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Kinetic Evidence of a Rapid Activation of Phosphatidylcholine Hydrolysis by Ki-*ras* Oncogene

POSSIBLE INVOLVEMENT IN LATE STEPS OF THE MITOGENIC CASCADE*

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A novel phospholipase C specific for phosphatidylcholine has been shown to be activated by several agonists. Also, recent evidence suggests that transformation mediated by the *ras* oncogene possibly involves the activation of this novel phospholipid degradative pathway which would account for the increased diacylglycerol levels associated with transformation. Here we use a mutant of Ki-*ras* which is temperature-sensitive for transformation to investigate the kinetics of activation of the phosphodiesterase-mediated turnover of phosphatidylcholine. Upon shift to the permissive temperature, products of the activated phosphatidylcholine-specific phospholipase C were detected by 30 min and reached maximal levels by 1–2 h. These results suggest that the product of the *ras* oncogene rapidly activates the phosphodiesteratic hydrolysis of phosphatidylcholine. Furthermore, the fact that at least 4 h are required for serum to activate this phospholipase C strongly suggests that the *ras* oncogene product might be involved in late steps of the mitogenic signaling cascade.

The phospholipase C-mediated degradation of inositol phospholipids has been shown to be an important step in the signal transduction cascades triggered by several agonists including growth factors (1–4). This pathway gives rise to two putative second messengers (1), diacylglycerol (DAG)[1] and inositol phosphates, which regulate protein kinase C (5) and cytosolic Ca^{2+} levels (6), respectively. Protein kinase C activation has been shown to play a key role in the control of the cell proliferative response (7, 8), which supports the notion that DAG generation is an important step in mitogenic signaling cascades. DAG has also been shown to be produced rapidly after microinjection of *Xenopus laevis* oocytes with the product of Ha-*ras* oncogene, *ras* p21 (9). These data are in good agreement with previous reports showing that cells transformed by this and other oncogenes displayed increased steady-state DAG levels (10–12).

The dramatic alterations in DAG levels after microinjection of oocytes with *ras* p21 were accompanied by little change in the products of phosphoinositide (PI) turnover (9). This, together with the fact that cells stably transformed by the *ras* oncogene did not show any increase in steady-state inositol phosphate levels (12), strongly suggests that sources of DAG other than PI turnover are activated by *ras* transformation. It has recently been shown that phosphocholine (PCho), which along with DAG is produced by the phosphodiesterase hydrolysis of phosphatidylcholine (PC), is significantly increased in cell lines stably transformed by *ras* oncogenes (13). This indicates that *ras* transformation may be activating a novel phospholipid degradative pathway which involves a PC-specific phospholipase C. This cascade has been shown to be triggered by several agonists (14–18).

Here we report kinetic data on the activation of the phosphodiesterase-mediated turnover of PC by the *ras* oncogene. We took advantage of the existence of a mutant of Ki-*ras* which is temperature-sensitive for transformation to undertake this investigation. This cell line (ts-6-315) has been shown to display a transformed phenotype at 32 °C whereas it assumes a flat morphology at 40.5 °C (19, 20). The ts-Ki-*ras* gene differs from the wild type by a single point mutation at residue 43 of the encoded p21 (21). This mutation is apparently responsible for the thermal instability of ts-p21 at 40.5 °C, which would explain the normal morphology of this cell line at high temperatures (22).

The results shown here clearly indicate that the *ras* oncogene product rapidly stimulates the phosphodiesterase-mediated turnover of PC, most probably through a phospholipase C. However, PCho release in response to serum was not observed until at least 4 h after the addition of this mitogen. The possible involvement of *ras* p21 in late steps of the mitogenic signaling cascade stimulated by serum is discussed.

MATERIALS AND METHODS

Cell Lines—Normal or transformed rat fibroblasts were routinely grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, penicillin (100 units/ml)/streptomycin (100 μg/ml), and 2 mM L-glutamine in standard tissue culture flasks in a humidified 95% air, 5% CO_2 incubator. ts-6-315 cells were grown at 32 °C displaying a transformed phenotype.

