Ca^{2+}-binding proteins of cilia and infraciliary lattice of *Paramecium tetraurelia*: their phosphorylation by purified endogenous Ca^{2+}-dependent protein kinases

Kwanghee Kim[1], Min Son[2], Joan B. Peterson[2] and David L. Nelson[2,*]

[1]Department of Oncology, McArdle Lab, University of Wisconsin-Madison, Madison, Wisconsin 53706, USA
[2]Department of Biochemistry, College of Agricultural and Life Science, University of Wisconsin-Madison, Madison, Wisconsin 53706, USA
*Author for correspondence (e-mail: nelson@biochem.wisc.edu)

This paper is dedicated to the memory of André Adoutte, a great scientist and a wonderful man, who died 18 March 2002

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Summary

We purified two small, acidic calcium-binding proteins (*Paramecium* Ca^{2+}-binding proteins, PCBP-25α and PCBP-25β) from *Paramecium tetraurelia* by Ca^{2+}-dependent chromatography on phenyl-Sepharose and by anion-exchange chromatography. The proteins were immunologically distinct. Monoclonal antibodies against PCBP-25β did not react with PCBP-25α, and antibodies against centrin from *Chlamydomonas* reacted with PCBP-25α but not with PCBP-25β. Like the centrins described previously, both PCBPs were associated with the infraciliary lattice (ICL), a fibrillar cytoskeletal element in *Paramecium*. Both were also present in isolated cilia, from which they could be released (with dynein) by a high-salt wash, and both PCBPs cosedimented with dynein in a sucrose gradient. PCBP-25β was especially prominent in cilia and in the deciliation supernatant, a soluble fraction released during the process of deciliation. The results of immunoreactivity and localization experiments suggest that PCBP-25α is a *Paramecium* centrin and that PCBP-25β is a distinct Ca^{2+}-binding protein that confers Ca^{2+} sensitivity on some component of the cilium, ciliary basal body or ICL.

We characterized these proteins and *Paramecium* calmodulin as substrates for two Ca^{2+}-dependent protein kinases purified from *Paramecium*. PCBP-25α and calmodulin were in vitro substrates for one of the two Ca^{2+}-dependent protein kinases (CaPK-2), but only PCBP-25α was phosphorylated by CaPK-1. These results raise the possibility that the biological activities of PCBP-25α and calmodulin are regulated by phosphorylation.

Key words: Centrin, Phosphorylation, *Paramecium*, Infraciliary lattice, Cilia, Dynein

Introduction

Unicellular eukaryotes such as *Paramecium* are unusual among the eukaryotes in that in a single cell they have all of the machinery necessary to carry out all of the functions of the whole organism – feeding, digestion, energy production, excretion, secretion, ionic homeostasis, signal reception, motility and sexual reproduction. Like multicellular eukaryotes, *Paramecium* uses Ca^{2+} as a second messenger in many of these processes. To avoid confusing crosstalk among the Ca^{2+}-regulated processes, the protozoan cell must either segregate the processes in space or use different Ca^{2+}-sensing proteins to trigger different functions, or both. In *Paramecium*, the processes that are known or thought to depend on intracellular Ca^{2+} concentration include regulated secretion of the trichocyst contents (Kerboeuf et al., 1993; Plattner and Klauke, 2001), reversal of the ciliary power stroke (Naitoh and Kaneko, 1972; Preston and Saimi, 1990), opening of several ion channels (Preston, 1990), reorganization of the cortical surface during and after conjugation and cytokinesis (Beisson and Ruiz, 1992), cortical contraction (Garreau de Loubresse et al., 1988), motile response to extracellular chemoeffectors (Wright et al., 1993; Clark et al., 1997) and circadian regulation of swimming motility (Hasegawa et al., 1999). The frequency of the ciliary beat is set indirectly by Ca^{2+}, which activates adenylyl cyclase (Gustin and Nelson, 1987) and guanylyl cyclase (Klumpp and Schultz, 1982) to produce cyclic nucleotides that regulate the ciliary beat (Nakaoka and Ooi, 1985; Bonini and Nelson, 1988).

The diverse functions of Ca^{2+}-regulated processes are reflected in the variety of Ca^{2+}-binding proteins (CaBPs), all members of the EF-hand superfamily (Plattner and Klauke, 2001), found in *Paramecium*. Calmodulin (CaM) is present (Walter and Schultz, 1981; Momayezi et al., 1986) and is known from genetic studies to be involved in the regulation of several ion channels. Missense mutations in the single *CaM* gene of *Paramecium* lead to striking defects in ion channel function, regulation of the ciliary beat and swimming behavior (Preston et al., 1991). CaM is the Ca^{2+} sensor for guanylyl cyclase (Klumpp et al., 1983a; Schultz and Klumpp, 1984) and probably for adenylyl cyclase (Gustin and Nelson, 1987), and binding studies have identified a number of other CaM-binding proteins in cilia and cell bodies (Evans and Nelson, 1989; Chan et al., 1999).

The infraciliary lattice (ICL), a contractile network of cytoskeletal filaments in the cell cortex (Garreau de Loubresse et al., 1988), contains and largely comprises six small, acidic Ca^{2+}-binding proteins (Klotz et al., 1997) that probably account for its ability to contract in response to Ca^{2+}. The genes

for these proteins have been cloned and found to be closely related to those for centrins of other organisms (Madeddu et al., 1996). Klotz et al. (Klotz et al., 1997) showed that mutations in one of these centrin-like genes result in disordered cortical structure. Allen et al. (Allen et al., 1998) used the same anti-centrin antiserum that we used here, as well as a monoclonal antibody they raised against a M_r 110,000 protein of the striated band, to show definitively that the ICL was immunologically distinct from the striated band, another filamentous cytoskeletal system in *Paramecium*.

It has been proposed that the cytoskeletal rearrangements at cell division in *Paramecium* may be associated with a wave of Ca^{2+}-induced phosphorylation of cytoskeletal proteins (Sperling et al., 1991; Beisson and Ruiz, 1992). Two Ca^{2+}-dependent protein kinases [CaPK-1, CaPK-2; (Gundersen and Nelson, 1987; Son et al., 1993; Kim et al., 1998)] that are unlike any found in animal cells are found in *Paramecium*; they require micromolar Ca^{2+}, but neither CaM nor phospholipids, for their activity. Their activation by Ca^{2+} results from direct binding of Ca^{2+} to the kinase (Gundersen and Nelson, 1987; Son et al., 1993). Cloning and sequencing of the genes encoding *Paramecium* CaPKs (Kim et al., 1998) revealed that they, like other CaPKs (Harper et al., 1991), contain a CaM-like regulatory domain fused to the highly conserved protein kinase catalytic domain. The endogenous substrates for these kinases in *Paramecium* are not known, and their physiological roles in mediating Ca^{2+} signaling are uncertain.

While purifying these kinases from *Paramecium* (Gundersen and Nelson, 1987; Son et al., 1993), we observed that several Ca^{2+}-binding proteins of M_r 20,000-25,000 copurified with the kinases and were substrates for Ca^{2+}-dependent phosphorylation. Here we describe two of these Ca^{2+}-binding proteins, PCBP-25α (*Paramecium* Ca^{2+}-binding protein of 25 kDa) and PCBP-25β, and compare them with CaM as substrates for phosphorylation by the purified Ca^{2+}-dependent protein kinases from *Paramecium*.

Materials and Methods

Materials

ATP (disodium salt, grade I), histones, casein, 5-bromo-4-chloro-3-indolyl phosphate (*p*-toluidine salt), phenyl-Sepharose CL-4B, Ponceau S concentrate, nitro blue tetrazolium, phenylmethylsulfonyl fluoride (PMSF), N-*p*-tosyl-L-arginine methyl ester (TAME), leupeptin, melittin and calmidazolium were purchased from Sigma (St. Louis, MO). Goat anti-mouse IgG coupled to alkaline phosphatase was from Kirkegaard and Perry (Gaithersburg, MD). [γ-^{32}P]ATP was from Amersham (Arlington Heights, IL). The recombinant centrin and antiserum against bacterially expressed *Chlamydomonas* centrin were generously provided by J. L. Salisbury (Mayo Clinic Foundation, Rochester, MN). The recombinant protein POK-2 was overproduced in *E. coli* (Kink et al., 1991) from a clone provided by Kit-Yin Ling. *Paramecium* cGMP-dependent protein kinase was a gift from Kyoungsook Ann (Ann and Nelson, 1995). The subciliary fractions and the sucrose gradient-purified dyneins were prepared by Claire Walczak following the published procedure (Travis and Nelson, 1988a). CaPK-1 and CaPK-2 were purified as described previously (Gundersen and Nelson, 1987; Son et al., 1993).

