UvA-DARE (Digital Academic Repository)

Characterisation and cloning of a calmodulin-like domain protein kinase from *Chlamydomonas moewusii* (Gerloff)

Siderius, M.; Henskens, H.; Porto-leBlanche, A.; van Himbergen, J.; Musgrave, A.; Haring, M.

DOI

[10.1007/s004250050105](https://doi.org/10.1007/s004250050105)

Publication date

1997

Document Version

Final published version

Published in

Planta

[Link to publication](#)

Citation for published version (APA):

Siderius, M., Henskens, H., Porto-leBlanche, A., van Himbergen, J., Musgrave, A., & Haring, M. (1997). Characterisation and cloning of a calmodulin-like domain protein kinase from *Chlamydomonas moewusii* (Gerloff). *Planta*, 202(1), 76-84.
<https://doi.org/10.1007/s004250050105>

General rights

It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations

If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: <https://uba.uva.nl/en/contact>, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.

UvA-DARE is a service provided by the library of the University of Amsterdam (<https://dare.uva.nl>)

Characterisation and cloning of a calmodulin-like domain protein kinase from *Chlamydomonas moewusii* (Gerloff)

Marco Siderius, Hans Henskens, Annette Porto-leBlanche, John van Himbergen, Alan Musgrave, Michel Haring

Institute for Molecular Cell Biology, Biocentrum Amsterdam, University of Amsterdam, Kruislaan 318, NL-1098 SM Amsterdam, The Netherlands

Received: 9 July 1996 / Accepted: 13 November 1996

Abstract. Calcium-stimulated protein kinase activity in the flagella of the green alga *Chlamydomonas moewusii* (Gerloff) was characterised. Using SDS-PAGE and an on-blot phosphorylation assay, a 65-kDa protein was identified as the major calcium-stimulated protein kinase. Its activity was directly stimulated by calcium, a characteristic of the calmodulin-like domain protein kinases (CDPKs). Monoclonal antibodies raised against the CDPK α from soybean cross-reacted with the 65-kDa protein in the flagella, and also with other proteins in the flagellum and cell body. The same monoclonal antibodies were used to screen a *C. moewusii* cDNA expression library in order to isolate CDPK cDNAs from *C. moewusii*. The CCK1 cDNA encodes a protein with a kinase and calmodulin-like domain linked by a junction domain typical of CDPKs. From Southern analyses, evidence was obtained for a CDPK gene family in *C. moewusii* and *C. reinhardtii*.

Key words: Calcium – Calmodulin-like domain protein kinase – *Chlamydomonas* – Flagellum – Gamete – Phosphorylation

Introduction

Calcium is an important cellular signal involved in the control of various processes in animal and plant cells. Since many cellular processes are controlled by reversible phosphorylation of proteins, calcium-regulated protein

kinases and phosphatases are implicated in transducing calcium signals by altering the phosphorylation status and, as a consequence, the enzymatic activity, localisation or binding characteristics of their substrates.

The unicellular green alga *Chlamydomonas* is a model system to study calcium signalling, since calcium is implicated in the control of several cellular processes such as flagellar beating (Witman 1993), surface transport of glycoproteins over the flagellum (Bloodgood 1991), de-flagellation (Quarmby and Hartzell 1994) and sexual signalling (Musgrave 1993; Quarmby 1994). *Chlamydomonas* cells are able to increase the cytoplasmic concentration of calcium either via rhodopsin-activated channels in the cell body, voltage-gated channels on the flagella (Hartz and Hegemann 1991; Beck and Uhl 1994) or by activating phospholipase C which hydrolyses phosphatidylinositol 4,5-bisphosphate into diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (InsP₃; Musgrave et al. 1992, 1993; Quarmby and Hartzell 1994). The InsP₃ can then induce the release of calcium from internal stores by binding to the InsP₃ receptor (Berridge 1993). Potential InsP₃-sensitive stores of calcium have been characterised in *Chlamydomonas* (Kaska et al. 1985; Siderius et al. 1996).

There are reports of proteins that respond to changes in calcium concentration in *Chlamydomonas*. Calmodulin has been characterised in flagella and cell bodies (Gitelman and Witman 1980). Furthermore, centrin (caltractin), a calcium-binding contractile protein, has been implicated in deflagellation (Sanders and Salisbury 1994) and has been cloned (Huang et al. 1988). Calcium-stimulated protein kinase and phosphatase activities have been proposed in the regulation of flagellar beating (Segal and Luck 1985) and surface transport of glycoproteins along the flagellum. Bloodgood (1992) suggested that a calcium-stimulated protein kinase activity in flagella of *C. reinhardtii* is involved in this mechanism. Since transport of agglutinins is an important sexual response for properly aligning gametes for cell fusion (Goodenough and Jurivich 1978; Homan et al. 1987), we set out to identify comparable kinase activity in *C. moewusii* gametes. Here we describe the characterisation of

Abbreviations: CDPK = Calmodulin-like domain protein kinase; DAG = diacylglycerol; FITC = fluorescein isothiocyanate; GST = glutathione S-transferase; InsP₃ = inositol 1,4,5-trisphosphate; IPTG = isopropyl β -D-thiogalactopyranoside; mAb = monoclonal antibody; PCR = Polymerase chain reaction; PVDF = polyvinylidene difluoride; TCA = trichloroacetic acid

Correspondence to: A. Musgrave; FAX: 31 (20) 5257934; E-mail: musgrave@bio.uva.nl

a calcium-stimulated protein kinase in the *Chlamydomonas* flagellum that is similar to calmodulin-like domain protein kinases (CDPKs; Roberts and Harmon 1992). A monoclonal antibody (mAb) raised against the CDPK α from soybean (Putnam-Evans et al. 1989) was used to examine the presence of this type of protein kinase in *Chlamydomonas*. The mAbs were then used to screen a *C. moewusii* cDNA expression library to isolate genes coding for CDPKs, thereby establishing their presence in this green alga.

