Role of Rac GTPase in the nuclear signaling by EGF

Byung-Chul Kim, Jae-Hong Kim*

Laboratory of Molecular and Cellular Genetics, Institute of Environment and Life Science, Hallym University, Chun-Cheon, Kangwon-do 200-702, South Korea

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Abstract The role of Rac in epidermal growth factor (EGF)-induced *c-fos* serum response element (SRE) activation was examined in Rat-2 fibroblast cells. By reporter gene analysis following transient or stable transfections with pEXV-RacN17 encoding a dominant-negative mutant of Rac, EGF-induced activation of *c-fos* SRE-luciferase gene was shown to be selectively inhibited, suggesting that Rac activity is necessary for the full activation of SRE by EGF. Our further study to analyze the downstream mediator of Rac in EGF-signaling cascade demonstrated that there is a functional link between Rac and phospholipase A_2 (PLA_2) activation and further that PLA_2 mediates, at least partly, the Rac signaling to SRE. Together, our results point to a critical role of Rac and Rac-activated PLA_2 in the EGF-signaling cascade to *c-fos* SRE. We propose that 'Rac-PLA_2' cascade is one of the major signaling pathways by which EGF stimulates *c-fos* SRE.

Key words: Signal transduction; Epidermal growth factor; Rac GTPase; *c-fos*; Serum response element; Phospholipase A_2

1. Introduction

The *c-fos* serum response element (SRE) has been shown to be necessary and sufficient for the rapid induction of *c-fos* gene transcription in response to serum stimulation and is a primary nuclear target for many extracellular signals such as growth factors, cytokines, and environmental stress [1,2]. Whether these signals lead to *c-fos* SRE activation through independent or convergent pathways is not clearly understood. A nuclear protein termed serum response factor (SRF) binds the CArG box motifs, the inner core of SRE, present in the promoter regions of many immediate early genes including *c-fos* and several actins [1,3]. At the *c-fos* SRE, SRF forms a ternary complex with TCF (ternary complex factor), which cannot bind the SRE by itself [2,4]. The Elk-1, one of TCFs, has been shown to regulate SRE in response to activation of the Ras–Raf–ERK (extracellular signal-regulated protein kinases) cascade, known as MAP kinase pathway [2,5]. Recently, Rac and other Rho family GTPases have been shown to play a role in the signaling pathway to *c-fos* SRE activation via MAP kinase-independent pathway which probably involves the direct activation of SRF [6]. Therefore, there is TCF/Elk-1-independent signaling pathway as well as TCF/Elk-1-dependent pathway for the SRE activation [6].

One of the immediate cellular responses to epidermal growth factor (EGF) is the transient transcription of *c-fos* via SRE [7]. Although Ras–Raf–ERK cascade represents the best-understood signaling pathway of EGF to *c-fos* SRE activation, the role of Rac GTPase, a member of Rho family GTPases, has never been examined. Previously, an essential role of Rac has been suggested in the signaling cascade of EGF leading to actin remodeling, known as membrane ruffling, or Ca^{2+} influx [8–10]. For example, in Swiss 3T3 cells, Rac was shown to mediate EGF-induced arachidonic acid (AA) release via phospholipase A_2 (PLA_2) activation [8] and it has been reported that AA, a principal product of Rac-activated PLA_2, is implicated in EGF-induced actin remodeling or stimulation of Ca^{2+} influx [9,10]. This connection between EGF and Rac activation therefore raises the possibility that Rac may also play an important role in the nuclear signaling by EGF.

In this study, we examined whether Rac GTPase activity is necessary for EGF-induced *c-fos* SRE activation in Rat-2 fibroblast cells. Our results showed that Rac activity and Rac-activated PLA_2 are critical for EGF-induced *c-fos* SRE activation. Therefore, 'Rac–PLA_2–AA' cascade could be regarded as one of the major signaling pathways by which EGF stimulates *c-fos* SRE.

