Roles of Phosphatidylinositol 3-Kinase and Rac in the Nuclear Signaling by Tumor Necrosis Factor-α in Rat-2 Fibroblasts*

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We investigated the extent to which phosphatidylinositol 3-kinase (PI 3-kinase) and Rac, a member of the Rho family of small GTPases, are involved in the signaling cascade triggered by tumor necrosis factor (TNF)-α leading to activation of c-fos serum response element (SRE) and c-Jun amino-terminal kinase (JNK) in Rat-2 fibroblasts. Inhibition of PI 3-kinase by LY294002 or wortmannin, two specific PI 3-kinase antagonists, or co-transfection with a dominant negative mutant of PI 3-kinase dose-dependently blocked stimulation of c-fos SRE by TNF-α. Similarly, LY294002 significantly diminished TNF-α-induced activation of JNK, suggesting that nuclear signaling triggered by TNF-α is dependent on PI 3-kinase-mediated activation of both c-fos SRE and JNK. We also found nuclear signaling by TNF-α to be Rac-dependent, as demonstrated by the inhibitory effect of transient co-transfection with a dominant negative Rac mutant, RacN17. Our findings suggest that Rac is situated downstream of PI 3-kinase in the TNF-α signaling pathway to the nucleus, and we conclude that PI 3-kinase and Rac each plays a pivotal role in the nuclear signaling cascade triggered by TNF-α.

Phosphatidylinositol 3-kinase (PI 3-kinase) is a lipid kinase involved in mitogenic signal transduction and cellular transformation. Evidence from intact cells suggests that PI 3-kinase is activated by a variety of growth factors and exerts its cellular effects by elevating phosphatidylinositol (3,4,5)-trisphosphate levels (1–3). In mammalian cells, PI 3-kinase is required for growth factor-induced changes of the actin cytoskeleton that are mediated by Rac, a member of the Rho family GTPases (2, 4, 5). For example, an inhibition of PI 3-kinase was shown to block growth factor induction of membrane ruffling, while activated PI 3-kinase is sufficient to induce membrane ruffling, acting through Rac (2, 4). Thus, Rac appears to lie downstream of PI 3-kinase within a signaling pathway that controls actin remodeling.

Rac is also crucially involved in the regulation of signal transduction cascades to the nucleus evoked by environmental stresses and proinflammatory cytokines; elements of such cascades include c-Jun amino-terminal kinase (JNK) (6, 7), c-fos serum response element (SRE) (8–10), p70S6 kinase (11), and the transcription factor NF-κB (12). For instance, in response to exogenous application of H2O2 or ceramide, a second messenger product of sphingomyelin hydrolysis by sphingomyelinasue, c-fos SRE, was activated via a Rac-dependent signaling pathway, suggesting a role of Rac in stress-induced gene regulation (9, 10). Although the role of PI 3-kinase in the regulation of Rac-mediated membrane ruffling has been well studied (2, 4, 5), almost nothing is known about the potential role of PI 3-kinase in Rac-mediated gene regulation in response to environmental stress or proinflammatory cytokines.

Tumor necrosis factor (TNF)-α is one of the most pleiotropic proinflammatory cytokines, signaling a large number of cellular responses, including cytotoxicity, antiviral activity, fibroblast proliferation, and the transcriptional regulation of various genes (14). It is known that a large majority of the pleiotropic activities of TNF are signaled by the TNF receptor-1 (TNFR1; Refs. 15–17). TNF engagement of TNFR1 leads to the recruitment of TNFR1-associated death domain protein, receptor-interacting protein, and TNFR-associated factor-2 (TRAF2) leads to the formation of a receptor complex (18–20) within which receptor-interacting protein and TRAF2, respectively, transduce signals required for TNF-mediated activation of NF-κB (21) and JNK (22–24). Nonetheless, little is known about the intracellular signaling mediating activation of nuclear transcription factors. In particular, the roles of PI 3-kinase and Rac in the nuclear signaling by TNF-α are as yet unclear. In the present study, we investigated the extent to which PI 3-kinase and Rac are involved in the TNF-α-induced activation of c-fos SRE and JNK. Our findings suggest that both PI 3-kinase and Rac have crucial functions within the intracellular signaling cascade triggered by TNF-α in Rat-2 fibroblasts.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—Lyposphatidic acid (LPA), mespacerin, and wortmannin (25), a PI 3-kinase antagonist, were purchased from Sigma. LY294002, another PI 3-kinase antagonist, and C6-ceramide were purchased from BioMol (Plymouth Meeting, PA). TNF-α was either purchased from Sigma or was obtained as a gift from Dr. D.-M. Jue (Catholic University Medical College, Seoul, Korea). Fetal bovine serum (FBS), gentamycin, and Dulbecco’s modified Eagle’s medium (DMEM) were purchased from Life Technologies, Inc. Control (GcTGCTCCATTAGTTTCATAsT) and antisense (GcTgcTGCTAAGAGATCAAAAsT) cytosolic phospholipase A2 (cPLA2) oligonucleotides were purchased from BioMol. The antisense oligonucleotide is directed against codons 4–9 of human cytosolic, Ca2+-dependent PLA2. Note that the linkages are

