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Ras Participates in the Activation of p38 MAPK by Interleukin-1 by Associating with IRAK, IRAK2, TRAF6, and TAK-1*

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Interleukin-1 (IL-1) activates p38 MAP kinase via the small G protein Ras, and this activity can be down-regulated by another small G protein Rap. Here we have further investigated the role of Ras and Rap in p38 MAPK activation by IL-1. Transient transfection of cells with constitutively active forms of the known IL-1 signaling components MyD88, IRAK, and TRAF-6, or the upstream kinases MKK6 and MKK3, activated p38 MAPK. Dominant negative forms of these were found to inhibit activation of p38 MAPK by IL-1. Dominant negative RasN17 blocked the effect of the active forms of all but MKK3 and MKK6, indicating that Ras lies downstream of TRAF-6 but upstream of MKK3 and MKK6 on the pathway. Furthermore, the activation of p38 MAPK caused by overexpressing active RasVHa could not be inhibited using dominant negative mutants of MyD88, IRAK, or IRAK-2, or TRAF6, but could be inhibited by dominant negative MKK3 or MKK6. In the same manner, the inhibitory effect of Rap on the activation of p38 by IL-1 occurred at a point downstream of MyD88, IRAK, and TRAF6, since the activation of p38 MAPK by these components was inhibited by overexpressing active Rap1AV12, while neither MKK3 nor MKK6 were affected. Active RasVHa associated with IRAK, IRAK2, and TRAF6, but not MyD88. In addition we found a role for TAK-1 in the activation of p38 MAPK by IL-1, with TAK-1 also associating with active Ras. Our study suggests that upon activation Ras becomes associated with IRAK, Traf-6, and TAK-1, possibly aiding the assembly of this multiprotein signaling complex required for p38 MAPK activation by IL-1.

As a central proinflammatory mediator, interleukin 1 (IL-1)[1] induces the expression of multiple genes involved in the inflammatory process. The signaling pathway leading to the activation of the transcription factor NFκB by IL-1 has been studied in great detail (reviewed in Ref. 1). IL-1 binds to the Type I IL-1 receptor (IL-1RI), after which the receptor forms a complex with the IL-1 receptor accessory protein (IL-1RAcP) (2, 3). This triggers the recruitment of the adapter protein MyD88, which associates with IL-1RI through its carboxyl-terminal Toll/IL-1 receptor (TIR) domain. Once recruited, MyD88 interacts with 2 IL-1 receptor-associated kinases, IRAK and IRAK-2 (4–6), which are pre-associated with the regulatory protein Toll-interacting protein (Tollip) (7). IRAK then becomes phosphorylated, dissociates from MyD88, and becomes associated with tumor necrosis factor receptor-associated factor 6 (8) which is preassembled in a complex with TAK1-binding protein (TAB)-2 (9). The mitogen-activated protein kinase kinase kinase (MAPKKK) transforming growth factor-β-activated kinase-1 (TAK1), constitutively associated with TAB1 (10), then associates with the TRAF6·IRAK complex, whereby TRAF6 in a ubiquitination-dependent manner triggers the phosphorylation and activation of TAK1 (11). TAK1 can then activate two kinase cascades: one leads to the activation of Jun N-terminal kinase (JNK), and the other cascade leads to the activation of the IκB kinase complex, and ultimately to the activation of NFκB (12, 13).

IL-1 also triggers a signaling cascade which leads to the activation of the stress activated mitogen-activated protein kinase (SAPK) p38(14). Activated p38 MAPK phosphorylates transcription factors in the nucleus that are responsible for the regulation of immediate-early genes. The p38 MAPK pathway also activates the transcription factors CREB, ATF1, ATF2, CHOP, and MEF-2C (15–19). In addition, p38 MAPK plays a role in the activation of other protein kinases such as MAPKAP kinase-2, MAPKAP kinase-3, and Mnk1/2 (20–22), as well as being involved in mRNA stabilization (23, 24). p38 MAPK also regulates cell differentiation, proliferation, and survival (reviewed recently in Ref. 25). Four isoforms make up the p38 MAPK family, p38α, p38β, p38γ, and p38δ (26). The signaling pathway leading to the activation of p38 by IL-1 is not as well elucidated as the pathway leading to the activation of NFκB, although early events in the two pathways share similarities, as both TRAF6 and IRAK have been shown to play a role (13, 27, 28). p38 MAPK is activated by the MAPK kinases MKK3b (the predominant isoform of MKK3) (29) and MKK6 (30). A variety of MAPKKK can activate MKK3 and MKK6, including PAK, TAK1, and ASK1 (31–33).

We recently demonstrated that the small G protein Ras plays a critical role in the activation of p38 MAPK by IL-1, with Rap having an antagonistic effect (34). In the present study, we have investigated the role of MyD88, IRAK, IRAK-2, TRAF6, and TAK1 in the activation of p38 MAPK. Furthermore, we sought to further elucidate the role of Ras on this pathway, and we show here that Ras and Rap act at a point downstream of MyD88, IRAK, IRAK-2, and TRAF6, but upstream of MKK3 and MKK6. In addition we show an association of active Ras with IRAK, IRAK2, TRAF6, and TAK1, suggesting that Ras may be involved in the assembly of this multiprotein complex.

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[1] The abbreviations used are: IL-1, interleukin-1; MAPK, mitogen-activated protein kinase; IL-1RI, IL-1 receptor type I; IL-1RAcP, IL-1 receptor accessory protein; MyD88, myeloid differentiation factor 88; IRAK, IL-1 receptor associated protein kinase; TRAF, tumor necrosis factor receptor-associated factor; TAK, transforming growth factor-β-activated kinase; TAB, TAK-1 binding protein; MKK, MAPK kinase; DMEM, Dulbecco's modified Eagle's medium; TBS, Tris-buffered saline.

EXPERIMENTAL PROCEDURES

Materials—Human recombinant IL-1α was a kind gift from The National Cancer Institute Biological Resources Branch (Rockville, MD). PhosphoPlus™ p38 MAPK (Thr^{180}/Tyr^{182}) Antibody Kit was obtained from New England BioLabs (United Kingdom) Ltd. The components of the PathDetect™ CHOP trans-Reporting System (pFA-CHOP, pFC2-dbd, pFR-Luc, and pFC-MEK3) were purchased from Stratagene (La Jolla, CA). The pyridinyl imidazole SB203580 was obtained from Alexis Corp. (Nottingham, UK). The expression vectors encoding constitutively active RasVHa, and dominant negative Ras N17 (35), were kind gifts from Dr. Doreen Cantrell (ICRF, London, UK). Full-length MyD88, the mutant truncated ΔMyD88 (amino acids 152–296) lacking the death domain, full-length IRAK2, COOH-terminal K-IRAK2, and NH_2-terminal death domain ddIRAK2 were all kind gifts from Marta Muzio (Mario Negri Institute, Milan, Italy (6). Dominant negative IRAK (amino acids 1–211) was a kind gift from Emma-Louise Cooke (Glaxo Wellcome, Stevenage, UK). Mutant ΔTRAF6, wild type TRAF6, and wild type IRAK1 were gifts from Tularik (South San Francisco). The pRK5 expression vectors encoding constitutively active Rap1AV12 and dominant negative Rap1AN17 were a kind gift from Dr. Jean de Gunzburg (Institute Curie, Paris, France). The vector encoding FLAG-tagged p38 MAPK was a kind gift from Dr. Jeremy Saklatvala (Kennedy Institute of Rheumatology, London, UK). The constructs encoding for MKK3, mutant MKK3(A), MKK6, and mutant MKK6(A), were all kind gifts from Dr. Jiahuai Han (The Scripps Research Institute, La Jolla, CA). MKK mutants have alanine as a replacement for the conserved lysine in the ATP-binding site.

