Regulated Targeting of a Protein Kinase into an Intact Flagellum

AN AURORA/Ipl1p-LIKE PROTEIN KINASE TRANSLOCATES FROM THE CELL BODY INTO THE FLAGELLA DURING GAMETE ACTIVATION IN CHLAMYDOMONAS*

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In the green alga Chlamydomonas reinhardtii flagellar adhesion between gametes of opposite mating types leads to rapid cellular changes, events collectively termed gamete activation, that prepare the gametes for cell-cell fusion. As is true for gametes of most organisms, the cellular and molecular mechanisms that underlie gamete activation are poorly understood. Here we report on the regulated movement of a newly identified protein kinase, Chlamydomonas aurora/Ipl1p-like protein kinase (CALK), from the cell body to the flagella during gamete activation. CALK encodes a protein of 769 amino acids and is the newest member of the aurora/Ipl1p protein kinase family. Immunoblotting with an anti-CALK antibody showed that CALK was present as a 78/80-kDa doublet in vegetative cells and unactivated gametes of both mating types and was localized primarily in cell bodies. In cells undergoing fertilization, the 78-kDa CALK was rapidly targeted to the flagella, and within 5 min after mixing gametes of opposite mating types, the level of CALK in the flagella began to approach levels normally found in the cell body. Protein synthesis was not required for targeting, indicating that the translocated CALK and the cellular molecules required for its movement are present in unactivated gametes. CALK was also translocated to the flagella during flagellar adhesion of nonfusing mutant gametes, demonstrating that cell fusion was not required for movement. Finally, the requirement for flagellar adhesion could be bypassed; incubation of cells of a single mating type in dibutyryl cAMP led to CALK translocation to flagella in gametes but not vegetative cells. These experiments document a new event in gamete activation in Chlamydomonas and reveal the existence of a mechanism for regulated translocation of molecules into an intact flagellum.

Cell-cell interactions leading to fusion between gametes of opposite sexes during fertilization are complex processes that involve dramatic changes in each of the interacting gametes. In most multicellular organisms, interactions between adhesion molecules/receptors on the sperm plasma membrane and ligands on the egg surface activate poorly understood signaling pathways that bring about transformation of the sperm into a fusion competent, activated gamete (1). For example, the sperm surface is remodeled as a consequence of the acrosome reaction, an event that accompanies gamete activation, and previously existing adhesion molecules are mobilized to new sites on the sperm (2–4). The molecular mechanisms that underlie and regulate signal transduction and movement of molecules between different gamete compartments during gamete activation largely are unknown (2).

As in multicellular organisms, gamete activation and fertilization in the unicellular green algae Chlamydomonas (5) depend on adhesion-induced signaling pathways and intercompartmental communication. At the completion of gametogenesis, during which asexually growing mt+ and mt− vegetative cells differentiate into sexually competent cells, the resulting mt+ and mt− gametes bear mating-type-specific adhesion molecules, agglutinins, on their flagellar membranes and on the plasma membrane of the cell body. The agglutinins on the flagella are in an active state and are capable of binding to flagellar agglutinins on gametes of the opposite mating type, whereas agglutinin molecules on the contiguous plasma membrane of the cell body are inactive (6).

When mt+ and mt− gametes are mixed together, random contacts between their highly motile flagella bring the mt+ and mt− flagellar agglutinins together. In addition to binding the flagella of gametes of opposite mating types to each other, these receptor/ligand-like interactions also initiate a complex series of events termed gamete activation. One of the earliest documented steps in gamete activation triggered by agglutinin interactions is activation of a gamete-specific flagellar adenyl cyclase and generation of cAMP (7–11). As part of an intricate feedback mechanism required to maintain and enhance flagellar adhesiveness and to keep the cells bound to each other until cell-cell fusion occurs, cAMP levels increase in the cell bodies of the gametes of both mating types during flagellar adhesion and lead to increased flagellar adhesiveness (6, 9, 12, 13).

The increased cellular levels of cAMP also induce additional events in the cell body, including regulated secretion of molecules required for release and degradation of the extracellular matrix (cell wall), activation of fusion organelles called mating structures on the apical ends of the interacting cells between their sets of flagella, and phosphorylation of an mt+ gamete-specific homeodomain protein, GPS1 (14, 15). Adhesion and fusion of the mating structures on the mt+ and mt− gametes are followed by complete merging of the two cell bodies. Fusion itself also induces the flow of signals from the cell body to the flagella, triggering inactivation and loss of the flagellar agglutinins (16, 17), flagellar resorption, and zygote maturation (reviewed in Refs. 18 and 19).

