In the present study, we show that Ras activity differentially controls interleukin (IL)-1-induced transcription factor activation by selective regulation of responses mediated by receptor complex components. Initial experiments revealed that stimulation with IL-1 caused a rapid, matrix-dependent activation of Ras. The effect was transient, peaking at 5 min and returning to base levels after 30 min. Activation correlated with pronounced changes in cell shape in EGFP-Ras-transfected cells. Transfection with the dominant negative mutant, Ras⁴⁰⁴¹⁷, inhibited IL-1-induced activation of the IL-8 promoter as well as of NF-κB and AP-1 synthetic promoters in transient transfection assays. Furthermore, overexpression of the IL-1 signaling proteins TRAF6 or MyD88 gave characteristic activation of IL-8, which was accentuated in the presence of IL-1. Co-transfection with Ras⁴⁰⁴¹⁷ gave a dose-dependent inhibition of TRAF6-induced responses in the presence and absence of IL-1, but had no effect on MyD88 mediated activity. Similarly, induction of NF-κB was abolished by Ras⁴⁰⁴¹⁷ only in TRAF6-transfected cells. In contrast, inhibiting Ras activity limited AP-1-mediated responses through both receptor complex proteins. Constitutively active Ras⁴⁰⁴¹² increased the TRAF6-induced activity of the NF-κB pathway similar to the effect induced by IL-1, while the Ras⁴⁰⁴¹²-induced activity was not inhibited by co-transfection with a dominant negative TRAF6. Our data show that activation of the Ras GTPase is an early, matrix-dependent response in IL-1 signaling which participates in structural regulation of IL-1-induced genes. In addition, they show that the Ras induced effect selectively regulates TRAF6-mediated activation of the NF-κB pathway, suggesting that Ras GTPase represents a convergence point in structural and cytokine responses, with distinct effects on a subset of downstream signaling events.

Cellular responses to cytokines and growth factors are influenced by the extracellular milieu (1). These regulatory effects are mediated to a large extent by receptors of the integrin family and involve modulation of signaling pathways that link receptor activation and changes in cell shape, the cytoskeleton, and gene expression (2, 3). Ras acts cooperatively and/or hierarchically with the Rho subfamily of guanine nucleotide triphosphatases (GTPases) to regulate focal adhesion and polymerized actin turnover (4). It is thought to be in their arrangement of signaling proteins and receptors via the cytoskeleton that the GTPases are able to channel a diverse range of stimuli, from growth factor and cytokines to cell attachment, into common downstream signaling cascades (5) such as the stress-activated protein kinase (6, 7) and nuclear factor-κB (NF-κB) pathways (8, 9).

The membrane proximal molecular events that take place following interleukin-1 receptor (IL-1R) binding and leading to transcription factor activation involves initially the heterodimerization of the IL-1R with accessory protein (IL-1RACP) (10), followed by binding of the adaptor protein MyD88 (11). MyD88 in turn recruits the Ser/Thr kinases: IL-1R associated kinase (IRAK) 1, IRAK-2, and IRAK-3 or M (11–13), which have recently been shown to be pre-associated with a further adaptor protein, Tollip (14). Subsequently, the IL-1R associated kinase 1s are freed and interact with the tumor necrosis factor receptor-associated factor (TRAF) 6, followed by activation of the stress-activated protein kinase and NF-κB pathways (15).

The majority of IL-1 receptors are located at focal adhesions (16), and matrix attachment results in alterations in IL-1 receptor function (17). Subsequent IL-1-induced signal transduction and gene activation are regulated by cell attachment and the cytoskeleton (18–20). Early alterations following IL-1 receptor binding in adherent cells include a rapid and transient phosphorylation of transmembrane linkage protein Talin and changes in the cytoketone (21), suggesting an immediate effect on the turnover of structural components at these sites. Aspects of Ras, Rho, Rac, and Cdc42 involvement in downstream IL-1 signaling pathways have recently been elucidated (22–25), suggesting they play a part in mediating these structural changes localized around the receptor complex. This may underpin the, as yet, unclear mechanism by which the GTPases interact with members of the classical IL-1 signaling cascade. So far, however, little is still known of how these effects are initiated, and how they may modulate IL-1 signaling and influence downstream cascades.

