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Scrape-loaded p21^{ras} down-regulates agonist-stimulated inositol phosphate production by a mechanism involving protein kinase C

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The effect of scrape-loaded [Val-12]p21^{ras} on agonist-stimulated phosphatidylinositol 4,5-bisphosphate (PIP_2) turnover in Swiss-3T3 cells was studied. Previously [Morris, Price, Lloyd, Marshall & Hall (1989) Oncogene 4, 27–31] we demonstrated that [Val-12]p21^{ras} activates protein kinase C within 10 min of scrape loading. Here, we show that [Val-12]p21^{ras} inhibits bombesin and platelet-derived growth factor-stimulated PIP_2 breakdown 1.5–4 h after scrape loading. This effect persisted for at least 18 h and could be mimicked in control cells by activation of protein kinase C with 12-*O*-tetradecanoyl 13-acetate (TPA) 15 min prior to ligand stimulation. When protein kinase C was down-regulated by chronic TPA treatment, [Val-12]p21^{ras} was no longer able to inhibit agonist-stimulated inositol phosphate production. These results indicate that changes in inositol phosphate levels caused by ras protein are probably due to activation of protein kinase C and not to an interaction of ras with phospholipase C.

INTRODUCTION

The three ras proteins (N-, Harvey- and Kirsten-ras) can bind and hydrolyse GTP (McGrath *et al.*, 1984) and are thought to act as guanine nucleotide binding regulatory proteins in the plasma membrane (Willingham *et al.*, 1983; Willumsen *et al.*, 1984). Oncogenically activated ras proteins contain single amino-acid substitutions and often have a decreased rate of GTP hydrolysis. More importantly, they are unresponsive to the GTPase activiting protein, a cellular protein which stimulates GTP hydrolysis by normal p21^{ras} (Trahey & McCormick, 1987). These two effects allow oncogenically activated ras mutants to remain in an 'active' (GTP-bound) conformation. Such mutants cause cell transformation (Barbacid, 1987) and are thought to act by constitutively activating internal mitogenic signals independently of exogenous growth factor stimulation.

Exactly which signalling system(s) ras is involved in is not clear. A number of reports indicate that some ras functions are mediated through protein kinase C (Kamata *et al.*, 1987; Wolfman & Macara, 1987; Morris *et al.*, 1989). Protein kinase C can be activated by diacylglycerol (Nishizuka, 1984), which in turn is generated by the breakdown of phospholipids, e.g. phosphatidylinositol 4,5-bisphosphate (PIP_2) (Berridge, 1984). Several groups have looked for changes in phosphatidylinositol turnover in ras-transformed cells, but the results have been inconsistent. Some reports have suggested that ras causes an elevation of both basal and growth factor-stimulated PIP_2 breakdown (Wakelam *et al.*, 1986; Fleischman *et al.*, 1986; Hancock *et al.*, 1988; Maly *et al.*, 1988), whereas others show a decrease in growth factor stimulation (Benjamin *et al.*, 1987; Parries *et al.*, 1987). There have also been reports that ras can stimulate an increase in phospholipase A_2 activity (Bar-Sagi & Feramisco, 1986) or elevate phosphatidylcholine breakdown (Lacal *et al.*, 1987*a*). The effect of p21^{ras} on phospholipid metabolism is therefore unclear.

The studies to date have utilized cell lines transfected with normal or oncogenically activated *ras* genes. The problem with this approach is that it is difficult to distinguish between the primary (short term) effects of ras and the secondary (long term) effects of clonal selection and cell transformation. Indeed, Alonso *et al.* (1988) have shown that cells transformed by a number of different oncogenes (including ras) display similar changes in levels of inositol phosphates.

We have recently utilized a novel method termed 'scrape loading' (McNeil *et al.*, 1984) to introduce oncogenically activated ras proteins into Swiss-3T3 cells (Morris *et al.*, 1989). This approach allows analysis of the immediate effects of adding a transforming protein to the cell. We have shown that: (1) protein kinase C is activated within 10 min of scrape loading [Val-12]p21^{ras} into the cells; (2) protein kinase C activation occurs by a mechanism which does not involve PIP_2 breakdown and (3) ras proteins can cause morphological transformation and initiate DNA synthesis 16–20 h after scrape loading (Morris *et al.*, 1989).

