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Deregulation of hamster fibroblast proliferation by mutated *ras* oncogenes is not mediated by constitutive activation of phosphoinositide-specific phospholipase C

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Stable expression of high levels of activated forms of Ha-*ras* (T24) or v-Ki-*ras* by transfection of Chinese hamster lung fibroblasts (CCL39) yielded cells highly tumorigenic in nude mice. Two classes of transformed cells were distinguished, one with moderate p21 expression (10-fold increased) had retained growth factor dependency, the second with higher level of p21 (>50-fold) appeared autonomous for growth. Neither class of transformants expressing Ki-*ras* or Ha-*ras* displayed a significant basal activity of polyphosphoinositide-specific phospholipase C, measured either in serum-starved cells or during exponential growth in the presence of growth factors of the tyrosine kinase family (EGF, FGF, insulin). In the growth-factor-dependent class of T24-Ha-*ras*-transfected cells (clone 39THaB), phospholipase C could be stimulated normally by serum, thrombin and AlF_4^-. In the more growth autonomous class (clones 39THaC and 39Ki9), release of inositol phosphates after stimulation with thrombin or serum was drastically reduced. This desensitization, apparently at the receptor level since the response to AlF_4^- persisted, is, however, not specific to *ras* expression. We observed it to the same degree in polyoma virus-transformed CCL39 cells. Finally, expression of mutated forms of p21 *ras* did not abrogate the sensitivity of phospholipase C activation to pertussis toxin. We conclude that the transforming potential of activated forms of $p21^{ras}$ does not result from persistent activation of phospholipase C and that *ras* GTP-binding proteins cannot substitute for Gp.

Key words: ras oncogene/growth factors/phosphoinositide breakdown/cell transformation

Introduction

Upon addition to quiescent cells, some growth factors activate a phosphoinositide-specific phospholipase C (Berridge and Irvine, 1984; Majerus *et al.*, 1986) which leads to the liberation of the second messenger molecules inositol triphosphate (IP_3) and diacylglycerol (DG) which in turn release Ca^{2+} from intracellular stores (Streb *et al.*, 1983) and stimulate protein kinase C (Nishizuka, 1984) respectively. Induction of these two events is sufficient to trigger platelet activation (Kaibuchi *et al.*, 1983) and exocytosis in neutrophils (Kajikawa *et al.*, 1983); whether they are necessary or sufficient steps mediating the mitogenic response of cells to the growth factors in question remains to be established.

Receptor activation of phospholipase C is mediated by a GTP-binding protein (Haslam and Davidson, 1984; Cockroft and Gomperts, 1985) sensitive to pertussis toxin in some experimental systems (Ohta *et al.*, 1985; Volpi *et al.*, 1985) including CCL39, a cell line of Chinese hamster lung fibroblasts (Paris and Pouysségur, 1986). In this system, the activation of the phospholipase C is a necessary step mediating the mitogenic effect of α-thrombin (Chambard *et al.*, 1987).

The proteins encoded by the *ras*-gene family are closely related proteins with a mol. wt of 21 kd ($p21^{ras}$). They bind guanine nucleotides (Scolnick *et al.*, 1979), share significant sequence homology to known GTP-binding proteins (Hurley *et al.*, 1984) and catalyze the hydrolysis of GTP (McGrath *et al.*, 1984). The mutated forms of $p21^{ras}$ which have tumorigenic potential are characterized by a reduced GTPase activity or increased GDP–GTP exchange rates (Walter *et al.*, 1986) *in vitro*. These properties are thought to increase the proportion of $p21^{ras}$ proteins in the GTP-bound, presumably active, state.

The discovery that *ras* proteins are GTP-binding proteins associated with plasma membranes has elicited a search for potential sites of action among known membrane-bound enzyme systems regulated by G-proteins. The homologue of $p21^{ras}$ in yeast regulates adenylate cyclase (Toda *et al.*, 1985), but no such activity has been demonstrated so far in higher eukaryotic cells (Beckner *et al.*, 1985).

Recently Wakelam *et al.* (1986) presented evidence for $p21^{N\text{-}ras}$ coupling bombesin receptors to the phospholipase C system in NIH 3T3 cells. Marshall (1987) extended these findings, reporting that normal $p21^{Ha\text{-}ras}$ specifically increased the phospholipase C response to PDGF in the same cells and that the mutated forms of $p21^{ras}$ enhanced phosphoinositide (PI) breakdown in the absence of agonists. These results were incorporated into a very attractive hypothesis which stated that mutated *ras* proteins deregulate growth by short-circuiting growth factor receptors and activating the phospholipase C signalling system persistently (Fleischman *et al.*, 1986; Marshall, 1987).

Several features make CCL39 hamster fibroblasts a very interesting cell system in which to analyze the phospholipase C signalling system: (i) in the absence of growth factors the phospholipase C pathway is essentially quiescent; (ii) serum or thrombin rapidly activate the phospholipase C in a GTP-dependent manner (L'Allemain *et al.*, 1986; Magnaldo *et al.*, 1987); (iii) AlF_4^- directly stimulates the coupling G-protein in intact cells, thereby circumventing all receptor-dependent processes; (iv) phospholipase C activatation in hamster fibroblasts by serum, thrombin and AlF_4^- is inhibited by pertussis toxin (Paris and Pouysségur, 1986, 1987).

Starting from the working hypothesis that *ras* proteins couple growth factor receptors to the phospholipase C, we expected to detect in *ras*-transformed CCL39 cells an increased basal and/or growth factor stimulated rate of PI

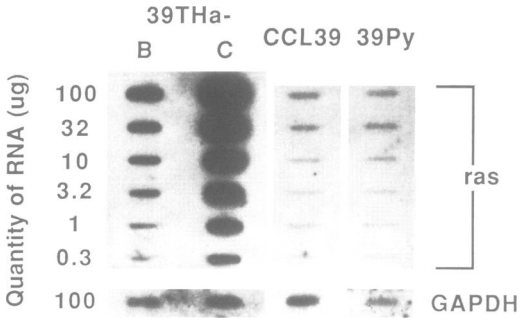

Fig. 1. Expression of *ras* mRNA in different cell clones. Total cellular RNA was prepared as decribed in Materials and methods. The quantities indicated on the left were spotted onto a Nytran membrane and hybridized with a *ras* or a GAPDH probe (indicated on the right).

turnover, or, as CCL39 cells may lack the appropriate growth factor receptors coupling to *ras* proteins, an increased AlF_4^- stimulation. In addition, we expected reduced sensitivity of phospholipase C activation to pertussis toxin, as the different forms of $p21^{ras}$ are not substrates of this ADP-ribosyl-transferase.

