Role of the Cytosolic Phospholipase A2-linked Cascade in Signaling by an Oncogenic, Constitutively Active Ha-Ras Isoform*

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Activation of Ras signaling by growth factors has been associated with gene regulation and cell proliferation. Here we characterize the contributory role of cytosolic phospholipase A₂ in the oncogenic Ha-RasV₁₂ signaling pathway leading to activation of c-fos serum response element (SRE) and transformation in Rat-2 fibroblasts. Using a c-fos SRE-luciferase reporter gene, we showed that the transactivation of SRE by Ha-Ras is mainly via a Rac-linked cascade, although the Raf-mitogen-activated protein kinase cascade is required for full activation. In addition, Ha-RasV₁₂-induced DNA synthesis was significantly attenuated by microinjection of recombiant Rac²¹⁷, a dominant negative mutant of Rac1. To identify the mediators downstream of Rac in the Ha-Ras signaling, we investigated the involvement of cytosolic phospholipase A₂. Oncogenic Ha-RasV₁₂-induced SRE activation was significantly inhibited by either pretreatment with mepacrine, a phospholipase A₂ inhibitor, or cotransfection with the antisense oligonucleotide of cytosolic phospholipase A₂. We also found that cytosolic phospholipase A₂ is required in the signaling pathway of Rac and cytosolic phospholipase A₂ in the signaling pathway by which Ha-RasV₁₂ transactivates c-fos SRE and transformation. Our findings point to cytosolic phospholipase A₂ as a novel potential target for suppressing oncogenic Ha-RasV₁₂ signaling in the cell.

Ras is a 21-kDa guanine nucleotide-binding protein that functions as a molecular switch linking upstream activators, such as growth factor receptors and nonreceptor tyrosine kinases, to several downstream effectors (1, 2). The best characterized Ras-activated pathway involves a Raf-MAPK cascade that includes Raf-1, MAPK kinase, and the mitogen-activated kinase extracellular signal-regulated kinases 1 and 2 (3–5), activation of which stimulates the transcriptional activity of p62TCF/Elk-1 (6–9). In addition, regulation of c-fos transcription by serum response element (SRE) is itself regulated by several proteins, including serum response factor (SRF) and p62TCF/Elk-1 (10–13). In that regard, activation of the MAPK cascade is known to stimulate interaction between p62TCF/Elk-1 and SRF at SRE, thus providing a direct link between MAPK activity and induction of c-fos (10, 11).

In addition to the Raf-MAPK cascade, an essential role of Rac, a member of Rho family GTPases, in the Ras signaling pathway has been demonstrated by several groups (14, 15). Rho family GTPases were once thought to be involved primarily in organizing the actin cytoskeleton (16). However, over the past several years, it has become evident that Rho GTPases also carry out critical functions in the control of cell proliferation and SRE activation (14, 15, 17–19). Unlike the Raf-MAPK cascade, which activates SRE in a p62TCF/Elk-1-dependent manner, Rac and other Rho family GTPases were shown to stimulate SRE largely via a p62TCF/Elk-1-independent pathway, which probably involves direct activation of SRF (6–10, 19). Thus, the Rac-linked pathway is suggested to act as another effector pathway of Ras in the cell (14, 15). Consistent with this, cooperation between Rac and Raf-MAPK cascades was shown to cause transformation synergistically (15). In addition, Rat-1 fibroblasts expressing RacV₁₂, a constitutively activated mutant of Rac1, displayed all the features of malignant transformation (14), again supporting the role of Rac as a downstream mediator of Ras in a signal pathway leading to transformation. However, the downstream elements of the Ras signaling cascade that mediates transformation remain to be identified. Although c-Jun N-terminal kinase (JNK) could be speculated as a downstream mediator, Rac mutants defective in activating JNK were still shown to induce transformation (20), suggesting that activation of JNK is probably not involved in Rac-mediated cell transformation. It has been reported that the p21-activated serine/threonine kinases might be involved in Rac transformation, because expression of a kinase-deficient p21-activated serine/threonine kinase 1 mutant inhibited Ras transformation (21). However, other groups reported that p21-activated serine/threonine kinase binding was dispensable for Rac-induced transformation, and thus the role of p21-activated serine/threonine kinases in transformation is still unclear (15).