Cell Culture—The murine thymoma cell line EL4.NOB-1 and the human HeLa cervical carcinoma cells were obtained from the European Collection of Animal Cell Cultures (ECACC, Wiltshire, UK). The EL4.NOB-1 cells were maintained in RPMI 1640 medium, supplemented with 100 IU/ml gentamycin, 2 mM L-glutamine, and 10% fetal calf serum. The HeLa cells were cultured in Dulbecco's modified Eagle's medium, supplemented with 100 IU/ml gentamycin, 2 mM L-glutamine, and 10% fetal calf serum. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2. For use in Western blotting 2×10^5 transfected HeLa cells in 4 ml of complete medium were incubated with medium alone or with the indicated doses of SB203580 for 1 h. Following this, where applicable, the cells were stimulated with 10 ng/ml IL-1α for 15 min. For use in transfection assays, transfected EL4.NOB-1 cells were seeded at 2×10^6 cells per ml, and pretreated as indicated in figure legends.

Transfection of EL4.NOB-1 Cells and Protocol for the GAL4-CHOP$^{(1–101)}$ Assay—Cells (7×10^6) were harvested (in exponential growth phase) and resuspended in a final volume of 0.6 ml of Tris-buffered saline (25 mM Tris (pH 7.4), 137 mM NaCl, 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.6 mM NaH_2PO_4) containing 5–15 μg of DNA (1.25 μg of pFA-CHOP, 5 μg of pFR-Luc, 1 μg of pEF-lacZ, and 1–5 μg of signaling component expression vector), 250 μg/ml DEAE-dextran, and 40 μg/ml chloroquine as described previously (37). Following incubation for 30 min at 37 °C, the cells were washed twice in complete RPMI 1640 medium and resuspended in 7 ml of RPMI, 20% (v/v) fetal calf serum medium. After a recovery period of 16–24 h, the cells were harvested, seeded at 1×10^6 cells/ml (samples assayed in triplicates or quadruples) and, when required, stimulated with IL-1α (10 ng/ml) for 6 h. Luciferase activity was measured using standard procedures according to manufacturers recommendations in cell lysates which were prepared using Passive lysis buffer (Promega Corp., Madison, WI) diluted 1:5. p38 MAPK activity was calculated when β-galactosidase activity was determined using standard protocols, and used to normalize luciferase activity according to transfection efficiency.

Transfecting Using FuGENE™ Transfection Reagent—HeLa cells were seeded at 1.5×10^5 cells in 3 ml of complete DMEM 24 h prior to transfection. 7.5 μl of FuGENE™ transfection reagent (Roche Molecular Diagnostics) per transfection in a total volume of 250 μl of serum-free DMEM was used according to the manufacturers recommendations. The FuGENE™/DMEM mixture was added to 2 μg of vector encoding FLAG-tagged p38 MAPK, together with 2.5–5 μg of vector encoding for components of the IL-1 signaling pathway, as indicated in the figure legends. Equal total amounts of DNA were added to samples that were to be directly compared, ensuring equal transfection efficiencies. The cells were harvested as described below, 48 h after transfection.

Immunoblotting—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) bromphenol blue. Cells were scraped and sonicated for 15 s. Equal amounts of lysates were subjected to SDS, 10% PAGE and then transferred onto nitrocellulose membranes (Sigma Co. Ltd., Dorset, UK) 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 dried milk in TBS, 0.1% Tween. A primary antibody recognizing phosphorylated (Thr^{180}/Tyr^{182}) p38 MAPK, or an antibody for p38 MAPK, independent of phosphorylation status, were each used at a dilution of 1:1000 in 1% fat-free dry milk in TBS, 0.1% Tween. The antibody-antigen complexes were detected using a horseradish peroxidase-coupled anti-rabbit antibody, used at a dilution of 1:2000 in 5% fat-free dry milk in TBS, 0.1% Tween, and the signal was developed using a kit for enhanced chemiluminescense substrate development (New England BioLabs (UK) Ltd.).

Immunoprecipitation—HeLa cells were seeded into 10-cm culture dishes (1×10^6 per dish) and transfected 24 h later with a total of 8 μg of DNA using FuGENE™ as described above. 4 μg of each expression vector was transfected into the cells, and the total amount of DNA was kept constant by supplementation with relevant empty vector. 24 h after transfection, the cells were lysed in 800 μl of lysis buffer (50 mM Hepes, pH 7.5, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% Nonidet P-40, leupeptin, aprotinin, phenylmethylsulfonyl fluoride, benzamidine, Na_3VO_4, β-glycerophosphate). 16 μl of each lysate was retained and used to control for transfection efficiency by Western blotting. The remaining cell extracts were normalized for protein content using the method of Bradford, and incubated for 2 h at 4 °C with relevant primary antibody (2–5 μg of antibody per point) pre-coupled to Protein G-Sepharose beads. The beads were subsequently washed 3 times in lysis buffer, 50 μl of 5 × sample buffer was added, and the protein was released from the beads by boiling the samples for 5 min. The supernatants were run on SDS-PAGE and transferred to nitrocellulose membranes. Western blotting was performed by blotting the membrane in 5% fat-free dry milk in TBS-Tween (0.05%) for 1–3 h at room temperature, after which the membrane was incubated with primary antibody diluted in 1% fat-free dry milk in TBS-Tween (0.05%), and subsequently secondary peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG diluted 1:1000 in 5% fat-free dry milk in TBS-Tween (0.05%). The resulting signals were detected using ECL (34).