Materials and methods

Cell cultures. *Chlamydomonas moewusii* (Gerloff) gametes (strains UTEX 10 and 17.17.2; Schuring et al. 1987) were grown in Petri dishes on agar-containing medium (Mesland and van den Ende 1978). Cultures were maintained at 20 °C in a 12 h light/12 h dark regime. An average light intensity of 2500 lx was provided by Philips (Eindhoven, The Netherlands) TL 65 W/33 fluorescent tubes. Gamete suspensions were obtained by flooding three- to four-week-old agar cultures with 10 mM Hepes (pH 7.4), 1 mM MgCl₂, 1 mM CaCl₂ and 1 mM KCl (HMCK).

Isolation of flagella and flagellar material. Flagella were isolated by pH-shock (Witman et al. 1972). Flagellar membrane vesicles, formed during deflagellation, were isolated as described in Kalshoven et al. (1990). Flagellar extracts were made by extracting isolated flagella with 1% Triton X-100 in buffer A [20 mM Hepes, pH 7.4; 5 mM MgCl₂; 1 mM dithiothreitol (DTT); 2 mM EGTA; 25 mM KCl] in the presence of the protease inhibitors phenylmethylsulphonyl fluoride (PMSF, 1 mM), pepstatin, leupeptin and aprotinin (each 5 $\mu\text{g} \cdot \text{ml}^{-1}$).

In-vitro phosphorylation. Flagellar extracts (20 μl) were incubated in 20 μl calcium/EGTA buffer containing 50 μM ATP, [γ -³²P]ATP (55 kBq $\cdot$ [μmol ATP]⁻¹) and 100 μM Na₃VO₄ resulting in 20 μM free calcium (+ Ca²⁺) or < 10⁻⁸ M free calcium (- Ca²⁺) as described in Bloodgood (1992). Incorporation of radioactivity was terminated by adding 1 ml of 10% trichloroacetic acid (TCA), 1 mM sodium pyrophosphate and 5 μg bovine serum albumin (BSA). The protein was then precipitated on ice for 15 min. The precipitate was washed with 10% TCA and 1 mM sodium pyrophosphate and taken up in 10 μl of 100 mM NaOH and an equal volume of SDS-PAGE sample buffer. The incorporation of label into the TCA precipitate was quantitated by liquid scintillation spectrometry. Labelled proteins were visualised by autoradiography after SDS-PAGE on 2–20% gradient gels.

On-blot phosphorylation assay. The protocol described by Ferrell and Martin (1991) was used with slight modifications. Proteins were separated by SDS-PAGE and blotted onto Immobilon polyvinylidene difluoride (PVDF) membrane filters (0.45 μm pore size; Millipore B.V., Etten-Leur, The Netherlands) for 1.5 h at 50 V $\cdot$ cm⁻¹ (4 °C). Filters were sequentially rinsed with methanol, water and transfer buffer (192 mM glycine base, 25 mM Tris base) prior to blotting. After blotting, they were incubated for 1 h in denaturation buffer (7 M guanidine hydrochloride, 50 mM Tris, 50 mM DTT, 2 mM EDTA, pH 8.3) at room temperature. The filters were then rinsed in Tris-buffered saline (pH 7.4) and incubated overnight at 4 °C while being gently rocked in renaturation buffer [140 mM NaCl; 10 mM Tris-HCl, pH 7.4; 2 mM DTT; 2 mM EDTA; 0.1% Nonidet P-40, Fluka, Amsterdam, The Netherlands]. They were then incubated for 1 h in renaturation buffer with calf thymus histone IIIs (1 mg $\cdot$ ml⁻¹) as kinase substrate. The renaturation buffer was removed and the blot covered with freshly prepared reaction buffer (30 mM Tris-HCl, pH 7.4; 10 mM MgCl₂, 2 mM MnCl₂; 3.7 MBq $\cdot$ ml⁻¹ [γ -³²P]ATP). The reaction was carried out in test tubes for 30 min at room

temperature. Filters were then washed sequentially (10 min per wash) in 30 mM Tris-HCl, pH 7.4 (2 $\times$); 30 mM Tris-HCl, pH 7.4, 0.05% Nonidet P-40 (2 $\times$); 1 M KOH and lastly 30 mM Tris-HCl, pH 7.4 (2 $\times$). Phosphorylated bands were visualised by autoradiography.

Western blotting and immunofluorescence. The anti-CDPK α mAb, kindly provided by Dr. A.C. Harmon (University of Florida, Gainesville, Fla., USA), was used as described by Putnam-Evans et al. (1989). Antibody-staining was visualised using a second antibody conjugated with horseradish peroxidase and visualised by enhanced chemoluminescence (ECL) according to the manufacturers protocol (Amersham's Hertogenbosch, The Netherlands).

Mating-structure balloons were induced by treatment of the 17.17.2 gametes with 8% ethanol (Schuring et al. 1990). Stimulated and non-stimulated cells were fixed in 1.5% glutaraldehyde, 0.5% formaldehyde and incubated 30 min on ice in phosphate-buffered saline (PBS) or PBS containing 1% Triton X-100. Cells were then incubated in PBS/3% BSA containing anti-CDPK α mAb for 1 h at room temperature. After washing with PBS, cells were incubated in PBS/3% BSA containing a fluorescein isothiocyanate (FITC)-labelled anti-mouse antibody for 1 h at room temperature. Excess FITC label was removed by washing with PBS. Cells were dried onto a coverslip and mounted in PBS/1% glycerol.

