Activation of the nuclear factor- κ B by Rho, CDC42, and Rac-1 proteins

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The Rho family of small GTPases are critical elements involved in the regulation of signal transduction cascades from extracellular stimuli to the cell nucleus, including the JNK/SAPK signaling pathway, the *c-fos* serum response factor, and the p70 S6 kinase. Here we report a novel signaling pathway activated by the Rho proteins that may be responsible for their biological activities, including cytoskeleton organization, transformation, apoptosis, and metastasis. The human RhoA, CDC42, and Rac-1 proteins efficiently induce the transcriptional activity of nuclear factor κ B (NF- κ B) by a mechanism that involves phosphorylation of I κ B α and translocation of p50/p50 and p50/p65 dimers to the nucleus, but independent of the Ras GTPase and the Raf-1 kinase. We also show that activation of NF- κ B by TNF α depends on CDC42 and RhoA, but not Rac-1 proteins, because this activity is drastically inhibited by their respective dominant-negative mutants. In contrast, activation of NF- κ B by UV light was not affected by Rho, CDC42, or Rac-1 dominant-negative mutants. Thus, members of the Rho family of GTPases are involved specifically in the regulation of NF- κ B-dependent transcription.

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In response to extracellular stimuli, several parallel and interconnected intracellular pathways are activated whose final destination is the regulation of transcription factors (Karin 1994). Among the components of these intracellular pathways, several independent but interconnected kinase cascades have been identified from yeast to mammals. These include the STE20/STE11/STE7/FUS3/KSS1 cascade, which regulates mating and morphology in *Saccharomyces cerevisiae*; STE6/GTPases/Byr2/Byr1/Spk1 cascade, which regulates mating in *Schizosaccharomyces pombe*; the PAK/MEKK/SEK/JNK/SAPK (P21 activating protein kinase/MAPK ERK kinase kinase/stress-activated ERK kinase/Jun N-terminal kinase/stress-activated protein kinase) cascade, which is involved in response to stress in mammalian cells; and the growth-regulating Raf/MEK/MAPK (Raf/MAPK ERK kinase/mitogen-activated protein kinase) cascade in mammals (Blumer and Johnson 1994; Karin 1994). Upon activation of these kinase cascades, transcription factors, such as c-Fos, c-Jun, NF- κ B, and others, are transcriptionally activated following a phosphorylation event (Karin 1994; Thanos and Maniatis 1995). Sev-

eral families of GTPases play a prominent role in the transmission of the signals from the extracellular environment to the cell nucleus (Bourne et al. 1990, 1991; Clapham and Neer 1993). Thus, the Ras GTPases, which are involved in the regulation of cell growth and differentiation, are activated in response to growth factors and hormones through specific factors, such as guanine nucleotide exchange factors (GEFs), that catalyze the exchange of GDP by GTP (Quilliam et al. 1995). Once in its active GTP-bound state, Ras proteins activate the Raf kinase by recruiting it to the plasma membrane by a mechanism that is still poorly understood (Boguski and McCormick 1993; Pawson 1995). Activation of the Raf kinase leads to the activation of MAPK and the further up-regulation of specific transcription factors (Karin 1994; Pawson 1995).

Rho proteins are members of the Ras superfamily of GTPases (Bourne et al. 1991). This family includes at least nine members designated as RhoA, RhoB, RhoC, RhoG, RhoE, CDC42, Rac-1, Rac-2, and TC10 (Bourne et al. 1991). Recently it has been reported that Rho proteins are either oncogenic themselves or are critical components of the Ras-dependent signaling pathways leading to transformation (Perona et al. 1993; Khosravi-Far et al. 1995; Prendergast et al. 1995; Qiu et al. 1995), apoptosis (Esteve et al. 1995; Jiménez et al. 1995; Moorman et al.

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1996; Na et al. 1996), invasion (Michiels et al. 1995), and stress (Fritz et al. 1995). Rac-1 and CDC42 proteins, but not RhoA, regulate the activation of JNK/SAPKs, a family of intracellular kinases that are part of the internal cascades leading to the nucleus through modulation of the Jun transcription factor (Coso et al. 1995; Minden et al. 1995) as well as the pp70 S6 kinase (Chou and Blenis 1996). Also, RhoA, CDC42, or Rac-1 proteins regulate the *c-fos* serum response element (SRE) by a still unknown mechanism (Hill et al. 1995) and are involved in the regulation of relevant enzymes for signal transduction such as PLA2 (Peppelenbosch et al. 1995), PLD (Bowman et al. 1993), PI3K and PI5K (Kumagai et al. 1993; Zhang et al. 1993; Chong et al. 1994), and other intracellular kinases (Manser et al. 1994; Martin et al. 1995; Amano et al. 1996; Ishizaki et al. 1996; Matsui et al. 1996; Watanabe et al. 1996). These results strongly implicate Rho proteins in the regulation of signaling pathways leading to the nucleus. Finally, several observations indicate that Ras- and Rho-mediated signaling pathways may be mutually dependent, because different members of the *rho* family induce alteration of growth properties and transformation of NIH-3T3 cells and dominant-negative mutants of the *rho* family block *ras* transformation (Perona et al. 1993; Khosravi-Far et al. 1995; Prendergast et al. 1995; Qiu et al. 1995).

We have demonstrated recently that overexpression of Rho proteins triggers apoptosis after serum removal (Jiménez et al. 1995), an effect that may be mediated through the generation of ceramides (Esteve et al. 1995). Ceramide production is strongly activated by tumor necrosis factor- α (TNF α), ionizing radiation, and interleukin-1 (IL-1), which are not only potent inducers of ceramide production but also activators of the transcription factor NF- κ B (Schütze et al. 1992; Grimm and Baeuerle 1993; Mathias et al. 1993; Obeid et al. 1993; Haimovitz-Friedman et al. 1994; Jarvis et al. 1994; Faucheu et al. 1995). In this report we have explored whether different members of the Rho family induce the activation of NF- κ B, including the *Aplysia* and the human RhoA, RhoB, RhoC, CDC42, and Rac-1 proteins. We have also investigated the mechanism of NF- κ B activation by expression of the Rho proteins with the NF- κ B inhibitor, I κ B α , or dominant-negative mutants of the Ras GTPase and the Raf-1 kinase. Finally, the physiological relevance of this effect has been investigated using dominant-negative mutants of different Rho proteins in response to TNF α or UV light.

