Mutant ras gene induces elevated levels of inositol tris- and hexakisphosphates in Dictyostelium

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Summary

Previous studies of Europe-Finner & Newell indicated that in amoebae of Dictyostelium discoideum, signal transduction used for chemotaxis to cyclic AMP involved transient formation of inositol tris- and polyphosphates. Evidence was also presented for the involvement of a GTP-binding G-protein. Here we report evidence for the involvement of a ras gene product in the D. discoideum inositol phosphate pathway. Use was made of strains of Dictyostelium transformed with a wild-type D. discoideum ras gene (ras-Gly12) or a mutant form of the gene (ras-Thr12). Experiments using separation of soluble inositol phosphates by Dowex anion-exchange resin chromatography indicated that cells transformed with the wild-type ras-Gly12 gene were unaffected in their basal levels of inositol polyphosphates and in the inositol phosphates formed in response to stimulation with the chemotactic agent cyclic AMP. In contrast, cells transformed with the mutant ras-Thr12 gene showed a basal level of inositol polyphosphate that was several-fold elevated over the controls and stimulation of these cells with cyclic AMP produced only a small further elevation. When the inositol phosphates were analysed by h.p.l.c. it was found that the basal level of inositol 1,4,5-trisphosphate was raised three- to fivefold in the ras-Thr12 strain compared to the strain transformed with ras-Gly12, and that inositol hexakisphosphate (which was found to be present in large amounts relative to other inositol phosphates in D. discoideum cells) was also raised to a similar extent in the ras-Thr12-transformed cells.

We propose that the Dictyostelium ras gene product codes for a regulatory protein involved in the inositol phosphate chemotactic signal-transduction pathway.

Key words: ras gene, inositol triphosphate, InsP3, G-protein, Dictyostelium, chemotaxis.

Introduction

Dictyostelium discoideum has been shown to possess a single ras gene that is highly homologous to the three human ras genes (Reymond et al. 1984; Pawson et al. 1985; Weeks & Pawson, 1987). This gene has been cloned and has been re-introduced into D. discoideum and shown to be expressed (Reymond et al. 1985). Using site-directed mutagenesis a missense mutation that codes for threonine at position 12 of the gene product instead of the wild-type glycine has been constructed and introduced into Dictyostelium amoebae (Reymond et al. 1986). In human ras genes amino acid substitutions at homologous positions produce neoplastic transformation and decreased GTPase activity. A study of the phenotypes of the D. discoideum amoebae transformed with the wild-type ras-Gly12 gene has revealed no measurable difference from the untransformed control, despite the fact that cells possess approximately fourfold higher levels of the ras gene product. In contrast, however, amoebae transformed with the mutant ras-Thr12 gene showed
aberrant development with defective chemotactic aggregation in low-density cultures. At high densities aggregates were formed but were aberrant in developing multiple organizing tips. When various components of the signal relay system connected with adenylate cyclase were measured, no aberration could be detected in the ras-Thr12-transformed cells that could explain the mutant phenotype (Reymond et al. 1987). However, the formation of cyclic GMP in response to pulses of cyclic AMP was found to be abnormally low, due apparently to an effect on the signal adaptation mechanism. These results suggested that the role of the ras gene product was connected with the signalling pathway concerned with chemotaxis via guanylate cyclase rather than with signal relay that operates via the adenylate cyclase pathway.

Evidence has previously been presented indicating that the increased activity of guanylate cyclase occurs by stimulation of InsP3, formation after binding of the chemotactic signal cyclic AMP to specific cell surface receptors; the InsP3 formed then releases Ca2+ from internal (non-mitochondrial) stores that is able to trigger cyclic GMP formation (Europe-Finner & Newell, 1985, 1986a,b, 1987a,b; Small et al. 1986; Newell et al. 1987). Evidence for the formation of the other product of the InsP3-forming reaction (1,2-diacyl glycerol) and its activation of protein kinase C is so far only indirect (Ludérus et al., personal communication).