RESULTS

MyD88, IRAK, TRAF6, RasVHa, MKK3, and MKK6 All Mediate the Activation of p38 MAPK by IL-1—To study the role of various known signaling components of the IL-1R signaling complex in the activation of p38 MAPK we used two approaches. Both approaches allow for transient transfection with different combinations of signaling proteins. The first technique is based on the specific ability of p38 MAPK to phosphorylate the transcription factor CHOP. The method is based on a *trans*-acting one-hybrid system where a construct encoding a fusion protein consisting of the activation domain of CHOP, and the GAL4 DNA-binding domain, are co-transfected together with a luciferase reporter gene under the control of a GAL4-activation domain promoter. Phosphorylation of the CHOP fusion protein by IL-1-activated p38 MAPK results in increased luciferase activity as a direct measurement of active p38 MAPK. This transfection assay was performed in the IL-1RI-rich murine thymoma cell line, EL4.NOB-1. The second approach involved overexpressing FLAG-tagged p38 MAPK in HeLa cells, and by Western blotting detecting the phosphorylation status of the transfected p38 MAPK after co-transfecting the cells with constructs encoding for wild type or mutant versions of MyD88, IRAK, TRAF6, Ras, Rap, MKK3, or MKK6. We found that both cell types were optimal for these respective assays and the use of HeLa cells provided a second IL-1 responsive cell line in which results from the CHOP reporter assay in EL4.NOB-1 cells could be confirmed.

IL-1 activated the CHOP-Gal4 reporter system in EL4.NOB-1 (Fig. 1*A*, *upper panel*). An increased luciferase activity could be detected after 1.5 h, with a maximum expression occurring after 6 h. Furthermore, IL-1 increased the phosphorylation of p38 MAPK in these cells within 5 min (Fig. 1*A*, *lower panel*), peaking at 10 min stimulation and reaching basal

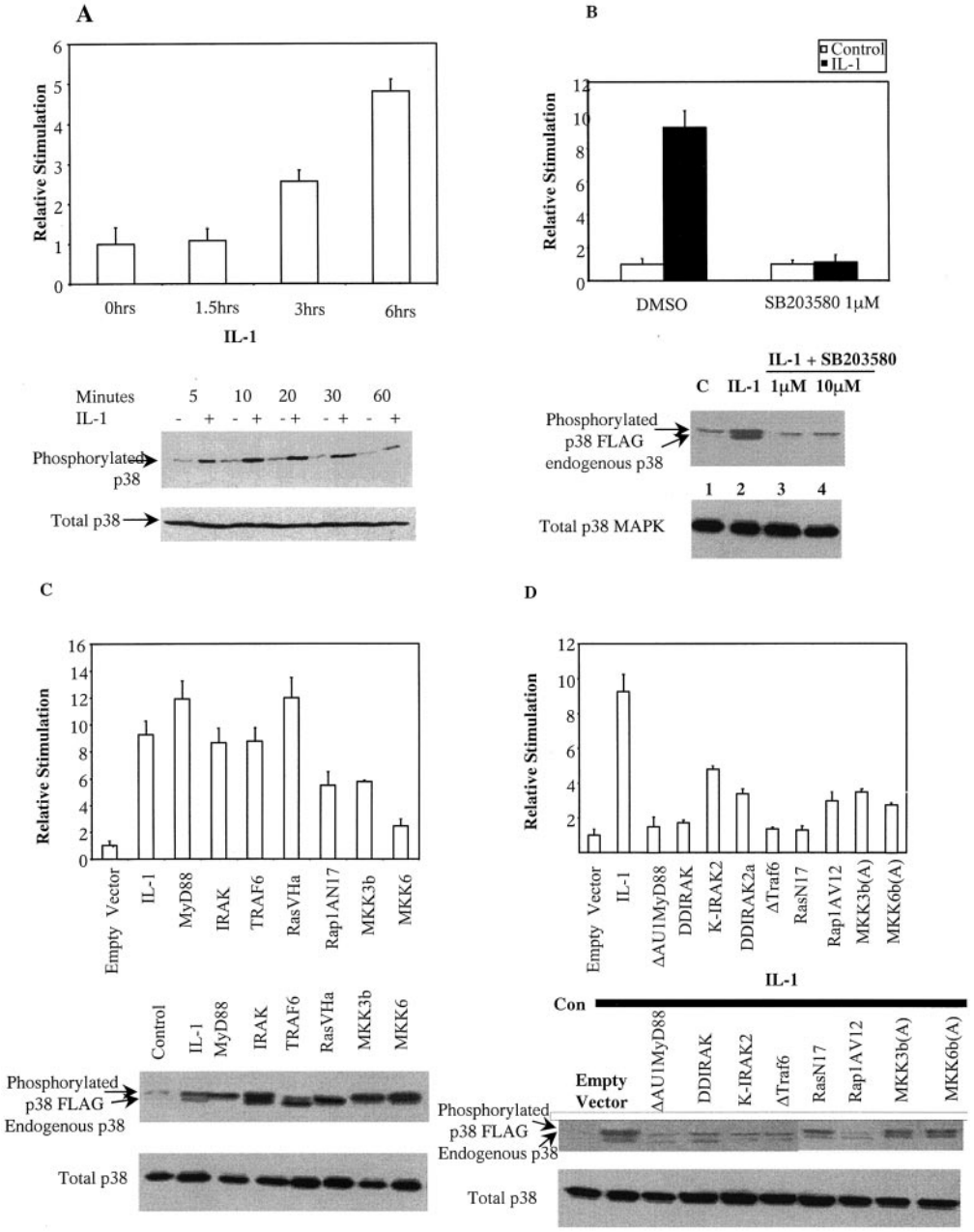

FIG. 1. **Effect of SB203580, MyD88, IRAK, TRAF6, Ras, Rap1A, MKK3, and MKK6 on p38 MAPK.** *A*, *B*, *C*, and *D*, *upper panels*, 7×10^6 EL4.NOB-1 cells were transfected with the components of the GAL4-CHOP$^{(1-101)}$ system as described under "Experimental Procedures." Transfected cells (5×10^5 per sample) were stimulated with 10 ng/ml IL-1α as indicated (*A*); pretreated with 1 μM SB203580 for 1 h prior to stimulation with IL-1α (10 ng/ml, 6 h) (*B*); co-transfected with plasmids encoding wild type MyD88, IRAK, TRAF6, MKK3, MKK6, constitutively active RasVHa, or dominant negative Rap1AN17 (*C*); or with constructs encoding dominant negative mutants of ΔMyD88, IRAK1-N, K-IRAK2, DDIRAK2, ΔTRAF6, MKK3A, MKK6A, RasN17, or constitutively active Rap1AN17 (*D*) as described under "Experimental Procedures." After 24 h, co-transfected cells (5×10^5 per sample) were stimulated with IL-1α for 6 h where indicated. All cells were subsequently lysed, the luciferase activity of each sample was measured, and readings were normalized for transfection efficiency by measuring β-galactosidase activity as described under "Experimental Procedures." One representative experiment of three to four identical experiments performed is shown expressed as mean ± S.E. of fold increase over control for samples assayed in quadruplicate. *A*, *lower panel*, EL4.NOB-1 cells (2.5×10^6 ml^{-1}, 4 ml per sample) were stimulated with 10 ng/ml IL-1 for indicated times (0–60 min). Cell extracts were prepared as described under "Experimental Procedures," and assayed for phosphorylated p38 MAPK (*upper panel*) or total p38 MAPK (*lower panel*) by Western blotting. Results show one representative experiment of three experiments performed. (*B*, *C*, and *D*, *lower panels*) HeLa cells (seeded at 1.5×10^5 cells in 3 ml of complete DMEM medium 24 h prior to transfection) were transfected with FLAG-tagged p38 MAPK, together with 2.5–5 μg of plasmids encoding wild type MyD88, IRAK1, TRAF6, RasVHa, MKK3, or MKK6 (Fig. 1*C*) or with 2.5–5 μg of plasmids encoding dominant negative mutants of ΔMyD88, IRAK1-N, K-IRAK2, ΔTRAF6, RasN17, MKK3A, or MKK6bA or constitutively active RapN17 (Fig. 1*D*) according to the method of FuGENE™ as described under "Experimental Procedures." 48 h after transfection the cells were stimulated with IL-1 for 10 min. In Fig. 1*B*, the cells were pretreated with 1 μM SB203580 for 1 h prior to stimulation. All cells were harvested, lysed, and Western blotting was performed, detecting activated transfected and endogenous p38 MAPK using an anti-phosphorylated p38 MAPK antibody, or an antibody for total p38 MAPK, as described under "Experimental Procedures." In each case, identical results were obtained in a further experiment.

