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Role of Cytosolic Phospholipase A_2 as a Downstream Mediator of Rac in the Signaling Pathway to *JNK* Stimulation

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Rac is an important regulatory molecule implicated in *c-jun* N-terminal kinase (JNK) activation in response to stress and cytokines. However, the signaling events that mediate the activation of JNK by Rac are not yet well characterized. To broaden our understanding of downstream mediators that link Rac signals to the JNK pathway, we investigated whether cytosolic phospholipase A_2 ($cPLA_2$) is involved in Rac activation of JNK. In this report we demonstrate that either co-transfection with antisense $cPLA_2$ oligonucleotide or pretreatment with arachidonyltrifluoromethyl ketone (AACOCF3), a potent and specific inhibitor of $cPLA_2$, inhibits Rac-mediated JNK activation, implying a potential role of $cPLA_2$ in Rac-signaling to JNK activation. In accordance with this observation, we demonstrate that the addition of exogenous arachidonic acid (AA), a principal product of Rac-activated $cPLA_2$, or leukotrienes, products of 5-lipoxygenase (5-LO) of AA, caused a specific stimulation of JNK. Together, our findings suggest that $cPLA_2$ mediates, at least partly, the signaling cascade by which Rac stimulates JNK.

Rac, a member of Rho family GTPases, mediates effects of growth factors on the actin cytoskeleton (1). For example, activation of Rac by platelet-derived growth factor (PDGF), epidermal growth factor (EGF), or insulin leads to an actin meshwork at the cell periphery producing lamellipodia and membrane ruffles (2–4). Besides its activity in actin remodeling, Rac has additional effects on the regulation of signal transduction cascades from environmental stress and proinflammatory cytokines to the cell nucleus, including the *c-jun* amino-terminal kinase (JNK) signaling pathway (5–6), the *c-fos* serum response factor (SRF) (7–9), the p70 S6 kinase (10), and the NF-κB (11). For example, recently, several groups reported that constitutively activated forms of Rac1 and Cdc42Hs activate JNK signaling cascade (also termed the stress-activated protein kinase pathway), composed of MEKK (MEK kinase-1), JNKK (JNK kinase) and JNK (5–6). JNKs are strongly activated in cells exposed to ultraviolet light (UV), γ irradiation, or osmotic shock (12–13) and are more modestly activated by various mitogenic growth factors and proinflammatory cytokines, including EGF, tumor necrosis factor (TNF)-α, and interlukin-1 (IL-1) (14–16). Especially, in response to various agents such as TNF-α, Fas ligand, or C6-ceramide, Racl was shown to mediate the JNK activation (14, 17–18). Also, consistent with the role as a mediator of JNK activation, Racl was shown to stimulate the transcriptional activity of *c-Jun* (6). Despite these emerging evidences for the role of Rac1 in JNK activation, the understanding of the downstream components of Rac1 mediating JNK activation still remains to be characterized. Although the p21-activated serine/threonine kinase (PAKs) or the mixed lineage kinase 3 (MLK-3) have been described as candidate effectors, the exact roles are still unclear (19–25).

It was recently demonstrated that Rac activation leads to stimulation of phospholipase A_2, especially cytosolic phospholipase A_2 ($cPLA_2$), and thus it was suspected that $cPLA_2$ may be one of the major downstream mediators of Rac-signaling in the cell (26–28). For example, Rac-activated $cPLA_2$ and subsequent arachidonic acid (AA) release have been implicated as

Abbreviations used: JNK, *c-jun* N-terminal kinase; $cPLA_2$, cytosolic phospholipase A_2; 5-LO, 5-lipoxygenase; AA, arachidonic acid; $AACOCF_3$, arachidonyltrifluoromethyl ketone; SRE, serum response element; PAK, p21-activated serine/threonine protein kinase; MLK-3, mixed lineage kinase 3.

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one of the major biochemical pathways by which Rac stimulates Ca^{2+} influx or Rho-dependent cytoskeletal organization in fibroblast cells (26, 29). In addition, our previous study demonstrated that 'cPLA$_2$-AA'-dependent cascade mediates Rac signaling to *c-fos* serum response element (SRE) activation (28). Recently, 'Rac-cPLA$_2$-AA' cascade was also shown to mediate the nuclear signaling in response to exogenous hydrogen peroxide (9) or ceramide (8), a proposed lipid second messenger, which is produced by sphingomyelin hydrolysis in response to various stress and proinflammatory cytokines such as TNF-α, Fas ligand, UV, or X-rays (30–31). In an approach to understand the molecular mechanism that account for Rac-mediated JNK activation, in the present study, we have tested whether cPLA$_2$ is involved in any of the JNK stimulation seen in response to Rac activation. Here, we present evidence that the activity of cPLA$_2$ is necessary for the JNK stimulation by Rac, suggesting that cPLA$_2$ may function as a major downstream mediator of Rac in the signaling pathway to JNK activation.