Partial purification of PCBP-25

Cells were extracted, and the extract was fractionated on phenyl-Sepharose, exactly as previously described (Son et al., 1993). Proteins that bound to phenyl-Sepharose in Ca^{2+} and were eluted with EGTA were concentrated, then applied to an anion-exchange FPLC column (MonoQ HR 5/5, Pharmacia, Piscataway, NJ) equilibrated in 20 mM bis-Tris propane-Cl, pH 6.7, 0.1 M sucrose, 0.1 mM EDTA and 1 mM dithiothreitol (DTT). Protein was eluted first with a 24 ml gradient from 0 to 0.3 M NaCl, then with a 24 ml gradient from 0.3 to 1 M NaCl in the same buffer.

Preparation of fractions enriched for ICL

ICL-enriched fractions were prepared as described by Garreau de Loubresse et al. (Garreau de Loubresse et al., 1988).

Preparation of ciliary fractions

Cells were washed and immobilized in a 1:1 mixture of Dryl's solution [2 mM Na_2HPO_4, 2 mM sodium citrate, 1.5 mM $CaCl_2$, pH 6.8 (Dryl, 1959)] and STEN (0.5 M sucrose, 20 mM Tris-Cl, 2 mM EDTA, 6 mM NaCl, pH 7.5) using 20 ml of Dryl's/STEN per ml of packed cells. The cilia were detached by the addition of $CaCl_2$ and KCl to final concentrations of 10 mM and 30 mM, respectively, plus 0.3 mM PMSF and 1 μg/ml leupeptin. Deciliation was monitored by phase contrast microscopy. Cell bodies were separated from cilia by centrifugation at 850 ***g*** for 2 minutes. The subsequent subciliary fractionations were performed by published methods (Travis and Nelson, 1988a).

Phosphorylation assays

Protein kinase activities (CaPK-1 and CaPK-2) were measured using casein as described (Son et al., 1993). A unit of protein kinase activity corresponds to 1 pmol of $^{32}P_i$ incorporated into substrate per minute. Phosphorylation of PCBP-25α and PCBP-25β by purified *Paramecium* protein kinases was performed under the following conditions without casein. In brief, the reaction mixture for the CaPK-1 and CaPK-2 contained 20 mM HEPES-NaOH, pH 7.2, 5 mM magnesium acetate, 1 mM DTT, 0.5 mM EGTA±0.51 mM $CaCl_2$ and 20 μM [γ-^{32}P]ATP (100 Ci/mol) in a final volume of 50 μl. The reaction was carried out at 30°C for 10 minutes and stopped with 15% trichloroacetic acid. Acid precipitates were subjected to SDS-PAGE followed by autoradiography. Assays for synthetic peptides were performed under the same conditions as for protein substrates except that 50 μM [γ-^{32}P]ATP (50 Ci/mol) was used and the assays were performed at room temperature. 40 μl of the reaction mixture was spotted onto Whatman phosphocellulose paper (P81), which was dropped into 75 mM phosphoric acid to quench the reaction. After four washes in the same solution, the filters were dried and counted for adsorbed radioactivity.

Immunoblots

Proteins were separated by SDS-PAGE, transferred to nitrocellulose (0.1 μm) and probed with either undiluted monoclonal antibodies or the 1000-fold diluted polyclonal serum. Transferred proteins were visualized by staining with Ponceau S (Salinovich and Montelaro, 1986). Blots were incubated in 0.1 μg/ml alkaline-phosphatase-conjugated goat anti-mouse or goat anti-rabbit IgG antibody 1000-fold diluted in wash buffer, and the reaction was detected with substrate solution (0.2 mg/ml 5-bromo-4-chloro-3-indolylphosphate and 0.2 mg/ml nitro blue tetrazolium, 5% methanol in 0.7 M Tris, pH 9.5).

Electron microscopy

Axenically grown *Paramecium* cells were washed in Dryl's solution (Dryl, 1959), resuspended in 50 mM HEPES, pH 7.3 and concentrated by low-speed centrifugation (2000 ***g***). They were resuspended in 0.5% glutaraldehyde in 50 mM HEPES, pH 7.3, at room temperature for

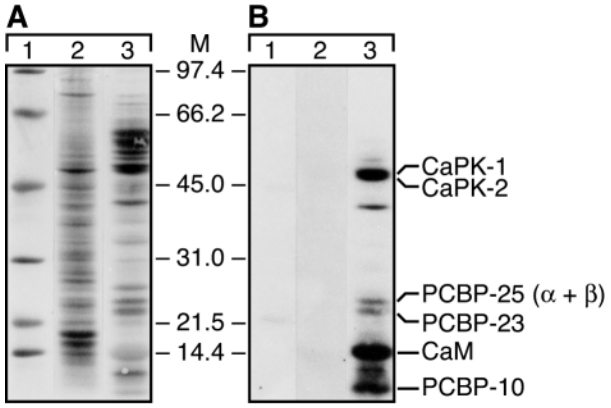

Fig. 1. Purification of Ca^{2+}-binding proteins on phenyl-Sepharose monitored by a $^{45}Ca^{2+}$ blot overlay assay. Samples were subjected to SDS-PAGE, transferred to nitrocellulose and probed with $^{45}Ca^{2+}$ as described in the Materials and Methods. The free Ca^{2+} concentration was 1.4 μM. After autoradiography (B), the membrane was stained with amido black (A). Lane 1, markers, 2 μg each; lane 2, phenyl-Sepharose load, 50 μg; lane 3, phenyl-Sepharose EGTA eluate, 20 μg; M, M_r of markers ($\times 10^3$).

1.5 hours. The cells were washed twice in buffer before incubation in 0.5% uranyl acetate for 30 minutes. They were washed once in distilled water, embedded in 1.5% agar at 40°C and then chilled on ice. The solidified agar pellet containing the cells was cut into small blocks. The cells were dehydrated in an ethanol series and embedded in LR White resin (EMS, Fort Washington, PA) according to the manufacturer's instructions. Silver-gray sections were cut on a Reichert Om U3 ultramicrotome and placed on Formvar-covered, carbon-coated gold mesh grids. Thin sections were contrasted with ethanolic uranyl acetate and lead citrate. Lead citrate staining was omitted in the immunolabeling studies.

For immunolabeling, sections were blocked in 10 mM Tris, 500 mM NaCl, 0.05% Tween-20, 1% bovine serum albumin and 0.02% sodium azide before transfer to buffer containing the primary antibody for 4 hours. Monoclonal antibody was undiluted tissue culture supernatant and the polyclonal antibody was diluted 800-fold in the wash buffer (10 mM Tris, 500 mM NaCl, 0.05% Tween-20, 0.3% bovine serum albumin and 0.02% sodium azide). Grids were washed in wash buffer and then incubated in goat anti-rabbit (or mouse) IgG linked to 10 nm gold particles diluted 1:30 (v/v) in blocking solution for 1 hour. Sections were stained for 10 to 30 minutes in 1% uranyl acetate. Micrographs were taken with a JEOL-100S electron microscope or with a Phillips 300 electron microscope.

Other procedures

$^{45}Ca^{2+}$ blot overlay assays (Maruyama et al., 1984) and silver staining (Poehling and Neuhoff, 1981) were performed as described by Son et al. (Son et al., 1993). The free [Ca^{2+}] was calculated using the COMICS program (Perrin and Sayce, 1967) translated to BASIC.

Results

Ca^{2+}-binding proteins of *Paramecium* resolved by Ca^{2+}-dependent chromatography on phenyl-Sepharose

While purifying two CaPKs from EGTA extracts of *Paramecium*, we found that several additional CaBPs copurified on phenyl-Sepharose. SDS-PAGE and $^{45}Ca^{2+}$ blot overlay assays revealed (Fig. 1) that the EGTA eluate was markedly enriched in CaBPs, including three that have been

