dase, and 3.3-diaminobenzidine-$Ni^{3+}$. As controls, either TUNEL enzyme or peroxidase converter enzyme was omitted.

25. Cryosections of embryonal mouse heads (10 μm) were fixed for 10 min in acetone, incubated with monoclonal antibody F4/80 [undiluted cell supernatant, D. A. Hume and S. Gordon, *J. Exp. Med.* **157**, 1704 (1983)] for 1 hour at room temperature, rinsed three times with PBS, incubated with peroxidase-coupled rabbit antibody to mouse secondary antibody for 1 hour, rinsed again three times with PBS, and stained with 3.3-diaminobenzidine. Sections were washed in 0.9% NaCl, incubated in $CuSO_4$ for 30 min, washed again, immersed in hematoxylin, and dehydrated.
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# A Subclass of Ras Proteins That Regulate the Degradation of IκB

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**Small guanosine triphosphatases, typified by the mammalian Ras proteins, play major roles in the regulation of numerous cellular pathways. A subclass of evolutionarily conserved Ras-like proteins was identified, members of which differ from other Ras proteins in containing amino acids at positions 12 and 61 that are similar to those present in the oncogenic forms of Ras. These proteins, κB-Ras1 and κB-Ras2, interact with the PEST domains of IκBα and IκBβ [inhibitors of the transcription factor nuclear factor kappa B (NF-κB)] and decrease their rate of degradation. In cells, κB-Ras proteins are associated only with NF-κB:IκBβ complexes and therefore may provide an explanation for the slower rate of degradation of IκBβ compared with IκBα.**

The transcription factor NF-κB plays an important role in the expression of a large number of inducible genes. In unstimulated cells, NF-κB remains in an inactive form in the cytoplasm bound to a member of the IκB family of inhibitors. Exposure of cells to various stimuli—including cytokines [such as tumor necrosis factor (TNF) and interleukin-1 (IL-1)], lipopolysaccharides, and ultraviolet light—results in the activation of signal transduction pathways that lead to the phosphorylation and degradation of the IκB proteins. The released NF-κB then translocates to the nucleus where it up-regulates the synthesis of genes involved in immune and inflammatory responses, including cytokines and adhesion molecules (*1*).

Another class of proteins that play a very important role in the transduction of cell surface signals is small guanosine triphosphatases (GTPases) such as Ras, Rap, Ral, and Rho (*2*, *3*). The Ras family (H-ras, K-ras, and N-ras) became the focus of intense interest when it was discovered that specific mutations in these proteins were associated with ~30% of all human tumors, including 50% of colon and 90% of pancreatic adenocarcinomas (*4*, *5*). These mutations, which lock the Ras proteins in a constitutively active, guanosine triphosphate (GTP)–bound form, are almost exclusively confined to three critical positions, 12, 13, and 61 (*6*). The ability of Ras proteins to transduce growth-stimulatory signals is dependent on their localization to the inner side of the plasma membrane and requires farnesylation of their COOH-termini (*7*).

Oncogenic Ras and the Rho GTPases can activate NF-κB, although the details of the signal transduction pathways responsible remain to be identified (*8*, *9*). Ras-activated NF-κB is proposed to act as an antiapoptotic factor that helps prevent transformed cells from undergoing p53-independent apoptosis (*8*). In an attempt to further explore the regulation of NF-κB signaling, we used yeast two-hybrid interaction screens with IκB proteins as bait to identify interacting molecules. In one such screen, using the COOH-terminal portion of IκBβ, IκBβΔ1 (amino acids 173 to 361), as bait, we isolated a series of mouse cDNAs (*10*), including a Ras-like protein that we named κB-Ras1 (for IκB-interacting Ras-like protein 1). In yeast, κB-Ras1 specifically interacted with full-length IκBβ and IκBβΔ1, but not with the IκB-like protein Bcl-3, the NF-κB subunits p50 and p65, or the transcription factors Myc and Bicoid (*11*–*13*). A full-length human cDNA clone of κB-Ras1 was then isolated, in which the initiator methionine is preceded by an in-frame stop codon. Using the sequence of κB-Ras1, we searched the expressed sequence tag (EST) databases and identified a related protein that was named κB-Ras2. Polymerase chain reaction was used to isolate the full-length human κB-Ras2 clone, which encodes a protein highly related to κB-Ras1 (71% identity, 85% similarity) (Fig. 1A). In vitro translation of the κB-Ras2 cDNA produced a protein of about 22 kD, which is slightly smaller than the human κB-Ras1 protein (Fig. 1B). In addition, a search of *Drosophila* EST databases identified a highly conserved homolog to κB-Ras (40% identity and 68% similarity to human κB-Ras1; Fig. 1A). This is of particular interest, considering that homologs to both components of NF-κB and Ras are present in *Drosophila*.