MATERIALS AND METHODS

Chemicals and plasmids. Antisense cPLA$_2$ oligonucleotide (GsTsGCTGGTAAGGATCTsAsT) is directed against codons 4–9 of the human cytosolic, Ca^{2+}-dependent PLA$_2$ and the two linkages are phosphothioated at both the 5′ and 3′ ends. Antisense and control (GsTsGCTCCTAAGTTTCTsAsT) cPLA$_2$ oligonucleotides were purchased from BIOMOL (Plymouth Meeting, PA). Arachidonyltrifluoromethyl ketone (AACOCF3), indomethacin, MK886, AA861, leukotrienes mixture, and AA were obtained from BIOMOL. Calf thymus DNA was purchased from Sigma Chemical Co. (St. Louis, MO). JNK assay kit (Cat. No. 9810) and MAP kinase assay kit (Cat. No. 9800) were purchased from New England Biolabs (Beverly, MA). Rabbit polyclonal antisera anti-hemagglutinin (HA) epitope, and anti-cPLA$_2$ as well as Myc monoclonal antibody 9E10 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Dulbecco's modified eagle's medium (DMEM), nonessential amino acids and fetal bovine serum (FBS) are purchased from Gibco-BRL (Gaithersburg, MD). All other chemicals were from standard sources and were molecular biology grade or higher. pEXV (Myc-tagged vector) and pEXV-RacV12 plasmids were from Dr. Alan Hall (University College, London, UK). pcDNA3-HA-JNK plasmid was described previously (6, 32).

Cell culture and DNA transfection. Rat-2 fibroblast cells were obtained from the American Type Culture Collection (CRL 1764). Cells were grown in DMEM (Cat. No. 11995; Gibco-BRL) supplemented with 0.1 mM non-essential amino acids (Gibco-BRL), 10% FBS, and penicillin (50 units/ml)-streptomycin (50 μg/ml) (Gibco-BRL) at 37°C in a humidified 95%/5% (v/v) mixture of air and CO_2. Transient transfection analysis was performed by plating approximately 5×10^5 cells in 100 mm dishes for 24 h before adding calcium phosphate:DNA precipitates. Calcium phosphate precipitates were prepared with a total of 20 μg DNA per 100 mm diameter dish. 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 structural gene is under the transcriptional control of the CMV promoter. The total amounts of DNA in each transfection were kept constant (20 μg) by adding sonicated calf thymus DNA. After 6 h incubation with calcium phosphate:DNA precipitates, cells were rinsed twice with phosphate buffered saline (PBS) before incubating with fresh DMEM supplemented with 0.5% FBS for an additional 36 h. To prepare cell extracts, cells were rinsed twice with PBS and then lysed in 0.2 ml of lysis solution (0.2 M Tris, pH 7.6 + 0.1% Triton X-100) per 100 mm plate. Lysed cells were scraped and spun for 1 min. Supernatants were assayed for both protein amount and β-galactosidase activities. β-Galactosidase assays were performed with 50 μl of extracts (diluted with 100 μl of H_2O), using 150 μl of 2 $\times$ reaction buffer (3 mg/ml 0-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, measured in a spectrophotometer at an optical density of 410 nm, and used to normalize the transfection efficiency. Protein concentrations were determined routinely using the Bradford procedure with Bio-Rad Dye Reagent (Bio-Rad Lab., Hercules, CA) and bovine serum albumin as a standard.

SDS-PAGE and immunoblot analysis. Protein samples were heated at 95°C for 5 min and analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). SDS-PAGE was performed on 8% acrylamide gels, followed by transfer to polyvinylidine difluoride (PVDF) membranes for 2 h at 100 V using a Novex wet transfer unit. Immunodetection was performed using the horseradish peroxidase (HRP) method. Briefly, the membrane was blocked overnight with TBS [PBS containing 0.01% (v/v) Tween 20] with 5% (w/v) nonfat dried milk. Blots were incubated for 2 h with primary antibody (1:1000 dilutions for Rac1, HA, and phospho-*c-Jun;* 1:2000 for cPLA$_2$) in TBS and then for 1 h with HRP-conjugated secondary antibody, prior to development using an enhanced chemiluminescence kit (Amersham Life Science, Inc.). Bands corresponding to cPLA$_2$, HA-JNK, or phospho-*c-Jun* (JNK assay) on XAR-5 film (Eastman Kodak Co.) were measured by densitometry.

