Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 9247–9251, October 1993
Biochemistry

Raf-1 protein kinase activates the NF-κB transcription factor by dissociating the cytoplasmic NF-κB–IκB complex

(protein phosphorylation/cell cycle/oncogene/signal transduction/gene expression)

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Communicated by Paul B. Sigler, July 2, 1993

ABSTRACT **Addition of mitogenic growth factors to quiescent cells triggers complex signal transduction cascades that result in the reprogramming of gene expression and entry into the cell cycle. We have found that an oncogenic variant of the c-Raf-1 protein kinase stimulated the expression of promoters containing NF-κB binding sites. *In situ* immunofluorescence analysis revealed elevated nuclear levels of the p65 subunit of NF-κB in v-*raf*-transformed NIH 3T3 cells. Incubation of HeLa cell cytoplasmic extracts with a purified recombinant glutathione *S*-transferase-raf fusion protein in the presence of ATP released active NF-κB that could be detected by electrophoretic gel mobility shift assay. Coincubation of purified recombinant IκB and glutathione *S*-transferase-raf in the presence of ATP resulted in the phosphorylation of IκB. Coexpression of GAL4 (activation domain)-IκB and GAL4 (DNA-binding domain)-raf fusion proteins in yeast resulted in stimulation of a GAL4-responsive reporter gene, indicating that IκB and Raf interact physically *in vivo*. These results indicate that the Raf-1 kinase functions in signal transduction in part by activating the NF-κB transcription factor by phosphorylating IκB in the cytoplasmic IκB–NF-κB complex to release active NF-κB.**

c-*raf-1* is the cellular homolog of v-*raf*, the transforming gene of murine sarcoma virus 3611, which encodes a cytosolic serine/threonine protein kinase (1). c-*raf-1* is a member of a small gene family conserved from mammals to *Drosophila* (2, 3). c-*raf-1* is expressed in all cell types, whereas other members are tissue specific. The C-terminal region of Raf contains the kinase domain, and the N-terminal portion contains a regulatory domain (4). The protooncogene can be converted into an oncogene by N-terminal fusion and/or truncation (5, 6). The C-terminal domain, but not the full-length protein, was shown to possess kinase activity *in vitro* and to induce transformation upon microinjection into quiescent NIH 3T3 cells (7).

c-Raf-1 has been demonstrated to be an essential transducer of mitogenic signals downstream of serum growth factor receptors and Ras (8). c-Raf-1 is phosphorylated within minutes of serum stimulation, leading to an increase in its intrinsic kinase activity (9, 10). c-Raf-1 is a substrate of the activated platelet-derived growth factor receptor (11) and protein kinase C (12). NF-κB is a transcription factor found as an inactive cytoplasmic complex with the inhibitor IκB (13). IκB in the IκB–NF-κB complex can be phosphorylated *in vitro* by protein kinase C, causing dissociation of the complex (14). Serum stimulation was found to cause the dissociation of the IκB–NF-κB complex *in vivo*, allowing active NF-κB to migrate into the nucleus (15, 16). The stimulation of Raf-1 activity and the appearance of NF-κB in the nucleus are very closely correlated after serum stimulation and both occur in the presence of cycloheximide.

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MATERIALS AND METHODS

Electroporation and Chloramphenicol Acetyltransferase (CAT) Assay. NIH 3T3 cells were electroporated as described (17) with a mixture of 50 μg of activator/control and 10 μg of reporter plasmid DNAs. Equal amounts of protein were assayed for CAT activity (18).

***In Situ* Immunofluorescence.** Cells were grown on glass coverslips, rinsed with phosphate-buffered saline (PBS), fixed in 100% methanol (5 min), and blocked with PBS/5% nonfat dried milk /0.2% Tween (19). Antibody incubations were at 37°C with anti-p65 NF-κB (Santa Cruz Biotechnology; 10 μg/ml, 1.5 hr) followed by biotin-conjugated anti-rabbit IgG and fluorescein-conjugated streptavidin (Jackson ImmunoResearch; 1 μg/ml, 1 hr each). For peptide competition, antibody was preincubated with peptide (10 μg/ml) at 4°C overnight. Cells were preblocked for 1 hr with peptide (10 μg/ml) and washed for 30 min with the same after incubation with primary antibody.