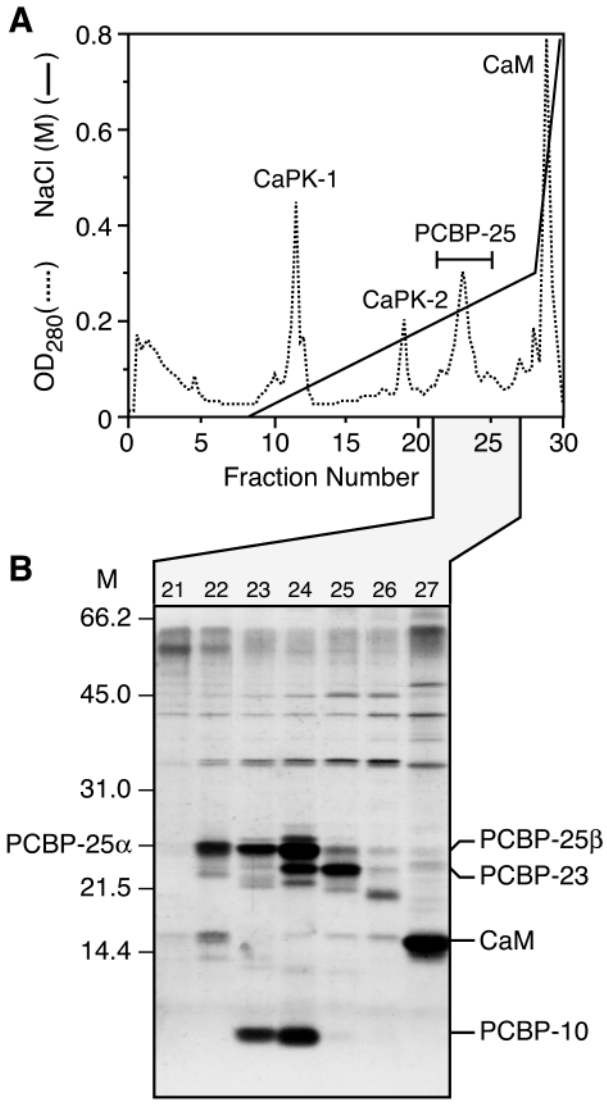

Fig. 2. Purification of PCBP-25 and CaM on MonoQ (A) and chromatographic separation of PCBP-25α and PCBP-25β visualized by silver stain (B). Chromatography was performed as described in the Materials and Methods. In A, the fractions containing PCBP-25 determined by on-blot $^{45}Ca^{2+}$-binding assay are indicated, as are the positions of two CaPKs and CaM. In (B), MonoQ fractions (15 μl each) were run on SDS-PAGE and silver stained as described in the Materials and Methods. The positions of Ca^{2+}-binding proteins and the fraction numbers are indicated. M, M_r of markers ($\times 10^3$).

previously described. Two proteins of M_r 52,000 and 50,000 were identified as CaPK-1 and CaPK-2, and a protein of M_r 17,000 was identified as CaM by their reactions with specific antibodies (Gundersen and Nelson, 1987; Son et al., 1993) (L. D. DeVito, MS thesis, University of Wisconsin-Madison, 1985). Three more unidentified bands of M_r 25,000, 23,000 and about 10,000 also bound Ca^{2+} strongly in the blot overlay assay. We refer to these CaBPs as PCBP-25 (α and β mixture), PCBP-23 and PCBP-10. In these experiments, the M_r markers served as negative controls; no binding above background was detected for any of these proteins, except the M_r 21,000 soybean trypsin inhibitor, for which others have also reported Ca^{2+} binding (Harmon et al., 1987). The PCBP-25 fractions

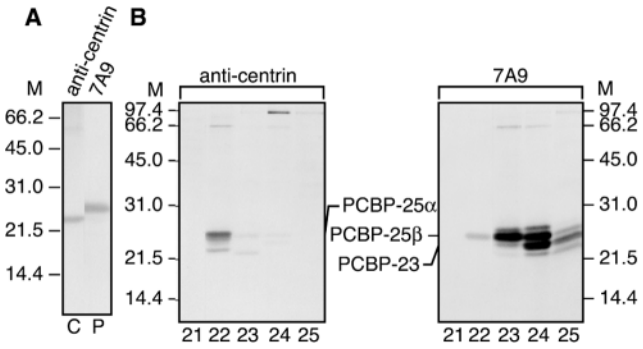

Fig. 3. Immunoblot analysis of (A) antibody specificity and (B) immunocrossreactivity of PCBP-25α and PCBP-25β. Samples were subjected to SDS-PAGE, transferred to nitrocellulose and probed with either polyclonal antibody against *Chlamydomonas* centrin or with monoclonal antibody 7A9 as indicated. (A) Lane C, *Chlamydomonas* whole cell, 15 μg; lane P, quick-killed *Paramecium*, 20 μg. (B) MonoQ fractions (20 μl) containing PCBP-25. Fraction numbers are indicated at the bottom of the blot. M, M_r of markers ($\times 10^3$).

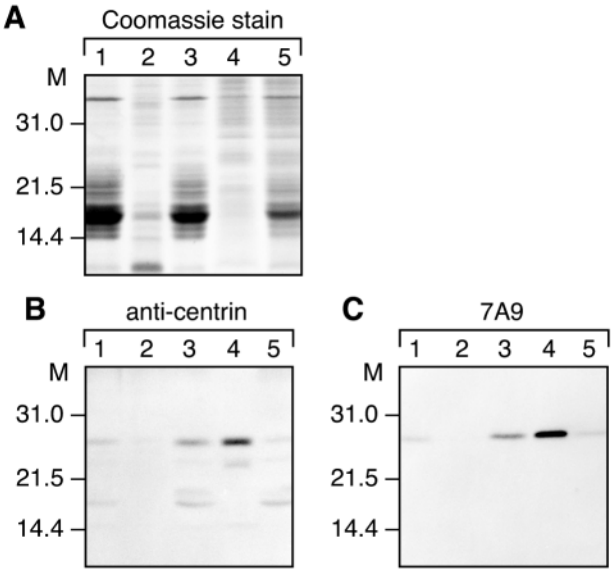

Fig. 4. Immunodetection of PCBP-25 in subciliary fractions. Subciliary fractions were generated as described in the Materials and Methods. (A) Coomassie-stained SDS-PAGE, (B) immunoblots with antibody against centrin and (C) with monoclonal antibody 7A9. The same amount of protein (15 μg) was loaded in each lane. Lane 1, whole cilia; lane 2, Triton-extracted ciliary membrane; lane 3, axoneme; lane 4, dynein fraction released by high salt from axoneme; lane 5, axoneme after high-salt extraction. M, M_r of markers ($\times 10^3$).

chosen for further study represented 5 to 10% of the total protein in the EGTA eluate from phenyl-Sepharose.

FPLC anion-exchange chromatography (on MonoQ) resolved the proteins of the EGTA eluate into four major peaks (Fig. 2). The third major peak contained PCBP-25 and PCBP-10. Subsequent immunological and phosphorylation studies showed PCBP-25 to consist of two components, PCBP-25α and PCBP-25β. PCBP-25α eluted at about 200 mM NaCl and PCBP-25β between 220 and 250 mM NaCl. Chromatography on MonoQ completely separated PCBP-25 from the two CaPKs, which were eluted with a salt gradient as two major earlier peaks. In the MonoQ peak containing PCBP-25, no protein kinase activity was detected (data not shown). Chromatography on MonoQ resulted in a considerable purification of PCBP-25α and PCBP-25β, as judged by the immunoreactivity of the whole cell homogenate and MonoQ fractions with antibodies that recognize the two proteins specifically (described below). From about 3 g of starting material (120,000 ***g*** supernatant), we routinely obtained at least 50 to 100 μg of PCBP-25α and 100 to 200 μg of PCBP-25β.

The amount of Ca^{2+} bound in overlay blots was similar for PCBP-25α and PCBP-25β when equal molar amounts of protein were compared, and both proteins bound Ca^{2+} to about the same extent as an equal amount of CaM (data not shown). The quantification of this binding was not precise enough to deduce the number of binding sites per molecule.

The mobility shift on SDS-PAGE in the presence and absence of Ca^{2+} that is characteristic of many EF-hand-type CaBPs was seen with PCBP-25β (which ran faster in the presence of Ca^{2+}). PCBP-25α showed no comparable shift under identical conditions (data not shown). In 10% or 12% Laemmli gels with no added Ca^{2+}, the M_r of both PCBP-25α and PCBP-25β was 25,000, and the addition or omission of β-mercaptoethanol in the sample buffer did not change the electrophoretic mobility for either protein (data not shown). In gel filtration on Sephacryl S-300 in 0.1 mM EDTA, PCBP-25β behaved like a protein of M_r 36,000 (data not shown), suggesting that its native aggregation state in the absence of Ca^{2+} is no larger than a dimer. Since most of the EF-hand CaBPs are highly asymmetric, the PCBP-25β may be a monomer like these CaBPs. Gel filtration clearly separated PCBP-25β from PCBP-10 [M_r (apparent) 19,000] and CaM [M_r (apparent) 17,000] (not shown).