JNK assays. To determine JNK activities mediated by RacV12, subconfluent Rat-2 cells were transiently co-transfected with pEXV-RacV12 (5 μg unless indicated), pcDNA3-HA-JNK (1 μg), and pCMV-β-GAL (1 μg) by calcium phosphate:DNA precipitation method. Total amount of transfected DNA was adjusted to 20 μg per plate (100 mm diameter dish) using sonicated calf thymus DNA, and at the next day, transfected Rat-2 cells were serum-starved in DMEM containing 0.5% FBS for 24 h unless indicated. For determining JNK activity induced by exogenous AA, TNF-α, or C2-ceramide, Rat-2 cells were serum-starved in serum-free DMEM for 18 h and stimulated with each agonist (AA, 20 μM; TNF-α, 10 ng/ml; C2-ceramide, 5 μM) for the indicated time period. Then, cells were washed with cold PBS, and lysed at 4°C in 0.5 ml ice-cold lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin) with 1 mM phenylethylsulfonyl fluoride (PMSF) to each 100 mm plate and incubated the plate on ice for 5 min, scraped into eppendorf tube, lysed by ten passes through 2.1-gauge needle on ice, and the cell lysate was harvested by microcentrifugation (14,000 rpm) for 10 min. Equal protein amounts were adjusted by normalizing with the protein level (assay by Bradford procedure with Bio-Rad Dye Reagent) and also with β-galactosidase activity (in case of RacV12-mediated JNK assays). JNK assays were done with 250 μl of the adjusted lysate samples. JNK activities were determined according to the manufacturer's protocol (JNK assay kit, New England Biolabs). Briefly, an N-terminal *c-Jun* (1–89) fusion protein bound to glutathione sepharose beads was used to pull down JNK from cell lysates and then the kinase reaction (50 μl) was carried out using *c-Jun* fusion protein as substrate in the presence of cold ATP. Phosphorylation of *c-Jun* fusion protein at Ser 63 was measured by Western blot using a phospho-specific *c-Jun* (Ser 63) rabbit polyclonal antibody that detects only catalytically activated *c-Jun* with phosphorylation at Ser 63. Separately, in case of RacV12-mediated JNK assays, the immunoblot analysis (HA-JNK or cPLA$_2$) were done with the remaining lysate. For example, the levels of HA-JNK were evaluated on Western blot analysis using HA-specific, rabbit polyclonal antibody.

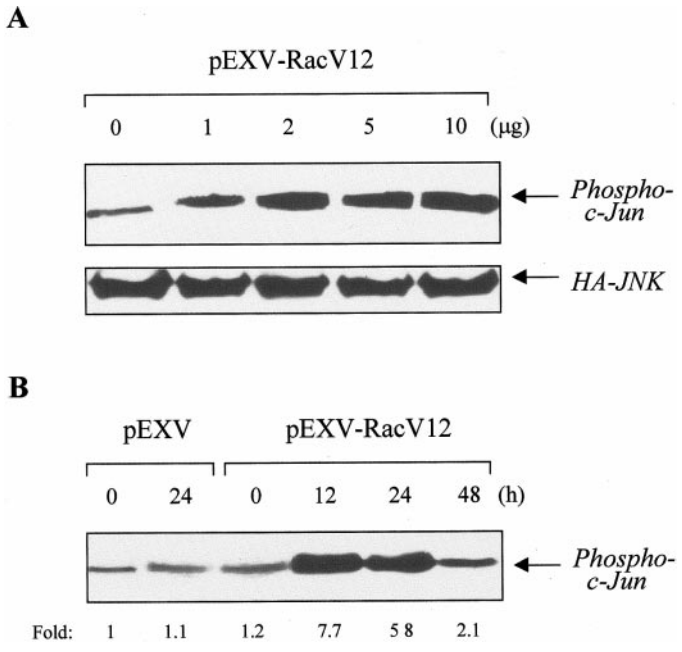

FIG. 1. JNK activation by RacV12 in a dose- and time-dependent manner. (A) Rat-2 cells were transiently transfected with indicated amounts (0, 1, 2, 5, and 10 μg) of pEXV-RacV12 together with 1 μg of pcDNA3-HA-JNK and 1 μg of pCMV-βGAL. Total amounts of expression vector were kept constant by adding decreasing amounts of pEXV. After 24 h of transfection, the transiently expressed HA-JNK was isolated and JNK activities were assessed by phosphorylation of *c-Jun* fusion protein (1–89) as a substrate as described in Materials and Methods. Separately, the level of HA-JNK expression was evaluated on Western blot analysis using a HA-specific antibody. (B) Rat-2 cells were transfected with 5 μg of pEXV or pEXV-RacV12 together with 1 μg of pcDNA3-HA-JNK and 1 μg of pCMV-βGAL and cultured for the indicated time periods (0, 12, 24, 48 h) before harvest for the JNK assays. Data are representative of three independent experiments and are expressed as fold increase with respect to control vector transfection (pEXV, 0 h).