However, we failed to detect any of these anticipated changes. On the contrary, we observed a marked inhibition of serum- or thrombin-stimulated phospholipase C activity in growth-factor-independent clones, including cells transformed by polyoma virus.

We conclude that CCL39 cells expressing the T24-Ha-*ras* or v-Ki-*ras* oncogene acquire tumorigenic potential without showing a 'constitutive' activity of the phospholipase C or increased activation in response to growth factors.

Results

Transfection of CCL39 fibroblasts with Ha-ras and Ki-ras oncogenes: characterization of isolated cell clones

CCL39 fibroblasts were transfected with plasmids pH06N1, pH06T1 and pE4 as described in Materials and methods. Transfected cell clones were isolated exclusively on the basis of G 418 resistance and immediately recloned. All clones isolated after transfection with activated Ha-*ras* (pH06T1) or v-Ki-*ras* (pE4) were morphologically clearly transformed, whereas only one out of >30 clones transfected with c-Ha-*ras* (pH06N1) showed some signs of transformation. The cells transfected with normal Ha-*ras* expressed only low levels of p21 (<10-fold over wild-type cells) and were undistinguishable from CCL39 with regard to anchorage and growth factor dependence as well as tumorigenicity in nude mice (data not shown).

Expression of *ras* mRNA and $p21^{ras}$ by different transformed cell clones is shown in Figures 1 and 2 respectively. Cells transfected with T24-Ha-*ras* (clones 39THaB, 39THaC) express very high levels (⩾10-fold over wild-type cells) of *ras* mRNA and p21 protein. Table I summarizes some of our results concerning the growth characteristics of these cells. The clones transfected with T24-Ha-*ras* or v-Ki-*ras* are transformed as judged by their ability to form tumors in nude mice with no latency period.

Figure 3 shows data on growth-factor-induced reinitiation of DNA synthesis after 24 h of growth factor deprivation. In wild-type CCL39 fibroblasts, [^{3}H]thymidine incorporation under these conditions is barely detectable in the absence of growth factors, but increases up to 100-fold in a

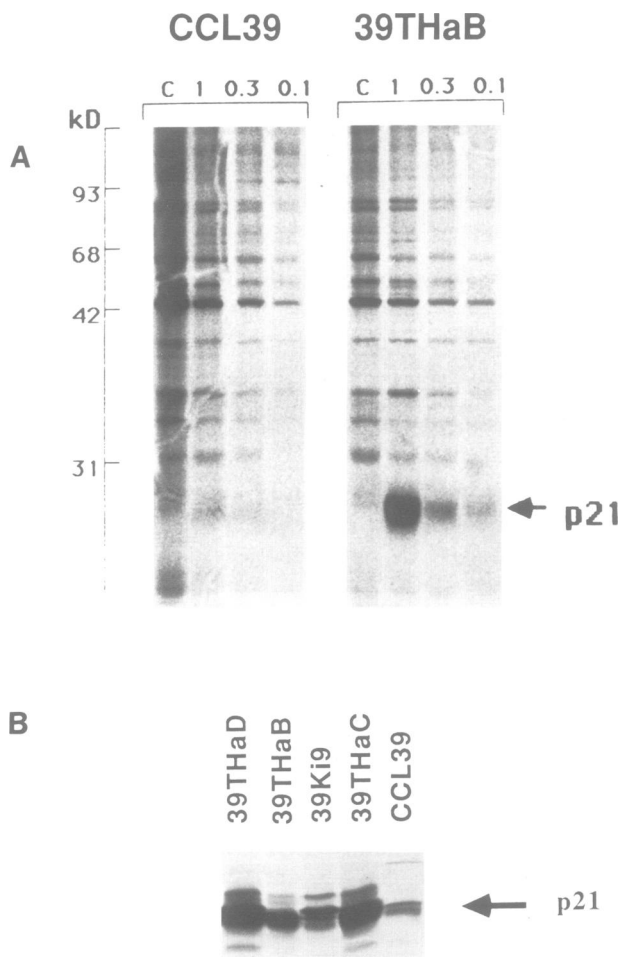

Fig. 2. Expression of p21 protein in different cell clones. $p21^{ras}$ was immunoprecipitated as described in Materials and methods. (**A**) Different quantities of ^{35}S-labeled extracts [relative amounts 1 ($\sim 10^6$ c.p.m.), 0.3, 0.1; as indicated on top] were immunoprecipitated. A control (C) experiment was performed using the same amount of extract as in (1), but omitting the antibody Y13-259. Left, CCL39 cells; right, 39THaB. (**B**) Comparative p21 expression in different cell clones.

Table I. Growth characteristics and tumorigenicity of CCL39 cells and derivatives

	CCL39	39THaB	39THaC 39THaD	39Ki9	39Py
Morphological transformation	−	+	+	+	+
Loss of contact inhibition of cell division	−	+	+	+	+
Growth in semi-solid medium[a]	−	+	+	+	+
Growth in minimal defined medium[b]	− (0)	± (<2)	+ (>3)	+ (>3)	+ (>3)
Tumorigenicity[c]	−	+	+	+	+

[a] −, <1%; +, >20% colony formation.
[b] Numbers in parentheses indicate the number of population doublings observed within 4 days in growth-factor-free medium (see Materials and methods).
[c] −, no tumor formation before 6 weeks; +, formation of rapidly growing tumors within 10 days of subcutaneous injection of 5×10^5 cells into nude mice.