Construction, screening and sequence analysis of cDNA library. Polyadenylated RNA was isolated out of total RNA from a synchronous culture (0.5 h after the end of the dark period) of *C. moewusii* UTEX 10 [mating type minus (mt⁻)] and used to construct a cDNA library in λ ZAP-II (Stratagene, La Jolla, Cal., USA). The library (1.4 $\cdot$ 10⁵ plaque-forming units) was immunoscreened as described by the manufacturers with the mAb against the soybean CDPK α (Putnam-Evans et al. 1989). Subclones of one isolated cDNA clone (CCK1) were generated by digestion with exonuclease III (Promega Corporation, Madison, Wis., USA) or by restriction-enzyme digestion. The double-stranded sequence was determined using T₇ polymerase (Pharmacia) essentially as described by the manufacturers. The longest open reading frame on the cDNA was translated into an amino-acid sequence which was aligned with other sequences using the MacDNAsis program (Hitachi, Pharmacia, Tilburg, The Netherlands). The CCK1 sequence is present in the EMBL/Genbank database under accession number Z49233.

Analyses of DNA and RNA. *Chlamydomonas* DNA was isolated and used for Southern analysis using standard techniques (Maniatis et al. 1982). The [α -³²P]ATP-labelled DNA probes were made by the random prime method (Feinman and Vogelstein 1983) using gel-purified DNA fragments (GeneClean; Bio 101 Inc., La Jolla, Cal., USA). Genomic DNA gel blots were hybridised with the [α -³²P]ATP-labelled 2001-bp PstI fragment (total coding sequence) of CCK1 at 55 °C and at 65 °C. The blots were washed under high-[0.1 $\times$ saline sodium citrate buffer (SSC) at 65 °C] and low-stringency (1 $\times$ SSC at 55 °C) conditions. Autoradiographs were exposed for 16–24 h.

Expression and purification of recombinant CCK1 protein. Polymerase chain reaction (PCR) primers were designed for the 5' coding region to include an *Xba* I restriction site to facilitate cloning (5'-GCTCTAGAGCCGATTTCGGCAGCAGATGGCAGC-3'). The 3' primer included the stop codon extended with a His6 tag and a *Xho* I site to facilitate cloning (5'-TGACTCGAGTCAGTGATGTGATGGTGATCAGGTTGCGCATCAT-CAG-3'). The PCR fragment generated with the CCK1 cDNA as template was cloned into the pGEX-KG vector (Guan and Dixon 1992) to generate an affinity sandwich construct (Binder et al. 1994) with a 5' glutathione S-transferase (GST) sequence and a 3' His tag. This construct was transformed into *Escherichia coli* DH5 α cells and the insert was checked by restriction-enzyme analysis. An overnight culture of these cells was diluted 15 times in 2 $\times$ YT (standard medium) and grown for about 2 h at 37 °C, after which they were diluted twice in

2 × YT and induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) for about 4–6 h. Induction of the CCK1 fusion protein was checked in whole-cell samples on Western blot with the anti-soybean CDPKα mAb. The protein was purified from inclusion bodies in *E. coli* by dissolving them in 6 M guanidine-HCl, followed by Ni²⁺-nitriloacetic acid (NTA) affinity chromatography as described by the manufacturer (Qiagen, Hilden Germany). The purified fusion protein was spotted onto PVDF membrane, kinase activity renatured, and protein kinase activity with histone IIIs as substrate was assayed on-blot in the presence and absence of calcium (Ferrell and Martin 1991). The incorporation of radioactive label into each spot was measured by liquid scintillation spectrometry after cutting the spots from the filter.

Results

Since calcium has been shown to regulate various flagellar processes, the initial characterisation of calcium-regulated protein kinases was confined to the flagella. An in-vitro phosphorylation assay was developed to analyse protein kinase activities in flagellar extracts. The in-vitro incorporation of ³²Pi from [γ-³²Pi]ATP into the TCA-precipitable fraction of the extract was time-dependent (Fig. 1A) and stimulated by calcium, with an optimum at a free calcium concentration of 20 μM (Fig. 1B). The increase in net protein phosphorylation activity did not result in hyperphosphorylation of

specific proteins in the extract, but in an overall increase in labelling.

In order to ascribe the in-vitro phosphorylation activity in flagellar extracts to specific protein bands, the proteins on Western blots were renatured and protein kinase activity detected using an on-blot phosphorylation assay (Ferrell and Martin 1991) in which histone IIIs was used as substrate. In this assay the labelling of a protein band can occur as a consequence of three different events: (i) phosphorylation of histone IIIs, that was applied over the whole blot, (ii) autophosphorylation of a renatured protein kinase or (iii) phosphorylation of a substrate co-migrating with the protein kinase in SDS-PAGE. By electrophoresing flagellar preparations in one broad lane over the width of a gel and, after blotting, cutting the membrane into strips, it was possible to analyse kinase activity in a single sample under various conditions. As is shown in Fig. 2 for both total flagella (Fig. 2; lanes 2, 4 and 6) and flagellar extracts (non-axonemal proteins, Fig. 2; lanes 8, 10 and 12), treatment of the blot membrane with histone IIIs enhanced labelling of specific bands. Bovine serum albumin could not substitute for histone IIIs. Labelling of a 65-kDa protein band (Fig. 2, see arrowhead) in the flagella and flagellar extract was clearly stimulated by calcium in the presence of histone IIIs (Fig. 2; compare lanes 2 and 4, and 8 and 10). It seems to be the major calcium-dependent protein kinase in the flagella and completely dominates activity in the extract. However, this assay is dependent upon the ability to renature activity on the blot and the capacity of a kinase to phosphorylate histone IIIs, and therefore some protein kinases may have been overlooked. The lack of a band of radioactivity at 65-kDa in the absence of histone IIIs