The involvement of a G-protein in the inositol phosphate pathway has been deduced from the stimulatory effects of GTP and non-hydrolysable analogues on inositol phosphate formation in saponin-permeabilized amoebae (Europe-Finner & Newell, 1987b).

In mammalian systems, recent evidence has implicated ras genes in coding for GTP-binding proteins involved in the inositol phosphate pathway of mammalian cells (Fleischman et al. 1986; Wakelam et al. 1986, 1987; Precis et al. 1986). It seemed appropriate, therefore, to investigate the effects of normal and oncogene-like mutant ras genes on inositol phosphate formation in D. discoideum.

**Materials and methods**

**Materials**

L-[^2H]inositol [1,2-[^2H]]inositol (sp. act. 58.5 Ci mmol⁻¹) was obtained from New England Nuclear and passed through Dowex 1-X8 column (formate form) prior to use to remove trace impurities. [³H]inositol 1,4,5-trisphosphate (potassium salt, sp. act. 1 Ci mmol⁻¹) was obtained from Amersham Int. PLC. Heps (N-2-hydroxyethyl-piperazine-N'-2-ethanesulphonic acid), Genetin and EDTA were from Sigma. Dowex 1-X8 (100-200 mesh chloride form) anion-exchange resin was obtained from Bio-Rad. The chloride form of the Dowex resin was converted to the formate form before use by passage of 3 M-ammonium formate through the resin in a 40 mm × 450 mm column, until acidified silver nitrate gave no reaction, followed by deionized water to neutrality.

**Harvesting of amoebae**

The axenic strain Ax3 and the Dictyostelium ras (Dd-ras)-transformed derivatives D. discoideum were grown axenically as described by Watts & Ashworth (1970) in medium supplemented with 250 μg ml⁻¹ dihydrostreptomycin sulphate. Amoebae were harvested during the exponential growth phase between 2 × 10⁶ and 5 × 10⁷ ml⁻¹ and the axenic medium was removed by washing in P buffer (17 mM-Na/K phosphate buffer, pH 6.1) and centrifugation at 190 g for 2 min. After three such washes the cells were resuspended in P buffer at 2 × 10⁸ cells ml⁻¹. To maintain the high copy-number of the plasmid bearing the Dd-ras genes the axenic medium was supplemented with 20 μg ml⁻¹ Genetin (also called G418) (Reymond et al. 1985).

**Labelling of amoebae with [³H]inositol: method 1 using starving cell suspensions**

Amoebae suspended in P buffer at 2 × 10⁸ cells ml⁻¹ were incubated in the presence of 170 nm-[^1,2-³H]inositol at a specific activity of 10 μCi mmol⁻¹. Dihydro-streptomycin sulphate and CaCl₂ were added to give final concentrations of 0.25 mg ml⁻¹ and 1 mM, respectively. Cells were incubated at 22°C with aeration in a rotary incubator at 170 revs min⁻¹. After 3 h cells were pulsed for 1 h with cyclic AMP (50 nM final concentration) at 15-min intervals so as to synchronize the amoebae. After a total incubation period of 4 h the amoebae were washed four times with 20 ml of P buffer to wash out [³H]inositol, and resuspended at 10⁸ cells ml⁻¹ in P buffer.

**Stimulation of labelled amoebae with cyclic AMP**

Samples (1 ml) of labelled washed amoebae (labelled by method 1) were incubated in the presence of 1 mM-CaCl₂ in plastic vials and shaken on an IKA-Vibrax platform shaker at 1400 revs min⁻¹ at 22°C or 4°C. Cells were stimulated with 20 μl of 2:6 μM cyclic AMP (to give a final concentration of 50 nM) and stopped at the appropriate time with 100 μl of 10% (v/v) HClO₄. The acid extracts were then left on ice for at least 30 min, neutralized with 135 μl of 1:53 M-KOH and then buffered to pH 7.2 with 40 μl of 75 mM-Heps. KClO₄ was precipitated at 0°C for 30 min on ice and removed by brief centrifugation.

