

Divergent Roles for Ras and Rap in the Activation of p38 Mitogen-activated Protein Kinase by Interleukin-1*

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We have found that lethal toxin from *Clostridium sordellii*, which specifically inactivates the low molecular weight G proteins Ras, Rap, and Rac, inhibits the activation of p38 mitogen-activated protein kinase (MAPK) by interleukin-1 (IL-1) in EL4.NOB-1 cells and primary fibroblasts. The target protein involved appeared to be Ras, because transient transfections with dominant negative RasN17 inhibited p38 MAPK activation by IL-1. Furthermore, transfections of cells with constitutively active RasVHa-activated p38 MAPK. Further evidence for Ras involvement came from the observation that IL-1 caused a rapid activation of Ras in the cells and from the inhibitory effects of the Ras inhibitors manumycin A and damnacanthal. Toxin B from *Clostridium difficile*, which inactivates Rac, Cdc42, and Rho, was without effect. Dominant negative versions of Rac (RacN17) or Rap (Rap1AN17) did not inhibit the response. Intriguingly, transfection of cells with dominant negative Rap1AN17 activated p38 MAPK. Furthermore, constitutively active Rap1AV12 inhibited p38 MAPK activation by IL-1, consistent with Rap antagonizing Ras function. IL-1 also activated Rap in the cells, but with slower kinetics than Ras. Our studies therefore provide clear evidence using multiple approaches for Ras as a signaling component in the activation of p38 MAPK by IL-1, with Rap having an inhibitory effect.

Interleukin-1 (IL-1)¹ is a potent proinflammatory cytokine that increases the expression of a wide variety of genes important for immunity and inflammation in target cells. The signaling pathways by which IL-1 mediates its effects have been the focus of much attention. IL-1 activates four protein kinase cascades in cells. The best characterized of these culminates in the activation of the transcription factor NF- κ B (reviewed in Ref. 1). The other three are mitogen-activated protein kinase (MAPK) cascades involving the stress-activated kinases p38 MAPK and c-Jun N-terminal kinase (JNK) and the classical MAPK cascade involving p42/p44 MAPK (2–4).

Early events involved in the activation of these pathways have been uncovered (reviewed in Ref. 1). IL-1 elicits its effects by binding to its Type I IL-1 receptor, which in a complex with

the IL-1 receptor accessory protein recruits the adapter protein MyD88. This in turn recruits IL-1 receptor-associated kinases 1 and 2. From there, the best characterized pathway—the one that leads to NF- κ B—involves the adapter TRAF-6, two additional kinases (TAK-1 and TAB-1 (5)), NF- κ B-inducing kinase, and the I κ B kinase complex. For the MAPK cascades, details are less clear. IL-1 has been shown to activate Raf-1 (6) and MKK-1 (7), which would lead to p42/p44 MAPK activation. The upstream kinases responsible for the activation of JNK by IL-1 are MKK7 (8) and MKK4 (9), whereas for p38 MAPK, MKK3 (10), MKK4 (9), and MKK6 (11) play this role. Transgenic studies have indicated that IL-1 receptor-associated kinase 1 (12) and MyD88 (13) are required for p38 and/or JNK activation, indicating that these proteins may be the means by which these cascades are triggered, although a direct link between any of these proteins and upstream kinases in these pathways has not been demonstrated.

A role for low molecular weight G proteins has also been explored, particularly in the activation of JNK and p38 MAPK. Both Rac and Cdc42 have been implicated, through the use of dominant negative mutants, in both pathways (14, 15), although a role for Rac in JNK activation has recently been disputed (16).

In this study, we have sought to investigate further the role of small G proteins in the activation of p38 MAPK by IL-1. We have used three distinct approaches: (i) treatment of cells with *Clostridium sordellii* lethal toxin (LT) and *Clostridium difficile* toxin B (ToxB) (virulence factors that specifically glucosylate and inhibit the small G proteins Ras, Rac, and Rap, or Cdc42, Rac, and Rho, respectively (17–20)); (ii) treatment of cells with two Ras inhibitors, manumycin A and damnacanthal, and finally (iii) transient transfection of cells with plasmids encoding mutant versions of Ras, Rac, and Rap. Our data strongly indicate a role for Ras in the activation of p38 MAPK by IL-1, with Rap having an antagonistic effect.

EXPERIMENTAL PROCEDURES

Materials—*C. sordellii* LT was obtained from culture supernatants of the pathogenic *C. sordellii* IP82 strain and purified as described previously (17). Toxin B was purified from *C. difficile* as described previously (21). The human recombinant IL-1 α was a kind gift from the NCI, National Institutes of Health, Biological Resources Branch (Rockville, MD). PhosphoPlus® p38 MAPK (Thr-180/Tyr-182) antibody kit was obtained from New England Biolabs Ltd. (Hitchin, United Kingdom). UDP-[¹⁴C]glucose in ethanol (300 mCi/mmol) was purchased from NEN Life Science Products. The mouse monoclonal antibody to human I κ B α recognizes an epitope between amino acids 21 and 48 (22) and was a kind gift from Dr. R. T. Hay (University of St. Andrews, Fife, United Kingdom). The antibody used for detecting apopain/ CPP32/pro-caspase 3, p12 subunit was supplied by Upstate Biotechnology (Lake Placid, NY). The components for the PathDetect® CHOP trans-reporting system (pFA-CHOP, pFC2-dbd, pFR-Luc, and pFC-MEK3) were purchased from Stratagene. The pyridinyl imidazole SB203580 was obtained from Alexis Corp. (Nottingham, United Kingdom). The expression vectors encoding constitutively active RasVHa, dominant negative RasN17 (de-

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¹ The abbreviations used are: IL-1, interleukin-1; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; LT, *C. sordellii* lethal toxin; ToxB, *C. difficile* toxin B; RBD, Ras binding domain; TBS, Tris-buffered saline; GST, glutathione S-transferase.

scribed previously (23)), constitutively active RacV12, and dominant negative RacN17 (described in Ref. 24), together with the expression vectors encoding amino acids 1–149 of human c-Raf1 in pGex-KG, *i.e.* glutathione *S*-transferase (GST)-Ras binding domain (RBD) (25) and pGex-4T3-GST-RalGDS-RBD (26), were all kind gifts from Dr. Doreen Cantrell (Imperial Cancer Research Fund, London, United Kingdom). Manumycin A and damnacanthal were purchased from Sigma and Calbiochem, respectively. The pan-Ras antibody is a product of Oncogene Research Products (Cambridge, MA), and the polyclonal anti-Rap1A antibody was a kind gift from Dr. Jean de Gunzburg (Institut Curie, Paris, France). The pRK5 expression vector encoding constitutively active Rap1AV12 and dominant negative Rap1AN17 were a gift from Dr. Jean de Gunzburg (Institut Curie).