Here, we have used the scrape-loading technique to monitor the basal and growth factor-stimulated levels of inositol phosphates over the time course required for initiation of DNA synthesis by scrape-loaded ras protein. We find that [Val-12]p21^{ras} has no effect on the basal level of inositol phosphates. However, activation of protein kinase C by [Val-12]p21^{ras} can significantly decrease both platelet-derived growth factor (PDGF)- and bombesin-stimulated inositol phosphate production 1.5–4 h after scrape loading ras. We suggest that activation of protein kinase C by ras can indirectly alter inositol phosphate levels in ras-transformed cells. This may go some way towards explaining the differences seen by various authors.

Abbreviations used: PIP_2, phosphatidylinositol 4,5-bisphosphate; PDGF, platelet-derived growth factor; DMEM, Dulbecco's modified Eagle's medium; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; PBS, phosphate buffered saline.

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MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), antibiotics, trypsin and foetal calf serum were from Gibco. *myo*-[2-^{3}H]Inositol and PDGF were from Amersham International. Bombesin, 12-*O*-tetradecanoylphorbol 13-acetate (TPA) and other chemicals were from Sigma.

Cells

Swiss-3T3 cells were grown in DMEM containing 10% foetal calf serum. Cells were seeded at 5×10^4 cells per 30 mm dish, and grown to confluence.

Labelling

Cells were labelled overnight (19 h) in serum- and inositol-free DMEM containing *myo*-[2-^{3}H]inositol (20 μCi/ml).

Scraping

The medium was removed and the cells were washed twice with phosphate-buffered saline (PBS; 138 mM-NaCl/2.7 mM-KCl/9.2 mM-Na_2HPO_4/1.8 mM-KH_2PO_4). Harvey-ras protein containing a Gly-12→Val-12 mutation {both [Val-12]p21^{ras} and [Ser-186,Val-12]p21^{ras} were purified from an *E. coli* expression system; Hall & Self (1986)} was layered over the cells (80 μl at 3 mg/ml). Cells were gently detached by scraping with a rubber policeman to allow protein uptake (McNeil *et al.*, 1984). Control cells were scraped in ras buffer (50 mM-NaCl, 5 mM-Tris/HCl, pH 7.0, and 5 mM-$MgCl_2$). Cells treated in this fashion take up [Val-12]p21^{ras} as assayed by immunofluorescence and are morphologically transformed after about 15 h (Morris *et al.*, 1989). After scraping, cells were either transferred to microcentrifuge tubes (for measurements in suspension) or allowed to re-attach to gelatin/fibronectin coated dishes and re-incubated with *myo*-[2-^{3}H]inositol (20 μCi/ml) in 1 ml of DMEM for 4–18 h.

Inositol phosphate assays

Attached cells were washed once in PBS, then incubated for 15 min in Ca^{2+}-free Hanks buffer, containing glucose (10 mM), LiCl (15 mM), bovine serum albumin (1 mg/ml) and Tris/HCl (10 mM, pH 7.0). Ligands were added after 15 min and the cells were incubated for the times indicated. Inositol phosphates were assayed as previously described (Morris *et al.*, 1989). Total inositol phosphates were eluted from Dowex-I columns with 1.2 M-ammonium formate/0.1 M-formic acid to ensure removal of inositol tetrakisphosphate (Berridge *et al.*, 1983). Results are expressed as c.p.m. in inositol phosphates per 1000 c.p.m. in inositol-containing lipids. All cell samples had similar levels of lipid labelling. Incubations typically contained 3000–5000 c.p.m.