2. Materials and methods

2.1. Chemicals and plasmids

Arachidonic acid, mepacrine, and lysophosphatidic acid (LPA) were obtained from Sigma Chemical Co. (St. Louis, MO). Epidermal growth factor (EGF) was purchased from Boehringer-Mannheim GmbH (Mannheim, Germany). All other chemicals were from standard sources and were molecular biology grade or higher. The pSRE-Luc was derived from pFos-lcf [11] and it contains sequences −53 to +45 of the *c-fos* promoter upstream of the luciferase coding sequences with *c-fos* SRE oligonucleotide inserted at the −53 position. pEXV and pEXV-RacN17 plasmids were gifts from Dr. A. Hall. pEXV-RhoN19 plasmid was constructed by subcloning RhoAN19 fragment into *Eco*RI site of pEXV vector. All Rac, Rho, Ras proteins were expressed as N-terminally 9E10 epitope-tagged derivatives under SV40 promoter.

2.2. Cell lines and cultures

Rat-2 fibroblast cells were obtained from the American Type Culture Collection (ATCC, CRL 1764). Rat-2 stable clones expressing RacN17 were prepared by co-transfecting pEXV-RacN17 with pSV-NeoR plasmid followed by selecting the clones in the presence of G418 antibiotics (400 μg/ml) for 2–3 weeks. Expression of RacN17 protein in the representative clone was confirmed by Western hybridization using anti-myc epitope antibody.

2.3. Transfections and luciferase assay

Transient transfection analysis was performed by calcium phosphate: DNA precipitation method [3,12]. To control for variations in both cell numbers and transfection efficiency, all clones were co-transfected with 1 μg of pCMV-βGAL, an eucaryotic expression vector in which *E. coli* β-galactosidase (lacZ) structural gene is under the

*Corresponding author. Fax: (82) 361-53-1953.
E-mail: jhkim@sun.hallym.ac.kr

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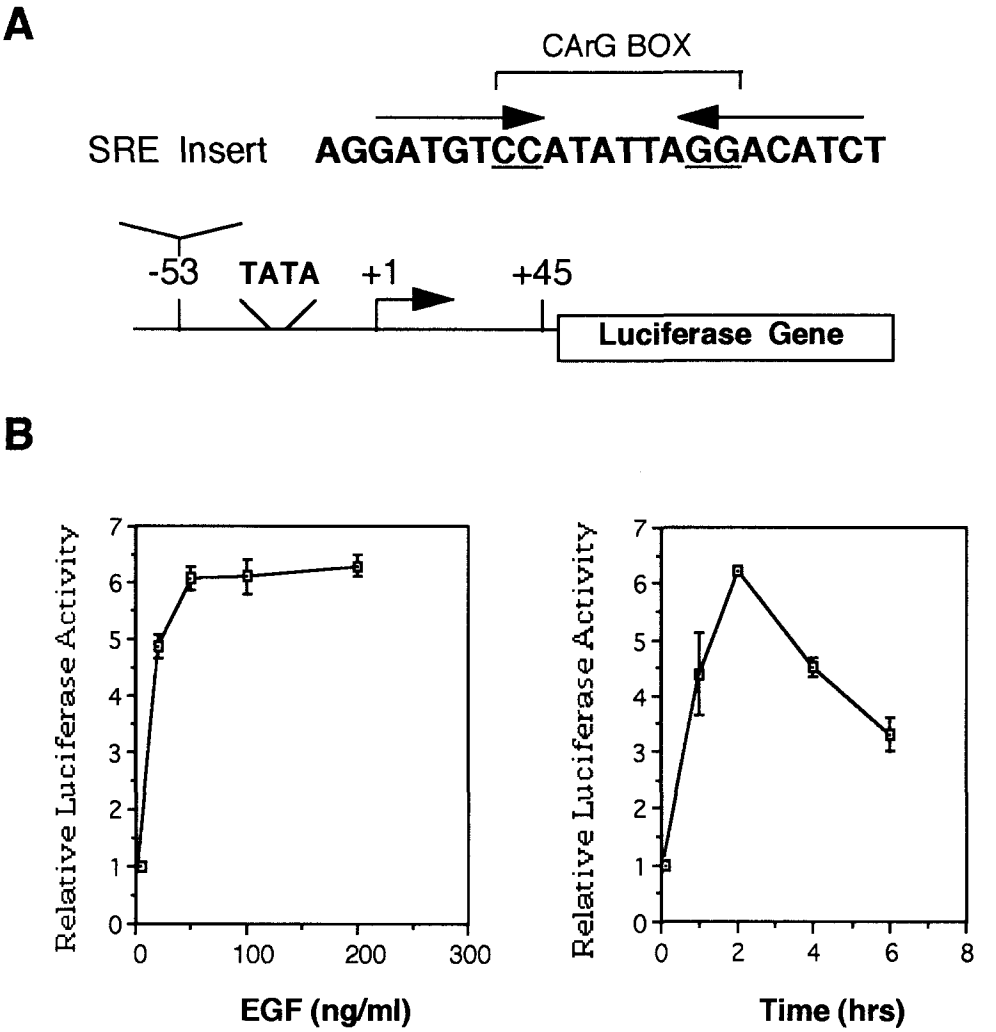