PCBP-25α and PCBP-25β are immunologically distinct

We used a monoclonal antibody (7A9) raised against the EGTA pool from phenyl-Sepharose (M. Son, PhD thesis, University of Wisconsin-Madison, 1991) and an antiserum raised against *Chlamydomonas* centrin (Baron and Salisbury, 1988) to distinguish several centrin-like proteins on immunoblots (Fig. 3). In quick-killed *Paramecium* samples (in which a small volume of living cells was squirted into boiling SDS-PAGE sample buffer and boiled more to minimize proteolysis), antibody 7A9 recognized a protein of M_r 25,000 and, as expected, the anti-centrin antibody stained a protein of about M_r 23,000 in whole cell extract of *Chlamydomonas* (Fig. 3A). Immunoblots of the individual MonoQ fractions containing PCBP-25 showed strong reaction of the early fractions containing PCBP-25α with the anti-centrin antibody. The later MonoQ fractions (containing PCBP-25β) reacted strongly with 7A9 but not with anti-centrin antibody (Fig. 3B). These two antibodies therefore defined two immunologically distinct proteins that have a similar size and chromatographic properties. Isoelectric focusing also separated PCBP-25α (pI 4.6) from PCBP-25β (pI 4.75–4.85) (data not shown). For comparison, the pI for CaM from *Paramecium* is about 4.0 (Plattner and Klauke, 2001), and the centrin-like proteins of the ICL focus at between pH 4.2 and pH 4.7 (Klotz et al., 1997).

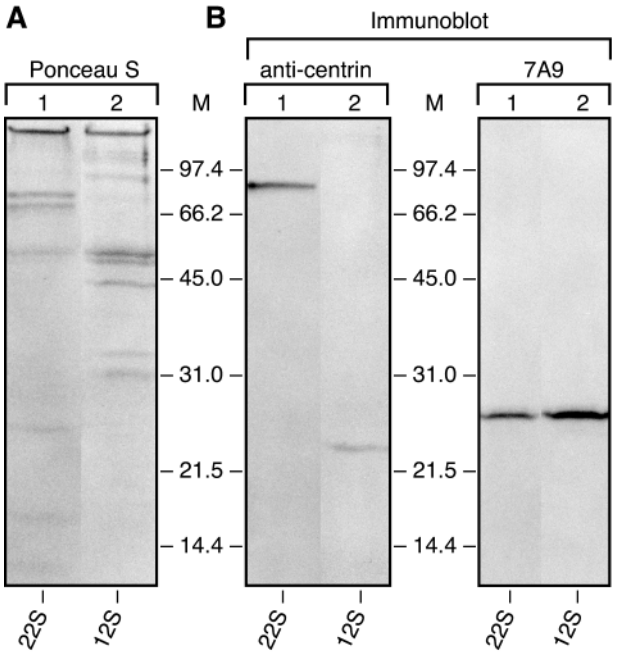

Fig. 5. Immunodetection of PCBP-25 in purified dynein fractions. *Paramecium* 22S and 12S dyneins were purified by sucrose gradient sedimentation from the high-salt extract (0.6 M KCl) of demembranated axonemes. Ponceau S-stained SDS-PAGE (A), immunoblots with antiserum against centrin or with monoclonal antibody 7A9 (B). The same amount of protein (10 μg) was loaded in each lane. Lane 1, 22S dynein; lane 2, 12S dynein; M, M_r of markers ($\times 10^3$).

Subcellular localization of PCBP-25α and PCBP-25β by blotting

Although PCBP-25α and PCBP-25β were antigenically distinct, their subcellular localizations, determined by immunoblotting, were similar. About two-thirds of each protein remained in the low-speed pellet after cell disruption and was not extractable with Triton X-100. Both PCBP-25α and PCBP-25β were present in the deciliation supernatant and in isolated cilia (data not shown). The deciliation supernatant contains proteins that are solubilized when cells are subjected to a Ca^{2+} shock in the procedure for releasing cilia (Adoutte et al., 1980). This subcellular fraction represents only 1 to 2% of the total protein of cells, but both PCBP-25α and PCBP-25β were found there in significant amounts.

Both PCBP-25α and PCBP-25β in isolated cilia remained associated with the axoneme after extraction of soluble proteins and membranes with Triton X-100 (Fig. 4). About two-thirds of the axoneme-associated PCBP-25α and PCBP-25β was released by high salt, which also solubilized dynein. When dynein was further separated into 22S and 12S forms by centrifugation through a sucrose gradient (see Travis and Nelson, 1988a), PCBP-25β was detectable in immunoblots of both fractions, but PCBP-25α was found only in 12S dynein and was much less prominent there than was PCBP-25β (Fig. 5). PCBP-25α in the 12S dynein fraction ran faster on SDS-PAGE than did PCBP-25α isolated on the MonoQ, probably as a result of proteolysis during isolation. The anti-centrin antibody also recognized a protein (M_r 90,000) in 22S dynein of unknown identity (data not shown). Baron and Salisbury (Baron and Salisbury, 1988) also noted crossreaction of their anti-centrin antibody with a larger polypeptide.

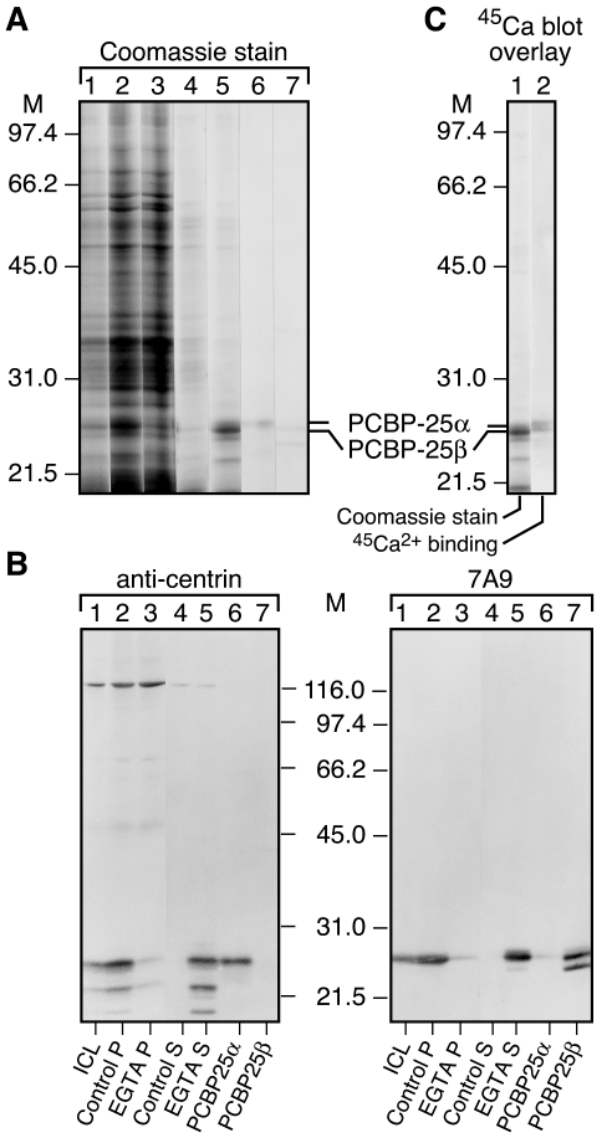

Fig. 6. Immunodetection and $^{45}Ca^{2+}$ binding of PCBP-25 in EGTA-extracted supernatant from infraciliary lattice (ICL) preparation. Purification and EGTA extraction (10 mM) of ICL were performed as described in the Materials and Methods. (A) Coomassie-stained SDS-PAGE and (B) immunoblots with antiserum against centrin and with monoclonal antibody 7A9. In (A) and (B), Lane 1, ICL, 40 μg; lane 2, ICL control pellet, 40 μg; lane 3, ICL EGTA pellet, 40 μg; lane 4, ICL control supernatant, 15 μg; lane 5, ICL EGTA supernatant, 15 μg; lane 6, PCBP-25α, 3 μg; lane 7, PCBP-25β, 5 μg. In (C), the $^{45}Ca^{2+}$ blot overlay assay was performed as described in the Materials and Methods at 1.4 μM free Ca^{2+} with 20 μg of ICL EGTA supernatant. Coomassie-stained SDS-PAGE (lane 1) and autoradiograph of $^{45}Ca^{2+}$ binding (lane 2). M, M_r of markers ($\times 10^3$).