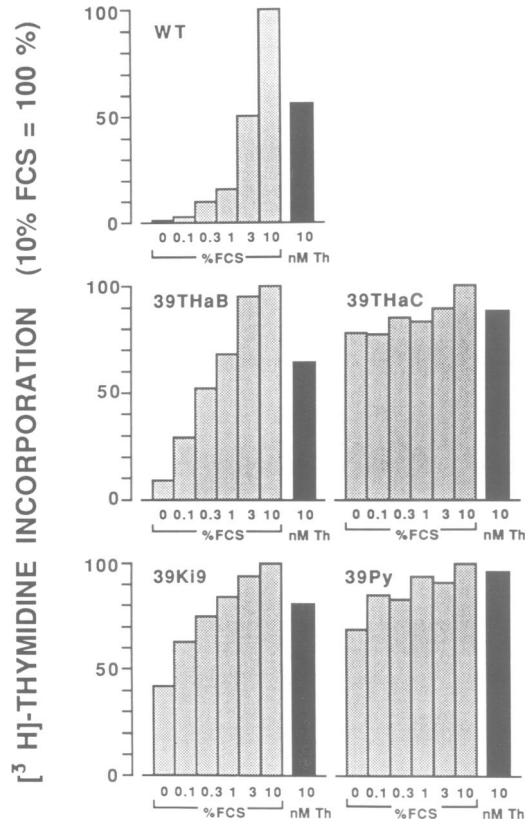

Fig. 3. Reinitiation of DNA synthesis as a function of serum concentration in different cell clones. Cells were deprived of growth factors and restimulated as described in Materials and methods. The data obtained from each clone were normalized to the [^{3}H]thymidine incorporation observed with 10% FCS (>20 000 c.p.m.). Mean values of duplicate determinations are shown (differences between determinations <10%). Thr: thrombin.

dose-dependent manner if serum or thrombin is added. However, in cells transfected with T24-Ha-*ras* or v-Ki-*ras* as well as in polyoma-virus-transformed cells (clone 39Py), substantially increased [^{3}H]thymidine incorporation can be observed in the absence of growth factors and high levels of incorporation are achieved with relatively low growth factor concentrations. Note the different patterns of [^{3}H]-thymidine incorporation observed in clones 39THaB and 39THaC which are representative of the growth-factor-dependent and independent classes of T24-Ha-*ras* transfectants respectively.

Phospholipase C activity in CCL39 cells transformed by activated Ha-ras or v-Ki-ras: no significant basal rate detectable

Following the hypothesis that mutated forms of p21^{ras} are G-proteins that are almost permanently in their activated state due to a defect in their intrinsic deactivation mechanism [lower rate of GTP hydrolysis or higher rate of GDP−GTP exchange (Lacal *et al.*, 1986; Walter *et al.*, 1986)] and capable of activating the PI cycle, an increased phospholipase C activity should be detectable in cells expressing mutated p21 even in the absence of growth factors.

We have therefore measured the basal rates of inositol phosphate accumulation by the addition of Li^+ in the absence of any stimulus in different cell clones transformed by T24-Ha-*ras* or v-Ki-*ras* and, for comparison, in wild-type CCL39 and polyoma virus-transformed cells.

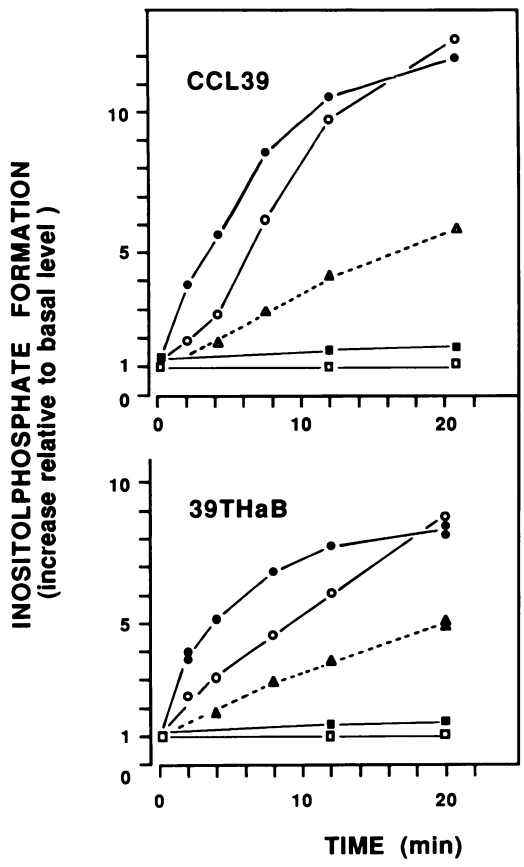

Fig. 4. Kinetics of inositol phosphate accumulation stimulated by thrombin, serum and AlF_4^- in CCL39 cells and in clone 39THaB. ●—● Li^+ + 1 nM thrombin; ○—○ Li^+ + 5% FCS; △---△ Li^+ + AlF_4^-; ■—■ Li^+ only (unstimulated rate of inositol phosphate production); □—□ no addition. Data are normalized to the label incorporated into inositol phosphates before stimulation ('basal level') in each cell type. This basal level represented 1.1% of the lipid incorporated label in CCL39 cells and 1.5% in 39THaB.

In addition, we stimulated the cells with serum, thrombin and AlF_4^- and recorded the kinetics of inositol phosphate formation. Typical experiments are shown in Figures 4 and 5.

The most important result of these experiments is that no significant rate of inositol phosphate production is detectable with Li^+ alone in the absence of a stimulus in either of these cell preparations.

Under these conditions inositol phosphate accumulation within a period of 30 min never amounted to more than a 80% increase over the basal level. We have made the same observations in other transformed derivatives of CCL39 cells, including cells expressing the *myc* and *fps* oncogene (K.Seuwen, A.Lagarde and J.Pouysségur, unpublished observation).