It was recently demonstrated that when activated, Rac in turn activates cytosolic phospholipase A₂ (cPLA₂), and there is a resultant release of arachidonic acid (AA), a principal product of cPLA₂ activity (22–24). This makes it likely that cPLA₂ is a downstream mediator of Rac signaling. Consistent with this, cPLA₂ has been shown to be necessary for Rac in mediating actin remodeling or c-fos SRE activation (23). For instance, the
Role of “Rac-cPLA2” Cascade in Oncogenic Ha-Ras Signaling

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—Antisense cPLA2 oligonucleotide (GsTsGCTTCTAAAGTCTAasT) is directed against codons 4–9 of the human cytosolic, Ca2+-dependent PLA2 gene, two linkages are phospho-thiolated at both the 5’ and 3’ ends. Antisense and control (GsTsGCTTCTAAAGTCTAeT) cPLA2 oligonucleotides were purchased from Biomol (Plymouth Meeting, PA). Mepacrine and wortmannin were from Sigma; nordihydroguaretic acid, indomethacin, and AA-chased from Biomol (Plymouth Meeting, PA). Mepacrine and wortmannin were added to each dish (29). To control for variations in cell number and transfection efficiency, all clones were cotransfected with 1 μg of a GTPase expression vector (e.g., pEXV-RacN17), pEXV-RacN17, and pEXV-RhoV14 (Rhoa14) expression vectors were from Dr. A. Hall (University College London, London, UK). Dominant negative mutants of PI 3-kinase (pSG5-3p85a) and Raf-1 (craf301, a kinase-defective form of Raf-1) were from Dr. J. Downward (Imperial Cancer Research Center) and Dr. U. R. Rapp (University of Wurzburg), respectively (26, 27).

Cell Culture, DNA Transfection, and Luciferase Assay—Rat-2 fibroblasts were obtained from the American Type Culture Collection (CRL 1764) and grown in DMEM supplemented with 0.1 mM nonessential amino acids (Life Technologies, Inc.), 10% fetal bovine serum (FBS), and penicillin (50 units/ml)-streptomycin (50 mg/ml) (Life Technologies, Inc.) at 37 °C under a humidified atmosphere of 95% air, 5% CO2 (v/v). The stable Rat2-HO6 clone expressing Ha-RasV12, a constitutively activated Ha-Ras mutant, has been described previously (28). Transient transfection was carried out by plating ~5 × 105 cells in 100-mm dishes for 24 h, after which calcium phosphate:DNA precipitates, the calcium phosphate:DNA precipitates prepared with a total of 20 μg of DNA, including 3 μg of pSPORT-Ha-Ras and 5 μg of a GTPase expression vector (e.g. pEXV-RacN17), were added to each dish (29). To control for variations in cell number and transfection efficiency, all clones were cotransfected with 1 μg of pCMV-bgal, a eukaryotic expression vector in which the Escherichia coli β-galactosidase (lac Z) structural gene is under the transcriptional control of the cytomegalovirus promoter. The total quantity of DNA in each transfection was kept constant at 20 μg by adding appropriate quantities of sonicated calf thymus DNA (Sigma). After incubating 6 h with the calcium phosphate:DNA precipitates prepared with a total of 20 μg of DNA, including 3 μg of pSPORT-Ha-Ras and 5 μg of a GTPase expression vector (e.g. pEXV-RacN17), were added to each dish (29). To control for variations in cell number and transfection efficiency, all clones were cotransfected with 1 μg of pCMV-bgal, a eukaryotic expression vector in which the Escherichia coli β-galactosidase (lac Z) structural gene is under the transcriptional control of the cytomegalovirus promoter. The total quantity of DNA in each transfection was kept constant at 20 μg by adding appropriate quantities of sonicated calf thymus DNA (Sigma). After incubating 6 h with the calcium phosphate:DNA precipitates, the cells were rinsed twice with phosphate-buffered saline before incubating in fresh DMEM supplemented with 0.5% FBS for an additional 36 h. Thereafter, cell extracts were prepared by rinsing each plate twice with phosphate-buffered saline and lysing the cells in 0.2 ml of lysis solution (0.2 M Tris, pH 7.6, 0.1% Triton X-100). The lysed cells

inhibition of cPLA2 by either pretreatment with mepacrine, a potent inhibitor of phospholipase A2, or cotransfection with antisense cPLA2 oligonucleotide dramatically repressed Rac-induced SRE activation (23). In addition, in actin remodeling, Rac was shown to stimulate growth factor-dependent actin stress fiber formation via cPLA2 and subsequent metabolism of AA metabolism by 5-lipoxygenase (25). Together, these observations place cPLA2 downstream of Rac in a pathway leading to SRE activation or actin remodeling. Thus, activated Rac may stimulate the Rac-cPLA2-dependent pathway as well as the Raf-MAPK-linked cascade to activate SRE and transformation.