Results

Rho proteins constitutively activate NF- κ B in NIH-3T3 cells

To test whether Rho proteins activate NF- κ B, we first used previously characterized NIH-3T3 cells stably transfected with the *Aplysia californica rho* gene and expressing high levels of the Rho product. To that end, two independent clones overexpressing the wild-type *rho* gene, WT15 and WT16, were used, which are trans-

forming in nude mice (Perona et al. 1993), have potent apoptotic activity after serum removal (Jiménez et al. 1995), and have been demonstrated to trigger ceramide production under these conditions (Esteve et al. 1995). As shown in Figure 1, extracts from both cell lines had a constitutive activation of the NF- κ B-dependent (-453/+80) HIV-luciferase (HIV-LUC) reporter (Devary et al. 1993). This activity was even more pronounced than the one produced by UV exposure of the corresponding control, G418-resistant cells generated by transfection with the empty vector (pZip-Neo). In contrast, cells overexpressing the PKC ζ isoenzyme did not present activation of the NF- κ B factor, as described previously (Genot et al. 1995; Montaner et al. 1995). Finally, *trans*-activation of the HIV promoter requires intact NF- κ B binding sites because no activation was observed when a HIV-LUC reporter containing 3-bp substitutions in each NF- κ B binding site was used (data not shown).

Activation of NF- κ B by Rho induces translocation of p65/RelA to the nucleus

NF- κ B transcription factor may be composed of homo- or

Figure 1. Constitutive activation of NF- κ B transcriptional activity by Rho expression. Cells stably overexpressing the *rho* gene from *A. californica* (WT15, WT16), or the wild-type PKC ζ gene (WT-MC3), or cells transformed by the H-*ras*-Val12 oncogene (*ras*), were analyzed for constitutive NF- κ B activation by transfection of the reporter plasmid HIV-LUC. Where indicated, control cells were treated with UV light (pZipNeo+UV) as indicated under Materials and Methods. The results are represented as fold induction above the basal (-453/+80) HIV-LUC expression in the control pZIP-Neo cells, and are the average $\pm$ S.D. of a single experiment performed in triplicate. The luciferase values were corrected by cotransfection with the pSV2-CAT plasmid and the stimulation of CAT activity as indicated in Materials and Methods. Similar results were obtained in three independent experiments.

Figure 2. Analysis of NF- κ B complexes activated by *rho* expression. Gel retardation analysis was carried out in the same cell lines as in Fig. 1. The arrows indicate the p50/p50 or p50/RelA complexes. (-TNF) Control cells; (+TNF) TNF α -treated cells (10 ng/ml). Samples from TNF α -treated cells were incubated with pre-immune serum (p.s.), or antibodies specific for either p50 (p50), RelA (p65a and p65b), or c-Jun (c-jun). (WT-MC3) A mass culture of NIH-3T3 cells overexpressing PKC ζ , characterized previously (Montaner et al. 1995). The experiment was repeated once more with similar results.

heterodimers of the different Rel/NF- κ B members, p50 (NF- κ B1), p52 (NF- κ B2), RelA (p65), c-Rel, and RelB proteins (Liou et al. 1993; Siebenlist et al. 1994; Thanos and Maniatis 1995; Verma et al. 1995). Thus, we investigated the composition of NF- κ B in the *rho* transfectants by electrophoretic mobility shift assay (EMSA) with a κ B-specific DNA probe and specific antibodies to each member of the family. A specific DNA binding was observed with nuclear extracts prepared from WT15 cells (Fig. 2) that shows the same mobility as control cells stimulated with TNF α . Similar results were also obtained with a second *rho*-transfected cell line, WT16 (not shown). In contrast, no complex formation was observed in cells transfected with the PKC ζ gene, as reported previously (Genot et al. 1995; Montaner et al. 1995). Both p50 and RelA proteins were found in the active complexes in nuclei extracts from TNF α -treated pZip-Neo cells, as well as the *rho*-transfected cells, as demonstrated by specific inhibition of the DNA-binding complexes by p50 and RelA antisera (Fig. 2). Nuclear translocation of p65/relA was demonstrated further by immunostaining of cells expressing the *Aplysia rho* gene, using a specific antibody against p65/relA (Fig. 3). Translocation of p65/relA was also observed in NIH-3T3 cells induced with TNF α , but not in untreated, control cells. Thus, activation of NF- κ B by Rho proteins is induced by its translocation to the nucleus. It is important to note that under these conditions not all the NIH-3T3 cells showed translocation of relA(p65) to the nucleus, an indication of a partial response of this specific NIH-3T3 cell line under these conditions.

Transient expression of different members of the human Rho family activate NF- κ B

To investigate whether the activation of NF- κ B by over-

expression of the Rho protein was a consequence of an indirect effect attributable to the generation of stable transfectants, transient transfection experiments were

Figure 3. Immunodetection of p65(RelA) in Rho-expressing cells. Control, pZIP-Neo, and WT16 cell lines were left untreated or treated with TNF α for 15 min, fixed, and the intracellular location of RelA(p65) determined by indirect immunofluorescence with the use of anti-RelA antibody. Typical fields of immunostained cells showing the exclusive cytoplasmic location of relA in untreated, pZIP-Neo control cells or its nuclear translocation in the pZIP-Neo cells treated with TNF α and the untreated WT16 cells are depicted. Note that not all NIH-3T3 cells treated with TNF α showed nuclear staining of relA(p65).

performed using different members of the *rho* family. Both wild-type and activated mutants of the human *rhoA*, *cdc42*, and *rac-1* genes were used under the control of the same cytomegalovirus (CMV) promoter in cotransfection experiments using the reporter plasmid pHIV-LUC. As shown in Figure 4A, all of the genes investigated activated the HIV reporter efficiently in an NF- κ B-dependent manner in COS-7 cells, a monkey kidney fibroblast-like cellular system. Similar results were observed when the *Aplysia* gene as well as the human *rhoB* and *rhoC* genes were used in this assay (data not shown). Finally, as positive controls, the activated H-Ras protein (Ras-Val¹²) and TNF α induction were also used with the expected results.

