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DEPHOSPHORYLATION PATHWAY OF D-MYO-INOSITOL 1,4,5-TRISPHOSPHATE IN THE UNICELLULAR GREEN ALGA *CHLAMYDOMONAS EUGAMETOS*

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Key Word Index—*Chlamydomonas eugametos*; Chlorophyceae; inositol phosphate; dephosphorylation; signal transduction.

Abstract—*In vitro* dephosphorylation of D-myo-inositol 1,4,5-trisphosphate [$\text{Ins}(1,4,5)\text{P}_3$] by vegetative cells, gametes and zygotes of the green alga *Chlamydomonas eugametos* was studied using a soluble cell fraction as enzyme source and labelled $\text{Ins}(1,4,5)\text{P}_3$ as substrate. This compound was dephosphorylated yielding predominantly $\text{Ins}(1,4)\text{P}_2$, but in most cell types $\text{Ins}(4,5)\text{P}_2$ could also be detected. Both products were subsequently dephosphorylated to $\text{Ins}(4)\text{P}$, $\text{Ins}(1)\text{P}$ and finally inositol. The study demonstrates that in these algae $\text{Ins}(1,4,5)\text{P}_3$ is degraded via a pathway that is characteristic of vertebrate rather than higher plant cells.

INTRODUCTION

It has been established in both animal and plant systems that D-myo-inositol 1,4,5-trisphosphate [$\text{Ins}(1,4,5)\text{P}_3$] is an important second messenger for receptor-mediated Ca^{2+} mobilization [1, 2]. The central role of this compound implies that its metabolism is well regulated. From previous work it appears that $\text{Ins}(1,4,5)\text{P}_3$ degradation might be dependent on the type and developmental stage of the cells studied. In vertebrate cells, it is dephosphorylated by a 5-phosphatase to the biologically inactive $\text{Ins}(1,4)\text{P}_2$. This product is then further dephosphorylated yielding $\text{Ins}(4)\text{P}$ and eventually inositol. Inositol is recycled into phosphatidylinositol 4,5-bisphosphate [$\text{PtdIns}(4,5)\text{P}_2$], the precursor of $\text{Ins}(1,4,5)\text{P}_3$. In this way, a cyclic metabolic pathway is established that is characteristic for this signalling system. In plant cells, on the other hand, $\text{Ins}(1,4,5)\text{P}_3$ degradation seems to occur mainly by a 1-phosphatase, yielding $\text{Ins}(4,5)\text{P}_2$ [3]. In *Dictyostelium discoideum*, both enzymes are operative, but their relative activity is dependent on the developmental stage. At the onset of development, both phosphatases are present in equal amounts, but during development the 5-phosphatase disappears [4, 5]. This raises the question whether specificity and developmental regulation of $\text{Ins}(1,4,5)\text{P}_3$ degradation can be demonstrated in other cell types. We have chosen the unicellular green alga *Chlamydomonas eugametos*, in which the

$\text{Ins}(1,4,5)\text{P}_3/\text{Ca}^{2+}$ signalling system has been shown to be present. *Chlamydomonas eugametos* proliferates vegetatively by binary fission, but under nitrogen starvation the cells undergo gametogenesis and become sexually competent. Gametes of opposite mating types (mt^+ and mt^-) agglutinate together via their flagellar surfaces. Receptor–receptor interaction triggers the formation of an intracellular signal that induces a mating structure to protrude through the anterior cell wall of each gamete [6]. Pairs of mt^+ and mt^- gametes eventually fuse together via their mating structures. There is evidence for the release of intracellular calcium during mating in *C. reinhardtii* [7, 8], suggesting a role for $\text{Ins}(1,4,5)\text{P}_3$. More recently, relative large quantities of $\text{Ptd}(4,5)\text{P}_2$ have been demonstrated in *C. eugametos*, with a relatively rapid turnover, as might be expected of a signal precursor [9–11]. It has also been shown that $\text{Ins}(1,4,5)\text{P}_3$ can induce mating structure activation in such gametes [12]. Thus, it is reasonable to believe that $\text{Ins}(1,4,5)\text{P}_3$ plays a role as second messenger in this species. In this study we have studied the degradation route of $\text{Ins}(1,4,5)\text{P}_3$ in extracts of gametes, zygotes and vegetative cells. It complements earlier work on the conversion of $\text{Ins}(1,4,5)\text{P}_3$ into higher phosphates [13].