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† The abbreviations used are: PI 3-kinase, phosphatidylinositol 3-kinase; TNF-α, tumor necrosis factor-α; JNK, c-Jun amino-terminal kinase; PLA2, phospholipase A2; cPLA2, cytosolic phospholipase A2; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; SRE, serum response element; PBS-T, PBS plus Tween 20; TNFR1, tumor necrosis factor receptor-1; TRAF2, TNFR-associated factor-2; FBS, fetal bovine serum.
phosphothioated at both the 5' and 3' ends (lowercase "s" in sequences). All other chemicals were from standard sources and were molecular biology grade or higher.

**Plasmids**—Reporter gene pSRE-Luc contains c-fos SRE oligonucleotide sequences (23-mer) inserted at the −53 position of a truncated base promoter region of 100 bp of pC台湾v, pEVTX-RacV12 (RacVal12), and pEVTX-RhoV14 (RhoValA14) plasmids were gifts from Dr. A. Hall (University College, London, United Kingdom). All Rac and Rho proteins were expressed as NH2-terminally 9E10 epitope-tagged derivatives driven by SV40 promoter (26, 27). pSG5-p53ASHis2-N (widely referred to as pSG5-3p53), which encodes a dominant negative mutant of p53, a regulator of PI 3-kinase (2), was a gift from Dr. J. Downward (Imperial Cancer Research Fund, London, United Kingdom). Amino acids 478–513 are deleted in the mutant, which, consequently, lacks the binding site for the catalytic subunit.

**Cell Culture, Transfections, and Luciferase Assay**—Rat-2 fibroblasts were obtained from the American Type Culture Collection (ATCC, CRL 1764). The cells were grown in DMEM supplemented with 0.1 mM nonessential amino acids (Life Technologies, Inc.), 10% FBS, 50 units/ml penicillin, and 50 μg/ml streptomycin at 37 °C under a humidified 95%, 5% (v/v) mixture of air and CO2. The Rat2-RacN17 stable clone expressing a dominant negative Rac inhibitor, RacN17, has been described previously (28).

Transient transfection was carried out by plating approximately 5×105-1×106 dishes for 24 h and then adding 30 μg of phosphatidylinositol (20 μg/ml) sonicated in 10 mM Tris-HCl (pH 7.5) containing 1 mM EDTA. After adding 10 μl of ATP solution (10 mM) containing 10 μM of [γ-32P]ATP, the immunoprecipitates were incubated for 20 min at room temperature with constant shaking. The reaction was stopped by addition of 100 μM of EDTA and 200 μM of CHC13-methanol (1:1). The samples were then centrifuged, and the lower organic phases were harvested and applied to silica gel TLC plates (Merck Co.) coated with 1% potassium oxalate. The TLC plates were developed in CHCl3/CH3OH-H2O-NH4OH (60:47:11:3.2), dried, and visualized autoradiographically.

**c-fos SRE-Activated Protein Kinase Assays**—To assay JNK activity mediated by TNF-α or C2-erase, subconfluent Rat-2 cells were serum-starved for 24 h in DMEM containing 0.5% FBS and then stimulated with TNF-α or C2-erase for 30 min. Each dish of cells was then washed with cold PBS, lysed by incubation for 5 min at 4 °C in 0.5 ml of 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 Na3VO4, 1 μg/ml leupeptin) with 1 mM phenylmethanesulfonyl fluoride, scraped into Eppendorf tubes, and triturated by 10 passes through a 21-gauge needle on ice. The supernatant (cell lysate) was harvested by microcentrifugation at 14,000 rpm for 10 min. Protein concentrations were equalized by normalizing them to the protein levels (assayed by Bradford procedure with Bio-Rad dye reagent) measured before the JNK assay.