Labeling Protocol—When seeded for experiments, cells were incubated at 40.5 °C (restrictive temperature) for 3 days in the presence of serum. For the last 24 h, the cells were labeled with either 2 μCi/ml [U-^{14}C]glycerol (Amersham International; specific radioactivity, 50–60 mCi/mmol), 2 μCi/ml [*methyl*-^{14}C]choline (Amersham International; specific radiactivity, 50–60 mCi/mmol), or 1 μCi/ml phosphorus-32, after which cells were shifted to serum-free label-contain-

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[1] The abbreviations used are: DAG, diacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PCho, phosphocholine; PEt, phosphatidylethanol; PI, phosphoinositide(s).

ing medium for 24 h. The medium was washed off, and fresh serum- and label-free medium was added. After a 30-min equilibration period, ts-6-315 cells were shifted to the permissive temperature, and phospholipids were extracted and analyzed at different times. Some cultures were incubated for different times in the presence of exogenous phospholipase D from peanut (type III, Sigma).

Analysis of Products of Phospholipid Metabolism—Reactions were stopped by removing the supernatants and adding ice-cold methanol to the cells. Methanolic cell extracts were fractionated into chloroformic and aqueous phases as previously described (23). In cultures labeled with [U-^{14}C]glycerol, chloroformic extracts were split in two halves, and DAG and phosphatidic acid content were quantified by using thin layer chromatography with the following solvent systems: hexane:diethyl ether:acetic acid (60:40:1, v/v/v) for DAG and the upper phase of ethyl acetate:trimethyl pentane:acetic acid:water (90:50:20:100, v/v/v/v) for phosphatidic acid and phosphatidylethanol. For the quantification of PCho levels, the aqueous phases from methanolic extracts from [*methyl*-^{14}C]choline-labeled cell cultures were fractionated by thin-layer chromatography (24), followed by autoradiography of plates in which standards corresponding to the different water-soluble metabolites were included. The corresponding spots were scraped off, and radioactivity content was determined by liquid scintillation counting.

[^{3}H]Thymidine Incorporation Assays—Cells grown for 3 days at 40.5 °C were made quiescent by serum starvation for 24 h at the same temperature. Afterward, dishes were shifted to the permissive temperature and *de novo* DNA synthesis was determined, as previously described (25), every 2 h by adding pulses of [^{3}H]thymidine (2 μCi/ml). In parallel experiments serum (10%) was added to normal parental cells and an identical protocol was followed.

RESULTS

Activation of Phospholipid Metabolism in Cells Transformed by a Ki-ras Temperature-sensitive Mutant—Cells transformed with a temperature-sensitive mutant of Ki-*ras* were routinely grown at 32 °C and were shifted to the restrictive temperature (40.5 °C) for 3 days. By this time, a completely flat morphology was observed (19–22). Afterward, cells were labeled with [U-^{14}C]glycerol and made quiescent by serum starvation. Labeled cells were shifted to the permissive temperature, and lipids were extracted and DAG levels determined at different times thereafter. Results shown in Fig. 1 clearly indicate that as early as 30 min after temperature shifting a reproducible increase in DAG was detected as compared with cultures carried out in parallel at 40.5 °C. Maximum levels of DAG were attained between 1 and 2 h after temperature shifting.

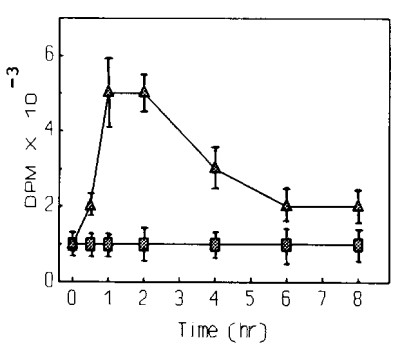

FIG. 1. **Time course of the production of DAG in ts-6-315 cells after temperature shifting.** ts-6-315 cells were labeled with [U-^{14}C]glycerol at the restrictive temperature (40 °C) under serum-free conditions as described under "Materials and Methods." Afterward, the label was removed and cells were equilibrated for 1 h in serum- and label-free medium after which cells were shifted to the permissive temperature (32 °C) for different times (△), and reactions were stopped and lipids extracted as described. Control dishes were kept at 40 °C and reactions terminated at different times (□). DAG levels were determined after fractionation of total lipids by thin-layer chromatography followed by autoradiography. The corresponding spots were scraped off and radioactivity content determined by liquid scintillation counting. Results are mean values ±S.D. of three independent experiments with incubations in duplicate.