The highest specific immunoreactivity of PCBP-25α and PCBP-25β was in the crude preparation of ICL, a cytoskeletal structure that lies beneath the pellicle of *Paramecium* (Garreau de Loubresse et al., 1988). For a given amount of protein, this fraction was stained at least ten times more intensely by both

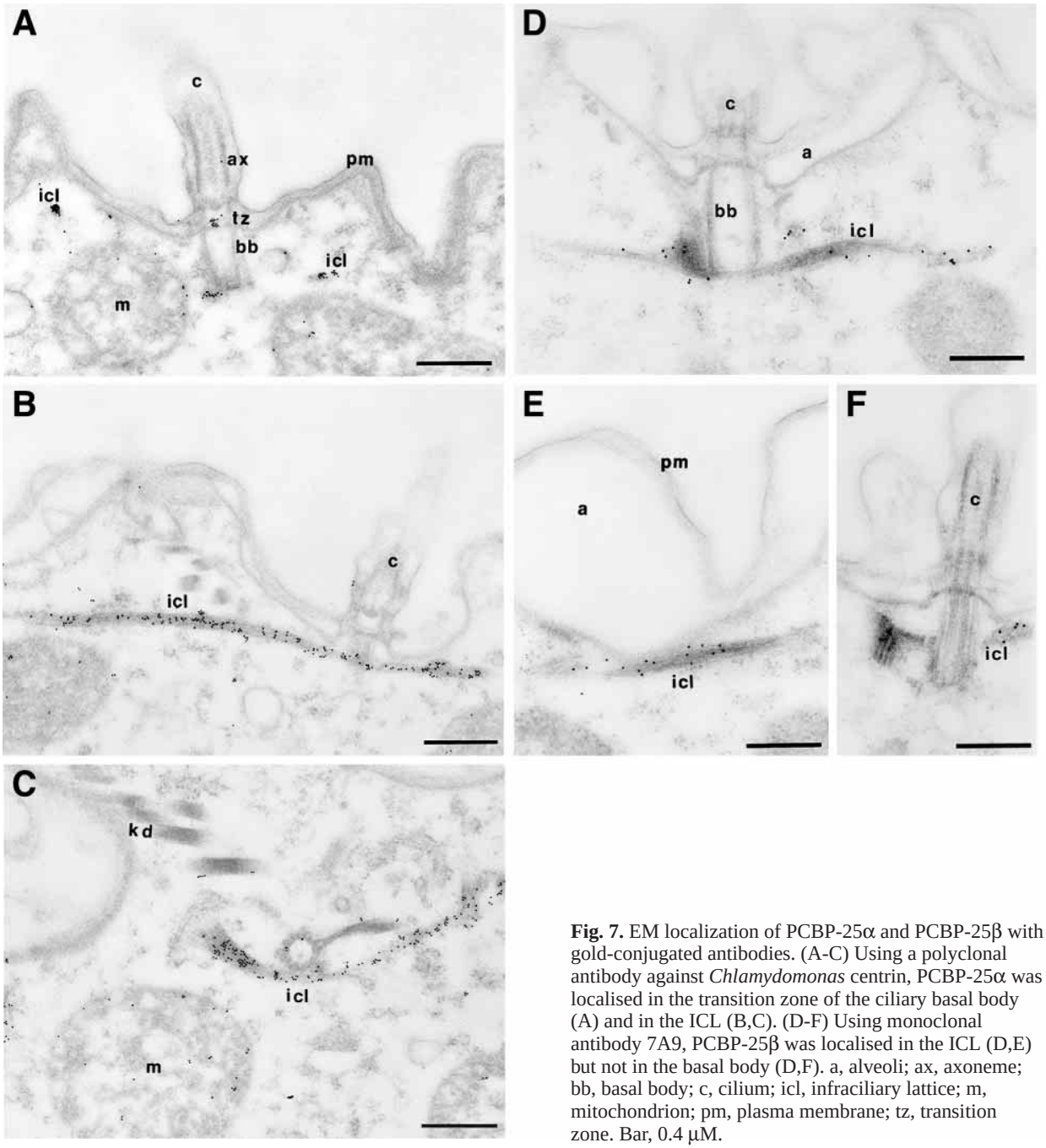

Fig. 7. EM localization of PCBP-25α and PCBP-25β with gold-conjugated antibodies. (A-C) Using a polyclonal antibody against *Chlamydomonas* centrin, PCBP-25α was localised in the transition zone of the ciliary basal body (A) and in the ICL (B,C). (D-F) Using monoclonal antibody 7A9, PCBP-25β was localised in the ICL (D,E) but not in the basal body (D,F). a, alveoli; ax, axoneme; bb, basal body; c, cilium; icl, infraciliary lattice; m, mitochondrion; pm, plasma membrane; tz, transition zone. Bar, 0.4 μM.

antibodies than were extracts of quick-killed cells (data not shown). Extraction of the crude ICL preparation with 10 mM EGTA or 1M KCl released nearly all of the PCBP-25α and PCBP-25β detectable in immunoblots or $^{45}Ca^{2+}$ overlay blots (Fig. 6B,C), but buffer containing Ca^{2+}, ATP, or urea produced little or no solubilization (data not shown). This solubility pattern parallels that of the Ca^{2+}-binding proteins identified as major components of the ICL (Garreau de Loubresse et al., 1988).

The anti-centrin antibody also reacted very specifically with a protein of M_r 115,000 in partially purified ICL preparations, which, unlike the PCBP-25α and PCBP-25β, was not solubilized with EGTA.

Subcellular localization of PCBP-25α and PCBP-25β by electron microscopy

To complement the studies of localization by subcellular fractionation, we determined the localization of PCBP-25α and PCBP-25β at the ultrastructural level using colloidal gold-conjugated secondary antibodies. The anti-centrin antibody labeled two distinct cellular structures – the ICL and the transition zone between the ciliary axoneme and basal body (Fig. 7A) – but did not label kinetodesmal fibers or other known microtubule-based subciliary structures. The labeling of the ICL was uniform. A small amount of label was also observed in mitochondria. Monoclonal antibody 7A9, which is specific for PCBP-25β, selectively labeled only the ICL (Fig. 7B),

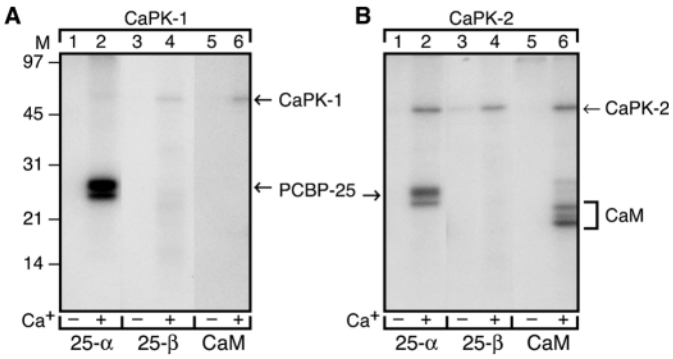

Fig. 8. Phosphorylation of PCBP-25α, PCBP-25β and CaM by purified Ca^{2+}-dependent protein kinases. CaPK-1 and CaPK-2 were purified by chromatography on phenyl-Sepharose and MonoQ. MonoQ-purified PCBP-25α, PCBP-25β and CaM were incubated with [γ-^{32}P]ATP and either (A) CaPK-1 or (B) CaPK-2 (20 units each) for 30 minutes in the absence or presence of Ca^{2+} (13.5 μM free) according to the Materials and Methods. Lanes 1 and 2, 1 μg PCBP-25α; lanes 3 and 4, 1 μg PCBP-25β; lanes 5 and 6, 2 μg concentrated, desalted CaM. Arrows indicate autophosphorylation of CaPK-1 or CaPK-2 and phosphorylation of PCBP-25β and CaM.

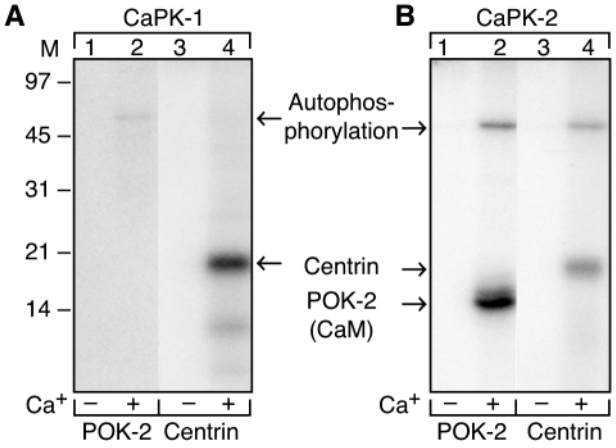

Fig. 9. Phosphorylation of purified POK-2 and centrin. Overexpressed CaM (POK-2) and engineered centrin of *Chlamydomonas* were purified and prepared for phosphorylation by CaPK-1 and CaPK-2. Lanes 1 and 2, 2 μg POK-2; lanes 3 and 4, 1 μg centrin. Autophosphorylation of each kinase and the phosphorylation of POK-2 and centrin are indicated.

confirming the results from subcellular fractionation. Negative controls performed with normal mouse or rabbit serum gave only very low-level background labeling (data not shown).