Fig. 1A,B. Stimulation of in-vitro protein phosphorylation in flagellar extracts by calcium. **A** Extracts of gametic flagella were used in an in-vitro phosphorylation assay plus (20 μM, closed squares) and minus calcium (< 10⁻⁸ M, open squares). **B** Various calcium concentrations were tested for stimulation of the ³²Pi incorporation into the TCA-precipitable fraction within 5 min. Equal amounts of the TCA precipitate were loaded onto the gel and incorporation of ³²Pi into the proteins was visualized by autoradiography

Fig. 2. On-blot phosphorylation of *C. eugametos* flagellar proteins. Total flagella protein (lanes 1–6) or 1% Triton X-100 flagellar extracts (lanes 7–12) were separated on a 2–20% SDS-polyacrylamide gel, then blotted onto PVDF membrane. Protein kinase activity was renatured, the membrane was cut into filter strips (1–12), and on-blot phosphorylation was carried out in the presence of EGTA, calcium (20 μM), calmodulin (15 μg · ml⁻¹), and histone IIIs (1 μg · μl⁻¹) as indicated. The arrow indicates the major renaturable, calcium-stimulated protein kinase activity at 65-kDa

indicates that this kinase cannot autophosphorylate under the applied conditions. This was confirmed by eluting a heavily labelled 65-kDa region from an assay blot. On subjecting the eluant to SDS-PAGE, only radiolabelled histone IIIs (30 kDa) was found and no radioactivity at 65-kDa. Addition of bovine brain calmodulin did not consistently increase labelling of the 65-kDa band and, what is more, the intensity of labelling in its absence (Fig. 2, + Calcium, – Calmodulin, lanes 4 and 10) suggests that calcium has a direct effect on the protein kinase. About seven other major bands of protein kinase activity were present in flagella, all of which were stimulated to a greater or lesser extent by calcium, except for the 40-kDa band which seemed unaffected.

Since the direct stimulation of protein kinase activity by calcium is typical of CDPKs (Harper et al. 1991; Roberts and Harmon 1992), a mAb directed against the soybean CDPK α (Putnam-Evans et al. 1989) was used to immunologically detect related proteins in extracts of *Chlamydomonas* flagella. A protein with an apparent MW of 65-kDa (Fig. 3, lane 3), co-migrating with the major calcium-stimulated protein kinase activity on blot (Fig. 3, lane 2), was detected in flagella, together with a minor band at about 80-kDa.

The presence of calcium-stimulated protein kinase activity, and cross-reactivity with an anti-CDPK mAb at the same position in the gel suggests the presence of CDPKs in *Chlamydomonas*. In order to analyse whether more cross-reacting CDPK-like bands were present, immunoblotting and immunofluorescence were performed on both flagellar and cell-body samples. Immunoblotting results illustrated that the immuno-reactive 65-kDa band was present in flagella but not detectable in cell bodies when equivalent amounts of protein were subjected to SDS-PAGE and blotted (Fig. 4, lanes 1 and 2). Much of that in flagella was readily extracted into Triton X-100 and seemed to be associated with the membrane, since purified flagellar membranes also contained a well-labelled 65-kDa band (Fig. 4A, lane 4).

Fig. 3. Cross-reaction of an anti-CDPK Mab with a 65-kDa protein kinase from *C. moewusii* gametic flagella. Proteins from isolated flagella were separated by SDS-PAGE and stained with Coomassie Brilliant Blue (lane 1), or blotted onto PVDF membrane and used in an on-blot phosphorylation assay in the presence of calcium (lane 2) or probed with the anti-CDPK α mAb (lane 3). Cross-reaction of the antibody was visualized using an anti-mouse alkaline phosphatase conjugate in an alkaline phosphatase assay with nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrate

Fig. 4A,B. Localization of antigens cross-reacting with an anti-CDPK α mAb in *C. moewusii* gametes. **A** Twenty microgram samples of protein from cell bodies (lane 1), total flagella (lane 2), Triton X-100 extract of flagella (lane 3) and flagellar vesicles (lane 4) were separated by SDS-PAGE, blotted onto PVDF membrane and probed with an anti-soybean CDPK α mAb (Putnam-Evans et al. 1989). Cross-reaction of the antibody was visualized using an anti-mouse antibody with a horseradish peroxidase conjugate using ECL for detection. **B** A similar procedure was followed, but this time using flagella (lane 1) and cell bodies (lane 2) from the same number of gametes (10^7 cells)

Immunofluorescence labelling of the flagella after, but not before permeabilizing the cells with Triton X-100, indicates that the labelled epitope lies within the flagellar membrane (Fig. 5A). Note that the fluorescence associated with the cell body is due to autofluorescence of the chlorophyll and not due to antibody labelling. Immunolabelling intact *C. moewusii* cell bodies is not possible because antibodies do not penetrate the cell walls. The presence of anti-CDPK epitopes within the protoplast

Fig. 5A,B. Immunofluorescent localization of CDPK antigens in *C. moewusii*. **A** Cells were fixed in 1.5% glutaraldehyde, 0.5% formaldehyde, then treated with 1% Triton X-100 for 30 min on ice. They were stained with anti-CDPK α , followed by anti-mouse FITC. Bar = 5 μ m. **B** Activated mating-structure balloons were formed by treating *C. moewusii* gametes with 8% ethanol. The cells were then stained as described above. Balloons are the smaller round fluorescent bodies at one end of each cell. Bar = 10 μ m

Having acquired evidence for the presence of CDPK antigens in *Chlamydomonas*, we set out to clone CDPKs using the anti-CDPK mAbs to screen a *C. moewusii* cDNA library. The 2633-bp cDNA (CCK1) isolated in this procedure contains an open reading frame after the first ATG sequence, coding for a protein of 591 amino acids (Fig. 6A). The characteristic polyadenylation signal for *Chlamydomonas* mRNAs, TGTAAG, is absent, for only a TGCAA sequence was found 14-bp upstream

