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Induction of Transformation and DNA Synthesis after Microinjection of *raf* Proteins

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Full-length and N-terminal deletion mutants of human c-*raf-1* cDNA were cloned into *Escherichia coli* expression plasmids. Bacterially expressed c-*raf* proteins were purified by anion-exchange, gel filtration, and affinity chromatography. Microinjection of mutant c-*raf* proteins into G_0-arrested NIH 3T3 cells induced DNA synthesis and morphological transformation, whereas microinjection of full-length c-*raf* had no effect. The amino terminus of the *raf* protein has an important negative regulatory influence; alteration of this region resulted in increased kinase activity and oncogenicity.

Oncogenes can be derived from normal cellular genes by point mutation, deletions, or other rare recombinant events that result in the constitutive expression or an activated version of the gene or both (1, 10, 11, 16). If the mutated gene is located in signal-transducing pathways that are involved in mitogenesis, transformation may occur. Several retroviral oncogenes are involved in and contribute to human malignancy (for a review, see reference 16). The v-*raf* oncogene was originally identified as the transforming element of murine sarcoma virus 3611 (20, 22). Southern blot analysis with v-*raf* cDNA probes identified two *raf* genes in humans, A-*raf* and c-*raf* (18, 21). A third member of the family, B-*raf*, was isolated by DNA transfection (11). This report will focus on c-*raf*, a 74-kilodalton cytoplasmic polypeptide with Ser-Thr protein kinase activity. All tissues and cell lines examined express c-*raf* mRNA, but the gene product is not transcriptionally regulated by growth factors or expressed in a cell cycle-dependent manner (18).

Full-length *Eco*RI-to-*Xba*I 2.1-kilobase human c-*raf-1* cDNA (Fig. 1A) (4) was engineered by site-directed mutagenesis from a Bluescript phasmid, with the addition of an *Nde*I site at the initiation codon (Fig. 1B). The *Nde*I-to-*Xba*I fragment containing the entire c-*raf* gene and 121 bases of 3′ noncoding sequence was inserted into the *Nde*I cloning site of the bacterial expression plasmid pAR-3039 (10, 29). The 3′ *Xba*I of c-*raf* and the remaining vector *Nde*I site were blunt-end ligated (Fig. 1B). This manipulation placed the c-*raf* gene in the reading frame coding for methionine, the first amino acid residue of the polypeptide. The *raf* gene was placed under the strict transcriptional control of the T7 polymerase promoter (Fig. 1B). The c-*raf* expression plasmid was transformed into protease-deficient *Escherichia coli*. Clones containing the c-*raf* gene in the proper orientation were identified by minilysate DNA purification and restriction analysis (6). The human c-*raf-1* cDNA was cleaved at unique restriction endonuclease sites (*Hgi*AI, *Hin*dIII, and *Bgl*II) in the 5′ end of the DNA strand (Fig. 1A) and ligated into the bacterial expression plasmid pJL6. Transformed *E. coli* expressed mutant c-*raf* polypeptides (13). Log-phase cultures of mutant and full-length c-*raf*-transformed bacteria were grown in ampicillin-containing medium to an optical density at 590 nm of approximately 1.0. Synthesis of *raf* proteins was induced by infection with T7 polymerase-expressing lambda bacteriophage (full length) or by heat shock (mutants). Cultures were maintained for 1 to 2 h before lysis and chromatographic purification of *raf* proteins.

