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Role of the Cytosolic Phospholipase A_2-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 A_2 in the oncogenic Ha-Ras^{V12} 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^{V12} is mainly via a Rac-linked cascade, although the Raf-mitogen-activated protein kinase cascade is required for full activation. In addition, Ha-Ras^{V12}-induced DNA synthesis was significantly attenuated by microinjection of recombinant Rac^{N17}, a dominant negative mutant of Rac1. To identify the mediators downstream of Rac in the Ha-Ras^{V12} signaling, we investigated the involvement of cytosolic phospholipase A_2. Oncogenic Ha-Ras^{V12}-induced SRE activation was significantly inhibited by either pretreatment with mepacrine, a phospholipase A_2 inhibitor, or cotransfection with the antisense oligonucleotide of cytosolic phospholipase A_2. We also found cytosolic phospholipase A_2 to be situated downstream of Ha-Ras^{V12} in a signal pathway leading to transformation. Together, these results are indicative of mediatory roles of Rac and cytosolic phospholipase A_2 in the signaling pathway by which Ha-Ras^{V12} transactivates c-*fos* SRE and transformation. Our findings point to cytosolic phospholipase A_2 as a novel potential target for suppressing oncogenic Ha-Ras^{V12} 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[1] cascade that includes Raf-1, MAPK kinase, and the mitogen-activated kinases extracellular signal-regulated kinases 1 and 2 (3–5), activation of which stimulates the transcriptional activity of p62^{TCF}/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 p62^{TCF}/Elk-1 (10–13). In that regard, activation of the MAPK cascade is known to stimulate interaction between p62^{TCF}/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 p62^{TCF}/Elk-1-dependent manner, Rac and other Rho family GTPases were shown to stimulate SRE largely via a p62^{TCF}/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 Rac 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 A_2 (cPLA$_2$), and there is a resultant release of arachidonic acid (AA), a principal product of cPLA$_2$ activity (22–24). This makes it likely that cPLA$_2$ is a downstream mediator of Rac signaling. Consistent with this, cPLA$_2$ has been shown to be necessary for Rac in mediating actin remodeling or c-*fos* SRE activation (23). For instance, the

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[1] The abbreviations used are: MAPK, mitogen-activated protein kinase; SRE, serum response element; SRF, serum response factor; JNK, c-Jun N-terminal kinase; PLA$_2$, phospholipase A_2; cPLA$_2$, cytosolic PLA$_2$; AA, arachidonic acid; PI 3-kinase, phosphoinositide 3-kinase; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; LT, leukotriene; WT, wild-type; Luc, luciferase; mt, mutant.

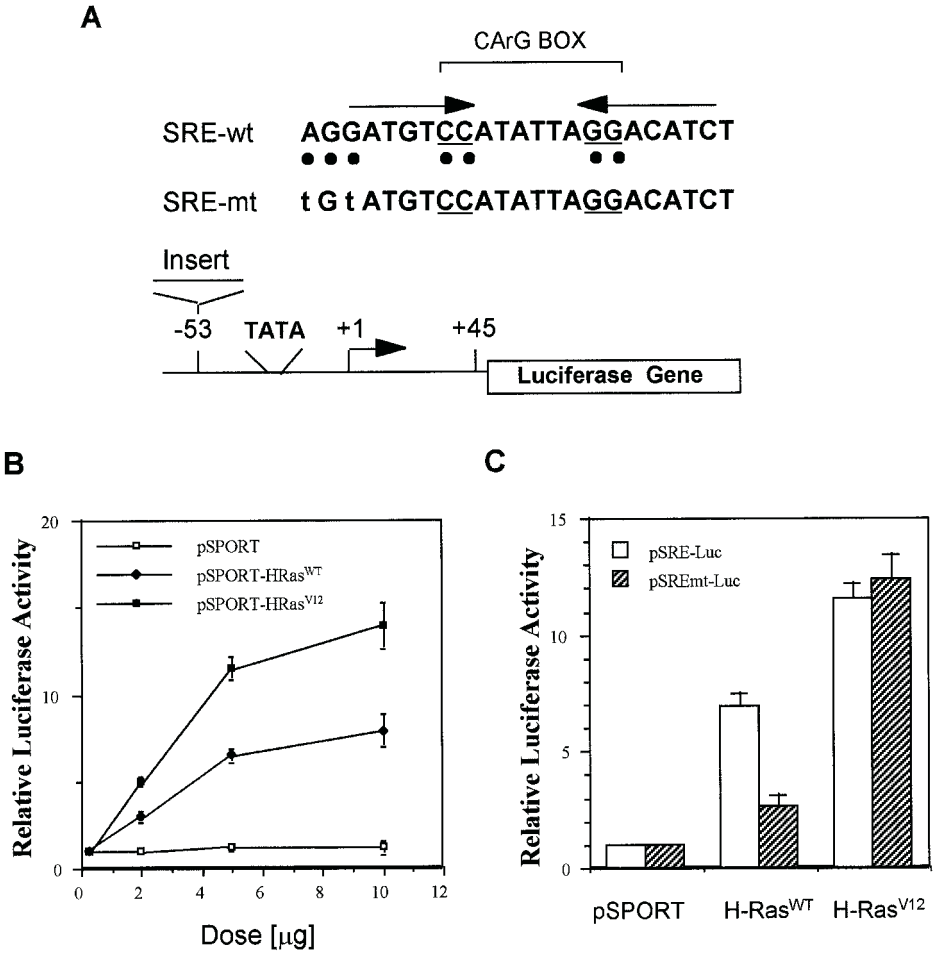

FIG. 1. **p62^{TCF}/Elk-1-independent activation of SRE by oncogenic Ha-Ras^{V12}.** *A*, schematic diagram illustrating the pSRE-luciferase reporter gene vectors used in the study. The structures of constructs containing either wild-type or mutant SRE oligonucleotide sequences (23-mer) inserted at the −53 position of a truncated c-*fos* promoter fused to the luciferase gene are shown. The *filled circles* denote the methylation interference pattern for the SRF ternary complex with p62^{TCF}/Elk-1. *B*, dose-dependent activation of SRE by transient cotransfection of pSPORT, pSPORT-Ha-Ras, or pSPORT-Ha-Ras^{V12}. Transfectants were serum-deprived in DMEM with 0.5% (v/v) FBS for 36 h before luciferase assay. *C*, p62^{TCF}/Elk1-independent SRE activation by Ha-Ras^{V12}. Reporter gene vectors, pSRE-Luc (3 μg) or pSREmt-Luc (3 μg), were transiently cotransfected with 5 μg of pSPORT, pSPORT-Ha-Ras, or pSPORT-Ha-Ras^{V12}. Relative activation of pSRE-Luc was calculated as described under "Experimental Procedures."