RESULTS

Effect of cPLA$_2$ antisense oligonucleotide on JNK activation by Rac. To study any contributing role of cPLA$_2$ in JNK activation by Rac, we examined whether antisense oligonucleotide to cPLA$_2$ has any inhibitory effect on Rac-induced JNK activation. Consistent with previous reports (5–6), RacV12 was shown to induce JNK activation (Fig. 1). By transient co-transfections with increasing amounts of pEXV-RacV12 along with pcDNA3-HA-JNK (6), a plasmid encoding HA epitope-tagged version of JNK1, RacV12 caused a dose-dependent stimulation of JNK (Fig. 1A). With 5 μg of the pEXV-RacV12, JNK activity was stimulated to as much as 7-fold of control (Fig. 1A). In addition, we have observed that the maximal JNK activity ($\approx$7.7 fold increase compared to control) was detected at 12 h after transient co-transfection with 5 μg of pEXV-RacV12 (Fig. 1B). To examine whether antisense cPLA$_2$ exhibits any inhibitory effect on the JNK activation by RacV12, Rat-2 cells were transiently co-transfected with pEXV-RacV12 and HA-JNK together with various amounts (0, 0.05 and 0.5 μM) of antisense or control cPLA$_2$ oligonucleotides. As shown in Fig. 2, co-transfection with antisense cPLA$_2$ led to a drastic inhibition of JNK activation by RacV12 (e.g., >90% inhibition by co-transfection with 0.5 μM of antisense cPLA$_2$). On the other hand, no inhibitory effect on RacV12-induced JNK activation was observed by control cPLA$_2$ at the same dose ranges that used (Fig. 2). Separately, the expression levels of HA-JNK and cPLA$_2$ were evaluated on Western blot analysis using a HA- and cPLA$_2$-specific rabbit polyclonal antibodies (Fig. 2). The level of cPLA$_2$ is clearly diminished by co-transfection with antisense, but not control, oligonucleotides in a dose-dependent manner. These results suggest that cPLA$_2$ is clearly involved in Rac-induced signaling to JNK activation.

To gain further evidence for the role of cPLA$_2$ in mediating Rac signal to JNK activation, we examined whether exogenous AA, a principal product of Rac-activated cPLA$_2$, could stimulate JNK. Consistent with the proposed role of cPLA$_2$ as a downstream mediator of Rac in the signaling to JNK activation, exogenous AA was shown to stimulate JNK (Fig. 3). After serum starvation in DMEM containing 0.5% FBS for 24 h, Rat-2 cells were treated with AA (20 μM) for the indicated time periods (0, 5, 10, 30, 60, and 120 min). Maximal JNK activity was observed at 30 min treatment of AA (Fig. 3). The stimulation of JNK activity by

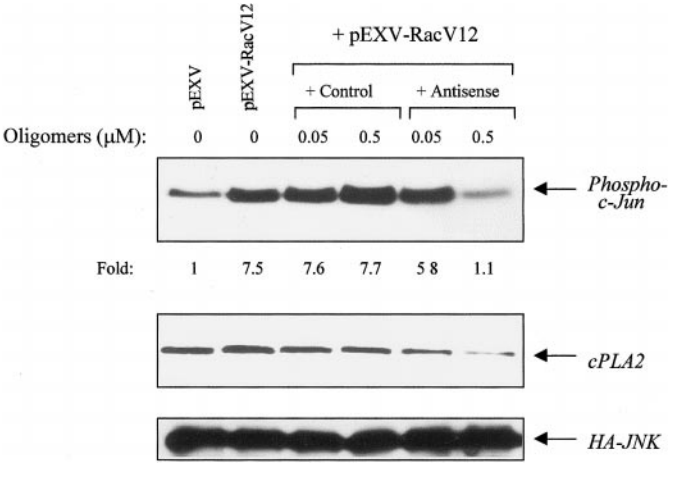

FIG. 2. Effect of antisense cPLA$_2$ oligonucleotide on JNK activation by RacV12. Rat-2 cells were transiently co-transfected with 5 μg of pEXV (C) or pEXV-RacV12 (V or RacV12) together with indicated amounts (0.05, 0.1, 0.5 μM) of control cPLA$_2$ oligomer (control) or antisense cPLA$_2$ oligomer (antisense). Also, 1 μg of pcDNA3-HA-JNK and 1 μg of pCMV-βGAL were co-transfected. After incubation for 24 h in DMEM containing 0.5% FBS, JNK activity was assessed using *c-Jun* fusion protein (1–89) as a substrate. Fold increase was calculated with respect to control vector transfection (pEXV, without oligonucleotide co-transfection). Levels of protein expression of HA-JNK and cPLA$_2$ are shown by immunoblots. Data are representative of three independent experiments.