Since the CCK1 gene is the first CDPK cloned from a lower plant, its sequence was compared with other representatives of this gene family, as well as with other protein kinases and calcium-binding proteins, in order to analyse the evolutionary conservation of the three domains. The CCK1 junction domain is 40–50% homologous with the junction domains of CDPKs from higher plants. In Fig. 7A, identical amino acids in the junction domain are boxed. The amino acids in this domain that have been implicated from studies with inhibitor peptides (Harmon et al. 1994) and deletion mutants (Harper et al. 1994) to be involved in regulating enzyme activity are conserved in the CCK1 junction domain, suggesting that its function is also conserved. When the kinase domains of CDPKs from higher plants and *Chlamydomonas* are compared with those of protein serine/threonine kinases, they clearly form a separate family (Fig. 7B), with calcium/calmodulin-dependent kinase II (CaMKII) and myosin light-chain kinase (MLCK) relatively more related to the CDPK family than to protein kinase A and C. The amino-acid sequence of the kinase domain in the algal CDPK is homologous to that of higher-plant types, ranging from 50% for the seed-development specific protein kinase SPK1 from rice (Kawasaki et al. 1993) to 57% (45.4%

Fig. 7A–C. Alignment of the CCK1 junction domain with that of other CDPKs and the relative homologies of the kinase- and calmodulin-like domains. **A** Three or more identical amino acids in the junction domains of CCK1, CDPK α (Harper et al. 1991), AK1 (Harper et al. 1993), AtCDPK1 (Urao et al. 1994), SPK1 (Kawasaki et al. 1993), ZmCDPK (Estruch et al. 1994) and DcCDPK (Suen and Choi 1991) are shown. **B** Phylogenetic relations of the kinase domains of the CDPKs from *Chlamydomonas* (CCK1), soybean (CDPK α), *Arabidopsis* (AK1) and carrot (DcCDPK) are shown compared with human PKA, PKC α (Finkenzeller et al. 1990), rat MLCK (Roush et al. 1988) and mouse calcium/calmodulin-dependent kinase II (Karls et al. 1992). **C** The calmodulin-like domains of four CDPKs are compared with the EF-hand-domains of wheat (*Ta*) calmodulin (Toda et al. 1985), *C. reinhardtii* (Cr) calmodulin (Zimmer et al. 1988) and *C. reinhardtii* (Cr) caltractin (Huang et al. 1988).

Fig. 8A–B. Expression, immunological characterization and isolation of the CCK1 fusion protein. **A** *Escherichia coli* cells, strain DH5 α containing the affinity-sandwich construct of CCK1, were grown and induced with IPTG. Total cell lysates of the non-induced (lane 1) and induced (lane 2) cells were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. **B** The same samples were blotted and probed with anti-CDPK α mAbs. **C** The His6-containing CCK1 protein was isolated with Ni^{2+} -NTA beads, and run on SDS-PAGE and stained with Coomassie Brilliant Blue. Molecular-weight standards are indicated on the right. Closed arrow indicates the position of the CCK1 protein, while the open arrow indicates that of the GST. **D** Isolated CCK1 was spotted onto PVDF filter and used in an on-blot phosphorylation assay. The incorporation of radioactivity in the presence (open squares) or absence of $20 \mu\text{M Ca}^{2+}$ (closed squares) is depicted

identity) for carrot DcCDPK (Suen and Choi 1991). The same high homology was found for the calmodulin-like domain ranging from 47% for the carrot CDPK to 50% (35.6% identity) for *Arabidopsis* AK1 (Harper et al. 1993). Interestingly, when the calcium-binding domains of calmodulin and caltractin (centrin) from *Chlamydomonas* are compared to those of CCK1, then the EF-hand domains in CCK1 are seen to be more closely related to those of higher plants than to those in other *Chlamydomonas* proteins, indicating that the CDPK family developed a distinct calcium-binding domain early in evolution.

To determine whether the CCK1 protein possesses calcium-stimulated kinase activity, a PCR fragment containing the entire coding region of CCK1, extended with a His6 tag at the carboxy-terminus, was cloned into the pGEX-KG expression vector (Guan and Dixon 1992) to generate an affinity-sandwich construct (Binder et al. 1994) that allowed us to rapidly purify the protein. This was done because CDPKs are known to be broken down rapidly and proteolytic fragments seriously interfere with measurements of calcium-stimulated kinase activity (Harper et al. 1994). When the *E. coli* DH5 α cells harbouring the expression construct were induced with IPTG, a protein of 55-kDa appeared (Fig. 8A, compare lanes 1 and 2, upper arrow). A protein migrating at the same position as free GST protein also appeared (lower arrow), suggesting that the GST affinity

tag had been lost from some of the expressed protein. Using Ni^{2+} affinity chromatography, a 55-kDa protein was partially purified (Fig. 8C, arrow) that was recognised by the anti-CDPK antibodies (Fig. 8B, lane 2). In non-induced samples only a weak cross-reacting band was detected (Fig. 8B, lane 1). The molecular weight of the purified protein was 55-kDa and not 90-kDa as expected for the fusion protein. This, together with the presence of lower-molecular-weight bands that were recognised by the anti-CDPK mAbs and the presence of free GST tag, indicate that the CCK1 protein was partially broken down. To test the protein for calcium-stimulated protein kinase activity, increasing amounts of the affinity purified-protein isolated from *E. coli* inclusion bodies (Fig. 8C) were spotted onto PVDF membrane, and kinase activity renatured on-blot using histone IIIs as substrate in the presence and absence of calcium (Ferrell and Martin 1991). The incorporation of radioactive label at each spot was measured. The activity of the CCK1 protein was clearly stimulated by the presence of calcium (Fig. 8D), reminiscent of the in-vitro activity isolated from *Chlamydomonas* (Fig. 1).