Studies on the flagellar adenyl cyclase, a key regulatory enzyme in gamete activation, have shown that, like the adenyl cyclases of gametes in multicellular organisms (20), the...
Chlamydomonas enzyme appears not to be regulated by G proteins. Instead, we have found that the flagellar adenyl cyclase is regulated by protein phosphorylation and dephosphorylation. A flagellar membrane-associated protein kinase activity in nonactivated gametes inhibits the adenyl cyclase (10). During gamete activation, agglutinin interactions relieve this inhibition and also stimulate a second protein kinase whose activity is required to activate adenyl cyclase (11). Concomitantly, flagellar adhesion leads to the inhibition of a third protein kinase, whose substrate itself is yet another protein kinase (GenBank™ accession number U36196) (11, 21). In addition to these protein kinase activities involved in the very early stages of gamete activation upstream of generation of cAMP, several protein kinases act downstream of cAMP. For example, the recently discovered homodomain protein GSP1 is phosphorylated within minutes after gametes of opposite mating types are mixed together. GSP1 phosphorylation can be induced by incubation of mt+ gametes in dibutyryl cAMP, thus bypassing flagellar adhesion in the gamete activation pathway (15).

To learn more about gamete activation and intercompart-mental communication in Chlamydomonas, we have begun to focus on a new protein kinase that undergoes activation-de- pendent changes during fertilization. Here we report that a novel member of the aurora/Ipl1p family of protein kinases is induced by flagellar adhesion in the absence of cell fusion and upon incubation of gametes of a single mating type in dibutyryl cAMP. Moreover, the regulated translocation of CALK is specific to gametes.

MATERIALS AND METHODS

Cells and Cell Culture—Chlamydomonas reinhardtii strains 21gr (mt−) (CC-1690), 6145C (mt−) (CC-1691), and impl−15 (mt+) (CC-462), available from the Chlamydomonas Genetic Center, Duke University, were cultured either with medium I or medium II of Sager and Granick (22) at 23°C on a 13:11 h light/dark cycle as described previ-ously (23). Vegetative cells were induced to become gametes by incuba- tion in medium without nitrogen (N-free medium) followed by culturing in contact with room temperature air.

Treatment of Cells with Dibutyryl cAMP—For experiments with dibutyryl cAMP, gametes in N-free medium and vegetative cells in medium II were incubated in 15 mM dibutyryl cAMP and 0.15 mM papaverine for 30 min with aeration. Cell wall loss, which is a measure of gamete activation, was assessed by determining whether cells be-came sensitive to disruption by incubation in 0.075% Triton 100X, 0.5 mM EDTA, 10 mM Tris, pH 8.0, as described earlier (24, 25).

Flagellar Isolation—Flagella were isolated essentially as described in Zhang et al. (10). Typically, 3–4 liters of cells were concentrated to 30 ml by centrifugation at 3500 × g for 5 min at 4°C, and ice-cold 25% sucrose in 10 mM Tris, pH 7.2, was added to yield a final concentration of 7% sucrose. While stirring the suspension, its pH was rapidly de-creased to 4.5 by the addition of 0.5 M acetic acid; after the flagella were detached (which typically required about 20 sec) the pH was raised to 7.2 with 0.5 M KOH. All subsequent steps were carried out at 4°C. The suspension of cells and flagella was underlayed with 25% sucrose in 10 mM Tris, pH 7.2, and centrifuged for 10 min at 2500 × g. The upper phase that contained flagella and a few remaining cell bodies was underlayed with 25% sucrose, 10 mM Tris, pH 7.2, and centrifuged again as above. The upper phase containing purified flagella was care-fully removed and centrifuged at 9000 × g for 8 min. The sedimented flagella were resuspended in buffer A (20 mM HEPES, pH 7.2, 5 mM MgCl2, 1 mM EDTA, 1 mM dithiothreitol) containing a 1/100 dilution of the Sigma protease inhibitor mixture for plant cells (Sigma catalogue number P9599) and were flash frozen in liquid nitrogen.

Protein Determination—Protein concentration was determined by use of a Bio-Rad protein assay kit with bovine serum albumin (Albumin Standard from Pierce) as a standard.