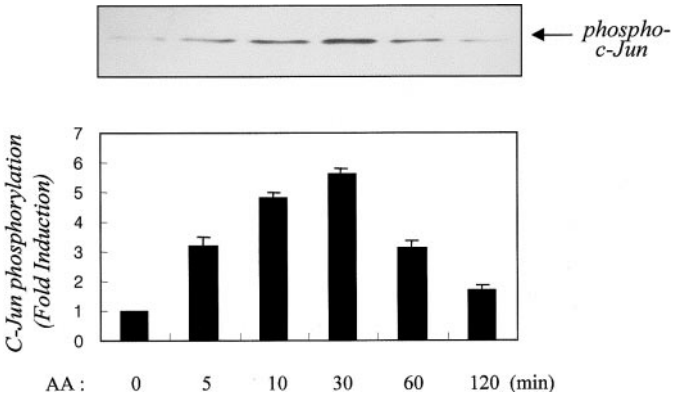

FIG. 3. Activation of JNK by exogenous AA. Rat-2 cells were serum-starved in serum-free DMEM for 18 h and then stimulated with AA (20 μM) for the indicated time periods (lane 1, 0 min, lane 2, 5 min; lane 3, 10 min; lane 4, 30 min; lane 5, 60 min; lane 6, 120 min). JNK activity was assessed using *c-Jun* fusion protein (1–89) as a substrate. Data are representative of three independent experiments and are expressed as fold increase with respect to control (0 min).

AA was as efficient as its stimulation by TNF-α (10 ng/ml) or C2-ceramide (5 μM) treatment of the same cells (data not shown).

Inhibition of cPLA$_2$ by AACOCF3 abrogates Rac-mediated JNK activation. Next, to further examine the role of cPLA$_2$ in Rac-mediated JNK activation, we determined whether RacV12-induced JNK activation is sensitive to pretreatment with arachidonyltrifluoromethyl ketone (AACOCF3), a specific inhibitor of cPLA$_2$. As shown in Fig. 4, pretreatment with 10 μM of AACOCF3 significantly inhibited RacV12-induced JNK activation without affecting AA-induced JNK stimulation (Fig. 4). In addition, pretreatment with AACOCF3 was shown to inhibit the activation of JNK in response to C2-ceramide (5 μM) of which intracellular signaling was previously reported to be mediated largely via Rac-dependent pathway (8, 17). Therefore, cPLA$_2$ appears to play a critical role in mediating JNK stimulation by Rac or agonists acting through Rac in the cell. This result is again well in accordance with the idea of cPLA$_2$ as a downstream mediator of Rac in the signaling cascade to JNK activation.

Exogenous AA stimulates JNK via 5-lipoxygenase-dependent manner. Our proposed mode of Rac activation of JNK via "cPLA$_2$-AA" cascade seems very similar to that of *c-fos* SRE activation (28). In *c-fos* SRE activation by Rac, an essential roles of cPLA$_2$ and subsequent AA production were already demonstrated (8, 28). Therefore, "cPLA$_2$-AA" signaling cascade appears to be commonly involved in the Rac-signaling pathways to either JNK or SRE regulation. Additionally, in previous study, we reported that lipoxygenase (LO), especially 5-lipoxygenase (5-LO), plays a critical role as a downstream mediator of "Rac-cPLA$_2$-AA" cascade to stimulate *c-fos* SRE (28). To determine whether "Rac-cPLA$_2$-AA"-signaling to JNK activation also involves the activity of 5-LO, we have tested the effects of MK886 (50 nM) or AA861 (1 μM), specific 5-LO inhibitors. As shown in Fig. 5, AA-induced JNK activation was dramatically inhibited by these inhibitors, while no inhibition was observed by indomethacin (10 μM), a cyclooxygenase inhibitor, or AACOCF3 (10 μM). Furthermore, the direct addition of leukotrienes $C_4/D_4/E_4$ mixture (200 nM), products of 5-LO metabolism of AA, induced a significant activation of JNK (3.2-fold) (Fig.