inhibition of cPLA$_2$ by either pretreatment with mepacrine, a potent inhibitor of phospholipase A$_2$, or cotransfection with antisense cPLA$_2$ 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 cPLA$_2$ and subsequent metabolism of AA metabolism by 5-lipoxygenase (25). Together, these observations place cPLA$_2$ downstream of Rac in a pathway leading to SRE activation or actin remodeling. Thus, activated Ras may stimulate the Rac-cPLA$_2$-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 cPLA$_2$ 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-Ras^{V12} is mainly mediated via the cPLA$_2$-linked cascade. In addition, we present evidence suggesting the role of cPLA$_2$ as a downstream mediator of Ha-Ras^{V12} in a signaling to transformation. Together, our findings point to cPLA$_2$ as a novel target for suppressing oncogenic Ha-Ras^{V12} signaling in the cell.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—Antisense cPLA$_2$ oligonucleotide (GsTs-GCTGGTAA GGATCTsAsT) is directed against codons 4–9 of the human cytosolic, Ca^{2+}-dependent PLA$_2$ gene; two linkages are phosphothiolated at both the 5′ and 3′ ends. Antisense and control (GsTsGCTCCTAAGTTTCTsAsT) cPLA$_2$ oligonucleotides were purchased from Biomol (Plymouth Meeting, PA). Mepacrine and wortmannin were from Sigma; nordihydroguaretic acid, indomethacin, and AACOCF$_3$ were from Biomol; PD 098059 was from Research Biochemical International (Natick, MA). Bromodeoxyuridine (BrdUrd) and monoclonal anti-BrdUrd antibody were purchased from Amersham Pharmacia Biotech. All other chemicals were from standard sources and were molecular biology grade or higher.

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-Ras^{V12} were from Dr. P. Kirshmeier (Schering-Plough Research Institute). pEXV, pEXV-Rac^{N17}, and pEXV-Rho^{V14} (RhoA^{val14}) expression vectors were from Dr. A. Hall (University College London, London, UK). Dominant negative mutants of PI 3-kinase (pSG5-Δp85α) 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% CO_2 (v/v). The stable Rat2-HO6 clone expressing Ha-Ras^{V12}, a constitutively activated Ha-Ras mutant, has been described previously (28). Transient transfection was carried out by plating ~5×10^5 cells in 100-mm dishes for 24 h, after which calcium phosphate:DNA precipitates prepared with a total of 20 μg of DNA, including 3 μg of pSRE-Luc and 5 μg of a GTPase expression vector (*e.g.* pEXV-Rac^{N17}), 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-βGAL, 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

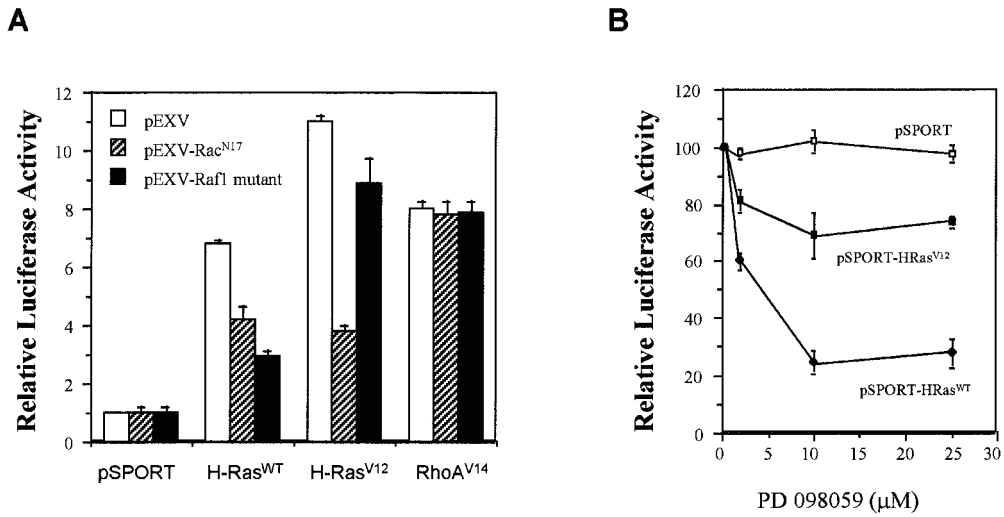

FIG. 2. **Preferential sensitivity of normal Ha-Ras^{WT} to inhibition of the Raf-MAPK cascade.** *A*, pSRE-Luc (3 μg) and expression vectors encoding Ha-Ras, Ha-Ras^{V12}, or RhoA^{V14} (RhoA^{val14}) (5 μg) were transiently cotransfected with 5 μg of dominant negative mutants, Rac^{N17}, or craf301. Total amounts of DNA were kept constant at 20 μg with calf thymus carrier DNA. Transfectants were serum-deprived for 36 h prior to luciferase assay. *B*, effect of PD 090859 on Ha-Ras or Ha-Ras^{V12}-mediated SRE activation. pSRE-Luc (3 μg) was transiently cotransfected with pSPORT, pSPORT-Ha-Ras, or pSPORT-Ha-Ras^{V12} (5 μg), after which the transfectants were exposed to the indicated concentrations of PD 098059 for 24 h before harvest for assay.

were scraped and spun for 1 min, and the supernatants were assayed for protein concentration and luciferase and β-galactosidase activities.