**Separation of inositol phosphates by Dowex anion-exchange resin chromatography.** Neutralized extracts, as prepared above, were diluted to 5 ml with 5 mM-sodium tetraborate/0.5 mM-EDTA and applied to columns (40 mm × 6 mm) of Dowex 1-X8 (100-200 mesh formate) (Bone et al. 1984; Berridge et al. 1983) as described by Europe-Finner & Newell (1987a). The peak eluting with 100 mM-formic acid/1 M-ammonium formate was verified to

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contain InsP₃ by co-elution with an authentic sample of 
³H]Ins(1,4,5)P₃ (1 Ci mmol⁻¹).

Labelling of amoebae with [³H]inositol: method 2 using development on filters
Amoebae suspended in P buffer at 2x10⁷ cells ml⁻¹ were dispensed as 0·5 ml samples onto 47 mm diameter Whatman 50 filter discs supported on 47 mm diameter absorbant pads (Millipore AP1004700) saturated in P buffer (Newell & Sussman, 1969). After the excess liquid that soaked through the filters had been removed, the filters and pads were incubated at 22°C in 90 mm Petri dishes in the dark. The filters were prevented from drying out (and any ammonia produced was absorbed) by 70 mm Whatman no. 17 filter circles soaked in P buffer attached to the undersides of the lids of the Petri dishes. After the required period of incubation (2 or 4 h) each filter was removed from its supporting pad, the pad discarded, and the filter and cells carefully placed on a 100 μl drop containing 100 μCi of L-my/o-[1,2,³H]inositol (sp. act. 58-5 Ci mmol⁻¹) placed in the centre of a 90 mm Petri dish so that the liquid spread evenly under the filter without excess. No supporting pads were used. The filters were incubated for 2 h at 22°C in the dark as before and then the cells were removed from the filters into 20 ml of P buffer and washed four times with 10 ml of P buffer to remove [³H]inositol, before being finally resuspended at 2x10⁷ cells ml⁻¹ in P buffer.

Preparation of samples for HPLC. Samples (5 ml) of 2x10⁷ cells ml⁻¹ labelled by method 2 were shaken on an IKA-Vibra platform shaker at 1000 revs min⁻¹ at 22°C (or at 4°C) in plastic scintillation vials. After 10 min incubation extracts were made by addition of 500 μl of 10% (v/v) HClO₄, and were then left on ice for at least 30 min. Cell debris was removed by brief centrifugation (200g for 2 min) and the supernatants were neutralized with 730 μl of 1·53 m-KOH, with 200 μl of 75 nm-Hepes buffer added to adjust the pH to 7·2. The precipitated KClO₄ was removed by brief centrifugation and the supernatant stored overnight at −20°C. Any further precipitate was removed by further brief centrifugation. Supernatants were then transferred to separate tubes containing 500 μl of 10 mm-EDTA (Downes et al. 1986).

HPLC separation of InsP₅, InsP₆, InsP₇ and InsP₈
Separation of inositol phosphates was essentially as described by Heslop et al. (1985). The flow rate was set at 2·5 ml min⁻¹ and fractions were collected at 30-s intervals using two linear ammonium formate gradients. For the first 7 min after sample injection, water was passed through the column. The eluant concentration was then increased linearly from 0 to 0·85 m ammonium formate (buffered to pH 3·7 with orthophosphoric acid) over a period of 23·5 min to elute InsP₅ and lower inositol phosphates. The eluant concentration was then increased linearly over the next 11·5 min to 3·4 M to elute InsP₆, InsP₇ and InsP₈. The eluant was held at this concentration for 10 min before returning to water. The identities of the peaks were verified by addition to the samples of authentic ³²P-labelled Ins(1,4,5)P₃, Ins(1,3,4,5)P₄ and Ins(1,3,4,5,6)P₅ generously supplied by Dr Philip J. Hawkins (Smith Kline & French Research Ltd, UK). InsP₈ was identified by Dr R. F. Irvine (AFRC Institute of Animal Physiology, Babraham, Cambridge, U.K.) using an electrophoretic technique (Seiffert & Agranov, 1965).

