C3 Toxin Activates the Stress Signaling Pathways, JNK and p38, but Antagonizes the Activation of AP-1 in Rat-1 Cells*

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Lysophosphatidic acid (LPA) stimulates the c-Fos serum response element (SRE) by activating two distinct signal pathways regulated by the small GTPases, Ras and RhoA. Ras activates the ERK cascade leading to phosphorylation of the transcription factors Elk-1 and Sap1a at the Ets/TCF site. RhoA regulates an undefined pathway required for the activation of the SRF/CARG site. Here we have examined the role of the Ras and RhoA pathways in activation of the SRE and c-Fos expression in Rat-1 cells. Pertussis toxin and PD98059 strongly inhibited LPA-stimulated c-Fos expression and activation of a SRE:Luc reporter. C3 toxin completely inhibited RhoA function, partially inhibited SRE:Luc activity, but had no effect on LPA-stimulated c-Fos expression. Thus, in a physiological context the Ras-Raf-MEK-ERK pathway, but not RhoA, is required for LPA-stimulated c-Fos expression in Rat-1 cells. C3 toxin stimulated the stress-activated protein kinases JNK and p38 and potentiated c-Jun expression and phosphorylation; these properties were shared by another cellular stress agonist the protein kinase C inhibitor Ro-31-8220. These data indicate that C3 toxin is a stress agonist the protein kinase C inhibitor Ro-31-8220. These data indicate that C3 toxin is a stress agonist the protein kinase C inhibitor Ro-31-8220. These data indicate that C3 toxin is a cellular stress which antagonizes activation of AP-1 at a point downstream of stress-activated kinase activation or immediate-early gene induction.

The AP-1 transcription factor complex coordinates cellular responses to growth and stress stimuli. AP-1 consists of dimers of Fos and Jun proteins (1); there are four fos genes and three jun genes which are expressed with different kinetics in response to stimulation (2, 3). Both c-Fos and c-Jun can form dimers with ATF/cAMP responsive element-binding protein transcription factors and c-Jun can also form homodimers (4–6) making AP-1 a versatile and dynamic transcriptional complex able to respond to a variety of different extracellular stimuli.

c-Fos expression serves as a paradigm for understanding how signals generated by growth factors are transduced to the nucleus to regulate gene expression (7, 8). The c-Fos promoter contains several enhancer elements including a cyclic AMP responsive element (9–12), a c-sis-inducible element (13), a serum response element (SRE) (14, 15), and an AP-1 site located immediately 3′ to the SRE (8). A direct signal pathway can be traced to the SRE which consists of adjacent binding sites for the Ets/TCF family of transcription factors (Elk-1 and Sap1a) and the serum response factor (SRF) (7); mutations in either site impairs responsiveness to serum and growth factors (16, 17). Growth factors such as lysophosphatidic acid (LPA) or epidermal growth factor (EGF) activate the Ras-dependent Raf-MEK-ERK kinase cascade (18–20). When activated, ERK accumulates in the nucleus, binds Elk-1 which is pre-bound to the Ets site, and forms a ternary complex with SRF. ERK phosphorylates and activates Elk-1 (21–25) thereby stimulating c-Fos expression.

Optimal expression of c-Fos requires the binding of SRF to the CARG/RF site adjacent to the Ets site in the SRE (24). SRF responds to growth factor signaling pathways and plays a vital role in mediating the effects of serum and LPA on c-Fos expression (16). Although the precise details of this pathway are unknown, it is thought that the Ras-related GTPase RhoA promotes TCF-independent SRF-dependent c-Fos expression in response to serum or LPA (16). Certainly a role for RhoA in LPA-stimulated signaling is well established. ADP-ribosylation of RhoA at Asn42, catalyzed by the Clostridium botulinum C3 toxin (25) converts endogenous RhoA into a dominant inhibitory form which is able to block LPA-stimulated stress fiber formation in several cell types (26, 27). In addition, C3 toxin prevents the rapid LPA-stimulated retraction of neurites in neuronal cells (28). Similarly, using isolated SRE or SRF sequences fused to a heterologous reporter gene in transient transfection assays, LPA-stimulated activation of the SRE is inhibited by C3 toxin in transient transfection assays while a mutationally activated RhoA (Val14Glu) stimulates the SRE in the absence of LPA or serum (16). Although these studies have focused attention on the SRE, it is unclear how well such transiently expressed reporters reflect the properties of the SRE in its physiological context (7, 8). For example, more recently it has been shown that Rho is not required for activation of a stably integrated c-Fos reporter construct (29).