Luciferase activity was assayed in 10-μl 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 β-galactosidase activity. β-galactosidase assays were carried out using 50-μl aliquots of extract diluted with 100 μl of H_2O and 150 μl of 2× reaction buffer (3 mg/ml *O*-nitrophenyl-β-galactopyranoside, 2 mM $MgCl_2$, 61 mM Na_2HPO_4, 39 mM NaH_2PO_4, 100 mM 2-mercaptoethanol). When a faint yellow color appeared, the reactions were stopped by the addition of 350 μl of 1 M Na_2CO_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) and using bovine serum albumin as a standard. Transfection experiments were performed in duplicate with two independently isolated sets, and the results were averaged.

[^{3}H]AA Release—Rat-2 cells were plated to a density of 1×10^5 cells/well in 6-well plates and maintained in DMEM supplemented with 10% FBS. After 4 h, 2 μCi/ml [^{3}H]AA (Amersham Pharmacia Biotech) was added to each well and incubated for an additional 36 h, after which the cells were washed at least three times with medium. The cells were then transfected with pSPORT-Ha-Ras^{WT} or pSPORT-Ha-Ras^{V12} using the calcium phosphate:DNA precipitation method. After incubating the cells for 6 h at 37 °C, the medium was exchanged for fresh DMEM supplemented with 0.5% FBS and incubated for another 6 h; [^{3}H]AA released into the medium during that period was assayed by scintillation counting. At the end of each experiment, the cells were solubilized in 0.5 ml of EtOH, and total intracellular incorporation was determined so that counts could be corrected to intracellular pools of AA.

Microinjection and Immunofluorescence Microscopy—The procedure for microinjection of purified fusion protein has been described. Briefly, Rat-2 cells were plated on scored 12-mm coverslips, incubated for 24 h, and then rendered quiescent by starvation for 48 h in serum-free DMEM. On the day of injection, the coverslips were transferred to 35-mm culture dishes, and Ha-Ras or Ha-Ras^{V12}, along with 2 mg/ml Rat IgG or Rac^{N17} protein, was microinjected using glass capillary needles, yielding about 150–200 microinjected cells/coverslip. Two h after microinjection, BrdUrd was added, and the cells were incubated for an additional 16 h at 37 °C. The cells were then fixed for 20 min at 22 °C in acid alcohol (90% EtOH, 5% acetic acid, 5% H_2O), after which they were incubated for 60 min at 22 °C with mouse anti-BrdUrd antibody, followed by 60 min with rhodamine-conjugated anti-mouse IgG antibody and then 60 min with fluorescein isothiocyanate-labeled anti-rat IgG. The coverslips were then washed intensively and mounted. DNA synthesis by individual cells was assessed as a function of BrdUrd incorporation, which was photographed and analyzed using an Axioskop fluorescence microscope (Carl Zeiss). The immunofluorescent staining of the injected cells indicated that about 80% of the cells were successfully microinjected.

Western Blot Analysis—Protein samples were heated at 95 °C for 5 min and subjected to SDS-polyacrylamide gel electrophoresis on 8% acrylamide gels, followed by transfer to polyvinylidine 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% (v/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 developed using enhanced chemiluminescence kits (ECL, Amersham Pharmacia Biotech). Bands on XAR-5 film (Eastman Kodak Co.) corresponding to cPLA$_2$ were measured by densitometry.

Soft Agar Analysis and Cell Growth Experiments—For the soft agar clonability assays, 10^3 or 10^4 cells suspended in 4 ml of agar (Noble, Difco; 0.3% in growth medium with 10% FBS) were poured onto a 6-ml basal 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 *p*-iodonitro tetrazolium 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 (3×10^5) 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 C2 reverse phase column (Amersham Pharmacia Biotech, RPN 1903). The methyl formate in the eluted samples was then removed by evaporation under vacuum, and the samples reconstituted in assay buffer were 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, RPN 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/well. The sensitivity, defined as the amount of $LTC_4/D_4/E_4$ needed to reduce zero dose binding, was 0.5 pg/well, 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) ($p < 0.01$).

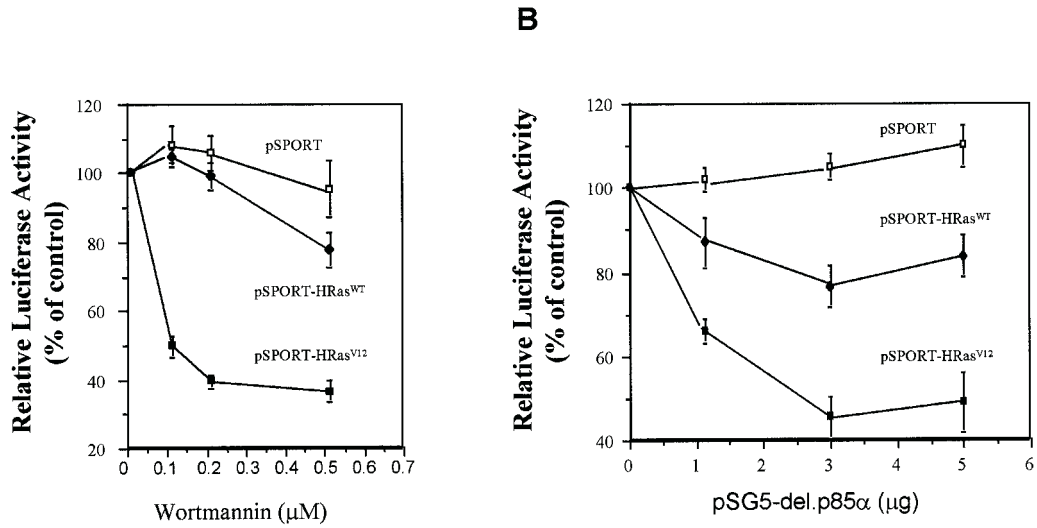

FIG. 3. **Preferential sensitivity of Ha-Ras^{V12} to inhibition of PI 3-kinase.** *A*, effect of wortmannin on Ha-Ras- and Ha-Ras^{V12}-induced SRE activation. Rat-2 cells transiently cotransfected with pSRE-Luc (3 μg) and pSPORT, pSPORT-Ha-Ras, or pSPORT-Ha-Ras^{V12} (5 μg) were exposed to the indicated concentrations of wortmannin for 24 h prior to harvest for assay. *B*, pSRE-Luc and pSPORT, pSPORT-Ha-Ras, or pSPORT-Ha-Ras^{V12} were transiently cotransfected with selected amounts (0, 1, 3, and 5 μg) of pSG5-del.p85α, an expression vector encoding a dominant negative PI 3-kinase mutant. Transfectants were serum-deprived in DMEM containing 0.5% (v/v) FBS for 36 h prior to luciferase assay.