Recently, the potent activation of the JNK/SAPKs by transient expression of *cdc42* and *rac-1* genes has been demonstrated (Coso et al. 1995; Minden et al. 1995). Under similar conditions the *rhoA* gene was shown to have very weak or no activity (Coso et al. 1995; Minden et al. 1995). A consequence of the activation of the JNK/SAPKs is the subsequent stimulation of the transcriptional activity of c-Jun. Thus, as a further control, the transcriptional activation of c-Jun was analyzed by cotransfection of the *rho* genes along with plasmid pGal4-c-Jun (1–223) encoding a fusion protein carrying the c-Jun activation domain. As shown in Figure 4B, *rac-1* and *cdc42*Hs (*Homo sapiens*) were potent inducers of c-Jun transcriptional activity, in keeping with previous obser-

Figure 4. Activation of NF- κ B and c-Jun transcriptional activity by different members of the rho family. (A) NF- κ B activity in transient experiments performed in the simian COS-7 cells. COS-7 cells were cotransfected by lipofection with 0.5 μ g of (–453/+80) HIV-LUC per 60 mm plate and 1 μ g of pCMV- β -gal along with 3 μ g of the expression vectors: pcDNAIII and the derived vectors expressing RhoA-WT, RhoA-QL, CDC42Hs-WT, and CDC42Hs-QL, Rac-1-WT, and Rac-1-QL as well as EXV-Ras-V12. When indicated, cells were stimulated with TNF α (10 ng/ml). Luciferase activity was determined 24 hr after transfection. The results were corrected for transfection efficiency against expression of the pCMV- β -Gal plasmid, by dividing the activity on HIV-LUC reporter by β -gal activity in the same sample. Ratios obtained for the empty vectors were considered 1. Data represent the means of a single experiment performed in triplicate $\pm$ s.d.. The experiments were repeated twice with similar results. (B) c-Jun transcriptional activation in COS-7 cells under identical conditions to those described in A for NF- κ B activation. COS-7 cells were transiently cotransfected with 1 μ g of 5 $\times$ Gal-LUC reporter and 1 μ g of expression vectors encoding either the GAL4-c-Jun (1–223) (solid bars), or GAL-c-Jun (1–223; A63/73) (hatched bars) fusion proteins, 2 μ g of the expression vectors described in A and 1 μ g of pCMV- β -gal. When indicated, the cells were stimulated with EGF (20 ng/ml) during the last 5 hr of incubation. Luciferase activity was determined 24 hr after transfection. The results are expressed relative to the level of luciferase activity in cells cotransfected with pcDNAIII and the fusion protein GAL4-c-Jun (1–223), which was given an arbitrary value of 1. Luciferase activity was normalized as described in A. Data represent the means of a single experiment performed in triplicate. Experiments were repeated twice with similar results. (C) NF- κ B activation in human T-cell lymphoma Jurkat cells under identical conditions to those described in A: Jurkat cells (5×10^6 cells) were cotransfected by lipofection with 0.5 μ g of (–453/+80) HIV-LUC and 1 μ g of pCMV- β -gal, along with the expression vectors described in A and B. Luciferase activity was determined 24 hr after transfections. Transfection efficiencies were corrected as in A. Ratios obtained for the empty vectors were considered 1. Data represent the means of a single experiment performed in triplicate. The experiments were repeated twice with similar results. Where indicated, Jurkat cells were stimulated with the phorbol ester PMA (100 nM) plus the Ca²⁺ ionophore A-23187 (1 mM) for the last 5 hr of incubation. (D) Western blots from COS-7 cells transfected with the Rho family expression vectors. Lysates from COS-7 transfected cells were subjected to Western blot analysis after SDS-PAGE and immunoblotted with the corresponding antiserum.

vations by other groups (Coso et al. 1995; Minden et al. 1995). A mutated version of the c-Jun fusion protein, carrying a double mutation Ala-63–Ala-73, was not responsive, a strong indication that the observed activation was dependent on c-Jun phosphorylation by the JNK cascade. A much less efficient induction of the c-Jun *trans*-activation was observed with *rhoA* in this cell line, in agreement with previous reports (Coso et al. 1995; Minden et al. 1995). Finally, as controls, the activated *ras* oncogene and epidermal growth factor (EGF)-treated cells showed activation of the transcription factor that was abrogated by the Ala-63–Ala-73 mutations.

To test whether there was any cell dependency, the human T-cell lymphoma Jurkat cell line was also used for the analysis of NF- κ B activation. All of the *rho* genes analyzed were potent activators of the transcriptional activity of the NF- κ B factor (Fig. 4C). Furthermore, similar results were also obtained with the murine fibroblast NIH-3T3 cell line (results not shown), suggesting that the results observed with the COS-7 cell system could be extrapolated to other cell systems and that this effect was not species-specific. Expression of the RhoA, Rac-1, and CDC42 proteins in transient experiments were determined by Western blotting, by use of specific antibodies. A typical analysis for protein expression in COS-7 cells is shown in Figure 4D, and similar results were observed with the other cell systems used (data not shown). Finally, the effect of transient expression of the human *rho* genes on NF- κ B activity was also investigated. As shown in Figure 5, expression of the constitutively active human *rhoA*, *rac-1*, and *cdc42* genes were able to efficiently induce nuclear translocation of the factor κ B, as demonstrated by gel retardation assays performed with nuclear extracts of transiently transfected NIH-3T3 cells. The extent of NF- κ B activity was similar to that observed in NIH-3T3 cells treated with TNF α , most likely as a result of a partial activation of NF- κ B by TNF α treatment (see Fig. 3).

