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16. Schneider S2 cells were maintained in Schneider's *Drosophila* medium (Gibco) supplemented with 10% fetal bovine serum (FBS) (Hyclone) and penicillin-streptomycin at 27°C. RPR cDNA was cloned into an expression vector containing the *Drosophila* metallothionein promoter [T. A. Bunch, Y. Grinblat, L. S. B. Goldstein, *Nucleic Acids Res.* **16**, 1043 (1988)] and cotransfected with a plasmid containing the hygromycin resistance gene under the control of the *Drosophila* actin 5C promoter. Stable transfections of S2 cells were performed by the calcium phosphate method [M. Ashburner, *Drosophila; A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989), pp. 142–145]. Because of the low adherence of the S2 cells, the transfection pool was grown up, and monoclonal cell lines were subsequently generated by limiting dilution (technique to subclone cells) in 96-well plates. The polyclonal transfection pool (Mt-RPR pool) and eight subclones (Mt-RPR1 to -8) were analyzed, and all gave similar results with regard to RPR induction and cell death in response to $CuSO_4$. In the rest of the study, we used the empty vector control pool, the Mt-RPR pool, and Mt-RPR1 and -2. For metallothionein promoter activation, cells were plated in Schneiders medium with 10% FBS, and 24 or 48 hours later, 0.5 M $CuSO_4$ was added to a final concentration of 0.5 mM. For the peptide inhibition experiments, cells were treated with 50 μM of the following peptides: Z-VAD-fmk, Z-FA-fmk, Z-AAD-cmk (Enzyme System Products, Dublin, CA), or ALLN (Sigma). After 90 min, $CuSO_4$ was added to a final concentration of 0.5 mM, and after 2 hours the percentage of apoptotic cells was determined by examination of at least 200 cells for the presence of plasma membrane blebs.
17. For the Northern (RNA) blot analysis, cells were treated with 0.5 mM $CuSO_4$ for the indicated times, after which total cellular RNA was isolated with the Ultraspec RNA isolation kit (Biotecx Laboratories, Houston, TX). Twenty-five micrograms of total RNA was used for the Northern blot analysis,which was performed according standard procedures. For the analysis of RPR protein, cells were treated as described above, and after a brief wash with ice-cold phosphate-buffered saline (PBS) the cells were lysed in Laemmli sample buffer. Fifty micrograms of protein was analyzed for the presence of RPR by immunoblotting with RPR peptide antiserum. The RPR peptide antiserum was raised in rabbits with the use of the COOH-terminal 15 amino acids of RPR ($NH_2$-CHPKTGRKSGKYRKP-COOH) (*27*), cross-linked to keyhole limpet haemocyanin (Animal Pharm Services, Healdsburg, CA). The RPR antiserum was affinity-purified on an RPR peptide column [E. Harlow and D. Lane, *Antibodies, A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1988), pp. 313–318].
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19. Vector control cells and Mt-RPR1 cells were grown on tissue culture slides (Nunc, Naperville, IL) and were left untreated or were treated with 0.5 mM $CuSO_4$ for 90 min. Cells were washed once with PBS and were subsequently fixed in 2% glutaraldehyde in PBS for 10 min, after which they were washed three times with PBS. After a 30-min incubation at room temperature in PBS, 0.1% Triton X-100, 2% bovine serum albumin (BSA), and 1% normal goat serum, the cells were incubated for 2 hours with preimmune serum or with affinity-purified RPR antiserum in PBS, 0.1% Triton X-100, and 0.2% BSA. Cells were washed three times with PBS and 0.1% Triton X-100, and bound RPR antibodies were visualized by incubation with a goat antibody to rabbit immunoglobulin G coupled to fluorescein isothiocyanate.
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25. For ceramide quantitation, cells were treated as described (*16*). Lipids were extracted, and ceramide amounts were subsequently determined by in vitro phosphorylation in the presence of [γ-$^{32}$P]ATP (adenosine triphosphate) by diacyl-glyceride kinase relative to total cellular phospholipid [P. P. van Veldhoven, T. J. Matthews, D. P. Bolognesi, R. M. Bell, *Biochem. Biophys. Res. Commun.* **167**, 209 (1992)]. C2-ceramide and C6-ceramide (Gibco) were disolved in ethanol at a concentration of 10 mM and diluted to a final concentration of 50 μM each in Grace's insect medium (Gibco) containing 0.5% FBS. The medium was sonicated in a bath sonicator for 5 s before being added to the cells. At different time points, the cells were stained with trypan blue, and the percent of positive cells was determined. The ethanol vehicle alone did not induce cell death.
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27. Single-letter abbreviations for the amino acid residues are as follows: C, Cys; G, Gly; H, His; K, Lys; P, Pro; R, Arg; S, Ser; T, Thr; and Y, Tyr.
28. We thank D. Schneider and J. Weiss for reagents and helpful discussions; and J. Escobedo, K. Giese, S. Harrison, M. Kavenaugh, and C. Reinhard for comments on the manuscript. G.J.P. was supported by a stipend from the Netherlands Organization for Scientific Research (NWO) during part of this work.