RESULTS

p62^{TCF}/Elk-1-independent Activation of c-fos SRE by Ha-Ras^{V12}—As a first step in characterizing the downstream signaling cascades elicited by constitutively activated, oncogenic Ha-Ras^{V12}, we investigated the mechanisms by which they stimulate c-*fos* SRE. Because activation of c-*fos* by normal Ha-Ras^{WT} was previously shown to be dependent upon p62^{TCF}/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-Ras^{V12} requires p62^{TCF}/Elk-1 binding to stimulate SRE (Fig. 1*A*). Cotransfection with either pSPORT-Ha-Ras^{WT} or pSPORT-Ha-Ras^{V12} caused dose-dependent activation of c-*fos* SRE (Fig. 1*B*). To assess the role of Elk-1/p62^{TCF}, pSREmt-Luc, containing a mutant oligonucleotide (*AGG* to *TGT*), with an intact SRF interaction site but lacking a p62^{TCF}/Elk-1 binding site (13, 31), was used as a reporter gene (Fig. 1*A*). Unlike transfection of pSPORT-Ha-Ras^{WT}, which activated c-*fos* SRE in a p62^{TCF}/Elk-1-dependent manner, transfection with pSPORT-Ha-Ras^{V12} stimulated both pSRE-Luc and pSREmt-Luc to similar degrees (~12-fold increase over a pSPORT control vector), indicating that Ha-Ras^{V12} acts independently of p62^{TCF}/Elk-1 (Fig. 1*C*).

Preferential Sensitivity of Ha-Ras^{V12} 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-Ras^{V12} mediates c-*fos* SRE activation, therefore, we examined the effect of cotransfecting vectors encoding dominant negative mutants of either Rac1 (Rac^{N17}) (17, 18) or Raf-1 (craf301) (27). As shown in Fig. 2*A*, both Rac^{N17} and craf301 significantly inhibited Ha-Ras^{WT}-induced SRE activation. On the other hand, although strongly inhibited by Rac^{N17}, transactivation of SRE by Ha-Ras^{V12} was only partially affected (~20% inhibition) by craf301 (Fig. 2*A*). Activation of SRE by RhoA^{V14}, a constitutively activated RhoA mutant transfected as a control, was unaffected by either Rac^{N17} or craf301 (Fig. 2*A*). SRE activation by Ha-Ras^{WT} 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-Ras^{V12}, 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-Ras^{WT} (*e.g.* ~75% inhibition at 10 μM) but had a substantially smaller effect on Ha-Ras^{V12}-induced activation (Fig. 2*B*). The levels of expression of Ha-Ras^{WT} and Ha-Ras^{V12} were similar (data not shown), meaning that the reduced sensitivity to inhibition of Raf-MAPK on the part of Ha-Ras^{V12} was not due to the differential expression of Ras isoforms. Together with the p62^{TCF}/Elk-1-independent nature (Fig. 1*C*), therefore, Ha-Ras^{V12} 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-Ras^{V12} 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-Ras^{V12}-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-Ras^{V12} but had minimal effects on activation by Ha-Ras^{WT} (Fig. 3*A*). As an example, pretreatment with 0.1 μM wortmannin inhibited Ha-Ras^{V12}-induced SRE activation by ~70% but had little effect on Ha-Ras^{WT}-induced SRE activation. Similarly, transient transfection with a dominant negative PI 3-kinase mutant, pSG5-Δp85α, dose-dependently inhibited the effects of Ha-Ras^{V12} but attenuated the effects of wild-type Ha-Ras to a much smaller degree (Fig. 3*B*).

Preferential Inhibition of Ha-Ras^{V12}-induced DNA Synthesis by Microinjection of Rac^{N17}—In another approach aimed at evaluating the role of Rac in Ha-Ras^{V12} signaling, recombinant Rac^{N17} protein was microinjected into cells, and Ha-Ras^{V12}-stimulated DNA synthesis was assessed by indirect immunofluorescence. Groups of 150–200 quiescent cells on coverslips were microinjected with Ha-Ras^{WT}, Ha-Ras^{V12}, or Rac^{N17} *plus* Ha-Ras^{V12} or Ha-Ras^{WT} along with control rat IgG and then labeled with BrdUrd. Ha-Ras^{WT} stimulated DNA synthesis in ~40% of the microinjected cells, as indicated by their BrdUrd-labeled nuclei, whereas Ha-Ras^{V12} stimulated 70% of cells to incorporate BrdUrd (Fig. 4). Coinjection of Rac^{N17} reduced the fraction of cells stimulated to initiate DNA synthesis by Ha-Ras^{V12} from 70 to 40% but had little effect on Ha-Ras^{WT}-induced DNA synthesis. The results of three independent experiments are graphically summarized in Fig. 4*B*; they provide