Cell Culture—The murine thymoma cell line EL4.NOB-1 was obtained from the European Collection of Animal Cell Cultures (Wiltshire, United Kingdom) and maintained in RPMI 1640 medium supplemented with 100 IU/ml penicillin, 100 IU/ml streptomycin, and 10% fetal calf serum. Chinese hamster Don diploid lung fibroblasts and the LT-resistant mutant cell line (Don Cdt^R-Q) derived from these cells (27) were cultured in minimal essential medium with Earle's salt supplemented with 100 IU/ml penicillin, 100 IU/ml streptomycin, and 10% fetal calf serum. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO₂. For use in Western blotting, 8 × 10⁶ EL4.NOB-1 or 2 × 10⁶ Don fibroblast cells in 4 ml of complete medium were incubated with medium alone or with the indicated doses of inhibitors for 1–4 h. Following this, the cells were stimulated with 10 ng/ml IL-1α for 10 min. For use in transfection assays, transfected EL4.NOB-1 cells were seeded at 2 × 10⁶ cells per ml and pretreated as indicated in the figure legends to Figs. 4–8.

Immunoblotting—Western blotting was performed essentially according to the method of Laemmli (28). Briefly, whole cell lysates were generated using a buffer consisting of 62.5 mM Tris-HCl (pH 6.8), 2% w/v SDS, 10% glycerol, 50 mM dithiothreitol, and 0.1% w/v bromophenol blue. Equal amounts of lysates were subjected to 10–15% SDS-polyacrylamide gel electrophoresis and then transferred onto nitrocellulose membranes (Sigma) in transfer buffer (25 mM Tris-HCl, pH 8.5, 0.2 M glycine, 20% methanol). Membranes were washed in Tris-buffered saline (TBS)/0.1% Tween (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% Tween-20). The membranes were blocked at room temperature for 2–3 h in 5% fat-free dry milk in TBS/0.1% Tween. The anti-IκB primary antibody was used at a dilution of 1:200 in 1% fat free dry milk in TBS/0.1% Tween. The antibodies recognizing dually phosphorylated (Thr-180/Tyr-182) p38 MAPK, anti-p38 MAPK, anti-CPP32, or anti-Rap1A were each used at a dilution of 1:1000 in 1% fat free dry milk in TBS/0.1% Tween. The pan-Ras antibody was used at a dilution of 1:20 in 1% fat-free dry milk in TBS/0.1% Tween. The antibody-antigen complexes were detected using a horseradish peroxidase-coupled anti-rabbit or a horseradish peroxidase-linked anti-mouse antibody, each used at a dilution of 1:2000 in 5% fat-free dry milk in TBS/0.1% Tween. The secondary antibody was subsequently detected using a kit for enhanced chemiluminescence substrate development (New England BioLabs Ltd).

Glucosylation of Extracts from EL4.NOB-1 and Don Cells—In order to assess the degree of glucosylation by LT in intact cells, 1 × 10⁷ cells (in 4 ml of RPMI 1640 medium/10% fetal calf serum) were incubated with medium alone or with LT at 500 ng/ml for various times or at the doses indicated in figure legends for 4 h. After washing the cells in phosphate-buffered saline, cell extracts were prepared as described previously (18). In brief, the cells were lysed by three cycles of freeze-thawing in 50 μl of 50 mM triethanolamine, 100 μM dithiothreitol, 10 μg/ml leupeptin. The extent to which glucosylation had occurred in intact cells was then measured by incubating 20 μl of the cell extracts, normalized for protein (as determined by the method of Bradford), with 2 μg/ml LT or ToxB together with 20 μl of dried ¹⁴C-labeled UDP-glucose (300 mCi/mmol). After 1 h of incubation at 37 °C, 5 μl of sample buffer was added, and the cell extracts were subjected to 15% SDS-polyacrylamide gel electrophoresis (28) and blotted onto nitrocellulose membranes in transfer buffer (25 mM Tris-HCl, pH 8.5, 0.2 M glycine, 20% methanol). In order to enhance the signal from the incorporated ¹⁴C-labeled UDP-glucose, the membranes were dipped in 20% 2,5-diphenylloxazole in toluene (w/v), dried, and exposed to radiographic film to detect the radiolabeled proteins. The degree of glucosylation in intact cells will be inversely proportional to the intensity of the bands obtained because there will be less target for LT to glucosylate *in vitro* if glucosylation has occurred in intact cells.

Transfection of EL4.NOB-1 Cells and Protocol for the GALA-CHOP¹⁻¹⁰¹ Assay—Cells (1.4 × 10⁷) were harvested (in exponential growth phase) and resuspended in a final volume of 1.2 ml of Tris-buffered

saline (25 mM Tris (pH 7.4), 137 mM NaCl, 0.7 mM CaCl₂, 0.5 mM MgCl₂, 0.6 mM NaH₂PO₄) containing 10–20 μg of DNA (5 μg of pFA-CHOP, 5 μg of pFR-Luc, and 2.5–10 μg of G protein mutant expression vector), 250 μg/ml DEAE-dextran, and 40 μg/ml chloroquine as described previously (29). Following incubation for 30 min at 37 °C, the cells were washed twice in complete RPMI 1640 medium and resuspended in 40 ml of RPMI/20% (v/v) fetal calf serum medium. After a recovery period of 16–24 h, the cells were harvested, seeded at 1 × 10⁶ cells/ml, and, when required for the experiment, incubated with medium alone or the indicated inhibitor for 1–4 h. Following this, the cells were washed and stimulated with IL-1α (10 ng/ml) for 4–6 h. Luciferase activity was measured in cell lysates, prepared using Passive lysis buffer (Promega Corp., Madison, WI) diluted 1:5, and samples were normalized for protein, determined by the method of Bradford (30). Luciferase activity of cell extracts were determined using standard procedures. In order to normalize for transfection efficiency, we co-transfected cells with a plasmid encoding β-galactosidase. In all samples tested, the levels of β-galactosidase expressed was negligible, and as a consequence, no normalization was performed. Cells were batch-transfected and aliquoted, and all results obtained were highly consistent with little between-experiment variation. Furthermore, all effects (including those that were inhibiting) were expressed relative to control values, with little effect being evident on the reporter system in control cells.