NF- κ B activation by Rho proteins is independent of the Ras/Raf signaling pathway

Recent evidence implicates the functional relationship of Ras- and Rho-dependent pathways. Although constitutively activated Rho and Rac proteins have weak transforming activity on their own (Perona et al. 1993; Khosravi-Far et al. 1995; Prendergast et al. 1995; Qiu et al. 1995), a dominant-negative mutant of Rac-1, RhoA, or RhoB efficiently blocked *ras*-mediated transformation (Khosravi-Far et al. 1995; Prendergast et al. 1995; Qiu et al. 1995). Furthermore, mutated, constitutively activated versions of *rho* and *rac-1* genes synergized with the *raf* oncogene for transforming activity, suggesting that the Ras/Raf1 pathway interacts with the Rho pathway at a still unknown point. Because Ras proteins have been shown previously to activate NF- κ B (Devary et al. 1993), we investigated whether activation of NF- κ B by Rho proteins was dependent on Ras and Raf1 kinase activity using the dominant-negative mutants Ras–Asn-17 (Feig and Cooper 1988) and Raf–C4 (Bruder et al. 1992). As

Figure 5. Gel retardation analysis of NF- κ B complexes induced by Rho in transiently transfected NIH-3T3 cells. NIH-3T3 cells were transiently transfected with 10 μ g of pcDNAIII empty vector (vector) or the derived vectors containing the activated *rhoA*, *rac-1*, and *cdc42* genes. After 24 hr cells were serum-starved and nuclear extracts assayed for gel retardation assay as indicated in Fig. 2. When indicated vector transfected cells were stimulated with TNF α (10 ng/ml). (Comp) Competition with oligonucleotides containing either NF- κ B or c-Fos SRE sites. The experiment was repeated twice with similar results.

shown in Figure 6, neither mutant significantly affected the basal, constitutive activation of NF- κ B observed in the NIH-3T3 cells expressing the *Aplysia rho* gene (Fig. 6A) or the transient activation of the transcription factor mediated by human *rhoA*, *cdc42*, or *rac-1* genes in COS-7 cells (Fig. 6B). In contrast, NF- κ B activation mediated by UV exposure (Fig. 6A) or EGF treatment (Fig. 6B) was sensitive to either mutant, as reported previously by others (Devary et al. 1993). These results indicate that Rho proteins affect NF- κ B by a mechanism independent of the Ras/Raf pathway.

Rho-dependent activation of NF- κ B is mediated by phosphorylation of I κ B α and is reversed by overexpression of I κ B α

Activation of NF- κ B by multiple agonists such as UV, viruses, phorbol esters, IL-1 β , lipopolysaccharides, and TNF α depends on phosphorylation and subsequent degradation of its inhibitory I κ B subunits (Miyamoto et al. 1994), with which it binds under nonstimulated conditions. Phosphorylation takes place at two serine residues in position Ser-32 and Ser-36 (Brockman et al. 1995; Brown et al. 1995). Because there are several alternative mechanisms described for the activation of NF- κ B (Liou et al. 1993; Siebenlist et al. 1994; Thanos and Maniatis

Figure 6. NF- κ B-dependent activation of HIV promoter by Rho proteins is independent of Ras and Raf. (A) NF- κ B activity in control NIH-3T3 cells and *rho*-transformed cells in the presence of dominant-negative mutants of Ras (ras-Asn-17) or Raf (rafC4). Control pZIP-Neo and WT15 cells were cotransfected with 4 μ g of (-453/+80) HIV-LUC along with carrier DNA or expression vectors encoding dominant-negative Ha-ras Asn-17 (10 μ g) and raf-C4 (10 μ g) per 100-mm plate. Luciferase activity was determined 24 hr later. When indicated, cells were irradiated with UVC light (40 J/m²) 2 hr after transfection. Results are expressed as percentage of the induction obtained in pZIP-Neo cells treated with UVC or the basal levels of WT15 cells transfected with the empty. Transfection efficiency was corrected by cotransfecting the pSV2-CAT plasmid. (B) NF- κ B activation in transient expression experiments in the presence of Ras (solid bars) and Raf (open bars) dominant-negative mutants using the COS-7 cell system and different Rho genes. COS-7 cells were transiently cotransfected by lipofection with 0.5 μ g of (-453/+80) HIV-LUC and 1 μ g of pCMV- β -gal per 60 mm plate together with either empty vector or dominant-negative Ha-ras Asn-17 (2 μ g) (solid bars) and raf-C4 (2 μ g) (open bars) and 2 μ g of the expression vectors: pcDNAIII-derived vectors expressing wild-type or activated RhoA, CDC42Hs, Rac-1, or the EXVrasV12. Luciferase activity was determined 24 hr after transfection. The results are expressed as percentage of luciferase activity obtained with each expression vector alone and represent the means of an experiment performed in triplicate. The experiments were repeated twice with similar results.

1995; Verma et al. 1995), we also investigated the effects of expression of the Rho protein on the known inhibitors for NF- κ B. As shown in Figure 7A, at least in the NIH-3T3 cell system, Rho proteins target I κ B α , because this factor is specifically accumulated in its phosphorylated form in the presence of the proteasome inhibitor PSI (Traenckner et al. 1994). As expected, similar results of accumulation of the phosphorylated form of I κ B α were observed when the cells were treated with TNF α . In contrast, no accumulation was observed in the case of I κ B β or p105 under identical conditions (data not shown) in NIH-3T3 cells. Furthermore, phosphorylation was not observed with Western blot analysis when a mutated I κ B α protein with substituted Ser-32 and Ser-36 to Ala-32 and Ala-36 was used in cotransfection experiments along with the *rho* genes or TNF α stimulation (Fig. 7B). In contrast, when wild-type I κ B α was cotransfected with RhoA, Rac-1, or CDC42 expression plasmids, an extra band was observed, indicating phosphorylation of the protein and retardation in the gel as reported previously (Brown et al. 1995; Whiteside et al. 1995). These results demonstrate that Rho-mediated phosphorylation of I κ B α is through residues Ser-32 and Ser-36.