Comparison of the sequences of the mammalian κB-Ras1 and κB-Ras2 with other small GTPases reveals their overall similarity, for example, 30% identity and 49% similarity to human K-ras (Fig. 1C) (*14*, *15*). Their assignment as a distinct subgroup of Ras-like proteins becomes apparent when small GTPases are sorted and classified with a dendrogram (Fig. 1D) (*16*). The major difference between κB-Ras and other Ras proteins (H-, K-, and N-Ras) is the lack of COOH-terminal membrane attachment sequences (Cys-a-a-X sequences, where "a" is any aliphatic amino acid and "X" is any amino acid) and the presence of alanine or leucine at position 13 (instead of glycine) and leucine at position 65 (instead of glutamine) (equivalent to positions 12 and 61, respectively, in H-Ras) (Fig. 1C) (*14*, *15*). The presence of alanine or leucine at position 12 and leucine at position 61 locks the known Ras proteins into a deregulated, active, GTP-bound conformation and underlies their oncogenicity (*17*, *18*). We therefore tested whether κB-Ras proteins also bound GTP. We produced COOH-terminal His-tagged κB-Ras1 protein in a bacterial overexpression system and used the GTP-binding protein Sec4 as a positive control for a nucleotide-binding assay (*19*). The κB-Ras1 protein specifically bound GTP, but not adenosine triphosphate (ATP) (Fig. 1E). The expression of κB-Ras1 and κB-Ras2 in various human tissues was examined with multiple tissue Northern (RNA) blot analysis. Both human κB-Ras1 and κB-Ras2 are widely expressed and are encoded by mRNAs of 1.6 and 2.5 kb, respectively (Fig. 1F). The nonoverlapping expression of the two κB-Ras proteins suggests that they might have distinct functions in different tissues.

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To explore the possible regulation of NF-κB activity by κB-Ras1 and κB-Ras2, we analyzed the interaction between κB-Ras and IκB proteins in in vitro pull-down assays. Both IκBα– and IκBβ–glutathione *S*-transferase (GST) fusion proteins interacted specifically with in vitro–translated $^{35}$S-methionine–labeled κB-Ras1 and κB-Ras2 and very weakly with K-Ras (Fig. 2A). *Drosophila* κB-Ras also interacted with the *Drosophila* IκB homolog, cactus, thus demonstrating the evolutionary conservation of this interaction (Fig. 2B). The region of κB-Ras that interacted with IκBα and IκBβ was localized with COOH- and $NH_2$- terminal truncations of κB-Ras1 to amino acids 58 to 93, which includes the switch II region of the potential GTP-binding domain (amino acids 64 to 79 in κB-Ras, which corresponds to amino acids 60 to 75 in H-Ras) (Fig. 2C) (*20*, *21*). In vitro–translated κB-Ras proteins loaded with guanosine diphosphate or GTP-γ−S interacted equally well with GST-IκB proteins, as demonstrated by GST pull-down analysis (*22*). Therefore, in agreement with the sequence of κB-Ras proteins that predicts a constitutively GTP-bound state, the interaction between κB-Ras and IκB does not appear to be regulated by nucleotide binding. The region of IκBα that interacts with κB-Ras1 was also mapped in His tag pull-down assays. In agreement with the two-hybrid interaction studies, it is the COOH-terminal PEST domain–containing region of IκBα that is mainly responsible for interaction with κB-Ras1 and κB-Ras2 (Fig. 2D) (*22*).

To assess the possible functional consequences of these interactions, we cotransfected κB-Ras1 and κB-Ras2 into 293 cells, along with a NF-κB reporter plasmid, pBIIx-luc (*23*, *24*). Addition of TNF-α (10 ng/ml) activated expression of the reporter gene by about 35-fold. However, cotransfected κB-Ras1 or κB-Ras2 inhibited the TNF-α–dependent activation of this reporter in a dose-dependent manner (Fig. 3A). Cotransfection of a Δ–κB-Ras2 protein lacking the $NH_2$-terminal 90 amino acids, which fails to interact with IκB, did not lead to inhibition of NF-κB activity in TNF-α– and IL-1–stimulated cells (*22*). In addition, the NFAT transcription factor, which is not regulated by IκB proteins, was relatively weakly inhibited by κB-Ras in a reporter assay (Fig. 3B).

To determine the point in the NF-κB activation pathway that is influenced by κB-Ras proteins, we transfected cells with either κB-Ras1 or κB-Ras2 and two intermediates from the IL-1 and TNF signal transduction pathways. We transfected 293 and HeLa cells (*11*) with the NF-κB–inducing kinase (NIK) and IκB-kinase β (IKKβ) (Fig. 3C). κB-Ras proteins inhibited NF-κB activation by both these signaling kinases, suggesting that κB-Ras–mediated inhibition of NF-κB takes place either at the IκB phosphorylation step or further downstream. However, transfected κB-Ras proteins did not affect the kinase activity of immunoprecipitated IKKα when tested by in vitro kinase assay on a GST-IκBα substrate (Fig. 3D).