Activation Assay for Ras/Rap—Ras and Rap1A activation assays using Raf-1-RBD and RalGDS-RBD, respectively, have been described elsewhere (25, 26, 31). In brief, pGEX-KG-Raf-RBD-GST and pGEX-4T3-Ral-GDS-GST were induced with isopropyl-1-thio-β-D-galactopyranoside, and the bacteria were sonicated for 5 min (pulses of 5 s) in phosphate-buffered saline containing 0.2 mg/ml lysozyme, 1 mM phenylmethylsulfonyl fluoride, and 0.1 μM aprotinin. The GST fusion proteins were isolated from glutathione-Sepharose beads with 10 mM reduced glutathione (pH 8) in 50 mM Tris-HCl.

2 × 10⁶ EL4.NOB-1 cells were incubated with IL-1α (10 ng/ml) as indicated in the figure legends. Whole cell extracts were prepared using a lysis buffer (50 mM HEPES, pH 7.4, 10 mM NaF, 10 mM iodacetamide, 75 mM NaCl, 1% Nonidet P-40, 10 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na₂VO₃), and equal amounts of protein per sample, as determined by the method of Bradford (30), were incubated with 10 μl of a 50% solution (v/v) containing GST-RalGDS-RBD or GST-RafRBD precoupled to glutathione-agarose beads (coupled by incubating 10 mg of fusion protein per ml of 50% slurry glutathione-agarose beads, for 2 h at 4 °C). The beads were subsequently washed in lysis buffer, and coupled protein was released by heating the samples to 95 °C while they were resuspended in sample buffer. The samples were subjected to 15% SDS-polyacrylamide gel electrophoresis (28) and detected by immunoblotting using monoclonal anti-pan-Ras antibody or polyclonal anti-Rap1 antibody as described above.

RESULTS

LT Inhibits the Activation of p38 MAPK by IL-1—LT from *C. sordellii* is a glucosyltransferase that elicits its effects by glucosylating and thereby inactivating Ras, Rac, and Rap in their effector domains (18). We first tested the effect of LT on p38 MAPK activation in EL4.NOB-1 cells. The Western blotting assay used here detects the dually phosphorylated form (and hence the active form) of p38 MAPK (3). Pretreating the cells with 500 ng/ml of LT blocked the ability of IL-1 to activate p38 MAPK (Fig. 1A, *top panel*). An optimal effect was observed with a pretreatment time of 4 h (Fig. 1A, compare *lanes 8* and *2*).

LT glucosylates at least two proteins of approximately 19 and 23 kDa in EL4.NOB-1, which are in the correct molecular mass range for low molecular weight G proteins (Fig. 1A, *bottom panel*, compare *lanes 2* and *1*). Similar to the inhibition of p38 MAPK activation, a 4-h pretreatment time was required to cause optimal glucosylation of these target proteins. This was determined by first treating the cells with LT for increasing times up to 4 h, following which cell extracts were treated with LT in the presence of ¹⁴C-labeled UDP-glucose. The degree of glucosylation in the extracts will be inversely proportional to that in intact cells, thereby giving a measure of the degree of glucosylation in intact cells. As can be seen in Fig. 1A, *bottom panel* (*lane 5*), a 4-h treatment of the cells was sufficient to cause significant glucosylation of target proteins, particularly the 23-kDa form, which was fully glucosylated by the toxin.

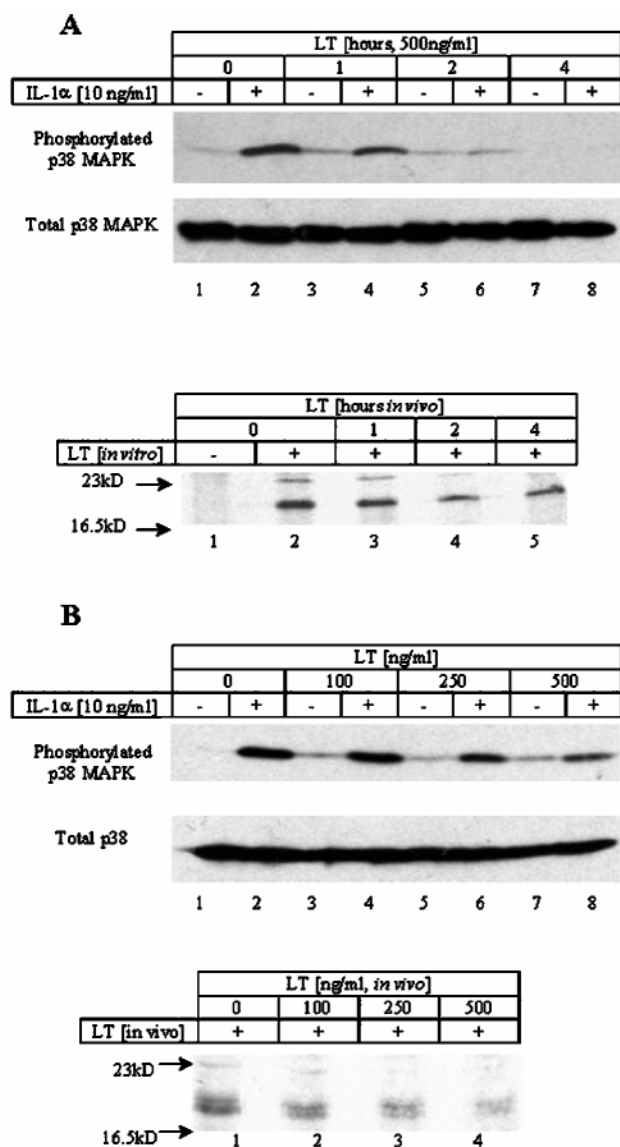


FIG. 1. Effect of LT on the activation of p38 MAPK by IL-1 in EL4.NOB-1 cells. EL4.NOB-1 cells ($2.5 \times 10^6 \text{ ml}^{-1}$, 4 ml per sample) were pretreated with LT for the indicated times (1–4 h (A)) or at the indicated doses (100–500 ng/ml (B)) at 37 °C, following which the cells were stimulated with IL-1 α (10 ng/ml) for a further 10 min. Cell extracts were prepared as described under “Experimental Procedures” and assayed for phosphorylated p38 MAPK or total p38 MAPK by Western blotting (A and B, top panels) or glucosylation of target proteins by LT *in vivo* (bottom panels). The position of molecular mass markers are indicated for the glucosylation assay. Results shown are representative of at least three experiments performed.