The aim of the present study, therefore, was to characterize the contribution made by cPLA2 to SRE activation and transformation induced by oncogenic Ras. With the aid of a c-fos SRE reporter plasmid, we found that transactivation of SRE by Ha-RasV12 is mainly mediated via the cPLA2-linked cascade. In addition, we present evidence suggesting the role of cPLA2 as a downstream mediator of Ha-RasV12 in a signaling to transformation. Together, our findings point to cPLA2 as a novel target for suppressing oncogenic Ha-RasV12 signaling in the cell.

Plasmids and DNA Manipulations—Reporter genes pSRE-Luc and pSREmt-Luc contain positions −53 to +45 of the c-fos promoter situated upstream of the luciferase gene, with wild-type or mutant SRE oligonucleotides (23-mers) inserted at the −53 position (23). pSPORT-Ha-Ras and pSPORT-Ha-RasV12 were from Dr. P. Kirshmeier (Scherer-Plough Research Institute). pEXV, pEXV-RacN17, and pEXV-Rhoe14 (Rhoa14) expression vectors were from Dr. A. Hall (University College London, London, UK). Dominant negative mutants of PI 3-kinase (pSG5-3p85a) and Raf-1 (craf301, a kinase-defective form of Raf-1) were from Dr. J. Downward (Imperial Cancer Research Center) and Dr. U. R. Rapp (University of Wurzburg), respectively (26, 27).

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were scraped and spun for 1 min, and the supernatants were assayed for protein concentration and luciferase and \(\beta\)-galactosidase activities.

Luciferase activity was assayed in 10-\(\mu\)l samples of extract using a luciferase assay system (Promega) according to the manufacturer’s protocol; luciferase luminescence was counted in a luminometer (Turner Design, TD-20/20) and normalized to cotransfected \(\beta\)-galactosidase activity. \(\beta\)-galactosidase assays were carried out using 50-\(\mu\)l aliquots of extract diluted with 100 \(\mu\)l of H\(_2\)O and 150 \(\mu\)l of 2× reaction buffer (3 mg/ml O-nitrophenyl-\(\beta\)-galactopyranoside, 2 mM MgCl\(_2\), 61 mM Na\(_2\)HPO\(_4\), 39 mM NaH\(_2\)PO\(_4\), 100 mM 2-mercaptoethanol). When a faint yellow color appeared, the reactions were stopped by the addition of 350 \(\mu\)l of 1 N Na\(_2\)CO\(_3\), and the optical density at 410 nm was measured in a spectrophotometer. The results were then used to normalize luciferase activity to transfection efficiency. Protein concentrations were routinely measured using the Bradford procedure with Bio-Rad dye reagent (Bio-Rad). Bands on XAR-5 film (Eastman Kodak Co.) corresponding to cPLA\(_2\) were measured by densitometry.

**Western Blot Analysis**—Protein samples were harvested at 95 °C for 5 min and subjected to SDS-polyacrylamide gel electrophoresis on 8% acrylamide gels, followed by transfer to polyvinylidene difluoride membranes for 2 h at 100 V using a Novex wet transfer unit. Membranes were then blocked overnight in Tris-buffered saline with 0.01% (w/v) Tween 20 and 5% (w/v) nonfat dried milk, after which they were incubated for 2 h with the primary antibody (anti-cPLA\(_2\) or anti-tubulin) in Tris-buffered saline and then for 1 h with horseradish peroxidase-conjugated secondary antibody. The blots were then exposed to X-ray film.