Phosphorylation of PCBP-25α, PCBP-25β and CaM by purified CaPKs

What originally drew our attention to PCBP-25α and PCBP-25β was the observation that a protein of M_r 25,000 in the phenyl-Sepharose eluate was phosphorylated in vitro by one of the CaPKs also present in that fraction. Although centrin is known to be phosphorylated in vivo (Salisbury et al., 1984; Salisbury, 1989; Martindale and Salisbury, 1990; Lutz et al., 2001), the kinase responsible for that phosphorylation has not been identified. We therefore tested four highly purified protein kinases from *Paramecium* [CaPK-1 and CaPK-2 (Fig. 8), cAMP-dependent protein kinase (PKA) and cGMP-dependent protein kinase (PKG) (data not shown)] for their ability to phosphorylate PCBP-25α and PCBP-25β as well as CaM from *Paramecium* in vitro.

Purified PCBP-25α was phosphorylated by both CaPK-1 and CaPK-2, whereas PCBP-25β was not significantly phosphorylated by either. Almost the same amount of ^{32}P was incorporated into each band of the doublet representing the purified fraction of PCBP-25α (lane 2), in which the lower band was probably a proteolytic fragment of PCBP-25α (M. Son, PhD thesis, University of Wisconsin-Madison, 1991). CaM was phosphorylated in a Ca^{2+}-dependent manner by CaPK-2 but not by CaPK-1. The CaM we prepare from axenically cultured cells of *Paramecium* always has several (usually three) electrophoretically distinct forms in a variable ratio, all recognized by a monoclonal antibody against CaM (B. C. Soltvedt, MS thesis, University of Wisconsin-Madison, 1985; L. D. DeVito, MS thesis, University of Wisconsin-Madison, 1985). All three forms bound $^{45}Ca^{2+}$ in blot overlay assays and were phosphorylated by CaPK-2 equally well (i.e., in proportion to their amounts).

PCBP-25α, PCBP-25β, and CaM were also tested as substrates for phosphorylation by purified PKA and PKG from *Paramecium* (Carlson and Nelson, 1995). PKG phosphorylated PCBP-25α but not PCBP-25β or CaM. None of the three proteins was a substrate for PKA (data not shown).

Substrate specificity of CaPK-1 versus CaPK-2

CaPK-2 phosphorylated PCBP-25α and CaM, whereas CaPK-1 used only PCBP-25α as a substrate, so we used these Ca^{2+}-binding proteins to explore further the substrate specificity of the kinases. *Paramecium* CaM overexpressed in *E. coli* (POK-2) and cloned centrin from *Chlamydomonas* were purified and also examined for phosphorylation by both CaPKs. CaM (POK-2) was phosphorylated by only CaPK-2, whereas centrin, which is immunologically related to PCBP-25α, was a substrate for both enzymes (Fig. 9), consistent with the results in Fig. 8. The extent of phosphorylation of POK-2 or centrin was almost the same as that for CaM or PCBP-25α, as determined by quantitative scans of autoradiograms (data not shown).

In preliminary experiments with casein as the substrate, we established that CaPK-1 phosphorylated mainly Thr but also Ser, whereas CaPK-2 preferred Ser over Thr. The requirement for basic residues near the Ser or Thr residue to be phosphorylated is common in second-messenger-dependent serine and threonine protein kinases. Several synthetic peptides of 7 to 17 residues containing the Lys/Arg-X-X-Ser motif were tested as substrates for CaPK-1 and CaPK-2 (Table 1). Peptides 1, 2 and 3 were designed from the sequence of *Tetrahymena* micronuclear phosphohistone by David Allis and were supplied by him. The remaining peptides were commercially available synthetic substrates for PKC, PKG or PKA, respectively. None of the peptides was stoichiometrically phosphorylated by CaPK-1, although a small amount of ^{32}P was incorporated into the peptide designed as a substrate for PKC (4). This peptide was also the best substrate among those tested for CaPK-2, which must therefore share some substrate

Table 1. Synthetic peptide substrates for CaPK-1 versus CaPK-2

Peptide	Sequence	Percentage of phosphorylation CaPK-1 –Ca^{2+}	CaPK-1 +Ca^{2+}	CaPK-2 –Ca^{2+}
1	K S R R N **S** M K E A R T K K A N K	0.64	0.38	0.43
2	R S K **S** K S A S K S R **S** K S	0.32	0.26	0.51
3	K R N **S** S S S S K R S **S** S S K	0.38	0.30	0.27
4	R F A R K G **S** L R Q K N V	0.38	3.20	0.17
5	R K R **S** R A E	0.11	0.04	0.14
6	L R R A **S** L G	0.11	0.12	0.10

Peptides 1, 2 and 3 were designed by David Allis on the basis of the phosphopeptide sequence of *Tetrahymena* micronuclear histone; peptide 4 is the substrate peptide for PKC (Gibco-BRL, Gaithersburg, MD); peptide 5 is the substrate peptide for PKG (Promega, Madison, WI); peptide 6 is the substrate peptide for PKA (Bachem, Bubendorf, Switzerland). Each peptide (1050 pmol) was added to the phosphorylation mixture and the percentage of phosphorylation was calculated from pmol P_i incorporated/pmol peptide. For peptides 2 and 3, half the amount of peptide was used because these peptides contain two S residues (bold) in the basic–X–X–S sequence. 100% phosphorylation means that one S per peptide was phosphorylated.

specificity with PKC from animal cells. The extent of phosphorylation on the other peptides was very low; the order of phosphorylation activity was: (peptide 4) >> (2), (6), (3), (1) >> (5).

Phosphorylation of purified CaM by CaPK-2

The amount of ^{32}P incorporated into CaM (POK-2, produced in *E. coli* from the cloned *Paramecium* CaM gene), increased with time and peaked at 60 minutes (Fig. 10A,C). The autophosphorylation of CaPK-2 also increased with time (Fig. 10; band at M_r 50,000). However, the maximum incorporation was less than 0.01 mol P per mol CaM. To determine if the CaM concentration affected the phosphorylation of CaM, increasing amounts of CaM were phosphorylated for 30 minutes (Fig. 10B). In this experiment, CaM phosphorylation by CaPK-2 was also substoichiometric (1%).

Does phosphorylation of CaM affect the function of CaM?

In purified preparations of *Paramecium* CaM, there were several electrophoretically separable forms of CaM, each of which could be phosphorylated by CaPK-2. After phosphorylation by CaPK-2, both natural CaM and POK-2 (CaM produced in *E. coli*) showed the Ca^{2+}-induced increase in mobility (of the labeled species) in SDS-PAGE that is typical of CaMs (data not shown). The smallest form of CaM showed the largest Ca^{2+}-dependent mobility shift. This Ca^{2+}-dependent mobility shift suggested that phosphorylation of CaM by CaPK-2 did not prevent interaction between CaM and Ca^{2+}.

Several CaM antagonists were tested for inhibition of CaM phosphorylation by CaPK-2. Neither melittin nor calmidazolium (CaM antagonists) blocked casein phosphorylation (Gundersen and Nelson, 1987; Son et al., 1993) or CaPK-2 autophosphorylation. However, both melittin and calmidazolium completely inhibited CaM phosphorylation at 5 μM (Fig. 11). In the presence of these antagonists, the extent of autophosphorylation was somewhat increased, as though CaM and CaPK-2 competed as substrates for phosphorylation.

Which residue of CaM is phosphorylated by CaPK-2?

Serine is the residue in CaM that is phosphorylated by CaPK-2 (data not shown), and this is consistent with our results with casein phosphorylation. *Paramecium* CaM contains five serines among its 148 amino acids, at positions 38, 70, 81, 101 and 147. In the mutant *cam*1, a Phe replaces Ser^{101} in Ca^{2+}-binding site III, and Ile^{136} (in Ca^{2+}-binding site IV) is mutated to Thr in the mutant *cam*2. The protein pjk-1 is a truncated CaM, consisting of only the N-terminal half (residues 1–75), including two Ca^{2+}-binding sites. Wild-type CaM isolated from *Paramecium* (PCaM), POK-2, cam1 and cam2 were all equally good substrates for CaPK-2, but pjk-1 was poorly

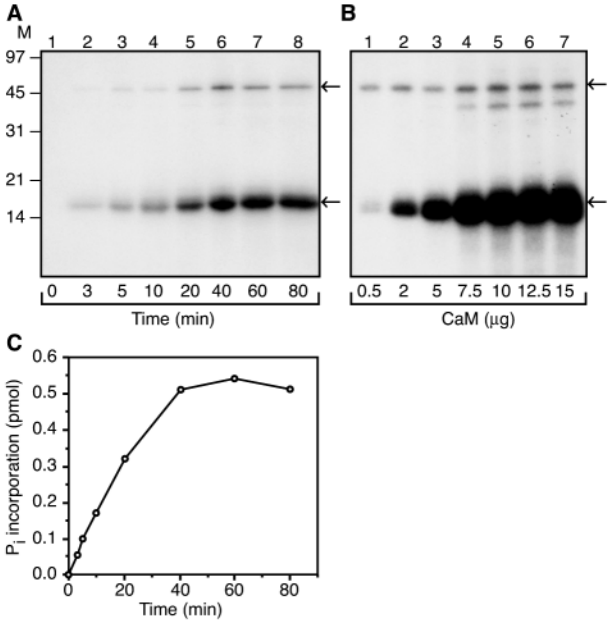

Fig. 10. Time dependence and substrate dependence of CaM phosphorylation by CaPK-2. (A) Phosphorylation by CaPK-2 was performed with 2 μg CaM and 20 units of kinase in the presence of Ca^{2+}. The same volume of reaction mixture was withdrawn at indicated time points, and the reaction was stopped by precipitating the protein with 15% TCA. (B) The amount of CaM for phosphorylation by 20 units of kinase was increased as indicated. The reaction was performed for 20 minutes at room temperature. Autophosphorylation of CaPK-2 (M_r 50,000) and CaM (M_r 17,000) phosphorylation are indicated by arrows. (C) The radioactivity incorporated into CaM was quantified from the dried gel using a Betagen detector system (Intelligenetics, Mountainview, CA) and plotted with time.