The association of κB-Ras proteins with the PEST domain of IκB proteins suggested that the PEST-dependent turnover of IκB proteins could be affected, leading to an increase in the amount of IκB and a consequent inhibition of NF-κB activation. To test this possibility, we transfected κB-Ras1 and κB-Ras2 and a mutant form of p65 (mutated at the protein kinase A phosphorylation site, $Ser^{276} \rightarrow$ Ala) that does not activate transcription (*25*) into 293 cells and examined the amount of transfected IκB proteins in these cells. Association of p65 with IκBα helps to stabilize it, thus allowing the accumulation of IκBα in cells (*26*). Overexpressed κB-Ras proteins were nearly as effective as p65 in stabilizing the transfected IκB proteins (Fig. 4A). Therefore, binding of κB-Ras proteins to the PEST regions of IκB proteins probably inhibits their continuous turnover in unstimulated cells. To test whether κB-Ras proteins could stabilize IκB proteins in stimulated cells, we transfected 293 cells with either

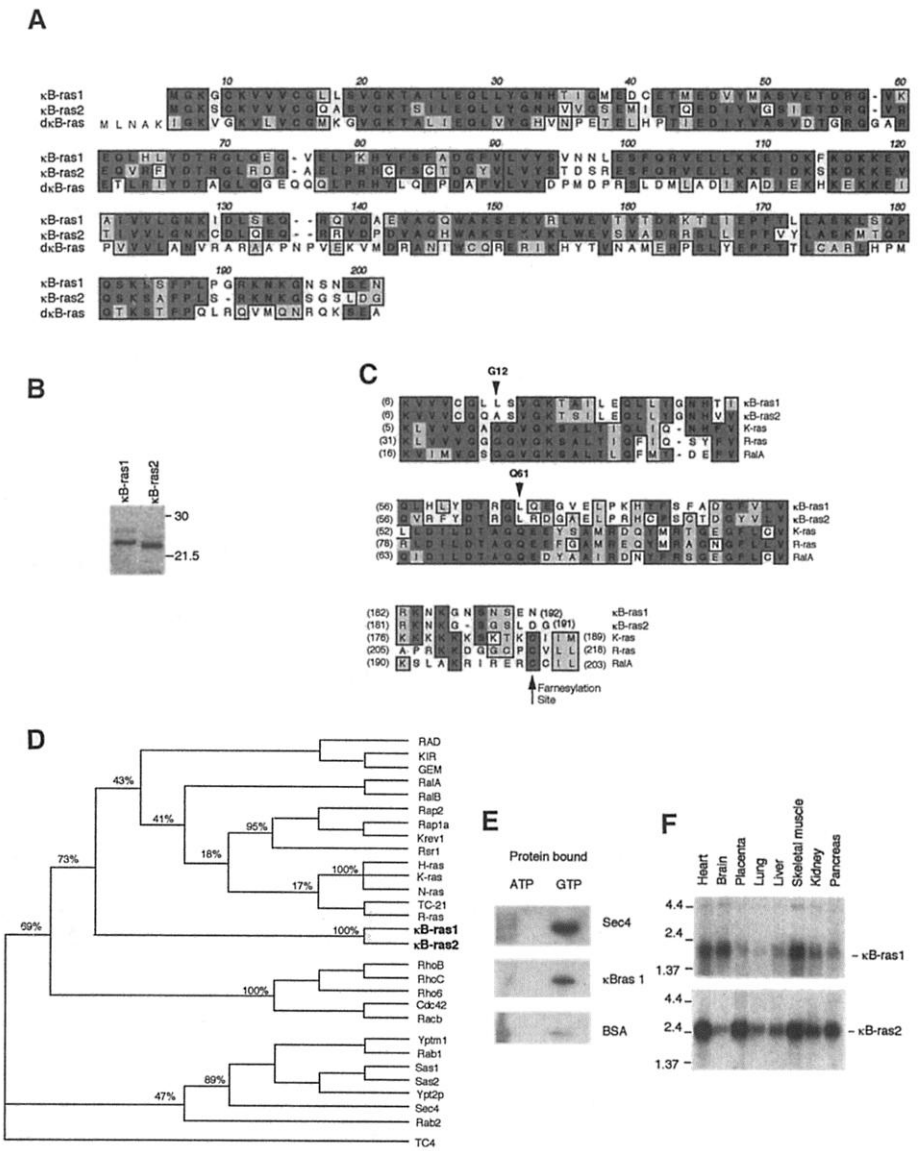


**Fig. 1.** Sequence alignments and expression of κB-Ras1 and κB-Ras2. **(A)** Alignment of the κB-Ras1, κB-Ras2, and *Drosophila* κB-Ras (dκB-Ras) polypeptide sequences (*33*). Identical and similar amino acids are indicated by light and dark shading, respectively. **(B)** In vitro translation of $^{35}$S-methionine–labeled κB-Ras1 and κB-Ras2, analyzed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (15%). **(C)** Alignment of κB-Ras polypeptide sequences with other Ras family members. Regions around the oncogenic mutations $Gly^{12}$ and $Gln^{61}$ (numbered according to the sequence of K-Ras) and the COOH-terminal farnesylation site are shown. **(D)** Classification of κB-Ras1 and κB-Ras2 in a dendrogram of small GTPases. Bootstrapping analysis determined the percentage of chance that protein members to the left of a specific branch were of the same group. **(E)** Specific binding of κB-Ras to GTP. Bacterially produced κB-Ras1, Sec4, and bovine serum albumin were incubated with $^{32}$P-labeled ATP and GTP and then immobilized on a slot blot. **(F)** A multiple tissue Northern blot (Clontech) probed with $^{32}$P-labeled κB-Ras1 and κB-Ras2.

