Identification of high levels of type 1 and type 2A protein phosphatases in higher plants

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Extracts of *Brassica napus* (oilseed rape) seeds contain type 1 and type 2A protein phosphatases whose properties are indistinguishable from the corresponding enzymes in mammalian tissues. The type 1 activity dephosphorylated the β -subunit of phosphorylase kinase selectively and was inhibited by the same concentrations of okadaic acid [IC_{50} (concentration causing 50% inhibition) $\approx$ 10 nM], mammalian inhibitor 1 (IC_{50} = 0.6 nM) and mammalian inhibitor 2 (IC_{50} = 2.0 nM) as the rabbit muscle type 1 phosphatase. The plant type 2A activity dephosphorylated the α -subunit of phosphorylase kinase preferentially, was exquisitely sensitive to okadaic acid (IC_{50} $\approx$ 0.1 nM), and was unaffected by inhibitors 1 and 2. As in mammalian tissues, a substantial proportion of plant type 1 phosphatase activity (40%) was particulate, whereas plant type 2A phosphatase was cytosolic. The specific activities of the plant type 1 and type 2A phosphatases were as high as in mammalian tissue extracts, but no type 2B or type 2C phosphatase activity was detected. The results demonstrate that the improved procedure for identifying and quantifying protein phosphatases in animal cells is applicable to higher plants, and suggests that okadaic acid may provide a new method for identifying plant enzymes that are regulated by reversible phosphorylation.

INTRODUCTION

Although it is well established that the reversible phosphorylation of proteins is a key regulatory mechanism in animal cells, the importance of this type of control in higher plants has only been recognized quite recently. Currently there is evidence that protein phosphorylation controls the switching between Photosystems I and II [1], and at least seven plant enzymes have been reported to be regulated by phosphorylation [2]. In several cases the protein kinases are Ca^{2+} /calmodulin-dependent [3–6], and Ca^{2+} is likely to be an important second messenger which mediates the effects of light on plant metabolism [7,8]. Even less is known about the dephosphorylation processes and their control. Apart from three reports describing an activity associated with pea (*Pisum sativum*) thylakoid phosphoproteins [9], the partial purification of a soybean (*Glycine max*) histone phosphatase [10] and two wheat-germ histone/casein phosphatases [11], to our knowledge no other paper on plant protein phosphatases has been published.

In the present study we have applied an improved procedure for identifying and quantifying the major serine/threonine-specific protein phosphatases in animal tissue extracts [12] to higher plants. Surprisingly, two of the four major protein phosphatase catalytic subunits found in mammalian tissues [13], type 1 and type 2A, were present in plant extracts at high levels, and their properties were essentially identical with those found in animal cells.

EXPERIMENTAL

Materials

Oilseed-rape (*Brassica napus* var. Jet Neuf) seed was provided by Dr. A. R. Slabas, Unilever Research, Sharnbrook, Bedford, U.K. Seeds were collected 38–44 days after flowering just at the onset of lipid accumulation [14], placed directly into liquid N_2 and stored at $-70^\circ C$. The catalytic subunits of protein phosphatases 1 and 2A (PP1 and PP2A) [15] and inhibitors 1 and 2 [16] were purified from rabbit skeletal muscle.

Preparation of rapeseed extracts

Seeds (10 g) were homogenized for 135 s (3×45 s) in 20 ml of ice-cold 50 mM-Tris/HCl/4 mM-EDTA, pH 7.0, containing 250 mM-sucrose, 1 mM-phenylmethanesulphonyl fluoride, 1 mM-benzamidine and 0.2% (v/v) 2-mercaptoethanol, using a Polytron (Ystral, Dottingen, Germany) homogenizer. The resulting homogenate was stirred with Triton X-100 (final concn. 2%, v/v) for 10 min, then centrifuged for 15 min at 20000 g ($4^\circ C$). The supernatant, termed 'cytosol', was filtered through glass wool and the pH was adjusted to 7.3 with 0.1 M-NaOH. The pale pellet was resuspended in the original volume of homogenization buffer. Protein concentrations of the cytosol and resuspended pellets, determined by the Bradford method [17], were 3.9 ± 0.1 and 17 ± 0.6 mg/ml respectively (three preparations).