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1 The abbreviations used are: NF-κB, nuclear factor-κB; IL-1R, interleukin 1 receptor; TRAF, tumor necrosis factor receptor-associated factor; IRAK, IL-1R associated kinase; CMV, cytomegalovirus; EGFP, epithelial growth factor; EGFP, enhanced green fluorescent protein.
We report here that IL-1-induced structural alterations in adherent cells involve activation of the Ras GTPase as an early signaling event, which regulates IL-1-induced NF-κB transcriptional activity of inflammatory genes. In addition, we show that Ras regulation of NF-κB affects TRAF6-mediated activation, but not that induced through MyD88, indicating that IL-1-induced pathways leading to NF-κB activation diverge upstream of TRAF6. Furthermore, the data suggest that Ras represents a convergence of IL-1 and matrix-mediated signaling events induced downstream of the adaptor proteins.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures and Transfection—**Human gingival (4) and skin fibroblast strains (1) (transfer 5–15) and HeLa cells were used. Cells were propagated in monolayer cultures in Dulbecco’s modified Eagle’s medium containing 5 mM sodium pyruvate, 100 μM penicillin and streptomycin, and 10% heat inactivated fetal calf serum (Life Technologies) in humidified atmosphere (95% air, 5% CO2) at 37 °C. Cells were plated subconfluently and grown for 1–3 days and transfected using the calcium phosphate co-precipitation method with glyceral shock (60 s, 20% glyceral in phosphate-buffered saline) or using Superfect (Qiagen). Transfection efficiency, measured by determining EGFP-C1 expression in confocal experiments or renilla levels using the TK-RL or CMV-RL vector: pEGFP-C1 (CLONTECH), to give fusion constructs: pEGFP-C1-(220–522) Rac1, pEGFP-C1-(289–522) TRAF6, pEGFP-C1-(289–522) TRAF6-(289–522) were constructed by subcloning a 220-base pair product of the primers: 5'-GGGGCTAGCATGACCGAATACAAGCTTGTTGTT-3' and 5'-GAAGATCTAACTTTCGTCATACTCCG-3'. The expression vectors pCMV-H-Ras and pCMV17Ras were constructed by PCR amplification of pEGFP-H-Ras and pEGFPN17Ras using the primers: 5'-GGGGCTAGCATGACCGAATACAAGCTTGTTGTT-3' (sense) and 5'-ACAGGCTATTTCAGGAGACGACACTT-3' (antisense); underlined residues indicate engineered restriction sites at 5'-GGTG-CAGCGGGTTCCTGGCCAC-3' and “Mutata-Gen” kit (Bio-Rad). The expression vectors pCMV-H-Ras and pCMV17Ras were constructed by PCR amplification of pEGFP-H-Ras and pEGFPN17Ras using the primers: 5'-GGGGCTAGCATGACCGAATACAAGCTTGTTGTT-3' (sense) and 5'-ACAGGCTATTTCAGGAGACGACACTT-3' (antisense); underlined residues indicate engineered restriction sites at 5'-GGTG-CAGCGGGTTCCTGGCCAC-3' and “Mutata-Gen” kit (Bio-Rad). The expression vectors pCMV-H-Ras and pCMV17Ras were constructed by PCR amplification of pEGFP-H-Ras and pEGFPN17Ras using the primers: 5'-GGGGCTAGCATGACCGAATACAAGCTTGTTGTT-3' (sense) and 5'-ACAGGCTATTTCAGGAGACGACACTT-3' (antisense); underlined residues indicate engineered restriction sites at 5'-GGTG-CAGCGGGTTCCTGGCCAC-3' and “Mutata-Gen” kit (Bio-Rad). The expression vectors pCMV-H-Ras and pCMV17Ras were constructed by PCR amplification of pEGFP-H-Ras and pEGFPN17Ras using the primers: 5'-GGGGCTAGCATGACCGAATACAAGCTTGTTGTT-3' (sense) and 5'-ACAGGCTATTTCAGGAGACGACACTT-3' (antisense); underlined residues indicate engineered restriction sites at 5'-GGTG-CAGCGGGTTCCTGGCCAC-3' and “Mutata-Gen” kit (Bio-Rad).

