

Identification of a 42 kDa protein as a substrate of protein phosphatase 1 in cilia from *Paramecium*

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Okadaic acid, a specific inhibitor of protein phosphatase 1 in *Paramecium* causes sustained backward swimming in response to depolarising stimuli (S. Klumpp et al. (1990) EMBO J. 9, 685). Here, we employ okadaic acid, tautomycin, microcystin LR and inhibitor 1 as phosphatase inhibitors to identify a 42 kDa protein in the excitable ciliary membrane that is dephosphorylated by protein phosphatase 1. Identification of the 42 kDa protein was facilitated by the finding that the protein kinase responsible for its phosphorylation uses Ca-ATP as a substrate just as effectively as Mg-ATP. Notably, dephosphorylation of the 42 kDa protein is specifically inhibited by cyclic AMP; cyclic GMP has no effect.

Protein phosphatase; *Paramecium*; Okadaic acid; cAMP; Ca-channel; Cilia

1. INTRODUCTION

The ciliary membrane of *Paramecium* carries ion channels, enzymes of cyclic nucleotide metabolism, protein kinases and protein phosphatases that play key roles in mediating sensory transduction mechanisms which regulate the direction of ciliary beat, and hence the motile behaviour of this protozoan (reviewed in [1,2]). The surface of *Paramecium* is covered by about 5000 cilia which propel the cell forward. However, when confronted by adverse stimuli, such as heat, light, touch at the anterior part and certain chemicals and ions, an avoidance reaction is triggered in which backward swimming takes place. These stimuli open a voltage-operated calcium channel in the ciliary membrane, resulting in an influx of calcium ions which drives the reversal of ciliary beat and initiates backward swimming (reviewed in [3]). This is a transient response, lasting for 10–15 s, at which point the calcium channel inactivates and forward swimming is resumed.

We recently found that in the presence of okadaic acid, a potent and specific inhibitor of protein phosphatases, backward swimming triggered by a rise in K^+ from 1 to 20 mM is no longer transient, but continues for several minutes, indicating that inactivation of the voltage-operated calcium channel involves a dephosphorylation event [4]. In all eukaryotic cells examined so far, except *Paramecium*, okadaic acid at nanomolar concentrations inhibits protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A), two structurally related enzymes which share 40% amino acid sequence identity [5,6]. However, while okadaic acid in-

hibits the PP1 of *Paramecium*, the PP2A-like activities present in the protozoan are unaffected [4]. Since at least 75% of the PP1 activity in *Paramecium* is located in the excitable ciliary membrane, these observations implicated PP1 in the inactivation of the voltage-operated calcium channel [4]. It therefore became important to identify the physiological substrates of this protein phosphatase. We now report that the dephosphorylation of a 42 kDa protein in the ciliary membrane is blocked specifically by okadaic acid and other inhibitors of PP1. The identification of this substrate has employed novel methodology which will also facilitate detection of the protein kinase responsible for phosphorylation of the 42 kDa protein.

2. EXPERIMENTAL

2.1. Materials

$[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was purchased from Amersham and molecular weight standards from Boehringer or Sigma. Okadaic acid, a gift from Dr Y. Tsukitani (Fujisawa Pharmaceutical Company, Tokyo, Japan) and tautomycin, kindly provided by Dr K. Isono (Antibiotics Laboratories, Saitama, Japan), were dissolved in dimethylsulphoxide to give a 5 mM solution and were further diluted with aqueous buffers before use. Microcystin-LR and activated, i.e. phosphorylated, inhibitor-1 (residues 9–41) were kindly provided by Dr P. Cohen (University of Dundee, Scotland).

2.2. Cell culture and tissue preparation

Paramecium tetraurelia wild-type 51s were grown, harvested and deciliated as described [7]. Cilia were purified by differential centrifugation and membranes were isolated on a sucrose gradient [7]. Cilia (10 mg/ml) or membranes (2 mg/ml) were homogenized in a Dounce homogenizer, dialysed extensively against 10 mM MOPS, pH 7.2, 10 mM 2-mercaptoethanol, 0.1 mM PMSF and stored at -80°C .

2.3. Protein phosphorylation

The reaction mixture for endogenous phosphorylation (0.1 ml) contained 50 mM MOPS, pH 7.2, divalent cations and cyclic nucleotides

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as indicated, and 40 μ g protein. Incubations were started by addition of 1 nmol [γ - 32 P]ATP (1–2 μ Ci) and terminated by heating for 4 min at 95°C. Protein pelleted by centrifugation (5 min, 10 000 $\times$ g) was redissolved in 25 μ l of 65 mM Tris-HCl, pH 6.8, 5% 2-mercaptoethanol, 5% SDS, 1% LiDS, 10% glycerol, 0.03% Bromophenol blue, heated for 4 min at 95°C and subjected to SDS-PAGE.