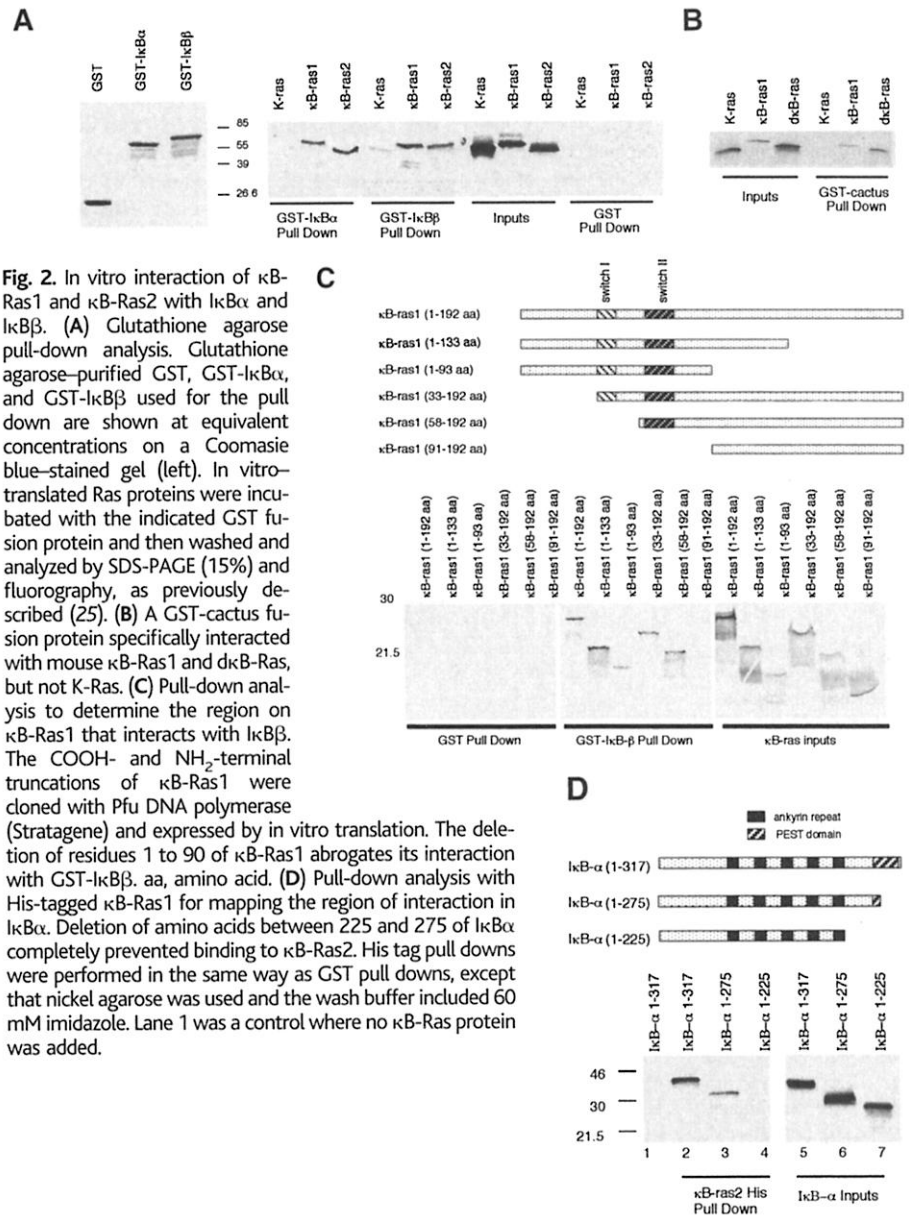


**Fig. 2.** In vitro interaction of κB-Ras1 and κB-Ras2 with IκBα and IκBβ. (**A**) Glutathione agarose pull-down analysis. Glutathione agarose–purified GST, GST-IκBα, and GST-IκBβ used for the pull down are shown at equivalent concentrations on a Coomasie blue–stained gel (left). In vitro–translated Ras proteins were incubated with the indicated GST fusion protein and then washed and analyzed by SDS-PAGE (15%) and fluorography, as previously described (*25*). (**B**) A GST-cactus fusion protein specifically interacted with mouse κB-Ras1 and dκB-Ras, but not K-Ras. (**C**) Pull-down analysis to determine the region on κB-Ras1 that interacts with IκBβ. The COOH- and $NH_2$-terminal truncations of κB-Ras1 were cloned with Pfu DNA polymerase (Stratagene) and expressed by in vitro translation. The deletion of residues 1 to 90 of κB-Ras1 abrogates its interaction with GST-IκBβ. aa, amino acid. (**D**) Pull-down analysis with His-tagged κB-Ras1 for mapping the region of interaction in IκBα. Deletion of amino acids between 225 and 275 of IκBα completely prevented binding to κB-Ras2. His tag pull downs were performed in the same way as GST pull downs, except that nickel agarose was used and the wash buffer included 60 mM imidazole. Lane 1 was a control where no κB-Ras protein was added.

κB-Ras1 or 2, stimulated them with TNF, and immunoblotted cell extracts for IκBα. Cells transfected with κB-Ras had increased amounts of IκBα protein, suggesting that κB-Ras proteins inhibited degradation of IκB proteins, probably by interfering with the PEST domain–mediated turnover (Fig. 4B). Although stimulation of cells led to the degradation of IκBα, phosphorylated IκBα was only detected in κB-Ras–transfected cells, indicating that degradation of the phosphorylated form of IκBα was slowed down (Fig. 4B). These results suggest that association of κB-Ras with IκBα (Fig. 4, A and B) and IκBβ (Fig. 4A), phosphorylated or unphosphorylated, slowed down their degradation. In cells transfected with p65, κB-Ras2 inhibited κB-dependent transcription from a pBIIx-luciferase reporter by about 70%, suggesting that the increased amount of IκB proteins in κB-Ras–transfected cells may have sequestered the transfected p65 in the cytoplasm (Fig. 4C).

To examine the association of κB-Ras with IκB proteins in cells, we raised a polyclonal antibody against recombinant κB-Ras1. This antibody detects both κB-Ras1 and κB-Ras2 in immunoblots of recombinant proteins or of proteins from cell extracts (Fig. 5A). We used antibodies to p65 to precipitate the majority of the NF-κB:IκBα and NF-κB:IκBβ complexes and analyzed the immunoprecipitates for associated proteins. As a control, we boiled the extracts with 1% SDS to disrupt noncovalent interactions, neutralized the SDS with 1% Triton X-100, and then completed the immunoprecipitation assays. The p65 antibody immunoprecipitated IκBα, IκBβ, and κB-Ras (Fig. 5B). Boiling the extracts disrupted the NF-κB:IκB complexes, and only p65 was precipitated (Fig. 5B).

To test whether κB-Ras was associated with both IκBα and IκBβ complexes, we immunoprecipitated proteins from 293 cell extracts with antibodies to IκBα and IκBβ. Both antibodies