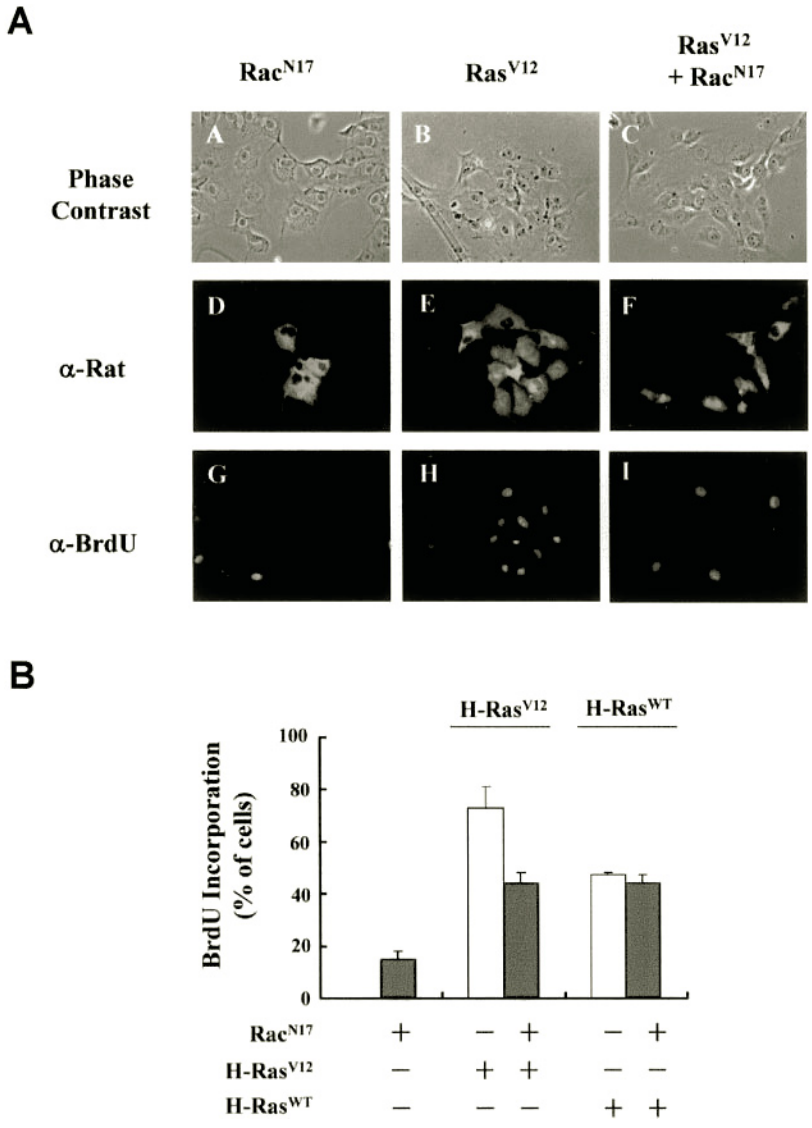

FIG. 4. **Effect of microinjected Rac^{N17} protein on Ha-Ras^{V12}-induced DNA synthesis.** *A*, immunofluorescence of microinjected Rat-2 cells. Phase contrast (*A–C*) and fluorescence (*D–I*) images of serum-starved cells injected with 7.25 mg/ml Rac^{N17} protein (*A*, *D*, *G*), Ha-Ras^{V12} protein (*B*, *E*, *H*), or both (*C*, *F*, *I*), along with Rat IgG (2 mg/ml). Injected cells were identified by cytoplasmic fluorescein isothiocyanate-labeling (*D–F*), and BrdUrd (*BrdU*) incorporation was identified by nuclear rhodamine labeling (*G–I*), as described under "Experimental Procedures." *B*, *shaded* and *open bars* represent injected and uninjected cells on the same coverslips, respectively. The data are the means ± S.E. of three independent experiments.

direct evidence that Rac is a critical link in the signal transduction pathway by which Ha-Ras^{V12} stimulates DNA synthesis and, presumably, cell proliferation.

cPLA$_2$ as a Downstream Mediator of Ha-Ras^{V12} Signaling—In fibroblasts, Rac stimulates growth factor-dependent actin stress fiber formation via PLA$_2$ activation and subsequent metabolism of AA by lipoxygenase (22). In addition, we observed that cPLA$_2$ 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 cPLA$_2$ is situated downstream of Ha-Ras^{V12} and mediates Rac-linked signals. To test this likelihood, the contributing role of cPLA$_2$ in Ha-Ras^{WT} or Ha-Ras^{V12}-induced SRE activation was examined using an antisense oligonucleotide against cPLA$_2$. As shown in Fig. 5*A*, cotransfection of the antisense cPLA$_2$ oligonucleotide, but not the control oligonucleotide, dose-dependently inhibited Ha-Ras^{V12}-induced SRE activation (*e.g.* ~70% inhibition by 0.5 μM antisense cPLA$_2$). The antisense oligonucleotide inhibited Ha-Ras^{WT}-induced SRE activation to a smaller degree. Separately, the expression level of cPLA$_2$ was evaluated on Western blot analysis using cPLA$_2$-specific rabbit polyclonal antibodies (Fig. 5*A*). The expression level of cPLA$_2$ is clearly diminished by cotransfection 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 cPLA$_2$ is clearly involved in Ha-Ras^{V12}-induced signaling to SRE activation. Similarly, pretreating cells with mepacrine (22), a specific inhibitor of PLA$_2$, dose-dependently inhibited Ha-Ras^{V12}-induced SRE activation but had a smaller effect on Ha-Ras^{WT}-induced SRE activation (Fig. 5*B*). 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 PLA$_2$ 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 cPLA$_2$, is indeed enhanced by Ha-Ras^{V12} in the cells. Consistent with the proposed role of cPLA$_2$ 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 cPLA$_2$ in Ha-Ras^{V12} signaling in the cell.