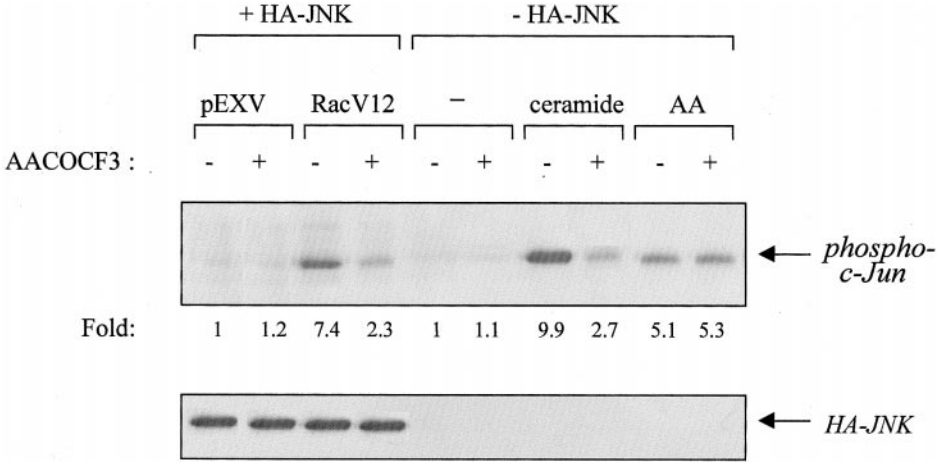

FIG. 4. AACOCF3 inhibits JNK activation induced by RacV12 or C2-ceramide. Rat-2 cells were transiently co-transfected with 5 μg of pEXV or pEXV-RacV12 together with 1 μg of pcDNA3-HA-JNK and 1 μg of pCMV-βGAL, followed by serum starvation. Then, AACOCF3 (10 μM) was treated for 6 h before the cell harvest for JNK assays. For determining the effect of AACOCF3 on ceramide- or AA-induced JNK activation, serum-starved Rat-2 cells were pre-treated with AACOCF3 (10 μM) for 20 min before the addition of C2-ceramide (5 μM) or AA (20 μM). After 30 min, cells were harvested for JNK assays. JNK activity was assessed using *c-Jun* fusion protein (1–89) as a substrate. Fold increase was calculated with respect to control (pEXV without mepacrine treatment) or control buffer (−) without AACOCF3 pretreatment. Levels of protein expression of HA-JNK are shown by immunoblots. Data are representative of three independent experiments.

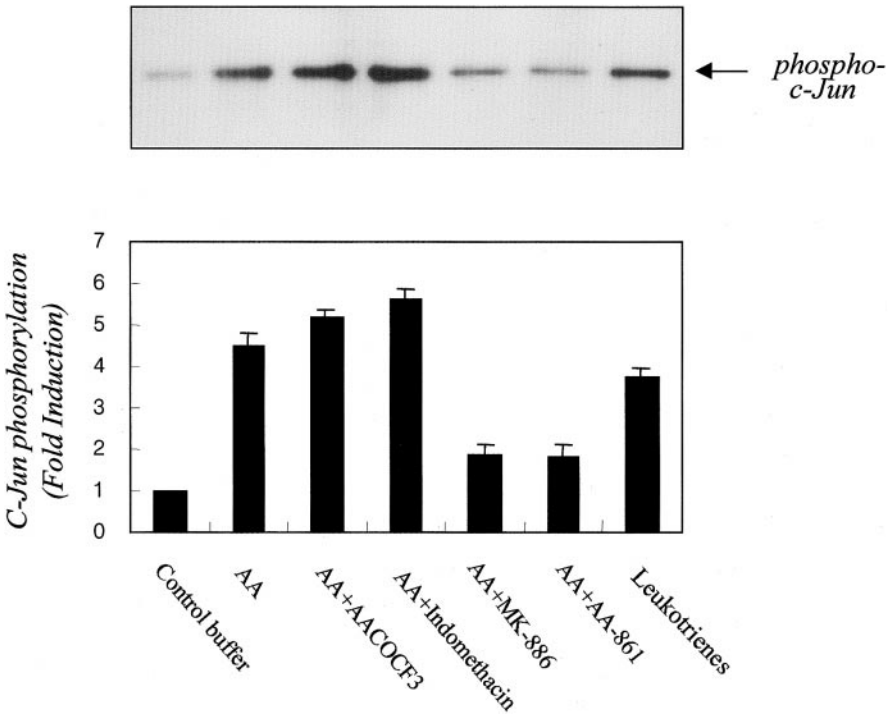

FIG. 5. Role of 5-LO in the JNK activation by exogenous AA. Serum-starved Rat-2 cells were pretreated with AACOCF3 (10 μM), indomethacin (Indo; 10 μM), AA861 (1 μM) or MK886 (50 nM) for 20 min before the addition of AA (20 μM). Separately, 200 nM of leukotrienes $C_4/D_4/E_4$ mixture (LTs; 200 nM mixture) was treated. After 30 min, cells were harvested for JNK assays. JNK activity was assessed using *c-Jun* fusion protein (1–89) as a substrate. Fold increase was calculated with respect to control (buffer treatment). Data are representative of three independent experiments.

5). These results suggest that 5-LO possibly mediates JNK activation downstream of "Rac-cPLA$_2$-AA" cascade. Together, our results indicate that cPLA$_2$ and subsequent AA release play important roles in mediating Rac signal to JNK stimulation.