Regarding inositol phosphate formation after addition of agonists, important differences between the cell types studied could be observed. Whereas in clones 39THaC and 39Ki9 serum- and thrombin-induced phospholipase C activation was suppressed to a large extent, an apparently normal activation was observed in clone 39THaB.

The desensitization observed in clones 39THaC and 39Ki9 is not specific for *ras*-transformed cells, as the same effect can be observed with CCL39 cells transformed by polyoma virus (Figure 5, bottom).

Sensitivity of the phospholipase C system to receptor

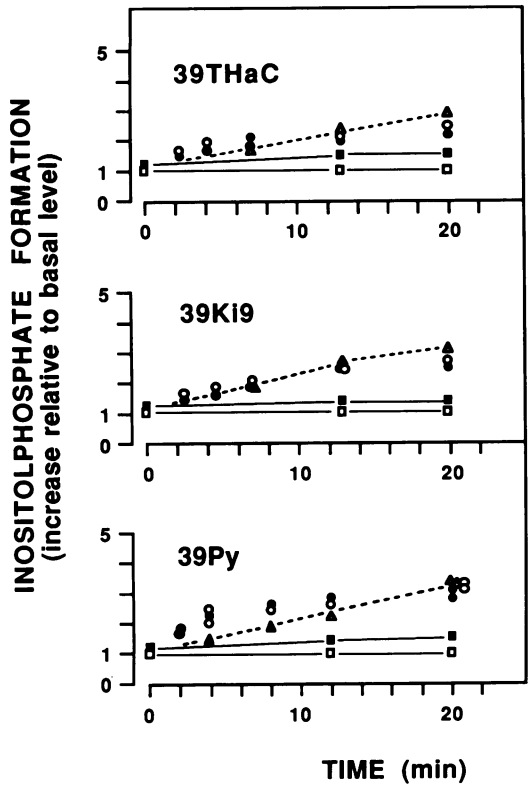

Fig. 5. Kinetics of inositol phosphate accumulation stimulated by thrombin, serum and AlF_4^- in clones 39THaC, 39Ki9 and 39Py. ●—● Li^+ + 1 nM thrombin; ○—○ Li^+ + 5% FCS; △---△ Li^+ + AlF_4^-; ■—■ Li^+ only (unstimulated rate of inositol phosphate production); □—□ no addition. Data are normalized to the label incorporated into inositol phosphates before stimulation ('basal level') in each cell type. This basal level represented 1.4% of the lipid incorporated label in 39THaC, 1.2% in 39Ki9 and 1.6% in 39Py.

stimulation seems to be correlated with the degree of growth factor dependence of the clones studied. We have verified that a lack of responsiveness is not paralleled by aberrantly high basal levels of inositol phosphates (note that we have normalized our data relative to these basal levels). The ratio of label incorporated into inositol phosphates relative to the label incorporated into lipids was not significantly different between wild-type and transformed cells or responsive and unresponsive cells (1.6 ± 0.4% for CCL39, 1.9 ± 0.6% for transformed cells; mean ± SE, >12 experiments); total incorporation of radioactivity per cell was usually ~50% higher in transformed cells than in normal cells. Note also that inositol phosphate formation can still be stimulated very well with AlF_4^- in every cell clone tested. This clearly indicates that the coupling between G-protein and phospholipase C still works in unresponsive cells and that all necessary elements of the PI cycle are in place (enzymes, substrates).

As a normal phospholipase C response to thrombin was measurable in clone 39THaB we decided to study the thrombin dose–response relationship in these cells because a possible modification of the phospholipase C response to growth factors induced by $p21^{ras}$ might manifest itself only at low growth factor concentrations. As the data in Figure 6A show, there is no significant difference relative to wild-type cells detectable.

In CCL39 cells, phospholipase C activity decreases sub-

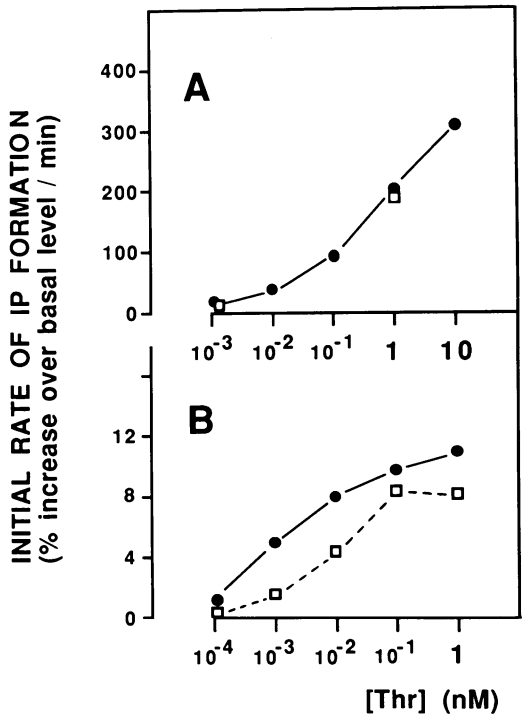

Fig. 6. Initial rate of inositol phosphate formation as a function of thrombin concentration. (● CCL39 cells; □ 39THaB.) **(A)** During the first minutes following stimulation. The assay was performed as described in Materials and methods. Li^+ was added 10 min before thrombin. The initial rate was measured over 1 min (10 nM Thr), 2 min (1 nM Thr), 8 min (0.1 nM Thr), 20 min (10 and 1 pM Thr). **(B)** After 90 min of stimulation. As in (A), cells were stimulated with different concentrations of thrombin. However, Li^+ was added 90 min later and the reaction stopped after a further 30 min, during which IP accumulation was linear for all thrombin concentrations. Means of duplicate or triplicate determinations are plotted (differences between determinations <5%). Data are normalized to the label incorporated into inositol phosphates before stimulation ('basal level') in each cell type.