RESULTS AND DISCUSSION

Dephosphorylation of $\text{Ins}(1,4,5)\text{P}_3$ in extracts of mt^+ gametes

When $[2\text{-}^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ was incubated with an extract of mt^+ gametes, three major products were formed:

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InsP₂, InsP and Ins (Fig. 1). This indicates that all the enzymes for Ins(1,4,5)P₃ degradation were present. A small decrease (*ca* 10%) in activity was found when Triton-X100 was omitted from the extraction buffer (not shown), suggesting that (part of) the enzymatic activity was membrane-associated. In non-dialysed extracts only 10% of the Ins(1,4,5)P₃ was degraded in 30 min and only 10% of the degraded products appeared as inositol. Dialysing the extract increased Ins(1,4,5)P₃ degradation to *ca* 65%, suggesting the presence of a phosphatase inhibitor of low molecular mass. Boiling the preparation did not destroy the inhibitor. It might be an inositol phosphate that was present in sufficient amount to compete with the radioactive substrates.

When intact living cells were incubated with [2-³H]Ins(1,4,5)P₃ for 20 hr, *ca* 5% of the substrate was degraded into products that co-eluted with InsP₂, InsP₁ and Ins. However, this was also found when the radioactive substrate was incubated without cells. Further analysis of the degradation products proved that the majority of them could not be degraded by phytase and were therefore considered artifacts. We therefore conclude that the phosphatase activities in the cellular extracts are of intracellular origin.

To determine which phosphate group was removed first from Ins(1,4,5)P₃, a mixture of [4,5-³²P]Ins(1,4,5)P₃ (with 12% of the ³²P label at the 4-position [4]) and [2-³H]Ins(1,4,5)P₃ was incubated with a gamete extract. In Fig. 2, the HPLC elution pattern of the products (B) is illustrated in comparison with a mixture of markers (A). A relatively large amount of ³H and a small amount of ³²P

co-eluted with Ins(1,4)P₂ and not with Ins(4,5)P₂. This indicates that most of the 5-phosphate is hydrolysed first, yielding Ins(1,4)P₂. The presence of ³²P label in this product is explained by its presence at the 4-position in the parent compound. However, this result does not exclude the possibility that some Ins(4,5)P₂ was also formed but was rapidly degraded, preventing its detection.

The parent compound [2-³H]Ins(1,4,5)P₃ was also used to characterize the second dephosphorylation product, assuming that Ins(1,4)P₂ is a major intermediate. The InsP product eluted after the Ins(1)P standard at the position of Ins(4)P (Fig. 3). Thus, Ins(4)P was identified as the second intermediate in the degradation of Ins(1,4,5)P₃ to Ins.

Metabolism of Ins(1,4,5)P₃ in other cell types

The possibility, mentioned above, that Ins(4,5)P₂ is an intermediate in Ins(1,4,5)P₃ degradation was reinforced by the fact that it could readily be detected in assays using

Fig. 1. HPLC elution pattern of [2-³H]Ins(1,4,5)P₃ and its degradation products InsP₂, InsP₁ and Ins. [2-³H]Ins(1,4,5)P₃ was incubated with *C. eugametos* extract for 30 min. The reaction products were separated on a Sorbax SAX column by elution with linear gradients formed from H₂O (X) and NH₄-Pi buffer, pH 3.7 (Y): 0 min, 0% Y; 10 min, 0% Y; 30 min, 70% Y; 35 min, 100% Y (dotted line). The flow rate was constant at 1 ml min⁻¹. Fractions were collected every 0.5 min and radioactivity in each fraction was determined by liquid-scintillation counting. The numbered peaks are 1, inositol; 2, InsP₁; 3, InsP₂; 4, InsP₃. Each peak was separately analysed by using shallow gradients. This allowed the different isomers to be identified.

Fig. 2. Analysis of the first degradation product of Ins(1,4,5)P₃ after incubation with *C. eugametos* gamete mt⁺ extract. A mixture of [5-³²P]Ins(1,4,5)P₃, [5-³²P]Ins(4,5)P₂ and [3-³H]Ins(1,4)P₂ were separated as markers and a mixture of [2-³H]Ins(1,4,5)P₃ and [5-³²P]Ins(1,4,5)P₃ was incubated with extract for 30 min. The markers (A) and degradation products (B) were separated and detected as described in Fig. 1. The column was eluted with linear gradients formed from H₂O (X) and NH₄-Pi buffer, pH 3.7 (Y) to separate the different isomers of InsP₂: 0 min, 0% Y; 10 min, 0% Y; 11 min, 8% Y; 23 min, 20% Y; 28 min, 100% Y; 38 min, 100% Y. The flow rate was 1 ml min⁻¹ and fractions of 0.5 ml were collected. Closed symbols, [³²P] label; open symbols, [³H] label.