Increased levels of DAG were observed even 4 h after temperature shifting, at which time a new plateau with a significantly higher steady-state level of DAG was observed. These results may indicate that *ras* p21 action is relatively proximal to the stimulation of a phospholipase C and are in very good agreement with recently reported data in *Xenopus* oocytes microinjected with *ras* p21 (9).

We searched for, but found no evidence of, stimulation of PI turnover in ts-6-315 cells labeled with [^{3}H]inositol and shifted to the permissive temperature for different times, either in the absence or in the presence of LiCl (data not shown). Taken together these results indicate that a source of DAG different from PI is activated by *ras* in ts-6-315 cells.

It has been shown in cell lines stably transformed by the Ha-*ras* oncogene that a phospholipase C specific for PC could account for the increased steady-state levels of DAG observed in these transformants (13). Conceivably, shifting ts-6-315 cells from the restrictive to the permissive temperature would activate the release of DAG from PC. To address this possibility the following series of experiments was performed; ts-6-315 cells labeled with [*methyl*-^{14}C]choline under restrictive conditions were shifted to the permissive temperature for different times. Reactions were then stopped, and levels of water-soluble choline metabolites (choline and PCho) and PC were determined. Results shown in Fig. 2 (*a* and *b*) indicate that temperature shifting in ts-6-315 cells leads to a prompt release both of PCho and choline. The time course of PCho release is compatible with the pattern of DAG production observed in Fig. 1. Concomitant with these effects in PCho and choline, a relatively rapid and transient decrease in PC levels was observed (Fig. 2*c*).

It has recently been suggested that PCho production in cells stably transformed by *ras* oncogene could be accounted for by an increased action of choline kinase on basal choline rather than by phospholipase C-mediated hydrolysis of PC (26). If this were the case, concomitant with the increase in PCho observed in ts-6-315 cells when shifted to the permissive temperature, a decrease in choline levels should have been detected. The fact that choline levels did not diminish but, on the contrary, significantly increased after temperature shifting strongly supports the notion that PCho changes in *ras* transformants derived from the breakdown of PC rather than synthesis from choline. Furthermore, we also measured PCho synthesis in ts-6-315 cells incubated either at the restrictive or at the permissive temperature for different times by labeling cells with [*methyl*-^{14}C]choline for 30 and 60 min prior to termination of the reactions. It is noteworthy that by using this experimental approach, the choline-containing phospholipid pool was not significantly labeled and, more importantly, no changes were detected in PCho labeling upon the shift of ts-6-315 cells to the permissive temperature (data not shown). This strengthens the idea that changes in PCho levels in transformed ts-6-315 cells are due to PC breakdown and not to PCho synthesis.

The fact that choline levels increased after the rise of PCho in ts-6-315 cells stimulated by temperature shifting could signify that choline derived by dephosphorylation of the PCho released as a consequence of *ras*-induced PC-phospholipase C activation. However, the stimulation by *ras* p21 of a PC-specific phospholipase D is also a conceivable possibility. In order to determine whether this enzymatic activity is stimulated by *ras* in ts-6-315 cells, cultures were labeled either with [U-^{14}C]glycerol or [^{32}P]phosphate and phospholipids extracted at different times after temperature shifting. Interestingly, no changes in phosphatidic acid, which would be produced along with choline by phospholipase D activation, were