In this study we have used biochemical inhibitors to examine the role of the Ras- and RhoA-regulated pathways in regulating the endogenous c-fos gene in LPA-stimulated Rat-1 cells. Se-
elective inhibition of the Ras-dependent ERK cascade inhibits LPA-stimulated activation of the SRE and c-Fos protein expression. In contrast, C3 toxin partially antagonizes RhoA-dependent signaling to the isolated SRE but has no effect on LPA-stimulated c-Fos expression. We also demonstrate that C3 toxin stimulates the SAPK family of protein kinases and induces phosphorylation of c-Jun at Ser 73 but actually inhibits AP-1 transcriptional activity. Our results show that LPA-stimulated c-Fos expression proceeds in the absence of functional RhoA and further indicate that C3 toxin, frequently used as a selective inhibitor of RhoA function in cells, exerts a variety of previously undescribed effects on pathways regulating gene expression.

EXPERIMENTAL PROCEDURES

Materials—All cell culture reagents were from Irvine Scientific. [32P]ATP was from NEN Life Science Products Inc. LPA was from Avanti Polar Lipids. EGF and protease inhibitors were from Boehringer-Mannheim. Ro-31-8220 was from LC Laboratories. Precast SDS-PAGE gels were from Novex Gel Systems. The GST-c-Jun fusion protein was obtained from Hibi et al. (30). The GST-C3 toxin-expressing plasmid was obtained from Marc Symons (ONXY Pharmaceuticals). The activity of recombinant GST-PDZ3 was from Test Pig. Gene Pulser cuvettes (0.4 cm electrode gap) were from Bio-Rad. All other reagents including myelin basic protein, protein A-Sepharose, and FITC-phalloidin were from Sigma.

Cell Culture, Stimulation, and Lysates—Rat-1 fibroblasts were maintained at 37 °C in an atmosphere of 5% CO 2 in DMEM supplemented with 0.6 mg/ml glutamine, 100 units/ml penicillin and streptomycin, and 10% fetal bovine serum. Rat-1 cells stably expressing either a SRE-Luc or AP-1/TRE-Luc were maintained in medium containing 2 μg/ml puromycin. Serum starvation, cell stimulation, and preparation of whole cell detergent lysates was performed as described previously (18, 37, 45, 50).

Immune Complex Kinase Assays—Equal quantities of cell lysate were used for ERK and JNK immunoprecipitation with 40 μl of protein A-Sepharose (30% slurry) and 3 μl of crude E1.2 serum (18), or 4 μl of anti-JNK1 serum (Santa Cruz Biotechnology). Immunoprecipitations and assay of ERK or JNK was as described previously (18, 37, 45, 50).

Western Blots—Samples to be blotted were electrophoretically transferred to Immobilon-P transfer membranes (Millipore) and developed using ECL detection reagents (Amer sham Life Science). Membranes were blocked with PBS containing 5% (w/v) nonfat milk and 0.1% (v/v) Tween 20. Primary antibody incubations were with anti-c-Jun antipeptide antibody (1:1000) from David Gillespie, Beatson CRC Laboratories (31); anti-c-Fos (1:250) and anti-RhoA (1:500) from Santa Cruz Biotechnology; and with anti-phospho-Tyr182-specific p38 (1:500), anti-phospho-Ser 9-specific c-Jun (1:500), and anti-phospho-Thr 9-specific ATF-2 (1:250) from New England BioLabs.

Electroporation—Rat-1 fibroblasts were trypanosmied and washed two times in electroporation buffer (20 mM Hepes, pH 7.05, 137 mM NaCl, 5 mM MgCl 2, 0.7 mM Na 2HPO 4, 6 mM dextrose) and resuspended at 2 × 10 5 cells/ml in electroporation buffer. 250 μl of cell suspension was placed in a Bio-Rad Gene-Pulser cuvette in the absence (mock) or presence (C3) of the indicated concentration of purified c-Jun toxin. Cells were then electroporated at 250 kV and 125 microfarads. Electroporated cells were left on ice for 10 min and then replated in fresh DMEM supplemented with 0.5% fetal bovine serum at 37 °C for 6 to 50 h prior to the application of external stimuli. Cells were stimulated by adding growth factors and other agents directly to the serum-free media. Cell lysis and luciferase assays were performed using the Tropix dual light system according to the manufacturer’s instructions. All luciferase values were normalized to total cellular protein as determined by Bradford analysis.

RESULTS

Inhibition of the Ras-dependent ERK Cascade Inhibits LPA-stimulated c-Fos Expression Rat-1 Cells—Rat-1 cells stably expressing the c-fos SRE-Luc reporter were serum starved and pretreated with either pertussis toxin or PD98059, a specific inhibitor of MEK1 (38), which completely inhibits ERK activation by either LPA or EGF in Rat-1 cells (39). The cells were then stimulated with LPA or EGF for 15 h and assayed for luciferase activity (Fig. 1A). The LPA response was strongly inhibited by both pertussis toxin and PD98059 whereas the EGF response was inhibited by PD98059 but not pertussis toxin. These results are consistent with LPA and EGF acting via the Ras-dependent ERK pathway to activate the c-fos SRE; in the case of LPA this pathway is regulated via a pertussis toxin-sensitive G protein (18–20, 36, 37).