**Soft Agar Analysis and Cell Growth Experiments**—For the soft agar clonability assays, \(10^5\) or \(10^6\) cells suspended in 4 ml of agar (Noble, Difco; 0.3% in growth medium with 10% FBS) were poured onto a 60-mm plate. The plates were incubated at 37 °C for 10 days, and the colonies were counted by staining them with p-isotrimethyl benzimidazolium violet dye as described previously (30). For the cell growth experiments, Rat-2 or Rat2-HO6 cells were plated onto a 6-well plate (\(10^5\) cells/plate) in 1 ml of DMEM containing 10% FBS. On the next day, the medium was replaced with serum-free medium or serum-free medium containing mepacrine. The viable cell number was counted at 36 h later.

**Leukotriene LTC\(_4\)/D\(_4\)/E\(_4\) Assays**—Rat-2 and Rat2-HO6 cells (3 \(\times\) 10\(^5\)) were plated on 60-mm dishes and incubated in DMEM supplemented with 10% FBS for 24 h. Then, the culture medium was replaced with DMEM containing 0.5% FBS for an additional 24 h. For the measurements of the level of LTC\(_4\)/D\(_4\)/E\(_4\), the plates were rinsed twice with cold phosphate-buffered saline and mixed with 4 times their volume of absolute ethanol and left at 4 °C for 30 min. The resulting precipitate was removed by centrifugation at 10,000 rpm for 30 min at 4 °C. The ethanolic supernatant and culture medium containing the leukotrienes were collected through a 2× reverse phase column (Amersham Pharmacia Biotech, RP 1803). The methyl formate in the eluted samples was then removed by evaporation under vacuum, and the samples were then reconstituted in assay buffer and stored under argon at −50 °C until assayed for LTC\(_4\)/D\(_4\)/E\(_4\) using a specific enzyme-linked immunosorbent assay (Amersham Pharmacia Biotech, RP 224) as instructed by the manufacturer. The enzyme immunoassay was calibrated with standard LTC\(_4\)/D\(_4\)/E\(_4\) from 0.75 to 48 pg/ml. The sensitivity, defined as the amount of LTC\(_4\)/D\(_4\)/E\(_4\) needed to reduce zero dose binding, was 0.5 pg/ml, which is equivalent to 10 pg/ml. The statistical significance of LTC\(_4\)/D\(_4\)/E\(_4\) assays was assessed with analysis of variance (ANOVA) (\(p < 0.01\)).
p62TCF/Elk-1-independent Activation of c-fos SRE by Ha-RasV12—As a first step in characterizing the downstream signaling cascades elicited by constitutively activated, oncogenic Ha-RasV12, we investigated the mechanisms by which they stimulate c-fos SRE. Because activation of c-fos by normal Ha-RasWT was previously shown to be dependent upon p62TCF/Elk-1 binding to SRF (3–5), we initially used a luciferase reporter gene under the control of a human c-fos minimal promoter fused to SRE oligonucleotide to assess the extent to which Ha-RasV12 requires p62TCF/Elk-1 binding to stimulate SRE (Fig. 1A). Cotransfection with either pSPORT-Ha-RasWT or pSPORT-Ha-RasV12 caused dose-dependent activation of c-fos SRE (Fig. 1B). To assess the role of Elk-1/p62TCF, pSREmt-Luc, containing a mutant oligonucleotide (AGG to TGG), with an intact SRF interaction site but lacking a p62TCF/Elk-1 binding site (13, 31), was used as a reporter gene (Fig. 1A). Unlike transfection of pSPORT-Ha-RasWT, which activated c-fos SRE in a p62TCF/Elk-1-dependent manner, transfection with pSPORT-Ha-RasV12 stimulated both pSRE-Luc and pSREmt-Luc to similar degrees (~12-fold increase over a pSPORT control vector), indicating that Ha-RasV12 acts independently of p62TCF/Elk-1 (Fig. 1C).