This therefore correlated with the degree of inhibition of p38 MAPK activation shown in Fig. 1A, top panel.

The effect of LT on p38 MAPK was also dose-dependent, as shown in Fig. 1B. 500 ng/ml of LT was required for optimal inhibition (top panel, lane 8). This again correlated with the degree of glucosylation, because 500 ng/ml LT at 4 h of incubation resulted in optimal glucosylation of protein substrates (bottom panel, lane 4). These results suggest that one or more of the target proteins for LT play a crucial role in the activation of p38 MAPK by IL-1.

It should be noted that the degree of inhibition differed between experiments, varying from total inhibition (as shown in Fig. 1A) to incomplete inhibition (as shown in Fig. 1B). Inhibition was always observed, however, and in general, it correlated with the degree of glucosylation of target substrates

by the toxin.

LT Does Not Inhibit the Activation of p38 MAPK by IL-1 in the UDP-Glucose-deficient Fibroblast Cell Line Don Cdt^R-Q—Although the inhibitory effect of LT on p38 MAPK activation by IL-1 correlated with the degree of glucosylation by the toxin, we wished to provide further evidence that the effect of LT was dependent on glucosylation. This involved testing the effect of the toxin on Cdt^R-Q, a mutant cell line (27) that, due to a single point mutation in the UDP-glucose phosphorylase gene, has an intracellular UDP-glucose level of just 26% of that of the parental strain (32). Because of this deficiency of the cofactor for glucosyl transferase toxins, the mutant cells are resistant to LT and ToxB (33).

Treatment of the parental line Don wild type with LT dose-dependently inhibited p38 MAPK activation by IL-1 (Fig. 2A, top panel), with 500 ng/ml causing an optimal inhibition (compare lanes 8 and 2). This indicated that the effect of LT was also evident in primary fibroblasts. Importantly, similar treatment did not inhibit the response in Don Cdt^R-Q, however (Fig. 2A, bottom panel), with doses of LT up to 500 ng/ml having no effect.

We next confirmed that that the differential effects on the Don wild type and the Cdt^R-Q by LT were not due to a difference in intracellular levels of the target proteins. As shown in Fig. 2B, top panel, treatment of intact Don cells leads to the glucosylation of a protein of 19 kDa, as is evident by the lack of substrate for LT during subsequent *in vitro* treatments with ¹⁴C-labeled UDP-glucose (Fig. 2B, top panel, compare lanes 2 and 5). On the other hand, although the substrates for LT are present in ample amounts in Cdt^R-Q, as assessed using ¹⁴C-labeled UDP-glucose supplied *in vitro* (Fig. 2B, bottom panel, lane 2), the toxin is unable to glucosylate these proteins in intact cells (Fig. 2B, bottom panel, lanes 3–5), because of the lack of UDP-glucose. These results therefore strongly indicate that the inhibitory effect of LT on p38 MAPK activation by IL-1 is dependent on glucosylation of target proteins. This has also been shown in the case of p42/p44 MAPK activation by epidermal growth factor in fibroblasts (18).

LT Does Not Affect the Ability of IL-1 to Induce Degradation of I κ B α and Does Not Induce Pro-caspase 3 Processing in EL4.NOB-1 Cells—We next tested the specificity of the effect of LT by examining its effect on another IL-1 response in the cells, I κ B α degradation, which, similarly to the p38 MAPK pathway, involves phosphorylation (22). As shown in Fig. 3A, increasing amounts of LT (up to 500 ng/ml for 4 h (lanes 5 and 6)) did not block IL-1-induced I κ B α degradation. In addition, as shown in Fig. 3B, LT did not induce processing of pro-caspase 3 (CPP32), which was used as a marker for apoptosis (34), verifying that at the concentrations used here, LT has no apoptotic effect on these cells. LT also did not induce an apoptotic or necrotic morphology in the cells under any of the conditions used in this study (not shown). These results indicate that the inhibitory effect on p38 MAPK was not due to nonspecific toxic effects.

LT Specifically Inhibits the Phosphorylation of CHOP by p38 MAPK in Response to IL-1—Our data with LT indicated that Ras, Rac, or Rap would appear to be critical for p38 MAPK activation by IL-1. We therefore focused on these three small G proteins using an assay that would allow the use of transient transfections with mutant constructs of each of these G proteins. The technique is based on a *trans*-acting one-hybrid system involving phosphorylation of the p38 MAPK-specific substrate CHOP (35) (as described under “Experimental Procedures”). IL-1 activated the CHOP-Gal4 reporter system in EL4.NOB-1, which was indicative of p38 MAPK activation, the effect being apparent from 1.5 h and peaking at 6 h (not shown). Activation of this response by IL-1 varied from 2-fold to

