Oncogenic Ha-Ras-induced Signaling Activates NF-κB Transcriptional Activity, Which Is Required for Cellular Transformation*

(Received for publication, June 16, 1997, and in revised form, July 22, 1997)

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Ras proteins function in stimulating cell proliferation and differentiation through the activation of Raf-dependent and Raf-independent signal transduction pathways and the subsequent activation of specific transcription factors. The transcription factor NF-κB has been widely studied as a regulator of genes involved in immune and inflammatory responses. A variety of stimuli activate NF-κB through the induced phosphorylation and degradation of the inhibitor IκB followed by nuclear translocation of NF-κB. We show here that oncogenic forms of Ha-Ras activate NF-κB, not through induced nuclear translocation, but rather through the activation of the transcriptional function of the NF-κB RelA/p65 subunit. Importantly, RelA/p65 −/− cells are inefficient in the activation of κB-dependent gene expression in response to oncogenic Ras expression. Furthermore, IκBα expression blocks focus formation in NIH3T3 cells induced by oncogenic Ras. These results demonstrate that NF-κB is a critical downstream mediator of Ha-Ras signaling and oncogenic potential.

Members of the Ras family of GTP-binding proteins serve as essential mediators in the ability of a variety of extracellular stimuli to regulate cellular proliferation and differentiation (1, 2). Oncogenic mutations in ras alleles, which occur in approximately 30% of human cancers, lead to chronic GTP binding, which initiates the activation of signal transduction cascades. In this regard, Ras is known to stimulate both the Raf/MEK/ERK pathway as well as the MEKK/SEK/JNK pathway (3–7). Activation of these and other protein kinase cascades (8–10) is critical for the ability of Ras to exert both its normal and oncogenic functions. The ultimate targets of the Ras-induced signal transduction pathways are transcription factors (see Ref. 4), which regulate the expression of genes involved in proliferation and oncogenesis. Two transcription factors, Ets and c-Jun, have been shown to be essential for Ras-induced gene expression and for Ras-mediated cell transformation in vitro and tumorigenesis (11, 12). In these cases, Ras-induced signaling pathways activate the transcriptional function of both Ets and c-Jun via induced phosphorylation of their transcriptional activation domains (Ref. 13 and reviewed in Ref. 4).

The NF-κB family of proteins has been studied largely for the ability of these transcription factors to regulate a variety of genes involved in immune and inflammatory responses (reviewed in Ref. 14). The activation of these genes in response to inflammatory cytokines, T cell activation signals, lipopolysaccharide, etc. involves the targeted phosphorylation and degradation of the NF-κB inhibitor IκB, allowing nuclear translocation of NF-κB (reviewed in Ref. 14). Additionally, growing evidence indicates that NF-κB may play an important role in controlling cellular proliferation. For example, the c-myc proto-oncogene has been shown to be transcriptionally regulated by NF-κB (15), and antisense inhibition of IκBα leads to cellular transformation of NIH3T3 cells (16). Furthermore, members of the NF-κB and IκB families are associated with chromosomal translocations found in certain lymphomas (for example, see Ref. 17).

We and others previously demonstrated that transient transfection of oncogenic forms of Ha-Ras or of Raf-1 leads to the activation of reporter gene expression controlled by multiple NF-κB sites (18, 19). Consistent with the previous co-transfection studies, κB-dependent gene expression was elevated significantly in both Ras- and Raf-transformed cells as compared with the parental 3T3 cells. Interestingly, increased NF-κB binding activity was not detected in the Ras- or Raf-transformed cells. However, the activity of the transcriptional activation domain of the NF-κB RelA/p65 subunit was significantly increased in these cells. p65 −/− fibroblasts exhibited a reduced κB-dependent transcription response to either oncogenic Ras or Raf but retained their ability to activate the p65/RelA transcriptional activation domain. Finally, oncogenic Ras focus-forming activity was blocked by IκBα expression. These data indicate that NF-κB is an important downstream target for Ras-activated signal transduction pathways.

