGAYYTNGARATYTTYGA and GTGAATTCSWYTCRTCVACRATRTARAT (where R is a purine, Y is a pyrimidine, N is any nucleotide, S is guanine or cytosine, W is adenine or thymine, and V is guanine, cytosine, or adenine), based on tryptic peptide sequences EDLEIFE and IYIVDE, respectively, were used to amplify a 400-bp fragment from *Chlamydomonas* gametic cDNA by reverse transcription-coupled PCR. The fragment was subcloned, sequenced, and used as a probe to screen a gametic cDNA library constructed in λ-ZapII (14). Four positive phage clones were excised *in vivo* to yield recombinant plasmids that were further analyzed by restriction enzyme digestions and nucleotide sequencing using standard methods (14, 15). Clone 4 (see below) was subjected to a series of exonuclease III deletions from both directions after digestions with *Sac* I and *Xba* I or *Kpn* I and *Xho* I. The exonuclease III-deleted plasmid constructs were blunted by treatment with S1 nuclease followed by T4 DNA polymerase and used to transform DH5α cells (Life Technologies). The plasmids were sequenced using T3 or T7 promoter primers (Life Technologies) and the USB Sequenase sequencing kit (United States Biochemical) (15, 16).

Expression of Recombinant Glutathione *S*-Transferase (GST) Fusion Protein. A 1926-bp *Bam*HI–*Xho* I fragment containing amino acids 12–408 from cDNA clone 4 was subcloned into pGEX-KG (17) in-frame with the coding sequence of a GST gene under control of an isopropyl β-D-thiogalactoside-inducible *tac* promoter. Soluble GST fusion protein ($M_r \approx 72{,}000$) was extracted from late logarithmic-phase DH5α cells harboring recombinant pGEX-KG plasmid after induction for 1.5 h in 1 mM isopropyl β-D-thiogalactoside by sonicating in PBS (150 mM NaCl/16 mM NaH_2PO_4/4 mM Na_2HPO_4, pH 7.3) containing 1% Triton X-100. The extract was incubated with glutathione-agarose beads (Sigma) overnight at 4°C. The beads were washed several times with PBS containing 1% Triton X-100, followed by PBS alone, and the fusion protein was eluted in 75 mM Hepes, pH 8.0/150 mM NaCl/5 mM dithiothreitol (DTT)/10 mM reduced glutathione. After elution, glycerol, $MgCl_2$, and $MnCl_2$ were added to a final concentration of 10%, 10 mM, and 1 mM, respectively, and aliquots of the fusion protein were stored at −80°C until use.

Protein Kinase Assays. For use as a substrate for flagellar protein kinases, ≈2.0 μg of fusion protein was incubated with 10 μl (≈38 μg) of flagellar supernatant in the presence of 50 μM [γ-^{32}P]ATP (1.5×10^4 dpm/pmol) in 37 mM Hepes, pH 7.8/15 mM $MgCl_2$/0.3 mM $MnCl_2$/0.6 mM EDTA/2.4% sucrose/50 mM NaCl/1.7 mM DTT/3.3 mM reduced glutathione/3.3% glycerol (final volume, 30 μl). Control samples containing GST alone or flagellar supernatant alone were incubated under identical conditions. After 15 min at 25°C, the reaction mixtures from all samples were incubated for an additional 15 min at 0°C in the presence of glutathione-agarose beads. The washed beads were resuspended in 50 mM Tris, pH 8.0/100 mM NaCl/2.5 mM $CaCl_2$/0.1% 2-mercaptoethanol/4× sample buffer [0.2 M Tris·HCl, pH 6.8/40% glycerol/8% (wt/vol) SDS/8% (wt/vol) bromophenol blue/400 mM DTT], and the samples were analyzed by SDS/PAGE and autoradiography. The GST fusion protein did not exhibit autophosphorylation activity under these conditions. For thrombin treatment, the GST fusion protein was phosphorylated, extracted as described above, and incubated with 2 units of thrombin in 50 mM Tris·HCl, pH 8.0/100 mM NaCl/2.5 mM $CaCl_2$/0.1% 2-mercaptoethanol for 10 min at room temperature.