DISCUSSION

Rac has been shown to mediate the activation of JNK signaling cascade (5–6), but the downstream effector molecules by which Rac mediates JNK activation remain largely unknown. Recently, several groups reported that Rac controls JNK activation, probably through a mechanism distinct from that involved in actin cytoskeleton organization (33–34). A serine/threonine kinase, p65 PAK, has been suggested to interact with the active forms of both Cdc42 and Rac (21–22), but not Rho, and possibly mediate Cdc42 or Rac activation of JNK (19–20, 24). However, the precise role of PAK in JNK activation still remains controversial. For example, an effector domain mutant of Rac that no longer interacts with PAK was identified (34) and surprisingly PAK binding was shown to be dispensable for the Rac-induced activation of JNK (34). Therefore, PAK is unlikely the downstream effector of Rac leading to JNK activation. Very recently, another novel target of Rac named MLK-3 was shown to lead to the activation of the JNK pathway (25), but the exact action mechanism remains to be characterized.

In an effort to broaden our understanding of the downstream mediators of Rac in the signaling cascade leading to JNK activation, we examined whether cPLA$_2$ is involved in the Rac activation of JNK. In the present study, we have shown that cPLA$_2$ activity is clearly necessary for the JNK activation by Rac, suggesting that Rac activate JNK, at least in part, via cPLA$_2$-dependent signaling pathway in Rat-2 fibroblast cells. Three types of experiments established the role of cPLA$_2$ in Rac-mediated JNK activation. First, we demonstrated that co-transfection with antisense cPLA$_2$ oligonucleotide blocks RacV12-induced JNK stimulation (Fig. 2). This effect is specific, as no inhibition was observed by control cPLA$_2$ oligonucleotide that contains the same sequence as the antisense cPLA$_2$ oligonucleotide except four mismatched. Second, we tested the effect of AACOCF3, a specific inhibitor of cPLA$_2$, on RacV12-induced JNK stimulation. As shown in Fig. 4, by pretreatment with AACOCF3, we observed a significant inhibition of the JNK stimulation in response to RacV12 or C2-ceramide which had been previously shown to require Rac for the nuclear signaling (8), thus suggesting a potential general mediatory role of cPLA$_2$ in the signaling pathways to JNK stimulation in response to various agonists acting through Rac.

Lastly, we demonstrated that exogenous addition of AA, a principal product of Rac-activated $cPLA_2$, caused a specific stimulation of JNK (Fig. 3), which is in agreement with the suggested action of $cPLA_2$ as a downstream mediator of Rac in the pathway leading to JNK stimulation. In this study, we also present evidence suggesting for the role of 5-lipoxygenase (5-LO) in JNK stimulation, possibly acting as a downstream of "Rac-$cPLA_2$-AA" cascade. Recently, Shin *et al.* (1999) reported that AA-induced JNK activation was partly prevented by RacN17, thus proposing that AA may act upstream of Rac for the JNK activation (35). Their results are quite in contrast to our and other previous reports (8, 28–29) and the reason for this discrepancy is not clear. The experimental conditions might be different or there could be a positive feedback loop regulation by AA acting on Rac, as claimed by the same authors (35). However, we predict that the role of feedback regulation would be, even if there is, minimal because we could not observe any severe inhibition on AA-induced JNK stimulation by RacN17 (data not shown). In any event, our present findings make us confident that $cPLA_2$ is essential for the JNK stimulation in response to Rac activation. In support of the role of "$cPLA_2$-AA" as a downstream cascade to mediate Rac activation of JNK, there is an increasing amount of evidence supporting the signaling-link between the JNK and $cPLA_2$-mediated AA metabolism (36–37). In summary, in the present study, we report on the critical role of $cPLA_2$ in the regulation of Rac-signaling to JNK activation. Although we do not know the detail downstream components of $cPLA_2$ in JNK signaling, we suspect that AA and subsequent production of leukotrienes by 5-LO are possibly involved in the JNK signaling downstream of Rac. Further studies aimed at the understanding the signaling link between $cPLA_2$ and JNK will lead to additional insights into the regulation of Rac-mediated signaling to JNK activation.