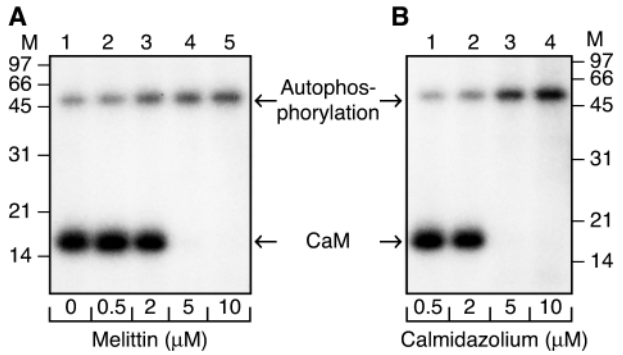

Fig. 11. The effect of CaM antagonists on CaM phosphorylation. CaM antagonists melittin or calmidazolium were added to a reaction mixture of CaM phosphorylation by 20 units of CaPK-2 (in the presence of 13.5 μM free Ca^{2+}; CaPK-2 is half-saturated at 0.2 μM free Ca^{2+}). (A) Lane 1, control – no antagonist; lane 2, 0.5 μM; lane 3, 2 μM; lane 4, 5 μM; lane 5, 10 μM melittin. (B) Lane 1, 0.5 μM; lane 2, 2 μM; lane 3, 5 μM; lane 4, 10 μM calmidazolium.

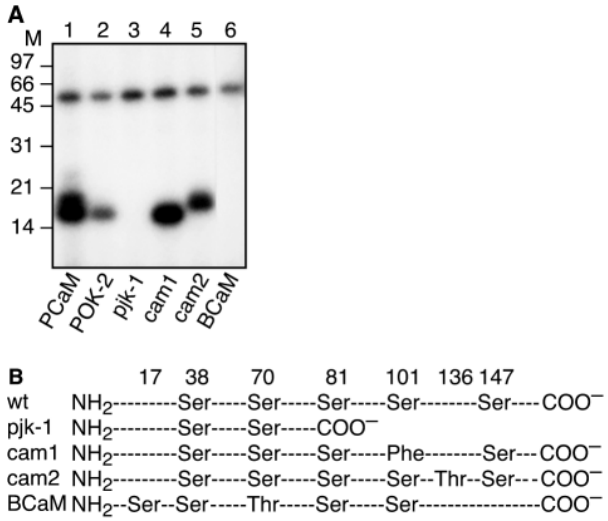

Fig. 12. Phosphorylation of several CaMs by CaPK-2. (A) Several *Paramecium* CaMs, including wild-type and behavioral mutants, and bovine CaM were tested for the substrate specificity of CaPK-2. (B) The relevant amino acid sequences are shown.

phosphorylated, eliminating the possibility that Ser^{101}, Ser^{38} or Ser^{70} is the residue phosphorylated. Bovine CaM, which has a Ser at position 81, was a poor substrate (Fig. 12), leaving Ser^{147} as the probable site of phosphorylation in PCaM.

Discussion

The diversity of Ca^{2+}-regulated function in *Paramecium* is reflected in the variety of Ca^{2+}-binding proteins present (for reviews, see Plattner and Klauke, 2001). CaM is localized in cilia, basal bodies, ICL, the membranes of the secretory organelles (trichocysts), phagosomes and the osmoregulatory system (contractile vacuoles) (Walter and Schultz, 1981; Klumpp et al., 1983b; Momayezi et al., 1986). The ICL is composed largely of several proteins related to centrin, a small, acidic, ubiquitous member of the EF-hand superfamily (Garreau de Loubresse et al., 1991). Two Ca^{2+}-dependent protein kinases (CaPK-1 and CaPK-2) containing EF hands are present in the cytosol and in cilia (Gundersen and Nelson, 1987; Son et al., 1993). Calcineurin, the Ca^{2+}- and CaM-dependent phosphoprotein phosphatase, is localized to the cell cortex and is also found in cilia (Klumpp et al., 1983b; Momayezi et al., 2000).

PCBP-25α and PCBP-25β are different proteins

PCBP-25α and PCBP-25β are different proteins, not simply two forms of the same gene product. Antiserum against centrin recognizes only PCBP-25α, and the monoclonal antibody 7A9 recognizes only PCBP-25β. A second, independently selected hybridoma (10D1), produced after immunization with a mixture of CaBPs from *Paramecium*, also recognized PCBP-25β and not PCBP-25α (M. Son, PhD thesis, University of Wisconsin-Madison, 1991). It is unlikely that two independently selected monoclonal antibodies would recognize the same epitope. Furthermore, a polyclonal serum with broad-enough specificity to recognize centrins from many species, from the alga *Chlamydomonas* to human (Salisbury, 1989), gave a strong reaction with PCBP-25α but did not react with PCBP-25β on immunoblots.

What are the relationships among PCBP-25α, PCBP-25β and other EF-hand proteins found in *Paramecium* and *Tetrahymena*?

The proteins of the ICL have been previously described (Garreau de Loubresse et al., 1988; Garreau de Loubresse et al., 1991; Klotz et al., 1997). Klotz et al. (Klotz et al., 1997) found 10 small, acidic proteins in crude preparations of ICL, including six that were stained on immunoblots with an antiserum that recognizes centrin from many species. Our PCBP-25α is probably related, or identical, to one of these six proteins. It has about the same M_r, and, like them, it reacts with anti-centrin antibodies, binds Ca^{2+} and is enriched in the ICL. It is most probably a product of one of the ~20 centrin genes of *Paramecium* (Madeddu et al., 1996; Vayssie et al., 1997).

Our PCBP-25β is not one of the six centrins of the ICL, as it does not cross-react with centrin. Neither is it one of the other four less-acidic proteins of the ICL, because PCBP-25β binds to Ca^{2+} and the four ICL proteins do not (Klotz et al., 1997). It is not yet clear whether PCBP-25β is encoded in one of the 20 genes in the centrin family described by Vayssie et al. (Vayssie et al., 1997).

In another ciliated protozoan, *Tetrahymen*a, three distinct EF-hand proteins have been described and localized (Vaudaux, 1976; Williams et al., 1995), then isolated and cloned. These are CaM (Suzuki et al., 1981) and two small, acidic proteins called *Tetrahymena* Ca^{2+}-binding proteins, TCBP-25 (Hanyu et al., 1995) and TCBP-23 (Hanyu et al., 1996). Both proteins have four putative EF hands and both bind to Ca^{2+}. Either could be a homolog of PCBP-25β: none of the three crossreacts with centrin but all bind Ca^{2+}. TCBP-23 and TCBP-25 do not crossreact with our monoclonal antibody against PCBP-25β (M. Son, PhD thesis, University of Wisconsin-Madison, 1991), but this is probably not surprising given the evolutionary distance between the two protozoans. TCBP-25 is present in cilia, and Watanabe et al. (Watanabe et al., 1990) have suggested that it may play a role in the regulation of the ciliary beat by Ca^{2+}.

Table 2. C-terminal sequences of calcium-binding proteins from several species

**Paramecium* CaM	DIDGDGHINYEE**F**VRMMV**S**K148
†*Paramecium* centrins (all)	ED**F**YNIMTKK**T**FA142
‡Human centrin 1	EE**F**LRIMKKT**S**LY172
Human centrin 2	QE**F**LRIMKKT**S**LY172
Human centrin 3	EE**F**IAIMGDI--167
Xenopus centrin	QE**F**LRIMKKT**S**LF172
Chlamydomonas centrin	DE**F**IRIMKKT**S**LF169
§*Chlamydomonas* dynein light chain	RDLNGFLSYDEFRALL**S**159
PKA consensus sequence	KKX**S**X
Good CaPK-2 substrate	RFARKG**S**LRQKNV

The S residues in bold in *Paramecium* CaM, human centrins and synthetic peptides are known to be phosphorylated; in the other proteins, S or T residues in analogous positions have not been shown to be phosphorylated. The first seven sequences are aligned with the conserved F near the C termini; the dynein light chain is arbitrarily aligned to put its terminal S in the same position as the other C-terminal Ser residues.