Preferential Sensitivity of Ha-RasV12 to Inhibition of Rac—Ras activates the MAPK and Rac pathways via interactions with Raf-1 and PI 3-kinase, respectively, and proper function of both pathways is required for efficient mitogenesis or transformation by Ras (14, 15, 26). To obtain further insight into the signaling mechanism by which Ha-RasV12 mediates c-fos SRE activation, therefore, we examined the effect of cotransfecting vectors encoding dominant negative mutants of either Rac1 (RacN17) (17, 18) or Raf-1 (craf301) (27). As shown in Fig. 2A, both RacN17 and craf301 significantly inhibited Ha-RasWT-induced SRE activation. On the other hand, although strongly inhibited by RacN17, transactivation of SRE by Ha-RasV12 was only partially affected (~20% inhibition) by craf301 (Fig. 2A). Activation of SRE by RhoA V14, a constitutively activated RhoA mutant transfected as a control, was unaffected by either RacN17 or craf301 (Fig. 2A). SRE activity by Ha-RasWT thus appears to be via a pathway dependent on both Raf-MAPK and Rac, although the contribution of the latter was relatively small. Activation by Ha-RasV12, by contrast, appears to be largely via the Rac-linked pathway.

Consistent with the aforementioned results, PD 098059, a specific MAPK kinase inhibitor (32), markedly inhibited SRE activation by Ha-RasWT (e.g. ~75% inhibition at 10 μM) but had a substantially smaller effect on Ha-RasV12-induced activation (Fig. 2B). The levels of expression of Ha-RasWT and Ha-RasV12 were similar (data not shown), meaning that the reduced sensitivity to inhibition of Raf-MAPK on the part of Ha-RasV12 was not due to the differential expression of Ras isoforms. Together with the p62TCF/Elk-1-independent nature (Fig. 1C), therefore, Ha-RasV12 signaling to SRE seems to be largely via the Rac-linked pathway, although the Raf-MAPK cascade seems to still be required for efficient signaling.

Role of PI 3-Kinase in Ha-RasV12 Signaling—It has been reported that PI 3-kinase is situated downstream of Ras in the pathway leading to Rac activation (26). Therefore, to further investigate the contributing role of Rac-linked signaling to Ha-RasV12-induced SRE activation, we tested the effect of wortmannin (33), a specific PI 3-kinase inhibitor, and observed that wortmannin selectively and dose-dependently inhibited SRE activation by Ha-RasV12 but had minimal effects on activation by Ha-RasWT (Fig. 3A). As an example, pretreatment with 0.1 μM wortmannin inhibited Ha-RasV12-induced SRE activation by ~70% but had little effect on Ha-RasWT-induced SRE activation. Similarly, transient transfection with a dominant negative PI 3-kinase mutant, pSG5-3p85α, dose-dependently inhibited the effects of Ha-RasV12 but attenuated the effects of wild-type Ha-Ras to a much smaller degree (Fig. 3B).

Preferential Inhibition of Ha-RasV12-induced DNA Synthesis by Microinjection of RacN17—In another approach aimed at evaluating the role of Rac in Ha-RasV12 signaling, recombinant RacN17 protein was microinjected into cells, and Ha-RasV12-stimulated DNA synthesis was assessed by indirect immunofluorescence. Groups of 150–200 quiescent cells on coverslips were microinjected with Ha-RasWT, Ha-RasV12, or RacN17 plus Ha-RasV12 or Ha-RasWT along with control rat IgG and then labeled with BrdUrd. Ha-RasWT stimulated DNA synthesis in ~40% of the microinjected cells, as indicated by their BrdUrd-labeled nuclei, whereas Ha-RasV12 stimulated 70% of cells to incorporate BrdUrd (Fig. 4). Coinjection of RacN17 reduced the fraction of cells stimulated to initiate DNA synthesis by Ha-RasV12 from 70 to 40% but had little effect on Ha-RasWT-induced DNA synthesis. The results of three independent experiments are graphically summarized in Fig. 4B; they provide
direct evidence that Rac is a critical link in the signal transduction pathway by which Ha-RasV12 stimulates DNA synthesis and, presumably, cell proliferation.