Results
Cyclic AMP stimulation of inositol phosphate accumulation in strain Ax3
Previous studies concerned with the formation of inositol phosphates in D. discoideum employed the wild-type NC4 strain grown in association with Klebsiella aerogenes (Europe-Finner & Newell, 1987a,b). However, the ras-transformed strains used in this study (Reymond et al. 1984) were derived from the axenic mutant strain Ax3 and it was therefore essential to examine first the formation of inositol phosphates of this parental strain after growth in axenic medium. In the initial series of experiments described below the inositol phosphates were determined by separation using Dowex anion-exchange resin chromatography. Under the conditions we employ, the fraction eluted from these columns by 1 m-ammonium formate (which was reported by Europe-Finner & Newell (1987a) to include InsP₃ and InsP₄) has been found by more recent work using h.p.l.c. separation (see below) to include InsP₅, InsP₆, InsP₇ and part of the InsP₈ fraction (which is present in large amounts in D. discoideum). As a consequence, it will be referred to in this paper as IP₈. The measurement of IP₈ is used in the experiments described below to reveal rapid

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changes in the flux through the inositol phosphate pathway prior to determination of individual inositol phosphates by h.p.l.c.

The results of experiments with strain Ax3 (Fig. 1) indicate that at both 22°C and 4°C the effect of cyclic AMP stimulation on InsP₆ formation was found to resemble closely the previously reported results for NC4. The timing of the peaks was similar to NC4, except that the axenic strain showed a somewhat slower first peak: at 22°C it could be reproducibly observed at 5 s (rather than being almost over at this time as observed for NC4) and at 4°C it appeared at 10 s (rather than at 5 s).

InsP₆ formation in ras-transformed Ax3 cells at 22°C

When Ax3 amoebae that had been transformed with the normal D. discoideum ras homologue, ras-Gly12, were stimulated with cyclic AMP at 22°C, the results strongly resembled those obtained with Ax3, showing the same basal and cyclic AMP-stimulated levels (Fig. 2A). However, when the same experiment was carried out using Ax3 amoebae transformed with the mutant ras-Thr12 gene, the basal level of InsP₆ was found to be greatly increased (Fig. 2B). Stimulation with cyclic AMP always produced oscillations but with only a small increase over the controls stimulated with water. The troughs of the oscillations were, however,
Fig. 3. Time course of \( \text{InsP}_5 \) accumulation at 4°C after stimulation of \( \text{ras} \)-transformed amoebae with cyclic AMP. The amoebae used were of strain Ax3 transformed with a high copy number of either the normal Dd \( \text{ras} \)-Gly12 gene (A) or the mutant Dd \( \text{ras} \)-Thr12 gene (B). The amoebae were incubated for 4 h with \( [1,2-^3\text{H}] \)inositol (sp. act. 58.5 Ci mmol\(^{-1}\)), then stimulated with 50 nM cyclic AMP (●) or water (○○○) and extracts made at the times shown. The \( \text{InsP}_5 \) that accumulated was determined as described for Fig. 1. In A results are means of three experiments (cyclic AMP) and five experiments (water control). In B results are means of five experiments (cyclic AMP) and four experiments (water control). Error bars represent S.E.M.

Noticeably below the controls, possibly suggesting the presence of a very active phosphatase such that brief cessation of \( \text{InsP}_5 \) synthesis led to its rapid degradation. Presumably these oscillations are sufficient to induce the weak cyclic GMP responses that have been observed in the \( \text{ras} \)-Thr12-transformed cells at 22°C.