Next we examined the effects of pertussis toxin and PD98059 on expression of the c-fos protein, an assay of the full-length native promoter. Serum-starved Rat-1 cells were pretreated with pertussis toxin or control buffer for 18 h and then stimulated with LPA. After 1 h whole cell detergent lysates were analyzed for the expression of c-Fos (Fig. 1B). LPA stimulated a strong increase in c-Fos expression and the c-Fos protein was also extensively phosphorylated as previously observed (40, 41). Pertussis toxin strongly inhibited both LPA-stimulated c-Fos expression and hyperphosphorylation. In addition, pretreatment with PD98059 resulted in a strong inhibition of c-Fos expression and phosphorylation in response to LPA, EGF, or the protein kinase C activator phorbol 12-myristate 13-acetate (Fig. 1F). Taken together these results indicate that the Ras-dependent ERK cascade is a major pathway required for LPA- and EGF-stimulated c-Fos expression.

Inactivation of RhoA Function by C3 Toxin in Permeabilized Rat-1 Cells—The importance of RhoA in c-fos expression appears to be dependent upon whether the reporter construct used is transiently, episomally expressed, or stably integrated (16, 29). To overcome this problem we investigated the role of RhoA in expression of the native c-fos gene by monitoring c-Fos
Regulation of AP-1 Activity by C3 Toxin

The ERK cascade is required for LPA stimulated c-Fos expression in Rat-1 cells. A, Rat-1 cells stably expressing a c-Fos SRE:Luc reporter construct were pretreated with 100 ng/ml pertussis toxin (PTx) or vehicle (control) for 12 h or with 40 μg PD98059 (PD) before stimulating with 50 μM LPA or 50 nM EGF for 16 h. Cell lysates were prepared and assayed for luciferase activity and values were normalized to total cellular protein. Data are shown from a single experiment in duplicate. B, wild type Rat-1 cells were pretreated with 100 ng/ml pertussis toxin for 12 h, 40 μg PD98059 for 30 min, or with the appropriate vehicle controls (C). Cells were then stimulated with 50 μM LPA, 50 nM EGF, or 50 nM phorbol 12-myristate 13-acetate (PMA) for 45 min. Detergent lysates were resolved by SDS-PAGE and immunoblotted for c-Fos. This blot is from a single experiment representative of three giving identical results.

protein expression. Such an approach requires ablation of all RhoA function in the cell population; however, interfering mutants of RhoA often only allow partial inhibition of RhoA function and this problem is compounded by the efficiency of transfection protocols. We elected to use the C3 ADP-ribosyl transferase exoenzyme of C. botulinum to ADP-ribosylate and functionally inactivate RhoA in situ thereby converting it to a dominant interfering form (26, 27, 32–34).

Rat-1 cells were electro-permeabilized in the presence of increasing doses of C3 toxin (0.03 ng ml−1 to 30 μg ml−1), repeated in media containing 0.5% serum and analyzed 8 h later for RhoA expression by immunoblotting. C3 toxin treatment caused the apparent molecular mass of RhoA to shift to a reduced mobility form which was apparent in all experiments (Fig. 2A, upper panel, and Fig. 3A) and is consistent with the covalent attachment of the ADP-ribose group (32–34). In addition, the quantity of immune reactive RhoA was severely reduced by C3 toxin treatment; this effect was apparent after 6–8 h and proceeded such that RhoA was barely detectable after 18 h. This apparent down-regulation of RhoA is consistent with previous reports (42).

To confirm that the reduced mobility of RhoA in C3 toxin-treated cells was due to ADP-ribosylation of the protein we performed an in vitro ADP-ribosylation reaction using recombinant purified C3 toxin and samples from control or C3 toxin-treated Rat-1 cells (32–34). Control reactions using recombinant purified RhoA and Ki-Ras proteins confirmed the specificity of C3 toxin for RhoA under our reaction conditions. Pretreatment in situ with increasing concentrations of C3 toxin resulted in a saturable and dose-dependent decrease in the subsequent incorporation of radioactive ADP-ribose into RhoA in vitro (Fig. 2A, lower panel) which matched the dose dependence of the mobility shift assay. The complete shift of RhoA to the lower mobility form suggested that ribosylation of endogenous RhoA was complete at 0.3–3 μg ml−1 C3 toxin. These effects were noted when the C3 toxin was added to whole cell monolayers over a 24-h period but under these conditions only 50–60% of the RhoA was ribosylated even at the highest concentrations used.2

Finally, we examined the state of actin stress fibers (26, 27) in C3 toxin-treated Rat-1 cells. After permeabilization Rat-1 cells were replated on coverslips in complete medium and 6 h later fixed and stained with phalloidin. In the control permeabilized cells abundant stress fibers were apparent (Fig. 2B) and likely reflect the fact that these cells are growing in 10% fresh serum which contains LPA. In C3 toxin-treated cells, stress fibers were completely absent (Fig. 2B). Based on these results we conclude that permeabilization with C3 toxin allows complete ADP-ribosylation and functional inactivation of all the endogenous RhoA in Rat-1 cells.