Purification of Raf-1 Kinase. The murine c-*raf-1* kinase domain was cloned into pGEX3X (ref. 20; pGST-raf). Lys-375 (in the ATP-binding site) was changed to Trp (pGST-raf-K375W; analogous to EH301, ref. 4). *Escherichia coli* cultures were grown and induced as described (20). Bacterial pellets (from 100 ml of culture) were resuspended in 1 ml of PBS containing 1 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride; sonicated; and centrifuged (10,000 rpm in a Sorvall SS-34 rotor, 4°C, 10 min). Triton X-100 was added (1% wt/vol) to the supernatants, which were slowly poured over glutathione-agarose (Sigma) columns (0.2-ml bed volume). The columns were washed extensively; Raf-1 kinase was eluted with 5 mM reduced glutathione, concentrated by ultrafiltration (Amicon PD-30), made 10% (vol/vol) in glycerol, flash-frozen in dry ice ethanol, and stored at −70°C.

Raf-1 Kinase and Electrophoretic Gel Mobility Shift Assays (EMSAs). Kinase conditions were 25 mM Tris·HCl (pH 7.4), 10 mM $MgCl_2$, 10 mM $MnCl_2$, 100 μM dithiothreitol, and 10–20 μCi of [γ-^{32}P]ATP (3000 Ci/mmol; 1 Ci = 37 GBq) in 10 μl for 15 min at 30°C. Assays were stopped using Laemmli sample buffer. Cytoplasmic extracts for EMSA were prepared as described (13, 19). Extract (8 μg) was incubated with probe (10,000 cpm; specific activity, 2×10^8 cpm/μg) in 10 mM Tris·HCl (pH 7.9), 50 mM NaCl, 0.5 mM EDTA, 10% glycerol, 1 mM dithiothreitol, and 3 μg of poly(dI-dC) in a total volume of 20 μl for 15 min at 22°C. Electrophoresis was on 5% gels in Tris/glycine/EDTA buffer at 22°C and 30 mA (19). Where indicated, 8 μg of extract was incubated with 50 ng of purified glutathione *S*-transferase (GST)-raf kinase under kinase reaction conditions except that [γ-^{32}P]ATP was replaced by 100 μM unlabeled ATP. EMSA was subsequently initiated by

Abbreviations: GST, glutathione *S*-transferase; EMSA, electrophoretic gel mobility shift assay; CAT, chloramphenicol acetyltransferase; PBS, phosphate-buffered saline; DOC, deoxycholate.

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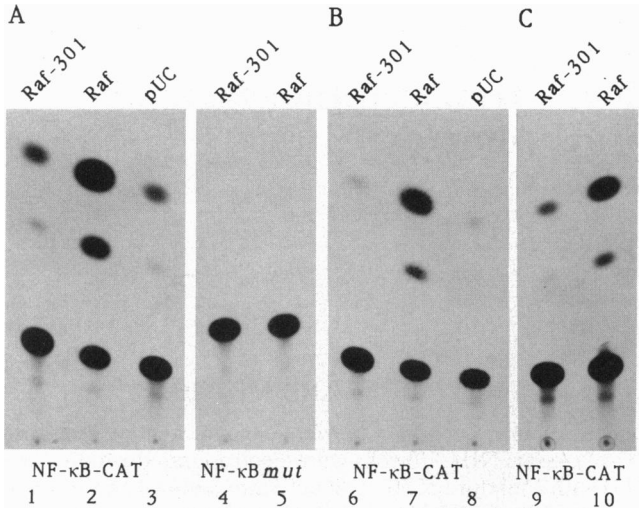