Cloning of the cdk cDNA—A 426-base pair cdk fragment was first cloned from a polymerase chain reaction product obtained by amplification of genomic DNA. Cloning DNA with degenerate primers (A/G/T/C/G-T/C/T/G/T/T/T/C/T/+/−) (A/G/C/G-G-T/A/G-T/A/G-T/C/G-T/R) was originally designed for amplification of adenyl cyclase. A probe for screening a Lambda II genomic library (kindly provided by Paul Lefebvre, University of Minnesota) was prepared from the cloned poly-merase chain reaction ampiclon by random labeling using a rapid labeling kit from Roche Molecular Biochemicals. The same probe was also used to screen a ZAP II cDNA library prepared from activated mt+ gametes (26, 27). The screen of the cDNA library yielded a single positive clone from 30,000 plaques. The plaque was picked and re-screened by polymerase chain reaction using unique primers designed from the sequence of the first cloned polymerase chain reaction product. The cdk phagemid clone was in vitro excised as recommended by the manufacturer (Stratagene, San Diego, CA), yielding a recombinant plBluescriptII plasmid containing cdk cDNA. The cDNA clone con-tained a 3.2-kilobase insert, which was sequenced in both directions by automated sequencing methods. Portions of 1 of 3 genomic clones ob-tained were used to confirm the sequence of the cDNA clone. The nucleotide sequence of the cDNA, which is termed CALK (see “Re- sults”), was submitted to GenBank™ (accession number AF199021). The cDNA contained a 768-amino acid open reading frame. The pres-ence of two stop codons upstream was confirmed by comparison to that region of the genomic clone. For routine propagation of cloned DNA, plasmid constructs were transformed into Escherichia coli DH5α cells.

Sequence Analysis—The amino acid sequences of CALK and related proteins were aligned with the ALLALL sequence alignment server, and the alignment shown in Fig. 2 was further refined by hand accord-ing to known protein kinase subdomains (28). The phosphorylation sites were predicted by analysis with PhosphoBase software (29).

Southern Blotting—Genomic DNA was isolated from 21gr cells by standard methods (27). Briefly, cells harvested from a 25-ml culture were resuspended in 1 ml of cetyltrimethyl-ammonium bromide lysis buffer (2% cetyltrimethyl-ammonium bromide, 100 mM Tris, pH 8.0, 20 mM EDTA, 1.4 mM NaCl, 2% freshly added β-mercaptoethanol) pre-warmed at 65°C. After vortexing and incubation at 65°C for 1 h with gentle shaking, DNA was extracted with phenol-chloroform followed by precipitation with isopropanol and washing in ethanol. DNA was dis-solved in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) containing 20 µg of RNase (Life Technologies, Inc.) ml−1.

A Southern blot analysis of DNA was digested with BamHI, HindIII, and KpnI, and the fragments were separated on 1% agarose gels and blotted onto nylon membranes (Schleicher & Schuell) using standard methods. The blot was probed with the random primer-la-beled (random labeling kit, Roche Molecular Biochemicals) genomic frag-ment of cdk in Bright-star hybridization solution (Ambion) at 55°C. The blot was washed in 0.1% SDS, 15 mM NaCl, 1.5 mM sodium citrate (0.1 X SSC) followed by washing in buffer containing 0.5% SDS, 0.1 X SSC at 65°C for 30 min. The blot was then exposed to x-ray film and developed.

Recombinant Protein Expression and Purification—To prepare a His-tagged, truncated, recombinant form of the protein, the Sph/PlaI frag-ment of the cDNA corresponding to amino acids 12–243, which includes the entire protein kinase domain, was cloned into expression vector pQE30 (Qiagen) in frame with the coding sequence for six consecutive His residues (6 × His) under control of an isopropyl β-D-thiogalac-to-side-inducible lac promoter. A 1-liter culture of M15 bacteria (Qiagen) harboring the recombinant plasmid was grown at 37°C with vigorous shaking until an A600 of 0.6 was reached. The temperature of the incubation was switched to 30°C, and 1 h later isopropyl β-D-thiogalacto-side was added to a final concentration of 0.5 mM and incubation was continued for 2 h. The His-tagged recombinant protein was purified by use of nickel-nitriotriacetic acid-agarose according to the instructions from the manufacturer (Qiagen). A nearly full-length recombinant, His-tagged CALK lacking the N-terminal 11 amino acids was also expressed and purified as described above.

In Vitro—For in vitro protein kinase assays, nearly full-length, His-tagged, recombinant CALK (100 ng in 20 mM HEPES, 1 mM EGTA, 1 mM dithiothreitol, 10 mM benzamidine, 0.2 mM phenylmethylsulfonyl fluoride, 10% glycerol) was added to 30 µl of protein kinase assay buffer (20 mM Tris, pH 8.0, 10 mM MgCl2, 0.1 mM dithiothreitol, 100 µM ATP, 2 µM [γ-32P]ATP (6000 Ci mmol−1, Amer-sham Pharmacia Biotech) and incubated for 30 min at 30°C. Some samples also contained 5 µg of dephosphorylated bovine casein (Sigma)
or bovine myelin basic protein (Sigma). The reactions were stopped by the addition of SDS-PAGE sample buffer and boiling, as described below, and analyzed by SDS-PAGE and autoradiography.