levels again after 60 min as detected by Western blotting.

Fig. 1*B*, *upper panel*, verifies that the response is p38 MAPK specific since treating the cells with the specific p38 MAPK inhibitor, SB203580 at 1 μM for 1 h inhibited the effect of IL-1 in the CHOP-Gal4 reporter system in EL4.NOB-1 cells. The same was true when studying the phosphorylation status of

transfected FLAG-tagged (*upper band*), and endogenous (*lower band*) p38 MAPK (Fig. 1*B*, *lower panel*) in HeLa cells, where pretreating the cells with SB203580 at 1 and 5 μM for 1 h potently inhibited the activation of p38 MAPK by IL-1. The ability of SB203580 to block phosphorylation of p38 MAPK has been shown previously by us (38) and others (39, 40). When using the GAL4 DNA-binding domain alone (lacking the CHOP activation domain) in the CHOP-Gal4 system, the system was unresponsive to the effects of IL-1, acting as a negative control (data not shown).

Transient transfection of EL4.NOB-1 cells with the components of the CHOP-Gal4 reporter system together with constructs encoding wild type versions of MyD88, IRAK, TRAF6, MKK3, MKK6, or active RasVHa (Fig. 1*C*, *upper panel*), demonstrated that all of these molecules are able to activate p38 MAPK. For MyD88, IRAK, TRAF6, and RasVHa, this activation was as potent as that of IL-1. The activation by dominant negative Rap1AN17 and MKK3 were both comparable to IL-1, while MKK6 was the weakest activator. This result suggests that these components are potential signaling molecules of the pathway leading to the activation of p38 MAPK by IL-1. In addition, when co-transfecting cells with a construct encoding wild type IRAK 2, although not sufficient to cause an activation of p38 MAPK by itself, greatly potentiated the activation of p38 MAPK in response to IL-1 (not shown).

The results obtained from using the CHOP reporter system were confirmed in HeLa cells using the phosphorylation of transfected FLAG-p38 MAPK as a readout (Fig. 1*C*, *lower panel*). MyD88, IRAK, TRAF6, MKK3, MKK6, or constitutively active RasVHa, caused a potent phosphorylation of FLAG-p38 MAPK. All of these components potently induced phosphorylation of the FLAG-tagged p38 MAPK (*upper band*), and to a varying degree endogenous p38 MAPK (*lower band*).

To further investigate the involvement of these signaling molecules in the activation of p38 MAPK by IL-1, constructs encoding dominant negative versions of MyD88 (ΔMyD88), IRAK (DDIRAK1), IRAK2 (K-IRAK2), TRAF6 (ΔTRAF6), Ras (RasN17), MKK3(A), MKK6(A), or active RapV12 were all co-transfected into EL4.NOB-1 cells together with the components of the CHOP-Gal4 reporter system. As shown in Fig. 1*D*, *upper panel*, IL-1 is unable to activate p38 MAPK in cells expressing any of the above mutant proteins, ranging from 50 to 100% inhibition, providing further evidence that they are signaling components mediating the activation of p38 MAPK by IL-1. Furthermore, as was shown previously (34), when transfecting cells with a constitutively active mutant of Rap1A (V12) (Fig. 1*D*), an inhibitory effect on the activation of p38 MAPK by IL-1 can be observed.

Again, the results from the CHOP-Gal4 assay were confirmed in HeLa cells. Fig. 1*D*, *lower panel*, shows the inability of IL-1 to activate p38 MAPK in HeLa cells transfected with the mutant dominant negative constructs ΔMyD88, DDIRAK1, K-IRAK2, ΔTRAF6, RasN17, MKK3(A), MKK6(A), or active RapV12. This points to a key role for these components in the activation of p38 MAPK by IL-1. Cells were also transfected with a dominant negative construct of TRAF2 as a negative control. No inhibitory effects was observed (not shown).

Ras Activity Is Required for the Signaling Effect of MyD88, IRAK, and TRAF6 on the Pathway Leading to the Activation of p38 MAPK by IL-1—We next sought to investigate the position of Ras on the pathway. To do this, we co-transfected constitutively active RasVHa with the dominant negative constructs ΔMyD88, DD IRAK1, K-IRAK2, ΔTRAF6, MKK3(A), and MKK6(A). As shown in Fig. 2*A*, dominant negative MyD88, IRAK, IRAK2, or TRAF6 were all unable to inhibit the activation of p38 MAPK by RasVHa. Dominant negative MKK3(A),