FIG. 1. *In vivo* activation of NF-κB-responsive promoters by Raf-1 kinase. (*A*) Transfection of growing cells followed by serum starvation. Cells were grown in 10% serum, harvested, electroporated, and incubated in 0.3% serum for 48 hr. (*B*) Transfection of quiescent cells. Cells were grown in 10% serum, starved for 24 hr (0.3% serum), and then treated as in *A*. (*C*) Transfection of growing cells. Cells were kept in 10% serum at all times. All experiments were performed on at least three separate occasions, and the data shown are a representative sample. A reporter plasmid containing the human immunodeficiency virus NF-κB binding site behaved in the same fashion. Raf, pRSV-Raf-BXB (wild-type Raf; ref. 23); Raf-301, pRSV-Raf-BXB-301 (kinase-inactive Raf; refs. 23 and 24); pUC, pUC19; NF-κB-CAT, pMHC-NF-κB CAT (wild-type NF-κB sites; ref. 15); NF-κB*mut*, pMHC-NF-κBmut CAT (mutant NF-κB sites; ref. 15).

adding the reaction to 10 μl of a 2-fold concentrated EMSA mixture and incubating for 15 min at 22°C.

Yeast Genetic Methods. The c-*raf-1* kinase domain was fused to the GAL4 DNA-binding domain in pGBD9 (ref. 21; pGBD-raf). IκB (MAD3; ref. 22) was fused to the GAL4 activation domain in pGAD1F (ref. 21; pGAD-IκB). The same fragments were also cloned in inverse orientations (pGBD-raf/AS, pGAD-IκB/AS). Strain Y526 (21) is deleted for *GAL4* and *GAL80* and contains an integrated *GAL1-lacZ* reporter gene. *lacZ* plate assays were performed by growing cells on nitrocellulose filters on selective synthetic medium (19) containing 2% Glc; transferring the filters for 24 hr to medium containing 2% Gal, ethanol, and glycerol; and then transferring the filters for 48 hr to medium without a carbon source containing 5-bromo-4-chloro-3 indolyl β-D-galactoside at 60 μg/ml. To assay β-galactosidase activity (19), cells were grown in selective synthetic medium containing 2% Glc; diluted 100-fold into medium containing 2% Gal, ethanol, and glycerol; and grown to midlogarithmic phase.

RESULTS

Plasmids encoding an oncogenic Raf-1 kinase and a CAT reporter with a NF-κB-responsive promoter were transiently cotransfected into NIH 3T3 cells. Raf-1 was observed to transactivate the minimal NF-κB promoter under several conditions (Fig. 1). Exponentially growing NIH 3T3 cells were electroporated and subsequently serum starved (Fig. 1*A*); these conditions were previously used to demonstrate serum inducibility of NF-κB sites (15) and the transactivation of the c-*fos* promoter by v-*raf* (25). Since an active Raf-1 kinase is known to prevent quiescence, in another experiment cells were first made quiescent (Fig. 1*B*). Transactivation was observed, showing that Raf-1 has the same effect on NF-κB activity as serum stimulation (15). Since injection of active Raf-1 kinase protein into quiescent cells is known to induce exit from G_0 (7), Raf-1 may have caused transactivation simply by pushing cells into the cell cycle. Exponentially growing cells were thus electroporated and further maintained in 10% serum (Fig. 1*C*). Transactivation was again observed, indicating that Raf-1 is capable of chronically