SDS-PAGE—Samples for SDS-PAGE were mixed with 1/3 volume of 4× SDS sample buffer (0.25% Tris, pH 6.8, 40% glycerol, 16% SDS, 0.4 mM dithiothreitol, 0.1% bromphenol blue) and boiled for 5 min. In some experiments sample buffer was used at a final concentration of 2×. The samples were subjected to electrophoresis in 9% acrylamide (15, 30) minislab gels at 30 mA in buffer containing 25 mM Tris, 192 mM glycine, 20% methanol at 100 V for 1 h, followed by destaining with 40% methanol, 10% acetic acid.

Immunoblot Analysis—For immunoblot analysis of CALK, sedimented whole cells or isolated flagella were subjected to electrophoresis. After SDS-PAGE, proteins were transferred to a polyvinylidene difluoride membrane (Immobilon P, Millipore, Bedford, MA) in buffer containing 25 mM Tris, 192 mM glycine, 20% methanol at 100 V for 1 h, or at 35 V overnight at 4 °C. The membrane was blocked with 5% Carnation dry milk (Nestles) in 20 mM Tris, pH 7.6, 137 mM NaCl, 0.05% Tween-20 (TBST) for 1 h and then incubated either with preimmune serum or with anti-CALK serum (obtained from a rabbit immunized with the truncated, His-tagged CALK) in 3% Carnation dry milk in TBST for 1 h. The membrane was washed as before and incubated in ECL immunoblotting reagents (Amersham Pharmacia Biotech) for 1 min as described by the manufacturer, exposed to Hyperfilm ECL (Amersham), and developed in an automatic film processor. In some experiments, after the immunoblot membrane was exposed, total proteins were visualized by staining with 0.1% Coomassie Brilliant Blue R-250 in 40% methanol, 10% acetic acid, followed by destaining with 40% methanol, 10% acetic acid.

RESULTS

Molecular Cloning of a cDNA Encoding a Protein Kinase—To investigate regulatory molecules in gamete activation, we characterized a newly obtained 3.2-kilobase cDNA that encodes a protein kinase (see “Materials and Methods”). The cDNA has an open reading frame of 2307 base pairs beginning with the ATG codon at nucleotide position 99 through the termination codon at position 2406. The ATG codon at position 99 was chosen because of two in-frame stop codons immediately upstream from it and because of its association with a conventional initiation sequence (31). The cDNA ends with a long poly(A) tail and contains a putative polyadenylation signal (AATGTA) 19 nucleotides upstream of the poly(A) sequence (see nucleotide sequence in the GenBank™), similar to those found in other Chlamydomonas transcripts (32, 33). The cDNA predicted a polypeptide containing 769 amino acids with a molecular mass of 80.6 kDa and a pI of 9.76 (Fig. 1A). The presence of the sequence DIK-PEN in subdomain VII indicates that the molecule belongs to the serine-threonine family of protein kinases (28). The sequence analysis also showed that the protein does not contain a putative signal peptide or a predicted transmembrane domain. The sequence analysis also showed that the protein does not contain a putative signal peptide or a predicted transmembrane domain, suggesting that it is a cytoplasmic protein.