Mepacrine, a PLA$_2$ Inhibitor, Suppresses Ha-Ras^{V12} Transformation—Considering the reported activity of Ha-Ras^{V12} as a transforming oncogene, the cPLA$_2$-linked cascade may also play a critical role in the transforming activity of Ha-Ras^{V12}. To test this possibility, we examined whether cPLA$_2$ 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. 7*A*,

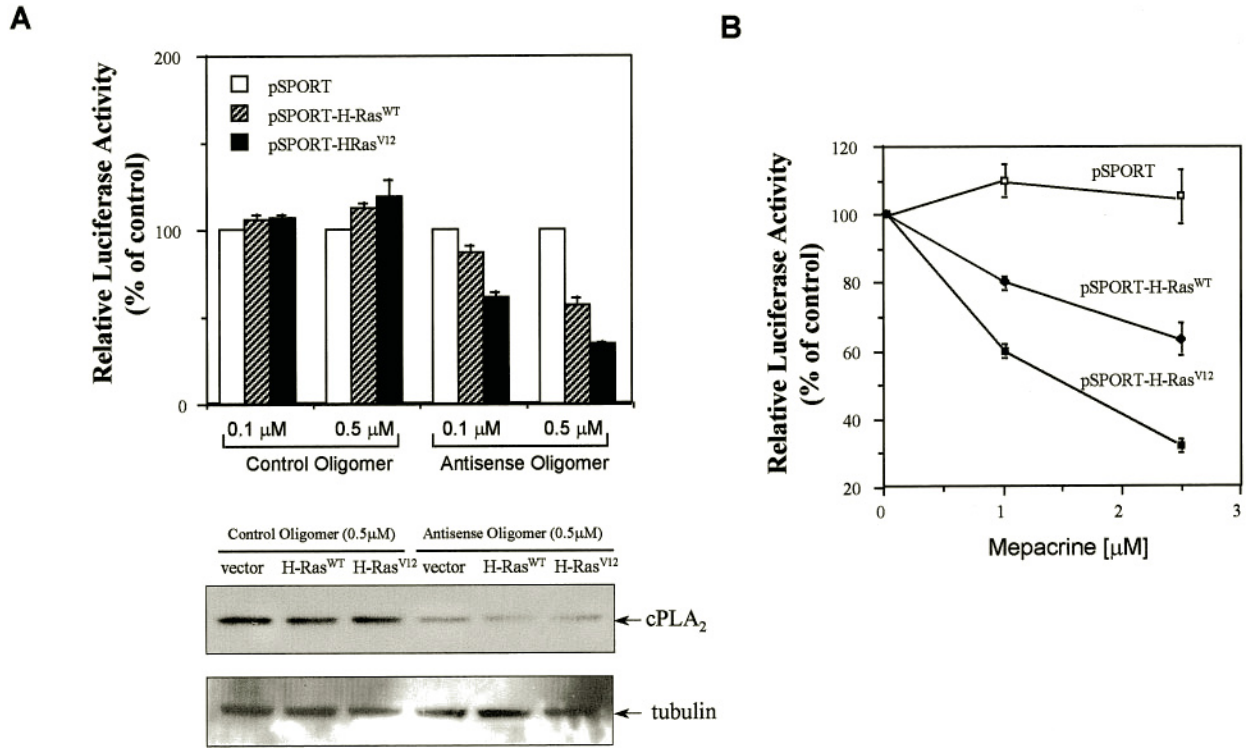

FIG. 5. **Preferential sensitivity of Ha-Ras^{V12}-induced SRE activation to cPLA$_2$ inhibition.** *A*, relative luciferase activity after pSRE-Luc (3 μg) and pSPORT, pSPORT-Ha-Ras, or pSPORT-Ha-Ras^{V12} (5 μg) were transiently cotransfected with the indicated quantities of antisense or control cPLA$_2$ oligonucleotides. Data are expressed as percents of each control (transfection without oligonucleotides). Levels of protein expression of cPLA$_2$ and tubulin (control) are shown by immunoblots. Data are representative of three independent experiments. *B*, effect of mepacrine (1, 2.5 μM) on Ha-Ras^{V12}-mediated SRE activation. pSRE-Luc (3 μg) was transiently cotransfected with pSPORT, pSPORT-Ha-Ras, or pSPORT-Ha-Ras^{V12} (5 μg), after which the transfectants were exposed to the indicated concentrations of mepacrine for 12 h before harvest for assay.

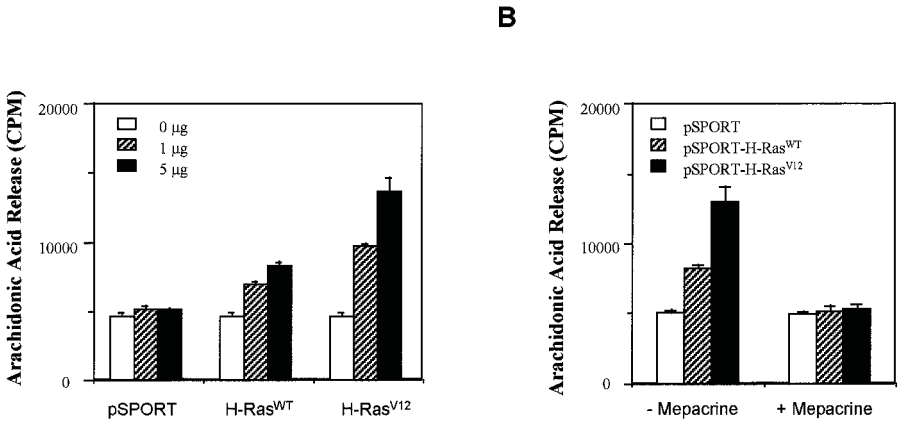

FIG. 6. **Preferential stimulation of cPLA$_2$ by Ha-Ras^{V12}.** *A*, [^{3}H]AA released from Rat-2 cells prelabeled for 36 h with [^{3}H]AA (0.5 μCi/ml) and transiently transfected with pSPORT, pSPORT-Ha-Ras, or pSPORT-Ha-Ras^{V12} (0, 1, or 5 μg). [^{3}H]AA released into the medium was quantified as described under "Experimental Procedures." *B*, mepacrine-sensitive release of [^{3}H]AA. Transfectants were incubated with 1 μM mepacrine for 6 h prior to collecting the medium for assay.