EXPERIMENTAL PROCEDURES

Cells and Transfections—NIH3T3 cells, the Ha-Ras and Raf-1-transformed counterparts, and the p65 +/− and p65 −/− mouse embryo fibroblasts were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum, penicillin, and streptomycin. DNA transfections were performed by the calcium phosphate precipitation method.**

1 The abbreviations used are: MEK, MAP/ERK kinase; ERK, extracellular signal-regulated kinase; JNK, Jun kinase; CAT, chloramphenicol acetyltransferase; HIV, human immunodeficiency virus; LTR, long terminal repeat; DHFR, dihydrofolate reductase; EMSA, electrophoretic mobility shift assay; CMV, cytomegalovirus.
method as described previously (18). The plasmid pGEM or salmon sperm DNA was used to equalize the amount of DNA transfected in each experiment to 15 μg. CAT analysis and luciferase assays were performed as previously reported (18, 20). In all cases, 1 unit of relative activity represents the CAT or luciferase activity obtained after transfection of the reporter gene alone. All experiments were performed at least three times with similar results.

**Plasmids**—The following plasmids have been described previously: activated Raf (RafBBX) and activated Ras (v-HaRas) expression vectors (18), the IκBα expression vector (21), the super-repressor IκBα expression vector (22), the expression vector encoding the Gal4 DNA-binding domain fused to the C-terminal domain of p65/RelA (Gal4p65aaS19–551 (23)), the reporters 5X-IκBα-CAT and 3X-mutB-CAT (18), the HIV LTR-CAT and HIV-ΔB-CAT reporters (18), 5X-Gal4-CAT (24), and DHFR-CAT (25).

**Extracts and Gel Mobility Shift Assays**—Nuclear and cytoplasmic extracts were prepared as described previously (26). For double sucrose pad purification, washed nuclei were resuspended in lysis buffer lacking Nonidet P-40 and layered on a sucrose pad (30% sucrose, 60 mM KCl, 15 mM NaCl, 15 mM Hepes, 2 mM EDTA, 0.75 mM spermidine, 0.15 mM spermine and 1 mM dithiothreitol) and centrifuged for 15 min at 3,000 rpm in an HB4 rotor. The sucrose pad was then removed, the nuclei resuspended, and the process repeated. Nuclear extracts were then prepared from the sucrose pad-purified nuclei. Gel mobility shift assays (EMSAs) were performed as described previously (26).

**Focus Formation Assays**—NIH3T3 cells were transfected by calcium phosphate coprecipitation essentially as described (27). For each 60-mm plate, 10 ng of Ras expression vector pZip-phosphate coprecipitation essentially as described (27). For each 60-mm plate, 10 ng of Ras expression vector pZip-phosphate coprecipitation essentially as described (27).

**RESULTS**

**Oncogenic Ras- or Raf-transformed Cells Exhibit Increased κB-dependent Transcription without Increased Nuclear Accumulation of NF-κB**—Previous transient cotransfection experiments indicated that expression of either oncogenic Ras or oncogenic Raf led to a significant activation of expression of a κB-dependent reporter (18). Consistent with the previous cotransfection data, the activity of a κB-dependent reporter was significantly elevated in both Ras- and Raf-transformed cells but not in the parental NIH3T3 cells (Fig. 1A). A reporter mutated in the NF-κB sites did not exhibit this enhanced activity, and expression of the NF-κB inhibitor IκBα blocked the Ras- and Raf-induced activation of κB-dependent reporter activity, indicating that NF-κB regulates the transcription response. A similar result was obtained with the NF-κB-dependent HIV-LTR reporter (data not shown). Additionally, expression of the non-Ras-responsive DHFR-CAT reporter was approximately equivalent in each of the three cell types (data not shown) showing that the differential responses observed in the transformed cells were not due to differential uptake of plasmids.

To determine if oncogenic Ras as well as oncogenic Raf activated nuclear accumulation of NF-κB, gel mobility shift assays (EMSAs) were performed with nuclear extracts from parental NIH3T3 cells, oncogenic Ras-transformed NIH3T3 cells, or Raf-transformed NIH3T3 cells. To demonstrate that binding activity was exclusively nuclear, extracts were prepared from double sucrose pad-purified nuclei. Immunoblotting for NF-κB/p105 (which is cytoplasmic) indicated that there was no cytoplasmic contamination in the nuclear preparations (data not shown). As shown in Fig. 2, NF-κB was detected in the nuclei of each of the different cells at similar levels. Antibody "supershift" experiments showed that this binding activity is authentic, p65/RelA-containing NF-κB (data not shown). These results were surprising and indicated that the activation of κB-dependent transcription observed in the transfection experiments shown in Fig. 1 was not controlled by the induced nuclear accumulation of NF-κB but suggested that this response was mediated by the relatively low levels of constitutively nuclear NF-κB in NIH3T3 fibroblasts. It should be noted that transient transfection of oncogenic Ras into 3T3 cells or the induction of oncogenic Ras in Rat-1 cells led to an approximate 3-fold increase in nuclear NF-κB (data not shown); however established Ras-transformed cells did not exhibit this property. These experiments indicated that oncogenic Ras or Raf can activate κB-dependent transcription without enhancing nuclear levels of NF-κB.

**Oncogenic Forms of Ras and Raf Activate the Transcriptional Function of NF-κB p65/RelA**—To explain the activation of κB-dependent transcription by oncogenic forms of Ras or Raf without an induction of NF-κB nuclear translocation, we asked whether the transcriptional activation function of NF-κB was stimulated...
in the transformed cells. A plasmid (Gal4p65) encoding a fusion protein between the DNA binding domain of the yeast transcription factor Gal4 and the C-terminal (TA1) transactivation domain of RelA was transfected into parental NIH3T3 cells, or Ras- or Raf-transformed cells, along with a luciferase reporter containing upstream Gal4-binding sites. Luciferase activity driven by p65/RelA transcriptional activation function controlled by the TA1 transcriptional activation domain.

The results described above suggested that the RelA subunit of NF-κB may function as a critical downstream transcriptional effector for the Ras oncoprotein. To test this hypothesis, we utilized immortalized RelA−/− and RelA+/− embryonic fibroblasts isolated from p65−/− or p65+/− mice for transfection and gene expression studies. Oncogenic Ras was ineffective at activating NF-κB-dependent gene expression in the p65−/− cells (approximately a 2-fold activation), whereas effective Ras activation of NF-κB-dependent gene expression (approximately 7-fold) was observed in the RelA+/− cells, as expected (Fig. 3B). To show that the Ras-responsive signal transduction pathway was still operative in the RelA−/− cells, the Gal4p65 construct was cotransfected with either activated Ha-Ras or activated Raf-1. Ras activated the Gal4p65 construct as effectively in RelA+/− cells as in RelA−/− cells. These results demonstrate that the RelA/p65 subunit of NF-κB is required for oncogenic Ras to effectively activate gene expression driven by consensus NF-κB-binding sites.

NF-κB Is Required for Ras-mediated Transformation—To determine whether NF-κB is required for cellular transformation controlled by oncogenic Ha-Ras, we determined whether the inhibition of NF-κB would affect the ability of Ras to cause formation of transformed foci in cultured NIH3T3 cells. To specifically inhibit NF-κB activity, we used an expression vector encoding IκBα, which can enter the nucleus and relocate NF-κB to the cytoplasm (29). Expression of pZIP-ras(61L) together with the empty CMV vector yielded an average of approximately 160 foci/plate (Fig. 4). Co-expression of oncogenic Ras with wild-type IκBα blocked focus formation activity by greater than 50%. Co-expression with a super-repressor form of IκBα (IκBα(AA)) that is unable to be inducibly phosphorylated or degraded in response to stimuli (see Ref. 22) blocked focus formation by approxi-
mately 70–75% (Fig. 4). Expression of IκBα did not block expression of the promoter driving Ras expression or Ras protein expression (data not shown). Interestingly, IκBα was unable to block the ability of activated Rho (Rho63L) to induce focus formation. In these experiments, activated Rho yielded approximately 20 foci/plate, and IκBα expression did not reduce this number of foci (data not shown).