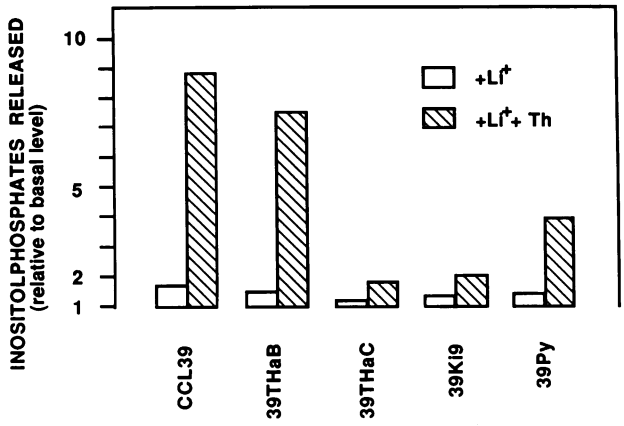

Fig. 7. Unstimulated and thrombin-stimulated inositol phosphate accumulation in different cell clones during exponential growth in a defined medium supplemented with EGF and FGF. Li^+ and thrombin (1 nM) were added to the cultures growing under CO_2 in a defined medium supplemented with EGF and FGF. Inositol phosphate accumulation during 30 min was measured. Data are normalized to the label incorporated into inositol phosphates before stimulation ('basal level') in each cell type. Mean values of duplicate determinations are shown (differences between determinations <10%).

Table II. Inhibition of thrombin- and AlF_4^--induced activation of phospholipase C by pertussis toxin in different cell clones

	Activation by	
	Thrombin (1 nM)	AlF_4^-
CCL39	55.9 ± 2.5 (6)	35.6 ± 1.9 (5)
39THaB	52.0 ± 0.9 (4)	38.2 ± 1.9 (3)
39THaC	64.5 ± 2.3 (6)	64.6 ± 3.3 (5)
39Ki9	56.0 ± 3.5 (5)	69.2 ± 4.2 (5)
39Py	69.0 ± 4.4 (3)	63.0 ± 2.8 (3)

In growth-factor-deprived cells pretreated or not with a saturating dose of pertussis toxin, thrombin- and AlF_4^--stimulated inositol phosphate release was measured (5–20 min incubation). The values ± SEM indicate the inositol phosphate accumulation in pertussis-toxin-pretreated cells relative to control (100%). Number of determinations in parentheses.

stantially with time after thrombin addition. After 90 min of stimulation, activity drops to $<10\%$ of the initial value that can be observed during the first minutes after stimulation. To see if T24-Ha-*ras* can influence this desensitization process and possibly maintain an elevated level of phospholipase C activity over time, we stimulated the cells with different concentrations of thrombin exactly as described for the previous experiment, but measured phospholipase C activity by a Li^+ pulse only after 90 min of stimulation. As the data plotted in Figure 6B show, T24-Ha-*ras* does not significantly change the degree of desensitization of the phospholipase C response, neither is a shift of the dose–response curve manifest. Similar results were obtained using FCS as activator of the PI cycle (data not shown). To show that the observed lack of a basal activity of phospholipase C is not an artefact of complete growth factor withdrawal, we have grown cells in a defined medium containing insulin, EGF and FGF as growth factors, which do not activate phospholipase C. Under these conditions the PI cycle of wild-type CCL39 fibroblasts is essentially quiescent even during exponential growth, but can be fully stimulated by thrombin or serum (L'Allemain and Pouysségur, 1986). As shown in Figure 7, T24-Ha-*ras* and v-Ki-*ras* transformed cells do not exhibit a phospholipase C activity exceeding that observed in wild-type cells when grown in the presence of insulin, EGF and FGF.

The phospholipase C response of different cell clones transfected with normal Ha-*ras* was indistinguishable from that of wild-type cells (data not shown).

Effect of pertussis toxin on thrombin- and AlF_4^--induced activation of phospholipase C

Activation of phospholipase C by thrombin in CCL39 fibroblasts is mediated by a G-protein sensitive to pertussis toxin (Paris and Pouysségur, 1986, 1987). The different forms of $p21^{ras}$ cannot be ADP-ribosylated by pertussis toxin (Beckner *et al.*, 1985). If p21 was expressed in sufficient quantities inside cells, it should render the cells less sensitive to the toxin, provided it can mediate the activation of the PI cycle by a specific agonist. We have already shown that the presence of $p21^{ras}$ does not increase the response of the phospholipase C to agonists. However, the effect of *ras* might be masked by the presence of the physiological coupling protein. We thought it would be instructive, therefore, to use pertussis toxin to neutralize this G-protein in order to unravel the stimulatory properties of $p21^{ras}$. We have compared the effect of pertussis toxin pretreatment on the stimulation of phospholipase C by AlF_4^- and thrombin in different cell clones studied. The results are shown in Table II. In clone 39THaB, AlF_4^-- and thrombin-induced activation of phospholipase C is inhibited to the same extent as in wild-type cells by pertussis toxin, whereas in the growth-factor-independent clones—39THaC, 39Ki9 and 39Py—a reduced degree of inhibition was found. Obviously, expression of activated *ras* proteins does not specifically modify the pertussis toxin sensitivity of phospholipase C activation.

Discussion

We have compared basal and activated rates of phospholipase C in hamster lung fibroblasts expressing different levels of normal and activated forms of Harvey and Kirsten *ras* proteins. None of the predictions expected if activated forms of *ras* were able to play the role of the G-protein activating phospholipase C have been observed. Our results can be summarized as follows. (i) Expression of mutated T24-Ha-*ras* ~10-fold over normal $p21^{ras}$ levels yielded clones fully transformed as judged by their ability to form tumors in nude mice with no latency and with occurrence of metastases (unpublished results), yet these clones, like 39THaB, retained growth factor dependence. The basal rate and thrombin- or AlF_4^--activated rates of PI turnover in the presence or absence of pertussis toxin did not markedly differ from those of normal CCL39 cells. (ii) Expression of high levels of T24-Ha-*ras* (>50-fold) or of v-Ki-*ras* yielded tumoral clones with almost complete relaxation of growth factor requirements. In these clones, however, although the basal rate was not increased, stimulation of phospholipase C by serum or thrombin was severely diminished, a property also observed in a CCL39 tumoral clone transformed by polyoma virus (39Py). In these growth-factor-relaxed tumoral clones, the phospholipase C is, however, still activatable by AlF_4^- and this activation remains sensitive to pertussis toxin. We would like to conclude from these results that neither Ha-*ras* nor Ki-*ras* has the potential to activate directly phospholipase C.