For autophosphorylation assays, 1.0 μg of GST fusion protein was incubated in 10 μM [γ-^{32}P]ATP (3.5×10^5 dpm/pmol)/42 mM Hepes, pH 8.0/84 mM NaCl/2.8 mM DTT/5.6 mM glutathione/5.6 mM $MgCl_2$/2.4 mM $MnCl_2$/5.6% glycerol for 30 min at 25°C and analyzed by SDS/PAGE and autoradiography. For casein phosphorylation experiments, 1.0 μg of GST fusion protein was incubated with 2 μg of bovine β-casein (Sigma) in 10 μM [γ-^{32}P]ATP (3.5×10^5 dpm/pmol) as above. For phosphoamino acid analysis, 0.5 μg of GST fusion protein was incubated with 38 μg of bovine β-casein for 30 min at 25°C under the conditions described above. Phosphoamino acid analysis was performed essentially as described by Kamps (18).

RESULTS

To determine whether the 48-kDa protein that was identified as a substrate for an adhesion-regulated protein kinase also was a protein kinase, we identified protein kinases by renaturation after SDS/PAGE (13). Fig. 1 *Left* shows an autoradiograph of the ^{32}P-labeled 48-kDa protein (arrow) and Fig. 1 *Right* shows that a renaturable protein kinase comigrated in SDS/PAGE with the 48-kDa protein. To further define the molecular properties of the 48-kDa protein, we purified the protein by HPLC. In ion-exchange chromatography of mt$^+$ flagellar supernatants, the ^{32}P-labeled 48-kDa protein was enriched in fractions 29 and 30 as shown in Fig. 2. Fractionation of flagellar supernatants by molecular sieve chromatography and sucrose gradients confirmed that only a single protein of 48 kDa could be phosphorylated (Y.Z. and W.J.S., unpublished data). By HPLC, the 48-kDa protein was purified and the amino acid sequences of six fragments were obtained.

To clone the cDNA for the 48-kDa protein, a 400-bp fragment was PCR-amplified from gametic *Chlamydomonas* cDNA by using degenerate oligonucleotide primers designed from two internal peptide sequences obtained from tryptic digestion products of the purified 48-kDa protein. Sequence analysis of the PCR fragment confirmed that it contained internal sequences predicted from the tryptic peptides. By using a nucleotide probe derived from this fragment, four cDNA clones were identified after screening ≈10,000 phage recombinants from a cDNA library constructed from *Chlamydomonas* mt$^+$ gametes. Fig. 3*A* shows the restriction map and relative alignment of the four cDNA clones. Nucleotide sequence analysis of the cDNA clones revealed a single open reading frame encoding a protein of 408 amino acids corresponding to a calculated relative molecular mass of 45 kDa

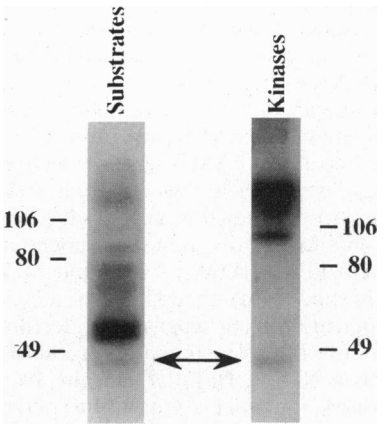

FIG. 1. *In vitro* phosphorylation and renaturable protein kinase activity of a 48-kDa protein. Renaturable protein kinase activities in mt$^+$ gametic flagella were detected (*Right*). A separate sample was *in vitro*-phosphorylated by incubation with [γ-^{32}P]ATP, transferred to the same poly(vinylidene difluoride) membrane, and exposed directly to x-ray film (*Left*). The double-headed arrow indicates the 48-kDa *in vitro*-phosphorylated protein (*Left*) and the 48-kDa renaturable protein kinase activity (*Right*).