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# Stimulation of Membrane Ruffling and MAP Kinase Activation by Distinct Effectors of RAS

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The RAS guanine nucleotide binding proteins activate multiple signaling events that regulate cell growth and differentiation. In quiescent fibroblasts, ectopic expression of activated H-RAS (H-RAS$^{V12}$, where V12 indicates valine-12) induces membrane ruffling, mitogen-activated protein (MAP) kinase activation, and stimulation of DNA synthesis. A mutant of activated H-RAS, H-RAS$^{V12C40}$ (where C40 indicates cysteine-40), was identified that was defective for MAP kinase activation and stimulation of DNA synthesis, but retained the ability to induce membrane ruffling. Another mutant of activated H-RAS, H-RAS$^{V12S35}$ (where S35 indicates serine-35), which activates MAP kinase, was defective for stimulation of membrane ruffling and induction of DNA synthesis. Expression of both mutants resulted in a stimulation of DNA synthesis that was comparable to that induced by H-RAS$^{V12}$. These results indicate that membrane ruffling and activation of MAP kinase represent distinct RAS effector pathways and that input from both pathways is required for the mitogenic activity of RAS.

RAS proteins are essential components of receptor-mediated signal transduction pathways that control cell proliferation and differentiation. RAS may control at least two signal transduction pathways, one regulating gene expression and the other controlling actin cytoskeleton organization (*1*, *2*). The first signaling pathway involves a series of cytoplasmic serine-threonine kinases, hereafter referred to as the MAP kinase pathway. The second pathway, hereafter referred to as the cell morphology pathway, is mediated by members of the Rho family of guanosine triphosphate (GTP) binding proteins, which regulate the organization of the actin cytoskeleton. The MAP kinase pathways and the cell morphology pathway can be dissociated (*3*, *4*). However, the point at which these pathways diverge has not yet been defined.

Morphological changes induced by activated forms of RAS proteins are accompanied by the induction of membrane ruf-

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fling (5). To examine whether the RAS effector RAF protein kinase is required for the activation of the cell morphology pathway, we tested the ability of activated forms of RAF to induce membrane ruffling. Quiescent rat embryo fibroblast (REF-52) cells were microinjected with expression plasmids encoding activated H-RAS (H-RAS$^{V12}$) or RAF-CAAX (a c-RAF1 kinase targeted to the plasma membrane by a COOH-terminal lipid modification signal from H-RAS) together with a CAT (chloramphenicol acetyltransferase) reporter gene controlled by five copies of the c-*fos* serum-response element (5XSRE) (6). Because activation of the SRE by RAS requires MAP kinase activation (7), SRE induction was used in this assay to monitor the effects of the injected plasmids on the MAP kinase pathway. Each of the expressed proteins was efficient in inducing SRE expression, which is consistent with their well-documented ability to induce MAP kinase activation (Fig. 1) (8). However, RAF-CAAX did not induce membrane ruffling (9). Similar results were obtained with the use of another form of activated RAF, v-RAF (10). These observations suggest that RAF activation is not sufficient to mediate the RAS-induced cell morphology pathway.