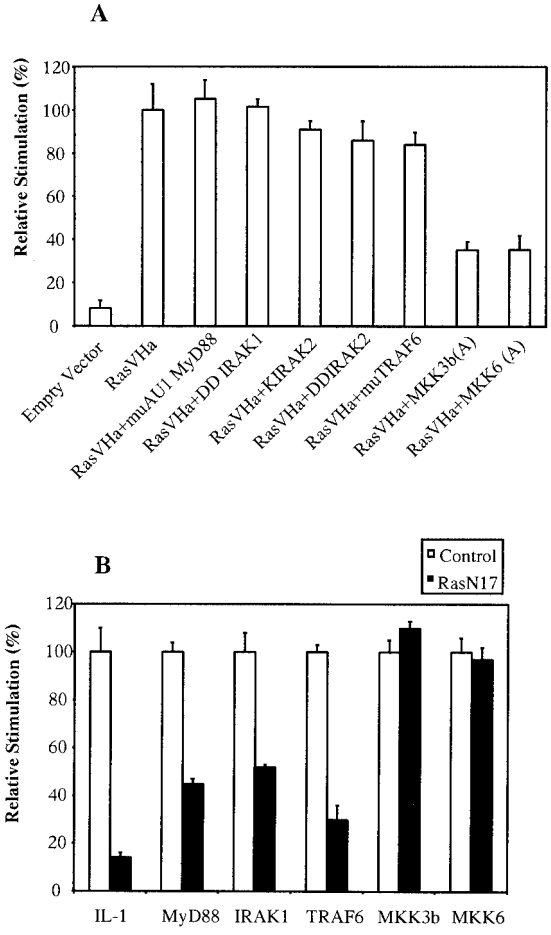

FIG. 2. **Role of Ras in p38 MAPK activation by IL-1 signaling components.** 7×10^6 EL4.NOB-1 cells were co-transfected with the components of the GAL4-CHOP$^{(1-101)}$ system together with (*A*) RasVHa and constructs encoding dominant negative mutants of ΔMyD88, IRAK1-N, KIRAK2, DDIRAK2, ΔTRAF6, MKK3A, or MKK6bA, or (*B*) RasN17 together with plasmids encoding wild type MyD88, IRAK1, TRAF6, MKK3, or MKK6b as described under "Experimental Procedures." Transfected cells (5×10^5 in 0.25 ml/sample) were stimulated with IL-1 for 6 h where required, the cells were lysed, the luciferase activity of each sample was measured, and readings were normalized for transfection efficiency by measuring β-galactosidase activity as described under "Experimental Procedures." For clarity, the stimulation by IL-1, RasVHa, or transfected wild type proteins are set at 100%, the inhibition by RasN17 or dominant negative proteins being shown relative to this, expressed as mean ± S.E. for quadruplicate determinations. Similar results were obtained in three further experiments.

and MKK6(A), however, were inhibitory, blocking the activation of p38 MAPK by RasVHa by more than 65%. This would indicate that Ras signals downstream of MyD88, IRAK, IRAK-2, and TRAF6 on this pathway, but upstream of MKK3 and MKK6.

The converse experiment is shown in Fig. 2*B*. Dominant negative RasN17 potently inhibited the activation of p38 MAPK caused by transfecting EL4.NOB-1 cells with the expression vectors for wild type MyD88, IRAK, and TRAF6, which provide further evidence that the effect of Ras occurs at a point in the signaling pathway which lies downstream of MyD88, IRAK, and TRAF6. The activation of p38 MAPK by MKK3 and MKK6, on the other hand, was not inhibited when co-transfecting these kinases with dominant negative RasN17. This strongly indicates that Ras lies upstream of the MKK3 and MKK6 but downstream of TRAF6.

Constitutively Active RapV12 Inhibits the Activation of p38 MAPK by MyD88, IRAK, and TRAF6, and Dominant Negative Rap1A Prolongs the Activation of p38 MAPK by IL-1—We next

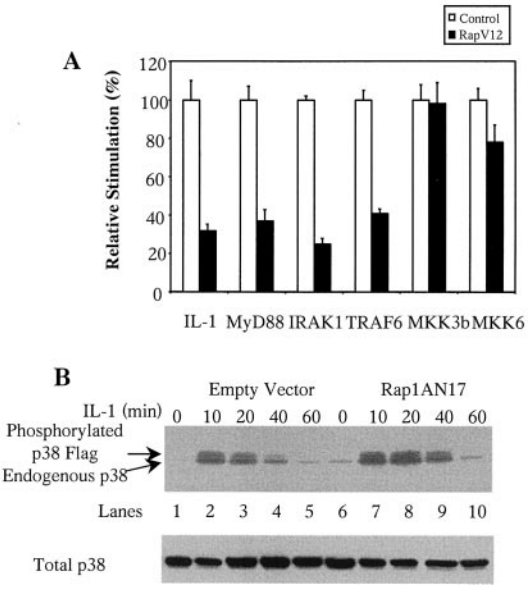

FIG. 3. **Effect of RapV12 on p38 MAPK activation by IL-1 signaling components.** *A*, 7×10^6 EL4.NOB-1 cells were co-transfected with the components of the GAL4-CHOP$^{(1-101)}$ system together with RapV12 and constructs encoding wild type MyD88, IRAK1, TRAF6, MKK3, or MKK6 as described under "Experimental Procedures." Transfected cells (5×10^5 in 0.25 ml/sample) were stimulated with IL-1 for 6 h where indicated, the cells were lysed, the luciferase activity of each sample was measured, and readings were normalized for transfection efficiency by measuring β-galactosidase activity as described under "Experimental Procedures." For clarity, the stimulation by IL-1 or transfected wild type proteins are set at 100%, the inhibition by RapV12 being shown relative to this, expressed as mean ± S.E. for quadruplicate determinations. Similar results were obtained in three further experiments. *B*, HeLa cells (seeded at 1.5×10^5 cells in 3 ml of complete DMEM medium 24 h prior to transfection) were co-transfected with FLAG-tagged p38 MAPK and 2.5 μg of RapN17 using FuGENE™ transfection reagent as described under "Experimental Procedures." 48 h after transfection the cells were stimulated with IL-1 for indicated times, harvested, and Western blotting was performed on the cell extracts, detecting transfected and endogenous p38 MAPK using an anti-phosphorylated p38 MAPK antibody, as described under "Experimental Procedures."

examined the position of Rap on the pathway, again utilizing the CHOP-Gal4 reporter system. As shown in Fig. 3*A*, co-transfecting EL4.NOB-1 cells with constitutively active RapV12 together with MyD88, IRAK, TRAF6, MKK3, and MKK6 indicated that RapV12 can inhibit the activation of p38 MAPK by all of these apart from MKK3 and MKK6. This suggests that the inhibitory effect of Rap occurs at a point between TRAF6 and MKK3/MKK6.

In addition, we investigated the effect of dominant negative RapN17 on the transient nature of the p38 MAPK activation in response to IL-1. We had previously shown that Rap is activated later than Ras and may act to limit p38 MAPK activation by IL-1 (34). To test this we examined the effect of RapN17 on the time course of p38 MAPK activation. As shown in Fig. 3*B*, p38 MAPK is activated in HeLa cells after a few minutes, peaking at 10–15 min after stimulation with IL-1. The signal is transient and reaches basal levels again after 40–60 min (Fig. 3*B*, *lanes 1–5*). When cells were transfected with dominant negative RapN17, this transient nature of the p38 MAPK response to IL-1 was delayed. Comparing *lane 4* to *lane 9* (Fig. 3) illustrates that RapN17 causes the phosphorylation of both endogenous as well as overexpressed FLAG-p38 MAPK to be more pronounced at the later time points, and also stronger at the earlier time points (compare *lane 3* to *lane 8*, Fig. 3*B*). Equal amounts of total p38 MAPK were ensured by blotting for p38 MAPK (Fig. 3*B*, *lower panel*). Rap may therefore play a role in the transient nature of this response.