Since we detected several proteins cross-reacting with the anti-CDPK mAbs in *C. moewusii* cells, genomic DNA gel blot analysis was performed to confirm the presence of a family of related genes in this organism. The distantly related species *C. reinhardtii* was included in the analysis to assess whether the algal CDPK genes had been conserved during evolution. Using the CCK1 5' 2001-bp *Pst* I fragment containing the coding region, several bands were detected on both *C. moewusii* and *C. reinhardtii* blots, when hybridised at low stringency (Fig. 9A). Washing the blot at high stringency reduced the number of bands (Fig. 9B), suggesting that at least two other CDPK isoforms are present in both species.

Discussion

In order to characterise *C. moewusii* proteins involved in calcium signalling, an in-vitro phosphorylation assay was used to detect calcium-stimulated protein kinases in

Fig. 9A,B. Southern analysis of genomic DNA from *C. moewusii* and *C. reinhardtii*. Genomic DNA of *C. moewusii* and *C. reinhardtii* (mt⁻) was digested with *Bam*HI (B), *Pst*I (P) and *Sac*I (S), and hybridized with the ³²P-labelled 5' *Pst*I fragment of the CeCDPK1 cDNA. Hybridization was performed under non-stringent (55 °C, 0.5 × SSC, A) or highly stringent conditions (65 °C, 0.1 × SSC, B)

extracts of gamete flagella. Since such a test only gives an idea of the total protein kinase plus phosphatase activity, we used an on-blot phosphorylation assay to characterise individual protein kinases. In this way a major band of calcium-stimulated activity was found to be located at 65-kDa. This kinase was the most prominent calcium-stimulated protein kinase in our assay of extracts of *C. moewusii* gametic flagella, although one should bear in mind that other, perhaps more prevalent protein kinases may not have been renatured on-blot. Calcium appeared to stimulate protein kinase activity directly as has been described for a relatively new family of protein kinases, CDPKs, that are present in plants and protists (Roberts and Harmon 1992; Zhao et al. 1993). The 65-kDa protein was immunologically characterised as CDPK-like using mAbs raised against the CDPK α from soybean. As was shown by immunoblotting experiments, the immuno-reactive 65-kDa flagellar protein was the most abundant cross-reacting protein in flagella, but was a minor component when compared with the cross-reacting bands in cell bodies. The possibility of multiple CDPKs in *Chlamydomonas* suggests a multifarious role for this type of protein kinase in calcium-controlled signalling routes. *Chlamydomonas* gametes contain calcium channels in their flagella as well as in their cell bodies (Hartz and Hegemann 1991) and the InsP₃/DAG/calcium signalling system has also been shown to be present (Musgrave 1993). Different CDPK isoforms could therefore function in the transduction of calcium signals at different locations in the cell. Also in higher plants, a multitude of CDPKs have been found at different locations in the whole plant and in plant cells (Roberts and Harmon 1992). Many CDPKs have already been cloned from *Arabidopsis* (Harper et al. 1993; Urao et al. 1994), therefore families of CDPKs must be expected in other plants as well.

Immuno-gold labelling studies have shown CDPKs to be present in most regions of the cytoplasm (data not shown), and this was reflected in the presence of the antigens in mating structure balloons. Interestingly, actin has been shown to be concentrated in these

structures (Kalshoven et al. 1994); therefore, an interaction between CDPKs and the cytoskeleton is possible. The presence of CDPKs in pollen tubes of maize (Estruch et al. 1994), where the actin cytoskeleton is implicated in outgrowth of these structures could indicate a common association of CDPKs with the cytoskeleton. In other plant systems, the interrelation between the cytoskeleton and CDPKs has also been studied (Putnam-Evans et al. 1989; McCurdy and Harmon 1992). In the green alga *Chara*, CDPK activity has been proposed to function in cytoplasmic streaming (McCurdy and Harmon 1992); thus, for example, the transport of cellular components via the cytoskeleton could be under the control of CDPK activity.

Using the anti-CDPK α mAbs, a CDPK cDNA was isolated from a *Chlamydomonas* library, suggesting that other antigens recognised in Western blots by the same antibody could be genuine CDPKs. Cloning CCK1 establishes the presence of CDPKs in lower plants. The protein encoded by this gene contains both conserved kinase-, junction- and calmodulin-like domains, indicating that this family of protein kinases was formed early in the evolution. A comparison of the EF-hands in the algal CDPK with those of other algal calcium-binding proteins such as caltractin and calmodulin and higher-plant calmodulin-like domains of CDPKs, again points to the early evolution of a distinct CDPK-type enzyme.

The CCK1 protein that was isolated by its carboxy-terminal His tag reacted with the anti-CDPK mAbs used in earlier studies and possessed protein kinase activity that was stimulated twofold by calcium. Nonetheless, the activity and the stimulation by calcium were low. This may have been due to partial breakdown because the protein was smaller than predicted (55 as opposed to about 90-kDa). Binder et al. (1994) also reported their inability to obtain a full-length protein when they expressed the *Arabidopsis* kinase-1 gene fused to that of GST. The protein constructs seem to be labile because we also witnessed the production of free GST protein. This was also observed when other *C. moewusii* proteins (e.g. VH-PTP13, Haring et al. 1995) were expressed as GST-fusions using this vector. Perhaps the glycine linker

that was created in order to release GST destabilizes the fusion. In order to further characterise the protein kinase activity of CCK1, another expression construct should be generated to ensure purification of the intact protein.