Because phosphorylation of I κ B α may be an obligatory step for NF- κ B activation (Miyamoto et al. 1994), and the results shown in Figure 7, A and B, demonstrated phosphorylation of I κ B α by overexpression of Rho, we investigated whether Rho-dependent activation of NF- κ B could be reverted by overexpression of the I κ B α inhibitor. To that end, we cotransfected plasmid pSVK3-I κ B α

along with the *rho*-carrying plasmids to overproduce I κ B α and analyzed the transient activation of the NF- κ B-dependent HIV-LUC reporter. As shown in Figure 7C, cotransfection of the *rho* genes with pSVK3-I κ B α induced a strong reduction in NF- κ B activation of ~70%, an indication that the Rho-mediated activation of NF- κ B follows the conventional I κ B α -dependent mechanism. These results are also in agreement with those shown in Figure 7, A and B, and further substantiate the conclusion that Rho proteins activate NF- κ B by targeting I κ B α . Furthermore, they can be extrapolated also to the human RhoA, CDC42, and Rac-1 proteins.

Physiological relevance of NF- κ B activation by Rho proteins

The above results indicate that Rho proteins efficiently activate NF- κ B transcriptional activity. However, these results do not indicate whether Rho proteins mediate NF- κ B activation as a response to physiological stimuli. Thus, we investigated whether Rho proteins were needed for the induction of NF- κ B activation in response to a physiological activator of the transcription factor such as TNF α . As shown in Figure 8A, inhibition of the RhoA- and the CDC42-dependent pathways by cotransfection of their respective dominant-negative mutants substantially reduced the activation of NF- κ B by TNF α treatment (between 50% and 70% inhibition). In contrast, the Rac-1 mutant did not affect this pathway. A dominant-negative mutant of Ras was also negative for

Figure 7. Activation of NF- κ B is mediated by targeting of I κ B α . (A) pZIP-Neo and WT15 cell lines were labeled with 32 P-labeled H_3PO_4 for 4 hr in the presence of the proteasome inhibitor PSI. The pZIP-Neo cells were either untreated (control) or stimulated with TNF α during 10 or 30 min. WT15 cells were left untreated. Cells were subsequently lysed, and I κ B α immunoprecipitated. The immunoprecipitates were analyzed by SDS-PAGE followed by autoradiography. Data shown are representative of two independent experiments with similar results. For simplicity, only the results with the I κ B α antibody are shown [see text]. (B) Western blot analysis of I κ B α proteins in transiently transfected COS-7 cells using 30 μ g of total protein from cells transfected with RhoA, Rac-1, or CDC42 expression plasmids or in cells treated with TNF α (10 ng/ml) along with 2 μ g of vectors expressing either wild-type human I κ B α or a mutated version where Ser-32 and Ser-36 have been changed to Ala (Whiteside et al. 1995). (●) Phosphorylated form of I κ B α . (C) Activation of NF- κ B induced by Rho proteins is blocked by I κ B α expression. COS-7 cells were transiently cotransfected by lipofection with 0.5 μ g of (-453/+80) HIV-LUC and 1 μ g of pCMV- β -gal per 60-mm plate along with either 2 μ g of pSVK3 vector (solid bars) or pSVK3I κ B α (open bars) and 2 μ g of the expression vectors: pcDNAIII-derived vectors expressing activated RhoA, CDC42Hs, or Rac-1, and EXVrasVal12 (rasV12). Luciferase activity was determined 24 hr after transfection. The results are expressed as percentage of luciferase activity obtained with each expression vector alone. Data represent the means $\pm$ S.D. of an experiment performed in triplicate. The experiments were repeated twice with similar results.

the activation of NF- κ B induced by TNF α , suggesting that in COS-7 cells, Ras is not involved primarily in this pathway, contrary to what has been reported previously in PC-12 cells (Devary et al. 1993).

Finally, to eliminate the possibility that inhibition by the RhoA and CDC42 dominant-negative mutants could be a consequence of nonspecific effects attributable to its interference with TNF α treatment, activation of NF- κ B induced by UV was also analyzed under similar conditions. Figure 8B shows that none of the dominant-negative mutants used showed a significant interference with NF- κ B activation by UV in NIH-3T3 cells. Similar results were also observed when COS-7 cells were used (data not shown). As controls for the specificity of the dominant-negative mutants, parallel assays for c-Jun transcriptional activity were performed under similar conditions. Both Ras (75% inhibition), Rac-1 (65% inhibition), and CDC42 (60% inhibition) but not RhoA (13% inhibition) dominant-negative mutants were able to block EGF-induced c-Jun transcriptional activation efficiently (Fig. 8C). The results shown are in agreement with the inhibitory effects of the CDC42 and Rac-1 dominant-negative mutants in the TNF α - and EGF-dependent activation of JNK activity, which has been re-

ported to be ~50% inhibition (Coso et al. 1995; Minden et al. 1995). In contrast with these results, UV-induced activation of NF- κ B is sensitive to Ras and Raf dominant-negative mutants (Fig. 6). Thus, the observed blockage of NF- κ B by RhoA and CDC42 mutants after TNF α induction was rather specific and not a consequence of nonspecific inhibition.

Discussion

NF- κ B plays a critical role in the regulation of the expression of specific sets of genes as a response to extracellular signals. NF- κ B has a primary role in immune function and inflammation, in lymphoid differentiation, and in embryonic axis determination in insects (Grimm and Baeuerle 1993; Liou and Baltimore 1993; Siebenlist et al. 1994; Thanos and Maniatis 1995; Verma et al. 1995). There is also evidence that NF- κ B plays a role in mitogenic signal transduction (Miyamoto et al. 1994) and programmed cell death (Baldwin et al. 1991; Beauparlant et al. 1994). Furthermore, knockouts of either p50, c-rel, or RelB indicate that NF- κ B is critical for specific and nonspecific immune response and cell division (Köntgen et al. 1995; Sha et al. 1995; Weih et al.

