Volume 334, number 1, 13–17 FEBS 13198 November 1993

Identification and characterisation of a type-1 protein phosphatase from the okadaic acid-producing marine dinoflagellate *Prorocentrum lima*

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Received 13 September 1993

The unicellular marine dinoflagellate, *Prorocentrum lima*, an established producer of okadaic acid (OA), was shown to contain a type-1 protein phosphatase (PP-1) the biochemical profile of which on Mono-Q and Superdex-75 fast protein liquid chromatography was identical to the catalytic subunit of PP-1 from rabbit skeletal muscle. Purified *P. lima* PP-1 (apparent molecular mass 37.5 kDa) was highly sensitive to inhibition by mammalian protein phosphatase inhibitor-1 and inhibitor-2, and to OA itself. A 6 7-fold increase in OA production by *P. lima*, when grown under controlled conditions, correlated with an up to 300-fold increase in *P. lima* PP-1 activity. Furthermore, *P. lima* did not contain any detectable type-2A protein phosphatase activity. This study represents the first identification of a serine/threonine protein phosphatase in a dinoflagellate.

Protein phosphatase-1; Marine dinoflagellate; *Prorocentrum lima*; Okadaic acid; Tumour promoter; Protein phosphatase inhibitor

1. INTRODUCTION

Reversible phosphorylation of proteins on phosphoserine, phosphothreonine and phosphotyrosine residues by protein kinases and phosphatases is widely accepted as a principal mechanism by which eukaryotic cells respond to extracellular signals [1,2]. There is increasing evidence that certain species of marine/freshwater cyanobacteria and dinoflagellates produce a variety of potent inhibitors of vertebrate and invertebrate serine/threonine protein phosphatases [3–5], which have been effective in the delineation of signal transduction pathways in higher eukaryotes [6,7]. One class of inhibitor includes C_{38} diarrheic polyether fatty acids of the okadaic acid (OA) family [6,8], and a second class includes the more recently identified hepatotoxic cyclic peptides of the microcystin and nodularin families [9–11]. The marine dinoflagellate, *Prorocentrum lima*, a single-celled benthic eukaryote, is one of a panel of eukaryotic dinoflagellate species which produce a variety of toxins, including OA [12]. The catalytic subunits of type-1 and 2A protein phosphatases (PP-1c and PP-2Ac) are potently inhibited by OA and related congeners in a diverse range of higher eukaryotes [6,8] and more importantly have been shown to be the targets for OA in vivo [7]. However, the role or target(s) of these toxins in the marine environment or in the flagellates which produce them has not been previously addressed.

The purpose of this study was to identify the target(s) of OA and begin to establish a role for this tumor promoter in a dinoflagellate which itself produces significant intracellular OA concentrations (up to 0.02% of total cell mass). The serine/threonine protein phosphatases are highly conserved proteins, with respect to both substrate and inhibitor specificity [13–15] and have been identified in a diverse range of eukaryotes [13] including plants [16,17] and some protists [18– 20]. In this paper, we report identification and characterisation of a type-1 protein phosphatase in the OA-producing marine dinoflagellate *P. lima*, which is biochemically indistinguishable from rabbit skeletal muscle PP-1c [21]. *P. lima* PP-1 is sensitive to inhibition by nanomolar concentrations of OA and its regulation by this inhibitor in vivo may represent a primitive signalling pathway involving reversible phosphorylation in this early evolved marine eukaryote.

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2. MATERIALS AND METHODS

2.1. *Cell strain and culture conditions*

Benthic marine dinoflagellate isolate, *Prorocentrum lima* strain 712, was from the North Eastern Pacific Culture Collection (NEPCC), Department of Oceanography, University of British Columbia. Cells were cultured in natural sea water with nutrient and vitamin supplements [22]. Cultures were maintained without agitation at a constant temperature of 16°C, with 14/10 h light/dark cycles.