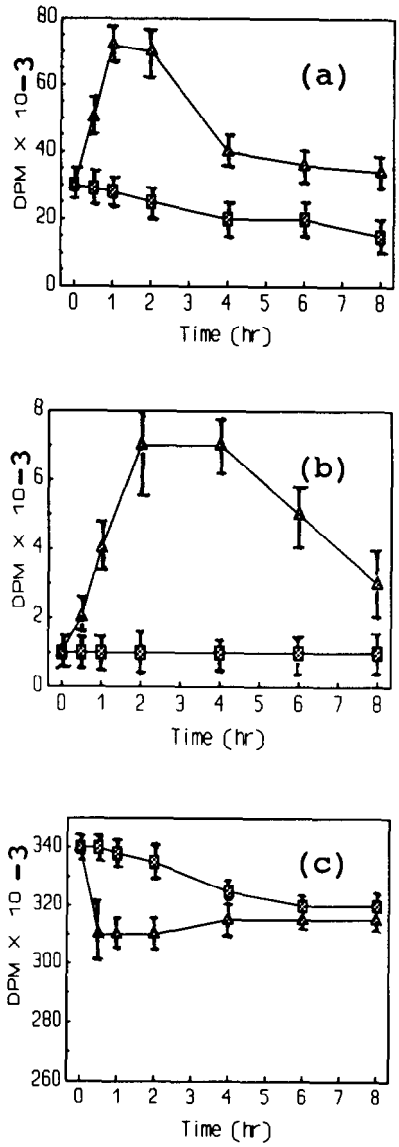

FIG. 2. **Time course of the production of PCho and choline in ts-6-315 cells after temperature shifting.** ts-6-315 cells were labeled with [*methyl*-^{14}C]choline as described under "Materials and Methods," and experiments were carried out as described in the legend to Fig. 1. Cells were incubated either at 32 °C (△) or at 40.5 °C (□) for different times after which reactions were terminated, and PCho (*a*), choline (*b*), and PC (*c*) were determined by thin-layer chromatography (21) followed by autoradiography of the aqueous and chloroformic phases of methanolic cell extracts. Results are mean values ± S.D. of three independent experiments with incubations in duplicate.

detected with either labeling protocol (data not shown). This may indicate that *ras* p21 is not activating a phospholipase D and that the increase in choline observed after temperature shifting of ts-6-315 cells could probably be due to dephosphorylation of the PCho generated by phospholipase C-mediated hydrolysis of PC. However, a rapid and therefore undetectable activation by *ras* p21 of phospholipase D could have occurred. This, together with a very active PA phosphohydrolase could theoretically account for the release of DAG observed in transformed ts-6-315 cells. If this were actually the case, treatment of ts-6-315 cells with propranolol (a very well established inhibitor of PA-phosphohydrolase (27) should inhibit the production of DAG by temperature shifting and favor the hypothetical accumulation of PA. When [U-^{14}C]glycerol-labeled ts-6-315 cells were shifted to the permissive temperature in the presence of up to 200 μM propranolol, no changes were detected in the production either of DAG or in the levels of PA (Table I). As a control, the exogenous addition of phospholipase D to [U-^{14}C]glycerol-labeled ts-6-315 cells at the permissive temperature led to the release of PA by 1 min and to the production of DAG by 10 min (Table I). Results shown in Table I also indicate that the presence of propranolol inhibited the production of DAG and promoted the accumulation of PA originated as a consequence of the exogenous addition of phospholipase D. Therefore, all these results argue against the idea of activation by *ras* p21 of phospholipase D-mediated degradation of PC. In order to further substantiate this notion, the following experiment was carried out. ^{32}P-Labeled ts-6-315 cells were stimulated by temperature shifting either in the absence or in the presence of 0.5% ethanol. The addition of this alcohol to cell incubations in which a phospholipase D has been activated leads to the accumulation of the metabolically stable phosphatidylethanol (PEt) (28). If PA was actually being originated as a consequence of transformation in ts-6-315 cells, PEt should be detected in those incubations performed in the presence of ethanol at the permissive temperature. Our results (Table II) clearly indicate that PEt is not formed in ^{32}P-labeled ts-6-315 cells upon a shift to the permissive temperature in the presence of 0.5% ethanol. As a control, the exogenous addition of phospholipase D to ^{32}P-labeled ts-6-315 cells led to the release of PA by 1 and 10 min (Table II). The presence of ethanol (0.5%) during the stimulation with phospholipase D promoted the appearance of PEt and significantly decreased the production of PA by phospholipase D (Table II). Taken together all these results strongly support a model whereby *ras* transformation is associated with the activation of PC breakdown, most probably through the stimulation of a phospholipase C.