RESULTS

Swiss-3T3 cells were scrape-loaded with [Val-12]p21^{ras} and reseeded onto gelatin/fibronectin-coated dishes for 18 h in the presence of *myo*-[2-^{3}H]inositol. The cells were then stimulated with PDGF, and inositol phosphate levels were monitored over the next 40 min. Fig. 1 shows that basal rates of inositol phosphate production were unaffected by the presence of [Val-12]p21^{ras} (Fig. 1; ○, □). PDGF gave a large stimulation of inositol phosphate production (●), but this was greatly reduced in cells scrape-loaded with [Val-12]p21^{ras} (Fig. 1; ■) prior to growth factor stimulation. Similar results were obtained with bombesin. The data are summarized in Table 1. Both the bombesin and PDGF inositol phosphate responses are markedly decreased in cells containing [Val-12]p21^{ras} (Table 1). This is similar to the decrease in the PDGF-stimulated inositol phosphate response seen in cells overexpressing mutant p21^{ras} (Benjamin *et al.*, 1987; Olinger & Gorman, 1988).

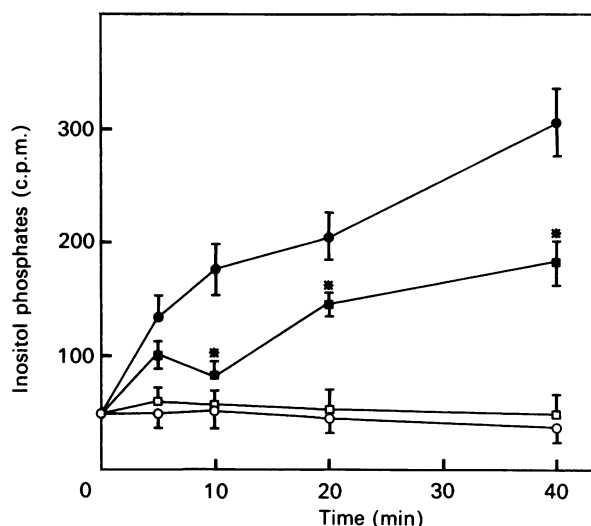

Fig. 1. PDGF stimulation of inositol phosphate turnover in cells scrape-loaded with [Val-12]p21^{ras}

Cells were labelled with [^{3}H]inositol (20 μCi/ml), scraped, allowed to re-attach and grown for 18 h. Inositol phosphate assays were carried out as in the Materials and methods section. LiCl was added 15 min before zero time points. ○ and ● are controls; □ and ■ are [Val-12]p21^{ras}. Open symbols, no addition; closed symbols, PDGF added (40 ng/ml). Results are expressed as c.p.m. in inositol phosphates per 1000 c.p.m. in inositol-containing lipids, and are means ± S.E.M. * indicates $P < 0.05$ in two-sided t test (n = 10–12) compared with controls (●).

Table 1. Growth factor stimulation of inositol phosphate production in [Val-12]p21^{ras} scraped cells

Conditions were as described in legend to Fig. 2. Cells were scraped in either buffer or buffer containing ras (3 mg/ml), and allowed to reattach for 18 h. Inositol phosphate assays were carried out as in the Materials and methods section. Cells were stimulated for 20 min. N.D. = not done. Results are means ± S.E.M. (n = 6), and are expressed as c.p.m. in inositol phosphates/1000 c.p.m. in inositol-containing lipids.

		Inositol phosphate production		
Cells scraped with:	Addition...	No addition	Bombesin (50 nM)	PDGF (40 ng/ml)
Buffer		72±12	157±14	205±11
[Ser-186,Val-12]p21^{ras}		63±4	N.D.	225±12
[Val-12]p21^{ras}		73±10	108±4	157±15

As a control for the effect of [Val-12]p21^{ras}, a biologically inactive protein, [Ser-186,Val-12]p21^{ras}, was scrape-loaded. This mutant is non-transforming since it lacks the palmitoylation site essential for membrane anchoring (Willumsen *et al.*, 1984). PDGF responses were unaffected in cells loaded with this defective protein (Table 1), indicating that the decreased PDGF response is due to the transforming activity of [Val-12]p21^{ras}.