Figure 8. Involvement of Rho proteins in the activation of NF- κ B induced by TNF α or UV light. COS-7 cells were cotransfected by lipofection with 0.5 μ g of (-453/+80) HIV-LUC per 60-mm plate along with the plasmids pCEV27 (Miki et al. 1991) or the derived expression vectors containing the dominant-negative mutants RhoA-Asn-19, Rac-1-Asn-17, CDC42-Asn-17, vector pZIP-Neo or pZIP-Ras-Asn-17 plasmid. Cells were stimulated 5 hr before harvesting with TNF α (10 ng/ml) (A), or treated with UVC (40 J/m²) 20 hr (B) before harvesting. Transfection efficiency was corrected with the plasmid pCMV- β -gal. Results are expressed as percentage of the luciferase activity obtained with the corresponding empty vector. Induction was of a 4.5-fold for TNF α and 10-fold for UVC. In A, the data presented are the mean values $\pm$ s.d. from $n=3$ (Ras-Asn-17), $n=4$ (CDC42-Asn-17), or $n=5$ (Rho-Asn-19 and Rac-Asn-17) each performed in triplicate. In B, the data presented are the mean values $\pm$ s.d. from two independent experiments each performed in triplicate. (C) COS-7 cells were transiently cotransfected with 1 μ g of 5 $\times$ Gal-LUC reporter and 1 μ g of expression vectors encoding the GAL4-c-Jun (1-223) fusion protein and 2 μ g of the expression vector pCEV27 or its derivatives containing mutants Ras-Asn-17, RhoA-Asn-19, Rac-1-Asn-17, or CDC42-Asn-17. The cells were stimulated with EGF (20 ng/ml) during the last 5 hr of incubation. Luciferase activity was determined 24 hr after transfection and the c-Jun transcriptional activity assayed as described in Materials and Methods. The results are expressed relative to the level of luciferase activity in cells cotransfected with pCEV27 and the fusion protein GAL4-c-Jun (1-223) and stimulated with EGF, which had a fourfold induction and was given an arbitrary value of 100. Data represent the means of three experiments performed in triplicate in parallel to those described in A and B.

1995). The specificity of gene transcription mediated by the Rel/NF- κ B family is complicated by its interaction with other families of transcriptional activators among which ATF2/c-Jun occupies a relevant role (Liou and Baltimore 1993; Siebenlist et al. 1994; Thanos and Maniatis 1995; Verma et al. 1995). Recent evidence indicates that several inducers of NF- κ B also activate JNK/SAPKs, which phosphorylate both ATF2 and c-Jun (Köntgen et al. 1995; Sha et al. 1995; Weih et al. 1995), and this could result in a synergistic transcriptional activation with NF- κ B of certain promoters as that of IFN β . The precise mechanism for NF- κ B activation from the extracellular stimuli to phosphorylation and degradation of the I κ B inhibitors remains to be elucidated. Recently, one of the kinases responsible for this effect has been identified (Chen et al. 1996), which will help to elucidate the pathway.

Although it was originally considered that the major function of the Rho family of proteins was to regulate the cellular cytoskeleton organization (Hall 1994; Takai et al. 1995), the overwhelming evidence generated during the last few years strongly supports the participation of different Rho proteins in signal transduction pathways

also governing cell growth, apoptosis and response to stress (Jahner and Hunter 1991; Bowman et al. 1993; Kumagai et al. 1993; Perona et al. 1993; Chong et al. 1994; Manser et al. 1994; Coso et al. 1995; Esteve et al. 1995; Fritz et al. 1995; Hill et al. 1995; Jiménez et al. 1995; Khosravi-Far et al. 1995; Martin et al. 1995; Minden et al. 1995; Peppelenbosch et al. 1995; Prendergast et al. 1995; Qiu et al. 1995; Amano et al. 1996; Ishizaki et al. 1996; Matsui et al. 1996; Watanabe et al. 1996). The recent findings that Rac-1 and CDC42 efficiently activate the JNK/SAPKs and the p70 S6 kinase (pp70S6K) and that RhoA, CDC42, and Rac-1 activate the *c-fos* serum response factor, along with the results shown in this study, is of great relevance for understanding the mechanism involved in the regulation of the biological activities attributed to Rho proteins.

CDC42Hs and Rac proteins activate the PAK65 kinase, most likely by direct, GTP-dependent, physical contact, whereas RhoA interacts with a different set of proteins (Manser et al. 1994; Martin et al. 1995; Amano et al. 1996; Ishizaki et al. 1996; Matsui et al. 1996; Watanabe et al. 1996), one of which may correspond to the I κ B kinase, responsible for NF- κ B activation (Chen et al.

1996). Our finding that RhoA has similar activity to that of CDC42Hs and Rac-1 for NF- κ B activation makes it unlikely that the JNK/SAPK pathway or the pp70S6K is responsible for this effect; rather, a common effector may be involved. Furthermore, similar results were observed with the human RhoB and RhoC proteins (results not shown), a further indication that it may be a common pathway for all Rho-related proteins. In keeping with this notion, it has been reported that at least in *S. cerevisiae*, a single protein, designated as BEM4, can interact physically with several members of the Rho family including Rho and CDC42 proteins (Mack et al. 1996), suggesting that different members of the Rho family may share some of their effectors.