Full-length human c-*raf*, p74, was purified from bacterial-cell lysates after ultrasonic disruption. Protein was concentrated with ammonium sulfate and suspended in lysis buffer (20 mM Tris hydrochloride [pH 7.4], 10 mM $MgCl_2$, 1 mM dithiothreitol, 100 μM polymethylsulfonyl fluoride, 100 μM leupeptin). After dialysis, crude protein was fractionated by anion-exchange chromatography and eluted from the column at a salt concentration of 0.36 M. Fractions containing c-*raf* were separated from lower-molecular-weight degradation products, as shown by immunoblot analysis (Fig. 2A, compare lanes 1 through 3). Sodium dodecyl sulfate-polyacrylamide gels (10% polyacrylamide) were loaded with 10 μg of protein per lane. Protein was transferred to nitrocellulose, blocked with 3% bovine serum albumin, and immunoabsorbed with anti-*raf* sp63 polyclonal antibody (20 μg/ml) (23). After being washed with phosphate-buffered saline, *raf* protein was identified by labeling the sp63 antibody with [^{125}I]protein A (10 nCi/ml; Amersham Corp.), and labeled bands were visualized on XAR-5 film (Eastman Kodak Co.). Induced bacterial-cell lysates transformed with the expression vector (with no *raf* insert) did not express proteins that were immunoreactive with the *raf*-specific sp63 antibody (Fig. 2A, compare lanes 1 and 4). The column fractions containing *raf* protein were further characterized by assays for kinase activity (Fig. 2B). Reaction cocktail consisting of 25 mM Tris hydrochloride (pH 7.4), 10 mM $MgCl_2$, 10 mM $MnCl_2$, 10 μM dithiothreitol, 0.1 μM ATP, and 5 μCi of [γ-^{32}P]ATP (3,000 ci/mM; Amersham) was incubated at 30°C for 30 min with 2 μg of protein in a total volume of 20 μl before being electrophoresed through sodium dodecyl sulfate-polyacrylamide gels (12% polyacrylamide). Gels were dried and exposed to X-ray film. Endogenous bacterial proteins labeled in the kinase reaction migrated on sodium dodecyl sulfate-polyacrylamide gels and showed apparent molecular masses of 45 and 57 kilodaltons; no phosphoproteins ran near 74 kilodaltons (Fig. 2B, lane 4). Anion-exchange chromatography resulted in complete purification

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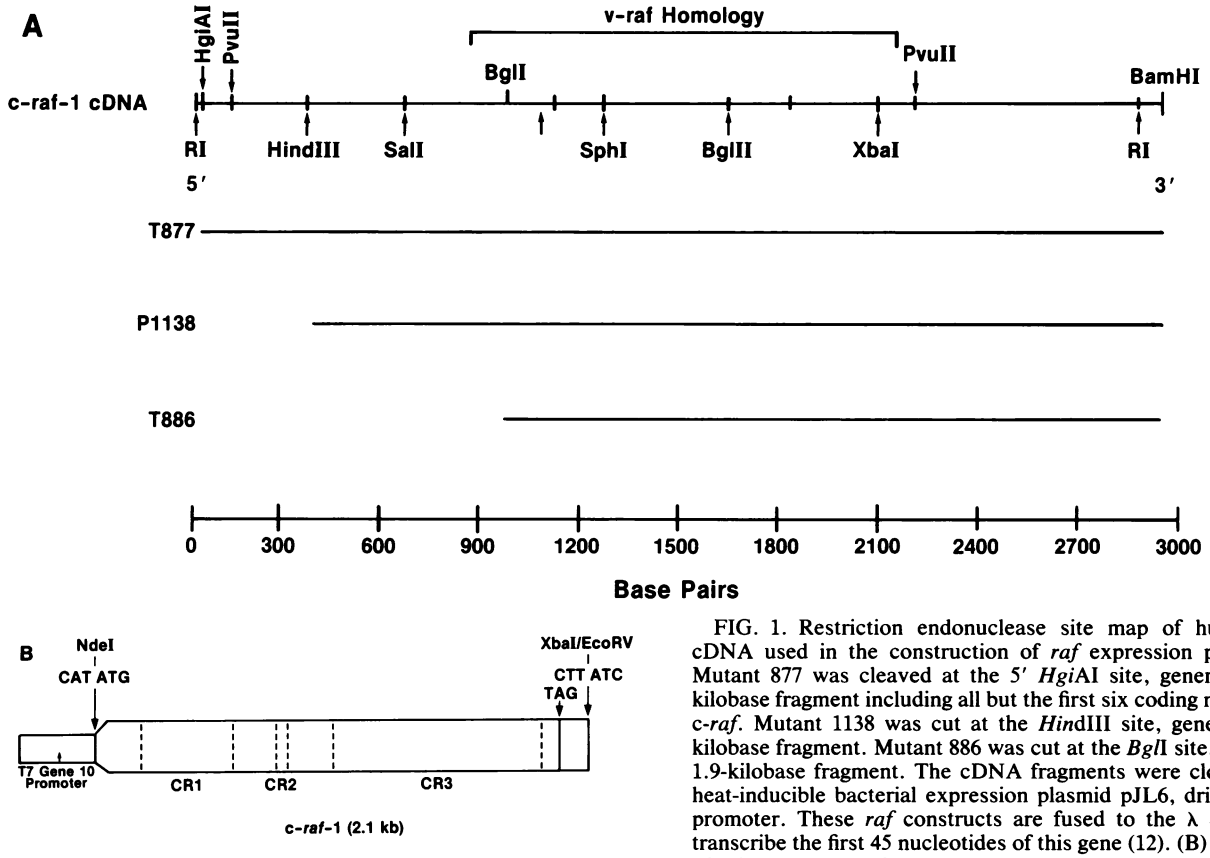