To see how rapidly down-regulation occurred after introducing ras, cells were scrape-loaded with [Val-12]p21^{ras} and reseeded onto gelatin/fibronectin coated dishes for either 4, 10 or 18 h, before addition of PDGF. The results are shown in Fig. 2. A small increase in the basal level of inositol phosphates occurs over the first 4 h after scraping (Fig. 2; ○, □). This is probably due to disruption of cellular metabolism and/or changes in ion fluxes during cell detachment. PDGF stimulation remains constant over the 18 h of the experiment in control cells (Fig. 2; ●). Cells scraped with [Val-12]p21^{ras} (Fig. 2; □) show no statistically significant changes in basal levels of inositol phosphates relative to controls.

The PDGF response in [Val-12]p21^{ras} scraped cells is, however, decreased compared to the control levels of inositol phosphates (Fig. 2; ●, ■). This effect is maximal after 4 h and persists for at least 18 h after scraping. Since cells require 1–2 h to re-attach after scraping, it is not possible to measure this effect less than 4 h after introducing [Val-12]p21^{ras}. However, cells scraped and maintained in suspension for up to 90 min can be used to look at earlier time points.

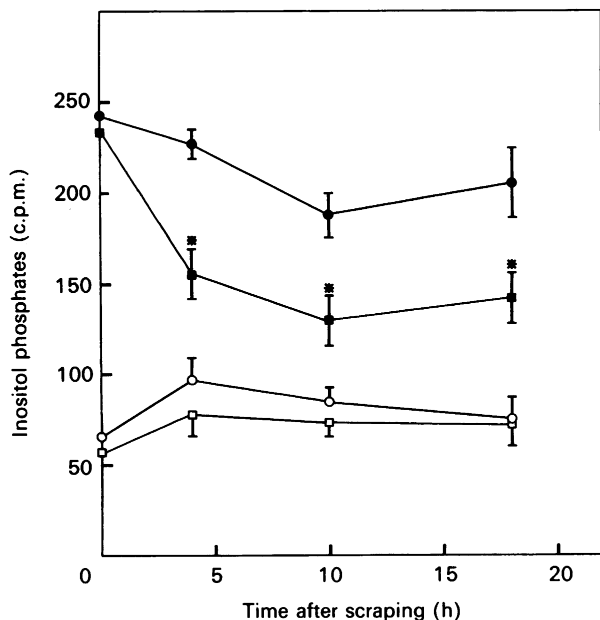

Fig. 2. Time-dependent decline in PDGF stimulation after scrape loading with [Val-12]p21^{ras}

Cells were labelled and scraped as described in the Materials and methods section and allowed to re-attach for 4, 10 or 18 h. Cells at $t = 0$ were assayed in suspension directly after scraping. Inositol phosphates were measured over 20 min. ○ and ● are controls; □ and ■ are [Val-12]p21^{ras}. Open symbols, basal rates; closed symbols, PDGF added. Results are expressed as c.p.m. in inositol phosphates per 1000 c.p.m. in inositol-containing lipids, and are means ± S.E.M. *indicates $P < 0.05$ in two-sided t test ($n = 10$–12), compared with controls (●).

Cells containing [Val-12]p21^{ras} kept in suspension for 40 min and then stimulated with PDGF have similar levels of inositol phosphates to control cells (control = 286 ± 45 c.p.m.; [Val-12]p21^{ras} = 279 ± 35 c.p.m.). Cells left 90 min apparently have a slightly decreased PDGF stimulation compared to controls (control = 300 ± 23 c.p.m.; [Val-12]p21^{ras} = 233 ± 24 c.p.m.), although this difference is not significant using a t test at $P = 0.05$. In all cases, basal rates were unaffected by [Val-12]p21^{ras} (results not shown). The data in Fig. 2 indicate that [Val-12]p21^{ras} causes a decrease in the levels of PDGF-stimulated phosphatidylinositol breakdown. This effect occurs between 90 min and 4 h after introduction of [Val-12]p21^{ras} and continues for at least 18 h.