**Confocal Fluorescence Microscopy—** Fibroblasts (10^4/10-cm dish) were transfected using 6.6 μg of total DNA/dish and replated onto fibronectin-coated or bare tissue culture plastic 8-well slides (Nunc). Cells were visualized using a Molecular Dynamics confocal laser scanning microscope with a 37 °C stage incubator coupled to a Nikon Diaphot microscope and a Silicon Graphics work station. Localization of the various agonists and inhibitors were consistent between the different treatments, and cross-sectional area was measured using NIH image. For the quantitation of RelA (p65) translocation, cells were transfected with EGFPRelA and CMV-H-Ras and analyzed as in Ref. 29. Briefly, nuclear and cytoplasmic protein levels were quantitated by scanning horizontal sections through the nucleus of transfected cells. Using NIH image, relative fluorescence was calculated by measuring the mean intensity of representative areas of nuclei and cytoplasm and averaging by the attending horizontal setting.

**Luciferase Reporter Assays—** Fibroblasts (1.5 × 10^4/well) were plated in 24-well plates and transfected with 2 μg of total DNA/dish, including 1 μg of luciferase reporter plasmid, containing the IL-8 promoter or NF-κB/AP-1 binding elements alone, and 1 μg of CMV expression vectors including 0.5 μg of CMV-H-Ras or CMV17Ras and 0.4 μg of CMV-RL internal control. Remaining DNA was made up using pcDNA3.1 (empty control vector). HeLa cells (6 × 10^6/100 mm) were plated in 24-well plates and transfected with 1.125 μg of total DNA/dish including 0.5 μg of luciferase reporter plasmid, 0.375 μg of expression vectors or pcDNA3.1 (empty control vector) and 0.3 μg of TK-RL internal control. Amounts of transfected IL-1 signaling proteins (1.25 μg of TRAF6 or MyD88) was based on previous data showing maximal stimulation of luciferase levels (30 ng (fibroblasts) or 24 h (HeLa cells)) after transfection, cultures were stimulated with 10^{-13}, 10^{-12}, or 10^{-9} M IL-1β, 6 h before lysis in Passive Lysis Buffer (Promega), transferred to microtiter plates (Dynex) and assayed for luminescence intensity using Standard Luciferase Assay and Stop “n” Glo Substrates (Promega) and an MLM3000 plate reader (Dynatech). Control for matrix requirement, using cultures plated on bare plastic, showed no induction of activity by IL-1. Induction of the IL-8, NF-κB, and AP-1 luciferase reporter constructs ranged between 15–50, 2–6.4, and 1.5–3-fold, on stimulation with 1 nM IL-1, 6 h, respectively. These differences primarily reflect variations in basal levels of activity. Importantly, the variations in absolute fold induction reflects the difference between separate experiments, however, the relative increase and reduction induced by the various agonists and inhibitors were consistent between experiments.

**RESULTS**

**IL-1 Induces an Attachment-dependent Activation of Ras—** The effect of IL-1 on activation of the Ras GTPase was deter-
Ras Mediates IL-1-induced Shape Changes in Attached Cells—Confocal microscopy and serial observations of single cells demonstrated that IL-1 stimulation resulted in pronounced and successive changes in shape of cells transfected with EGFPRas, correlating with the IL-1 induced Ras activity (Fig. 2A). The reduction in cross-sectional area coincided with an apparent concentration of the fusion protein at the plasma membrane, which in unstimulated cells, was the same as that of the endogenous protein (4). In comparison, transfection with EGFRac1, EGFPcde42, or EGFPRhoadA had no effect on cell shape or localization of the protein (data not shown). The Ras-induced changes were nontransient and constituted retraction of the peripheral flattened areas followed by progressive rounding, initially occurring at 5–15 min, and resulting in reduction in cross-sectional area of about 50% after 60 min (Fig. 2B). Less pronounced effects were observed following Ras transfection alone without matrix attachment or IL-1 stimulation, and total inhibition of the IL-1 induced changes was noted following transfection with a dominant negative mutant of EGFP-Ras (EGFPN17Ras) (Fig. 2B).