**RESULTS**

c-fos SRE Is One of the Nuclear Target Sequences of TNF-α—As an initial approach to understanding the role of PI 3-kinase in the signal transduction pathway between TNF-α and the nucleus, we assessed the capability of TNF-α to stimulate c-fos SRE, which is a primary nuclear target for various extracellular signals (9–11, 30). To accomplish this, Rat-2 cells were transiently transfected with reporter plasmid pSRE-Luc (5 μg) containing c-fos SRE oligonucleotides inserted upstream of the c-fos minimal promoter fused to luciferase coding sequences (9). TNF-α-induced SRE activation was monitored by measuring luciferase activities normalized to co-transfected reporter gene (pSRE-Luc) and 5 μg of JNK activity was determined using a JNK assay kit according to the manufacturer’s protocol (New England Biolabs). Brieﬂy, an aminoterminal c-Jun (amino acid residues 1–99) fusion protein bound to glutathione-Sepharose beads was used to pull down JNK from cell lysates. The kinase reaction (50 μl) was then carried out using the c-Jun fusion protein as a substrate in the presence of cold ATP. Phosphorylation of the c-Jun fusion protein at Ser-63 was measured by Western blot using an anti-phospho-c-Jun rabbit polyclonal antibody that detects only catalytically activated c-Jun phosphorylated at Ser-63. Protein samples were heated to 95 °C for 5 min and subjected to SDS-polyacrylamide gel electrophoresis on 5% acrylamide gels, followed by transfer to polyvinylidene difluoride membranes for 2 h at 100 V using a Novex wet transfer unit. Membranes were then blocked overnight in PBS-T (PBS containing 0.1% (v/v) Tween 20) with 5% (v/v) nonfat dried milk, after which they were incubated for 2 h with primary antibody (anti-phospho-c-Jun) in PBS-T and then for 1 h with horse-radish 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 phospho-c-Jun were measured by densitometry.

**JNK/Stress-activated Protein Kinase Assays**—To determine the role of PI 3-kinase in the phosphorylation of c-Jun, which, consequently, lacks the binding site for the catalytic subunit.

**PI 3-Kinase Activity Is Essential for TNF-α Signaling to c-fos SRE**—To assess the role of PI 3-kinase, we examined the effects of PI 3-kinase antagonists, LY294002 (Fig. 2A) and wortmannin (Fig. 2B) on TNF-α-induced c-fos SRE activation. Both compounds dose-dependently inhibited TNF-α-evoked SRE luciferase activity at levels that selectively inhibit PI 3-kinase activity (31, 32). As examples, 25 μM LY294002 reduced TNF-α-evoked SRE luciferase activity by ~40%, while 100 nM—
mannin reduced the activity by 50%. C2-Ceramide-induced SRE activation was similarly attenuated by PI 3-kinase inhibition (Fig. 2, A and B). Further, co-transfection with pSG5-Δp85 encoding a dominant negative PI 3-kinase mutant significantly and dose-dependently diminished TNF-α-induced stimulation of SRE-luciferase activity (Fig. 2C). Of the quantities tested, co-transfection with 5 μg of pSG5-Δp85 reduced TNF-α-induced stimulation of SRE-luciferase activity by 75%, whereas lysophosphatidic acid (LPA)-induced SRE activation was little affected. Taken together, these results are strongly suggestive of the participation of PI 3-kinase in TNF-α signaling to c-fos SRE.

Encouraged by above results, we next directly assayed TNF-α-evoked PI 3-kinase activity by measuring the levels of the product, phosphatidylinositol phosphate, in serum-starved Rat-2 cells exposed to TNF-α for 10 min (Fig. 3). Consistent with the above results, addition of TNF-α-stimulated PI 3-kinase activity significantly and dose-dependently diminished TNF-α-induced stimulation of SRE-luciferase activity by 75%, whereas lysophosphatidic acid (LPA)-induced SRE activation was little affected. Taken together, these results are strongly suggestive of the participation of PI 3-kinase in TNF-α signaling to c-fos SRE.