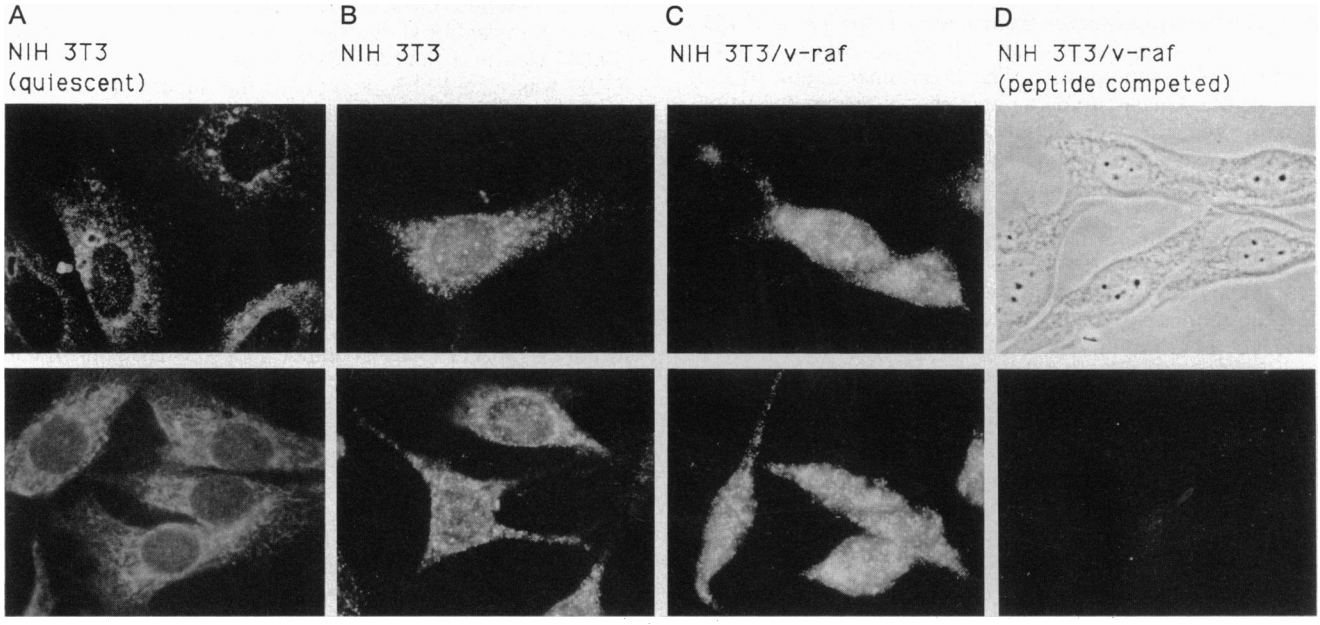

FIG. 2. Localization of the NF-κB p65 subunit in normal and v-*raf*-transformed NIH 3T3 (NIH 3T3/v-raf) cells. (*A–C*) *In situ* immunofluorescence. Two micrographs are shown for each cell line and/or growth condition. (*A*) Serum-starved NIH 3T3 cells. (*B*) NIH 3T3 cells grown continuously in 10% serum. (*C*) v-*raf*-transformed NIH 3T3 cells grown continuously in 10% serum. Examination of a second independent v-*raf*-transformed cell line produced the same results. Serum-starved v-*raf*-transformed NIH 3T3 cells appear identical to cells in 10% serum. (*D*) Competition of the anti-p65 antibody with its cognate peptide antigen. Exponentially growing v-*raf*-transformed NIH 3T3 cells were stained with antibody preincubated with peptide. (*Upper*) Phase-contrast view. (*Lower*) Corresponding view under fluorescence illumination.

elevating the activity of NF-κB binding sites above the level found in growing cells.

Clonal lines of MSV3611 v-*raf* (26)-transformed NIH 3T3 cells were examined for the subcellular localization of the p65 subunit of NF-κB by *in situ* immunofluorescence. NIH 3T3 cells displayed a very low nuclear p65 signal under quiescent conditions and a slightly elevated but discernable signal under exponential growth conditions (Fig. 2). v-*raf*-transformed NIH 3T3 cells under exponential growth conditions displayed a significantly elevated p65 nuclear signal. These results indicate that an active Raf-1 kinase increases the localization of p65 NF-κB to the nucleus.