At least two reports in the literature favor the opposite hyothesis. Fleischman *et al.* (1986) have shown differences in the DG/PIP_2 ratio and IP/PIP_2 ratio between *ras*-transformed and control cells. These differences observed only in confluent cells may reflect the fact that normal cells at high cell density enter a quiescent state, whereas transformed cells continue to proliferate. Working on a specific clone of NIH 3T3 fibroblasts transfected with the normal N-*ras* gene under control of a dexamethasone-inducible promoter, Wakelam *et al.* (1986) have shown that in cells pretreated with dexamethasone a substantially higher phospholipase C response to bombesin, bradykinin and serum could be observed, which correlated with elevated $p21^{N\text{-}ras}$ expression. This observation was recently extended to other forms of *ras* (Marshall, 1987). For instance, overproduction of normal Ha-*ras* in NIH 3T3 cells amplified the phospholipase C response to PDGF, whereas expression of the mutated *ras* proteins raised the basal rate of PI turnover in the absence of added growth factors. Along the same lines we recently observed that serotinin (5-hydroxytryptamine, 5HT) elicits a relatively strong PLC response in several transformed

CCL39 derivatives, including the *ras*- and *Py*-transformed cells described in this study, whereas it is much less active in normal cells. As in the case of bombesin described by Wakelam *et al.* (1986), this increased response is not due to an increase in receptor number. Because the increased 5HT response is not specific for cells expressing *ras*-oncogenes, it cannot be explained by an increased coupling exerted by $p21^{ras}$.

In view of the contradictory results found in different cell systems a few points need to be discussed to validate the results obtained with CCL39 cells. First, although not an obligatory event preceding growth-factor-induced reinitiation of DNA synthesis in CCL39 cells, activation of the phospholipase C is crucial to mediate α-thrombin mitogenesis. Indeed, attenuating the activation of phospholipase C with pertussis toxin severely inhibits thrombin-induced DNA synthesis (Chambard *et al.*, 1987). A second major point of importance is the fact that a basal rate of PI turnover is only barely detectable in CCL39 cells in the absence of growth factors. This is not due to a lack of sensitivity of the assay since with the Li^+ pulse we can detect an increased rate of IP release with a non-mitogenic concentration of thrombin as low as 10 pM. The phospholipase C is simply 'shut off' in CCL39 cells arrested in G_0 or growing in a defined medium supplemented with EGF/FGF and insulin. Wakelman *et al.* (1986) and Marshall (1987) have implied that the different forms of *ras* could interact with different receptors. According to this view one might argue that thrombin receptors are not adequate for interaction with Ha-*ras* as the phospholipase C response to thrombin was not affected in the corresponding clones expressing normal or T24-Ha-*ras* (39THaB). This interpretation was ruled out by the use of AlF_4^-. AlF_4^- interacts with G-proteins in their GDP-bound (inactive) state, probably substituting for the γ-phosphate group of GTP, thus inducing the active state of the protein (Sternweis and Gilman, 1982; Bigay *et al.*, 1985). As the nucleotide-binding domains of the different G-proteins known (including $p21^{ras}$) are very conserved, we anticipated that AlF_4^- can activate $p21^{ras}$ independently of a receptor, as it activates the putative physiological G-protein (Paris and Pouysségur, 1986, 1987). In fact, using fluorescent GDP analogs, J.Feurstein and R.Goody (personal communication) recently showed that AlF_4^- induces conformational changes in the $p21^{ras}$ molecule. However, AlF_4^--stimulated phospholipase C activity was not significantly enhanced in T24-Ha-*ras*-transfected cells, even after pretreatment of cells with a maximal dose of pertussis toxin in order to inactivate G-protein [$p21^{ras}$ cannot be ADP-ribosylated by pertussis toxin (Beckner *et al.*, 1985)].

The phospholipase C system of CCL39 cells does not respond to bombesin, bradykinin and vasopressin probably because CCL39 cells do not express receptors for these growth factors. But, as another possible defect could be the lack of the appropriate coupling G-protein, we have none the less tested whether a phospholipase C response to these agonists was detectable in c-Ha-*ras*- or T24-Ha-*ras*-transfected cells. The phospholipase C response to PDGF was not different from wild-type cells in clone 39THaB and in cells transfected with normal Ha-*ras* (not shown). This result was also expected because the PDGF mitogenic response in CCL39 cells is not coupled to phospholipase C activation. Thus, there is no evidence for an interaction of $p21^{Ha\text{-}ras}$ or $p21^{Ki\text{-}ras}$ with the phospholipase C system of CCL39 fibroblasts.

Another point of interest which deserves some discussion is the finding that the phospholipase C response to thrombin and serum was almost abolished in the growth-factor-independent clones 39THaC and 39Ki9. As similar results were obtained with polyoma virus-transformed cells, this desensitization cannot be considered as a specific consequence of the high level of expression of mutated $p21^{ras}$, but fits rather well to an observation made earlier in this laboratory, namely a diminished phospholipase C response in derivatives of CCL39 fibroblasts with relaxed growth factor requirements (L'Allemain *et al.*, 1986). So far, the phenomenon of the reduced phospholipase C responsiveness observed in growth-factor-independent cells cannot be explained in a straightforward manner. It could be explained by a loss of substrates or enzyme activity resulting from a permanent activation of the PI breakdown. Evidently this is not the case as substantial stimulation of inositol phosphate formation is elicited by AlF_4^-. This seems to indicate a disturbance at the receptor level or at the level of interaction between receptor and the putative coupling G-protein. Indeed a similar desensitization of the phospholipase C response can be observed in wild-type CCL39 cells that are continuously exposed to α-thrombin or serum (L'Allemain *et al.*, 1986). After 24 h in the presence of the agonists, no activity can be detected by addition of Li^+, even after re-addition of serum or thrombin. This is a homologous desensitization at the receptor level as stimulation by AlF_4^- is still possible and cells preincubated in the presence of serum can be stimulated by thrombin and vice versa (unpublished observation).