2.4. Miscellaneous

For SDS-PAGE 12.5% polyacrylamide 'minigels' (100 $\times$ 80 $\times$ 0.45 mm) were used. Gels were stained with Coomassie brilliant blue, dried and autoradiographed for 6 days at -30°C. The amount of radioactivity detected was 1 nCi per band. In order to discriminate between phosphoserine/phosphothreonine residues labelled by protein kinases and phosphoaspartic acid formed as an enzyme intermediate of certain ATPases, the proteins that had been precipitated by heat-treatment at 95°C were incubated for 10 min with 150 mM hydroxylamine to hydrolyse phosphoaspartic acid, prior to SDS-PAGE [8,9]. To distinguish phosphorylation of serine/threonine residues (alkali-labile) from phosphorylation of tyrosine residues (alkali-stable), the stained gels were incubated for 1 h at 40°C in either 10% acetic acid or 1 M KOH, fixed, dried and autoradiographed [10].

3. RESULTS AND DISCUSSIONS

3.1. Identification of a 42 kDa phosphoprotein as a substrate of PPI

Phosphorylation of ciliary proteins by endogenous protein kinases is optimal in the presence of Mg^{2+} , and at least 30 polypeptides became labelled in one minute upon incubation of cilia with 10 mM Mg^{2+} and 10 μ M

[γ - 32 P]ATP [11,12]. As reported earlier, further incubation for 30 min greatly decreased the extent of phosphorylation (Fig. 1B). This is explained by the rapid hydrolysis of ATP by ciliary ATPases which stops phosphorylation within a few minutes, allowing dephosphorylation to dominate [11,12]. Thus one-minute incubations provide a measure of endogenous protein kinases, while 30-min incubations predominantly reveal protein phosphatase activities.

Because okadaic acid only inhibits PP1 in *Paramecium* [4], this toxin was added to the 30 min incubations to potentially reveal specific substrates of this protein phosphatase. Surprisingly, the inclusion of 1 μ M okadaic acid led, after 30 min, to enhanced phosphorylation of just one band with an apparent molecular mass of 42 kDa (Fig. 1B). Okadaic acid also enhanced phosphorylation of the same protein in isolated ciliary membrane vesicles (Fig. 1A), indicating that the 42 kDa protein and the protein kinase and phosphatase that catalyse its reversible phosphorylation, are spatially associated within the excitable ciliary membrane as a functional trimeric complex in such a neighbourly way that allows the 42 kDa protein to be substrate for both membrane-bound enzymes. Subsequent experiments were carried out with cilia, although qualitatively similar results were obtained using ciliary membranes.