The responses observed in ts-6-315 cells after shifting from the restrictive to the permissive temperature were not detected when either the normal parental cells or cells transformed by the wild-type Ki-*ras* gene were temperature-shifted (data not shown).

Evidence That the ras Oncogene May Be Involved in Late Steps of the Mitogenic Cascade Stimulated by Serum—It has long been known that *ras* p21 is an important intermediary in the mitogenic pathway(s) stimulated by serum. It has been shown that microinjection of a neutralizing anti-*ras* antibody

TABLE I

Effect of propranolol on diacylglycerol and phosphatidic acid levels of ts-6-315 cells activated by temperature shifting and after the addition of phospholipase D

[U-^{14}C]Glycerol-labeled ts-6-315 cells were either maintained at the restrictive temperature or shifted to the permissive temperature for 30 min or 2 h, either in the absence or in the presence of 200 μM propranolol (Prop). Exogenous phospholipase D (PLD, 5 units/ml) was added to parallel cultures either 1 or 10 min prior to termination of the reactions. Afterward, reactions were stopped, and DAG and PA levels were determined as described under "Materials and Methods." Results are expressed as -fold stimulation over control which consisted of dishes kept at 40.5 °C in the absence of propranolol or phospholipase D. Control values were 950 ± 25 dpm/well for DAG and 2200 ± 250 dpm/well for PA. Results are mean ± S.D. of three independent experiments with incubations in duplicate.

Treatment	30 min at 32 °C		2 h at 32 °C	
	DAG	PA	DAG	PA
	-fold over control		*-fold over control*	
None	2.0 ± 0.1	1.0	5.0 ± 0.2	1.0
Prop	2.2 ± 0.2	1.0 ± 0.1	4.8 ± 0.3	1.0 ± 0.1
PLD (1 min)	2.1 ± 0.2	3.1 ± 0.2	5.2 ± 0.2	3.2 ± 0.3
PLD (1 min) + Prop	2.2 ± 0.2	3.2 ± 0.2	4.8 ± 0.3	3.4 ± 0.4
PLD (10 min)	4.1 ± 0.3	5.2 ± 0.5	7.2 ± 0.5	5.3 ± 0.7
PLD (10 min) + Prop	2.7 ± 0.3	8.2 ± 1.0	5.1 ± 0.2	8.3 ± 0.8

TABLE II

Effect of ethanol on phosphatidic acid and phosphatidylethanol levels of ts-6-315 cells activated by temperature shifting and after addition of phospholipase D

^{32}P-Labeled ts-6-315 cells were either maintained at the restrictive temperature or shifted to the permissive temperature for 30 min or 2 h, either in the absence or in the presence of 0.5% ethanol (EtOH). Exogenous phospholipase D (PLD, 5 units/ml) was added to parallel cultures either 1 or 10 min prior to termination of the reactions. Afterward, reactions were stopped, and PA and PEt were determined as described under "Materials and Methods." Results are expressed as dpm/well and are means ± S.D. of three independent experiments with incubations in duplicate. The control consisted of dishes kept at 40.5 °C in the absence of ethanol or phospholipase D. Control values were 2150 ± 125 dpm/well for PA and 1540 ± 115 dpm/well for PEt.