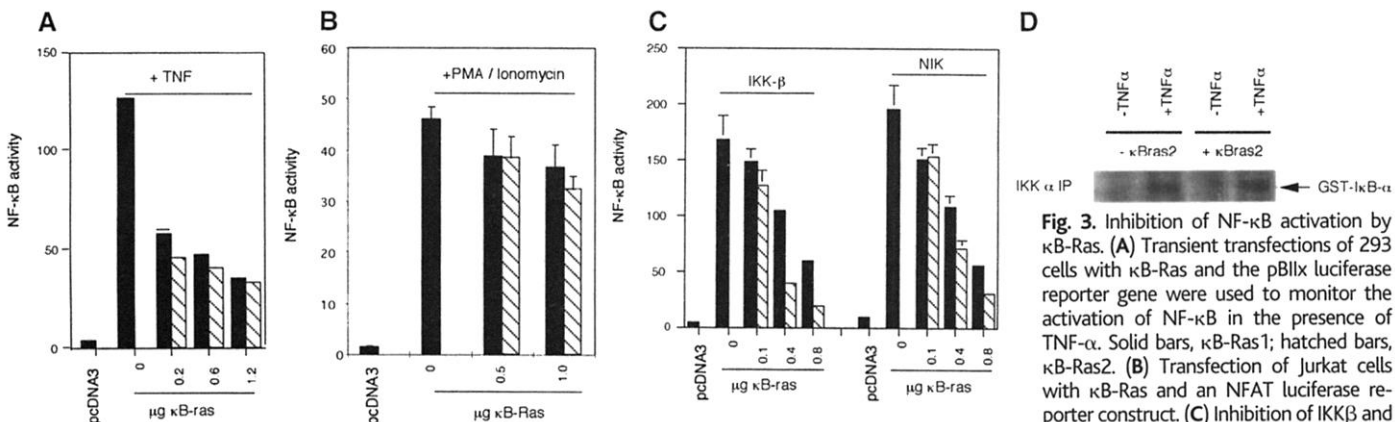


**Fig. 3.** Inhibition of NF-κB activation by κB-Ras. (**A**) Transient transfections of 293 cells with κB-Ras and the pBIIx luciferase reporter gene were used to monitor the activation of NF-κB in the presence of TNF-α. Solid bars, κB-Ras1; hatched bars, κB-Ras2. (**B**) Transfection of Jurkat cells with κB-Ras and an NFAT luciferase reporter construct. (**C**) Inhibition of IKKβ and NIK induced NF-κB activation by κB-Ras. 293 cells were transfected with IKKβ (0.4 μg) or NIK (0.4 μg) and the indicated amount of κB-Ras1 or κB-Ras2, and then harvested after 24 hours for the luciferase assay. (**D**) κB-Ras does not affect the kinase activity of TNF-α–activated IKK. 293 cells were transfected with either pcDNA3 or κB-Ras, incubated for 24 hours, treated with TNF-α (10 ng/ml) as indicated for 15 min, and then harvested in TNT buffer. Proteins from cell lysates were then immunoprecipitated (IP) with an antibody to IKKα, precipitated with protein A–Sepharose, washed, and used in a kinase assay with purified GST-IκBα and [$\gamma$-$^{32}$P]ATP as substrates. After a 30-min incubation at 30°C, GST-IκBα was precipitated with glutathione Sepharose beads, washed, resolved by 10% SDS-PAGE, and then dried and visualized by autoradiography.

immunoprecipitated the NF-κB:IκB complex, as indicated by the presence of other components of this complex; for example, p65 coprecipitated both IκBα and IκBβ (Fig. 5B). However, κB-Ras was only detected in IκBβ complexes, suggesting that κB-Ras proteins are predominantly associated with NF-κB:IκBβ complexes in cells. Therefore, although direct interaction experiments (in vitro GST pull downs or overexpression in cells) indicated that κB-Ras proteins could associate with the PEST domains of both IκBα and IκBβ, in cells, κB-Ras proteins appear to be associated only with NF-κB:IκBβ complexes. The specificity of κB-Ras proteins for IκBβ may therefore explain a long-standing question in NF-κB regulation, namely, why IκBβ is degraded more slowly than IκBα or sometimes not degraded at all (*27*, *28*), despite the fact that both IκB's are phosphorylated equally well by activated IKKβ (*29*, *30*).

We tested whether the ability of κB-Ras to discriminate between IκBα and IκBβ only man-

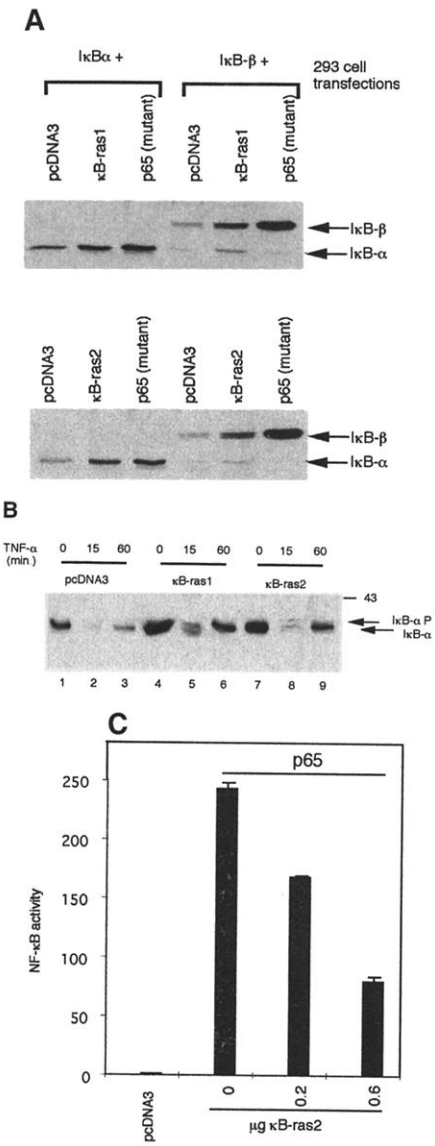


**Fig. 4.** κB-Ras proteins increase cellular IκB protein levels. (**A**) Stabilization of IκBα and IκBβ by cotransfected κB-Ras1 and κB-Ras2. 293 cells were transfected with either IκBα or IκBβ (0.4 μg), κB-Ras1 or κB-Ras2, or mutant p65 (Ser$^{276}$ → Ala) (1.2 μg). (**B**) Stabilization of endogenous IκBα in 293 cells treated with TNF-α. Cells were transfected with pcDNA3 or κB-Ras1 or κB-Ras2, incubated for 24 hours, and then treated with TNF-α (10 ng/ml) before being harvested at 0, 15, and 60 min. Immunoblot analysis was done on the cell extracts to examine the stabilization of IκBα by κB-Ras1 and κB-Ras2 in the presence of TNF-α. The doublet bands seen in the κB-Ras lanes are the phosphorylated and unphosphorylated forms of IκBα. (**C**) Inhibition of p65-mediated transcription by κB-Ras2 in pBIIx luciferase reporter assay.