Fig. 1. A: Diagram of pSRE-luciferase reporter gene plasmid. The structure of construct containing SRE oligonucleotide sequences (23mers) inserted to −53 position of the truncated *c-fos* promoter fused to luciferase gene is shown. B (left): Dose-dependent SRE activation by EGF. Various concentrations of EGF (0, 25, 50, 100, 200 ng/ml) were treated for 2 h to the transfected cells with pSRE-Luc (3 μg) plasmid. B (right): Time-dependent response of EGF (50 ng/ml) on SRE activation. Serum-starved, transfected Rat-2 cells were treated with EGF for the lengths of time indicated. Luciferase activities were measured as described in Section 2.

transcriptional control of the CMV promoter. Lysates prepared from the harvested cells were assayed for both luciferase activity and β-galactosidase activity, which was used as an internal standard to normalize the luciferase activity directed by the test plasmid. Luciferase activity was determined as described previously [13,14]. Transfection experiments were performed in triplicate with two independently isolated sets, and the results were averaged.

2.4. [^{3}H]Arachidonic acid release

Rat-2 or Rat2-RacN17 cells in DMEM medium containing 10% fetal bovine serum (FBS) were plated to 1×105 cells per well (12 well plate). Three hours later, 0.5 μCi/ml of [^{3}H]AA (250 μCi; Amersham) was supplemented to each well and incubated for 24 h, then washed at least 4 times with DMEM containing 0.5% FBS. Then, cells were labeled with [^{3}H]AA at 0.5 μCi/ml for 48 h in DMEM/0.5% FBS, washed 8 times with DMEM/0.5% FBS, and finally added with 2 ml of DMEM/0.5% FBS containing EGF (50 ng/ml) or control buffer for 1–2 h. The released [^{3}H]AA into the medium was quantitated by scintillation counting of 0.5 ml of medium each time. At the end of experiments, the cells were solubilized with 0.5 ml of ethyl alcohol (EtOH) for the determination of total intracellular incorporation. Counts were corrected for total incorporation.

3. Results and discussion

3.1. EGF-induced SRE activation is inhibited by a dominant negative mutant, RacN17

To determine the SRE-stimulating activity by EGF, we carried out transient transfection analysis using a reporter plasmid, pSRE-Luc, containing *c-fos* SRE fused to luciferase coding sequences, described in Fig. 1A. Following transient transfection, Rat-2 cells were serum-starved in DMEM containing 0.5% FBS for 36 h before the addition of EGF. SRE activation was monitored by luciferase activities normalized with co-transfected β-galactosidase activity. As shown in Fig. 1, EGF stimulated the promoter activity of *c-fos* SRE in a dose- and time-dependent manner. By dose-responsive analysis, maximal luciferase activity was reached at 50 ng/ml concentration of EGF (Fig. 1B). Also, by time-dependent analysis, a 6-fold increase in the luciferase activity occurred 2 h after the addition of EGF and by 6 h, the *c-fos* SRE luciferase level declined.