Treatment	30 min at 32 °C		2 h at 32 °C	
	PA	PEt	PA	PEt
	dpm × 10^{-3}/well		*dpm × 10^{-3}/well*	
None	2.0 ± 0.1	1.5 ± 0.1	2.1 ± 0.2	1.4 ± 0.1
EtOH	2.0 ± 0.1	1.6 ± 0.2	2.0 ± 0.1	1.7 ± 0.2
PLD (1 min)	6.5 ± 0.4	1.7 ± 0.2	6.3 ± 0.7	1.5 ± 0.2
PLD (1 min) + EtOH	4.1 ± 0.3	3.4 ± 0.2	4.0 ± 0.4	3.5 ± 0.3
PLD (10 min)	10.2 ± 0.8	1.7 ± 0.2	10.9 ± 0.9	1.7 ± 0.2
PLD (10 min) + EtOH	6.7 ± 0.5	5.7 ± 0.4	6.5 ± 0.6	5.9 ± 0.6

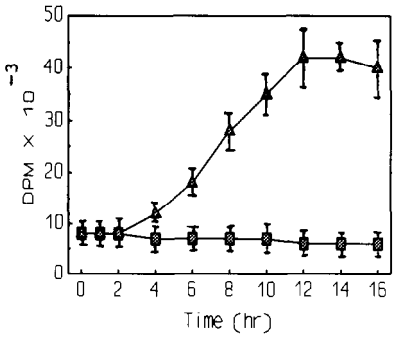

FIG. 3. **Time course of the production of PCho in normal rat fibroblasts stimulated by 10% serum.** Normal rat fibroblasts labeled with [*methyl*-^{14}C]choline as described were either unstimulated (□) or treated with 10% serum (△) for different times, after which reactions were stopped and PCho levels determined. Results are mean values ±S.D. of three independent experiments with incubations in duplicate.

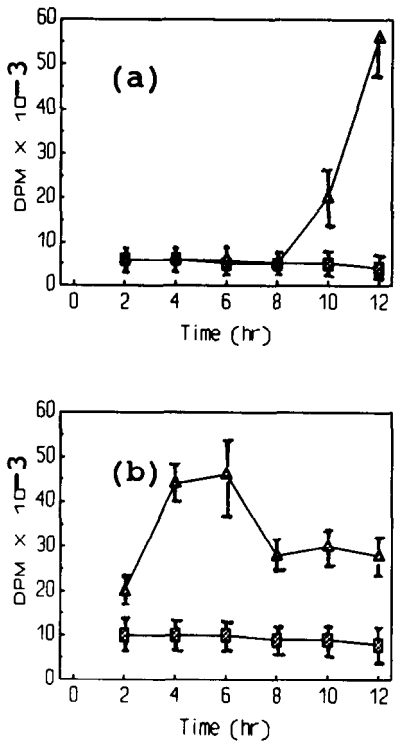

FIG. 4. **DNA synthesis in ts-6-315 cells after temperature shifting and in normal rat fibroblasts in response to serum.** *a*, quiescent normal rat fibroblasts were either unstimulated (□) or treated with 10% serum for different times (△), and DNA synthesis was determined by the addition of 2 μCi/ml [^{3}H]thymidine 2 h prior to the termination of the reactions. *b*, quiescent ts-6-315 cells were either kept at 40.5 °C (□) or shifted to 32 °C (△) for different times, and DNA synthesis was determined as described above. Results are mean values ±S.D. of three independent experiments with incubations in duplicate.

blocks the ability of serum to induce DNA synthesis in fibroblasts (29). Therefore, if the PCho release stimulated by a *ras* oncogene is an important event in mitogenic signaling, serum should activate this phospholipid degradative pathway. It has also been reported that a small increase in PCho occurs shortly after the addition of certain growth factors (14).

Results shown in Fig. 3 demonstrate that little or no change in PCho levels was observed until 4 h after the addition of serum to normal fibroblast cultures. A maximal increase in PCho production (4-fold) was attained by 12 h. These results suggest that PC hydrolysis is a late event in the signal transduction pathway triggered by serum. Since *ras* p21 stimulation of the phosphodiesteratic hydrolysis of PC is a rapid event (see above), these results would suggest that *ras* might be acting late in the mitogenic signaling cascade. To address whether this has any significance on the ability of *ras* p21 to couple the signals leading to DNA synthesis, the following experimental approach was carried out. The time course of [^{3}H]thymidine incorporation was measured in the normal parental cell line in response to 10% serum. Results shown in Fig. 4*a* indicate that for serum to induce DNA synthesis at least 10 h of stimulation are required. If *ras* p21 is acting late in the cascade activated by serum, as the PCho data suggest, DNA synthesis induced by *ras* p21 should require much less time than by serum. Results shown in Fig. 4*b* demonstrate that this is exactly the case, measurable [^{3}H]thymidine incorporation is detected as early as 2 h after temperature shifting in ts-6-315 cells and reaches the maximal level by 6 h. This finding is more remarkable when one considers that temperature-shifted ts-6-315 cells are metabolizing more slowly at 32 °C than serum-stimulated normal fibroblasts at 37 °C.