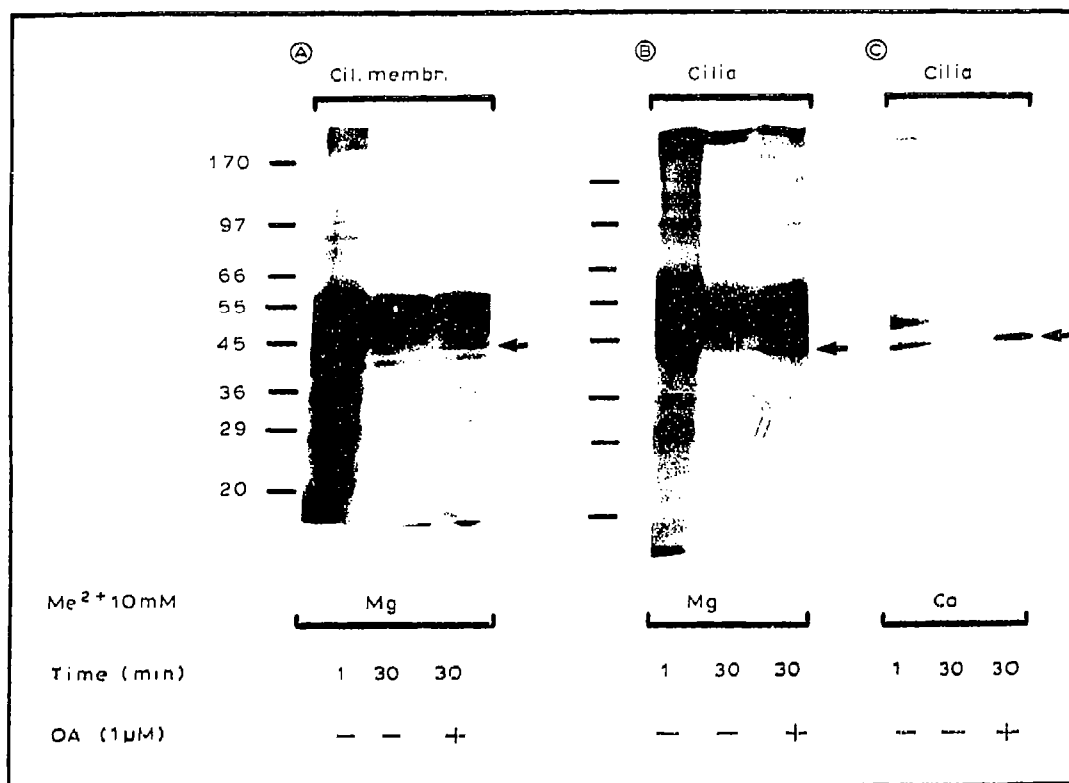


Fig. 1. Effect of okadaic acid (OA) on protein phosphorylation by endogenous protein kinases in *Paramecium* ciliary membranes (A) and cilia (B) using Mg ATP, and (C) cilia using Ca ATP as a substrate. Divalent cations were present at 10 mM. In (A), a 10–20% gradient polyacrylamide gel was used, in (B) and (C) 12.5% linear gels were employed. Only the autoradiographs are shown, molecular weight markers are indicated.

The ^{32}P -radioactivity associated with the 42 kDa protein was not lost upon incubation with hydroxylamine, indicating that phosphate was not linked to aspartic acid residues [8,9], and disappeared upon incubation for 1 h with 1 M KOH (but not 10% acetic acid), indicating phosphorylation on serine or threonine residues, but not tyrosine (data not shown, [10]).

3.2. Phosphorylation of the 42 kDa protein by Ca-ATP

In order to selectively enhance phosphorylation of the 42 kDa protein in comparison to other proteins, 10 mM Mg^{2+} was replaced by either Mn^{2+} or Ca^{2+} . The intensity and pattern of phosphorylation observed with 10 mM Mn^{2+} was essentially the same as with Mg^{2+} (data not shown). In contrast, one-minute incubations employing 10 mM Ca^{2+} resulted in the ^{32}P -labelling of only two proteins with apparent molecular masses of 42 kDa and 47 kDa (Fig. 1C). The ^{32}P -labelling of both proteins almost disappeared after incubation for 30 min, but dephosphorylation of the former species was prevented by okadaic acid, indicating its possible identity with the 42 kDa protein detected after incubation with Mg^{2+} in the presence of okadaic acid (compare Fig. 1C with A and B).

Next, the calcium requirement of the 42 kDa phosphorylation was examined. Ca^{2+} introduced with the assay constituents was 20 μM , as estimated by titration with the Ca-indicator dye arsenazo III, and presumably explains why phosphorylation of the 42 kDa and 47 kDa proteins could just be detected in the absence of added divalent cations (Fig. 2A). Phosphorylation of both proteins increased up to 10 mM Ca^{2+} (Fig. 2A). This indicates that phosphorylation is not catalysed by the known Ca-dependent protein kinase in *Paramecium* which requires in addition to Mg^{2+} only micromolar concentrations of Ca^{2+} for optimal activity [13].

These observations suggest that the protein kinase which phosphorylates the 42 kDa protein is unusual in being able to use Ca-ATP as a substrate as well as Mg-ATP, a finding which should greatly facilitate detection of this enzyme. In view of the simple pattern of phosphorylation observed in the presence of calcium, all further analyses of 42 kDa phosphorylation were carried out using Ca-ATP instead of Mg-ATP. It should be noted, that phosphorylation in the presence of 10 mM Mg^{2+} was not affected by up to 1 mM Ca^{2+} , and even 10 mM Ca^{2+} only reduced overall phosphorylation in the presence of 10 mM Mg^{2+} by about 25% (data not shown). Dephosphorylation of the 42 kDa protein occurred with a half-time of about 10 min (Fig. 2B) and was essentially complete after 30 min (Fig. 1C). Notably, dephosphorylation of the 47 kDa protein was much slower (Fig. 2B) and little affected by the presence of okadaic acid (Figs. 1C and 2B). This suggests that this protein is not a good substrate of PP1.