To examine any contributing role of Rac GTPase in the

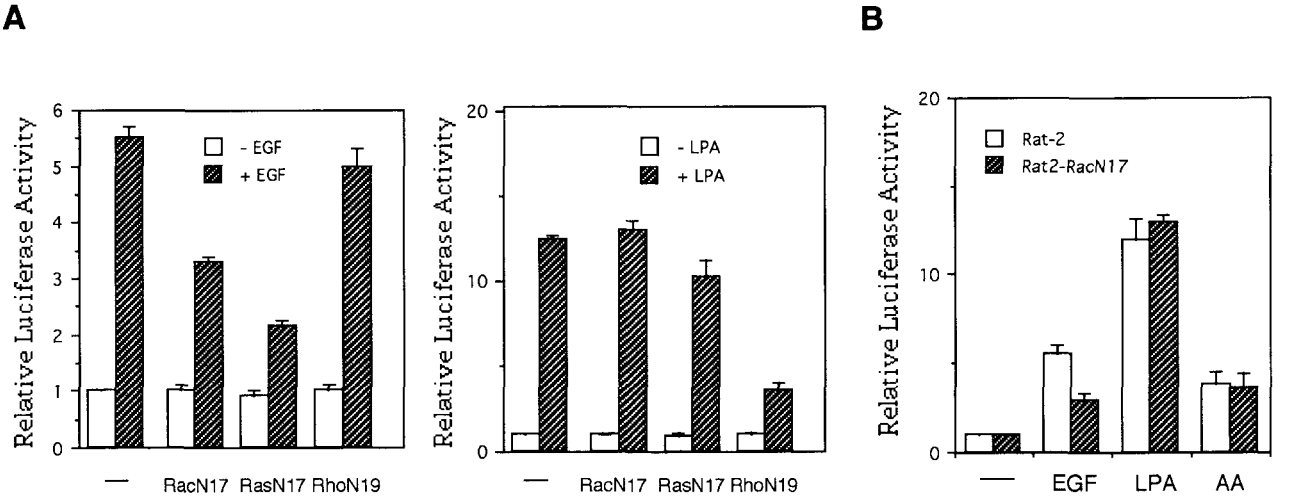

Fig. 2. A: Inhibition of EGF-induced SRE activation by RacN17. A reporter gene plasmid, pSRE-Luc (3 μg) was transiently co-transfected with 5 μg of pEXV, RasN17, RacN17, or RhoN19 to Rat-2 cells. Total amounts of DNA was kept at 20 μg with calf thymus carrier DNA. Transfected cells were serum-deprived for 36 h before EGF (50 ng/ml) or LPA (10 μM) treatment. After 2 h of treatment, luciferase activities were measured and the relative luciferase activities were calculated. B: Rat-2 expressing RacN17 (Rat2-RacN17) shows relative inhibition to EGF-induced SRE activation. Following transfection with pSRE-Luc plasmid (3 μg), Rat-2 and Rat2-RacN17 cells were serum-starved in DMEM containing 0.5% FBS for 36 h before treating control buffer (−), EGF (50 ng/ml), LPA (10 μM), or AA (100 μM). After 2 h of treatment, SRE activation was monitored by luciferase activities normalized with co-transfected β-galactosidase activity.