A significant fraction of NF-κB activity in HeLa cells is cytoplasmic and can be detected by EMSA after treatment with deoxycholate (DOC), which causes dissociation of IκB (refs. 13 and 27; Fig. 3*A*). Identity of the complex was confirmed by supershifting with the NF-κB p65 antibody (Fig. 3*B*). A GST-*raf* fusion gene was constructed and expressed in *E. coli*, and the GST-raf hybrid polypeptide was purified. The GST-*raf* gene should encode an activated kinase, since only the kinase domain portion was cloned into the GST vector. The purified GST-raf polypeptide was added to the HeLa cytoplasmic extract, and the mixture was incubated under kinase assay conditions and subsequently analyzed by EMSA for NF-κB DNA-binding activity (Fig. 3*C*). The same band that was released by DOC treatment was also visualized after incubation with the GST-raf polypeptide. The band was not generated after incubation without GST-raf under kinase assay conditions, omission of ATP from the assay, or incubation with a kinase-inactive GST-raf protein (GST-raf-K375W).

Purified GST-raf protein displayed autokinase activity and phosphorylated histone H1 (Fig. 4*A*); both activities have been shown to be associated with Raf-1 kinase immunoprecipitated from mammalian cells (28). GST-raf was also capable of phosphorylating purified recombinant IκB (Fig. 4*A*). GST-raf-K375W protein did not show detectable autophosphorylation or histone H1 or IκB phosphorylation (Fig. 4*B*). The GST-raf and IκB preparations were estimated to be ≈90% and ≈50% pure, respectively (Fig. 4*C*). GST-raf did not show detectable phosphorylation of the other protein species found in the IκB preparation, intact GST, or bovine serum albumin. Elution of GST-raf protein from glutathione beads without extensive washing resulted in contamination with numerous *E. coli* proteins; in kinase assays, such partially purified preparations did not display an apparent increase in phosphorylated species. Phosphoamino acid analysis revealed that autophosphorylated GST-raf and GST-raf-phosphorylated IκB contained phosphoserine and phosphothreonine but no detectable phosphotyrosine. The phosphorylation of IκB by GST-raf *in vitro* thus appears not to be due to a gross loss of specificity of the kinase for its substrates.

The yeast two-hybrid system (21, 29) was employed to demonstrate that Raf-1 and IκB can interact *in vivo*. This method can detect the interaction of SNF1 and SNF4, a protein kinase and substrate (30). Fusions of the GAL4