Active Ras Associates with IRAK, IRAK2, and TRAF6—We next tested whether Ras could associate with any of the components of the IL-1RI complex. We transiently expressed constructs encoding tagged versions of MyD88, IRAK, IRAK2, and TRAF6 together with active RasVHa or dominant negative RasN17 into HeLa cells. Using a Ras antibody, overexpressed Ras could be immunoprecipitated, and associated components of the IL-1RI complex could be detected by immunoblotting. When RasVHa or RasN17 were coexpressed with AU1-tagged MyD88, no association could be detected when Ras was immunoprecipitated and the samples were blotted for MyD88, suggesting that active Ras is not part of a complex containing MyD88 (not shown). Also, the same results were obtained when the converse experiment was performed whereby AU1-tagged MyD88 was immunoprecipitated and Ras was detected by Western blotting (not shown). On the other hand, when RasVHa was co-expressed with IRAK, myc-IRAK2, or Flag-TRAF6 a clear co-immunoprecipitation could be detected (Fig. 4*A*, *C*, and *E*, *upper panels*, *lane 3*). Immunoprecipitation using an antibody for IRAK, myc-IRAK-2, or Flag-Traf-6 demonstrated that each were present in the immunoprecipitate (Fig. 4, *A*, *C*, and *E*, *upper panels*, *lanes 1*), and a negative control was provided by immunoprecipitation using a mouse IgG (Fig. 4, *A*, *C*, and *E*, *upper panels*, *lanes 2*). Equal transfection efficiencies was ensured by blotting cell lysates for IRAK, myc-IRAK2 and Flag-TRAF6 (Fig. 4, *A–F*, *lower panels*) or for total Ras (not shown). Several bands were detected when immunoblotting for IRAK, with the major bands occurring at molecular weights corresponding to phosphorylated (100,000) and the faster migrating unphosphorylated IRAK (83,000) as shown. RasVHa here associates with both forms of IRAK (Fig. 4*A*, *lane 3*).

The same co-immunoprecipitation experiments were performed using dominant negative RasN17 (Fig. 4, *B*, *D*, and *F*). RasN17 has been shown to have a higher affinity for GDP compared with GTP, but is also known to be unable to interact with downstream target proteins within the cell. We used it here to investigate for associations with resting, GDP-bound Ras. When overexpressed, RasN17 was not able to co-immunoprecipitate IRAK or TRAF6 (Fig. 4, *B* and *F*, *upper panels*, *lanes 3*), although a weak association with IRAK2 could be detected (Fig. 4*D*, *upper panel*, *lane 3*).

TAK1 Is Required for the Activation of p38 MAPK by IL-1 and TAK1 Associates with Active Ras—Several recent studies have reported on a signaling complex consisting of IRAK, TRAF6, TAB1, TAB2, and TAK1 forming in response to IL-1 (11, 41). TAK1 is a kinase, which has been shown to directly phosphorylate MKK6, an upstream activator of p38 MAPK. In light of this we next investigated the role of TAK1 here. Overexpressing TAK1, alone (not shown) or in combination with TAB1, caused an increased activation of p38 MAPK as seen in Fig. 5*A*. Activation of p38 MAPK by IL-1 was inhibited in the presence of kinase inactive ΔTAK1, which acts as a dominant negative. Overexpression of TAK1 also increased the phosphorylation of both FLAG-tagged p38 and endogenous p38 (Fig. 5*B*, *lane 4*). In addition, ΔTAK1 inhibited the effect of IL-1 on the phosphorylation of p38 (Fig. 5*B*, compare *lane 3* to *lane 2*), agreeing with the data shown in Fig. 5*A*.

As TAK1 has been shown to be part of a complex together with TRAF6 and IRAK, which forms in response to IL-1, we next investigated if Ras could associate with a TAK-1-containing complex. As can be seen in Fig. 5*C*, *upper panel*, *lane 3*, RasVHa coimmunoprecipitates with Flag-TAK1. RasN17 on the other hand showed no such association TAK1 (Fig. 5*D*, *upper panel*, *lane 3*). Equal transfection efficiencies were ensured by blotting the cell extracts for Flag-TAK1 (Fig. 4, *B* and *C*, *lower panels*) and Ras (not shown).