other cell types. Extracts were prepared from zygotes as well as from vegetative cells and gametes of both strains of *C. eugametos* and incubated with $\text{Ins}(1,4,5)\text{P}_3$. The phosphatase activity was highest in extracts of gametes and vegetative mt^+ cells and lowest in the vegetative mt^- cells and zygotes (Table 1). When the latter two extracts were mixed with that of mt^+ gametes, intermediate levels of activity were found, indicating that low activities were not due to inhibitors in the extracts (not shown). The first degradation product was mainly $\text{Ins}(1,4)\text{P}_2$, but $\text{Ins}(4,5)\text{P}_2$ was clearly present, except in extracts of mt^+ gametes, as shown above. It is apparent that there is an inverse correlation between the contribution of 1-phosphatase activity and the overall $\text{Ins}(1,4,5)\text{P}_3$ degradation activity (Table 1). This suggests that a low level of 1-phosphatase activity is present in all cell types, but is masked in those cells with high 5-phosphatase activity, such as mt^+ gametes.

The second degradation product in the cell extracts was mainly $\text{Ins}(4)\text{P}$. In mt^- vegetative cells the high proportion of $\text{Ins}(1)\text{P}$ was remarkable. This suggests the

presence of a very active 4-phosphatase, which as far as we know, has not been described previously. In all extracts $\text{Ins}(1,4,5)\text{P}_3$ was finally degraded to inositol (not shown).

Taken together, these results indicate that in *C. eugametos*, the dephosphorylation of $\text{Ins}(1,4,5)\text{P}_3$ is of a mixed type, with a 1-, 4- and 5-phosphatase being involved, dependent on the strain. Generally, the action of the 5-phosphatase was predominant, corresponding to the dephosphorylation pattern found in vertebrates. This is in agreement with the fact that the route by which higher inositol phosphates are synthesized in extracts of *C. eugametos* gametes also resembles that of higher animals rather than *Dictyostelium* or plants [13]. Differences between the various developmental stages were apparent, suggesting that the dephosphorylation pattern is modulated, as found in *Dictyostelium*. This may result in changes in the steady state level of $\text{Ins}(4,5)\text{P}_2$ and $\text{Ins}(1,4)\text{P}_2$ that may be significant in analogy with erythrocytes, where $\text{Ins}(4,5)\text{P}_2$ as opposed to $\text{Ins}(1,4)\text{P}_2$ has been shown to have signal properties [14]. Thus, by favouring a particular degradation pathway, the level of biologically active intermediates can be modulated.

Fig. 3. Analysis of the second (InsP) products. The HPLC conditions were as described in Fig. 1. $[^3\text{H}]\text{Ins}(4)\text{P}$ and $[^{14}\text{C}]\text{Ins}(1)\text{P}$ were used as markers. To separate the isomers the column was eluted with linear gradients formed from H_2O (X) and $\text{NH}_4\text{-Pi}$ buffer, pH 3.7 (Y): 0 min, 0% Y; 10 min, 0% Y; 11 min, 8% Y; 23 min, 20% Y; 28 min, 100% Y; 38 min, 100% Y. The flow rate was 2 ml min^{-1} and fractions of 0.1 ml were collected. Closed symbols, $[^{14}\text{C}]$ label; open symbols, $[^3\text{H}]$ label.

EXPERIMENTAL

Materials. $[2\text{-}^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$, $[5\text{-}^{32}\text{P}]\text{Ins}(1,4,5)\text{P}_3$ (with 12% of the label at the 4-position) and $[^3\text{H}]\text{Ins}(4)\text{P}$ were from New England Nuclear (Den Bosch, The Netherlands); $[^3\text{H}]\text{Ins}(1,4)\text{P}_2$, $[^3\text{H}]\text{Ins}(4)\text{P}$ and $[^{14}\text{C}]\text{Ins}(1)\text{P}$ were from Amersham Int. $[^{32}\text{P}]\text{Ins}(4,5)\text{P}_2$ was prepred from $[5\text{-}^{32}\text{P}]\text{Ins}(1,4,5)\text{P}_3$ using 1-phosphatase isolated from *D. discoideum* [4]. Phenylmethylsulphonyl fluoride (PMSF), aprotinin, leupeptin, dithiothreitol, Dowex-1 and phytase were from Sigma.