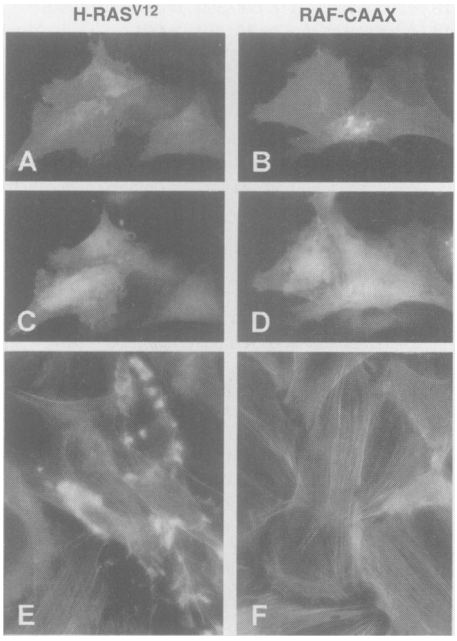


**Fig. 1.** Induction of 5XSRE-CAT but not membrane ruffling by RAF-CAAX. Quiescent REF-52 cells were microinjected with 5XSRE-CAT reporter construct and plasmids expressing either H-RAS$^{V12}$ (**A** and **C**) or RAF-CAAX (**B** and **D**) (6). Cells were immunostained simultaneously with a mixture of antibodies [(A) and (C)] to RAS [fluorescein staining (A)] and CAT [rhodamine staining (C)] or with a mixture of antibodies [(B) and (D)] to RAF [fluorescein staining (B)] and CAT [rhodamine staining (D)] (21). (**E** and **F**) Cells injected with either H-RAS$^{V12}$ (E) or RAF-CAAX (F) were stained with rhodamine-labeled phalloidin to visualize membrane ruffling (9).

To investigate whether RAF activation is required for RAS-induced membrane ruffling, we screened a panel of RAS effector binding loop mutants for their ability to activate MAP kinase and stimulate membrane ruffling. We reasoned that if the morphology pathway and the MAP kinase pathway are differentially controlled by RAS, it should be possible to identify mutations in RAS that selectively interfere with these activities. The effector binding loop mutant of activated H-RAS, H-RAS$^{V12C40}$, contains a substitution of Cys for Tyr at position 40 and is defective for RAF binding in the two-hybrid system (11). REF-52 cells were cotransfected with H-RAS$^{V12}$ and H-RAS$^{V12C40}$ expression plasmids and a plasmid encoding an epitope-tagged version of MAP kinase (12). After 48 hours, the transiently expressed ERK2 was isolated by immunoprecipitation and its activity was measured in an immunocomplex kinase assay with myelin basic protein (MBP) as a substrate. Expression of H-RAS$^{V12C40}$ had no measurable effect on MAP kinase activity, whereas in the same assay the expression of H-RAS$^{V12}$ resulted in the activation of MAP kinase (Fig. 2A).

Likewise, microinjection of H-RAS$^{V12C40}$ did not stimulate SRE-CAT expression (13). However, the H-RAS$^{V12C40}$ mutant did induce membrane ruffling with an efficiency similar to that induced by H-RAS$^{V12}$ (Fig. 2C). The subcellular localization of the H-RAS$^{V12C40}$ mutant appeared to be identical to that of H-RAS$^{V12}$, as judged by immunofluorescent staining of REF-52 cells that were microinjected with expression plasmids encoding these proteins (Fig. 2B). Together, these results indicate that the MAP kinase pathway and the cell morphology pathway bifurcate at the level of RAS itself and that RAF activation is neither necessary nor sufficient for the RAS-mediated effects on cell morphology. Induction of membrane ruffling by activated RAS is dependent on RAC proteins (14). The stimulation of membrane ruffling by H-RAS$^{V12C40}$ also required RAC, as indicated by the fact that membrane ruffling was prevented by the co-injection of H-RAS$^{V12C40}$ with a dominant-interfering form of RAC, RAC$^{N17}$ (where N17 is Asn$^{17}$) (13). Thus, it is likely that H-RAS$^{V12}$ and H-RAS$^{V12C40}$ use the same signaling mechanisms to induce membrane ruffling.

We examined the relative contribution of the MAP kinase pathway and the cell morphology pathway to the mitogenic activity of RAS. To assess the contribution of the MAP kinase pathway, we have used the RAS effector binding loop mutant H-RAS$^{V12S35}$, a transformation-attenuated mutant that retains the ability to activate the MAP kinase pathway (15). In REF-52 cells, ectopic expression of the H-RAS$^{V12S35}$ mutant stimulated MAP kinase activation (Fig. 2A) and SRE-CAT expression (13) but failed to induce membrane ruffling (Fig. 2C). Microinjection of an expression plasmid encoding the H-RAS$^{V12S35}$ mutant into quiescent REF-52 cells did not stimulate DNA synthesis, as measured by 5-bromodeoxyuridine (BrdU) incorporation (Fig. 3) (16). This observation suggests that in REF-52 cells, RAS-mediated activation of the MAP kinase pathway is not sufficient to induce a mitogenic response. This interpretation is further supported by our observation that RAF-CAAX did not stimulate DNA