Sequence Analysis Shows That CALK Is a Member of the Aurora/Ipl1p Protein Kinase Family—A BLAST (34) search of the GenBank™ data bases indicated that the molecule showed highest similarity to members of the aurora/Ipl1p-like protein kinase family, many of which are regulators of chromosome segregation and cytokinesis (reviewed in Refs. 40 and 41). The first protein on the list of similar proteins found in the BLAST search was the human protein kinase, aurora2 (accession number AF011468). Within the protein kinase catalytic domain, the Chlamydomonas polypeptide exhibited 36% identity and 56% similarity over 279 amino acids with aurora2. Aurora2 (also...
FIG. 2. Comparison of CALK to other aurora/Ipl1p protein kinases. A, the protein kinase catalytic domains from the sequences indicated in the tree were aligned using Clustal W (92). The distance matrix was calculated from the alignment using the PROTDIST program in the PHYLIP package, and the tree was built by the neighbor-joining method using the NEIGHBOR program in the PHYLIP package (93). The tree was viewed with TreeViewPPC.1 (94). The accession numbers of the proteins in the tree are as follows: Ipl1p, U07163; AIRK-Sp, CAA18315; AIR2-Ce, AF071207; IAL-Dm, AF121358; aurora-Dm, X84365; aurora2-Hs, AF008551; pEg1-Xl, AF071206; aurora1-Hs: AF008552; AIE1-Mm: AF054620; AIRK-At, AC003680; AIR1-Ce, AF071206; protein kinase A (PKA)-Sc, P11792; PKA-Hs, P17612; PKA-Ce, P21137; protein kinase C (PKC)-Hs, P17592; PKC-Ce, AF078781; extracellular signal-regulated kinase (ERK)-Sc, P16892; ERK1-Hs, P27361; ERK Ce, A36978; STE7-Sc, P06784; mitogen-activated kinase/extracellular signal-regulated kinase 2 (MEK2)-Hs, P36507; MEK-Ce, AF054620; AIRK-At, AC003680; AIR1-Ce, AF071206; aurora1-Hs: AF008552; AIE1-Mm: AF054620; AIRK-At, AC003680; AIR1-Ce, AF071206; protein kinase A (PKA)-Sc, P11792; PKA-Hs, P17612; PKA-Ce, P21137; protein kinase C (PKC)-Hs, P17592; PKC-Ce, AF078781; extracellular signal-regulated kinase (ERK)-Sc, P16892; ERK1-Hs, P27361; ERK Ce, A36978; STE7-Sc, P06784; mitogen-activated kinase/extracellular signal-regulated kinase 2 (MEK2)-Hs, P36507; MEK-Ce, AF054620.

The asterisks indicate deduced protein sequences obtained from genome sequencing programs. B, diagrammatic representation of CALK and other aurora/Ipl1p protein kinases.

Because of the similarity of this new Chlamydomonas protein kinase to members of the aurora/Ipl1p family, we have named it Chlamydomonas aurora/Ipl1p-like protein kinase, CALK. A sequence alignment of the protein kinase subdomains of CALK with aurora2 and with the two original members of this emerging family, aurora from Drosophila and Ipl1p from Saccharomyces cerevisiae, is shown in Fig. 1B. The protein kinase domains of CALK, several aurora/Ipl1p family members, and several other types of protein kinases were used to make the phylogenetic tree shown in Fig. 2A, which is consistent with the BLAST search results indicating that CALK is more related to aurora/Ipl1p protein kinases than it is to other members of the protein kinase superfamily.

The BLAST search and visual examination indicated that the similarity between CALK and other members of the aurora/Ipl1p protein kinase family was restricted to the protein kinase domain as depicted in Fig. 2B. For most of the members of this family, the protein kinase catalytic domain represents the majority of the polypeptide. Those family members that contain significant numbers of noncatalytic domain amino acids usually have them in an N-terminal extension, and some family members share limited sequence similarity in these N-terminal domains (41). Interestingly, CALK has few amino acids N-terminal to the catalytic domain; but, it has an extensive noncatalytic, C-terminal domain that makes it nearly twice as large as all other known aurora/Ipl1p family members.

The large C-terminal domain of CALK has several features that are notable. Two regions are enriched in basic amino acids. The sequence from 491–578 has a pI of 12.8 and shows similarity to the Ser, Pro-rich microtubule-binding domain of the microtubule-associated protein MAP4 (Fig. 1A, single underline) (49–51). The very C-terminal portion of CALK contains an acidic region that includes a PEST sequence (amino acids 738–755) with a PEST score of 27 (Fig. 1B, double underline) (49). Several consensus phosphorylation sites are present in the CALK sequence, including sites for cAMP-dependent protein kinase, protein kinase C, calcium/calmodulin-dependent protein kinase II, and p34cdc2 (not shown).

Southern blot hybridizations of Chlamydomonas genomic DNA with a nucleotide probe derived from calk genomic DNA (Fig. 3) indicated that CALK is encoded by a single copy gene. By use of RFLP mapping, the calk gene maps near the Gs2 and Lc3 loci on linkage group XII.2

Recombinant CALK Has Protein Kinase Activity—To determine if CALK had protein kinase activity, we evaluated the ability of the nearly full-length (lacking the N-terminal 11

2 P. Kathir, C. Silflow, and P. Lefebvre, personal communication.
CALK is encoded by a single copy gene. Genomic DNA was digested by KpnI, HindIII, and BamHI, separated on an agarose gel, transferred to a nylon membrane, and probed with a calc-specific genomic probe. DNA size markers showing molecular mass in kilobases was digested by KpnI. Myelin basic protein in the autoradiograph in Fig. 4, CALK underwent autophosphorylation and to phosphorylate casein and icasein kinase activity toward casein (Fig. 4).