C3 Toxin Does Not Block c-Fos Protein Expression in Rat-1 Cells—We examined the role of RhoA in regulating the endogenous c-Fos promoter by examining c-Fos protein levels in C3 toxin-treated cells. Rat-1 cells maintained in 0.5% serum for 24 h were permeabilized in the presence or absence of 1 μg ml−1 C3 toxin (control and C3). These cells were replated and maintained in medium containing 0.5% serum for 4 h to allow reattachment; the cells were then serum starved for 4 h before stimulating with LPA. Immunoblotting confirmed that all the RhoA was ADP-ribosylated and therefore functionally inactivated under these conditions (Fig. 3A). LPA-stimulated c-Fos expression proceeded normally in the mock permeabilized cells indicating that the permeabilization protocol itself did not compromise the cells ability to respond to agonist (Fig. 3B). Remarkably, the ability of LPA to stimulate c-Fos expression was not affected by permeabilization in the presence of C3 toxin even though all the RhoA was inactivated and much of the RhoA had disappeared from these cells (Fig. 3A and B). In all these experiments RhoA mobility and immune reactivity were assayed in parallel to confirm the efficiency of the C3 toxin treatment and an aliquot of cells from each permeabilization reaction was plated on coverslips and used to assess the presence or absence of actin stress fibers. In all cases complete inactivation of RhoA was confirmed.

In the course of these experiments we also examined the effect of C3 toxin on the isolated SRE in Rat-1 cells stably expressing a SRE:Luc reporter and found that C3 toxin caused a partial inhibition of both LPA- or EGF-stimulated SRE:Luc activity (60%) (Fig. 3D). In addition, we were able to demonstrate activation of this same reporter by RhoA-V14 in transient assays.3 These results suggest that in response to LPA, RhoA is required to maximally stimulate the isolated SRE but not the endogenous full-length c-Fos promoter whereas the Ras-dependent ERK cascade is required for both the stimulation of the isolated SRE and c-Fos expression in Rat-1 cells.

Ras and RhoA are proposed to activate parallel, independent pathways which cooperate to regulate the c-Fos SRE (16). As the ERK cascade contributes significantly to c-Fos expression and activation of the SRE (Fig. 1) it was crucial to establish that C3 toxin was inhibiting RhoA function without inhibiting the coupling of LPA to the ERK cascade. We found that C3 toxin treatment did not affect the ability of LPA to activate either ERK1 or ERK2 and that ERK1 and ERK2 protein levels

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2 S. J. Cook, unpublished observations.

3 G. A. Martin, unpublished observations.
were not affected by C3 toxin treatment (Fig. 3C and data not shown). This is a notable difference between our work and previous studies which have shown that C3 toxin inhibits LPA stimulated ERK activation by 50% in Swiss 3T3 cells and 80–90% in NIH3T3 cells (16, 43).

C3 Toxin Stimulates the Expression of c-Jun and Its Phosphorylation at Ser^73 in Rat-1 Cells—In the course of these studies we also examined the effect of C3 toxin on c-Jun expression. We found that LPA stimulated only a modest increase in c-Jun levels in control permeabilized cells but that this response was amplified and more sustained in cells which had been treated with C3 toxin (Fig. 4A). Furthermore, if cells were
weak increase in JNK1 activity observed in response to LPA in Rat-1 cells were electro-permeabilized in the absence or presence of 1 µg/ml C3 toxin, replated in DMEM + 0.5% FBS for 4 h and then maintained in serum-free DMEM for 4 h before stimulating with 50 µM LPA for the times indicated. Whole cell detergent lysates were analyzed by immunoblotting for (A) c-Jun expression and (B) phosphorylation of c-Jun at Ser73. In A some cells were permeabilized in the absence or presence of C3 toxin and received no subsequent LPA stimulation as indicated. C, Rat-1 cells were electro-permeabilized in the presence of increasing concentrations of C3 toxin as indicated and replated in medium containing 0.5% FBS. After 8 h cells were harvested and whole cell lysates resolved by SDS-PAGE. Samples were then immunoblotted with a c-Jun Phospho-Ser73-specific antibody.

left unstimulated by LPA the introduction of C3 toxin alone was sufficient to induce the expression of c-Jun (Fig. 4A). Control permeabilized cells did not exhibit an elevation of c-Jun levels.