Ras Mediates Activation of IL-1 Responsive Genes—The involvement of Ras in IL-1-induced gene activation was demonstrated by co-transfection experiments. These experiments showed a pronounced enhancement in IL-1 induced activation of the IL-8 promoter in the presence of excess Ras (Fig. 3A) demonstrating additive effects over a range of IL-1 concentrations. Transfection of cells with RasAON-17 prior to stimulation with IL-1 abolished activation of the IL-8 promoter, and caused a marked decrease in basal levels of transcription in fibroblasts (Fig. 3A) showing a direct effect of Ras in IL-1 mediated gene regulation. RasAON-17 similarly inhibited induction of the IL-8 promoter in HeLa cells (Fig. 3B), resulting in a reduction in transcription of around 80%. Similar experiments using an NF-κB synthetic promoter showed the same level of increase by Ras transfection, demonstrating additive effects in the fibroblast cell lines (Fig. 3C) but with no net effect on nuclear translocation of EGFPRelA assessed by confocal microscopy (data not shown). In addition, comparing the effects of NF-κB and AP-1 using synthetic promoters showed that the negative mutant RasAON-17 caused a reduction of the IL-1β-mediated responses at saturated levels of ligand of about 60% for both transcription factors (Fig. 3D), demonstrating involvement of Ras in IL-1-induced gene regulation through both the NF-κB and AP-1 pathways.

Ras Regulated IL-1-induced Gene Transcription Is Mediated through TRAF6—Further analyses focused on the upstream regulatory mechanism involved in mediating the Ras-induced response and the role of the adapter proteins TRAF6 and MyD88 in structural regulation of the IL-1-induced pathways. For these experiments, cells were transfected with the IL-8 promoter reporter and signaling protein constructs. Consistent with previous results from this laboratory, transfection with both TRAF6 and MyD88 typically resulted in activation of the IL-8 promoter corresponding to 70–120-fold and 20–30-fold induction, respectively (30) (Fig. 4A). Co-transfection with RasAON-17 demonstrated a dose-dependent inhibition of the TRAF6-mediated response of up to about 60%. In contrast, transfection with RasAON-17 had no effect on the MyD88 induced activity (Fig. 4A). Furthermore, we tested potency of the RasAON-17 inhibitory effect in the presence of IL-1 stimulation and adaptor protein overexpression. Similar to the observations in unstimulated cultures, co-transfection with the TRAF6 containing construct resulted in enhancement of the IL-1 induced activity. Thus, addition of IL-1 in the presence of TRAF6 showed massively elevated levels of activation, increasing induction from 70–120- to 300–370-fold (Fig. 4B), suggesting a

Data shown are the averages of two experiments.

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**Fig. 1.** IL-1 induces activation of Ras in fibronectin-attached cells. A, primary fibroblasts were replated subconfluently on fibronectin coated 10-cm Petri dishes for 3–4 h, and labeled with [32P] (200 μCi/ml, 1 h) prior to stimulation with 1 nm IL-1β or 40 ng/ml EGF, at the times indicated, and subsequently lysed and immunoprecipitated at the times indicated with primary and secondary antibody or with 2a ab alone (control lane), as described under “Experimental Procedures.” GTP and GDP-bound Ras fractions were separated by thin layer chromatography and detected by autoradiography. B, quantitation of autoradiographs obtained as in A was carried out using NIH image. Data are expressed as fold increases in GTP/GDP-bound Ras after stimulation for various times, as indicated, relative to nonstimulated samples. Data shown are the averages of two experiments. FN, fibronectin; TC, tissue culture plastic.