cPLA2 as a Downstream Mediator of Ha-Ras V12 Signaling—In fibroblasts, Rac stimulates growth factor-dependent actin stress fiber formation via PLA2 activation and subsequent metabolism of AA by lipoxygenase (22). In addition, we observed that cPLA2 is a principal downstream mediator of Rac-induced activation of c-fos SRE, JNK, and reactive oxygen species (23, 24, 34). It seems probable, therefore, that cPLA2 is situated downstream of Ha-Ras V12 and mediates Rac-linked signals. To test this likelihood, the contributing role of cPLA2 in Ha-Ras WT or Ha-Ras V12-induced SRE activation was examined using an antisense oligonucleotide against cPLA2. As shown in Fig. 5A, cotransfection of the antisense cPLA2 oligonucleotide, but not the control oligonucleotide (cPLA2), dose-dependently inhibited Ha-Ras V12-induced SRE activation (e.g., 70% inhibition by 0.5 μM antisense cPLA2). The antisense oligonucleotide inhibited Ha-Ras WT-induced SRE activation to a smaller degree. Separately, the expression level of cPLA2 was evaluated on Western blot analysis using cPLA2-specific rabbit polyclonal antibodies (Fig. 5A). The expression level of cPLA2 is clearly diminished by co-transfection with 0.5 μM antisense, but not control, oligonucleotides, whereas no change was observed in the level of tubulin, which was used as a control. These results suggest that cPLA2 is clearly involved in Ha-Ras V12-induced signaling to SRE activation. Similarly, pretreating cells with mepacrine (22), a specific inhibitor of PLA2, dose-dependently inhibited Ha-Ras V12-induced SRE activation but had a smaller effect on Ha-Ras WT-induced SRE activation (Fig. 5B). As an example, 2.5 μM mepacrine reduced Ha-Ras V12-induced SRE activation by ~70% but reduced Ha-Ras WT-induced SRE activation by only 25–30%, indicating that PLA2 activity is preferentially involved in the Ha-Ras V12-signaling pathway. Encouraged by the above results, we tested whether the level of AA, a principal product of cPLA2, is indeed enhanced by Ha-Ras V12 in the cells. Consistent with the proposed role of cPLA2 as a downstream mediator of Ha-Ras V12, transient transfection with Ha-Ras V12 expression plasmid significantly elevated levels of AA in a dose-dependent manner, an effect that was selectively inhibited by mepacrine (Fig. 6). Together, our results strongly suggest the mediatory role of cPLA2 in Ha-Ras V12 signaling in the cell.

Mepacrine, a PLA2 Inhibitor, Suppresses Ha-Ras V12 Transformation—Considering the reported activity of Ha-Ras V12 as a transforming oncogene, the cPLA2-linked cascade may also play a critical role in the transforming activity of Ha-Ras V12. To test this possibility, we examined whether cPLA2 inhibition shows any transformation suppression activity to Rat2-HO6, a transformed Rat-2 cell line stably expressing Ha-Ras V12 (28). By dose-dependent analysis as shown in Fig. 7A,
mepacrine (1 μM) was shown to cause a significantly reduced cell growth in Rat2-HO6, with little effect on the growth of Rat-2 normal cells. In addition, morphological reversion of Rat2-HO6 by mepacrine (1 μM) was observed, but there was no effect on the morphology of Rat-2 cells (Fig. 7B). Clearly, the morphology of the oncogenic Ras-transformed Rat2-HO6 cells was reverted to that of Rat-2 parental cells, showing a flat and dispersed phenotype. In accordance with this result, mepacrine clearly diminished the colony formation in soft agar plates of Rat-HO6 cells (Fig. 7C), suggesting that cPLA2 is critical for the transforming activity of Ha-Ras V12. Thus, the cPLA2-linked cascade by Ha-Ras V12 appears to be commonly essential for the signaling cascades induced by Ha-Ras V12 leading to c-fos SRE expression and transformation. Importantly, the resulted preferential sensitivity of Ha-Ras V12-transformed cells to cPLA2 inhibition led us to suggest that cPLA2 could be an ideal target against Ha-Ras V12-induced transformation.

**DISCUSSION**

We showed that the Rac-linked cascade apparently plays a crucial role in Ha-Ras V12 signaling leading to transactivation of c-fos SRE and transformation. Several approaches were taken to show that the Rac-linked cascade is required for Ha-Ras V12-induced signaling. First, cotransfection of Rac N17 dramatically inhibited SRE stimulation by Ha-Ras V12 but had only minor effects on Ha-Ras WT-induced signaling (Fig. 2A). Besides c-fos SRE activation, Ha-Ras V12-induced DNA synthesis is also preferentially mediated by the Rac-linked pathway, as shown in the microinjection experiment (Fig. 4). The aforementioned findings provide direct evidence that Rac is a crucial link in the signal transduction pathway mediating Ha-Ras V12-induced transformation.
DNA synthesis and, presumably, cell proliferation.