**InsP\(_5\) formation in ras-transformed cells at 4°C**

Previous experiments had shown that at 22°C, rapidly formed \( \text{InsP}_5 \) peaks could be missed if they occurred within 5 s of stimulation, but that such peaks could be observed if the events were slowed down by performing the experiments at 4°C (Europe-Finner & Newell, 1987\(^a\)). To ascertain whether such rapid effects were being missed in the experiments described above with the \( \text{ras} \)-Thr12 strain, the \( \text{InsP}_5 \) assays were repeated on cells incubated at 4°C. The results shown in Fig. 3 were obtained. As at 22°C the \( \text{ras} \)-Gly12-transformed cells showed basal \( \text{InsP}_5 \) levels similar to the parental Ax3 strain and could be stimulated by a pulse of cyclic AMP. The \( \text{ras} \)-Thr12-transformed amoebae, however, showed approximately a threefold higher basal level of \( \text{InsP}_5 \) and cyclic AMP did not show a significant increase or any evidence for oscillations.

When the mean values for the basal \( \text{InsP}_5 \) levels were calculated from all of the water controls shown in Figs 1–3, the results shown in Fig. 4 were obtained. It is evident that the \( \text{ras} \)-Gly12-transformed cells showed only very minor differences from the Ax3 parental cells but the elevation of \( \text{InsP}_5 \) levels in the \( \text{ras} \)-Thr12-transformed cells was highly significant, particularly in experiments performed at 4°C.

![Time course of InsP\(_5\) accumulation at 4°C after stimulation of \( \text{ras} \)-transformed amoebae with cyclic AMP](image)

**Fig. 4.** Mean basal levels of \( \text{InsP}_5 \) in amoebae of strain Ax3, and the \( \text{ras} \)-transformed strains of Ax3, \( \text{ras} \)-Gly12 and \( \text{ras} \)-Thr12. A. At 22°C; B, at 4°C. Conditions and methods were as described for Fig. 1. The means were calculated from the water stimulated time points over 60 s incubation shown in Figs 1–3. Errors bars represent S.E.M.

**Basal levels of \( \text{InsP}_3 \) and \( \text{InsP}_6 \) separated by h.p.l.c. in ras-transformed cells**

In order to ascertain which of the inositol phosphates were elevated in the mutant \( \text{ras} \)-transformed cells, cell extracts from \( [^3\text{H}] \)inositol-labelled cells were separated by h.p.l.c. using 25 cm Partisil Sax 10 anion-exchange columns (Irvine et al., 1985; Batty et al., 1985). In order to get sufficient \(^3\text{H}\) label into this fraction for accurate determinations, the labelling conditions were altered from the conditions described for \( \text{InsP}_5 \) determination (see Materials and methods) with the cells being deposited for development on filters rather than in starving suspension culture. Using these techniques the amounts of labelled \( \text{InsP}_3 \), \( \text{InsP}_2 \), \( \text{InsP}_1 \) and \( \text{InsP}_4 \) were determined in the \( \text{ras} \)-Gly12 strain. It was found that the \( \text{InsP}_3 \) and \( \text{InsP}_6 \) levels corresponded well with determinations made using Dowex anion-exchange columns.
Fig. 5. Mean basal levels of Ins(1,4,5)P₃ in amoebae of ras-Gly12- and ras-Thr12-transformed strains of Ax3 as determined by anion exchange h.p.l.c. The amoebae were incubated for 4 h at 22°C on filters with [1,2-³H]inositol (sp. act. 58.5 Ci mmol⁻¹) present from 2-4 h, before harvesting and washing the cells and incubation for 10 min at either 4°C or 22°C. A. Amoebae incubated at 22°C; B, amoebae incubated at 4°C. Results are the means of three experiments with error bars representing S.E.M.

columns on the same samples and were very similar to values reported previously for strain NC4 (Europe-Finner & Newell, 1987). It was found, however, that the amounts of InsP₂ and InsP₄ were much less than the InsP₃ peak seen with Dowex chromatography, suggesting that a significant amount of a higher inositol phosphate was present. When the eluant conditions that were used with the h.p.l.c. column were altered to those reported to elute higher inositol phosphates such as InsPs and InsP₆ (Heslop et al. 1985) a small amount of a ³H-labelled compound was found, which was identified as InsP₅ on the basis of its co-elution with authentic ³2P-labelled Ins(1,3,4,5,6)P₅ added to the sample. Further elution revealed an additional large ³H-labelled peak that corresponded in its elution time to that of InsP₅ reported by Heslop et al. (1985). This identification was verified by Dr R. F. Irvine (AFRC Institute of Animal Research, Babraham, Cambridge, U.K.) using the electrophoretic method of Seiffert & Agranoff (1965).