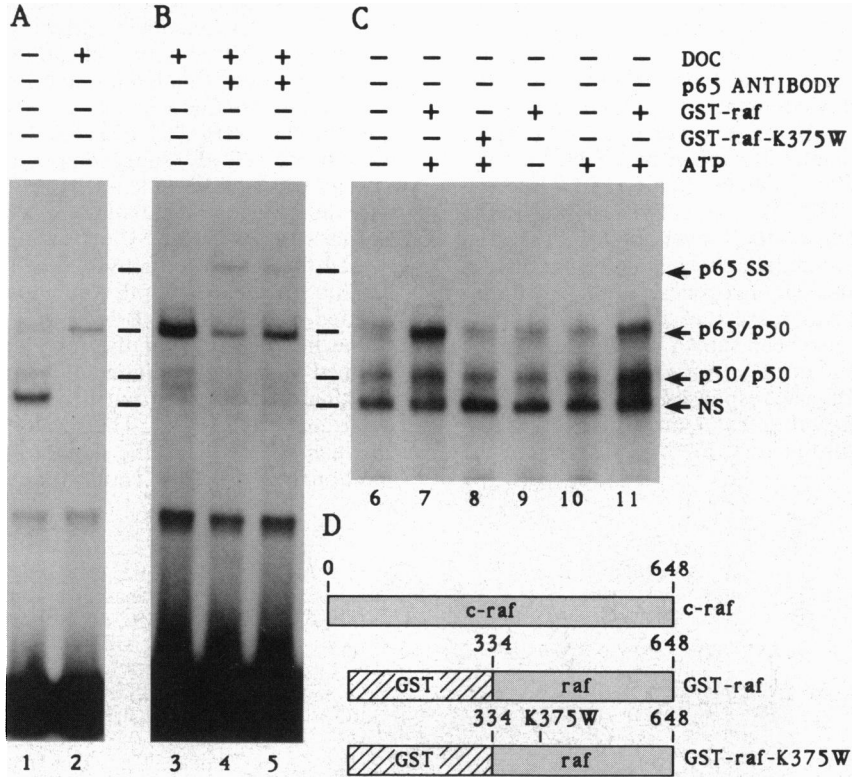

FIG. 3. *In vitro* activation of NF-κB DNA-binding activity by Raf-1 kinase. (*A*) Activation of NF-κB DNA-binding activity by DOC. Cytoplasmic extracts were subjected to EMSA using an NF-κB-binding site from the major histocompatibility complex promoter as the probe (15) either in the absence (lane 1) or presence (lane 2) of 0.2% DOC. This concentration of DOC does not interfere with DNA binding of the p65/p50 heterodimer but eliminates the binding of the p50/p50 homodimer (27). The band visualized in the absence of DOC is nonspecific (marked NS in the right margin) since it also appeared with a mutant major histocompatibility complex probe (15). (*B*) Detection of the p65 subunit in the DOC-released NF-κB activity. Cytoplasmic extracts were subjected to EMSA in the presence of 0.2% DOC and 0 ng (lane 3), 400 ng (lane 4), or 100 ng (lane 5) of anti-p65 antibody. (The antibody was the same as that used in Fig. 2.) The supershifted band is identified as p65 SS in the right margin. (*C*) Activation of NF-κB activity by Raf-1 kinase. Cytoplasmic extracts were incubated in various combinations with purified GST-raf protein, mutant GST-raf-K375W protein, and ATP as indicated, prior to being subjected to EMSA. Lanes 7 and 11 are duplicates using different batches of GST-raf. The faint band migrating above the p65/p50 band (lanes 7 and 11) is probably either a p65/Rel heterodimer or a p65 homodimer. The same band is detected after DOC treatment (lane 3) and is characteristic of an activated NF-κB state. (*D*) Diagram of the constructs used to produce GST-raf and GST-raf-K375W proteins. Numbers refer to amino acid residues of murine c-Raf-1.

DNA-binding domain with the Raf-1 kinase domain (pGBD-Raf) and the GAL4 activation domain with IκB (pGAD-IκB) were transformed into a strain containing a *lacZ* gene controlled by a *GAL4*-responsive element. Clones containing either pGBD-Raf or pGAD-IκB alone were white on 5-bromo-4-chloro-3-indolyl β-D-galactoside plates, whereas clones containing both plasmids displayed a distinct blue color. As controls, pGAD-IκB was tested with the GAL4 DNA-binding domain only (pGBD-Raf/AS) and with the GAL4 DNA-binding domain fused to lamin; in both cases white colonies were obtained. Assays of β-galactosidase activity (Fig. 5) revealed a 4-fold higher activity in cells containing pGBD-Raf and pGAD-IκB relative to cells containing pGBD-Raf and pGAD-IκB/AS. The activity produced by pGBD-Raf and pGAD-IκB is quantitatively similar to that observed with SNF1 and SNF4 (30). pGBD-raf cannot be activated by an unusually large number of activation domain fusion constructs. A mammalian cDNA expression library was constructed in the pGAD vector and screened in combination with pGBD-raf; blue colonies due to specific interactions were recovered at a frequency of ≈1/150,000. Similar frequencies have been observed by others using the two-hybrid system to screen expression libraries.