EGF-induced SRE activation, pSRE-Luc (3 μg) was transiently co-transfected with 5 μg of pEXV (control vector) or pEXV-RacN17 encoding a dominant negative mutant of Rac1. As shown in Fig. 2A, EGF-induced SRE activation is significantly inhibited by co-transfection with pEXV-RacN17 (≈40% reduction of luciferase activity), suggesting that Rac activity is necessary for the full activation of SRE by EGF. A more drastic inhibition was observed (60% reduction of luciferase activity) by co-transfection with pEXV-RasN17 encoding a dominant negative H-Ras. In contrast, no detectable inhibition was observed by co-transfection with pEXV-RhoN19 encoding a dominant negative RhoA (Fig. 2A). On the other hand, lysophosphatidic acid (LPA)-induced SRE activation was dramatically inhibited only by RhoN19, not by RasN17 or RacN17, indicating that LPA signaling to *c-fos* SRE is primarily through Rho-linked pathway (Fig. 2A). This result is in well agreement with the previous report showing that LPA activates *c-fos* SRE principally via Rho-dependent pathway [6]. Together, these results suggest that EGF-induced SRE activation requires Rac as well as Ras activity in Rat-2 fibroblast cells.

To further investigate the role of Rac in EGF-induced SRE

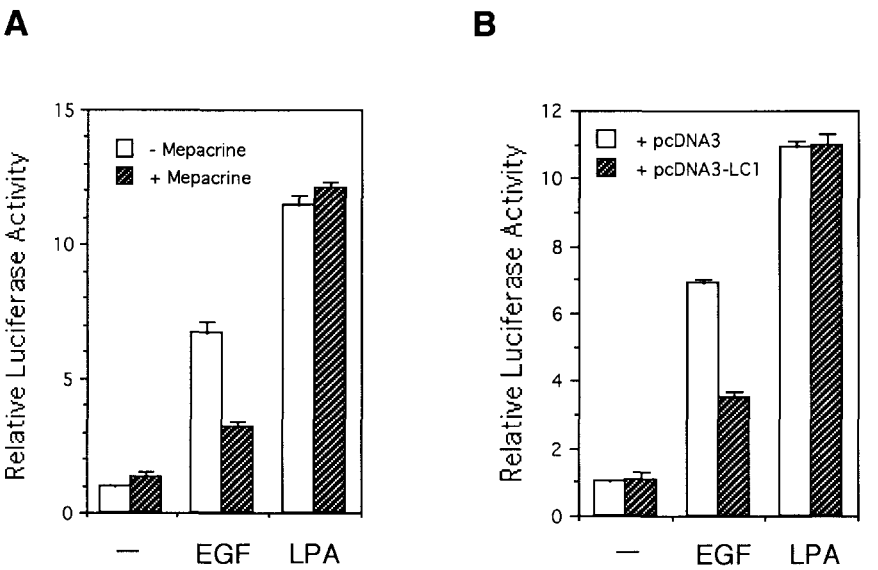

Fig. 3. PLA2 is necessary for the full activation of SRE by EGF. A: Rat-2 cells were transiently transfected with 3 μg of pSRE-Luc plasmid and then serum-starved for 36 h in DMEM medium containing 0.5% FBS. Mepacrine (1 μM) was pretreated 30 min before the addition of control buffer (−), EGF (50 ng/ml) or LPA (10 μM). At 2 h later, luciferase activities were measured and normalized with β-galactosidase activities. B: A reporter gene plasmid, pSRE-Luc (3 μg) was transiently co-transfected with 2.5 μg of pcDNA3 (empty vector) or pcDNA3-LC1, a lipocortin-1 expression plasmid. Total amounts of DNA was kept at 20 μg with calf thymus carrier DNA. Transfected cells were serum-deprived in DMEM with 0.5% FBS for 36 h before addition of control buffer (−), EGF (50 ng/ml) or LPA (10 μM). At 2 h later, luciferase activities were measured and normalized with β-galactosidase activities. Values were representative of multiple transfections.