The c-Jun promoter includes AP-1 sites which allow autoregulation of c-Jun levels by pre-existing c-Jun and ATF-2 (1, 5, 44). Such autoregulation requires that c-Jun be phosphorylated at Ser63 and Ser73 in its transactivation domain. In order to determine if c-Jun was phosphorylated at these sites we performed immunoblots with a phospho-Ser73-specific antibody. The experiments revealed that LPA caused little or no increase in c-Jun Ser73 phosphorylation (Fig. 4B) consistent with the weak increase in JNK1 activity observed in response to LPA in Rat-1 cells (45). However, stimulation of C3 toxin-treated cells with LPA now resulted in a strong and sustained phosphorylation of c-Jun at Ser73 (Fig. 4B). Further experiments revealed that C3 toxin alone was able to promote a strong phosphorylation of c-Jun at Ser73 in a dose-dependent manner even in the absence of a growth factor stimulus (Fig. 4C). The EC50 for C3 toxin-stimulated increase in c-Jun Ser73 phosphorylation was between 30 and 300 ng ml-1; the same as that required for half-maximal ADP-ribosylation of Rho (Fig. 2).

C3 Toxin Stimulates the Stress-activated Protein Kinase JNK1—In addition to the ERK cascade there are at least two other well described proline-directed kinase signaling cassettes in eukaryotic cells. These are known collectively as the stress-activated kinases (SAPKs). SAPKs are composed of two families, the c-Jun N-terminal kinases (JNKs) (46) and the p38 or RK kinase (47). JNKs and p38 are activated in response to a variety of extracellular insults (reviewed in Ref. 47) and proinflammatory cytokines resulting in the phosphorylation of c-Jun at Ser63 and Ser73 and ATF-2 at Thr69 and Thr71 (46–49). These sites lie within the transactivation domains of c-Jun and ATF-2 and their phosphorylation serves as an activating event.

Because C3 toxin alone was able to induce c-Jun expression and phosphorylation, we examined what effect C3 had on JNK activity. Permeabilization of Rat-1 cells in the presence of C3 toxin resulted in a time- and dose-dependent increase in JNK1 activity (Fig. 5). JNK1 activity was elevated at the first time point tested after permeabilization (6 h) and persisted above the corresponding control level for at least 30 h (Fig. 5A) and 50 h. Control permeabilized Rat-1 cells did not exhibit any elevation of JNK activity indicating that it was not the act of permeabilization but the presence of C3 toxin which resulted in JNK activation (Fig. 5A). Half-maximal activation of JNK1 was also observed at doses between 10 and 100 ng ml-1 C3 toxin (Fig. 5B), the same as that required for phosphorylation of c-Jun at Ser73. In the same experiments C3 toxin did not activate ERK.

C3 Toxin and Ro-31-8220 Activate p38/RK but Only Ro-31-8220 Induces ATF-2 Phosphorylation—We previously demonstrated that the protein kinase C inhibitor Ro-31-8220 is a

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4 J. Beltman and S. J. Cook, unpublished observations.

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FIG. 4. C3 toxin stimulates c-Jun expression and phosphorylation and amplifies the response to LPA. Rat-1 cells were electro-permeabilized in the absence or presence of 1 µg/ml C3 toxin, replated in DMEM + 0.5% FBS for 4 h and then maintained in serum-free DMEM for 4 h before stimulating with 50 µM LPA for the times indicated. Whole cell detergent lysates were analyzed by immunoblotting for (A) c-Jun expression and (B) phosphorylation of c-Jun at Ser73. In A some cells were permeabilized in the absence or presence of C3 toxin and received no subsequent LPA stimulation as indicated. C, Rat-1 cells were electro-permeabilized in the presence of increasing concentrations of C3 toxin as indicated and replated in medium containing 0.5% FBS. After 8 h cells were harvested and whole cell lysates resolved by SDS-PAGE. Samples were then immunoblotted with a c-Jun Phospho-Ser73-specific antibody.