FIG. 1. Restriction endonuclease site map of human c-*raf-1* cDNA used in the construction of *raf* expression plasmids. (A) Mutant 877 was cleaved at the 5' *Hgi*AI site, generating a 2.85-kilobase fragment including all but the first six coding nucleotides of c-*raf*. Mutant 1138 was cut at the *Hin*dIII site, generating a 2.5-kilobase fragment. Mutant 886 was cut at the *Bgl*I site, generating a 1.9-kilobase fragment. The cDNA fragments were cloned into the heat-inducible bacterial expression plasmid pJL6, driven by the λ promoter. These *raf* constructs are fused to the λ *c*II gene and transcribe the first 45 nucleotides of this gene (12). (B) Construction of a full-length c-*raf* bacterial expression plasmid. Cloning sites for c-*raf-1* cDNA which were used to insert the c-*raf* gene into the T7 gene 10 promoter-driven expression plasmid pAR-3039 are shown. The TAG termination signal and three highly conserved consensus regions, CR1 (divalent cation or DNA binding site or both), CR2 (Ser-Thr-rich phosphorylation site), and CR3 (kinase domain), are shown. kb, Kilobases.

of p74 from immunoreactive degradation products (Fig. 2A, compare lanes 1 and 3) and partial purification of phosphoproteins from the ammonium sulfate fraction (Fig. 2B, compare lanes 1 and 3).

Gel filtration desalted the partially purified protein preparation yet resulted in little purification. Several low-molecular-weight bands disappeared from the sodium dodecyl sulfate-polyacrylamide gels, but immunoblot and kinase activity results were similar to those from ion-exchange chromatography (data not shown).

Affinity chromatography through threonine-Sepharose resulted in purification of *raf* p74 to near homogeneity. Immunoblot analysis (Fig. 2A, lanes 5 and 6) verified that p74 was separated from lower-molecular-weight degradation fragments of the *raf* protein, and silver-stained gels contained two minor contaminating bands near 50 and 19 kilodaltons (data not shown). These bands may have been *raf* degradation products that were not visible on immunoblots (Fig. 2A, lane 6), because they were detectable in the more-sensitive kinase assay (Fig. 2B, lane 6). p74 required divalent cations for kinase activity (Fig. 2B), and the only contaminating *E. coli* phosphoprotein, p45 (lane 5), was separated from p74 (lane 6).