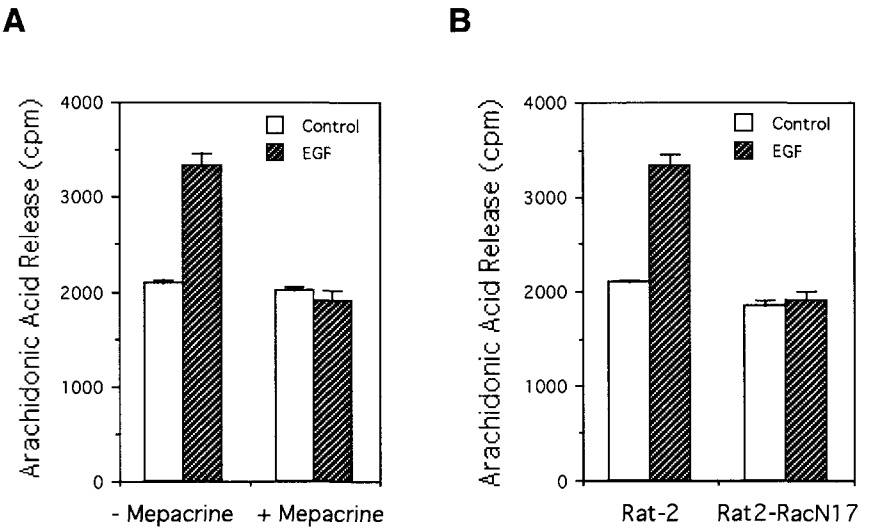

Fig. 4. Rac-activated PLA2 mediates the release of [^{3}H]AA in a mepacrine-sensitive manner. A: Rat-2 cells were labeled with [^{3}H]AA (0.5 μCi/ml; 48 h) and washed 8 times with DMEM/0.5% FBS as described in Section 2. Mepacrine (1 μM) was pretreated 30 min before the addition of control buffer (−) or EGF (50 ng/ml). At 2 h later, the released [^{3}H]AA into the medium was quantitated by scintillation counting. B:Rat-2 and Rat2-RacN17 cells were labeled with [^{3}H]AA and washed as same as above. Control buffer or EGF (50 ng/ml) was added 2 h before the harvest for determination of [^{3}H]AA release. The released [^{3}H]AA into the medium was quantitated by scintillation counting and normalized by the total counts as described in Section 2.

activation, we prepared Rat-2 stable cells expressing RacN17 as described in Section 2. Following transient transfection with pSRE-Luc plasmid, Rat-2 and Rat2-RacN17 cells were serum-starved in DMEM containing 0.5% FBS for 36 h before treating EGF, LPA, or AA. As shown in Fig. 2B, in Rat2-RacN17 cells, EGF-induced SRE activation was significantly inhibited up to 50% level of that in Rat-2 parental cells. On the other hand, the addition of either LPA or AA, a principal product of PLA_2, induced the same level of SRE stimulation in Rat-2 and Rat2-RacN17 cells, respectively (Fig. 2B). From these and earlier results (Fig. 2A), we conclude that EGF signaling to *c-fos* SRE is, at least partly, mediated by Rac-linked pathway.

3.2. *PLA_2 activity is necessary for EGF-induced SRE activation*

Recently, AA was shown to be released via Rac activation in response to EGF, implying a potential role of 'Rac-activated PLA_2 and subsequent AA release' in the signaling pathway of EGF leading to actin remodeling [8]. In a further study to understand the downstream element of Rac in EGF-signaling pathway leading to *c-fos* SRE, we examined any possible role of PLA_2 as a downstream mediator of Rac in this signaling cascade. As an initial approach toward this study, we first determined whether PLA_2 activity is necessary for EGF-induced *c-fos* SRE activation. To do this, we tested the effect of mepacrine, a potent inhibitor of PLA_2, on the EGF-induced SRE activation. Rat-2 cells were transiently transfected with pSRE-Luc, followed by EGF (50 ng/ml) or LPA (10 μM) treatment. The SRE activation was monitored by luciferase activities, normalized with co-transfected β-galactosidase activity. As shown in Fig. 3A, mepacrine (1 μM) inhibited EGF-induced SRE activation significantly (≈50% inhibition of luciferase activity) without affecting LPA-induced SRE activation, suggesting a specific role of PLA_2 activity in EGF-signaling cascade to *c-fos* SRE.