In one set of experiments, Triton was not added to the homogenate. After centrifugation at 20000 g, two dis-

crete fractions were observed in the pellet: a 'dark-green pellet' overlaying the 'pale pellet' observed in the presence of Triton. The dark-green pellet could be removed without disturbing the pale pellet, and both fractions were therefore resuspended separately.

Preparation of ^{32}P -labelled substrates, and phosphatase assays

^{32}P -labelled rabbit skeletal-muscle phosphorylase kinase (1.66 mol of phosphate/ $\alpha\beta\gamma\delta$ unit) [18] and bovine casein [19] were prepared by phosphorylation with cyclic AMP-dependent protein kinase and ^{32}P -labelled muscle glycogen phosphorylase (1.0 mol of phosphate/mol of subunit) [15] by phosphorylation with phosphorylase kinase. The specific radioactivity of each substrate was $\approx 10^6$ d.p.m./nmol. Type 1 and type 2A protein phosphatases were assayed in the absence of bivalent cations and in the presence of 0.1 mM-EGTA [15] and type 2C phosphatase in the presence of 20 mM- Mg^{2+} [19] using 10 μM -phosphorylase, 6 μM - ^{32}P -casein and 1 μM -phosphorylase kinase. One unit of activity was that amount which catalysed the dephosphorylation of 1.0 μmol of substrate in 1 min. When inhibitor 1 or inhibitor 2 were included, diluted extracts were preincubated with these proteins for 15 min before initiating the reactions with substrate [16]. All assays were carried out at 150-fold dilution of each fraction, unless stated otherwise.

RESULTS AND DISCUSSION

Type 1 and type 2A protein phosphatases are the only enzymes in mammalian tissues with significant activity towards glycogen phosphorylase in the absence (or presence) of bivalent cations, and recent studies have demonstrated that these enzymes can be quantified in dilute tissue extracts by inclusion of either inhibitor 1 or inhibitor 2 (to inhibit type 1 phosphatases) or the tumour promoter okadaic acid, which inhibits type 2A phosphatases specifically at 1 nM [12].

The cytosol of *Brassica napus* showed high levels of phosphorylase phosphatase activity, of which $62 \pm 4\%$ could be inhibited by 0.1 μM -inhibitor 1 and $35 \pm 4\%$ by 1 nM-okadaic acid. In the presence of both 1 nM-okadaic acid and 0.1 μM -inhibitor 1, activity was reduced to $1.5 \pm 2\%$ (mean $\pm$ s.d. for five experiments with three different extracts). Thus activity unaffected by inhibitor 1 was inhibited almost completely by 1 nM-okadaic acid, whereas that resistant to 1 nM-okadaic acid was inactivated by inhibitor 1. Inhibitor 1 could be replaced by inhibitor 2 with identical results (not shown). These results indicated that the phosphorylase phosphatase activity in *Brassica* cytosol was contributed by two enzymes resembling the type 1 and type 2A phosphatases of mammalian tissues.

Phosphorylase phosphatase activity insensitive to inhibitor 1 was inhibited by okadaic acid with an IC_{50} (concentration causing 50% inhibition) of 0.1 nM, almost identical with that observed using the same concentration (0.15 munit/ml) of purified PP2A from rabbit skeletal muscle (Fig. 1). Conversely, activity that was sensitive to inhibitor 1 and unaffected by 1 nM okadaic acid was inactivated by higher concentrations of the tumour promoter. The IC_{50} value (≈ 10 nM) was identical with that observed with purified PP1 from rabbit skeletal muscle (Fig. 1).

Activity insensitive to 1 nM-okadaic acid was inhibited

Fig. 1. Effect of okadaic acid on plant and mammalian type 1 and type 2A protein phosphatases

The cytosol of *Brassica napus* was assayed at 150-fold dilution with ^{32}P -labelled phosphorylase as substrate. Type 2A activity (PP2A) was measured after preincubation for 15 min with 100 nM-inhibitor 1 (○). Type 1 activity (PP1) was determined in the absence of inhibitor 1 (▽) and the graph plotted after subtracting activity measured at 0.3 nM-okadaic acid to correct for type 2A activity. The closed triangles and circles show experiments with the homogeneous catalytic subunits of rabbit skeletal-muscle PP1 and PP2A respectively.

by rabbit muscle inhibitor 1 and inhibitor 2 with IC_{50} values of 0.6 nM and 2 nM respectively (Fig. 2). These concentrations are virtually identical with those observed for inhibition of type 1 phosphatase activity in rabbit skeletal-muscle extracts (inhibitor 1, 1 nM; inhibitor 2, 2 nM [12]).