Bacterially expressed mutant *raf* proteins were purified by the procedures described for the full-length c-*raf*. Immunoblot approximations of the molecular weights of the mutant polypeptides (Fig. 3A, lanes 2, 4, and 6) corresponded to those predicted on the basis of the primary nucleotide sequence (12). The purified proteins were shown to be *raf* by adding the peptide used to generate the sp63 antibody to the immunoabsorbent step. Antibody association to the protein on the immunoblot was blocked by peptide addition (Fig. 3A, lanes 1, 3, and 5).

Mutant *raf* proteins had very high intrinsic kinase activities, and autophosphorylation was prevalent in the reaction cocktail without divalent cation. Polyacrylamide gels showed that the partially purified preparations contained no contaminating *E. coli* phosphoproteins (Fig. 3B). In addition, several well-known kinase substrates (histone IIIS, myosin, tropomyosin, and actin) were not phosphorylated by any of the *raf* proteins. This observation suggests that *raf* phosphorylation is not a result of contaminating *E. coli* protein kinase activity. To date, no specific biological substrate has been identified for *raf* kinases.

Microinjection of oncogenic protein into quiescent NIH 3T3 cells often results in morphological transformation and induction of DNA synthesis (7, 26, 27). In this report, we have purified bacterially expressed c-*raf* proteins and used single-cell microinjection to study the intracellular oncogenic potential of full-length and truncated c-*raf* proteins.

Affinity-purified mutant *raf* proteins were found to induce DNA synthesis (Fig. 4) and to morphologically transform NIH 3T3 cells (Fig. 5) after direct microinjection. NIH 3T3 cells (3×10^5) were plated onto etched-glass cover slips in 35-mm-diameter culture plates. The cultures were grown to confluent monolayers for 1 to 2 days in Dulbecco modified Eagle medium (GIBCO Laboratories) supplemented with

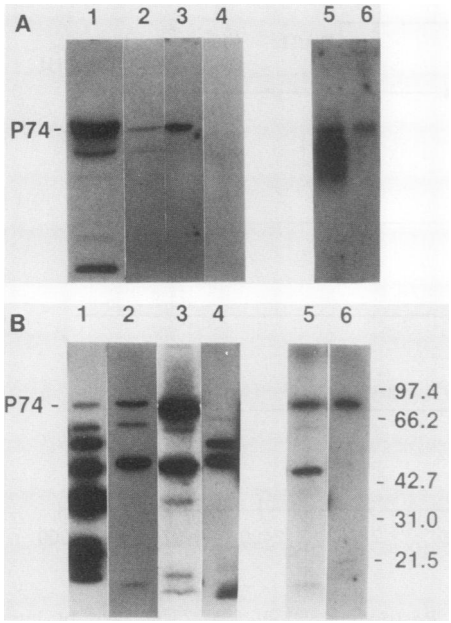


FIG. 2. Immunoblot (A) and kinase activity (B) of full-length c-*raf* protein expressed in *E. coli*, demonstrating purification of human c-*raf* protein. Lanes 1, Ammonium sulfate-precipitated protein; lanes 2, anion-exchange fraction containing *raf* p74 and p58; lanes 3, anion-exchange fraction with peak p74 *raf* activity; lanes 4, ammonium sulfate-precipitated control bacterial protein from *E. coli* lysates expressing the plasmid vector with no c-*raf* insert; lanes 5, pooled *raf* protein from gel filtration chromatography; lanes 6, purified p74 *raf* from threonine-Sepharose column. Numbers on the right indicate molecular masses measured in kilodaltons.