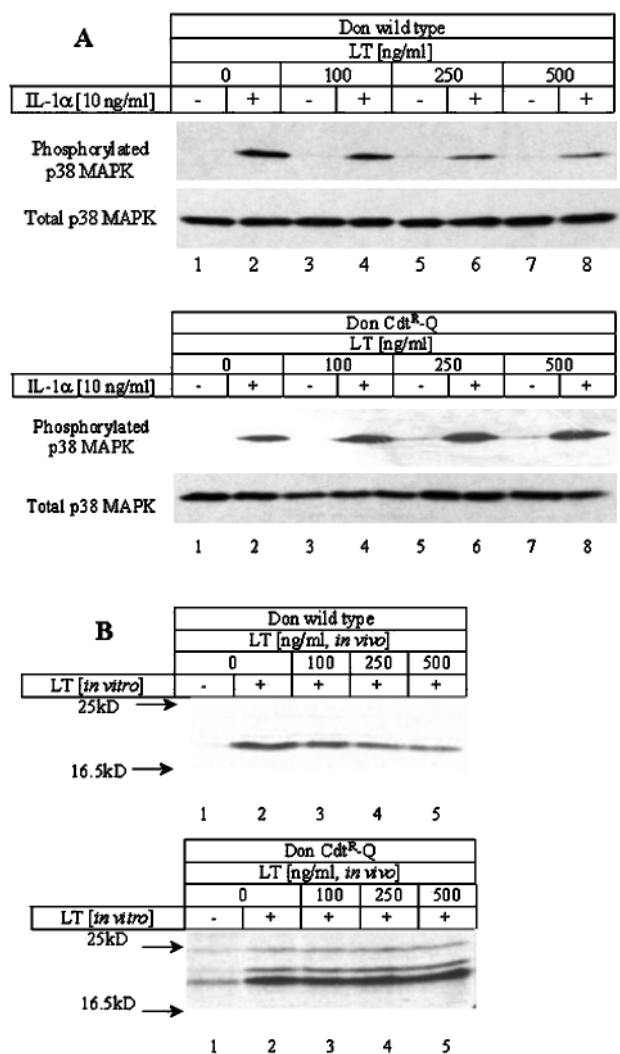


FIG. 2. Effect of LT on the activation of p38 MAPK by IL-1 in the UDP-glucose-deficient cell line Don-Cdt^{RQ}. A, cells (2×10^6 in 4 ml of medium, Don wild type (top panel) or Don Cdt^{RQ} (bottom panel)) were incubated with the indicated concentrations of LT (100–500 ng/ml) for 4 h at 37 °C and were subsequently stimulated with IL-1 α for a further 10 min. Cell extracts were prepared as described above and assayed for phosphorylated p38 MAPK and total p38 MAPK as indicated by Western blotting. Results show one representative of three experiments performed. B, cells (1×10^7 cells in 4 ml of medium, Don wild type (top panel) or Don Cdt^{RQ} (bottom panel)) were incubated with the indicated concentrations of LT (100–500 ng/ml) for 4 h at 37 °C. Cell extracts were subsequently prepared and subjected to glucosylation assay *in vitro* (18) in the presence of ¹⁴C-labeled UDP-glucose, and incorporated radioactivity could be detected by autoradiography. Results show one representative experiment of two experiments performed. Molecular mass markers are indicated.

8-fold over control levels, depending on the passage number of the cells. Later passage cells ($p > 25$) were generally less responsive (not shown).

Fig. 4A shows that the response requires p38 MAPK because treating the cells with the specific p38 MAPK inhibitor SB203580 at 1 μ M inhibited the effect of IL-1, verifying that the assay is specific for p38 MAPK (36). When using the GAL4 DNA binding domain alone, rather than the GAL4-CHOP coupled construct, the system is unresponsive to the effects of IL-1, acting as a negative control (data not shown).

LT also proved inhibitory in this assay. Fig. 4A illustrates how 500 ng/ml LT causes a nearly total inhibition of the IL-1-induced expression of luciferase. This verifies the role played by the small G proteins targeted by LT in the activation of the

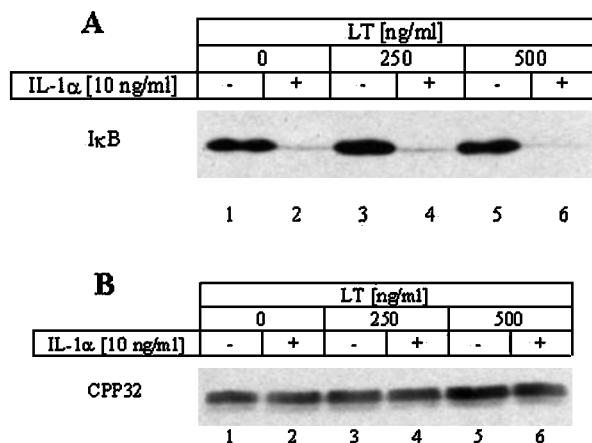


FIG. 3. Effect of LT on IL-1 α -induced degradation of IκB α and on the degradation of pro-caspase 3 (CPP32), as a marker of apoptosis. EL4.NOB-1 cells (8×10^6 in 4 ml) were incubated with the indicated concentrations of LT (250–500 ng/ml) for 4 h at 37 °C. Extracts were prepared as described under “Experimental Procedures,” after which the levels of IκB α , and pro-caspase 3 could be measured by Western blotting. Identical results were obtained in an additional experiment.

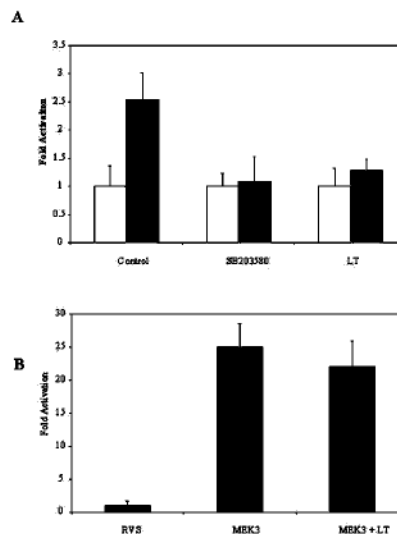


FIG. 4. Effect of SB203580 and LT on IL-1-induced phosphorylation of CHOP by p38 MAPK in EL4.NOB-1 cells. A, 1.4×10^7 EL4.NOB-1 cells were transfected with the components of the GAL4-CHOP^(1–101) system as described under “Experimental Procedures.” Transfected cells (5×10^5 per sample) were pretreated with SB203580 for 1 h or LT for 4 h at the indicated doses (1 μ M and 500 ng/ml, respectively), following which cells were stimulated with IL-1 α for a further 4 h (filled bars) or left unstimulated (open bars). B, cells co-transfected with the components of the GAL4-CHOP^(1–101) system and constitutively active MEK3 were pretreated with LT (500 ng/ml; 4 h) and stimulated with IL-1 α for 4 h (filled bars) or left unstimulated (open bars). In all cases (A and B), cells were subsequently lysed, the luciferase activity of each sample was measured, and readings were corrected for protein measured according to the method of Bradford (30). Results show one representative experiment of three identical experiments performed expressed as mean \pm S.E. for samples assayed in quadruplicate.

p38 MAPK pathway by IL-1, using this independent assay.