To obtain further insight into the role of PLA_2 in EGF signaling to SRE, we have examined whether EGF-induced SRE activation is sensitive to other type of PLA_2 inhibitor. For this analysis, lipocortin-1 [15], which is also called annexin-1, expression was used. Lipocortin-1 has been shown to specifically inhibit PLA_2 via still-unknown mechanism in various cases [15–17]. Cells were transiently co-transfected with pcDNA3 (vector) or pcDNA3-LC1 (lipocortin-1), and determined whether lipocortin-1 transfection could block the EGF-induced SRE activation. We observed that by pcDNA3-LC1 co-transfection, EGF-induced SRE activation was significantly inhibited (50% reduction of luciferase activity) whereas LPA-induced SRE activation was not affected (Fig. 3B). These results again indicate that the signaling pathway of EGF to *c-fos* SRE is partly dependent upon functional PLA_2 activity.

3.3. *Role of 'Rac-activated PLA_2' in EGF-signaling cascade*

Although the above results suggest a critical role of PLA_2 in EGF-signaling cascade to *c-fos* SRE, it still does not give any evidence regarding the role of Rac in the activation of PLA_2, thus we cannot exclude the possibility that EGF-induced PLA_2 activation may result from alternative routes independent of Rac. To demonstrate the functional existence of 'Rac-activated PLA_2' as downstream element of Rac in EGF signaling to SRE, we examined if EGF-induced PLA_2 activation is mediated mainly through Rac. To do this, we first determined to measure AA liberation in response to EGF treatment to Rat-2 cells which had been pre-labeled with [^{3}H] AA as described in Section 2. As shown in Fig. 4A, EGF induced a significant liberation of AA (≈40% stimulation of AA release over control), but, this over-induced liberation of AA was completely diminished by 30 min pretreatment of mepacrine (1 μM), suggesting that PLA_2 mediates EGF-induced AA release (Fig. 4A). Next, we determined whether this PLA_2-mediated AA liberation requires Rac activity. By comparing the level of AA liberation in between Rat-2 and Rat-2 stable cells expressing RacN17 (Rat2-