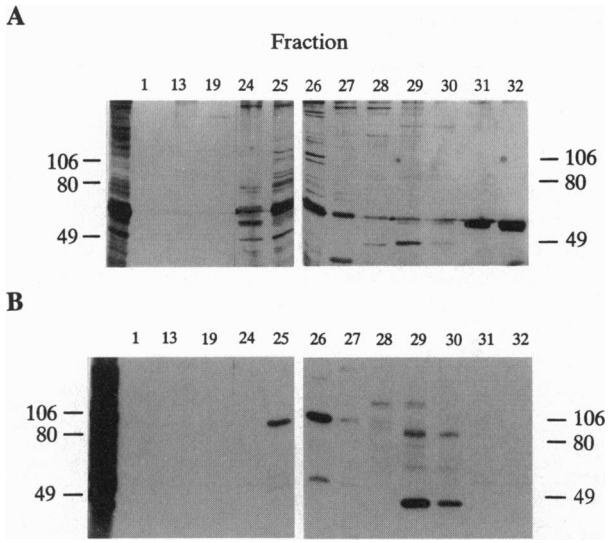

FIG. 2. Purification of the 48-kDa protein. A sample of *in vitro*-phosphorylated supernatant from nonadhering (mt^+) flagella was fractionated on a DEAE 5PW HPLC column. The ^{32}P-labeled 48-kDa protein was enriched in fractions 29 and 30 as shown in *A* (silver stain) and *B* (autoradiograph). Lanes on the left of *A* and *B* contain material loaded onto the column.

(Fig. 3*B*), consistent with the 48-kDa mass estimated from migration in SDS/PAGE. The presence of a stop codon (TAA) 15 bp upstream of the ATG codon suggested that the open reading frame was complete. All six tryptic peptides obtained from the purified 48-kDa protein were present in the deduced amino acid sequence of the cDNA (underlined sequences, Fig. 3*B*). Comparison of the deduced amino acid sequence (ref. 19; Genetics Computer Group) to protein sequences in the Swiss-Prot data base by using the FASTA program did not indicate strong homology with any known proteins. Northern blot hybridization identified a transcript of ≈2.3 kb in *Chlamydomonas* gametic poly(A)$^+$ RNA, a size consistent with that expected for a 48-kDa polypeptide (V.K. and W.J.S., unpublished data). Since this molecule was identified as a substrate for a protein kinase involved in *Chlamydomonas* sexual signaling, it is referred to as Sks-C (sexual signaling kinase substrate in *Chlamydomonas*).

When the predicted amino acid sequence was searched for potential phosphorylation sites with the MOTIFS program (Genetics Computer Group), consensus phosphorylation sites for both serine/threonine and tyrosine protein kinases were identified (Fig. 3*B*). The molecule contains two consensus phosphorylation sites for mitogen-activated protein (MAP) kinases, three sites for protein kinase C, and 10 sites for casein kinase II but none for protein kinase A. Consistent with our previous biochemical studies that this protein is tyrosine-phosphorylated in nonadhering gametes, a potential phosphorylation site (GEYLG) for tyrosine kinases (20) was present at position 62 in the N-terminal portion of the protein (Fig. 3*B*). In other systems, similar phosphotyrosine-containing motifs have been shown to interact with SH2 domains of phospholipase C-γ (21), leading to the activation of protein kinase C and further downstream phosphorylation events (22).

To determine whether the cDNA encoded a molecule that could be phosphorylated by a *Chlamydomonas* flagellar protein kinase, we prepared a bacterially expressed GST fusion protein. The GST fusion protein was incubated with a flagellar supernatant in the presence of [γ-^{32}P]ATP and was recovered by glutathione-agarose beads. Fig. 4 shows that a protein kinase in the soluble fraction of *Chlamydomonas* flagella phosphorylated the GST fusion protein (lanes FP+FL). Under these conditions, the fusion protein did not undergo autophos-