Essential Role of Rac in the Nuclear Signaling by TNF-α—PI 3-kinase activity induces repertoire of Rac-mediated responses
inhibited by co-transfection with 5 μg of pEXV-RacN17 (~65% reduction in luciferase activity), suggesting that Rac activity is crucial for TNF-α-induced signaling to c-fos SRE. On the other hand, SRE activation induced by 10 μM LPA was unaffected by pEXV-RacN17 transfection.

The role of Rac was further investigated by comparing the SRE-luciferase activities in Rat-2 and Rat2-RacN17 cells. Fig. 4B shows TNF-α-induced SRE activation was inhibited by 50% in serum-starved Rat2-RacN17 cells. In contrast, levels of LPA-induced SRE activation were similar in Rat-2 and Rat2-RacN17 cells (Fig. 4B), while epidermal growth factor-evoked activation of SRE was reduced somewhat in Rat2-RacN17 cells. We, therefore, conclude that TNF-α signaling to c-fos SRE is mediated, at least in part, by a Rac-dependent cascade.

**Pretreatment with LY294002 Inhibits JNK Activation by TNF-α**—The effect of LY294002 on TNF-α-induced JNK activation was assessed to determine the extent to which it is dependent on PI 3-kinase and Rac activities. Serum-starved Rat-2 cells were pretreated with LY294002 (+) or control buffer (−) for 30 min before adding TNF-α (10 ng/ml), C2-ceramide (5 μM), or arachidonic acid (AA; 100 μM), a principal product of Rac-activated phospholipase A2 (33). TNF-α and C2-ceramide each induced a ~5-fold increase of JNK activity as compared with control buffer, an effect that was dramatically inhibited by LY294002 (Fig. 5A). On the other hand, LY294002 had no inhibitory effect on AA-induced JNK activation, which suggests that PI 3-kinase is specifically required for activation of JNK by TNF-α or C2-ceramide and implies a common, essential role for PI 3-kinase in TNF-α-induced activation of both JNK and c-fos SRE.

To determine the function of Rac in TNF-α signaling to JNK, levels of JNK activation were compared between control cells and cells stably expressing RacN17. As shown in Fig. 5B, TNF-α- and C2-ceramide-induced JNK activation was dramatically reduced in Rat2-RacN17 cells, indicating the importance of Rac activity in those cases. On the other hand, JNK activation induced by 100 μM AA was unaffected by RacN17 expression.

The signaling hierarchy between PI 3-kinase and Rac was investigated further by assessing the LY294002 sensitivity of SRE activation by RacV12, a constitutively activated form of Rac1. LY294002 had no inhibitory effect on SRE activation by RacV12 or RhoV14 (Fig. 6), whereas RasV12-induced SRE activation was significantly and dose-dependently inhibited by LY294002. This is consistent with previous reports showing that PI 3-kinase acts as a downstream mediator of H-Ras within the signaling cascades leading to actin remodeling and transformation (2, 3), and is further evidence that Rac is situated downstream of PI 3-kinase in the nuclear signaling cascade leading to activation of c-fos SRE or JNK. In a separate
Role of cPLA₂ in TNF-α Signaling to SRE Activation—We previously reported that cytosolic phospholipase A₂ (cPLA₂) plays an essential role in mediating Rac signaling to c-fos SRE and thus acts as an important downstream mediator of Rac (34). Considering the linkage between TNF-α and Rac signaling, it seems reasonable to hypothesize that cPLA₂ may be involved in TNF-α signaling to SRE. To test this possibility, we assessed the extent to which mepacrine, a potent PLA₂ inhibitor, inhibited TNF-α-induced activation of SRE. Fig. 7A shows that pretreatment with 1 μM mepacrine inhibited TNF-α-induced SRE activation by approximately 50% without affecting LPA-induced activation, suggesting PLA₂ is specifically required for TNF-α signaling to c-fos SRE.