The open reading frame of CCK1 codes for a 65-kDa protein and a protein of the same size was detected in Western analyses of CDPKs in flagella. However, there is no reason to believe they are the same protein, for in general mRNA levels of flagellar proteins increase after deflagellation while the flagella are being regenerated (Bernstein et al. 1994). The CCK1 mRNA levels did not exhibit this pattern of expression after deflagellation, and the level was maintained in zygotes (data not shown) that do not have flagella.

High- and low-stringency Southern hybridisations of genomic DNA demonstrated that *C. moewusii* and *C. reinhardtii* possess at least two to three different CDPK isoforms. We think that their characterisation in these alga could help establish a role for CDPKs because Ca^{2+} -signalling has already been shown to be involved in photo-shock, deflagellation and the induction of mating responses in gametes. Thus CDPK expression and activity can be readily coupled to phenotypic responses.

We thank Dr. A.C. Harmon (Dept. Botany, University of Florida, Gainesville, Fla., USA) for the initial western analysis of *Chlamydomonas* flagella and for providing the anti-soybean CDPK α antibody. We also thank Peter van der Gulik for his help in the initial in-vitro phosphorylation experiments.

References

- Beck C, Uhl R (1994) On the location of voltage-sensitive calcium channels in the flagella of *Chlamydomonas reinhardtii*. *J Cell Biol* 125: 1119–1125
- Bernstein M, Beech PL, Katz SG, Rosenbaum JL (1994) A new kinesin-like protein (Klp1) localized to a single microtubule of the *Chlamydomonas* flagellum. *J Cell Biol* 125: 1313–1326
- Berridge MJ (1993) Inositol trisphosphate and calcium signalling. *Nature* 361: 315–325
- Binder BM, Harper JF, Sussman MR (1994) Characterization of an *Arabidopsis* calmodulin-like protein kinase purified from *Escherichia coli* using an affinity sandwich technique. *Biochemistry* 33: 2033–2041
- Bloodgood RA (1991) Transmembrane signalling in cilia and flagella. *Protoplasma* 164: 12–22
- Bloodgood RA (1992) Calcium-regulated phosphorylation of proteins in the membrane-matrix compartment of the *Chlamydomonas* flagellum. *Exp Cell Res* 198: 228–236
- Estruch JJ, Kadwell S, Merlin E, Crossland C (1994) Cloning and characterization of a maize pollen-specific calcium-dependent calmodulin-independent protein kinase. *Proc Natl Acad Sci USA* 91: 8837–8841
- Feinman AP, Vogelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* 132: 6–13
- Ferrell JE, Martin GS (1991) Assessing activities of blotted protein kinases. *Methods Enzymol* 200: 430–435
- Finkenzeller G, Marme D, Hug H (1990) Sequence of human protein kinase C α . *Nucleic Acids Res* 18: 2183
- Gitelman SE, Witman GP (1980) Purification of calmodulin from *Chlamydomonas*: calmodulin occurs in the cell bodies and flagella. *J Cell Biol* 98: 764–770
- Goodenough UW, Jurivich D (1978) Tipping and mating structure activation induced in *Chlamydomonas* gametes by flagellar membrane antisera. *J Cell Biol* 79: 680–693
- Guan K, Dixon JE (1992) Eukaryotic proteins expressed in *Escherichia coli*: an improved thrombin cleavage and purification procedure of fusion proteins with glutathione-S-transferase. *Anal Biochem* 192: 262–267
- Hanks SK, Quinn AM, Hunter T (1988) The protein kinase family: Conserved features and deduced phylogeny of catalytic domains. *Science* 241: 42–52
- Haring MA, Siderius M, Walton KM, Jonak C, Hirt H, Musgrave A (1995) Tyrosine phosphatase signalling in a lower plant: cell-cycle and oxidative stress-regulated expression of the *Chlamydomonas eugametos* VH-PTP13 gene. *Plant J* 7: 981–988
- Harmon A, Yoo B-C, McCaffrey C (1994) Pseudosubstrate inhibition of CDPK, a protein kinase with a calmodulin-like domain. *Biochemistry* 33: 7278–7287
- Harper JF, Sussman MR, Schaller GE, Putnam-Evans C, Charbonneau H, Harmon AC (1991) A calcium-dependent protein kinase with a regulatory domain similar to calmodulin. *Science* 252: 951–953
- Harper JF, Binder BM, Sussman MR (1993) Calcium and lipid regulation of an *Arabidopsis* protein kinase expressed in *Escherichia coli*. *Biochemistry* 32: 3282–3290
- Harper JF, Huang J-F, Lloyd SJ (1994) Genetic identification of an autoinhibitor in CDPK, a protein kinase with a calmodulin-like domain. *Biochemistry* 33: 7267–7277
- Hartz H, Hegemann P (1991) Rhodopsin-regulated calcium currents in *Chlamydomonas*. *Nature* 351: 489–491
- Homan WL, Sigon C, van den Briel W, Wagter R, de Wit D, Kolk A, van den Ende H (1987) Transport of membrane receptors and the mechanics of sexual cell fusion in *Chlamydomonas eugametos*. *FEBS Lett* 215: 323–326
- Huang B, Mengersen A, Lee VD (1988) Molecular cloning of a cDNA for caltractin, a basal body associated calcium-binding protein: homology in its protein sequence with calmodulin and the yeast CDC31 gene product. *J Cell Biol* 107: 133–140
- Kalshoven HW, Musgrave A, van den Ende H (1990) Mating receptor complex in the flagellar membrane of *Chlamydomonas moewusii* gametes. *Sex Plant Reprod* 3: 77–87
- Kalshoven HW, Brederoo J, Emons AMC, Musgrave A, Schuring F, Wells B, van den Ende H (1994) Actin in the mating structures of *Chlamydomonas eugametos* gametes. *Acta Physiol Plant* 91: 657–664
- Karls U, Muller U, Gilbert DJ, Copeland NG, Jenkins NA, Harbers K (1992) Structure, expression, and chromosome location of the gene for the beta subunit of brain-specific calcium/calmodulin-dependent protein kinase II identified by transgene integration in an embryonic lethal mouse mutant. *Mol Cell Biol* 12: 3644–3652
- Kaska DD, Piscopo IC, Gibor A (1985) Intracellular calcium redistribution during mating in *Chlamydomonas reinhardtii*. *Exp Cell Res* 160: 371–379
- Kawasaki T, Hayashida N, Baba T, Shinozaki K, Shimada H (1993) The gene encoding a calcium-dependent protein kinase located near the *sbe1* gene encoding starch branching enzyme I is specifically expressed in developing rice seeds. *Gene* 129: 183–189
- Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York
- McCurdy DW, Harmon AC (1992) Calcium-dependent protein kinase in the green alga *Chara*. *Planta* 188: 54–61
- Mesland DAM, van den Ende H (1978) The role of flagellar adhesion in sexual activation of *Chlamydomonas eugametos*. *Protoplasma* 98: 115–129
- Musgrave A (1993) Mating in *Chlamydomonas*. In: Round FE, Chapman DJ (eds) Progress in phycological research. Biopress Ltd, Bristol, pp 193–239