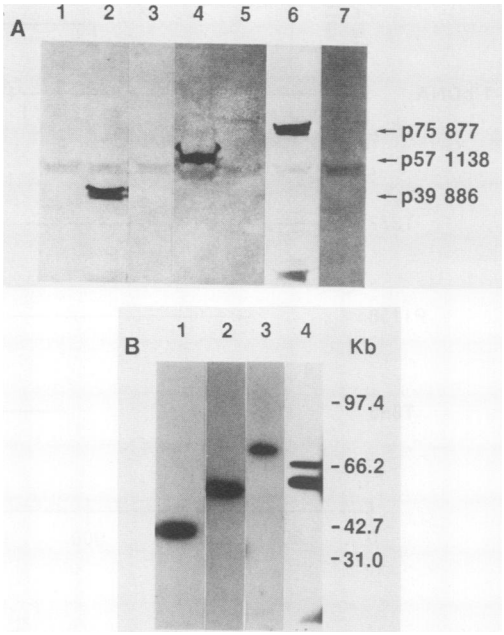
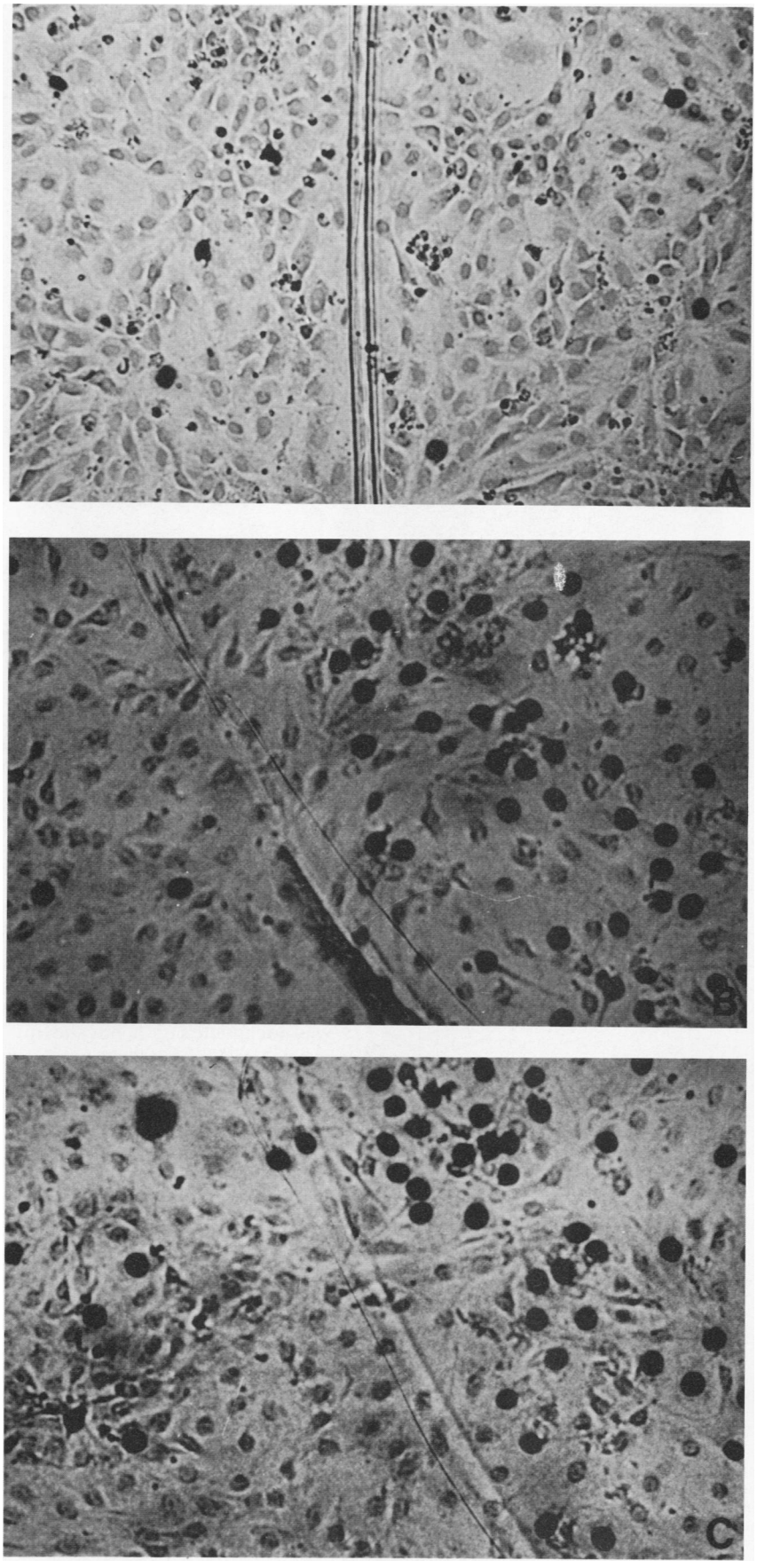