FIG. 5. Activation of JNK1 by C3 toxin in electro-permeabilized Rat-1 cells. A, Rat-1 cells were electro-permeabilized in the absence (control) or presence (C3 tox) of 1 µg/ml C3 toxin and replated in DMEM + 0.5% FBS. At the indicated times cells were harvested and assayed for JNK1 activity by an immune complex kinase assay using GST-c-Jun as a substrate. Data are expressed as the mean of duplicate time points from a single experiment representative of five giving similar results. B, Rat-1 cells were electro-permeabilized in the presence of increasing concentrations of C3 toxin as indicated and replated in medium containing 0.5% FBS. After 8 h cells were harvested and JNK1 or ERK1 assayed in an immune complex kinase assay using GST-c-Jun or myelin basic protein as substrate, respectively. Data are from a single experiment representative of two giving similar results.
Regulation of AP-1 Activity by C3 Toxin

**FIG. 6.** Differential effects of C3 toxin and Ro-31-8220 on Jun and ATF-2 phosphorylation. A, serum-starved Rat-1 cells were stimulated with 10 μM Ro-31-8220 for the indicated times and whole cell detergent lysates were analyzed by immunoblotting for c-Jun phosphorylation using the phospho-Ser73-specific antibody (upper panel), a phospho-Tyr182-p38 antibody (center panel), or a phospho-Thr71-ATF-2 antibody (lower panel). B, Rat-1 cells were electro-permeabilized in the absence or presence of C3 toxin and replated in DMEM at 0.5% FBS for 4 h and then serum-free DMEM for 4 h. Cells were then stimulated with 50 μM LPA for the indicated times and harvested. Whole cell detergent lysates were analyzed by immunoblotting for phospho-Tyr182-p38 (upper panel) or phospho-Thr71-ATF-2 (lower panel). C, two separate experiments are shown. In the left panel Rat-1 cells were electro-permeabilized in the absence (Con) or presence (C3) of 1 μg/ml C3 toxin, replated in DMEM + 0.5% FBS for 4 h, and then maintained in serum-free DMEM for 4 h. Some cells were then stimulated with 10 μM Ro-31-8220 as indicated. In the right panel Rat-1 cells were permeabilized in the absence or presence of 1 μg/ml C3 toxin, replated in DMEM + 0.5% FBS, and maintained for 8 h. In both cases detergent lysates were immunoblotted for phospho-ATF-2 (upper panel) or with a nondiscriminating ATF-2 antibody as a control (lower panel).

potent activator of the stress-activated kinase JNK1 (50). The pattern of post-translationally modified c-Jun observed with Ro-31-8220 was remarkably similar to that described here for C3 toxin. Immunoblotting with a phospho-Ser73-specific antibody confirmed that Ro-31-8220 did indeed stimulate the phosphorylation of c-Jun at Ser73 (Fig. 6A) and this was temporally consistent with the activation of JNK1 (50).

Many stresses also induce the activation of p38 concomitant with JNK activation. p38 is implicated as a kinase responsible for phosphorylation and activation of ATF-2 (47–49). We assessed p38 activation using an anti-Tyr182 phospho-specific antibody since phosphorylation at this site is required for p38 activity. Immunoblotting with the phospho-Tyr182-specific p38 antibody confirmed that Ro-31-8220 did induce the activation of p38. This was accompanied by phosphorylation of ATF-2 at Thr71 (Fig. 6A). Indeed, activation of JNK (50) and p38 and phosphorylation of c-Jun and ATF-2 proceeded with virtually identical kinetics in response to Ro-31-8220 (Fig. 6A).

In addition to JNK activation, C3 toxin was also able to amplify the weak and transient activation of p38 observed in response to LPA (Fig. 6B) and induce the activation of p38 in the absence of a growth factor stimulus. However, in contrast to Ro-31-8220, C3 toxin had little or no effect on ATF-2 phosphorylation despite inducing robust activation of p38 (Fig. 6, B and C). ATF-2 phosphorylation was weakly induced in LPA-stimulated C3 toxin-treated cells but this was a late event which did not correlate with p38 activation (Fig. 6B).

When mock permeabilized Rat-1 cells were treated with Ro-31-8220, immunoreactivity against phospho-Thr71 was observed but when the same cells were treated with C3 toxin alone only a weak and barely detectable response was observed (Fig. 6C). The inability of C3 toxin to promote ATF-2 phosphorylation was due to a deleterious effect of the toxin which somehow obstructed ATF-2 phosphorylation since Ro-31-8220 could still promote phosphorylation of ATF-2 in C3 toxin-treated cells (Fig. 6C). In conclusion, both C3 toxin and Ro-31-8220 strongly activate p38 but only in the case of Ro-31-8220 was this accompanied by a reproducible increase in phosphorylation of ATF-2 at Thr71. This suggests that phosphorylation of ATF-2 at Thr71 cannot be considered a reliable marker of p38 activation.