Type 1 and type 2 protein phosphatases can also be distinguished by their selective dephosphorylation of the β (type 1) or α (type 2) subunits of phosphorylase kinase. *Brassica* cytosol also had high phosphorylase kinase phosphatase activity. Consistent with the results shown above, the β -subunit was dephosphorylated specifically in the presence of 2 nM-okadaic acid, whereas the α -subunit was dephosphorylated preferentially in the presence of 0.1 μM -inhibitor 1 (Fig. 3).

The resuspended 20000 g pellet had 40% of the type 1 phosphorylase phosphatase activity present in the cytosol (Table 1). This particulate activity was inhibited 80–95% by inhibitor 1 or inhibitor 2 and only 5–20% by 1 nM-okadaic acid (Table 1). The IC_{50} values for inhibitor 1, inhibitor 2 and okadaic acid were similar to those observed for the cytosolic type 1 phosphatase and, as expected, the particulate enzyme dephosphorylated the β -subunit of phosphorylase kinase selectively (not shown). These observations are similar to those made in several mammalian tissues [20]. For example, 75% of the type 1 phosphorylase phosphatase activity in rat liver homogenates, but almost no type 2A activity, is found in the 16000 g pellet (see Table 1 in [21]).

Extracts of mammalian tissues [12] or yeast [21] contain two further serine/threonine-specific protein phosphatases, termed type 2B and type 2C, which can be distinguished from type 1 and type 2A phosphatases by

Fig. 2. Effect of inhibitor 1 (I-1, ●) and inhibitor 2 (I-2, ○) on the phosphorylase phosphatase activity of protein phosphatase 1 in the cytosol of *Brassica napus*

Assays were performed in the presence of 1 nM-okadaic acid to inactivate PP2A.

their absolute requirement for Ca^{2+} and Mg^{2+} respectively [13]. In mammalian cells, type 2B activity is detected as a Ca^{2+} or Mn^{2+} -dependent, calmodulin-stimulated and trifluoperazine-inhibited activity, using ^{32}P -labelled inhibitor 1 as substrate [22]. However, no such activity could be detected in *Brassica* cytosol. Type 2C activity (which is resistant to okadaic acid) is most conveniently measured as a Mg^{2+} -dependent casein phosphatase in the presence of 5 μM -okadaic acid (to inhibit both type 1 and type 2A phosphatases completely [12]). No type 2C

Fig. 3. Dephosphorylation of the α - and β -subunits of phosphorylase kinase by the cytosol of *Brassica napus*

Experiments were carried out at 16-fold dilutions of the extracts in the presence of 0.1 mM-EGTA and either 2 nM-okadaic acid or 100 nM-inhibitor 1. The rabbit skeletal-muscle phosphorylase kinase substrate contained 0.79 mol of phosphate/mol of α -subunit and 0.89 mol of phosphate/mol of β -subunit. Release of phosphate from the α - and β -subunits was quantified as in [18].

casein phosphatase activity could be detected in *Brassica* cytosol, even though the expected level of type 2C was observed in parallel experiments with yeast extracts (results not shown).

The relative activities of the cytosolic and particulate type 1 phosphatases towards phosphorylase, phosphorylase kinase and casein were similar (Table 1) and resembled type 1 phosphatases of mammalian extracts in having very low activity towards casein [21]. Type 2A phosphatases accounted for nearly all the casein phosphatase activity in plant extracts (Table 1). The specific activities in cytosol with phosphorylase as

Table 1. Specific activities of type 1 and type 2A protein phosphatases in the cytosol and resuspended 20000 g pellet (pellet) prepared from *Brassica napus* extracts

Fractions were diluted 150-fold for measurements of phosphorylase phosphatase (PhP) and casein phosphatase (CP) activity, and 90-fold for measurement of phosphorylase kinase phosphatase (PhKP) activity. Assays were performed in the absence of bivalent cations as described in the Experimental section. Values are given as $\pm$ S.D. for the number of experiments shown in parentheses. Type 1 protein phosphatase activity was measured as both activity sensitive to 100 nM-inhibitor 1 and as activity not inactivated by 1 nM-okadaic acid, and these two values were averaged. Type 2A activity was the average of the inhibitor-1-insensitive activity and the okadaic acid (1 nM)-sensitive activity. The protein concentrations in the cytosol and pellet were 3.9 ± 0.1 and 17 ± 0.6 mg/ml (see the Experimental section)