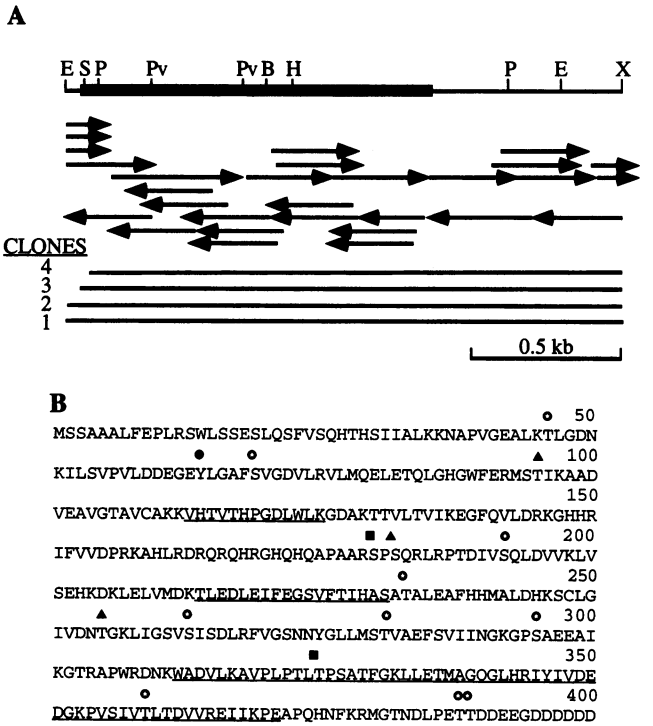

FIG. 3. Characterization of cDNA for the 48-kDa soluble flagellar protein from *Chlamydomonas*. (*A*) Restriction map and sequencing strategy. A composite map of an ≈2.0-kb cDNA derived from the four cDNA clones (numbered 1 through 4) is shown along with restriction enzyme sites. The thick line represents an open reading frame of 408 amino acids. Arrows indicate the relative lengths and directions of the sequences obtained from exonuclease III-deleted plasmid derivatives of cDNA clone 4. E, *Eco*RI; S, *Sac* I; P, *Pst* I; Pv, *Pvu* II; B, *Bgl* II; H, *Hinc*II; X, *Xho* I. (*B*) Predicted amino acid sequence. Peptide sequences corresponding to those obtained from tryptic digestion products (VHTVTHPGDLXLK, TLEDLEIFEGSVFTIXAS, LLETMAGQGLHRIYIVDESGK, SADLK, AVPLPTLTPSATFGK, and PVSIVTLTDVLREIXGPE) are underlined. Potential phosphorylation sites are represented as follows: ●, tyrosine kinase; ■, MAP kinase; ▲, protein kinase C; ✪, casein kinase II.

phorylation (lanes FP). Phosphorylation by the flagellar supernatant occurred only on the fusion protein since GST was not phosphorylated (lanes GST+FL). Furthermore, when the phosphorylated fusion protein was incubated with thrombin to separate the Sks-C fragment from GST, only the Sks-C fragment of the fusion protein was phosphorylated (lanes FP+FL+T). These results indicate that Sks-C was a specific substrate for phosphorylation by a flagellar protein kinase.

Because the 48-kDa protein comigrated on SDS/PAGE with a renaturable protein kinase, we wanted to determine whether the fusion protein itself had protein kinase activity. By using assay conditions that were optimized for detecting autophosphorylation, we found that the purified fusion protein had autophosphorylating activity (Fig. 5*A*, lanes FP). Phosphoamino acid analysis (Fig. 5*B*) of autophosphorylated fusion protein indicated that phosphorylation occurred primarily on serine and to a lesser extent on tyrosine. In separate experiments, we determined that myelin basic protein (V.K. and W.J.S., unpublished data) and bovine β-casein could serve as exogenous substrates (Fig. 5*C*, lanes FP+Cas). Phosphoamino acid analysis (Fig. 5*D*) of phosphorylated casein showed the presence of radiolabeled serine.