2.2. *Preparation of cell extracts*

Cells were harvested during late exponential growth phase by centrifugation at 2,500 × *g*. Extraction buffer (3 ml) comprising 50 mM Tris-HCl, pH 7, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% β-mercaptoethanol and 0.01% Brij 35, was added per g wet weight of either fresh cell pellet or pellet which had been flash frozen and stored at −20°C. A proteinase inhibitor cocktail (Boehringer-Mannheim) comprising antipain (50 μg/ml), (4-amidinophenyl)methanesulfonyl fluoride (10 μg/ml), aprotinin (2 μg/ml), bestatin (10 μg/ml), chymostatin (20 μg/ml), E-64 (10 μg/ml), EDTA-Na_2 (50 μg/ml), leupeptin (0.5 μg/ml),

pepstatin (0.7 μg/ml) and phosphoramidon (60 μg/ml) was also present. Cell suspensions were sonicated employing 45 s pulses with equivalent rest periods on ice. Resulting homogenates were centrifuged at 15,850 × *g* for 20 min at 4°C. Supernatants were subsequently dialysed against 12 vols. extraction buffer employing Amicon 10 concentrators at 1,000 × *g* and 4°C. After addition of 10% v/v glycerol, retentates were flash frozen and stored at −20°C. Protein concentrations were determined by BioRad dye binding assay employing bovine albumin as standard.

2.3. *PP-1c assay*

Aliquots (10 μl) of dinoflagellate extract were analyzed for their ability to dephosphorylate ^{32}P-radiolabelled glycogen phosphorylase *a* (EC 2.4.1.1). Preparation of components for this phosphorylase phosphatase assay was described previously [23]. To establish the identity of type 1 and/or 2A protein phosphatases, activity was measured in the presence and absence of a panel of inhibitors frequently used to characterise these enzymes. Protein phosphatase inhibitor-1 active peptide (PPIP-46) phosphorylated by cAMP-dependent protein kinase [24], and inhibitor-2 [25] were employed at final concentrations of 0.5 μM and 1.0 μM, respectively. OA (5 μM and 5 nM), microcystin-LR [4] (1.7 nM) and non-specific NaF (30 mM) were also employed. Assays were conducted such that phosphorylase phosphatase activity was not greater than 1.0 mU/ml.

2.4. *Fast protein liquid chromatography (FPLC)*

Dinoflagellate extracts were fractionated employing Mono-Q anion exchange columns developed with a linear gradient of 100 to 700 mM NaCl in running buffer (20 mM triethanolamine-HCl pH 7, containing 0.1 mM EGTA, 10% glycerol and 0.01% Brij 35) [21]. Column fractions were screened for phosphorylase phosphatase activity in the presence and absence of inhibitor-1 peptide (10 nM). Active fractions were pooled and subjected to Centricon-10 concentration at 1,000 × *g* and 4°C. Concentrates were then chromatographed on a Superdex-75 gel filtration column developed with 20 mM triethanolamine-HCl, pH 7, containing 100 mM NaCl, 0.1 mM EGTA, 10% glycerol, 0.1% β-mercaptoethanol and 0.01% Brij 35, at a flow rate of 0.1 ml/min. Molecular weight standards (13,700–200,000) were from Pharmacia.

2.5. *IC_{50} value determinations*

IC_{50} values for the inhibition of *P. lima* PP-1 by OA and inhibitor-1 peptide were determined, with final enzyme activity between 0.8 and 1.6 mU/ml per assay, where 1 unit is defined as the amount of enzyme activity required to dephosphorylate 1 μmol of phosphorylase *a* per min [26].

2.6. *Liquid chromatography and capillary electrophoretic analyses of OA*

P. lima cell filtrates were fractionated on a Vydac C_{18} reverse phase column and active fractions analysed by capillary electrophoresis (Beckman PACE 2100), as described previously [4].