The most important inositol phosphate peak in terms of those with known functions in *D. discoideum* is Ins(1,4,5)P₃, which releases Ca²⁺ from non-mitochondrial stores (Europe-Finner & Newell, 1986b). When this peak was analysed in cell extracts from the ras-transformed strains the results shown in Fig. 5 were obtained, indicating that at both 22°C and 4°C the ras-Thr12-transformed amoebae had a three- to fivefold higher level of Ins(1,4,5)P₃ compared to amoebae transformed with ras-Gly12.

It was also of interest to determine whether the InsP₆ fraction, which was found to be the major ³H-labelled inositol polyphosphate in these cells (approximately 50- to 100-fold greater than the InsP₃ fraction), also showed the same pattern. The results (Fig. 6) indicate that, while considerable variation in the amount of InsP₆ was observed for different batches of cells (shown by the error bars) the ras-Thr12-transformed amoebae showed between three- and fivefold elevated levels compared to those of the ras-Gly12-transformed cells at both 22°C and 4°C.

**Basal levels of other inositol phosphates seen by h.p.l.c.**

Analysis of other peaks seen by h.p.l.c. indicated that a peak provisionally identified as glycerophosphoinositol was very similar in magnitude in both the ras-Gly12- and ras-Thr12-transformed strains. While the levels of InsP₁, InsP₂, InsP₄ and InsP₅ showed too much variation between batches of cells to permit meaningful assessment of differences, an unidentified peak that eluted after InsP₅ showed a significant two- to threefold elevation in the ras-Thr12-transformed amoebae at 22°C and 4°C.

**Effect of labelling at different times on InsP₃ and InsP₆ levels**

As a control to ensure that the differences in the InsP₃ and InsP₆ levels described above were not due to any subtle differences in the rate of development of the two ras-transformed strains, these two peaks were analysed in amoebae of both strains after labelling with [³H]inositol for different periods (either 2-4 h or 4-6 h) during development on filters. It was found that for both labelling times the pattern of differences between the two strains was identical and was as described above.

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Discussion

The data presented in this paper indicate that transformants bearing an oncogene-like mutation in the *D. discoideum* ras gene leading to substitution of threonine for glycine at position 12 of the ras gene product show a three- to fivefold elevated steady state level of Ins(1,4,5)P$_3$, and a similarly raised level of InsP$_6$, which is the major inositol polyphosphate of *D. discoideum*. These findings implicate the ras gene product in the signal transduction system leading from the cell surface cyclic AMP receptors to formation of InsP$_3$ and polyphosphates. The observed effects are not due simply to the fourfold increase in the level of ras protein in the transformants as cells transformed with the normal (Gly) ras gene under similar conditions behaved indistinguishably from the untransformed Ax3 strain.

In mammalian systems, similar studies using transformation with mutant ras genes have been found to lead to elevation of inositol phosphates (Fleischman et al. 1986; Wakelam et al. 1987; Preiss, 1986) although in some systems only elevation of diacyl glycerol has been reported (Wolfman & Macara, 1987). From the results presented here and from previous studies it is evident that the *Dictyostelium* signal-transduction pathway closely resembles that of mammalian cells both in its components and in at least some aspects of its regulation. It is a striking (and from an evolutionary point of view instructive) finding that such a strong similarity exists between both the inositol phosphate and the adenylate cyclase signalling systems of higher multicellular organisms and those of a 'primitive' eukaryote.

The nature of the regulatory action of the ras gene product is, however, unknown. It seems, from its size, to be unlikely to be identical with any of the known subunits of the G-proteins of signal-transduction pathways, and further understanding of its role must await the identification of protein(s) with which it interacts.

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