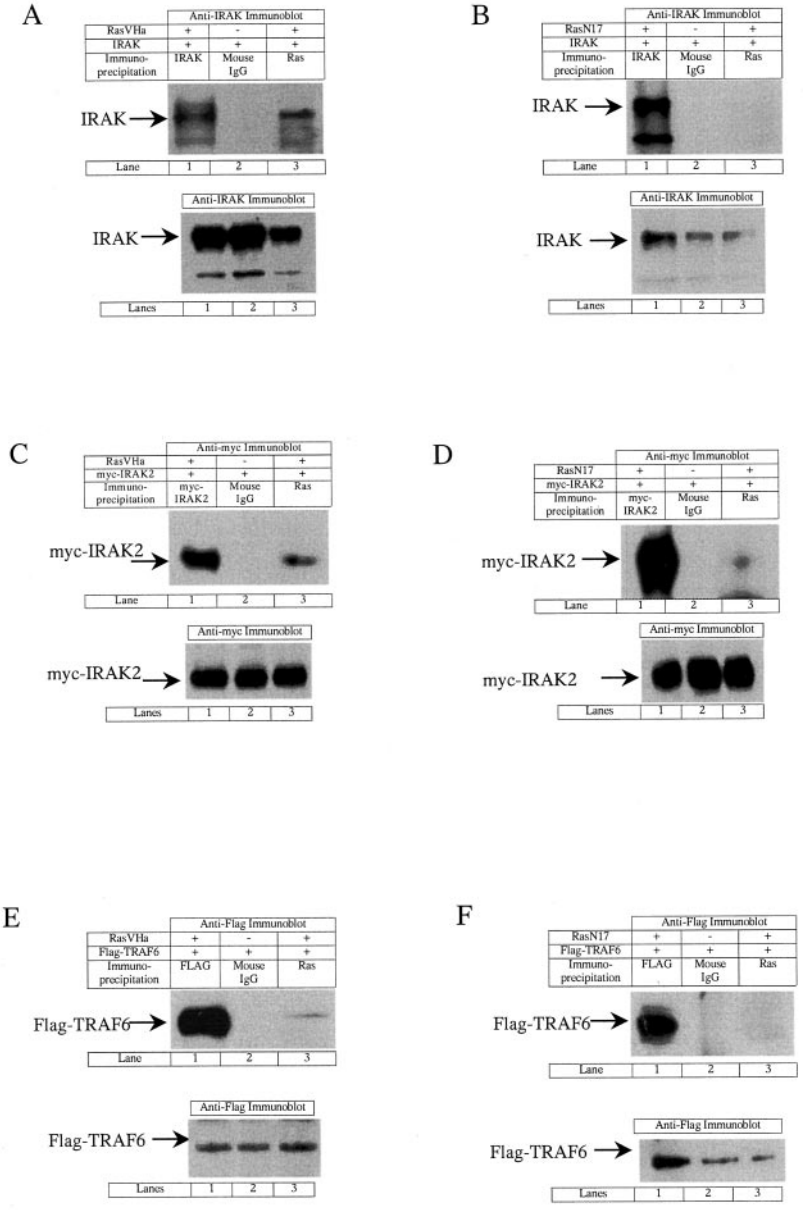

FIG. 4. **RasVHa associates with overexpressed IRAK1, IRAK2, and TRAF6 in HeLa cells.** HeLa cells were seeded at 1×10^6 cells per sample. The cells were transfected 24 h later with indicated expression vector constructs. *A–F*, *upper panels*, HeLa cells were transfected with 4 μg of plasmids encoding RasVHa (*A*, *C*, and *E*) or RasN17 (*B*, *D*, and *F*) (*lanes 1* and *3* in each panel) and 4 μg of plasmids encoding IRAK, myc-IRAK-2, or Flag-TRAF6 (all lanes) as indicated. Antibodies recognizing Ras (each *lane 3*), mouse preimmune IgG control (each *lane 2*), or IRAK, Myc, or FLAG (positive control, each *lane 1*) were used for immunoprecipitation and IRAK, myc-IRAK-2, and Flag-TRAF6 were detected by immunoblotting using an anti-IRAK, anti-Myc, or anti-FLAG antibodies, respectively. *A–F*, *lower panels*, equal transfection efficiencies were ensured by blotting cell extracts for IRAK, IRAK2, or TRAF6 using an anti-IRAK, anti-Myc, or anti-FLAG antibody. Results show one representative of three identical experiments performed.

We also attempted to order the relationship between Ras and TAK-1 by examining the effects of dominant negative mutant forms on corresponding active forms. Dominant negative ΔTAK1 inhibited the activation of p38 in response to RasVHa by 30–40% (not shown). Similarly, dominant negative RasN17 blocked the stimulatory effect of TAK1 overexpression by 30–40% (not shown). This implied that there was a co-dependence between the 2 proteins in driving this signal, which is being explored further. Taken together our data suggest that active Ras can associate with a multiprotein complex containing IRAK, IRAK-2, Traf-6, and TAK-1.

DISCUSSION

Although IL-1 is a well known activator of p38 MAPK, the signaling cascade leading to p38 MAPK activation by IL-1 has not been fully elucidated. The small G protein Ras has been shown to play a key role in the activation of p38 MAPK by multiple stimuli, including hemopoietic cytokines, platelet-derived growth factor, and fibroblast growth factor (15, 42, 43) and we recently demonstrated that Ras plays an important role in the activation of p38 MAPK by IL-1, with Rap having an inhibitory role (34). Here we have attempted to further elucidate the cascade leading from IL-1RI to the activation of p38 MAPK, and to determine the roles and relative positions of Ras and the negative regulator Rap on this pathway.

Dominant negative mutants of the IL-1 signaling components MyD88, IRAK, IRAK-2, and TRAF6 all inhibited the activation of p38 MAPK by IL-1. Furthermore, overexpressing wild type versions of these proteins caused an increase in p38 MAPK activity. These proteins therefore participate in both NF-κB and p38 MAPK activation by IL-1. p38 MAPK was activated by overexpressing constitutively active RasVHa, and this activation was inhibited by overexpressing dominant negative MKK3(A) or MKK6(A). This suggests that Ras activates p38 MAPK from a point in the pathway that occurs upstream of these kinases. Dominant negative MyD88, IRAK, IRAK-2, and TRAF6 were unable to inhibit the activation of p38 MAPK by RasVHa. Accordingly, overexpression of dominant negative RasN17 not only inhibited the activation of p38 MAPK by IL-1, but also inhibited the activation caused by overexpressing wild type MyD88, IRAK1, and TRAF6, but not MKK3 and MKK6. Taken together, these results show that IL-1 activates p38 MAPK on a pathway involving MyD88, IRAK, IRAK-2, TRAF6,