Visual inspection of the deduced amino acid sequence indicated that Sks-C exhibited sequence homology with catalytic domain sequences in the eukaryotic protein kinase superfamily (refs. 23 and 24; Fig. 6). Several amino acids in

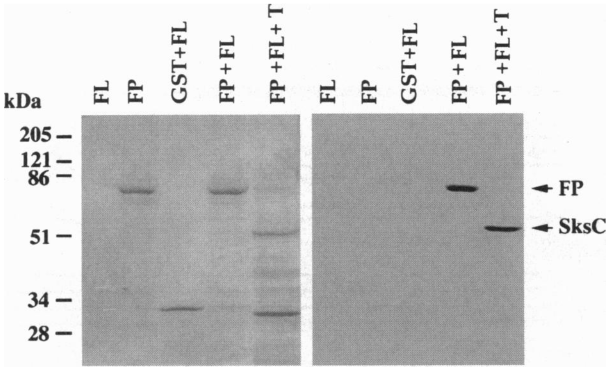

FIG. 4. Analysis of Sks-C as a substrate for a flagellar protein kinase. GST–Sks-C was incubated with [γ-^{32}P]ATP in the presence (lanes FP+FL) or absence (lanes FP) of *Chlamydomonas* flagellar supernatant. A separate sample was prepared for thrombin cleavage (lanes FP+FL+T). As additional controls, flagellar supernatant was incubated with [γ-^{32}P]ATP with (lanes GST+FL) or without GST (FL). All samples were recovered by the addition of glutathione-agarose beads prior to electrophoresis and autoradiography. (*Left*) Silver-stained gel. (*Right*) Autoradiograph. Arrows indicate the positions of GST–Sks-C and the Sks-C fragment after cleavage by thrombin.

subdomains known to be conserved among most protein kinases were present in Sks-C, although residues were substituted in two of the most highly conserved consensus regions in the catalytic domain; the asparagine in subdomain VI and the phenylalanine and the glycine in subdomain VII were not present in the Sks-C sequence. Based on these observations, this protein may represent a class of eukaryotic protein kinases.

DISCUSSION

Although protein kinases are important regulators of cellular events, with the exception of their importance in yeast mating (1–3), the role of protein kinases in fertilization is not yet clearly defined. In higher plants, S-locus receptor protein kinases may mediate early events during pollen germination (27–29). Phosphorylation on tyrosine residues of a 95-kDa sperm surface protein, a hexokinase that also has protein kinase activity (30), may be an important event during fertilization in mouse (31, 32). In sea urchin spermatozoa, cell surface guanylyl cyclases, which produce the second messenger cGMP in response to binding by egg peptides, are regulated by phosphorylation/dephosphorylation events (33, 34). In sea urchin eggs, a plasma membrane cell adhesion protein is