Ras determined by immunoprecipitation and thin layer chromatography, comparing fibroblasts plated on fibronectin-coated plates or on bare tissue culture plastic. Analysis of the ratio of GTP to GDP bound Ras showed that stimulation with 1 nm IL-1 induced a matrix dependent increase in endogenous Ras activity. Thus, in the presence of fibronectin attachment IL-1 induced a 4–5-fold increase in activity within 5 min (Fig. 1, A and B). The response was transient and the activity returned to base levels by 30 min. The level of activity was comparable, but less pronounced than that induced by stimulation with EGF, a potent activator of Ras (31). EGF also caused a more sustained activity, which was maintained throughout the experiment. In contrast, cells on bare tissue culture plastic showed no change in the levels of GDP-bound Ras during 60 min stimulation with IL-1 but demonstrated the same base levels prior to stimulation as cells plated for 4 h on the fibronectin matrix (Fig. 1B).
synergistic effect with the soluble agonist, while transfection with MyD88 had no significant effect on the IL-1 response. Furthermore, the presence of Ras<sub>N17</sub> caused a pronounced reduction in the levels of TRAF6 + IL-1 induced activity corresponding to around 50%. In contrast, and similarly to the nonstimulated levels of activity, activation induced by IL-1 in MyD88-transfected cells was unaffected by co-transfection with Ras<sub>N17</sub>. Levels of TRAF6 + IL-1 activation of IL-8 promoter activity, even in the presence of Ras<sub>N17</sub>, were still significantly higher than those induced by 1 nM IL-1 or TRAF6 alone, agreeing with the notion that Ras regulation of IL-1 responses is mediated through particular facets of the signaling complex.

**NF-κB Regulation by Ras Is Induced through TRAF6**—Identical experiments using a synthetic NF-κB responsive promoter demonstrated lower fold induction due to higher base levels, and resulted in a 15–20-fold increase in activity in cells co-transfected with a TRAF6 containing construct. The dominant negative Ras construct, as previously, caused a concentration dependent inhibition of this TRAF6 induced activation, corresponding to 60 and 80% at 0.125 and 0.25 µg of CMVN17Ras, respectively (Fig. 5A). Again, the lower level of induction of NF-κB activity by MyD88 was unaffected by co-transfection with Ras<sub>N17</sub>; demonstrating a selectivity of Ras regulation of NF-κB responses at the level of receptor complex components. The correlation between the observations using the NF-κB and the complete construct agrees with a primary regulation of this promoter through NF-κB (32). In contrast, in similar experiments using an AP-1 promoter, inhibition of Ras activation had a pronounced effect on both TRAF6- and MyD88-induced responses resulting in levels of 10 and 20% of that in the controls at higher concentrations of Ras<sub>N17</sub> (Fig. 5B). The specific involvement of TRAF6 in Ras-mediated NF-κB responses was further confirmed by using a constitutively active Ras mutant, Ras<sub>Val-12</sub>, which gave a weak induction of NF-κB activity (2–3-fold) (Fig. 5C). Co-transfection in cells transfected with TRAF6 had a pronounced effect resulting in a 25–35-fold enhancement of the response, significantly higher that would be induced by additive effects, and indicating synergy of the response. In contrast, the constitutively active Ras construct had no effect on the levels induced by MyD88, supporting the notion that Ras regulation of NF-κB involves selective interactions between receptor complex components.

**Ras Regulation of the NF-κB Pathway Is Induced Downstream of TRAF6**—IL-1 stimulation of TRAF6-transfected cells resulted in a more than additive effect on induction of NF-κB activity (Fig. 6). Furthermore, to the effect on the non-IL-1 induced levels, co-transfection with Ras<sub>N17</sub> resulted consistently in a 75–80% reduction of the NF-κB activity. In contrast, the IL-1-mediated response induced following MyD88 transfection was no more than additive and consistent with results using the IL-8 promoter, no significant inhibition was observed on co-transfection with Ras<sub>N17</sub>. In comparison to the pronounced effects induced on the TRAF6-mediated response by blocking Ras activity, a dominant negative TRAF6, ΔTRAF6(289–522) (15), had no effect on Ras induced activation of NF-κB. Thus, co-transfection with ΔTRAF6(289–522), failed to inhibit the 3-fold enhancement in NF-κB transcription mediated by the constitutively active Ras<sub>Val-12</sub> (data not shown), suggesting that while Ras involvement is mediated through TRAF6 at the level of the receptor, its subsequent regulation of the NF-κB pathway is induced downstream of the adaptor proteins.