A Comparison of the Effects of C3 Toxin and Ro-31-8220 on AP-1:Luc Activity in Rat-1 Cells—We have previously shown that Ro-31-8220 inhibits c-Fos expression in Rat-1 cells (50), whereas C3 toxin does not (Fig. 3). We were interested in relating the differential effects of Ro-31-8220 and C3 toxin on c-Fos expression (50) and c-Jun and ATF-2 phosphorylation to their effects on AP-1 transcriptional activity. For these experiments we used four different clonal Rat-1 cell lines stably expressing an AP-1:Luc reporter gene. All four lines gave similar results and data from a single line are presented here. The growth factors, EGF and LPA, elicited a modest 2–3-fold activation of AP-1:Luc (Fig. 7A). Treatment with Ro-31-8220 resulted in a 5-fold increase in AP-1:Luc activity whereas permeabilization of C3 toxin into Rat-IAP-1:Luc cells had no significant effect upon AP-1:Luc activity (Fig. 7A). Strikingly, treatment with a combination of Ro-31-8220 and EGF or LPA resulted in strong synergistic potentiation of AP-1:Luc activity. No such synergy was observed when EGF or LPA was combined with C3 toxin. Thus, Ro-31-8220 but not C3 toxin, is able to stimulate AP-1-dependent transcription and synergize with growth factors in this response.

Finally, we compared the effect of C3 toxin on the synergy we observed between growth factors and Ro-31-8220 for induction of AP-1:Luc activity. Rat-IAP-1:Luc cells were permeabilized in the absence or presence of C3 toxin, replated, and subsequently stimulated with growth factors alone or in combination with Ro-31-8220. The results, shown in Fig. 7B, revealed that C3 toxin treatment inhibited by approximately 60% the synergy between Ro-31-8220 and growth factors and to a lesser extent the response to Ro-31-8220 alone.

**DISCUSSION**

LPA-stimulated c-Fos Expression Requires the Ras-regulated ERK Cascade but Not a RhoA-dependent Pathway in Rat-1 Cells—The role of RhoA in LPA-stimulated c-Fos expression was first defined using transiently expressed reporter constructs (16) but subsequent studies using stably integrated
Regulation of AP-1 Activity by C3 Toxin

Our results suggest that the Ras pathway is required for LPA-stimulated c-Fos expression (16, 51, 52). Our results confirm previous studies in as much as there is a C3 toxin-inhibitable, RhoA-dependent pathway to the SRE but it appears that in Rat-1 cells there are either alternative RhoA-independent pathways to the SRF site or other enhancer elements in the c-Fos promoter (16). The Ras and RhoA pathways are proposed to act cooperatively at the TCF and SRF sites of the c-Fos SRE but such a model is difficult to test or interpret in NIH3T3 cells where C3 toxin inhibits both of these pathways. In Rat-1 cells C3 toxin blocks RhoA function without inhibiting ERK activation and when we inhibit each pathway independently of the other, we find that the Ras-ERK cascade, but not the RhoA cascade, is required for LPA-stimulated c-Fos expression.

These results do not imply that the SRF site is not required for optimal c-Fos expression induced by LPA or serum (16, 51, 52). Our results confirm previous studies in as much as there is a C3 toxin-inhibitable, RhoA-dependent pathway to the SRE but it appears that in Rat-1 cells there are either alternative RhoA-independent pathways to the SRF site or other enhancer elements in the c-Fos promoter (16). The Ras and RhoA pathways are proposed to act cooperatively at the TCF and SRF sites of the c-Fos SRE but such a model is difficult to test or interpret in NIH3T3 cells where C3 toxin inhibits both of these pathways. In Rat-1 cells C3 toxin blocks RhoA function without inhibiting ERK activation and when we inhibit each pathway independently of the other, we find that the Ras-ERK cascade, but not the RhoA cascade, is required for LPA-stimulated c-Fos expression.

The role of RhoA in regulating c-Fos expression appears to be distinct in different cell types since RhoA was not required for expression of the native c-fos gene in Rat-1 cells whereas C3 toxin completely inhibited LPA-stimulated c-Fos expression, as monitored by RNase protection, in NIH3T3 cells (16). In considering this disparity the role of RhoA in regulating ERK appears to be important. In Rat-1 cells, C3 toxin did not impair or enhance LPA-stimulated ERK activation indicating that RhoA is not required for this response. This is in striking contrast to the studies in NIH3T3 cells where the ability of LPA to stimulate ERK activity was inhibited by at least 80% by C3 toxin (16). The Ras and RhoA pathways are proposed to act cooperatively at the TCF and SRF sites of the c-Fos SRE but such a model is difficult to test or interpret in NIH3T3 cells where C3 toxin inhibits both of these pathways. In Rat-1 cells C3 toxin blocks RhoA function without inhibiting ERK activation and when we inhibit each pathway independently of the other, we find that the Ras-ERK cascade, but not the RhoA cascade, is required for LPA-stimulated c-Fos expression.

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Our results suggest that the Ras pathway is required for LPA-stimulated c-Fos expression since pertussis toxin and PD98059, which prevent ERK activation by LPA (36–39), strongly inhibit activation of the c-fos SRE and the expression of c-Fos protein. By the same criteria RhoA appears to play a minor or redundant role in LPA-stimulated c-Fos expression. Although we observed that RhoA is required for maximal activation of the isolated c-fos SRE by LPA, as reported by others (16), C3 toxin treatment exerted no effect on the ability of LPA to stimulate c-Fos protein expression from the endogenous promoter despite complete functional inactivation of RhoA.