FIG. 3. Immunoblot and kinase activity of mutant c-*raf* proteins. (A) Immunoblot of purified bacterially expressed mutant *raf* protein. Duplicate nitrocellulose strips were incubated with sp63 anti-*raf* antibody (lanes 2, 4, 6, and 7) or with sp63 antibody plus sp63 peptide (lanes 1, 3, and 5). Lanes 1 and 2, Mutant 886 p39; lanes 3 and 4, mutant 1138 p57; lanes 5 and 6, mutant 877 p75; lane 7, pJL6 expression vector transformed in *E. coli* with no *raf* insert. (B) Autophosphorylation of purified mutant *raf* proteins. Lane 1, Mutant 886 p39; lane 2, mutant 1138 p57; lane 3, mutant 877 p75; lane 4, pJL6 expressed in *E. coli*. Numbers on the right indicate molecular masses (measured in kilodaltons).

10% calf serum. Cultures were serum starved in 0.5% calf serum medium for 24 h before microinjection (10^{-11} ml) of coded samples. After injection (16 h), cultures were photographed and/or pulsed with [^{3}H]thymidine (1 μCi/ml [1 Ci = 37 GBq]; Amersham) for 4 h, washed with isotonic phosphate-buffered saline, and fixed in 3.7% (vol/vol) formaldehyde. The cover slips were mounted onto glass slides, coated with nuclear-track emulsion (NTB2; Eastman Kodak), and exposed for 24 h. The uptake of label into nuclei was monitored by microscopic observation. The induction of DNA synthesis and the transformations observed from several microinjection experiments are summarized in Table 1. Bovine serum albumin had no effect in injected cells. To control for bacterial proteins that may copurify with *raf* protein and induce DNA synthesis, the host bacteria transformed with pAR-3039 and pJL6 vectors minus the c-*raf* gene were grown and purified (Fig. 2 and 3). At all stages of purification, the induced-vector-transformed bacterial preparations were found not to activate DNA synthesis or to transform NIH 3T3 cells. Every injection experiment was controlled with transforming Ha-*ras* protein purified from soluble bacterial lysates. Full-length *raf* protein did not induce DNA synthesis or transform resting cells (Fig. 4A). Mutant *raf* p75 induced levels of DNA synthesis slightly above background levels (sixfold) but did not transform. Both *raf* mutants, p57 (22-fold) and p39 (26-fold), induced DNA synthesis (Fig. 4B and C) and morphologically transformed NIH 3T3 cells after microinjection (Fig. 5). Thus, mutant *raf* proteins are intracellular mitogens.