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. 7*B*). 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. 7*C*), suggesting that cPLA$_2$ is critical for the transforming activity of Ha-Ras^{V12}. Thus, the cPLA$_2$-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 cPLA$_2$ inhibition led us to suggest that cPLA$_2$ 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. 2*A*). 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

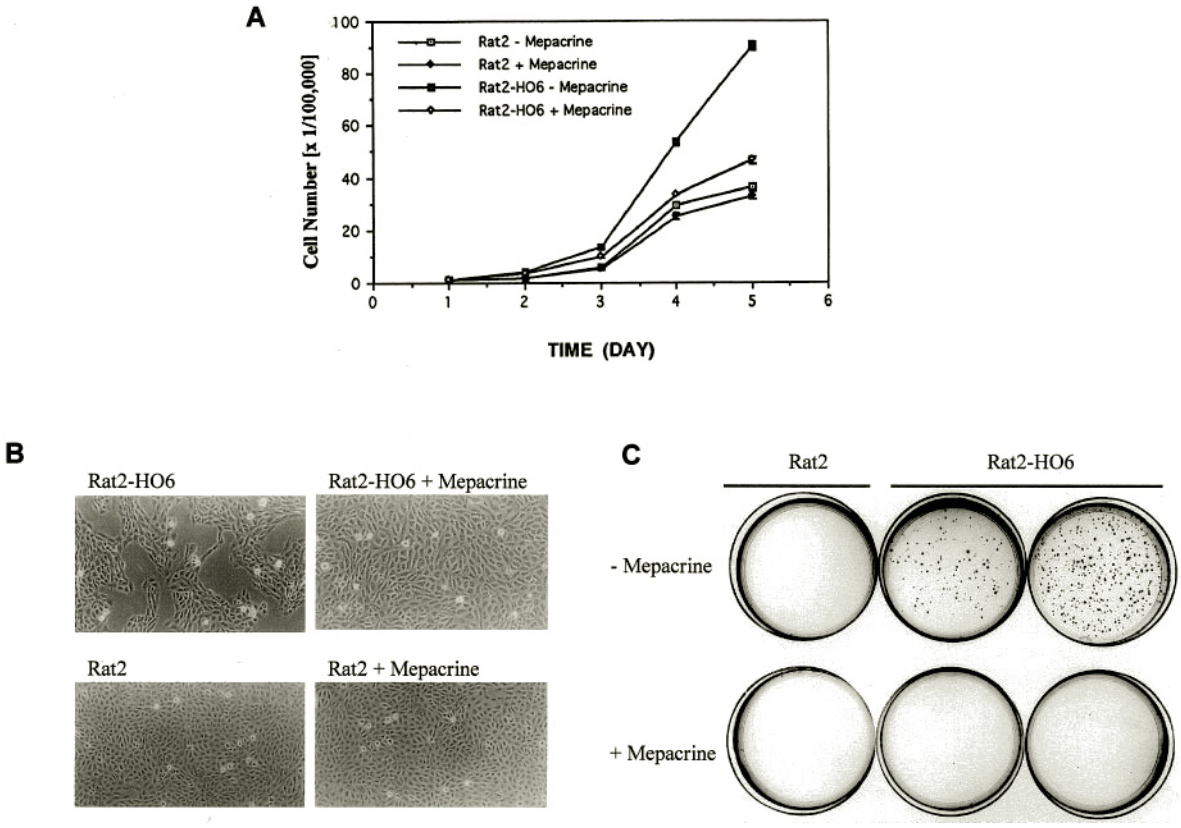

FIG. 7. **cPLA$_2$ inhibition by mepacrine causes a selective inhibition to the transforming activity by Ha-Ras^{V12}.** *A*, suppression of cancerous cell growth of Rat2-HO6 by mepacrine in a dose-dependent manner. Rat-2 and Rat2-HO6 cells were placed to a density of 1×10^5 cells per plate and added with mepacrine (1 μM) or control buffer (phosphate-buffered saline) after 6 h of cell splitting, and the cell number was counted every day. This experiment was performed in duplicate with two independently isolated sets, and the results were averaged. *B*, morphological reversion of Rat2-HO6 cells by mepacrine. Rat-2 and Rat2-HO6 cells growing with and without mepacrine (1 μM) for 24 h were photographed with a phase contrast microscope. *C*, inhibition of soft agar growth of Rat2-HO6 in the presence of mepacrine (1 μM). For the soft agar clonability assays, 10^3 or 10^4 cells (*last panel*) suspended in 4 ml of agar (Noble, Difco; 0.3% in growth medium with 10% FBS) 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 *p*-iodonitro tetrazolium violet dye. Data are representative of two independent experiments.

DNA synthesis and, presumably, cell proliferation.

In addition, our findings suggest that cPLA$_2$ is situated downstream of Ha-Ras^{V12}, mediating Ha-Ras^{V12} signaling to transformation. For example, cotransfection of antisense oligonucleotide against cPLA$_2$ 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 cPLA$_2$ is preferentially involved in the signaling by Ha-Ras^{V12}. The preferential involvement of cPLA$_2$ in oncogenic Ha-Ras^{V12} signaling points to cPLA$_2$ 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 cPLA$_2$ 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 cPLA$_2$ in oncogenic Ha-Ras^{V12} signaling. In support of the suggested role of cPLA$_2$ 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 cPLA$_2$, an effect that was selectively inhibited by mepacrine (Fig. 6*A*). Interestingly, we also observed that the expression level of cPLA$_2$ 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 cPLA$_2$ 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 PLA$_2$ activity and that the effects of Ras protein on the activity of PLA$_2$ reflect a critical aspect of the mitogenic activity of Ras proteins (37). In addition, the increased expression of cPLA$_2$ 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 cPLA$_2$ stimulation remains obscure. Indeed, it has been well characterized that a Raf-MAPK-linked cascade, in addition to the Rac-linked cascade, contributes to cPLA$_2$ stimulation (39, 40). For example, according to a report from Leslie and coworkers (39), extracellular signal-regulated kinases phosphorylate cPLA$_2$ on Ser-505, which modestly increases its catalytic activity. Recent reports also show that p38 kinase is the MAPK responsible for cPLA$_2$ 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 cPLA$_2$ by MAPK is not sufficient to induce AA release. For example, phosphorylation of cPLA$_2$ 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 cPLA$_2$ activation, although Raf-MAPK is generally