3. RESULTS

3.1. *Identification of a type-1 protein phosphatase (PP-1) in the cytosolic extract of the OA-producing marine dinoflagellate,* Prorocentrum lima

Employing a panel of inhibitors which have been used to characterise serine/threonine protein phosphatase activity in tissue extracts [26,27], it was determined that a type-1 protein phosphatase was present in the dinoflagellate *P. lima* (Fig. 1). *P. lima* PP-1 dephosphorylated glycogen phosphorylase, a known physiological substrate of this enzyme in higher eukaryotes [13,28]. This activity was strongly inhibited in the presence of a fully active peptide from protein phosphatase

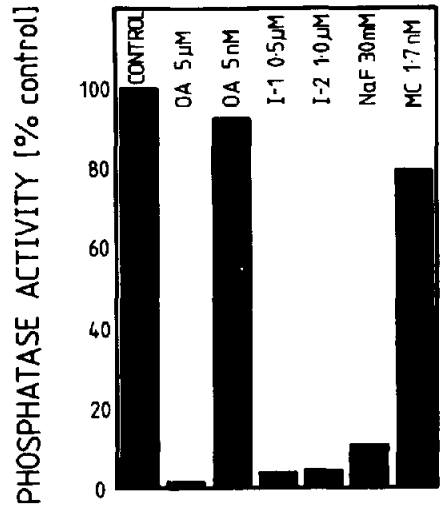

Fig. 1. The identification of type-1 protein phosphatase activity in a dialysed extract from *P. lima*. Serine/threonine type protein phosphatase activity was assayed employing [^{32}P]glycogen phosphorylase as substrate in the presence of a number of inhibitors (concentrations indicated). The activity is reported as a percentage of that observed in the absence of added inhibitors relative to the control.

inhibitor-1 and by protein phosphatase inhibitor-2, which are potent and specific inhibitors of type-1 protein phosphatases from mammalian sources [13,24]. *P. lima* PP-1 was sensitive to inhibition by OA at concentrations (5 μM) expected to inhibit both PP-1 and PP-2A, but only slightly inhibited (less than 10%) at concentrations (5 nM) expected to inhibit PP-2A only. These data are consistent with the identification of the *P. lima* enzyme as a type-1 protein phosphatase. Type 2A protein phosphatases (IC_{50} for OA = 0.1 nM), if active, would have been completely inhibited at the concentrations of OA utilised in these experiments [6,8,29]. *P. lima* PP-1 was also sensitive to inhibition by the cyclic heptapeptide toxin microcystin-LR, although the concentration employed (1.7 nM), suggested that *P. lima* PP-1 may not be as potently affected as the form of the enzyme present in mammalian tissues [26]. It is important to note that no phosphorylase phosphatase activity was detectable in extracts in *P. lima* prior to dialysis (see section 2). This observation was consistent with the presence of an endogenous protein phosphatase inhibitor in the dinoflagellate. The identification of this endogenous inhibitor as OA in all *P. lima* cultures utilised for phosphorylase phosphatase determinations (up to 215 μg/g wet weight) was confirmed by capillary electrophoresis, following extensive buffer exchange of the extract (Fig. 2).

3.2. *Further characterisation of* P. lima *PP-1 by Mono-Q anion exchange chromatography and Superdex-75 gel permeation chromatography*

The purified catalytic subunits of PP-1 and PP-2A (PP-1c and PP-2Ac) from rabbit skeletal muscle have