In addition, our findings suggest that cPLA2 is situated downstream of Ha-Ras V12, mediating Ha-Ras V12 signaling to transformation. For example, cotransfection of antisense oligonucleotide against cPLA2 or pretreatment with mepacrine markedly inhibited Ha-Ras V12-induced SRE activation but inhibited Ha-Ras WT-induced activation to a much smaller degree (Fig. 5), suggesting that cPLA2 is preferentially involved in the signaling by Ha-Ras V12. The preferential involvement of cPLA2 in oncogenic Ha-Ras V12 signaling points to cPLA2 as a possible target for suppressing the transforming activity of Ha-Ras V12. Consistent with this idea, treatment of Rat2-HO6 cells with mepacrine significantly reduced the growth and colony formation in soft agar plates of Rat2-HO6 (Fig. 7). Furthermore, we observed that by transient cotransfection with plasmids expressing Ha-Ras V12 and annexin-1, which was shown to specifically inhibit cPLA2 by direct interaction (35, 36), the number of transformed foci formations was significantly reduced compared with that by Ha-Ras V12 alone (data not shown), thus again suggesting the mediatory role of cPLA2 in oncogenic Ha-Ras V12 signaling. In support of the suggested role of cPLA2 as a downstream mediator of Ha-Ras V12, transient expression of Ha-Ras V12 induced a dose-dependent generation of AA, a principal product of cPLA2, an effect that was selectively inhibited by mepacrine (Fig. 6A). Interestingly, we also observed that the expression level of cPLA2 protein is elevated in Rat2-HO6 cells (data not shown). Thus, longer-term exposure of Ha-Ras V12 is suggested to induce the regulation of cPLA2 at the level of gene expression as well as activity. Similar to our result, it has been reported that microinjection of Ras oncogene protein results in the stimulation of PLAs activity and that the effects of Ras protein on the activity of PLAs reflect a critical aspect of the mitogenic activity of Ras proteins (37). In addition, the increased expression of cPLA2 protein was reported in human cancer cell lines harboring oncogenic Ras mutations (38).

From the results of the present study, we speculate that the Ha-Ras V12-evoked cascade leading to SRE activation or transformation may be somewhat different from that evoked by wild-type Ha-Ras, although the exact mechanism by which the differential effects are accomplished is not clear. In addition, the details of the Ha-Ras V12-mediated signaling pathway to cPLA2 stimulation remains obscure. Indeed, it has been well characterized that a Raf-MAPK-linked cascade, in addition to the Rac-linked cascade, contributes to cPLA2 stimulation (39, 40). For example, according to a report from Leslie and co-workers (39), extracellular signal-regulated kinases phosphorylate cPLA2 on Ser-505, which modestly increases its catalytic activity. Recent reports also show that p38 kinase is the MAPK responsible for cPLA2 phosphorylation in thrombin- and collagen-activated platelets and in tumor necrosis factor-α-stimulated neutrophils (41–43). However, there is increasing evidence that in some cell types, phosphorylation of cPLA2 by MAPK is not sufficient to induce AA release. For example, phosphorylation of cPLA2 on Ser-505 is not required for AA release from thrombin-stimulated platelets, but it may be involved in the platelet response to collagen (41, 42). Thus phosphorylation does not provide definitive proof of a role for Raf-MAPK in cPLA2 activation, although Raf-MAPK is generally
assumed to contribute at least somewhat. More information will be required to clarify the role of MAPK pathways in the regulation of evoked cPLA₂ activity.

On the other hand, a number of reports have suggested that cPLA₂ is stimulated via Rac (22, 23, 34, 44, 45). As reported previously, cPLA₂ mediates a variety of cellular activities (e.g. stimulation of c-fos SRE or JNK and generation of reactive oxygen species, among others) that are induced by Rac activation, thus suggesting stimulation of cPLA₂ by Rac1. This means that cPLA₂ stimulation may be either Rac-dependent or Raf-MAPK kinase-MAPK-dependent (Rac-independent), and our earlier findings indicate that in certain cases, the former predominates. For example, C2-ceramide stimulates cPLA₂ activity in Rat-2 fibroblasts (about a 4.2-fold increase, as measured by AA release), and the effect is dramatically inhibited by RacN17 expression (46). Similarly, we observed that epidermal growth factor-evoked cPLA₂ activity in Rat-2 fibroblasts is largely Rac-dependent (31), that RacN17 inhibits cPLA₂ activation induced by hydrogen peroxide (47), and that phorbol 12-myristate 13-acetate stimulation of SRE is selectively suppressed by inhibiting cPLA₂ (35). More recently, we observed that phorbol 12-myristate 13-acetate induces SRE activation primarily via a Rac-cPLA₂-dependent cascade, because phorbol 12-myristate 13-acetate-induced cPLA₂ activation was shown to be dramatically inhibited by RacN17 expression.