CALK exists in two forms in vegetative cells and gametes of both mating types and is present primarily in cell bodies—To learn more about the cellular properties of CALK, we investigated its expression in vegetative cells and gametes. A rabbit polyclonal antiserum raised against a His-tagged, recombinant, truncated CALK containing the protein kinase domain was used in immunoblot analysis of whole cell lysates (see “Materials and Methods”). Fig. 5A shows that the anti-CALK antibody reacted with a protein doublet of 78/80 kDa in synchronously growing vegetative cells of both mating types and in gametes of both mating types. Antibody reactivity with the doublet was blocked when the primary antibody was absorbed with recombinant CALK protein, indicating the specificity of the antibody (Fig. 5B, bottom panel). As expected, the apparent molecular mass of the protein in the doublet was similar to the mass of CALK (80 kDa) predicted from the amino acid composition.

With few exceptions (see “Discussion”), most of the aurora/ Ipl1p family members are expressed only in dividing cells and are associated with microtubule-containing structures such as centrosomes and the mitotic spindle. On the other hand, even though Chlamydomonas gametes are nondividing cells (unless returned to N-containing medium), they expressed CALK. To determine if CALK was associated with one of the most prominent microtubule-containing structures in Chlamydomonas, the flagella, we analyzed the cellular distribution of CALK. Our results indicated that it was present primarily in cell bodies, with very low amounts detectable in flagella (Fig. 6).

CALK moves to the flagella during gamete activation—Previous studies from our laboratory have demonstrated that protein kinases play key roles in Chlamydomonas fertilization (10, 11, 15, 54, 55). To determine if CALK underwent changes during cell-cell interactions, we investigated the levels of this protein kinase in flagella of mt+ and mt− gametes undergoing fertilization. To do this, mt+ and mt− gametes were mixed together and at various times after mixing, we harvested the cells and isolated their flagella. Surprisingly, as shown in Fig. 7 (immunoblot, upper panel), gamete adhesion led to a rapid and striking accumulation of CALK in the flagella. Moreover, only the 78-kDa form of CALK appeared in the flagella (Fig. 7). The lower panel in Fig. 7 shows the immunoblot after staining with Coomassie Blue.

In this experiment with mt+ and mt− wild type gametes, phase contrast examination showed that nearly 70% of the cells had fused within 30 min after mixing. To test if CALK appearance in the flagella required cell fusion or if flagellar adhesion and gamete activation were sufficient to induce translocation, we examined flagellar levels of CALK during cell-cell adhesion of impI-15 mt+ gametes. These impotent cells undergo flagellar adhesion and gamete activation after being mixed with mt− gametes but are unable to fuse (56). When we mixed the gametes together, they underwent normal flagella adhesion (not shown), and immunoblotting showed that CALK translocated to the flagella (Fig. 8A). These results indicated that cell-cell fusion was not required for appearance of CALK in the flagella.

In earlier studies we showed that flagellar adhesion and gamete activation induce new protein synthesis (57). To determine if the appearance of increased CALK in the flagella during activation required synthesis of new proteins, we mixed mt+ and mt− gametes together in the presence of the protein synthesis inhibitor, cycloheximide, and then analyzed CALK by immunoblotting. The results shown in Fig. 8B demonstrated that cycloheximide treatment did not block the appearance of CALK in the flagella. Thus, the increased CALK in flagella during flagellar adhesion resulted from movement of pre-existing CALK and did not require synthesis of new CALK. Moreover, the results indicated that all of the cellular machinery required for CALK translocation was present in the gametes before they were mixed together.

CALK translocation can be induced by dibutyril cAMP—One of the important molecules in gamete activation is the cyclic nucleotide, cAMP, which undergoes 10-fold increases in cellular levels within minutes after gametes are mixed together. Because many of the cellular events that comprise gamete activation can be induced by incubation of gametes of a single mating type in dibutyril cAMP, we investigated the influence of this molecule on CALK. To do this, mt+ gametes were incubated with dibutyril cAMP and the phosphodiesterase inhibitor, papaverine; after 30 min, the cells were harvested and their flagella were isolated and immunoblotted for CALK. An assay for cell wall loss, one cellular response to
cAMP and an indicator of gamete activation, indicated that the dibutyryl cAMP/papaverine indeed induced gamete activation (data not shown). Moreover, as shown in Fig. 8C (upper panel), dibutyryl cAMP also induced movement of CALK to the flagella. These results indicated that flagellar adhesion per se was not required for translocation and also showed that CALK translocation is a unique property of gametes.