Cell cultures. *Chlamydomonas eugametos* strains 17.17.2 (mt^+) and 5.39.4 (mt^-) were grown in Petri dishes on agar-containing medium [15]. Gamete suspensions were obtained by flooding 2- to 4-week-old cultures with H_2O . Vegetative cells of the same strains were produced by growing them for 4 days in 500 ml of liquid medium. The cells were tested for the presence of gametes by mixing them with gametes of opposite mating type. The absence of agglutination indicated that the cells were vegetative. Primary zygotes were produced by mixing mt^+ and mt^- gamete suspensions in equal amounts in 15-cm Petri

Table 1. Dephosphorylation of $[2\text{-}^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ in extracts of different cell types of *C. eugametos* and the relative abundance of the InsP_1 and InsP_2 isomers recovered

Cell type	Per cent of InsP_3 degraded ($\mu\text{g}^{-1}\text{ protein min}^{-1}$)	Ratio of degradation products	
		$\text{Ins}(1,4)\text{P}_2 : \text{Ins}(4,5)\text{P}_2$	$\text{Ins}(1)\text{P} : \text{Ins}(4)\text{P}$
mt^+ Gametes	1.8	100:0	0:100
mt^- Gametes	1.6	84:16	31:69
mt^+ Veg. cells	1.5	90:10	33:67
mt^- Veg. cells	0.5	71:29	50:50
Zygotes	0.2	64:36	0:100

dishes. After 4–6 hr, vis-a-vis pairs began to aggregate in large patches on the bottom of each dish. After 18 hr, free-swimming gametes and agglutinating gametes were removed by suction in a pipette. Developing zygotes remained adhered to the bottom of the Petri dish. They were washed twice with H₂O and then brought into suspension by scraping them off with a piece of plastic tubing. *Ca* 3 ml of a dense suspension of these cells (*ca* 10⁷ ml⁻¹) was then inoculated on to agar medium in a Petri dish. After drying the agar surface in a stream of sterile air, the culture was left for 24 hr at 20° under continuous light (100 µmol photons m⁻² sec⁻¹). The pairs had then developed into round or sausage-shaped primary zygotes.

Enzyme extracts. *Ca* 2 × 10⁹ cells were centrifuged for 5 min at 1000 g. The pellet was washed with 10 vol of ice-cold buffer (20 mM HEPES–NaOH, 0.5 mM EDTA, pH 7), and resuspended in 5 vol of extraction buffer (20 mM HEPES–NaOH, 0.5 mM PMSF, 1 µg ml⁻¹ aprotinin, 1 µg ml⁻¹ leupeptin, 1 mM dithiothreitol, 1% Triton X-100, pH 7). Cells were then disrupted by sonication and the resulting homogenate was centrifuged for 10 min at 10 000 g. The supernatant was recentrifuged for 10 min at 150 000 g and the clear extract was dialysed twice for 6 and 16 hr against 100 vol of extraction buffer. During the whole extraction procedure the temp. did not exceed 4°.

Phosphatase assay. The dephosphorylation of inositol phosphates was assayed in extraction buffer in the presence of 5 mM MgCl₂ at 20°. Incubation was started by adding 10 µl of the *Chlamydomonas* extract to 10 µl assay mixt. containing 60 000–180 000 Bq radioactive substrate. The reaction was stopped after 10–60 min by boiling for 5 min (for HPLC analysis) or by adding 0.5 ml CHCl₃–MeOH–35% HCl (20:40:1) prior to chromatography on Dowex-1.

HPLC analysis. Labelled inositol phosphates and their isomers were sepd by HPLC on a Sorbax SAX column (Du Pont, Wilmington, U.S.A.) using a gradient system with increasing conc of a NH₄-Pi buffer, pH 3.7, in H₂O. The column was equilibrated with H₂O before each run. Aliquots (5 µl) of 5 mM AMP, ADP and ATP were included as int. standards in each sample and their elution was monitored by *A* at 259 nm. Eluant samples of 0.25, 0.50 or 1 ml were collected and the radioactivity determined after adding 2 ml scintillation fluid (Emulsifier 299, Packard).

Dowex anion exchange chromatography. The method was essentially as described earlier [4]. Phosphatase assays were stopped with CHCl₃–MeOH–35% HCl (20:40:1). To each sample 200 µl of H₂O was added, the mixt. shaken vigorously and the phases sepd by centrifuga-

tion (1 min, 10 000 g). The aq. phase was applied to a 0.5 ml Dowex-1 anion exchange column (formate form). The different reaction products were sepd by stepwise elution with: (i) 10 ml of H₂O (Ins); (ii) 10 ml of 150 mM NH₄-formate–5 mM Na₂B₄O₇ (InsP and Pi); (iii) 10 ml 300 mM NH₄-formate–100 mM formic acid (InsP₂), and (iv) 10 ml 750 mM NH₄-formate–100 mM formic acid (InsP₃). Radioactivity in the frs was measured after adding 10 ml of scintillation fluid.

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