**DISCUSSION**

In the present study, we demonstrate that IL-1 transiently activates Ras in a matrix-dependent fashion, correlating with translocation of EGFP-H-Ras fusion protein and structural effects on IL-1 responses in transfected cells. We also show that the regulation of IL-1-mediated NF-κB activity by Ras is induced through TRAF6.

The IL-1-induced Ras activation occurs during the initial steps of signal transduction involving heterodimerization of the receptor and the accessory protein and in matrix-attached cells, recruitment of a heparan sulfate to the receptor complex. Association of the adaptor protein MyD88 results in activation of the receptor IRAKs and association of TRAF6 (see Fig. 7). The observed Ras activity correlate with the similarly rapid, matrix-dependent IL-1 induced serine phosphorylation of the transmembrane linkage protein, Talin preceding the alterations in cell structure and the changes in the cytoskeleton (20, 21). Such alterations are known to be directly associated with events related to GTPase activation (33). These data agree with recent
findings of essential roles for other members of the GTPase family in IL-1 signaling (22–25), and together with the known interdependence between members of the Ras and Rho subfamilies suggest that their roles in IL-1-mediated responses to be interconnected.

Our data further suggest that the IL-1 effect involves an increase in the concentration of the GTPase at the plasma membrane during activation. This is likely, to a significant extent, to be a consequence of the reduction in cell shape. However, Ras isoforms have been shown to have the ability to rapidly diffuse through the plasma membrane (34) and an increase in local concentration at the cell surface could reflect recruitment to specific areas to facilitate signal transduction. This could involve a mechanism similar to that demonstrated for Ras, Rac, and Rho during integrin and growth factor signaling, when localization to caveolin-rich regions in pre-assem-
bled complexes is a prerequisite for signal transduction (35–37).

The rapid induction of Ras activation suggests that it is induced through an immediate, receptor-associated event, and could be a direct consequence of the early IRAKs and TRAF6 association. This likely involves receptor-associated regulators of the GTPases such as the putative Rap/GTPase activating protein (GAP), IIP-1 (38). Furthermore, the attachment dependence suggests that activation could be induced as a consequence of recruitment of the matrix-dependent, accessory receptor component to the IL-1 receptor complex (17) and thus result from co-regulation through integrin mediated activities. Such effects could be induced following selective activation of signaling components by structural events, as suggested by our data demonstrating specificity for the activities mediated through receptor-associated proteins MyD88 and TRAF6. This type of collaboration would thus be similar to that reported for MyD88 and Tollip during recruitment to the IL-1 receptor (14).

We show that IL-1-induced NF-κB mediated transcription of IL-8 is dependent on Ras activity, demonstrated by using wild type and dominant negative mutant forms of Ras in transient transfection reporter assays. This agrees with previous findings from this laboratory, showing a direct dependence of IL-1 induced inflammatory genes (19) and NF-κB activation (18, 20) on cell architecture and attachment. These effects of Ras could be mediated through MEKK (39) with subsequent effects on the NF-κB pathway (40–42) resulting from induction of IκBα phosphorylation (43). However, the lack of effect of nuclear translocation of RelA could reflect that Ras, as has been suggested for Rac1 (24), may regulate subsequent transactivation events such as phosphorylation of nuclear NF-κB (44). In addition, the burgeoning evidence of complexity of NF-κB activity, involving association of nuclear IκBα-NF-κB complexes subsequent to independent translocation (45) and regulation through RelA shuttling suggests that significant activation of the pathway may be induced without any net effect on the level of nuclear RelA. This is also supported by results from studying the endogenous protein demonstrating that activation of the pathway does not necessarily correlate with an absolute in-

\[ \text{FIG. 4. The dominant negative Ras}_{\text{Asn-17}} \text{ inhibits TRAF6-dependent transcription of the IL-8 promoter.} \]