In searching for I κ B α kinase candidates responsible for NF- κ B activation, some contradictory results have been reported. Thus, MEK kinase 1 (MEKK1), an activator of Jun kinase, has been involved in the activation of NF- κ B by TNF α (Hirano et al. 1996). These results suggest an interaction between MEKK activation and NF- κ B signaling. Supporting these findings, an association of JNK with c-Rel in vivo has been reported in Jurkat cells, suggesting a significant role for the MEKK1 kinase cascade in NF- κ B-dependent transcription (Meyer et al. 1996). In contrast with these results, it has been reported that although activation of JNK and NF- κ B by TNF α treatment is mediated by recruitment of TNF α receptor activating protein (RIP) and TRAF2, activation of JNK is mediated by MEKK1, whereas activation of NF- κ B is mediated by an alternative pathway (Liu et al. 1996). Thus the pathway leading to NF- κ B activation may be rather complex and dependent on the cellular context.

Altogether, these results suggest that a common novel effector may be acting downstream of Rho, Rac, and CDC42 leading to the NF- κ B pathway. Also, a specific, unknown member of the family of JNK/SAPK kinases may be activated by the Rho proteins, but not by CDC42Hs or Rac-1, and converge at the NF- κ B level in the same pathway. A similar mechanism has been proposed for the activation of SRE by the RhoA, Rac-1, and CDC42 proteins (Hill et al. 1995). Finally, the fact that RhoA and CDC42 interfered efficiently with the activation of NF- κ B induced by TNF α suggests that at least some of the known intracellular targets of TNF receptor (Chinnaiyan et al. 1995; Cleveland and Ihle 1995; Hsu et al. 1995, 1996) may be responsible for this effect. A similar situation has been reported for the TNF α -dependent activation of JNK, which is inhibited ~50% by Rac-1 or CDC42 dominant-negative mutants (Coso et al. 1995; Minden et al. 1995). Research aimed at the elucidation of the specific mechanisms for each member of the Rho family and their effectors is extremely interesting.

The possibility of a single family of GTPases activating several intracellular pathways necessary for their biological activity has been demonstrated recently for Ras (White et al. 1995). A proper combination of these alternative cascades seems necessary for Ras transforming activity and some depend on Rho proteins (Khosravi-Far et al. 1995; Prendergast et al. 1995; Qiu et al. 1995). Furthermore, Rho proteins have been shown to interact

with several effector molecules (Manser et al. 1994; Martin et al. 1995; Amano et al. 1996; Ishizaki et al. 1996; Matsui et al. 1996; Watanabe et al. 1996), further supporting that this notion may be also true for Rho proteins.

The results presented in this study clearly demonstrate that Rho proteins induce the transcriptional activity of NF- κ B. Either RhoA, RhoB, RhoC, CDC42Hs, or Rac-1 each seem to be equally effective, with a better response with the corresponding activated mutants. We also show that the potent activation of NF- κ B transcriptional response is not dependent on the presence of active Ras protein or the Raf1 kinase, suggesting a pathway independent of that mediated by the Ras/Raf1/MAPK cascade. Finally, we demonstrate that Rho dominant-negative mutants interfere with NF- κ B activation in response to physiological stimuli. The observed blockage of NF- κ B by RhoA and CDC42 mutants after TNF α induction was rather specific and not a consequence of nonspecific inhibition. Moreover, although these results are in keeping with the notion that Rho proteins may be downstream of Ras in some signaling pathways, they also suggest that activation of NF- κ B may be a far more complex system than anticipated previously, with several alternative pathways, as already demonstrated for other transcription factors.

From these results it can be proposed that different members of the family of Rho GTPases mediate specific cell responses to stress similar to the way the family of Ras GTPases mediates cell responses to a variety of cell growth and differentiating stimuli. In keeping with this, our previous studies demonstrate that Rho proteins can induce apoptosis under certain conditions (Jiménez et al. 1995) and that this effect may be related to ceramide production (Esteve et al. 1995). Some of the Rho-mediated signals may lead to a protective response (UV light, heat shock, etc.) and others to cell suicide (cytokines, serum deprivation), dependent of cell type and the specific extracellular stimulus. Our hypothesis can be tested and will constitute one of the goals of our group.

Materials and methods

Cell culture and transfections

Murine NIH-3T3 fibroblasts and derived cell lines expressing the *Aplysia rho* gene (WT15, WT16), the mouse PKC ζ gene (WT-MC3), and the Ha-ras Val12 gene (*ras*) were grown in Dulbecco's modified Eagle medium (DMEM) with 10% newborn calf serum (NCS) and 1 mM glutamine. Simian COS-7 fibroblast-like cells were cultured in DMEM supplemented with fetal bovine serum (FCS). Human T-cell lymphoma Jurkat cells were maintained in RPMI supplemented with 10% FCS and 1 mM glutamine. For transient transfection assays of NIH-3T3-derived cell lines (WT15, WT16, WT-MC3, and *ras*) the calcium phosphate method was used as described (Perona et al. 1993), keeping the amount of DNA at 30 μ g/100-mm plate with calf thymus DNA. Transfections using COS-7 cells were performed in 60-mm dishes using DOTAP (Boehringer), according to the protocol of the manufacturer. The total amount of DNA was kept constant at 5–6 μ g per plate. Jurkat cells were also transfected with

DOTAP in a final volume of 2 ml using 5 µg of total DNA. COS-7 and NIH-3T3 cells were incubated in serum-free medium and Jurkat cells in RPMI/10% FCS. When indicated, the cells were stimulated during the last 5 hr of culture and harvested 24 hr after transfection. Transfection efficiency was normalized by cotransfection using the plasmids pCMV-β-gal or pSV2-CAT.