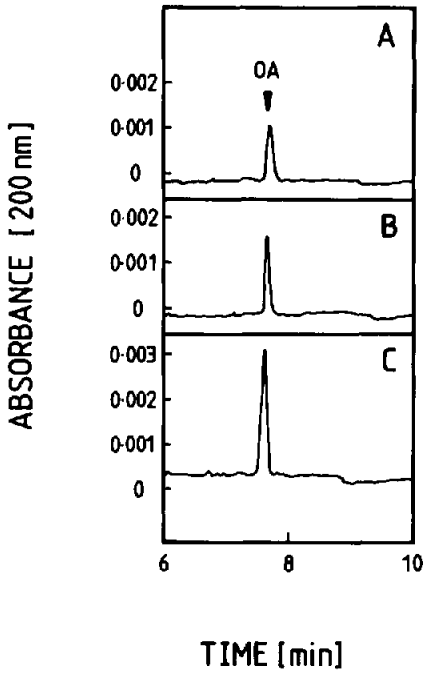

Fig. 2. Optical resolution and identification of OA present in *P. lima* by micellar capillary electrophoresis. Panel A represents the elution position of OA standard (0.5 ng), panel B represents the resolution of OA from the *P. lima* sample (0.54 ng) and panel C represents the confirmation of its identity by a characteristic increase in peak height upon spiking with the standard sample (1.04 ng, total).

characteristic elution positions when chromatographed on Mono-Q anion exchange FPLC columns. An extract from *P. lima* was subjected to Mono-Q chromatographic analysis (Fig. 3, panel A). Its elution position was almost identical to that of rabbit muscle PP-1c (Table I), and its identity as a type-1 protein phosphatase was further confirmed by complete inhibition of the phosphorylase phosphatase activity of this enzyme in the presence of inhibitor-1 peptide (10 nM). These results were suggestive of a similar net charge and primary sequence between the two forms of the enzyme. The elution position of *P. lima* PP-1 was characteristic of the catalytic subunit, with no bound regulatory subunits being detected, as are present in higher eukaryotes [13,30]. No type-2A protein phosphatase activity was detected in *P. lima*, in direct contrast to the closely related dinoflagellate *Prorocentrum micans* (which is not a producer of OA) where type-1 and -2A protein phosphatase activity could be resolved on Mono-Q FPLC columns and eluted at salt concentrations close to PP-1c and PP-2Ac from rabbit skeletal muscle (data not shown).

Superdex-75 gel filtration of the active PP-1 fractions from Mono-Q anion exchange chromatography, revealed this protein to have an apparent molecular mass of 37.5 kDa (Fig. 3, panel B). This data compares well with values reported for PP-1c from a variety of higher eukaryotes, including mammals (Table I) [13]. *P. lima* PP-1 purified from Superdex-75 chromatography was also strongly inhibited in the presence of inhibitor-1 peptide (Fig. 3, panel B).

3.3. *Inhibition of purified* P. lima *PP-1 by marine toxins and mammalian inhibitor proteins*

The concentrations of OA and inhibitor-1 peptide required to inhibit phosphorylase phosphatase activity