In order to ensure that LT does not interfere with the one-hybrid system nonspecifically, the effects of LT on constitutively active MEK3, an upstream activator of p38 MAPK (10), were studied. Fig. 4B shows the strong activation of p38 MAPK by constitutively active MEK3. Treatment with LT at 500 ng/ml (a dose causing nearly total inhibition of the IL-1-induced effect) had no effect on the ability of MEK3 to drive the

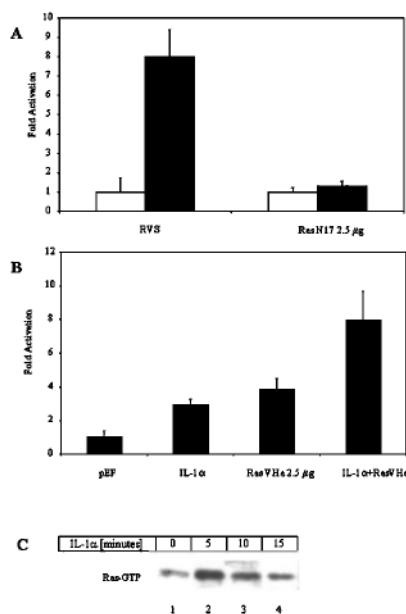


FIG. 5. Effects of RasN17 and RasVHa on IL-1 α -induced phosphorylation of CHOP by p38 MAPK in EL4.NOB-1 cells. 1.4×10^7 EL4.NOB-1 cells were co-transfected with the components of the GAL4-CHOP⁽¹⁻¹⁰¹⁾ system and the indicated amounts of RasN17 (A) or RasVHa (B) as described under "Experimental Procedures." Transfected cells (5×10^5 in 0.25 ml of medium per sample) were stimulated with IL-1 α for 6 h at 37 °C (filled bars) or left unstimulated (open bars), following which cells were lysed, the luciferase activity of each sample was measured, and readings were normalized for protein as measured according to the method of Bradford (30). Results shown are from a single experiment (mean \pm S.E., $n = 4$). Identical results were obtained in three additional experiments. C, cell extracts from 2×10^6 EL4.NOB-1 cells pretreated with IL-1 α for the indicated times (5–15 min) were incubated with GST-RafRBD precoupled to glutathione-agarose beads for 2 h at 4 °C as described under "Experimental Procedures." Following subsequent washing of the beads, bound Ras-GTP was detected by Western blotting, using an anti-pan Ras antibody. Identical results were obtained in an additional experiment.

expression of the luciferase gene.

Ras Is Involved in p38 MAPK Activation by IL-1—Having validated the transfection-based assay, we next examined the effect of dominant negative and constitutively active Ras, a prominent target for LT, on the response. As depicted in Fig. 5A, transfection of cells with 2.5 μ g of plasmid encoding RasN17 inhibited IL-1-induced luciferase expression. Transfection of cells with constitutively active RasVHa, on the other hand, activated p38 MAPK (Fig. 5B). As little as 2.5 μ g of DNA of the RasVHa expression vector gave rise to a 4-fold increase in expression of luciferase. Fig. 5B also shows how treatment of transfected cells with IL-1 caused a further increase in the response.

We next tested whether IL-1 could activate Ras in the cells, using the Ras binding domain of Raf-1 (which binds GTP-bound Ras) as a means of isolating active Ras, which can then be detected by Western blotting (31). Fig. 5C shows that the incubation of EL4.NOB-1 cells with IL-1 gave rise to a rapid increase in active GTP-bound Ras, reaching a maximum activation after 5 min (lane 2). Also evident is the transient nature of this response, which returned to basal levels after 15 min.

Further evidence for Ras involvement was next sought using two Ras inhibitors, manumycin A and damnacanthal. Treatment of cells with manumycin A, which inhibits Ras by acting as a farnesyl transferase inhibitor (37, 38), dose-dependently inhibited the activation of p38 MAPK by IL-1, with optimum inhibition occurring at 5 μ M manumycin A (Fig. 6A). Damnacanthal, a Ras function inhibitor with an unknown mechanism, also inhibited the response, the optimum effect being evident at

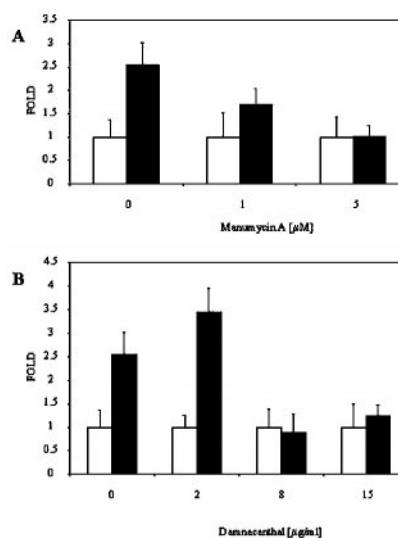


FIG. 6. Effects of manumycin A and damnacanthal on IL-1-induced phosphorylation of CHOP by p38 MAPK in EL4.NOB-1 cells. 1.4×10^7 EL4.NOB-1 cells were transfected with the components of the GAL4-CHOP⁽¹⁻¹⁰¹⁾ system as described under "Experimental Procedures." Transfected cells (5×10^5 in 0.25 ml of medium per sample) were pretreated with manumycin A (A) or damnacanthal (B) at the indicated concentrations for 1 h at 37 °C, following which cells were stimulated with IL-1 α for a further 6 h (filled bars) or left unstimulated (open bars). Cell extracts were subsequently prepared, after which the luciferase activity of each sample was measured, and readings were normalized for protein as measured according to the method of Bradford (30). Results show one representative experiment of three identical experiments performed expressed as mean \pm S.E. for samples assayed in quadruplicate.

8 μ g/ml (Fig. 6B). Taken together, these data strongly implicate Ras in p38 MAPK activation by IL-1.

Rac1 Is Not Involved in p38 MAPK Activation by IL-1—The data with Ras indicated that this was the G protein being targeted by LT in the IL-1 pathway. As stated above, Rap and Rac are the other two major substrates for the toxin. We therefore next examined these two G proteins, starting with Rac. We first utilized another large clostridial glucosyltransferase toxin, ToxB, a toxin that glucosylates and thereby inhibits the small G proteins Rho, Cdc42, and Rac (19). Treating EL4.NOB-1 cells with this toxin prior to stimulation with IL-1 did not affect the ability of IL-1 to phosphorylate and thereby activate p38 MAPK (Fig. 7A), as measured by Western blotting (as described above).