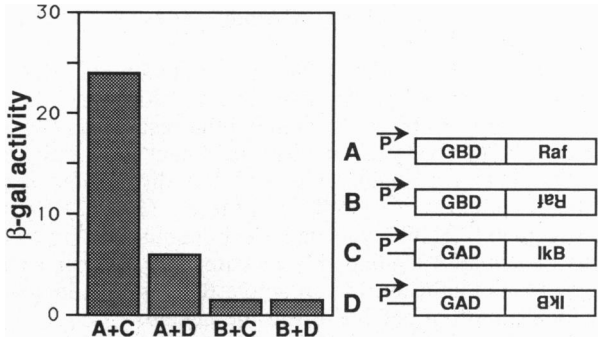

FIG. 5. Reconstitution of GAL4 activity by Raf-1 and IκB hybrid proteins. β-Galactosidase (β-gal) activities are expressed in Miller units (19). The values are averages of three independent transformants. A, pGBD-Raf; B, pGBD-Raf/AS; C, pGAD-IκB; D, pGAD-IκB/AS. pGBD-Raf has a very low intrinsic activity; such low-level activity is frequently observed when heterologous proteins are fused to the GAL4 DNA-binding domain (21). In the plate assay, this activity produced white colonies. Abbreviations: GBD, GAL4 DNA-binding domain; GAD, GAL4 activation domain.

DISCUSSION

Molecular interactions that follow mitogenic stimulation of quiescent cells and lead to the activation of immediate early genes occur very rapidly and independently of *de novo* protein synthesis. Raf-1 is known to be positively regulated by phosphorylations initiated by several growth factor receptors (9, 11). Clues have also been obtained from the analysis of *Drosophila* development (31); polehole, a c-*raf-1* homolog, acts genetically downstream of torso, a receptor tyrosine kinase of the platelet-derived growth factor family. Several lines of evidence indicate that c-*raf-1* also acts downstream of *ras* (24, 32–34). The activity of the PEA1 enhancer element, which contains overlapping AP-1- and Ets-related sites, can be stimulated *in vivo* by the expression of v-*raf* (6). Several nonnuclear oncogenes, serum, and tumor promoters are known to increase the activity of the PEA motif (35, 36). v-*raf* has also been shown to transactivate the c-*fos* and β-actin promoters (25). Activation of these elements is one of the primary responses to growth factor-induced stimulation, suggesting that some transcription factors, or interacting cellular factors, are likely targets of the Raf-1 kinase.

The only physiological substrate of Raf-1 known to date is mitogen-activated protein kinase kinase (37, 38). This communication presents several lines of evidence indicating that IκB (MAD3) is also a substrate of Raf-1. Cotransfection of plasmids encoding active Raf-1 and a CAT reporter gene with an NF-κB-responsive promoter into quiescent cells resulted in significant stimulation of CAT activity. The same cotransfection into exponentially growing cells also elicited an increase in CAT activity, indicating that Raf-1 is capable of chronically overstimulating the NF-κB-responsive element. *In situ* immunofluorescence analysis revealed that v-*raf*-transformed NIH 3T3 cells contained elevated levels of nuclear p65 NF-κB, suggesting that the increase in NF-κB activity was caused, at least in part, by relocalization of p65 into the nucleus. Incubation of a HeLa cell cytoplasmic extract with purified GST-raf under kinase assay conditions caused the release of active NF-κB DNA-binding activity. Finally, purified GST-raf was capable of phosphorylating purified recombinant IκB *in vitro*.

The interpretation of the foregoing experiments is constrained by two uncertainties. First, in all experiments except the kinase assay using purified components, the action of Raf-1 could be indirect. The results thus show that Raf-1 is upstream of NF-κB in the signal transduction pathway, but additional components could be involved in mediating the