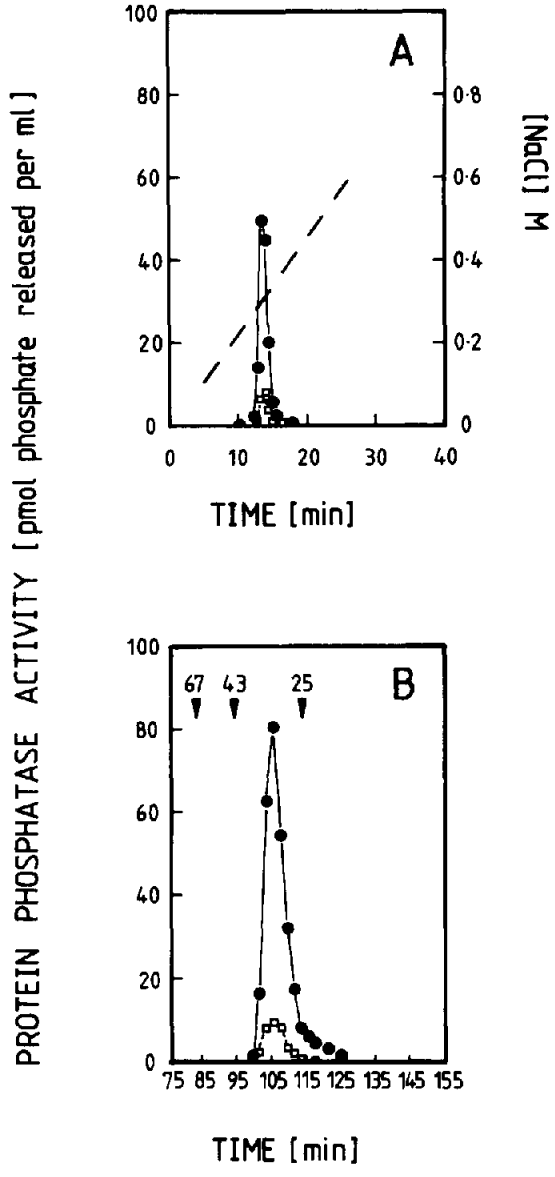

Fig. 3. Chromatographic analysis of phosphorylase phosphatase activity from *P. lima*. Panel A represents anion exchange chromatographic analysis on a Mono-Q column. Protein phosphatase activity is measured in the presence (□-□) and absence (●-●) of phosphorylated PPIP-46 (10 nM), a specific inhibitor of type-1 protein phosphatase. The broken line represents the salt gradient. Panel B represents Superdex-75 gel permeation chromatographic analysis of the active fractions from anion exchange chromatography. The elution of a single peak of activity in the presence (□-□) and absence (●-●) of phosphorylated PPIP-46 (10 nM) is shown in relation to relevant molecular weight standards.

Table I

Comparison of rabbit skeletal muscle PP1-c and *P. lima* PP1

	Rabbit skeletal muscle PP-1c	*Prorocentrum lima* PP-1
Substrate		
Phosphorylase *a*	Yes	Yes
Inhibitors		
Okadaic acid	IC_{50} = 10 nM	IC_{50} = 10–20 nM
Inhibitor-1	IC_{50} = 0.5 nM	IC_{50} = 3.0 nM
Inhibitor-2-microcystin-LR NaF	Yes	Yes
Chromatographic properties		
Elution position on Mono-Q FPLC	295 mM NaCl	310 mM NaCl
Apparent molecular mass on Superdex-75 and/or SDS-PAGE	33–37 kDa	37.5 kDa

IC_{50} values were determined using the purified enzyme (from active fractions) following anion exchange and gel permeation chromatography. Determinations were as detailed in section 2.

of *P. lima* PP-1 by 50% were determined. Employing a range of concentrations of both inhibitors, IC_{50} values were determined to be 10–20 nM and 3 nM for OA and inhibitor-1 peptide, respectively (Table I). These values compare well with IC_{50} values reported for PP-1c isolated from diverse eukaryotic sources [6,13,24].

3.4. *Comparative analysis of intracellular* P. lima *OA concentration and PP-1 activity*

Production of OA by different *P. lima* isolates was found to vary significantly, depending upon the sea water used as a nutrient source. It was therefore of interest to establish whether or not PP-1 activity might fluctuate in conjunction with altered levels of OA in *P. lima*. Comparative analyses of equivalent g/ml dialysed extracts prepared from two separate cultures of *P. lima* (NEPCC strain #712) (grown under moderate vs. high salt) natural sea water nutrient conditions, consistently revealed a 6–7-fold increase in the levels of OA between these extracts (typically 35 μg/g vs. 215 μg/g, respectively) as determined by LC and CE analysis, concomitant with a 300-fold increase in PP-1 activity (0.17 and 54 mU/ml, respectively).

4. DISCUSSION

At least four major types of eukaryotic serine/threonine protein phosphatase catalytic subunits are recognised which have been characterized according to their substrate specificity [13], association with regulatory/targeting subunits [13,30], and regulation by specific thermostable inhibitory proteins [13] and environmental toxins including OA [6]. According to this convention and direct comparative biochemical analysis with the catalytic subunit of rabbit skeletal muscle PP-1, we have characterised a type-1 protein phosphatase in the OA-producing marine dinoflagellate *Prorocentrum lima*. PP-1 purified from *P. lima* is biochemically indistinguishable from the rabbit skeletal muscle form of the enzyme (PP-1c) with respect to substrate (phosphorylase *a*), inhibitor specificity and chromatographic behaviour [13,21,28]. To our knowledge this study represents the first identification and characterisation of a protein phosphatase in a marine phytoflagellate and its presence in this early evolved eukaryote concurs with a growing body of evidence for the presence of these highly conserved enzymes in a number of protists [18–20].