By exposing cell extracts to ToxB in the presence of UDP-[¹⁴C]glucose, at least one protein with an approximate mass of 21 kDa was detected, showing that the toxin was active in the cells (Fig. 7B). Furthermore, morphological changes, such as clumping of EL4.NOB-1, which is characteristic of the effects of ToxB, confirmed that the toxin was taken up by the cells (data not shown). ToxB also had no effect on p38 MAPK activation in Don fibroblasts, but again caused clumping and rounding of the cell bodies (not shown). Rac, the only common substrate for LT and ToxB, was therefore not involved in p38 MAPK activation by IL-1 here.

This was further supported when we tested plasmids encoding mutant versions of Rac1 in the p38 MAPK transactivation assay. Fig. 7C shows that transfection of cells with 5 or 10 μ g of plasmid encoding dominant negative RacN17 did not inhibit the response to IL-1. As shown in Fig. 7D, however, overexpression of constitutively active RacV12 activated p38 MAPK, inducing a 4-fold increase over control levels. This confirmed that Rac could activate the p38 MAPK pathway. It was unlikely to be involved in p38 MAPK activation by IL-1, however.

Rap Inhibits p38 MAPK Activation and Is Activated by IL-

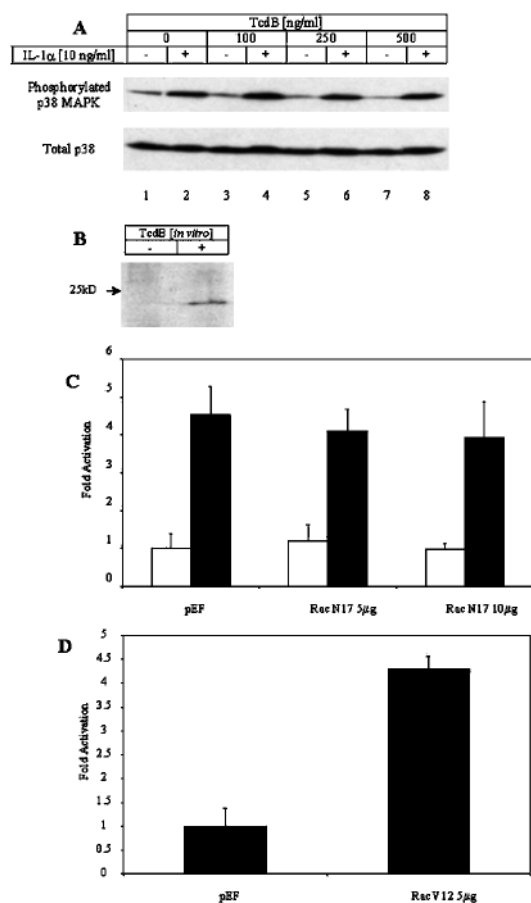


FIG. 7. Effect of ToxB, RacN17, and RacV12 on the activation of p38 MAPK by IL-1 α in EL4.NOB-1 cells. A, cells (8×10^6 in 4 ml) were incubated with the indicated concentrations of ToxB (100–500 ng/ml) for 4 h at 37 °C. Extracts were prepared as described under “Experimental Procedures,” and phosphorylated and total p38 MAPK was detected by Western blotting. Results show one representative experiment of three experiments performed. B, cell extracts of 1×10^7 EL4.NOB-1 cells were incubated with ToxB in the presence of 14 C-labeled UDP-glucose for 1 h at 37 °C. Radiolabeled protein from incorporation of UDP-glucose were subsequently visualized by SDS-polyacrylamide gel electrophoresis and autoradiography as described under “Experimental Procedures.” C and D, 1.4×10^7 EL4.NOB-1 cells were co-transfected with the indicated amounts of RacN17 (C) or RacV12 (D) as described under “Experimental Procedures.” Transfected cells (5×10^5 in 0.25 ml per sample) were stimulated with IL-1 α for 6 h at 37 °C (filled bars, C) or left unstimulated (open bars, C), following which cells were lysed, the luciferase activity of each sample was measured, and readings were normalized for protein as measured according to the method of Bradford (30). Results show one representative experiment of three identical experiments performed expressed as mean \pm S.E. for samples assayed in quadruplicate.

1—Finally, we investigated the role of Rap on p38 MAPK activation by IL-1. Intriguingly, transfection of dominant negative Rap1AN17 activated the reporter system (Fig. 8A). Transfection of cells with 2.5 μ g of expression plasmid encoding Rap1AN17 induced a response similar to stimulation with IL-1. Adding IL-1 to transfected cells further enhanced this response. This result implied that Rap was having a negative effect on p38 MAPK activation by IL-1. In agreement with this, the same experiment performed with a constitutively active version, Rap1AV12 (Fig. 8B), showed a clear inhibition of the activation of p38 MAPK by IL-1. This suggested that Rap has an antagonistic effect on p38 MAPK activation by IL-1.

We also found that IL-1 activated Rap in the cells. The activation of Rap can be measured by using the Ral-GDS-RBD domain coupled by GST to glutathione-agarose beads (25). Only

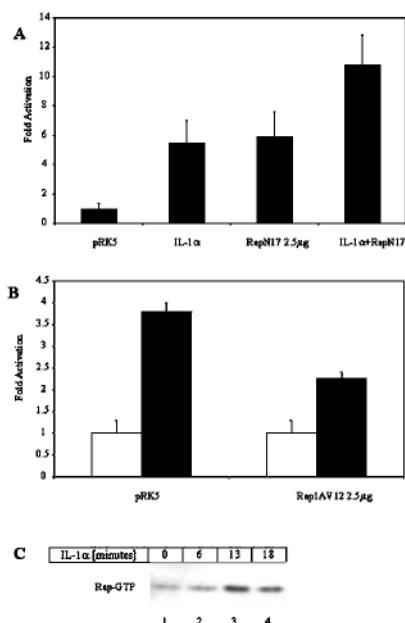


FIG. 8. Effects of RapN17 and RapV12 on IL-1 α -induced phosphorylation of CHOP by p38 MAPK in EL4.NOB-1 cells. 1.4×10^7 EL4.NOB-1 cells were co-transfected with the components of the GAL4-CHOP⁽¹⁻¹⁰¹⁾ system together with RapN17 (A) or RapV12 (B) as described under “Experimental Procedures.” Transfected cells (5×10^5 in 0.25 ml per sample) were stimulated with IL-1 α for 6 h at 37 °C (B, filled bars) or left unstimulated (B, open bars), following which cells were lysed, the luciferase activity of each sample was measured, and readings were normalized for protein as measured according to the method of Bradford (30). Results show one representative experiment of four identical experiments performed expressed as mean \pm S.E. for samples assayed in quadruplicate. C, cell extracts from 2×10^6 EL4.NOB-1 cells pretreated with IL-1 α for the indicated times (0–18 min) were incubated with GST-RalGDS-RBD precoupled to glutathione-agarose beads for 2 h at 4 °C as described under “Experimental Procedures.” Following subsequent washing of the beads, the bound Rap-GTP was detected by Western blotting, using a polyclonal anti-Rap antibody. Identical results were obtained in an additional experiment.