- Musgrave A, Kuin H, Jongen M, de Wildt P, Schuring F, Klerk H, van den Ende H (1992) Ethanol stimulates phospholipid turnover and inositol 1,4,5-trisphosphate production in *Chlamydomonas eugametos* gametes. *Planta* 186: 442–449
- Musgrave A, Schuring F, Munnik T, Visser K (1993) Inositol 1,4,5-trisphosphate as fertilization signal in plants: test case *Chlamydomonas*. *Planta* 171: 280–284
- Putnam-Evans C, Harmon AC, Palevitz BA, Fechtmeier M, Cormier MJ (1989) Calcium-dependent protein kinase is localized with F-actin in plant cells. *Cell Mot Cytoskel* 12: 12–22
- Quarmby LM (1994) Signal transduction in the sexual life of *Chlamydomonas*. *Plant Mol Biol* 26: 1271–1287
- Quarmby LM, Hartzell HC (1994) Two distinct, calcium-regulated, signal transduction pathways can trigger deflagellation in *Chlamydomonas reinhardtii*. *J Cell Biol* 124: 807–815
- Roberts DM, Harmon AC (1992) Calcium-modulated proteins: targets of intracellular calcium signals in higher plants. *Annu Rev Plant Physiol Plant Mol Biol* 43: 375–414
- Roush CL, Kennelly PJ, Glaccum MB, Helfman DM, Scott JB, Krebs EG (1988) Isolation of the cDNA encoding rat skeletal muscle myosin light chain kinase, sequence and tissue distribution. *J Biol Chem* 263: 10510–10516
- Sanders MA, Salisbury JL (1994) Centrin plays an essential role in microtubule severing during flagellar excision in *Chlamydomonas reinhardtii*. *J Cell Biol* 124: 795–806
- Schuring F, Smeenk JW, Homan WL, Musgrave A, van den Ende H (1987) Occurrence of O-methylated sugars in surface glycoconjugates in *Chlamydomonas eugametos*. *Planta* 170: 322–327
- Schuring F, Brederoo J, Musgrave A, van den Ende H (1990) Increase in calcium triggers mating structure activation in *Chlamydomonas eugametos*. *FEMS Microbiol Lett* 71: 237–240
- Segal RA, Luck DJ (1985) Phosphorylation in isolated *Chlamydomonas* axonemes: a phosphoprotein may mediate the Ca^{2+} -dependent photophobic response. *J Cell Biol* 101: 1702–1712
- Siderius M, Musgrave A, Koerten H, Cambier P, van der Meer P, van den Ende H (1996) *Chlamydomonas eugametos* (Chlorophyta) stores phosphate in polyphosphate bodies together with calcium. *J Phycol* 32: 402–409
- Suen KL, Choi JH (1991) Isolation and sequence analysis of a cDNA clone for a carrot calcium-dependent protein kinase: homology to carrot calcium/calmodulin-dependent protein kinases and to calmodulin. *Plant Mol Biol* 17: 581–590
- Toda H, Yazawa M, Sakiyama F, Yagi K (1985) Amino acid sequence of calmodulin from wheat germ. *J Biochem* 98: 833–842
- Urao T, Katagiri T, Mizoguchi T, Yamaguchi-Shinozaki K, Hayashida N, Shinozaki K (1994) Two genes that encode Ca^{2+} -dependent protein kinases are induced by drought and high-salt stresses in *Arabidopsis thaliana*. *Mol Gen Genet* 244: 331–340
- Witman GB (1993) *Chlamydomonas* phototaxis. *Trends Cell Biol* 3: 403–408
- Witman GB, Carlson K, Berliner J, Rosenbaum JL (1972) *Chlamydomonas* flagella. 1. Isolation and electrophoretic analysis of microtubules, matrix, membranes and mastigonemes. *J Cell Biol* 54: 507–539
- Zhao Y, Kappes B, Franklin RM (1993) Gene structure and expression of an unusual protein kinase from *Plasmodium falciparum* homologous at its carboxy terminus with the EF hand calcium-binding proteins. *J Biol Chem* 268: 4347–4354
- Zimmer WE, Schloss JA, Silflow CD, Youngblom J, Watterson DM (1988) Structural organization and expression of the calmodulin gene. *J Biol Chem* 263: 19370–19383