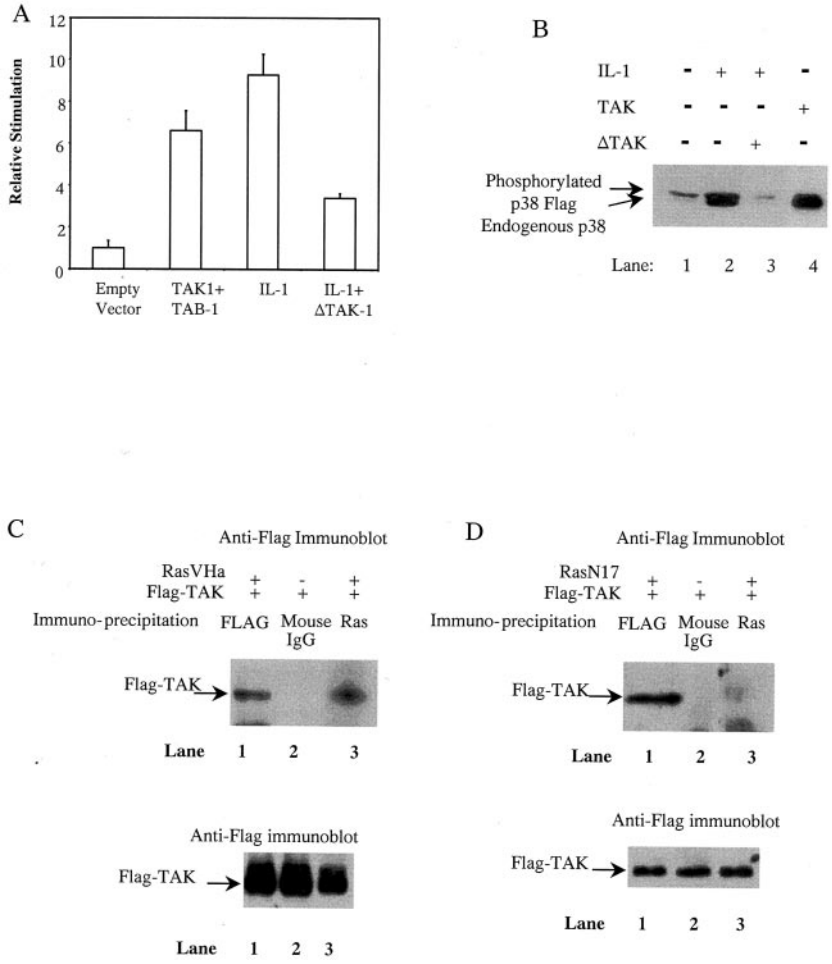

FIG. 5. **TAK-1 plays a role in the activation of p38 MAPK by IL-1, and associates with active Ras in HeLa cells.** *A*, 7×10^6 EL4.NOB-1 cells were transfected with the components of the GAL4-CHOP$^{(1-101)}$ system together with wild type TAK-1 together with TAB-1, or a mutant version of TAK-1, ΔTAK-1, as described under "Experimental Procedures." Transfected cells (5×10^5 per sample) were stimulated with 10 ng/ml IL-1α for 6 h where indicated after which the cells were lysed, the luciferase activity of each sample was measured, and readings were corrected for transfection efficiency by measuring β-galactosidase activity as described under "Experimental Procedures." One representative experiment of three identical experiments performed is shown expressed as mean ± S.E. fold increase over control for samples assayed in quadruplicate. *B*, HeLa cells (seeded at 1.5×10^5 cells in 3 ml of complete DMEM 24 h prior to transfection) were transfected with FLAG-tagged p38 MAPK, together with 5 μg of plasmids encoding wild-type TAK-1 or mutant ΔTAK-1 according to the method of FuGENE™ as described under "Experimental Procedures." 48 h after transfection the cells were stimulated with IL-1 for 10 min. Cells were harvested, lysed, and Western blotting was performed, detecting activated transfected and endogenous p38 MAPK using an anti-phosphorylated p38 MAPK antibody as described under "Experimental Procedures." In each case, identical results were obtained in a further experiment. *C* and *D*, HeLa cells were seeded at 1×10^6 cells per sample. The cells were transfected 24 h later with indicated expression vector constructs. *Upper panels*, HeLa cells were transfected with plasmids encoding 4 μg of RasVHa or RasN17 (*lanes 1* and *3*) and 4 μg of plasmid encoding Flag-TAK-1 (all lanes) as indicated. Antibodies recognizing Ras (each *lane 3*), mouse preimmune IgG control (each *lane 2*), or FLAG (positive control, each *lane 1*) were used for immunoprecipitation and Flag-TAK-1 were detected by immunoblotting using an anti-Flag antibody. *Lower panels*, equal transfection efficiencies were ensured by blotting cell extracts for TAK-1 using an anti-FLAG antibody or Ras (not shown). Identical results were obtained in a further experiment.

Ras, MKK3, and MKK6, with Ras participating at a point downstream of TRAF6 but upstream of MKK3 and MKK6.

Our study also suggests that the inhibitory effect of Rap on p38 MAPK activation by IL-1 occurs at the same level as that of Ras, since active RapV12 blocked the effect of MyD88, IRAK, and TRAF6 but not MKK3 or MKK6. Of the dominant negative versions of these proteins, only MKK3 and MKK6 inhibited the stimulatory effect of dominant negative RapN17. Rap activation by IL-1, which we have previously demonstrated occurs at a later time point to Ras (34), may be responsible for the transient activation of p38 MAPK by IL-1. This hypothesis was supported by the observation that transfecting cells with dominant negative RapN17 led to a prolonged activation profile of p38 MAPK in response to IL-1. The basis for the inhibitory effect of Rap is not clear. Rap has been shown to interfere with p42/p44 MAPK activation by Ras (44) and in a recent study by Mochizuki *et al.* (45), the activation of Rap by epidermal growth factor was found to occur in the center of the cell, as opposed to the plasma membrane, where Ras activation occurs. The authors therefore suggested a mechanism whereby Rap1 may interfere with Ras signaling by retaining Ras effectors away from the site of active Ras, reducing the effect of Ras on downstream events. This may also be the case in IL-1 signaling.

Precisely how IL-1 is activating Ras and Rap is also not known. The best studied mechanism of Ras activation is in growth factor signaling where ligands cause receptors to autophosphorylate, and bind SH2 domains of adapters such as Grb2, which recruits guanine nucleotide exchange factors for Ras (46, 47). When activated, the effector domain of Ras interacts with the Ras-binding domain of Raf-1 (48), which then interacts with the downstream kinases MKK 1 and MKK 2 (49), leading to p42/p44 MAP kinase activation. Although IL-1 signaling differs from growth factor signaling in that no receptor tyrosine phosphorylation has been found (50), we and others have previously shown that IL-1 activates Ras. Caunt *et al.* (51) have implicated the focal adhesion complex in Ras activa-

tion by IL-1 consistent with studies demonstrating that IL-1RI and IRAK are recruited into focal adhesions (52, 53). Ras may then activate a MAPKKK which would lead to activation of MKK3 and/or MKK6. MAPKKKs that have been shown to play a role in p38 MAPK signaling include PAK (31), TAK1 (32), ASK1 (33), and mixed-lineage kinase MLK-3 (36).

Recent findings by Wang *et al.* (11) illustrate that signaling pathways elicited by IL-1 may not be of a simple linear fashion, but rather dependent on the formation of a multiprotein complex. In their study they show that the signaling activity of a complex consisting of TAK1, TAB1, and TAB2 is directly dependent on TRAF6 and a ubiquitin-conjugating enzyme complex (composed of Ubc13 and Uev1A). Furthermore, the direct phosphorylation of MKK6 at Ser^{207} and Thr^{211} by ubiquitinated TAK1 has been demonstrated. Takaesu *et al.* (41) have recently shown that IL-1 can induce the association of a complex consisting of IRAK, TRAF6, TAB2, and TAK1. In this study we found that RasVHa (which is in the GTP-bound state) but not RasN17 (which is GDP-bound) associates with IRAK, IRAK2, TRAF6, and TAK1, indicating that Ras is in the multiprotein complex formed in response to IL-1. We are currently investigating whether Ras plays a role in the formation of this multiprotein complex, promoting activation of TAK-1 by Traf-6, Ubc13, and Uev1A. Our evidence that dominant negative Ras could partially block p38 activation by TAK1, and that dominant negative TAK1 could partially block p38 activation by active Ras would imply a co-dependence between the proteins in this pathway. The inability of either dominant negative protein to fully inhibit the response of corresponding active forms is not clear, but suggests that in the case of dominant negative TAK1, other MAPKKKs can still activate MKK3/6 in response to Ras. This is currently under investigation. Another example of redundancy in the pathway would be in the failure of lethal toxin (which inactives Ras), to inhibit degradation of IκB, as shown in a previous study (34). The would suggest that although TAK1 may be involved in this process, an alternative route to the IKK complex must exist in IL-1-treated cells, as treatment with lethal toxin would be expected to block TAK1 activation. This is also being studied further.

In conclusion, our study illustrates a novel signaling pathway leading to the activation of p38 MAPK by IL-1. The pathway involves MyD88, IRAK, IRAK2, TRAF6, Ras, TAK1, MKK3, and MKK6, with a negative down-regulatory role for Rap, occurring at the level of Ras on the pathway. The study suggests that Ras takes part in the formation of a multiprotein signaling complex made up of IRAK, IRAK2, TRAF6, Ras, and TAK1, which conveys the signal leading to the activation of p38 MAPK by IL-1.

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