Therefore it could be proposed that the unresponsive, transformed cells secrete growth factors that bind to their appropriate receptors inducing a pleiotypic down-regulation. At least for thrombin, however, we could demonstrate that the desensitization is not due to secretion of thrombin or a thrombin-like activity, as preincubation of cells for 24 h in the presence of hirudin, which specifically inactivates thrombin, did not increase the phospholipase C response (not shown). We propose that a mechanism leading to heterologous desensitization is working in growth-factor-independent cells, that is not, or much less, active in normal cells. It could touch not only receptors coupled to PI turnover, but also receptors activating other biochemical signalling pathways, for instance the adenylate cyclase system (Tarpley *et al.*, 1986; K.Seuwen and J.Pouysségur, in preparation). A permanently activated protein kinase C might be involved in this process, as was described recently by Wolfman and Macara (1987), who observed substantially elevated diacylglycerol levels and kinase C activities in Ha-*ras*- and Ki-*ras*-transformed cells, but no detectable phospholipase C activity. The diacylglycerol activating the kinase C could stem from other sources than PI breakdown, as recently reported by Irving and Exton (1987) and Farese *et al.* (1987).

Growth-factor-independent derivatives of CCL39 fibroblasts are not the only examples of transformed cells showing the described desensitization of the phospholipase C to agonists. In accordance with our results, a decrease of the phospholipase C response to PDGF in *ras*-transfected NIH 3T3 cells has been reported by Parries *et al.* (1987) and Benjamin *et al.* (1987).

In conclusion, no evidence could be obtained for the hypothesis that mutated *ras* proteins are positive modulators of the phospholipase C system in CCL39 cells. Because *ras* proteins are highly conserved, we propose that this conclusion is not restricted to the hamster cells we have used but to all types of cells, including NIH 3T3 in which growth is deregulated by expression of activated *ras* proteins. The signalling pathway activated by the *ras* GTP-binding proteins in mammalian cells remains to be discovered.

Materials and methods

Cell culture

The Chinese hamster lung fibroblast line CCL39 (American Type Culture Collection) was maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 5% fetal calf serum (FCS), antibiotics (50 U/ml of penicillin and 50 μg/ml of streptomycin) and 25 mM bicarbonate, at 37°C in 5% CO_2/95% air.

Clones 39THB, C, D and 39Ki9 are CCL39 cells transfected with mutated (T24) Ha-*ras* and Ki-*ras* as described in this study. 39Py is a subclone of CCL39 transformed with polyoma virus.

When cells had to be grown in a defined medium, a 1:1 mixture of DMEM and Ham's F12 (Gibco) was used, which was supplemented with a mixture of insulin, sodium selenite and transferrin (Sigma) yielding final concentrations of 5 μg/ml, 20 nM and 5 μg/ml respectively. If used, EGF and FGF were added at a concentration of 50 ng/ml.

Transfection of CCL39 cells with Ha-ras and Ki-ras

The plasmids used for transfection of CCL39 cells were those constructed by Spandidos and Wilkie (1984), containing the normal (pH06N1) and activated (pH06T1) form of the human Ha-*ras* oncogene, and p4E described by Tsuchida *et al.* (1982), containing the entire Ki-MuSV genome. Both pH06N1 and pH06T1 contain the bacterial *aph* gene, conferring on eukaryotic cells resistance to the antibiotic geneticin (G 418) and eliminating the need for co-transfection with a selectable marker. p4E was co-transfected with the pSV*neo* plasmid.

Transfection was carried out applying the calcium phosphate co-precipitation technique (Graham and Van der Eb, 1973) using calf thymus DNA (Boehringer) as carrier. Five different plates were transfected to ensure the isolation of absolutely independent clones. Transfected cell clones were isolated exlusively on the basis of resistance to the antibiotic G 418 following the protocol published by Southern and Berg (1982), but using 600 μg/ml of G 418, and were immediately recloned, expanded and frozen. Experimental cultures of these cells were maintained for no more than five passages and always in the presence of 400 or 600 μg/ml G 418.

A systematic assay of the stability of plasmid DNA integration was undertaken with clone 39THaC. After >20 passages in the absence of G 418 these cells had not lost G 418 resistance and still expressed p21 as judged by immunofluorescence.

Preparation of RNA and quantification of ras-mRNA

Total RNA was purified from confluent cultures in 15-cm dishes ($0.8-1 \times 10^8$ cells). The monolayer was lysed with 4 ml solubilization buffer (5 M guanidinium isothiocyanate, 10 mM EDTA, 50 mM Tris−HCl, pH 7.5, 8% v/v 2-mercaptoethanol) and the RNA was precipitated by addition of 30 ml of 4 M LiCl and overnight incubation at 4°C. Following centrifugation at 10 000 *g* for 2 h, the RNA pellet was dissolved in 3 ml TES (10 mM Tris−HCl, pH 7.5, 1 mM EDTA, 0.1% SDS), extracted once with phenol−chloroform (1:1), once with chloroform, ethanol precipitated and redissolved in 10 mM sodium phosphate, pH 7.5, 1 mM EDTA, 50% formamide. The RNA was denatured by heating this solution to >70°C for 10 min and the indicated quantities were spotted onto wet Nytran membranes (Schleicher and Schuell) using a Schleicher and Schuell Minifold II microsample filtration apparatus. The slots were washed with 10 × SSC (20 × SSC is 3 M NaCl, 0.3 M sodium citrate, pH 7.0). The Nytran membranes were baked at 80°C for 2 h and prehybridized in 3−4 ml of hybridization solution (6 × SSC, 5 × Denhardt's solution, 100 μg/ml denatured herring sperm DNA, 0.1% SDS, 50% formamide) for 2 h at 42°C in a sealed plastic bag. Thereafter the hybridization buffer was changed and the membranes were incubated together with the *ras* or GAPDH probe (100 ng) for another 24 h at 42°C. The *ras* probe used was the 3-kb *Sac*I fragment (Santos *et al.*, 1982) cut out of pH06N1, labeled to $>10^8$ d.p.m./μg using the Amersham nick-translation kit. The GAPDH probe was used to obtain an internal marker of input RNA. It was a partial cDNA of the human gene cloned into pBR322 (Dani *et al.*, 1984). After hybridization, the membranes were washed several times in 2 × SSC, 0.1% SDS at 30°C followed by 0.1 × SSC, 0.1% SDS at 68°C for 1 h and exposed to Kodak XAR-5 film for 24−72 h at −70°C.