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FIG. 7. Ro-31-8220, but not C3 toxin, activates an AP-1-Luc reporter in Rat-1 cells. A, Rat-1 cells stably expressing an AP-1-Luc reporter construct were permeabilized in the presence of vehicle (control) or 1 μg/ml C3 toxin and then replated in DMEM + 0.5% FBS and maintained for 8 h or stimulated with 10 μM Ro-31-8220 for 30 min and then were stimulated with 50 μM LPA or 50 μM EGF for 16 h. Cell lysates were prepared, assayed for luciferase activity, and values were normalized to total cellular protein. Data are shown from a single experiment in duplicate and are typical of three to six additional experiments in three different clones of Rat-1 AP-1:Luc cells giving similar results. B, the experiment was repeated as in A except that the effect of growth factors and/or Ro-31-8220 was also examined in C3-treated cells. Note that while C3 toxin does not impair c-Fos (Fig. 3) or c-Jun (Fig. 4) expression or block Ro-31-8220-stimulated ATF-2 phosphorylation it inhibits the synergy between growth factors and Ro-31-8220 for activation of AP-1-Luc.
of both growth (ERK) and stress (JNK and p38) signaling pathways is required for the optimal activation of AP-1 transcriptional activity. Second, while both C3 toxin and Ro-31-8220 caused phosphorylation and activation of p38, only in the case of Ro-31-8220 was this accompanied by significant phosphorylation of ATF-2 at Thr71. We had intended to use ATF-2 phosphorylation as a marker of p38 activation but our results suggest that ATF-2 may not be the relevant substrate for p38 in all cases. Finally, we previously showed that Ro-31-8220 completely inhibited LPA-stimulated c-Fos expression (50) whereas C3 toxin has no effect on LPA-stimulated c-Fos expression (50). Hence, the inhibitory effect of C3 toxin seems to be downstream of SAPK and promoting c-Jun phosphorylation. Furthermore, c-Jun:ATF-2 dimers can also bind ATF/CAMP responsive element sites (which are similar to AP-1 sites) and this is repressed by c-Fos (6).

The inability of C3 toxin to cooperate with growth factors in stimulating AP-1:Luc may relate to C3 toxin exerting an additional inhibitory effect on AP-1 activation which is independent or downstream of the early biochemical events studied here. For example, C3 toxin inhibited the synergy between growth factors and Ro-31-8220 for activation of AP-1 despite activating the SAPKs and promoting c-Jun phosphorylation. Furthermore, while C3 toxin did not induce ATF-2 phosphorylation it did not block the ability of Ro-31-8220 to do so. Thus the inhibitory effect of C3 toxin seems to be downstream of SAPK or ERK activation and the phosphorylation of immediate-early transcription factors. The AP-1 family is not confined to c-Fos, c-Jun, and ATF-2; indeed, studies indicate that from 6 to 8 h onwards after growth factor stimulation c-Fos is no longer expressed and the major AP-1 proteins include Fra-1, Fra-2, c-Jun, and JunB (2,3,5) Since the AP-1:Luc assay entails a prolonged stimulation time it is possible that C3 may exert some effect on these other AP-1 components and future studies will aim to address this.

Alternatively, the inhibition of AP-1 by C3 toxin may reflect a more nonspecific effect. There is evidence to suggest that the cytoskeleton may influence gene expression by coordinating the spatial distribution of transcription factors and other signaling elements in the cell (60). It is possible that the profound disruption of the actin cytoskeleton by C3 toxin (26, 32, 54) may undermine this spatial arrangement leading to a nonspecific inhibitory effect on gene expression. The ability of C3 toxin to inhibit activation of the SRE by LPA is consistent with a wealth of data demonstrating that RhoA plays a role in LPA signaling (16, 26, 28, 43). The partial inhibition of the EGF response is more difficult to rationalize as there is less evidence of a role for RhoA in EGF signaling. The ability of C3 toxin to inhibit activation of the c-Fos SRF site by LPA, platelet-derived growth factor, 12-O-tetradecanoylphorbol-13-acetate, hyperosmolarity, arsenite, and Ras is consistent with RhoA playing a vital role as a signal transducer in the diverse pathways used by these stimuli in signaling to c-Fos (16). However, these observations are equally consistent with C3 toxin exerting a more general nonspecific effect. Our observation that C3 toxin activates JNK and p38 but antagonizes AP-1 activation suggests that future studies employing C3 toxin will require careful interpretation to ensure that the results are due to loss of RhoA function rather than activation of the SAPKs.

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