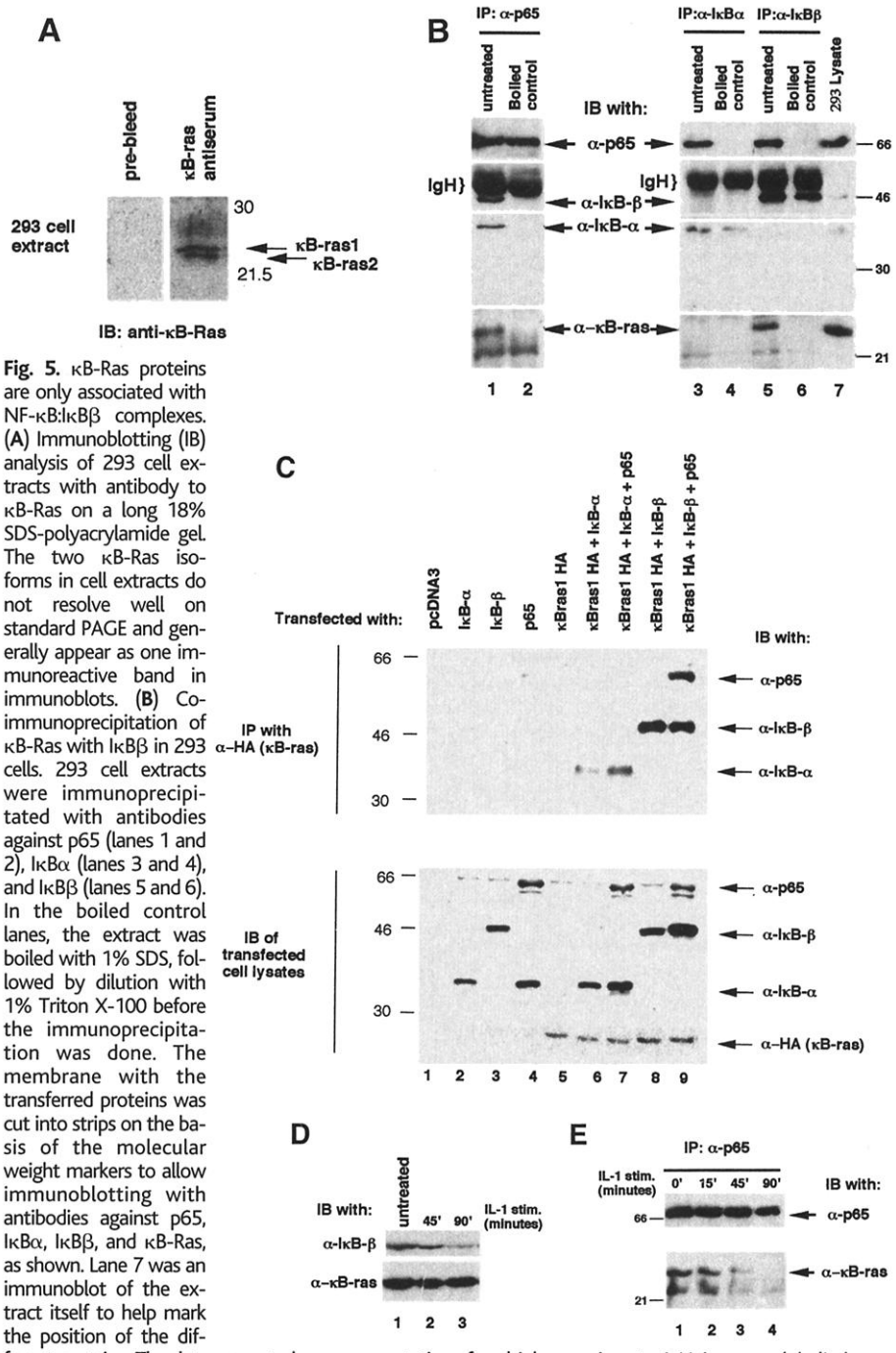


**Fig. 5.** κB-Ras proteins are only associated with NF-κB:IκBβ complexes. (**A**) Immunoblotting (IB) analysis of 293 cell extracts with antibody to κB-Ras on a long 18% SDS-polyacrylamide gel. The two κB-Ras isoforms in cell extracts do not resolve well on standard PAGE and generally appear as one immunoreactive band in immunoblots. (**B**) Coimmunoprecipitation of κB-Ras with IκBβ in 293 cells. 293 cell extracts were immunoprecipitated with antibodies against p65 (lanes 1 and 2), IκBα (lanes 3 and 4), and IκBβ (lanes 5 and 6). In the boiled control lanes, the extract was boiled with 1% SDS, followed by dilution with 1% Triton X-100 before the immunoprecipitation was done. The membrane with the transferred proteins was cut into strips on the basis of the molecular weight markers to allow immunoblotting with antibodies against p65, IκBα, IκBβ, and κB-Ras, as shown. Lane 7 was an immunoblot of the extract itself to help mark the position of the different proteins. The data presented are representative of multiple experiments. IgH, immunoglobulin heavy chain. (**C**) Transfection of COS cells with the indicated plasmids, followed by immunoprecipitation with antibody to HA and immunoblotting with antibodies to p65, IκBα, and IκBβ. Immunoblots with antibody to HA of the antibody to HA immunoprecipitates were uninformative because of background immunostaining. The data presented are representative of multiple experiments. (**D**) Immunoblot analysis of extracts from 293 cells stimulated for the indicated times with IL-1. (**E**) Immunoprecipitation of p65 from 293 cells stimulated with IL-1, followed by immunoblotting of the precipitates with the antibody to κB-Ras.

ifested itself in the context of NF-κB:IκB complexes and not free IκB proteins. We transfected hemagglutinin (HA)–tagged κB-Ras1, p65, IκBα, and IκBβ by themselves or in combination into COS cells (*31*). COS cells were used because they have extremely low amounts of NF-κB and related proteins, and hence only the transfected proteins are detected in immunoblotting assays. Cotransfected HA-tagged κB-Ras1 associated efficiently with coexpressed IκBβ but very poorly with coexpressed IκBα (Fig. 5C). Cotransfection of p65 along with κB-Ras1 and IκBα or IκBβ resulted in p65 being immunoprecipitated only with the κB-Ras–IκBβ complex (Fig. 5C). Thus, κB-Ras appears to associate only with p65-bound IκBβ protein in these overexpression experiments.

If the association of κB-Ras proteins with NF-κB:IκBβ complexes is mediated mainly through interaction with IκBβ, then degradation of IκBβ should lead to a loss of κB-Ras protein that can be immunoprecipitated with NF-κB. To test this hypothesis, we stimulated 293 cells with IL-1, an inducer that causes IκBβ degradation in about 90 min (Fig. 5D). Immunoblotting with an antibody to κB-Ras revealed that the amount of κB-Ras proteins was not altered upon stimulation (Fig. 5D). We then immunoprecipitated NF-κB p65 from the stimulated extracts, followed by immunoblotting of the immunoprecipitates with the κB-Ras antibody. The amount of coimmunoprecipitated κB-Ras decreased in a manner proportional with the decrease in IκBβ levels (Fig. 5E), consistent with the possibility that κB-Ras is anchored to the NF-κB:IκBβ complex through IκBβ.