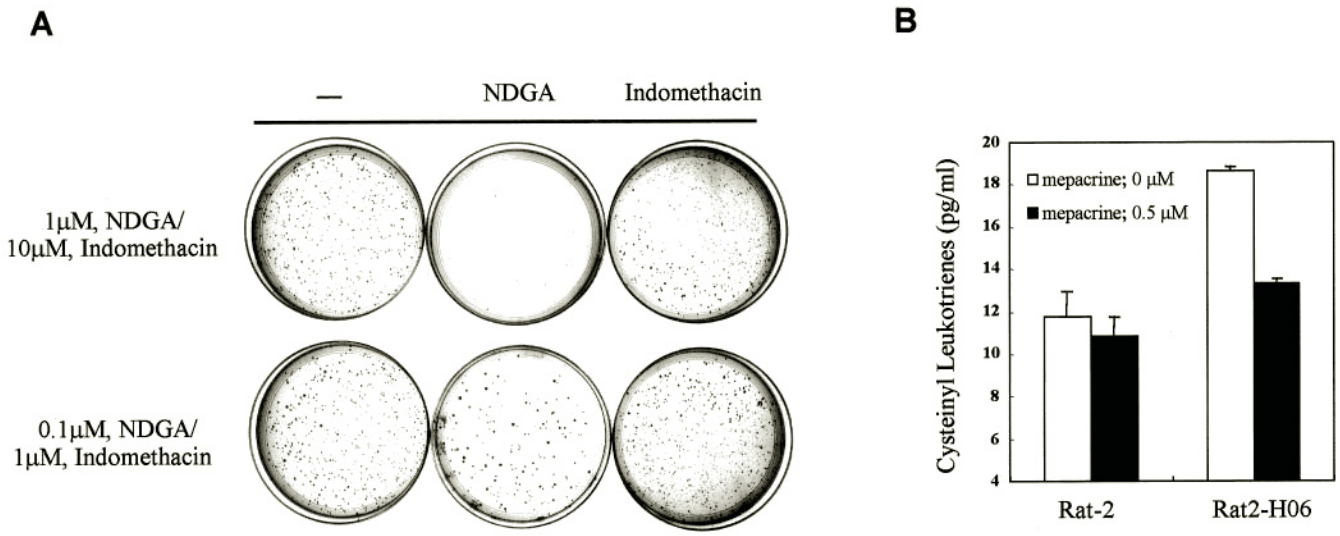

FIG. 8. **Leukotriene synthesis by lipoxygenase is possibly involved in the transforming activity by Ha-Ras^{V12}.** *A*, inhibition of soft agar growth of Rat2-HO6 in the presence of nordihydroguaretic acid (1 μM). For the soft agar clonability assays, 10^4 cells of Rat2-HO6 suspended in 4 ml of agar (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 *p*-iodonitro tetrazolium violet dye. Data are representative of two independent experiments. *B*, enhanced generation of leukotriene $C_4/D_4/E_4$ 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 $C_4/D_4/E_4$ mixture as described under "Experimental Procedures." Values represent the average of three independent experiments.

assumed to contribute at least somewhat. More information will be required to clarify the role of MAPK pathways in the regulation of evoked cPLA$_2$ activity.

On the other hand, a number of reports have suggested that cPLA$_2$ is stimulated via Rac (22, 23, 34, 44, 45). As reported previously, cPLA$_2$ 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 cPLA$_2$ by Rac1. This means that cPLA$_2$ 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 cPLA$_2$ 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 cPLA$_2$ activity in Rat-2 fibroblasts is largely Rac-dependent (31), that RacN17 inhibits cPLA$_2$ activation induced by hydrogen peroxide (47), and that phorbol 12-myristate 13-acetate stimulation of SRE is selectively suppressed by inhibiting cPLA$_2$ (35). More recently, we observed that phorbol 12-myristate 13-acetate induces SRE activation primarily via a Rac-cPLA$_2$-dependent cascade, because phorbol 12-myristate 13-acetate-induced cPLA$_2$ activation was shown to be dramatically inhibited by RacN17 expression.[2]

The aforementioned findings strongly indicate that Rac is a principal mediator of cPLA$_2$ stimulation in some cases, although Raf-MAPK may contribute to full activation. We would predict a similar scenario for cPLA$_2$ stimulation by oncogenic Ha-Ras^{V12}. Interestingly, Goldschmidt-Clermont (48) and coworkers reported that reactive oxygen species generated by Ha-Ras^{V12} 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-Ras^{V12} signaling to transformation. Our recent results suggest that Rac signaling to reactive oxygen species generation is through cPLA$_2$ activation in Rat-2 fibroblasts (44), thus suggesting a mediatory role of a Rac-cPLA$_2$ cascade for the efficient transformation by oncogenic Ha-Ras^{V12}. In any event, there is an apparent signaling link between Ha-Ras^{V12} and cPLA$_2$ stimulation. In support of the signaling link between Ras and cPLA$_2$, 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 cPLA$_2$ mediates oncogenic H-Ras^{V12} signaling. Nonetheless, because nordihydroguaretic acid, a general lipoxygenase inhibitor, markedly inhibited the colony formation in soft agar plates of Rat2-HO6 (Fig. 8*A*), 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. 8*A*). Therefore, leukotriene synthesis by lipoxygenase is possibly situated downstream of cPLA$_2$, mediating Ha-Ras^{V12} signaling to transformation. Consistent with the proposed role of leukotriene as downstream mediator, Rat2-HO6 cells show a significantly enhanced level of leukotriene $C_4/D_4/E_4$ compared with Rat-2 cells, an effect that was selectively inhibited by mepacrine (Fig. 8*B*).

In summary, our results clearly indicate that Ha-Ras^{V12} is selectively sensitive to cPLA$_2$ inhibition, and thus it may be appropriate to evaluate cPLA$_2$ as a novel target for suppressing Ha-Ras^{V12} transformation. Given that cPLA$_2$ is probably a downstream mediator of Ha-Ras^{V12}-induced transformation, further characterization of the cPLA$_2$ signaling cascade would appear to be a pivotal step toward a better understanding of oncogenic, Ha-Ras^{V12}-mediated signal transduction.

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