**DISCUSSION**

The current studies have uncovered a novel cellular mechanism in gamete activation, regulated translocation of a protein kinase into intact flagella. Whereas only small amounts of CALK were detectable in flagella isolated from nonadhering gametes, flagella of adhering gametes acquired significantly increased amounts of the aurora/Ipl1p-like protein kinase, amounts that approximated levels normally found in the cell body. Results from experiments with nonfusing mutant gametes and with gametes of a single mating type incubated with dibutyryl cAMP, however, we found that movement of CALK to the flagella was not induced (Fig. 8C, lower panel). These results indicated that cAMP-induced CALK translocation is a unique property of gametes.

The initial experiments with adhering wild type mt+ and mt− gametes showed that CALK appeared in the flagella very soon after the cells were mixed, becoming detectable within 1 min after mixing, and appearing in significant quantities by 5 min after mixing. That only the 78-kDa form of CALK was translocated remains enigmatic. It is possible that both forms were translocated but that the 80-kDa form underwent a post-translational modification that rendered it unable to bind to the anti-CALK antibodies. This seems unlikely, though, because the anti-CALK antibody is a polyclonal antibody raised against the entire protein kinase domain of the protein. Another possibility is that both forms were translocated, and the 80-kDa form was rapidly converted to the 78-kDa form in the flagella.
flagella, or the 80-kDa form may not be able to interact with the cellular machinery that is responsible for translocation. Future experiments examining the mechanisms of translocation should help to distinguish among these and other possible explanations for the apparent selectivity of the translocation process.

Studies with an impotent, nonfusing mutant demonstrated that cell fusion was not required for CALK movement. When imp1–15 mt+ gametes were mixed with wild type mt– gametes, the appearance of CALK in the flagella was indistinguishable from that observed in wild type cells (Fig. 8A). These results indicating that CALK translocation was part of gamete activation and did not require cell fusion were confirmed in related experiments. Incubation of mt+ gametes in dibutyryl cAMP for 30 min also led to movement of CALK to the flagella (Fig. 8C). Moreover, the effects of dibutyryl cAMP were unique to gametes. Incubation of vegetative cells in this cyclic nucleotide did not induce CALK translocation to the flagella (Fig. 8C).

Because Chlamydomonas gametes are transcriptionally active, it was possible that the appearance of CALK in the flagella was because of targeting of newly synthesized protein to these organelles. This possibility was ruled out by the experiments showing that CALK appeared in the flagella of gametes that were mixed together in the presence of the protein synthesis inhibitor, cycloheximide (Fig. 8B). Thus, CALK appearance in the flagella was because of movement of pre-existing cell body CALK into the flagella. Moreover, the results showed that the cellular machinery required for translocation also was present in unactivated gametes.

**CALK Is an Aurora/Ipl1p-like Protein Kinase—**Database searches and analysis by protein alignment methods indicated that CALK is a member of the aurora/Ipl1p family of serine-threonine protein kinases (Figs. 1 and 2). Aurora/Ipl1p-related protein kinases (or AIRKs, as suggested by Giet and Prigent (40)) are present in *S. cerevisiae*, *C. elegans*, *Drosophila melanogaster*, *Xenopus laevis*, mouse, rat, and humans; AIRK sequences also are present in *Arabidopsis* (accession numbers AC003680.1 and AC0053851) and *Schizosaccharomyces pombe* (accession number AL022445.2). In those cells in which AIRKs have been studied, these protein kinases are localized in centrosomes, the midbody, and at the poles of the bipolar spindle in mitotic cells and are necessary for completion of many mitotic events. The overexpression of AIRKs in several tumor cell types and the observations that ectopic expression of AIRKs in rat cells and human cells produces a transformed phenotype also indicate that AIRKs play key roles in cell division. Experiments with the *Xenopus* AIRK pEg2 have shown that the protein binds to spindle microtubules in the cell in anaphase and to taxol-stabilized microtubules in vitro. Moreover, dominant negative constructs of the AIRK pEg2 provoke inhibition of mitotic spindle assembly in *Xenopus* egg extracts. To date, the only known substrate for any AIRK is the *Xenopus* oocyte kinesin-related protein, XIEg5 (40), a motor protein also known to be involved in assembly of a bipolar spindle (44, 61, 62).