Cellular *raf* kinases are believed to be intermediates in the biochemical pathways of membrane signal transduction (9). The *raf* protein can be directly activated through tyrosine phosphorylation by the platelet-derived growth factor β receptor (13), and platelet-derived growth factor binding to its receptor increases *raf* phosphorylation and kinase activity (14). The mitogenic signal induced on quiescent NIH 3T3 cells by serum growth factors was inhibited by anti-*ras* monoclonal antibody microinjection (15). Injection of the anti-*ras* antibody into *raf*-transformed NIH 3T3 cells did not repress mitogenesis (25), implying that *raf* acts downstream or independently of *ras* in mitogenic-signal-transducing pathways (18). Addition to and removal of phosphate from proteins has been recognized as a mechanism to regulate enzymatic activity. Rapp et al. have proposed that one biological role of *raf* kinases may be to modulate transcription factor activity via phosphorylation (19). Direct experimental evidence comes from transient transfection of activated *raf* genes, which demonstrates an increased transcriptional activity from AP-1-dependent promoters, im-

FIG. 4. Induction of DNA synthesis after microinjection of *raf* proteins into quiescent NIH 3T3 cells. Cells on the right side of the photomicrograph were injected with full-length c-*raf* p74 (A), mutant 1138 *raf* p57 (B), and mutant 886 *raf* p39 (C). Cells on the left side of the photomicrograph were not injected. At 16 h after injection, cultures were pulsed with [^{3}H]thymidine for 4 h, fixed in 3.7% formaldehyde, and autoradiographed.


A
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C

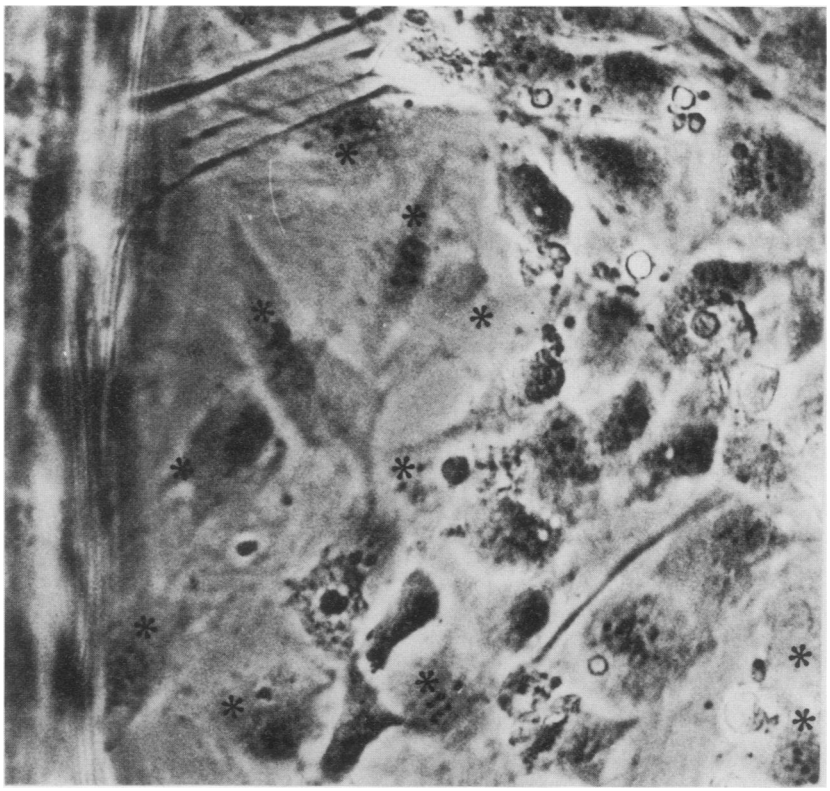

FIG. 5. Morphological transformation of NIH 3T3 fibroblast cells injected with mutant *raf* protein. Mutant *raf* p39 (886) was microinjected into flat confluent NIH 3T3 cells. After injection (16 h), living cells were photographed. Morphologically transformed cells are round, refractile, and three-dimensional compared with the adjacent uninjected flat cells (*). Similar morphological transformation was observed with injection of *raf* p57.