A regulatory subunit associated form of PP-1 in *P. lima* was not directly observed. Regulatory subunits play a role in determining the subcellular location and modifying the specificity of the serine/threonine protein phosphatases in mammalian cells [30], and have also been observed in higher plants [16,17]. In addition, type-2A protein phosphatase activity was not detected, however, the absence of this protein phosphatase in the dinoflagellate awaits further confirmation as the possibility exists that it may be permanently inactivated in *P. lima* extracts by the strong interaction with its inhibitor OA [31].

The biochemical similarity between *P. lima* PP-1 and rabbit skeletal muscle PP-1c reported here is consistent with the strong conservation of the primary sequence of this enzyme throughout evolution [13,15]. Furthermore, employing degenerate primers corresponding to highly conserved regions in the catalytic domain of mammalian PP-1c, we have amplified a portion (10%) of the cDNA corresponding to the *P. lima* PP-1 protein which exhibits a high degree of homology (> 65% identity) with cDNA for rabbit skeletal muscle PP-1c. The complexity of the dinoflagellate genome [32] coupled with prohibitively long doubling times for these organisms, has at present facilitated only one other study of a protein at the cDNA level in these organisms [33].

The dinoflagellates are related to the ciliates [34], both having diverged well before the Metazoa. In the ciliate *Paramecium*, exogenously added OA has been shown to enhance backward swimming in response to depolarizing stimuli [35], suggesting a role for reversible phosphorylation in the regulation of voltage gated Ca^{2+} channels controlling swimming behaviour in this organism. Co-localization of a type-1 protein phosphatase (PP-1) to voltage-dependent Ca^{2+} channels in excitable ciliary membranes [19,35], and identification of a putative PP-1 substrate (p42), which is hyperphosphorylated directly in response to OA and other specific PP-1 inhibitors [36], supports a role for PP-1 in regulating motility in this ciliate.

The identification of a type-1 protein phosphatase in *P. lima* is clearly significant owing to the endogenous

presence of OA, a potent and specific inhibitor of eukaryotic PP-1c/PP-2Ac [6] and of *P. lima* PP-1 itself. If *P. lima* PP-1 represents an intracellular target for OA, an obvious potential role for OA in inhibiting PP-1 in vivo might be to regulate behavioural and physiological responses in this flagellate which are controlled by reversible phosphorylation. The intracellular regulation of *P. lima* PP-1 by endogenous OA or the apparent resistance of the protist to this toxin represents an important question to be addressed, since reversible protein phosphorylation is recognized as one of the most widely used mechanisms for signal transduction in eukaryotic cells [37]. In this regard, we have obtained preliminary evidence for a relationship between OA levels and *P. lima* PP-1 activity in this marine dinoflagellate. In a number of eukaryotic cells, exogenously added OA enhances the presence of the AP-1 transcription factor complex by inhibition of protein phosphatase activity, a mechanism which occurs via transcriptional activation of *jun* and *fos* genes [38,39]. The apparent upregulation of PP-1 activity which occurs concomitantly with increased levels of OA, might be explained by indirect transcriptional regulation of the *P. lima* PP-1 gene by intracellular OA. We are currently attempting to test this hypothesis further.

Acknowledgements: This work was supported by the Medical Research Council of Canada and the Alberta Heritage Foundation for Medical Research. We are grateful to Mrs. Jeanette Ramirez at the North Eastern Pacific Culture Collection for providing the cultured dinoflagellate isolate, *Prorocentrum lima* strain 712, and to Dr. Dawn Renfrew at Bamfield Marine Station on Vancouver Island for supplying natural seawater for the culturing of phytoplankton. We would also like to acknowledge the expert technical assistance of Ms. Kathy He Wang.

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