The absence of a putative signal sequence or transmembrane domain in the CALK sequence was consistent with the idea that, like all the other aurora/Ipl1p protein kinases studied, CALK is a cytoplasmic protein. Most of the AIRKs have been found only in cells in mitosis, although aurora2 (also known as STK15 and BTAk) (53) localizes to centrosomes in interphase cells in addition to being found at each spindle pole during mitosis. Because our experiments were carried out with synchronized cells that were not in mitosis, we do not know whether *Chlamydomonas* cells express CALK during cell division. The vegetative cells used in our studies were harvested around the middle of the light period of the light/dark cycle and were in the G1 phase of the cell cycle (63). Such synchronously growing cells undergo mitosis only during the dark period. Moreover, gametes are in the G1 phase of their life cycle and remain in an undividing state for weeks unless a nitrogen source is added back to their medium (64). Thus, CALK has been recruited by *Chlamydomonas* for uses not previously described for AIRKs.

The molecular mass of CALK and several features of its sequence also distinguish it from other AIRKs. For example, the CALK sequence is identical in only 11 of 16 (69%) of the amino acids between subdomains VII and VIII of the protein kinase catalytic domain that Giet and Prigent (40) identified as a consensus sequence signature domain for AIRKs (marked by asterisks in Fig. 1B). Even the *Arabidopsis* and *S. pombe* sequences have substitutions in at most two of the signature sites. This particular divergence of the CALK protein kinase domain from that of the other AIRKs is one example of the overall divergence reflected in the tree shown in Fig. 2A.
The nearly 2-fold larger molecular mass of CALK compared with other AIRKs suggests that the nonprotein kinase region, mostly in the C terminus (Fig. 2B), contains domains that are important for its nonmitotic functions. For example, the putative microtubule-binding domain might be important for the gamete activation-associated localization to gametic flagella. In addition, whereas CALK does not have the putative D-box (degradation box) set of consensus amino acids found in the protein kinase domain of other AIRKs (40), the presence of a putative PEST sequence (Fig. 2B) at the C terminus of CALK suggests that proteolysis might be an important aspect of its regulation. It is also possible that the C terminus is involved in regulation of the protein kinase activity of CALK. The recombinant, nearly full-length CALK that was used for the in vitro protein kinase assays showed a much greater preference for myelin basic protein as a substrate than casein (Fig. 4). Assays using the truncated form of CALK should indicate if the C terminus plays a role in this specificity.

The immunoblot result showing ~78- and ~80-kDa forms of CALK in cells (Fig. 5) was unexpected, and the presence of the two antigens remains unexplained. It is unlikely that the two forms of CALK are different gene products, because Southern blot analysis was consistent with a single calk gene in Chlamydomonas (Fig. 3). The two proteins might arise from differential splicing, an internal ATG start site, or posttranslational modifications such as proteolysis, phosphorylation, ubiquitination, or addition of lipid moieties.

CALK and Gamete Activation during Fertilization—It is likely that the function of CALK in flagella is more related to the sensory and signaling properties of Chlamydomonas flagella than to their motile functions (65, 66). For example, it could be one of the protein kinase activities implicated in known steps in gamete activation and fertilization. As discussed in the Introduction, our laboratory has shown that the coupling of mt+ and mt− agglutinin interactions to activation of adenyl cyclase during flagellar adhesion depends on the activity of several protein kinase activities. In nonadhering gametes, the gamete-specific, non-G protein-dependent adenyl cyclase is inhibited by a membrane-bound protein kinase and agglutinin interactions relieve this inhibition (10, 11). A second protein kinase activity is required for adhesion-induced activation of adenyl cyclase (11), and a third protein kinase activity regulates the phosphorylation of a fourth, soluble protein kinase, SksC (21, 55). In other experiments Pan et al. (67) have shown that protein phosphorylation is involved in the light-dependent regulation of agglutinin activity.

In addition to those just described, other gamete-specific events could require CALK. For example, CALK may be involved in agglutinin mobilization during gamete activation. Indirect evidence has been presented from several groups that the agglutinin undergoes gamete activation-induced increases on the flagella. Greater than 90% of the total cellular agglutinin molecules are present in an inactive or cryptic form associated with the cell bodies of unactivated gametes. During gamete activation, levels of cell body agglutinins fall and flagellar adhesiveness increases, providing indirect evidence that the agglutinins are moving from the cell body to the flagella (6, 9, 12, 68). The flagellar tips of gametes, which are normally tapered, become bulbous as amorphous material accumulates just underneath the flagellar membrane during gamete activation, and the A microtubules of the outer doublets lengthen (69). This process, termed flagellar tip activation, also coincides with accumulation of flagellar adhesion sites at the flagellar tips (68). It is possible that CALK participates in flagellar tip activation and becomes localized at the flagellar tips in association with agglutinin or even with adenyl cyclase.

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