Fraction	Activity (munits/mg)		
	PhP	PhKP	CP
Cytosol			
Type 1 phosphatase	2.38 ± 0.21 (5)	0.12 ± 0.02 (3)	0.026 ± 0.03 (3)
Type 2A phosphatase	1.36 ± 0.19 (5)	0.08 ± 0.01 (3)	0.35 ± 0.02 (3)
Pellet			
Type 1 phosphatase	0.34 ± 0.04 (3)	0.02 ± 0.002 (3)	< 0.001 (3)
Type 2A phosphatase	0.016 ± 0.005 (3)	< 0.001 (3)	0.004 ± 0 (2)

Table 2. Effect of Triton on the distribution of type-1 and type-2A protein phosphatases in extracts prepared from *Brassica napus*

The cytosol, dark green pellet and pale pellet were prepared as described in the Experimental section. Assays were carried out using phosphorylase as substrate and the proportion of type-1 and type-2A activity determined as in Table 1. The results show activity in munits derived from 1 ml of homogenate. Values are given as means $\pm$ s.d. for three preparations

Fraction	Activity (munits)			
	+ Triton		- Triton	
	PP1	PP2A	PP1	PP2A
Homogenate	16.2 $\pm$ 1.3	6.2 $\pm$ 1.5	15.0 $\pm$ 1.7	4.4 $\pm$ 0.8
Cytosol	9.1 $\pm$ 0.9	5.3 $\pm$ 0.6	1.6 $\pm$ 0.2	0.7 $\pm$ 0.06
Dark green pellet	0*	0*	10.5 $\pm$ 0.8	2.9 $\pm$ 0.3
Pale pellet	4.0 $\pm$ 0.6	1.3 $\pm$ 0.4	4.4 $\pm$ 1.0	1.2 $\pm$ 0.5

* The dark green pellet does not exist in extracts prepared with Triton (see the Experimental section).

substrate (2.4 munits/mg for type 1 and 1.4 munits/mg for type 2A) are high. The type 1 phosphorylase phosphatase specific activity is similar to that in rabbit skeletal-muscle extracts [23], which is the highest of all mammalian tissues [24]. The type 2A specific activity is greater than in several mammalian tissues, such as muscle or liver [21].

All the experiments described above were carried out using rapeseed homogenates that had been incubated with 2% Triton. If Triton was omitted, type 1 and type 2A phosphatases in the 20000 g supernatant were greatly decreased, these activities now being recovered in a new dark-green pellet (absent in homogenates incubated with Triton) which overlays the pale pellet (Table 2; also see the Experimental section). The finding that total type 1 and type 2A activity in the supernatant plus pellets was unchanged in the presence of Triton indicates that the phosphatases associated with the dark green pellet are accessible to substrate and therefore not contained within intact organelles. In addition, dissolution of the dark green pellet with Triton did not alter activity. In fact homogenization of the seeds with a Polytron homogenizer most likely destroys all subcellular organelles such as chloroplasts.

The results presented here have demonstrated that rape seeds contain high levels of type 1 and type 2A protein phosphatases with properties remarkably similar to those of the corresponding enzymes in animal cells. Similar observations have been made with leaf extracts prepared from maize (*Zea mays*) and pea (C. MacKintosh & H. G. Nimmo, unpublished work). The observation that the plant type 1 phosphatase is just as sensitive to mammalian inhibitor 1 and inhibitor 2 is particularly intriguing, because it raises the possibility that analogous regulatory proteins may be present in plants.

Okadaic acid was shown to inhibit type 1 and type 2A phosphatases specifically by Bialojan & Takai [25]. This C₃₈-fatty-acid polyether [26] can enter cells and, when added to intact mammalian adipocytes and hepatocytes, alters intracellular protein phosphorylation and carbohydrate and lipid metabolism in the manner expected of a specific protein phosphatase inhibitor [27]. It will be extremely interesting to examine the effects of this substance on protein phosphorylation and metabolism in intact plant cells. This approach may identify plant

enzymes that are regulated by phosphorylation, as well as those proteins which are dephosphorylated by the type 1 and type 2A protein phosphatases.

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