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REFERENCES

1. Hall, A. (1994) *Ann. Rev. Cell. Biol.* **10,** 31–54.
2. Nobes, C. D., and Hall, A. (1995) *Cell* **81,** 53–62.
3. Ridley, A. J., and Hall, A. (1992) *Cell* **70,** 389–399.
4. Ridley, A. J., Paterson, H. F., Johnston, C. L., Diekmann, D., and Hall, A. (1992) *Cell* **70,** 401–410.
5. Coso, O. A., Chiariello, M., Yu, J. C., Teramoto, H., Crespo, P., Xu, N., Miki, T., and Gutkind, J. S. (1995) *Cell* **81,** 1137–1146.
6. Minden, A., Lin, A., Claret, F. X., Abo, A., and Karin, M. (1995) *Cell* **81,** 1147–1157.
7. Hill, C. S., Wynne, J., and Treisman, R. (1995) *Cell* **81,** 1159–1170.
8. Kim, B. C., and Kim, J. H. (1998) *Biochem. J.* **330,** 1009–1014.
9. Kim, J. H., Kwack, H. J., Choi, S. E., Kim, B. C., Kim, Y. S., Kang, I. J., and Kumar, C. C. (1997) *FEBS Lett.* **406,** 93–96.
10. Chou, M. M., and Blenis, J. (1996). *Cell* **85,** 573–583.
11. Perona, R., Montaner, S., Saniger, L., Sanchez-Perez, I., Bravo, R., and Lacal, J. C. (1997) *Genes & Dev.* **11,** 463–475.
12. Chen, Y-R., Wang, X., Templeton, D., Davis, R. J., and Tan, T-H. (1996) *J. Biol. Chem.* **271,** 31929–31936.
13. Rosette, C., and Karin, M. (1996) *Science* **274,** 1194–1197.
14. Min, W., and Pober, J. A. (1997) *J. Immunol.* **159,** 3508–3518.
15. Natoli, G., Costanzo, A., Ianni, A., Templeton, D. J., Woodgett, J. R., Balsano, C., and Levrero, M. (1997) *Science* **275,** 200–203.
16. Reinhard, C., Shamoon, B., Shyamaia, V., and Williams, W. T. (1997) *EMBO J.* **16,** 1080–1092.
17. Brenner, B., Koppenhoefer, U., Weinstock, C., Linderkamp, O., Lang, F., and Gulbins, E. (1997) *J. Biol. Chem.* **272,** 22173–22181.
18. Gulbins, E., Coggeshall, K. M., Brenner, B., Schlottmann, K., Linderkamp, O., and Lang, F. (1996) *J. Biol. Chem.* **271,** 26389–26394.
19. Bagrodia, S., Taylor, S. J., Creasy, C. L., Chernoff, J., and Cerione, R. A. (1995) *J. Biol. Chem.* **270,** 22731–22737.
20. Brown, J., Stowers, L., Baer, M., Trejo, J., Coughlin, S., and Chant, J. (1996) *Curr. Biol.* **6,** 598–605.
21. Manser, E., Leung, T., Salihuddin, H., Zhao, Z-S., and Lim, L. (1994) *Nature* **367,** 40–46.
22. Martin, G. A., Bollag, G., McCormick, F., and Abo, A. (1995) *EMBO J.* **14,** 1970–1978.
23. Teramoto, H., Coso, O. A., Miyata, H., Igishi, T., Miki, T., and Gutkind, J.S. (1996) *J. Biol. Chem.* **271,** 27225–27228.
24. Zhang, S., Han, J., Sells, M. A., Chernoff, J., Knaus, U. G., Ulevitch, R. J., and Bokoch, G. M. (1995) *J. Biol. Chem.* **270,** 23934–23936.
26. Peppelenbosch, M. P., Tertoolen, L. G. J., Hertog, J. D., and de Laat, S. W. (1992) *Cell* **69,** 295–303.
27. Peppelenbosch, M. P., Tertoolen, L. G. J., Hage, W. J., and de Laat, S. W. (1993) *Cell* **74,** 565–575.
28. Kim, B. C., and Kim, J. H. (1997) *Biochem. J.* **326,** 333–337.
29. Peppelenbosch, M. P., Qiu, R. G., de Vries-Smits, A. M. M., Tertoolen, L. G. J., de Laat, S. W., McCormick, F., Hall, A., Symons, M. H., and Bos, J. L. (1995) *Cell* **81,** 849–856.
30. Hannun, Y. A. (1994) *J. Biol. Chem.* **269,** 3125–3128.
31. Verheij, M., Bose, R., Lin, X. H., Yao, B., Jarvis, W. D., Grant, S., Birrer, M. J., Szabo, E., Zon, L. I., Kyriakis, J. M., Haimovitz-Friedman, A., Fuks, Z., and Kolesnick, R. N. (1996) *Nature* **380,** 75–79.
32. Shim, J., Lee, H., Park, J., Kim, H., and Choi, E. J. (1996) *Nature* **381,** 804–806.
33. Lamarche, N., Tapon, N., Stowers, L., Burbelo, P. D., Aspenstrom, P., Bridges, T., Chant, J., and Hall, A. (1996) *Cell* **57,** 519–529.
34. Westiwick, J. K., Lambert, Q. T., Clark, G. L., Symons, M., Van Aelst, L., Pestell, R. G., and Der, C. J. (1997) *Mol. Cell. Biol.* **17,** 1324–1335.
35. Shin, E. A., Kim, K. H., Han, S. I., Ha, K. S., Kim, J. H., Kang, K. I., Kim, H. D., and King, H. S. (1999) *FEBS Lett.* **452,** 355–359.
36. Cui, X. L., and Douglas, J. G. (1997) *Proc. Natl. Acad. Sci.* **94,** 3771–3776.
37. Rizzo, M. T., and Carlo-Stella, C. (1996) *Blood* **88,** 3792–3800.