*(Kink et al., 1990), †(Madeddu, 1996), ‡(Lutz et al., 2001), §(King and Patel-King, 1995).

Is PCBP-25β a regulator of the ciliary beat?

One well-characterized Ca^{2+}-dependent function in *Paramecium* is the reorientation of the ciliary power stroke, which causes backward swimming. The Ca^{2+} receptor protein for this process remains unknown; it is conceivable that the Ca^{2+}-binding proteins we describe here play that role.

There is a complex interplay between cyclic nucleotides and Ca^{2+} in ciliary regulation. Detergent-permeabilized cell models swim forward when reactivated with Mg^{2+}-ATP but backward when micromolar Ca^{2+} is also present (Naitoh and Kaneko, 1972; Eckert and Brehm, 1979). Cyclic nucleotides (cAMP and cGMP) antagonize this effect of Ca^{2+} on models, and the fast forward swimming induced by cyclic nucleotide addition is antagonized by micromolar [Ca^{2+}] (for reviews, see Bonini et al., 1991; Pech, 1995). Both adenylyl cyclase and guanylyl cyclase of *Paramecium* are tightly regulated by micromolar [Ca^{2+}] (Klumpp and Schultz, 1982; Klumpp et al., 1983a; Gustin and Nelson, 1987). Dynein ATPase is stimulated two-fold by micromolar [Ca^{2+}], but CaM does not co-sediment with either 22S or 12S dynein (Travis and Nelson, 1988b). PCBP-25β is associated with both 22S and 12S dynein, primarily with the 22S species. The outer arm dynein of another unicellular organism, *Chlamydomonas reinhardtii*, has a subunit of M_r 18,000 that binds to Ca^{2+} (King and Patel-King, 1995). It is 42% identical in sequence to *Chlamydomonas* CaM and 37% identical to centrin, and it may be the Ca^{2+} sensor that mediates the transition from asymmetric to symmetric waveform in the flagellar beat. Centrin is a component of a dynein regulatory complex of *Chlamydomonas* flagella, which associates with the inner arm dyneins (LeDizet and Piperno, 1995). It is possible that PCBP-25β confers Ca^{2+} sensitivity on ciliary dynein. It was not seen along the axoneme by EM immunocytochemistry, but its enrichment in the deciliation supernatant suggests that it may be loosely associated with some ciliary structure.

Is the phosphorylation of PCBP-25α and CaM functionally significant?

In a number of cases, phosphorylation of EF-hand proteins has been observed in vivo, and this covalent alteration is presumed to be functionally significant. The centrin of *Chlamydomonas* undergoes phosphorylation in vivo in response to rapid changes in the extracellular milieu or in intracellular [Ca^{2+}] that lead to deflagellation (Martindale and Salisbury, 1990). The kinase responsible for centrin phosphorylation in *Chlamydomonas* has not been identified, but it is of interest that *Chlamydomonas* has a CaM-domain kinase like the CaPKs of *Paramecium* (Siderius et al., 1997). Garreau de Loubresse et al. (Garreau de Loubresse et al., 1991) reported preliminary evidence for the phosphorylation of polypeptides of M_r 23,000-24,000 of the ICL in vitro by bovine PKA. Lutz et al. (Lutz et al., 2001) recently reported that the phosphorylation of centrin in cultured vertebrate cells varied strikingly over the cell cycle, peaking at the G2/M phase. Phosphorylation is on Ser^{170}, the third residue in from the C terminus, in a sequence typical of those preferred by many Thr/Ser kinases, with two basic residues preceding the Ser residue: KKTSLY. Experiments with permeant analogs of cAMP implicate PKA in this phosphorylation.

PCBP-25α was a substrate for phosphorylation in vitro by CaPK-1, CaPK-2 and PKG of *Paramecium*, and *Paramecium* CaM was phosphorylated by CaPK-2. CaPK-1 and CaPK-2 from *Paramecium* also phosphorylated centrin from *Chlamydomonas*. The interaction between CaM and CaPK-2 was sensitive to CaM antagonists, even though the kinase activity on other proteins is unaffected by these compounds (Son et al., 1993). The phosphorylated C-terminal sequences in *Paramecium* CaM and two of three human centrins are similar; of the 10 residues at the C terminus of CaM, six are identical to the corresponding residues in human centrin, and two others represent conservative substitutions (Table 2). The C-terminal sequences of the three reported centrins from *Paramecium* are identical to each other, and of the 13 residues at the C terminus, seven are identical to those in human centrin and two more are conservative substitutions. The centrins from *Paramecium* have a Thr residue near their C termini (not a Ser as in the other proteins). If this is the residue phosphorylated in *Paramecium* centrin, it matches the position of Ser^{170} in two of the human centrins. The third human centrin also lacks a Ser residue near its C terminus (Table 2) and is presumably not subject to the same regulation by phosphorylation that occurs with the other two human centrins.

Why is the phosphorylation in vitro of CaM by CaPK-2 substoichiometric? *Paramecium* CaM might be already phosphorylated in vivo and therefore not capable of accepting phosphoryl groups in vitro. This seems unlikely, as even the *Paramecium* CaM overexpressed in *E. coli* could not be phosphorylated stoichiometrically in vitro. Another possible explanation for the low stoichiometry is heterogeneity at the C terminus of CaM, produced by carboxypeptidase action in extracts during CaM purification. Although there is clearly

only one copy of the CaM gene in *Paramecium* (Kink et al., 1990), we have always observed several electrophoretic forms of CaM in our highly purified preparations (B. C. Soltvedt, MS thesis, University of Wisconsis-Madison, 1985). If the penultimate residue (Ser^{147}) is the one phosphorylated, and if most molecules lack several residues at their C termini, low stoichiometry would be expected.

The location of the phosphorylated Ser^{147} residue in CaM is similar to that of the Ser^{170} residue phosphorylated in vertebrate centrin (Lutz et al., 2001) – just at the C-terminal boundary of the fourth EF hand. In both cases, the negative charges on the phosphorylated Ser residue would be expected to force the C-terminal tail away from the negatively charged residues of the EF hand, or to attract a calcium ion, and might thereby alter Ca^{2+} binding to that domain. The M_r 18,000 dynein light chain described by King and Patel-King (King and Patel-King, 1995) is a member of the EF-hand superfamily and also has a C-terminal Ser (Table 2), but the sequence around this Ser lacks the paired basic residues that often demarcate a phosphorylation site.

The dramatic reorganization of the cytoskeleton of *Paramecium* during cell division and after conjugation (Iftode et al., 1989) may involve Ca^{2+}-dependent phosphorylation as an integrating signal to disassemble, then reassemble the cytoskeleton, including the ICL (Keryer et al., 1987; Sperling et al., 1991; Klotz et al., 1997; Prajer et al., 1997). Garreau de Loubresse et al. (Garreau de Loubresse et al., 1991) reported preliminary results showing that the general protein kinase inhibitor 6-dimethylaminopurine selectively inhibited disassembly of the ICL. The same kinase inhibitor interfered with the rearrangement of cytoplasmic microtubule organizing centers and cytokinesis in *Paramecium* (Kaczanowska et al., 1996). The protein kinase implicated in these events has not been identified, but there are only three known Ca^{2+}-dependent protein kinases in *Paramecium:* the two CaPKs described here and a PKC-like activity (K. Kim, PhD thesis, University of Wisconsin-Madison, 1994). The ability of CaPK-2 to phosphorylate centrin, a major component of the ICL, is consistent with a role for the enzyme in triggering cytoskeletal rearrangements. The Ca^{2+} sensitivity of CaPK-2 is also appropriate for a cytosolic enzyme activated by Ca^{2+}; the enzyme is half-saturated at 0.2 μM Ca^{2+} (Son et al., 1993), and the cytosolic [Ca^{2+}] is believed to vary between about 0.1 μM to 1 μM during Ca^{2+}-mediated signaling (Plattner and Klauke, 2001). Transformation with appropriate constructs produces gene silencing in *Paramecium* (Ruiz et al., 1998), and we have cloned the CaPK genes from *Paramecium* (Kim et al., 1998), so it may be possible to obtain cells without CaPKs and to test the effects of CaPK action on ciliary reversal and cytoskeletal rearrangement.

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