activated Rap-GTP will bind to the Ral-GDS-RBD, and Rap-GTP can be precipitated and detected by Western blotting using a Rap-specific antibody. An increase in Rap-GTP was evident after 6 min (Fig. 8C, lane 2), reaching maximum activation after 13 min (lane 3), and was shown to be transient reaching nearly basal levels after 18 min (lane 4). This slower kinetics to Ras activation implies that the later activation of Rap may be responsible for the transient nature of p38 MAPK activation in IL-1 treated cells.

DISCUSSION

Our study indicates that Ras is required for p38 MAPK activation by IL-1, with Rap having an antagonistic effect. That low molecular weight G proteins play an important role in IL-1 signaling has been indicated by various other studies, most of which have relied on the use of constitutively active or dominant negative mutants of various G proteins. We took a similar approach but, importantly, also utilized various inhibitors of G protein function, namely the clostridial toxins LT and ToxB (which are highly specific) and two Ras inhibitors, manumycin A and damnacanthal. We feel that these multiple approaches have yielded more definitive and reliable results on G protein involvement in IL-1 action.

Studies using dominant negative RacN17 and Cdc42N17 have implicated these G proteins in p38 MAPK activation, as well as in the activation of JNK. However, more recently, a role for Ras in p38 MAPK activation by hemopoietic cytokines has been demonstrated (39, 40). In addition, a role for Ras in p38

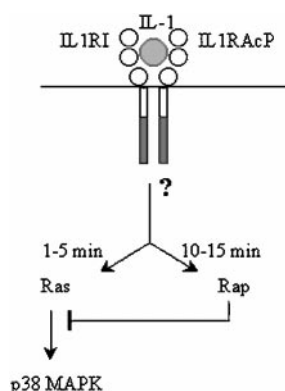


FIG. 9. A model depicting the divergent roles of Ras and Rap in p38 MAPK activation by IL-1. Further details are given in the text.

MAPK activation by both platelet-derived growth factor (41) and fibroblast growth factor (42) has been shown. Our data clearly add IL-1 to the list of p38 MAPK activators that require Ras. A role for Ras in IL-1 signaling has been indicated in other studies. Induction of the collagenase promoter by IL-1 in chondrocytes has been shown to require Ras (43), as has induction of the brain natriuretic peptide in myocytes (44). Others, however, have failed to demonstrate activation of Ras by IL-1 (45). Our data, in contrast, clearly show rapid and transient activation of Ras in EL4.NOB-1. How IL-1 activates Ras and the means by which Ras activates the p38 MAPK cascade are at present unclear.

Our results also indicate that Rac is not involved in this response. Dominant negative Rac has been shown by others to inhibit NF- κ B and p38 MAPK activation by IL-1, however (14, 15). The basis for the discrepancy with our results is not clear, but given that both ToxB and transfection of RacN17 failed to have any effect, we conclude that Rac is not important in our system. A lack of effect of Rac in p38 MAPK activation by IL-1 in cardiac myocytes has also recently been shown (44), and a role for Rac in JNK activation by IL-1 has recently been disputed (16). We therefore conclude that Rac may not be an important regulator of p38 MAPK in the IL-1 system.

Apart from the activation of Ras, we also found IL-1 to be an activator of Rap. A number of other extracellular signals, including platelet-derived growth factor, epidermal growth factor, endothelin, and 1-oleoyl-lyso-phosphatidic acid, have recently been shown to activate Rap (46, 47), although a role for Rap in downstream events was not investigated. Recent reports have pointed to Rap as a positive regulator in cell signaling, although the major role of Rap in cell signaling appears to be as an antagonist toward Ras (48–52). The mechanism of this antagonism is likely to be due to competition for effectors. Evidence has been presented for Rap interacting with Raf-1, blocking its activation by Ras (52). Furthermore, RapV12 has been shown to inhibit p42/p44 MAPK activation by 1-oleoyl-lyso-phosphatidic acid and epidermal growth factor (51). Because Rap activation occurred at a later time than Ras activation, it is possible that the transient nature of p38 MAPK activation by IL-1 may be due to Rap inhibiting the response, as depicted in the suggested model presented in Fig. 9. Furthermore, although LT would block both Rap and Ras, the inhibiting effect on Ras would predominate, because Ras is crucial for the signal to occur.

The activation of Rap may also be important for the induction of anergy in T cells. Boussiotis *et al.* (52) showed that Rap-GTP was present in anergic T cells and proposed a negative regulatory role for Rap in T-cell Receptor mediated IL-2 gene transcription, suggesting that Rap may be responsible for the specific defect in IL-2 production in T cell anergy. This may

be relevant for IL-1 signaling, because IL-1 induces IL-2 in EL4 cells. It is therefore possible that the activation of Rap by IL-1 would lead to an inhibition of signaling initiated as a result of Ras activation, thereby limiting the effects of IL-1 in IL-2 production (and as stated above in p38 MAPK activation), acting as a negative feedback loop. Our data also suggest that in unstimulated cells, Rap is maintaining the p38 MAPK pathway in an inactive state, because transfection of a plasmid encoding RapN17 activates this pathway.

In conclusion, our study identifies Ras as a key signal in the activation of p38 MAPK by IL-1. Furthermore, this is the first demonstration of Rap activation by IL-1, which, given its inhibiting effect, provides further evidence for a role for Ras in the effect of IL-1 and suggests a mechanism for the transient nature of IL-1 signaling.

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