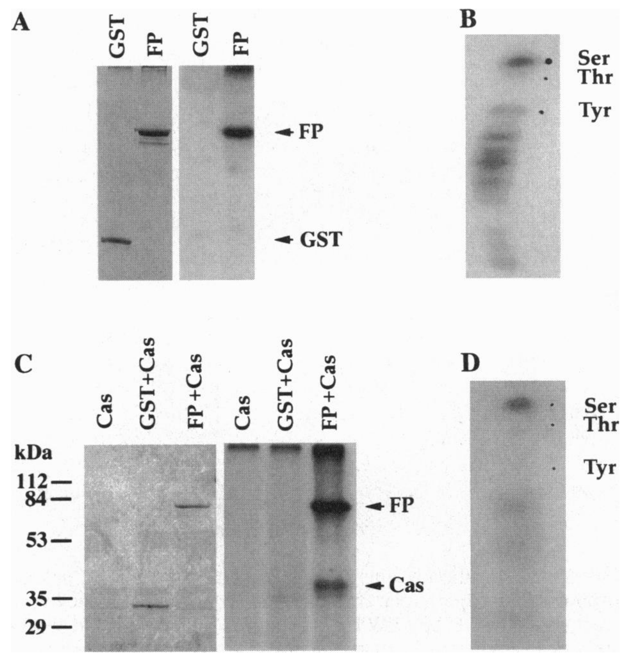

FIG. 5. Protein kinase activity of GST–Sks-C. (*A*) The GST fusion protein and GST alone were incubated with [γ-^{32}P]ATP and analyzed by SDS/PAGE and autoradiography. (*Left*) Silver-stained gel. (*Right*) Autoradiograph. The arrows indicate the positions of GST and GST–Sks-C (FP). (*B*) The fusion protein was autophosphorylated as in *A* and analyzed for phosphoamino acids. The positions of phosphoamino acid standards are indicated. Lower spots on the autoradiograph probably represent partial hydrolysis products. (*C*) The GST fusion protein and GST alone were incubated in the presence of bovine β-casein and [γ-^{32}P]ATP. As an additional control, bovine β-casein (Cas) also was incubated with [γ-^{32}P]ATP. (*Left*) Coomassie blue staining. (*Right*) Autoradiograph. (*D*) Casein was phosphorylated as in *C* and phosphoamino acid analysis was performed as described (17). The positions of phosphoamino acid standards are indicated.

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                                            I                                      II
Sks_C          51 KILSVPVLDD..EGEYLGAFSVGDVLRVLMQELETQLGHGWFERMSTIKAADVEAVGTAVCAKKVHTVTHPGDL
Ste7_Yeast    181 SNGNYIQLQDLVQLGKIGAGNSGTVVKALH........................VPDSKIVAKKTI.PVEQNNS
Fus3_Yeast      9 SSD..FQLKSL.....LGEGAYGVVCSATH........................KPTGEIVAIKKI.EPF.DKP
Ypk1_Yeast    336 SRNKPLSIDDFDLLKVIGKGSFGKVMQVRK........................KDTQKVYALKAIRKSYIVSK
Csk_Mouse     189 ....ALNMKELKLLQTIGKGEFGDVMLGDY................. .....RGNK..VAVKCIKN.....D

                              III          IV
Sks_C             WLKGDAKTTVLTVIKEGFQVLDRKGHHR.IFVVDPRKAHLRDRQRQHRGHQHQAPAARSPSQRLRPTDIVSQLDV
Ste7_Yeast        .......TIINQLVRELSIVKNVKPHENIITFYGAYYNQHIN..NEIIILMEYSDCGSLDKILSVYKRFVQRGTV
Fus3_Yeast        .......LFALRTLREIKILKHFK.HENIITIFNIQRPDSFENFNEVYIIQELMQTD.LHRVIS...........
Ypk1_Yeast        .......SEVTHTLAERTVLARV..DCPFIVPLKF....SFQSPEKLYFVLAFINGGELFYHLQKEGRFD.....
Csk_Mouse         .......ATAQAFLAEASVMTQLR.HSNLVQLLGV....IVEEKGGLYIVTEYMAKGSLVDYLRSRGRSV.....

                                                                   VI                  VII
Sks_C             VKLVSEHKDKLELVMDKTLEDLEIFEGSVFTIHASATALEAFHHMALDHKSCLGIVDNTGKLIGSVSISDLRFVG
Ste7_Yeast        SSKKTWFNELTISKIAYGVLNGLDHLYRQYKI............IHRDIKPSNVLINSKGQIKLC....DFGVS
Fus3_Yeast        ...TQMLSDDHIQYFIYQTLRAVKVL.HGSNV............IHRDLKPSNLLINSNCDLKVC....DFGLA
Ypk1_Yeast        ......LSRARF..YTAELLCALDNL.HKLDV............VYRDLKPENILLDYQGHIALC....DFGLC
Csk_Mouse         ......LGGDCLLKFSLDVLEAMEYL.EGNNF............VHRDLAARNVLVSEDNVAKVS....DFGLT

                                           VIII              IX
Sks_C             SNN.YGLLMSTVAE.....FSVIINGKGPSAEEAIKGTRAPWRDNKWADVLKAVPLPTLTPSATFGKLLETMAGQ
Ste7_Yeast        KKLINSIAD............TFVGTSTYMSPE.RIQGNVYSIK...GDVWSLGLMIIELVTGEFPLGGHN....
Fus3_Yeast        RIIDESAADNSEPTGQQSGMTEYVATRWYRAPEVMLTSAKYSRA...MDVWSCGCILAELFLRRPIFPGRDYRHQ
Ypk1_Yeast        K....LNMKDDDK .....TDTFCGTPEYLAPEL.LLGLGYTKA...VDWWTLGVLLYEMLTGLPPYYDEDVPKI
Csk_Mouse         K....EASSTQDT ......GKL..PVKWTAPEA.LREKKFSTK...SDVWSFGILLWEIYS............