The κB-Ras proteins specifically associate with PEST domains and influence their degradation. It is therefore possible that they might also regulate the turnover of other PEST domain–containing proteins besides IκBβ. In fact, we detected κB-Ras proteins in high–molecular weight complexes, distinct from the cytoplasmic NF-κB/IκB complexes (*22*). Identification of these other κB-Ras–associated proteins will therefore be crucial in understanding the overall biological role of κB-Ras proteins.

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31. Cells were harvested and blotted as previously described (*25*) with membranes probed with antibody to IκBα or IκBβ (C21 and C20; Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal rabbit antibodies, or monoclonal mouse antibody to HA (12CA5). Immunoprecipitations were performed on transfected COS or 293 cells (3.5- and 10-cm plates, respectively), as previously described, with antibody to HA.
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33. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.


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# Signaling and Circuitry of Multiple MAPK Pathways Revealed by a Matrix of Global Gene Expression Profiles


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Genome-wide transcript profiling was used to monitor signal transduction during yeast pheromone response. Genetic manipulations allowed analysis of changes in gene expression underlying pheromone signaling, cell cycle control, and polarized morphogenesis. A two-dimensional hierarchical clustered matrix, covering 383 of the most highly regulated genes, was constructed from 46 diverse experimental conditions. Diagnostic subsets of coexpressed genes reflected signaling activity, cross talk, and overlap of multiple mitogen-activated protein kinase (MAPK) pathways. Analysis of the profiles specified by two different MAPKs—Fus3p and Kss1p—revealed functional overlap of the filamentous growth and mating responses. Global transcript analysis reflects biological responses associated with the activation and perturbation of signal transduction pathways.


Mitogen-activated protein kinase (MAPK) cascades control changes in gene expression, cytoskeletal organization, and cell division (*1*). Genome-wide DNA microarrays are emerging as a powerful tool for broad correlation of gene activity with alterations in physiological or developmental states (*2–4*). Global expression profiles assembled from diverse ex-