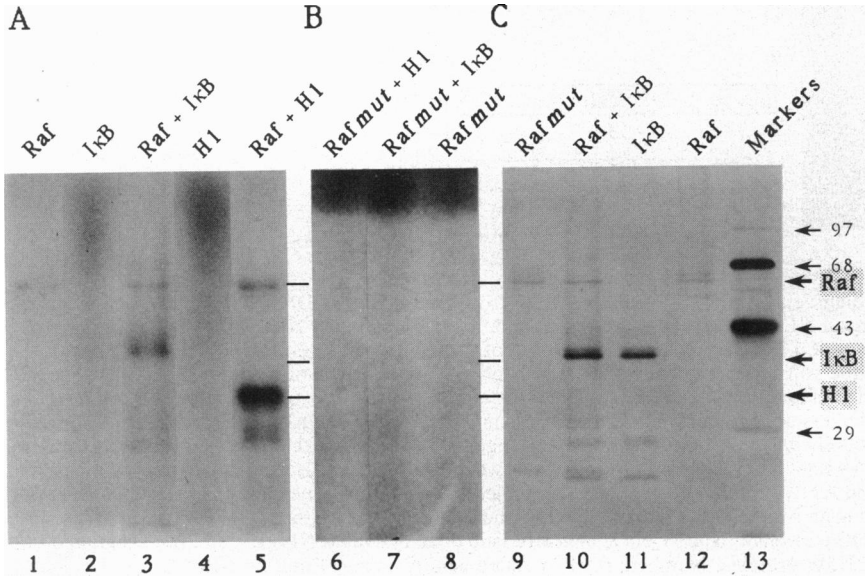

FIG. 4. *In vitro* phosphorylation of IκB with Raf-1 kinase. (*A*) Kinase assays using recombinant GST-raf protein. (*B*) Kinase assays using mutant GST-raf-K375W protein. (*C*) Silver-stained gel of protein preparations. Lane 9, 100 ng of GST-raf-K375W; lane 10, 100 ng of GST-raf and 400 ng of IκB (MAD3; refs. 15 and 16); lane 11, 400 ng of IκB (MAD3); lane 12, 100 ng of GST-raf; lane 13, molecular size markers. The faint, fast migrating doublet (lanes 3 and 5) is due to breakdown products of the GST-raf fusion polypeptide that contains the Raf kinase domain. The amount of the breakdown products varies from preparation to preparation and can often be visualized by silver staining (lane 10, the doublet immediately above the 29-kDa marker). These breakdown products possess autokinase activity. The breakdown product containing the GST domain (27-kDa band in lanes 9, 10, and 12) has never been observed to become phosphorylated. Raf, GST-raf; Raf*mut*, GST-raf-K375W; H1, histone H1.

transmission of the signal. The enzyme–substrate relationship between Raf-1 and IκB using purified recombinant proteins is in turn compromised by the often-demonstrated propensity of certain protein kinases to lose substrate specificity *in vitro*. The fact that GST-raf does not indiscriminately phosphorylate proteins *in vitro* is reassuring, but not absolutely convincing. The ability of Raf-1 and IκB to interact *in vivo* was demonstrated using the yeast two-hybrid system (21, 29). Coexpression of a GAL4 DNA-binding domain-Raf-1 kinase domain fusion (pGBD-raf) and a GAL4 activation domain-IκB fusion (pGAD-IκB) elicited a 4-fold stimulation of a *lacZ* reporter gene controlled by a *GAL4*-responsive element. The activity seen with the combination of pGBD-Raf and pGAD-IκB was quantitatively very similar to that observed with SNF1 and SNF4, a previously identified protein kinase and substrate (30).

The two-hybrid experiment, by itself, does not demonstrate that IκB is a substrate of Raf-1. Given, however, that a protein–protein interaction can be demonstrated between IκB and Raf-1 in a heterologous *in vivo* system makes it very unlikely that the kinase–substrate relationship seen *in vitro* is a mere artifact. When all the experiments presented in this communication are considered together, the simplest explanation is that IκB is a substrate of Raf-1 and that the mechanism of signal transduction is release of active NF-κB from the inactive IκB–NF-κB cytoplasmic complex. Since NF-κB is expressed in a wide variety of cells and functional NF-κB binding sites have been found in many genes (39), signal transduction between c-Raf-1 and NF-κB is likely to be a physiologically important component of the mitogenic response.

We thank U. R. Rapp and A. S. Baldwin for plasmid clones and C. Rosen for purified recombinant IκB (MAD3). This work was supported by National Institutes of Health Grant GM-R01-41690 and National Science Foundation Presidential Young Investigator Grant DMB-9057715 to J.M.S.

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