Plasmids

pCDNAIII (Invitrogen) vectors containing cDNAs encoding RhoA, CDC42Hs, Rac-1, and the corresponding activated genes (QL) have been described previously (Coso et al. 1995). pCEV27 (Miki et al. 1991) or the derived expression vectors containing the dominant-negative mutants RhoA-Asn-19, Rac-1-Asn-17, CDC42-Asn-17, vector pZIP-Neo, or pZIP-Ras-Asn-17 plasmids have been described previously (Feig and Cooper 1988; Coso et al. 1995). (-453/+80) HIV-LUC contains the NF-κB sites of the HIV promoter and ΔNF-κB HIV-LUC contains a 3-bp substitution in each of the NF-κB binding sites (Devary et al. 1993). The following plasmids have been described previously: SVK3-IκBα (Beuparlant et al. 1994), pRSV-C4-Raf (Bruder et al. 1992), pZIP-Neo Ras-Asn17 (Feig and Cooper 1988), GAL4-c-Jun (1-223) and Gal4-c-Jun (1-223 A63/73) (Dérjard et al. 1994; Minden et al. 1994; Su et al. 1994; Lin et al. 1995), and pRC-CMV-IκBα and pRC-CMV-IκBα-Ala-32/Ala-36 (Whiteside et al. 1995).

Gene expression analysis

Cells were transfected as described above and harvested 24 hr after transfection. Protein extracts were prepared by three consecutive cycles of freezing and thawing. Luciferase activity was determined with a commercial kit (Promega) using a Berthold luminometer. Results are expressed as fold induction considering as 1 the luciferase activity of the transfection with the corresponding vector. The chloramphenicol acetyltransferase (CAT) activity was analyzed as described previously (Gorman et al. 1982).

Analysis of NF-κB activity has been performed by transfecting the plasmid containing the wild-type HIV site, (-453/+80) HIV-LUC, and the one containing the ΔNF-κB site, ΔNF-κB HIV-LUC. Fold induction was calculated for each reporter considering as 1 the values obtained for the empty expression vector. To obtain the activity corresponding to the κB sites of the reporter, we calculated the ratio of the fold induction obtained using the plasmid with the wild-type HIV site, (-453/+80) HIV-LUC, and the one containing the ΔNF-κB site, ΔNF-κB HIV-LUC. To assay for c-Jun transcriptional activity corresponding to JNK we used the plasmid 5 × Gal-LUC reporter and the plasmids encoding a GAL4-c-Jun (1-223) fusion protein containing the c-Jun activation domain. As a control we used the same plasmid with a mutation in the sites of JNK phosphorylation of c-Jun (A63/A73). Transfection efficiencies were corrected routinely by dividing the activities on the luciferase assays by β-gal activity obtained in the same samples by cotransfection with the pCMV-β-gal. None of the proteins used in this study affected the levels of transcription activation of pCMV-β-gal or pSV2-Neo-CAT because similar levels were always observed when compared with the respective empty plasmids. Only small variations of 10–15% were observed in all the experiments presented in this study among control and Rho-expressing plasmids for β-gal or CAT assays.

Immunofluorescence

Cells were plated in coverslips and serum starved during 24 hr

of culture. After treatments, cells were fixed with paraformaldehyde and permeabilized with Triton X-100. RelA was detected with a polyclonal RelA antibody and subsequently with a biotinylated anti-rabbit followed by streptavidin Texas Red.

Immunoprecipitation and Western blot assays

For immunoprecipitation assays, cells were plated and serum-starved for 24 hr and, after phosphate depletion, were labeled with ³²P-labeled H₃PO₄ in the presence of proteasome inhibitor PSI (Traenckner et al. 1994). The labeling medium was removed and the cells were washed with PBS and lysed in the presence of proteinase and phosphatase inhibitors as reported (Pérez et al. 1995). Cell lysates were first cleared with preimmune serum and then immunoprecipitated with IκBα-, IκBβ-, or p105-specific antisera and incubated with protein A-Sepharose CL4B. The immunocomplexes were washed, samples were boiled in Laemmli buffer, and proteins were separated in a 12% polyacrylamide gel as reported previously (Pérez et al. 1995). Fixed gels were autoradiographed.

For protein expression assays, COS-7 cells were transfected with 5 µg of expression plasmids by the lipofection method. After 24 hr cells were harvested and lysates obtained in 1× SDS Laemmli buffer. Thirty micrograms of total cellular protein was subjected to western blot analysis after SDS-polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose, and immunoblotted with the corresponding rabbit (Rac-1, CDC42, or IκBα) or mouse (RhoA) antiserum (Santa Cruz Laboratories). Immunocomplexes were visualized by enhanced chemiluminescence detection (Amersham) using a biotinylated anti-rabbit or anti-mouse antibody and streptavidin-peroxidase.

EMSA

NIH-3T3 and stable derived cell lines were serum starved 24 hr before extract preparation. For transient expression NIH-3T3 cells were transfected by the calcium phosphate method and 24 hr later serum starved 24 hr more. Positive controls for the assays were obtained by incubating the cells with TNFα (10 ng/ml). Nuclear extracts were prepared essentially as described (Montaner et al. 1995). Briefly, plates were washed twice with ice-cold PBS and scraped in buffer A (20 mM HEPES, 10 mM KCl, 0.15 mM EGTA, 0.15 mM EDTA, 0.15 mM spermidine, 0.15 mM spermine, 1 mM DTT, 1.25% NP-40 at pH 8). Nuclei were obtained by centrifugation at 400g for 5 min and washed in buffer B (20 mM HEPES, 50 mM NaCl, 25% glycerol, 0.15 mM EGTA, 0.15 mM EDTA, 1.5 mM MgCl₂, 1 mM DTT at pH 8). After centrifugation, nuclear proteins were extracted by incubation for 30 min in buffer C (buffer B plus 400 mM NaCl). Two micrograms of nuclear extract was used for incubation with a ³²P-labeled probe containing the NF-κB site. Samples from TNFα-treated and NF-κB-positive cells were incubated for supershift assays with preimmune serum, or antibodies specific for either p50 (p50), two different for RelA (p65a and p65b), or c-Jun (c-jun). Extracts were incubated 15 min with the corresponding antibody before probe binding. Specificity of binding was checked using cold oligonucleotides corresponding with the NF-κB of c-fos SRE binding sites. The free and bound oligonucleotides were separated in a 4% polyacrylamide gel in 0.5% (90 mM Tris-borate, 90 mM boric acid, 2 mM EDTA) TBE buffer. The gels were dried and exposed to X-ray films.

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