                                                                          XI
Sks_C             GLHRIYIVDEDGKPVSIVTLTDVVREIIKPEAPQHNF.................KRMGTNDLPETTDDEEGD 395
Ste7_Yeast        DTPDGILDLLQRIVNEPSPRLPKDRIYSKEMTDFVNRCC..........IKNERERSSIHELLHHDLIMKYV 470
Fus3_Yeast        LLLIFGIIGTPHSDNDLRCIESPRAREYIKSLPMYPAAP..........LEKMFPRVNPKGIDLLQRMLVFD 293
Ypk1_Yeast        YKKILQEPLVFPDGFDRDAKDLLIGLLSRDPTRRLGYNGADEIRNHPFFSQLSWKRLLMKGYIPPYKPAVSN 625
Csk_Mouse         FGRVPYPRIPLKDVVPRVEKGYKMDAPDGCPPAVYDVM.......KNCSHLDAATRPTFL............ 434
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FIG. 6. Comparison of Sks-C amino acid sequence to sequences of known protein kinases. Ste7 (23), a dual specificity (Thr/Tyr) protein kinase involved in yeast mating; Fus3 (23), a MAP kinase (Erk) homolog from yeast; Ypk1 (25), a Ser/Thr protein kinase from yeast in Swiss-Prot data base that had the highest homology score among protein kinases (22.6% identity and 46.5% similarity) to Sks-C; Csk (26), a protein tyrosine kinase from mouse involved in adhesion-induced signaling events. Conserved amino acid residues in selected protein kinase subdomains (indicated by Roman numerals) are shown in boldface type. The protein kinase sequences were first aligned by using the PILEUP program from Genetics Computer Group and then manually adjusted to maximize homology.

phosphorylated on tyrosines in rapid response to interactions with the sperm protein bindin, and an Abl-related protein tyrosine kinase also may play a role during fertilization in this organism (35, 36). Even though characterization of these molecules has begun, the importance of their phosphorylation and whether or not they are elements of a signaling pathway during fertilization is not yet clear.

To our knowledge, Sks-C is the first protein kinase to be cloned from *Chlamydomonas*, an organism with potential as a model system for signal transduction studies (9, 37, 38). That Sks-C itself is a protein kinase suggests that this molecule and the activity that phosphorylates it may constitute central elements of a protein kinase signaling cascade. According to our working model, an as yet unidentified protein kinase activity ("Sks-C kinase") normally phosphorylates Sks-C in nonadhering gametes and may be part of a pathway that normally inhibits the flagellar adenylyl cyclase (Y.Z., Y.L., and W.J.S., unpublished data, and ref. 7). The adhesion-dependent change in phosphorylation of Sks-C protein may regulate its protein kinase activity and bring about further downstream events. In this context, it is useful to recall that this molecule contains several consensus phosphorylation sites for eukaryotic protein kinases, many of which lie within the putative catalytic domain (Fig. 3*B*). For example, the potential tyrosine phosphorylation site at position 62 is in close proximity to the glycine-rich putative ATP-binding region (Figs. 3*B* and 6), which may be significant in regulation of this protein. In the case of many protein tyrosine kinases and MAP kinases, phosphorylation of residues on regulatory sequences within the catalytic domains may regulate their protein kinase activity (39–41). Whatever its immediate partners, we believe that Sks-C plays a crucial role in signal transduction initiated by gametic interactions during fertilization. Its sequence and structural elements should prove valuable in structure–function studies on eukaryotic protein kinases.

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