Scrape-loading cells with [Val-12]p21^{ras} has been shown to activate protein kinase C approx. 10 min after loading (Morris *et al.*, 1989). Protein kinase C is known to modulate the inositol phosphate response in 3T3 cells (Brown *et al.*, 1987). We have used two approaches to see if the effects of [Val-12]p21^{ras} on growth factor stimulated PIP_2 breakdown are due to protein kinase C activation: first, activation of protein kinase C with TPA prior to ligand stimulation; and secondly, removing protein kinase C by prolonged (40 h) exposure to TPA (Rodriguez-Pena & Rozengurt, 1984) prior to stimulation.

Pretreatment of cells for 15 min with TPA (100 nM) caused a decease in the inositol phosphate response of control cells to both PDGF and bombesin (Fig. 3). Basal rates were unaffected (Fig. 3; A, B). This decrease in inositol phosphate levels is similar to that seen in cells scrape-loaded with [Val-12]p21^{ras} (Fig. 3; C). So both

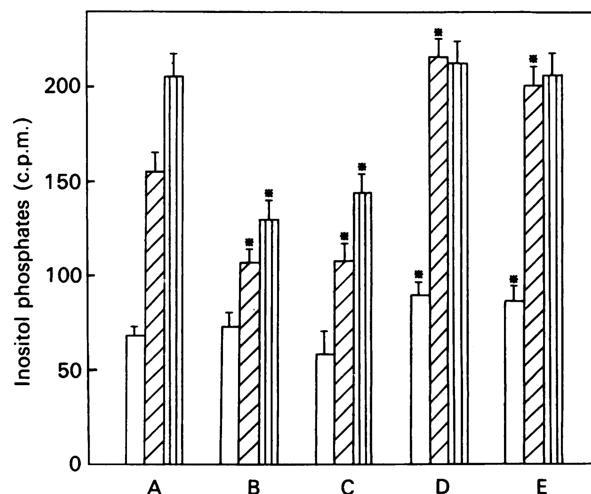

Fig. 3. Effect of protein kinase C down-regulation on the [Val-12]p21^{ras} inhibition of inositol phosphate production

Cells were labelled with [^{3}H]inositol, scraped and allowed to re-attach for 18 h in the presence of [^{3}H]inositol. Cells were either untreated (A, C), pretreated for 15 min with TPA (100 nM) before ligand stimulation (B), or treated for 22 h with TPA (400 nM), scraped, and then treated with TPA for a further 18 h (D, E). A, B and D were scraped in buffer; C and E were scrape-loaded with buffer plus [Val-12]p21^{ras}. □, Control; ▨, bombesin; ▥, PDGF. Results are expressed as c.p.m. in inositol phosphates per 1000 c.p.m. in lipid, and are means ± S.E.M. * Indicates $P < 0.005$ in two-sided t test ($n = 12$) compared with A.

activation of protein kinase C (by TPA) or addition of [Val-12]p21^{ras} can cause a decrease in PDGF- and bombesin-stimulated inositol phosphate production. To down-regulate protein kinase C, cells labelled with [^{3}H]inositol were pretreated with TPA for 22 h before scraping. After 18 h to allow reattachment, cells were tested for their response to PDGF and bombesin. It can be seen that both basal and bombesin-stimulated responses were enhanced in control cells (Fig. 3; A, D), suggesting that protein kinase C may normally have a negative effect on inositol phosphate production. PDGF stimulation was unaffected. However, when cells in which protein kinase C had been down-regulated were scrape-loaded with [Val-12]p21^{ras}, there was no longer any decrease in PDGF- or bombesin-stimulated inositol phosphate production compared with controls. These results indicate that decreases in growth factor responses are caused by activation of protein kinase C, and suggest that protein kinase C may negatively regulate both the basal and growth-factor-stimulated levels of inositol phosphates.

DISCUSSION

It has been proposed that ras may be a guanine nucleotide regulatory protein involved in the coupling of growth factor receptors to PIP_2 breakdown (Fleischman *et al.*, 1986; Wakelam *et al.*, 1986). Cell transformation by oncogenic forms of ras would then occur by constitutively elevating the basal levels of inositol phosphates and diacylglycerol, causing continuous protein kinase C activation. We (Morris *et al.*, 1989) and others (Kamata *et al.*, 1987; Wolfman & Macara, 1987) have demonstrated that protein kinase C is activated in cells transformed by ras and that this activation is essential for the initiation of DNA synthesis by ras (Lacal *et al.*, 1987*b*).