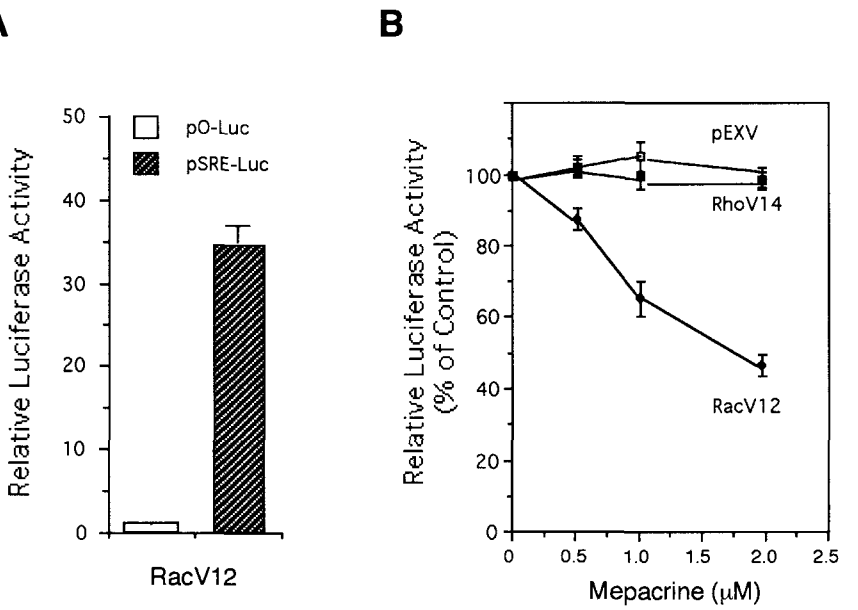

Fig. 5. RacV12 co-transfection activates SRE in a mepacrine-sensitive manner. A: Transient co-transfection of pEXV-RacV12 (5 μg) with 3 μg of either pO-Luc (vector without SRE insert) or pSRE-Luc. The relative activation of pSREwt-Luc to pO-Luc promoter activity was calculated and histograms of the results of the luciferase are shown. Values were representative of multiple transfections. B: Inhibition of RacV12-induced SRE activation by mepacrine. A reporter gene plasmid, pSRE-Luc (3 μg) was transiently co-transfected with 5 μg of pEXV, RacV12, or RhoV14 to Rat-2 cells. Total amounts of DNA was kept at 20 μg with calf thymus carrier DNA. Transfected cells were serum-deprived for 30 h before treatment of various concentration of mepacrine (0, 0.5, 1.0, 2.0 μM). At 6 h later, luciferase activities were measured as described in Section 2. The relative activity was calculated as percentage (%) activity of the control (without mepacrine treatment).

RacN17), it was shown that EGF-induced AA liberation is fully dependent on Rac activity (Fig. 4B). Taken together, these results indicate that the EGF-induced AA liberation is through PLA_2 activation and further that this PLA_2 activation is mediated mainly through Rac. Thus, Rac-activated PLA_2 is suggested to mediate EGF-induced AA release.

To gain further evidence for the functional link between Rac and PLA_2 in EGF-signaling cascade to *c-fos* SRE activation, we undertook the analysis of Rac-induced activity to stimulate SRE and examined whether PLA_2 activity is required for Rac-induced SRE activation. In agreement with the proposed role as an important mediator of EGF signaling to *c-fos* SRE, co-transfection of Rac-expression plasmid is sufficient to stimulate the SRE-linked promoter activity via Ras/MAP kinase-independent pathway (data not shown). pEXV-RacV12 encoding a constitutively active GTPase-deficient allele of Rac1, termed RacV12 was transiently co-transfected with a reporter plasmid, pO-Luc (a control vector without SRE) or pSRE-Luc. As shown in Fig. 5A, by pEXV-RacV12 (5 μg) co-transfection, a remarkable stimulation (≈35-fold) of the relative activity of pSRE-luciferase to pO-luciferase was observed. Next, we determined whether RacV12-induced SRE activation is mediated by PLA_2. To do this, pSRE-Luc (3 μg) was transiently co-transfected with 5 μg of pEXV, pEXV-RacV12, or pEXV-RhoV14 to Rat-2 cells. Transfected cells were serum-deprived in DMEM containing 0.5% FBS for 30 h before the treatment of various concentration of mepacrine (0.5, 1.0, 2.0 μM). As shown in Fig. 5B, mepacrine exhibited a dose-dependent inhibition on the RacV12-induced SRE stimulation, not on RhoV14-induced SRE activation. At 2.0 μM concentration of mepacrine pretreatment, RacV12-induced SRE stimulation was reduced up to 50% level of control whereas RhoV14-induced activation of SRE was not affected (Fig. 5B). This result indicates that Rac-signaling cascade leading to *c-fos* SRE activation is significantly mediated via PLA_2 activation which will induce the release of AA.

In the present study, our results provide evidence for a critical role of Rac in EGF-induced activation of *c-fos* SRE in Rat-2 fibroblast cells. Although the detail mechanism of Rac action in EGF signaling to SRE remains unclear yet, our results indicate that the action of Rac is exerted, at least partly, through the activation of PLA_2 in Rat-2 fibroblasts. Therefore, Rac and Rac-activated PLA_2 clearly participate in the signaling pathway from EGF to *c-fos* SRE in nucleus.

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