discussion

The data presented here indicate that oncogenic ras alleles activate NF-κB-dependent transcription, not through the induced nuclear translocation of NF-κB, but rather through the stimulation of the transcriptional activation function of NF-κB via the targeting of the RelA/p65 subunit. Furthermore, the data indicate that NF-κB is required for Ras to initiate efficient cellular transformation and that NF-κB plays a role in mediating certain essential aspects of cellular transformation. Thus, NF-κB joins Etα family members (13) and c-Jun (4, 12) as downstream targets of oncogenic Ras that are required for Ras-mediated cellular transformation.

How does Ras activate NF-κB functional activity? Our data strongly indicate that the transcriptional activation function of RelA/p65 NF-κB is potentiated in both Ras- as well as Raf-transformed cells, and at least two mechanisms exist to explain this phenomenon. First, a Ras-initiated signal transduction pathway may target the p65 transcriptional activation domain for phosphorylation, which may allow enhanced interactions with a transcriptional co-activator or with basal transcriptional machinery. Such a mechanism appears to be operative for both Etα-1 and -2 and for c-Jun (4, 13). A second mechanism may be that a transcriptional co-activator is modified such that it interacts functionally with p65 transcriptional activation domain. Also of importance is identification of the signal transduction pathway that is initiated by Ras to stimulate NF-κB transcriptional activity. Since both oncogenic Ras as well as oncogenic Raf stimulate IκB-dependent activity, it may be assumed that the relevant pathway is downstream of Raf and is, therefore, the MEK/ERK pathway. However, inhibitors of this pathway did not block the ability of Ras to activate IκB-dependent transcription, and dominant negative forms of kinases in the SEK/JNK pathway were able to block this response. Thus the ability of Raf to activate IκB-dependent gene expression in a MEK/ERK-independent pathway may be explained by the recent observation that Raf stimulates JNK activity via an auto-crime mechanism (30).

Prior studies have shown that the major regulatory mechanism involved in regulating IκB-dependent transcription is induced nuclear translocation (see Ref. 14). Our data indicate that significant IκB-dependent transcription can be realized without enhancing the constitutive, low nuclear levels of NF-κB. This suggests that under some circumstances the functional activity of NF-κB can be separated from induction of nuclear translocation. Consistent with this concept are the recent observations that the tyrosine kinase inhibitor genistein blocks the ability of NF-κB to stimulate transcription of an NF-κB-dependent reporter but is not able to block nuclear translocation of NF-κB (31) and that phorbol 12-myristate 13-acetate can activate the TA2 transcriptional activation domain of RelA/p65 (23).

Evidence that NF-κB is required for Ras-mediated cellular transformation is consistent with several observations indicating a role for NF-κB in controlling cell growth. First, it has been shown that NF-κB can regulate c-myc gene expression. Second, antisense studies indicate that NF-κB can control oncogenesis. These experiments utilized antisense to p65 to block oncogene-controlled transformation (32, 33) and antisense to IκBα to induce transformation of NIH3T3 cells (16). Additionally, other oncogenes such as Her2/NEU are known to activate NF-κB (34). Thus, the activation of NF-κB may be common to a number of oncogenes, particularly those that utilize Ras-controlled signaling pathways. Additionally, we have been able to show NF-κB activation is required to block a Ras-induced apoptotic response. This result is consistent with recent data (22, 35–38) that NF-κB activation can block the induction of apoptosis. Further experiments are required to establish the exact role that NF-κB plays in controlling Ras-mediated oncogenesis.

acknowledgment—We gratefully acknowledge Dr. P. Baeuerle for the kind gift of the GaI4p65 construct.

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