To further analyze the role of PLA₂ in TNF-α signaling, especially that of cPLA₂, we examined the effect of transfecting cells with antisense cPLA₂ oligonucleotide on TNF-α-induced SRE activation. Co-transfection with the antisense oligonucleotide but not the control oligonucleotide significantly inhibited TNF-α-induced SRE activation (Fig. 7B). For example, cotransfection with 0.5 μM cPLA₂ antisense oligomer reduced SRE activation by ~45%, which suggests that a Rac-cPLA₂-linked cascade is involved in TNF-α signaling to c-fos SRE. In contrast, LPA-induced SRE activation was unaffected by transfection of the antisense oligonucleotide, suggesting that the involvement of cPLA₂ is specific to TNF-α-induced signaling to c-fos SRE.

DISCUSSION

In the present study, we provide evidence supporting novel roles for PI 3-kinase and Rac in the nuclear signaling cascade triggered by TNF-α in Rat-2 fibroblasts. TNF-α was previously reported to rapidly induce protooncogene c-fos in the adipogenic 3T3-L1 cell line, although the exact target promoter sequences by which TNF-α stimulates c-fos transcription remain unknown (35). Our results clearly indicate that SRE is at least one of the nuclear target sequences by which TNF-α stimulates c-fos expression. Consistent with this conclusion, c-fos SRE is also reported to be a nuclear target of ceramide, a putative second messenger for certain stresses (e.g. ultraviolet and X-rays) and inflammatory cytokines such as TNF-α (9). In addition, our results suggest a role for cPLA₂ that is in good agreement with the earlier report of Haliday et al. (35) showing that AA and its lipoxygenase-generated metabolite are downstream elements in the TNF-α signaling pathway to c-fos. The function of AA as a downstream mediator of TNF-α signaling was also demonstrated in stromal cells, where AA mediates TNF-α-induced activation of JNK (36).

The involvement of PI 3-kinase in TNF-α-induced signaling to c-fos SRE was confirmed by the significant inhibitory effects of LT294003 and wortmannin, specific PI 3-kinase antagonists, and of transient transfection with pSG5-Dp85 encoding a dominant negative PI 3-kinase mutant. Consistent with this conclusion, JNK activation by TNF-α was dramatically inhibited by LY294002, implying PI 3-kinase functions broadly as a downstream TNF-α mediator in the signaling pathways leading to SRE and JNK activation. That TNF-α stimulates PI 3-kinase activity in vitro lends additional support to this idea.

![Fig. 6. RacV12-induced SRE activation is not inhibited by LY294002.](image)

![Fig. 7. cPLA₂ activity is involved in TNF-α-induced c-fos SRE activation.](image)
We do not yet know the TNF-α target molecule(s) that mediates PI 3-kinase activation; nonetheless, since the mode of action of C2-terminus is quite similar to that of TNF-α, especially with respect to inhibition by LY294002, we postulate that enhanced production of ceramide might be involved. On the other hand, although further characterization is needed for confirmation, our evidence suggests the role of TRAF2 in the TNF-α signaling to SRE or JNK is minimal. For example, a dominant negative mutant of TRAF2 does not inhibit activation of either JNK or SRE in cells exposed to TNF-α (data not shown). This finding is in contrast to previous reports (22, 24) in which TRAF2 was shown to be essential for TNF-α-induced JNK activation in lymphocytes, suggesting the function of TRAF2 differs in Rat-2 fibroblasts and lymphocytes. In any event, our present findings make us confident that PI 3-kinase is essential for mediating the nuclear signaling cascades triggered by TNF-α and ceramide, which is consistent with increasing evidence indicating that PI 3-kinase is activated by environmental stresses and growth factors (37–40).

We also found evidence for the role of Rac in TNF-α signaling to the nucleus, which is consistent with earlier findings demonstrating an essential role of Rac in the nuclear signaling by C2-terminus, cytokines and environmental stresses (6, 7, 9). Thus, the present study shows that TNF-α stimulates c-fos SRE and JNK vi a signaling cascade involving PI 3-kinase and Rac. Although precise determination of the mechanisms of action of PI 3-kinase and Rac will require further study, we postulate a hierarchical relationship among these proteins (TNF-α → PI 3-kinase → Rac), whereby Rac serves as a PI 3-kinase downstream molecule in a TNF-α-triggered nuclear signaling pathway. Future studies elucidating the linkage between PI 3-kinase and Rac will likely be pivotal to a complete understanding of TNF-α-evoked intracellular signaling.

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