Paramecium calmodulin (0.1 mg/ml) had no detec-

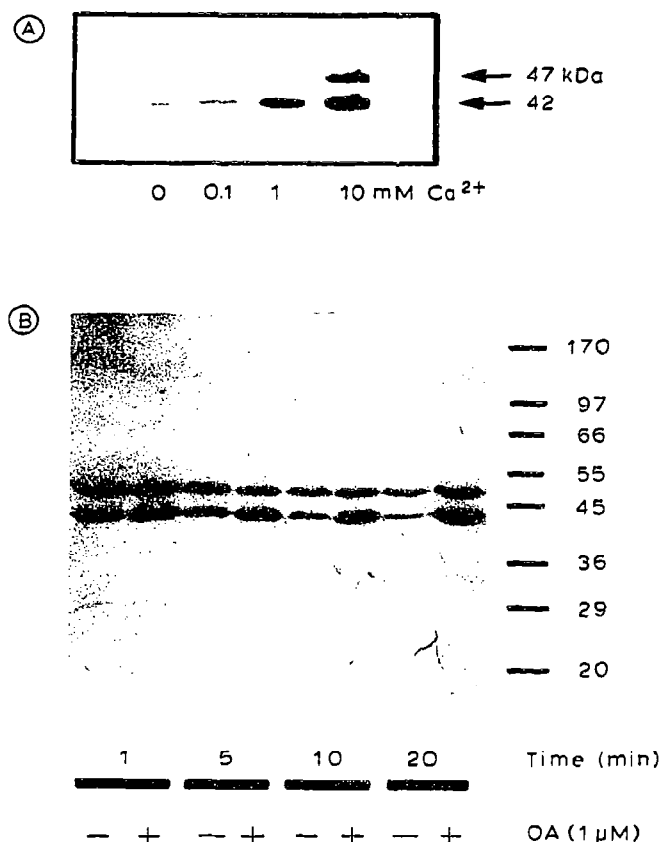


Fig. 2. Ca^{2+} requirement of phosphorylation (A) and time dependence of dephosphorylation (B) of a 42 kDa protein in cilia from *Paramecium* in the presence (+) or absence (-) of okadaic acid. Incubations in (B) contained 10 mM Ca^{2+} .

table effect on phosphorylation or dephosphorylation in the presence of Mg-ATP or Ca-ATP. Performing SDS-PAGE in the presence of 5 mM Ca^{2+} or 5 mM EGTA failed to shift electrophoretic mobility of the 42 kDa band, suggesting that it is neither a Ca^{2+} -binding nor a calmodulin-binding protein.

3.3. Inhibition of dephosphorylation of the 42 kDa phosphoprotein by cyclic AMP

Irrespective of the added divalent cation, phosphorylation of the 42 kDa protein was only slightly enhanced by addition of cyclic GMP or cyclic AMP (Fig. 3). A maximal effect (20–50%) was observed at 10 μM cyclic GMP and 1 μM cyclic AMP. In contrast, phosphorylation of the 47 kDa protein was dependent on the presence of cyclic nucleotides (Fig. 3). This suggests that phosphorylation of the 47 kDa protein is catalysed by a cyclic nucleotide-dependent protein kinase and that the major protein kinase phosphorylating the 42 kDa protein is unaffected by cyclic nucleotides. This was also indicated by the failure of the isoquinolinesulfonamide compound H7 (100 μM , [14]) to inhibit phosphorylation of the 42 kDa protein (data not shown).

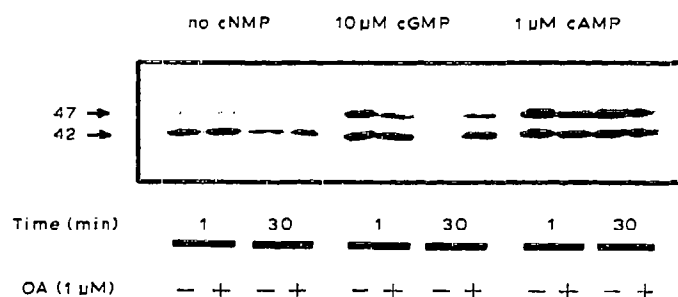


Fig. 3. Effect of cyclic nucleotides on in vitro protein phosphorylation in *Parametium* cilia. Samples containing 10 mM Ca^{2+} and okadaic (OA) as indicated were incubated for 1 or 30 min, respectively, in the absence of cyclic nucleotides (left), presence of 10 μM cGMP (middle) or 1 μM cAMP (right).