Taken together, all these results strongly suggest that *ras* p21 may be controlling the mitogenic response to serum as a late step in the signal transduction cascade. Also these results suggest that *ras* might be performing such a role through the activation of a PC-phospholipase C.

DISCUSSION

The fact that microinjection of *X. laevis* oocytes with transforming *ras* p21 triggered the prompt release of DAG with little changes in inositol phosphates (9) strongly suggested the notion that *ras* p21 rapidly activates a source of DAG distinct from PI turnover. Evidence that chronic *ras*-transformed cells exhibit increased DAG and PCho levels (13) led to the speculation that *ras* p21 may influence PC turnover at the level of phospholipase C. However, stably transformed cells are not adequate when a detailed kinetic relationship between oncogene action and mitogenic signal transduction mechanisms is to be established. First, the use of stable transformants precludes the investigation of any temporal relation between oncogene function and phospholipid metabolism. Furthermore, it is possible that cells stably transformed may have devised means to compensate for initial alterations in mitogenic signaling cascades which, together with the pleiotropic actions of transformation events, make difficult the

interpretation of coupling issues in this system.

The ts-6-315 cell line is a suitable system in which to carry out investigations on the kinetics of *ras*-induced alterations in phospholipid-mediated signal transduction pathways. First, ts-6-315 is a fibroblast cell line; mitogenic signaling pathways subverted by oncogenes in fibroblasts are being progressively unveiled. Second, *ras*-mediated transformation is rapidly triggered upon temperature shifting (19).

The results reported here using this temperature-sensitive transformation mutant clearly show that *ras* action and the stimulation of a PC-specific phospholipase C are proximal events. The exact mechanism used by *ras* to activate this novel phospholipid degradative pathway remains unclear. We and others have recently demonstrated that the stimulation of PC-phospholipase C by receptor-dependent mechanisms is mediated by a G protein (17, 30). The *ras* oncogene product belongs to this class of coupling proteins (31). A possible model could be that *ras* p21 may act as the G protein that couples certain ligands to this novel phospholipase C. However, recent studies suggest that the activation by Ha-*ras* p21 of PC-phospholipase C may require protein kinase C activation (32). Undoubtedly, further work is necessary to clarify the exact mechanism utilized by *ras* to trigger PC turnover.

The activation by serum of PC hydrolysis takes place at least 4 h after the agonist stimulus (Fig. 3), which indicates that PC-phospholipase C is a late event in the signal cascade activated by this mitogen. Taken together, these results suggest the possibility that *ras* p21 might be coupling late steps in mitogenic signal pathways. The fact that significant DNA synthesis is detected 2 h after temperature shifting in ts-6-315 cells whereas the addition of serum to normal fibroblasts induces measurable DNA synthesis by 10 h strongly supports the notion that *ras* may be acting late in the mitogenic cascade. As far as we know this is the first report suggesting the involvement of *ras* oncogene in late steps of the route leading to DNA synthesis. Other oncogenes, like $pp60^{src}$, may also be involved in the coupling of not only early signals (33) but also in very late events in the mitotic pathway, namely at the G_2/M point of control in the cell cycle (34–36). But, unlike to $pp60^{src}$, late actions of *ras* take place before detectable DNA synthesis (compare Figs. 2*a* and 4*b*), which would suggest that this oncogene, possibly through the activation of PC hydrolysis, may act by relieving restrictions to S-phase entry.

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