The situation with inositol phosphates is not so clear. A wide range of cell types containing either oncogenic or proto-oncogenic *ras* genes have been used to look for changes in inositol phosphate levels. NRK cells containing v-Ki-*ras* show an increased basal level of both diacylglycerol and inositol phosphates (Fleischman *et al.*, 1986; Kamata *et al.*, 1987) but v-Ki-*ras*-transformed NIH-3T3 cells apparently show no change in basal levels of inositol phosphates, but elevated diacylglycerol levels (Parries *et al.*, 1987; Wolfman & Macara, 1987). v-Ha-*ras* (in NIH-3T3 cells) shows no change in basal levels of inositol phosphates, but elevated diacylglycerol levels and a greatly decreased PDGF stimulation of both PIP_2 hydrolysis and Ca^{2+} release (Benjamin *et al.*, 1987, 1988; Olinger & Gorman, 1988). c-Ha-*ras* show partial loss of PDGF stimulation (Benjamin *et al.*, 1988). The N-*ras* proto-oncogene shows an amplified response to bombesin and other growth factors in the T15 cell line, whilst *ras* oncogenes show an elevated basal level of inositol phosphates in NIH-3T3 cells, and after transient expression in COS cells (Wakelam *et al.*. 1986; Hancock *et al.*, 1988).

In a previous paper we were able to eliminate the difficulty inherent in using transformed cell lines by using a scrape-loading technique to introduce ras protein directly into the cells. We have demonstrated that activation of protein kinase C occurs within 10 min of treatment with TPA or scrape-loading [Val-12]p21^{ras} (Morris *et al.*, 1989). Here, we have shown that this activation of protein kinase C by TPA or [Val-12]p21^{ras} leads to a marked inhibition of growth factor stimulated inositol phosphate release. This suggests that the loss of PDGF and bombesin responses in cells over-expressing oncogenic Ha-*ras* (Benjamin *et al.*, 1987, 1988) is a consequence of protein kinase C activation. However, TPA acts within 15 min to block ligand-stimulated inositol phosphate release, whereas ras required 1.5–4 h. This implies that there is some difference in the mechanism by which TPA and ras down-regulate inositol phosphate responses, perhaps because TPA is a more potent activator of protein kinase C than ras. Some authors have shown increased levels of inositol phosphates due to ras (Wakelam *et al.*, 1986). Our results do not show any ras-induced increases. However, chronic treatment of cells with TPA to remove protein kinase C leads to an increase in both basal and growth factor stimulated levels of inositol phosphates, presumably due to the loss of down-regulation by protein kinase C (Fig. 3; Brown *et al.*, 1987).

We speculate that chronic stimulation of protein kinase C by ras could therefore give rise to two effects: (1) activation of protein kinase C leading to inhibition of growth factor stimulated PIP_2 breakdown; and (2) down-regulation and loss of protein kinase C resulting in increased PIP_2 breakdown (in a similar fashion to that caused by prolonged TPA treatment) leading to an elevation of basal and growth factor stimulated rates. In support of this, Wolfman *et al.* (1987) and Kamata *et al.* (1987) have shown that some ras-transformed cells have reduced protein kinase C activity and reduced [^{3}H]-phorbol ester binding capacity, indicating that ras is capable of down-regulating protein kinase C.

In conclusion, we suggest that the differing results of the effects of ras on PIP_2 turnover in the literature can be exlained in the following way. In some cell systems, ras may activate protein kinase C, which in turn down-regulates PIP_2 hydrolysis. Secondly, ras may down-regulate protein kinase C and relieve this inhibitory effect, leading to elevated growth factor-stimulated and basal levels of inositol phosphates. Which of these effects is dominant is likely to depend on the cell line, the levels and types of protein kinase C present and perhaps the particular *ras* gene used. In any case, it is apparent that ras does not directly activate a PIP_2-specific phospholipase C.

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