A surprising observation was that cyclic AMP (1 μM), but not cyclic GMP (10 μM), considerably attenuated the dephosphorylation of the 42 kDa protein occurring between 1 and 30 min (Fig. 3). This highly reproducible effect is ascribed to the inhibition of dephosphorylation, rather than to the stimulation of phosphorylation because after incubation for one min the extent of labelling in the presence of either cyclic AMP or cyclic GMP was only marginally enhanced. Actually it is conceivable that the inhibition of dephosphorylation by cyclic AMP might contribute to the slight enhancement of 42 kDa phosphorylation (Fig. 3). However, PP1 that has been purified from cilia, is not inhibited by cyclic AMP using ^{32}P -labelled phosphorylase α as a substrate (data not shown). This suggests that the strikingly specific effect of cyclic AMP in the crude membrane preparation is mediated by a component distinct from the catalytic subunit of PP1. This might be an, as yet, unidentified protozoan protein analogous to inhibitor-1 in mammalian cells, which becomes a potent inhibitor of PP1 only after phosphorylation by cyclic AMP-dependent protein kinase [15]. Alternatively, it is possible that the phosphorylated 42 kDa protein might interact with cyclic AMP causing a conformational change that prevents dephosphorylation by PP1.

3.4. Effect of other inhibitors of PP1 on 42 kDa dephosphorylation

Apart from okadaic acid, PP1 from *Parametium* and other eukaryotic cells is also potentially inhibited by tautomycin, an antifungal agent produced by *Streptomyces* bacteria, the structure of which resembles that of okadaic acid [16], and by microcystin-LR, a cyclic heptapeptide produced by the cyanobacterium *Microcystis aeruginosa* which is a potent hepatotoxin [17]. Like okadaic acid, these substrates do not inhibit the PP2A-like enzymes of *Parametium*, although they are powerful inhibitors of PP2A from other eucaryotic cells [16,17]. Tautomycin and microcystin-LR prevented dephosphorylation of the 42 kDa band in a similar manner to okadaic acid (Fig. 4), further establishing that this protein is a specific substrate for PP1. Furthermore, the phosphorylated inhibitor 1 from metazoans which also inhibits PP1 *Parametium* [4], blocked dephosphorylation of the 42 kDa protein (Fig. 4).

4. CONCLUSIONS

We have identified a 42 kDa protein as a specific substrate for PP1 in the excitable ciliary membrane of *Parametium*. These studies will pave the way for the purification and characterisation of this protein. Autophosphorylation of protein kinases is commonly observed. Judging from the molecular weights and cellular localization reported for the protozoan cyclic AMP-, cyclic GMP-, and Ca^{2+} -dependent protein kinases and their subunits [13,18-20], we conclude that these proteins have no relation to the 42 kDa protein. Investigation of whether it is a component of the voltage-operated calcium channel that ultimately controls backward swimming is now a feasible possibility. It must be noted, however, that substrates for PP1, that concurrently are also dephosphorylated by okadaic acid insensitive phosphatases in the cilia of *Parametium* (PP2A, PP2C [4]), would probably not be detected in our experiments. Our observations have facilitated identification of the protein kinase which

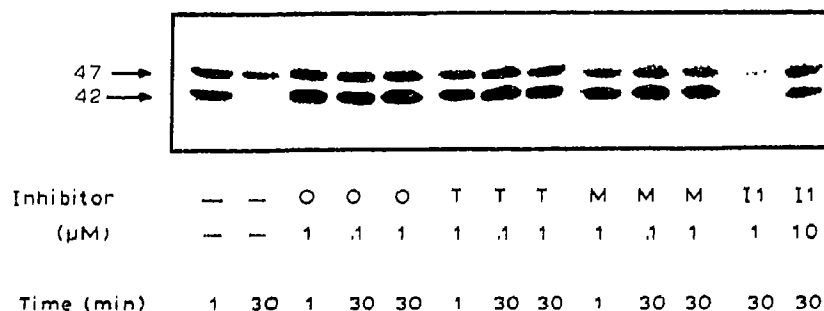


Fig. 4. Effect of inhibitors of protein phosphatase I on phosphorylation and dephosphorylation in *Parametium* cilia. Incubations containing 10 mM Ca^{2+} and 10 μM cGMP were incubated for 1 or 30 min, respectively. Okadaic acid (O), tautomycin (T), microcystin LR (M), and inhibitor 1 peptide (I1), were present as indicated.

phosphorylates the 42 kDa component. The introduction of Ca-ATP instead of Mg-ATP as a substrate may prove to be of general utility. Finally, the observation that the 42 kDa dephosphorylation is blocked by cyclic AMP suggests that this second messenger may control the activity of PP1 in *Paramecium* and, thus, may implicate cyclic AMP to modulate behavioural responses of this creature [21,22].

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