TABLE 1. Induction of DNA synthesis and transformation of NIH 3T3 fibroblasts after injection of *raf* proteins

Injected sample (amt [mg/ml])[a]	% Thymidine incorporation		Fold induction ± SD[b]	Transformation[c]
	BK[d]	INJ[e]		
BSA (2)	2.3	4.1	1.8 ± 0.8	−
Control (2.7)[f]	2.0	3.2	1.6 ± 0.7	−
c-*raf* p74 (2.8)	2.5	8.1	3.4 ± 1.1	−
c-*raf* p75 (2.2)	2.3	14.3	6.2 ± 2.2	−
c-*raf* p57 (2.3)	3.2	69.2	21.6 ± 6.1	+
c-*raf* p39 (2.0)	3.1	81.6	26.3 ± 5.8	+
Ha-*ras* p21 (2.2)	2.8	46.8	16.7 ± 4.3	+

[a] Coded samples were microinjected into 150 to 200 cells contained within a defined area of a cover slip. The protein concentration in the injection inoculum was quantitated by immunoblot and spectrophotometric protein assays. Each *raf* protein has a specific dose response, as was shown previously for phospholipase C-γ (28) and *ras* (27) Injection of these concentrations introduced 2×10^5 to 3×10^5 molecules per cell. Injection of two to three times more p74 did not induce DNA synthesis or transformation. Transforming Ha-*ras* protein was purified from the soluble fraction of bacterial lysates. BSA, Bovine serum albumin.

[b] Computed as the ratio of percent thymidine incorporation in the injected area to that in the background. Standard deviations are for at least four separate determinations.

[c] The injected area was scored for transformation by microscopic examination 16 h after microinjection. −, No transformation; +, transformation occurred.

[d] Percentage of cells in an area adjacent to injected area that incorporated [^{3}H]thymidine into DNA. BK, Background.

[e] Number of injected cells that incorporated label divided by total number of injected cells. INJ, Injected area.

[f] Control purified protein was isolated from induced bacterial cells containing the T7 promoter-driven or pJL6 expression vectors minus the c-*raf* gene and was partially purified.

plying that *raf* phosphorylates transcription factors of the AP-1 complex (30).

Affinity-purified *raf* proteins were autophosphorylated when supplied with divalent cations and [γ-^{32}P]ATP (Fig. 2B); however, when divalent cations were omitted from the reaction cocktail, the autokinase activity of full-length c-*raf* was greatly reduced, while the activity of mutant *raf* proteins was not affected (data not shown). These results suggest that purified p74^{raf} is not an active kinase until acted upon by ligands and that deletion of amino-terminal sequences results in deregulation of kinase activity.

The microinjection data support popular models for *raf* activation which correlate kinase activity to oncogenicity (10, 14, 19). The mechanism of *raf* activation is directly related to alteration or deletion of N-terminal amino acids that contain several regulatory motifs (Fig. 1B). This region of the polypeptide normally forms a negative regulatory region which hinges over the kinase domain, blocking activity (2, 4, 5, 21). Several human malignancies have been shown to express a transforming *raf*-like gene with amino-terminal deletions similar to those of the mutants generated and studied in this report (8, 24, 28). A similar mechanism has been implicated in the generation of an active catalytic fragment for protein kinase C (17). Protein kinase C also contains regulatory regions with cysteine-rich zinc finger structures that are involved in divalent cation or DNA binding or both (3).

The observation that full-length c-*raf* protein does not transform or induce DNA synthesis in quiescent NIH 3T3

cells is not surprising, since these cells normally express the protein. High concentrations of microinjected $p74^{raf}$ were not mitogenic. Amino-terminal deletion mutants of c-*raf* were found to induce transformation and DNA synthesis in NIH 3T3 cells. DNA transfection of full-length c-*raf* cDNA into NIH 3T3 cells did not result in transformation, while several mutant *raf* genes with deleted 5′ termini were found to be transforming (10). These results suggest that the *raf* protein is not oncogenic in its normal biologic form and that some mechanism of activation via continuous phosphorylation, ligand binding, or truncation of the amino terminus of the polypeptide is required to activate the oncogenicity of the kinase.

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