The aforementioned findings strongly indicate that Rac is a principal mediator of cPLA₂ stimulation in some cases, although Raf-MAPK may contribute to full activation. We would predict a similar scenario for cPLA₂ stimulation by oncogenic Ha-RasV₁₂. Interestingly, Goldschmidt-Clermont (48) and co-workers reported that reactive oxygen species generated by Ha-RasV₁₂ somehow mediate oncogenic signaling in fibroblasts, and they proposed that Rac, not Raf-MAPK kinase-MAPK, is involved in the signaling to reactive oxygen species generation, thus mediating Ha-RasV₁₂ signaling to transformation. Our recent results suggest that Rac signaling to reactive oxygen species generation is through cPLA₂ activation in Rat-2 fibroblasts (44), thus suggesting a mediatory role of a Rac-cPLA₂ cascade for the efficient transformation by oncogenic Ha-RasV₁₂. In any event, there is an apparent signaling link between Ha-RasV₁₂ and cPLA₂ stimulation. In support of the signaling link between Ras and cPLA₂, Warner et al. (49) reported that Ras is essential for epidermal growth factor-induced AA release in Rat-1 fibroblasts.

We do not yet know in detail the downstream molecule(s) by which cPLA₂ mediates oncogenic H-RasV₁₂ signaling. Nonetheless, because nordihydroguaretic acid, a general lipoxygenase inhibitor, markedly inhibited the colony formation in soft agar plates of Rat2-HO6 (Fig. 8A), we predict that leukotriene synthesis by lipoxygenase is probably involved. In contrast, no detectable inhibition was observed by treatment with indomethacin, a cyclooxygenase inhibitor (Fig. 8A). Therefore, leukotriene synthesis by lipoxygenase is possibly situated downstream of cPLA₂, mediating Ha-RasV₁₂ signaling to transformation. Consistent with the proposed role of leukotriene as downstream mediator, Rat2-HO6 cells show a significantly enhanced level of leukotriene C₄/D₄/E₄ compared with Rat-2 cells, an effect that was selectively inhibited by mepacrine (Fig. 8B).

In summary, our results clearly indicate that Ha-RasV₁₂ is selectively sensitive to cPLA₂ inhibition, and thus it may be appropriate to evaluate cPLA₂ as a novel target for suppressing Ha-RasV₁₂ transformation. Given that cPLA₂ is probably a downstream mediator of Ha-RasV₁₂-induced transformation, further characterization of the cPLA₂ signaling cascade would appear to be a pivotal step toward a better understanding of oncogenic, Ha-RasV₁₂-mediated signal transduction.

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FIG. 8. Leukotriene synthesis by lipoxygenase is possibly involved in the transforming activity by Ha-RasV₁₂. A, inhibition of soft agar growth of Rat2-HO6 in the presence of nordihydroguaretic acid (1 μM). For the soft agar clonability assays, 10⁴ cells of Rat2-HO6 suspended in 4 ml of agar (Noble, Difco; 0.3% in growth medium with 10% FBS), added with nordihydroguaretic acid (0.1 or 1 μM) and indomethacin (1 or 10 μM), were poured onto a 6-ml base layer (0.6% agar in DMEM) in 100-mm plates. The plates were incubated at 37 °C for 10 days, and the colonies were counted by staining them with 4-iodonitro tetrazolium violet dye. Data are representative of two independent experiments. B, enhanced generation of leukotriene C₄/D₄/E₄ by Rat2-HO6 and its suppression by mepacrine. Rat-2 and Rat2-HO6 cells were grown for 24 h in DMEM containing 10% FBS and then harvested for the quantitation of levels of the leukotriene C₄/D₄/E₄ mixture as described under “Experimental Procedures.” Values represent the average of three independent experiments.