A

HeLa cells (6 × 10^4) were co-transfected in triplicate wells as described under “Experimental Procedures” with an IL-8 promoter-luciferase reporter and with 0.125 μg of TRAF6 or MyD88 together with the indicated amounts of Ras_{Asn-17}. Remaining DNA was made up with pcDNA3.1 (control). Twenty-four hours after transfection, cells were lysed and assayed for firefly and renilla (internal control) luciferase activity. B, a similar experiment in which, 24 h after transfection, cells were stimulated with IL-1β (1 nm), 6 h, 37°C as indicated. Cells were subsequently lysed and assayed for luciferase activity as in A. Data in each panel represent one of four experiments showing the same results and are expressed as fold induction relative to the activity in unstimulated cells transfected with reporter ± S.D. for triplicate wells.
crease in nuclear NF-κB. Finally, it is possible that the lack of RelA translocation reflects that structural induction activates only a subset of NF-κB-dependent genes, as has been shown for glycogen synthase kinase-3β (46).

This type of specificity is also in agreement with the suggested divergence of the pathway upstream of TRAF6, and indicate that MyD88 can activate NF-κB via a TRAF6 independent pathway. The effect on both TRAF6 and MyD88 induced AP-1 regulation indicate that Ras may be able to influence activation of specific genes through selectivity of adaptor protein function, reflected in distinct sets of upstream regulators. The total lack of effect of the dominant negative TRAF6 on NF-κB activation induced through transfection with RasVal-12 suggests that structural regulation through Ras feeds into the NF-κB pathway downstream of TRAF6 or is induced through second messengers totally separate from that regulated by TRAF6. Thus, activation through TRAF6 via TAB-2 and TAK-1 constitutes an IL-1-regulated pathway controlling NF-κB, while other regulators, such as NIK and NAK (48) have been shown not to directly mediate IL-1 induced NF-κB activity. The indicated synergy in the observed response suggests, however, that whatever the upstream discrepancies in signaling, the Ras-induced effect on the NF-κB pathway is not totally distinct from the IL-1 mode of activation but rather that, the two activities converge upstream of induction of transcription.

This type of receptor proximal divergence of TRAF6/MyD88-regulated pathways has been reported in in vitro studies on Toll signaling (49). Studies on TRAF6 and MyD88 knockout mice have also shown a degree of compensation and/or redundancy and partial overlapping at the level of the adaptor pro-

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3 E. E. Qwarnstrom and S. K. Dower, unpublished data.

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FIG. 5. Ras regulates TRAF6-induced transcription of NF-κB. A, HeLa cells (6 × 10⁴) were co-transfected in triplicate wells, as described under “Experimental Procedures,” with a synthetic NF-κB luciferase reporter and 0.125 μg of constructs containing TRAF6 or MyD88, together with the indicated amounts of RasVal-12 containing constructs. Remaining DNA was made up with pcDNA3.1 (control). B, cells were transfected as in A, but with an AP-1 synthetic promoter luciferase reporter and 0.125 μg of constructs containing TRAF6 or MyD88, together with the indicated amounts of RasVal-12. Remaining DNA was made up with pcDNA3.1 (control). C, cells were transfected as in A with 0.125 μg of RasVal-12 expression vector in the presence of 0.125 μg of vectors containing TRAF6 or MyD88 using the NF-κB synthetic promoter as a readout. Cell extracts were analyzed as described in the legend to Fig. 4. For each figure, data shown represent one experiment of four giving the same results, and are expressed as fold induction relative to the activity in unstimulated cells transfected with reporter ± S.D. for triplicate wells.
cells transfected with 0.125 μg of dominant negative mutant RasAsn-17 and a construct containing the synthetic NF-κB promoter were stimulated with 1 nM IL-1 and NF-κB-regulated kinases 1, 2, and 3 or MyD88, Tollip, TRAF6; TRAF6, Ras, fate proteoglycan; integrin; IRAK1, IRAK2, IRAK3/M; interleukin-1 receptor complex components (see Fig. 7).

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