Immunoprecipitation of p21^{ras}

Cells in 20-cm^2 dishes were labeled overnight in methionine-free DMEM supplemented with 10% FCS and 200 μCi/ml [^{35}S]methionine. After removal of the radioactive medium, the plates were rapidly washed twice in ice-cold PBS and the cells were lysed and scraped off the plate in 3 ml of extraction buffer (50 mM Tris−HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1% sodium deoxycholate, 1% aprotinine) at 0°C. The extract was homogenized using a Dounce homogenizer and centrifuged at 100 000 *g* for 45 min at 4°C.

The supernatant was used for immunoprecipitation. About 10^7 trichloroacetic acid precipitable c.p.m. were diluted in 500 μl extraction buffer and incubated with monoclonal antibody Y13-259 (Furth *et al.*, 1982) for 1 h at 0°C.

The immunocomplexes were collected overnight at 4°C with Protein A−Sepharose (Pharmacia) coated with rabbit anti-rat IgG (Bio-Yeda Scientific). The Sepharose was washed four times in extraction buffer, thereafter the proteins were dissolved in electrophoresis sample buffer (0.25 M Tris−HCl, pH 6.8, 0.1 M dithiothreitol, 10% glycerol, 2% SDS) at 95°C, 2 min, and separated in 12% polyacrylamide gels which were dried and exposed to Kodak XAR-5 films for 1−3 days.

Measurement of DNA synthesis reinitiation

Confluent cells in 24-well plates were deprived of growth factors by a 20-h incubation in serum free DMEM/Ham's F12 medium (1:1). Then fresh medium containing [^{3}H]thymidine (3 μM, 1 μCi/ml) was added and cells were stimulated with growth factors. [^{3}H]Thymidine incorporation was terminated 24 h after mitogen addition and radioactivity incorporated into trichloroacetic acid-precipitable material was measured.

Soft agar assay

Cells (2000) were suspended in 3 ml DMEM containing 10% FCS and 0.2% freshly melted agarose and seeded into 20-cm^2 culture plates covered with a solid basal layer (DMEM + 10% FCS + 0.4% agarose). Colonies were counted after ~2 weeks. When the assay was performed with Ha-*ras*-transfected cells, both agarose layers were supplemented with 600 μg/ml G 418.

Measurement of [^{3}H]inositol phosphate formation

Confluent cultures in 4.5-cm^2 wells were labeled to equilibrium with [^{3}H]inositol (2 μCi/ml) for 20 h in serum-free DMEM or in a defined medium as indicated in the figure legends. When used, pertussis toxin was added for the last 4 h of prelabeling. Cells were either stimulated directly in this medium by addition of agonists and Li^+ (20 mM) and maintained under CO_2 atmosphere (Figure 7) or in Hepes (20 m)-buffered DMEM (pH 7.4) after 15 min of preincubation. Unless otherwise stated, Li^+ was added 10 min before the agonists. To stimulate with the AlF_4^- complex, 5 μM $AlCl_3$ was added together with Li^+ 10 min before the addition of 10 mM NaF.

Inositol phosphate formation was stopped by aspiration of the medium and addition of 750 μl of ice-cold 10 mM formic acid (pH 3). After 30 min this solution containing the extracted inositol phosphates and inositol was diluted into 3 ml of 5 mM NH_3 solution (yielding a final pH of 8−9), and applied to a column containing 0.7 ml Bio-Rad anion exchange resin (AG 1-X8, formate form, 200−400 mesh). Free inositol, glycerophosphoinositol and inositol mono-, bis- and triphosphates were successively eluted with (i) 5 ml of H_2O, (b) 4 ml of 40 mM, (c) 4 ml of 200 mM, (iv) 4 ml of 600 mM, (v) 4 ml of 2 M ammonium formate/formic acid, pH 5 respectively. Alternatively, total inositol phosphates were eluted in a single step with 4 ml of 2 M ammonium formate/formic acid, pH 5. The high ionic strength of this medium also allows elution of inositol tetrakisphosphate.

Radioactivity remaining on the plates after formic acid extraction could be totally recovered with chloroform/methanol and therefore represented [^{3}H]myoinositol incorporated into lipids.

Materials

[^{3}H]Thymidine, Myo-2-[^{3}H]inositol and [^{32}P]dCTP were obtained from Amersham, [^{35}S]methionine from NEN. Highly purified α-thrombin (2660 NIH units/mg) was a gift from Dr J.W.Fenton (New York State Department

of Health). Mouse EGF and the basic form of bovine brain FGF were purified to homogeneity in our laboratory by the methods of Savage and Cohen (1972) for EGF and Gospodarowicz *et al.* (1984) for basic FGF Pertussis toxin (islet-activating protein) was purchased from List Biological Laboratories. Highly purified PDGF was a gift from Dr C.H.Heldin, Institute of Medical and Physiological Chemistry, University of Uppsala, Sweden. Bombesin, bradykinin and vasopressin were from Peninsula Laboratories, Inc. Antibiotic G 418 was from Sigma. Antibody Y13-259 was kindly provided by Dr J.C.Lacal (NIH, National Cancer Institute, Bethesda).

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