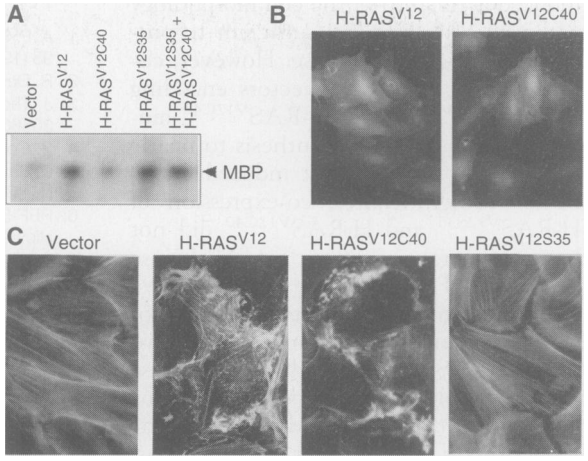


**Fig. 2.** Effects of H-RAS$^{V12}$, H-RAS$^{V12C40}$, and H-RAS$^{V12S35}$ on MAP kinase activity and membrane ruffling. (**A**) Effects of H-RAS$^{V12}$, H-RAS$^{V12C40}$, and H-RAS$^{V12S35}$ on MAP kinase activity. REF-52 cells were cotransfected with expression plasmids encoding HA-tagged p42$^{MAPK}$ and the indicated H-RAS mutants. HA-tagged p42$^{MAPK}$ was isolated from cell lysates by immunoprecipitation with monoclonal antibody 12CA5, and MAP kinase activity was measured in an immunocomplex kinase assay with myelin basic protein (MBP) as a substrate (12). Radioactivity incorporated into MBP was visualized by phosphorimaging. This experiment was repeated three times with similar results. (**B**) Subcellular localization of H-RAS$^{V12}$ and H-RAS$^{V12C40}$. REF-52 cells were microinjected with the indicated expression plasmids (50 μg/ml) . Six hours after injection, cells were fixed and immunostained with antibody to RAS (21). (**C**) Effects of H-RAS$^{V12}$, H-RAS$^{V12C40}$, and H-RAS$^{V12S35}$ on membrane ruffling. REF-52 cells were microinjected with the indicated expression vectors (50 μg/ml). Six hours after injection, cells were fixed and stained with rhodamine-labeled phalloidin to visualize the actin cytoskeleton (9).

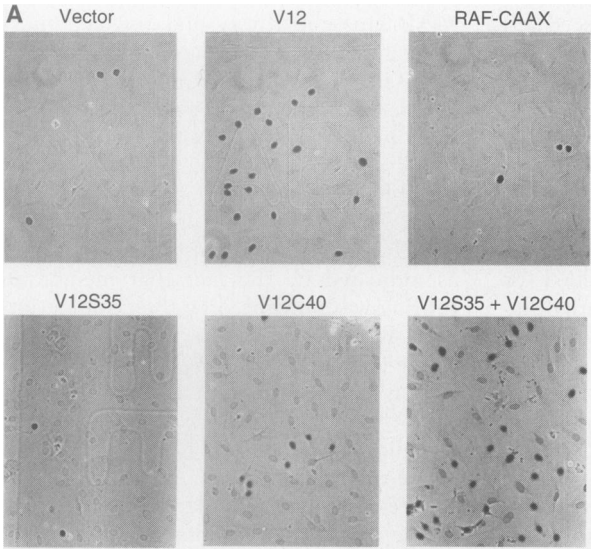


**Fig. 3.** Effects of H-RAS effector mutants on DNA synthesis. (**A**) Quiescent REF-52 cells were microinjected with the indicated pDCR-H-RAS expression plasmids (5 μg/ml), and DNA synthesis was monitored 30 hours after injection by immunostaining of BrdU incorporated into newly synthesized DNA (*16*). (**B**) Percentage of BrdU-positive REF-52 cells 30 hours after microinjection of the indicated plasmids. Values correspond to the averages of three independent assays in which at least 100 injected cells were scored per condition in each assay. Error bars correspond to the standard deviation.

synthesis when microinjected into REF-52 cells (Fig. 3). Constitutively active MAP kinase kinase can induce transformation in NIH 3T3 cells (*4*, *17*). The apparent discrepancy between these findings and ours might be due to cell type differences or could reflect the possibility that the signaling mechanisms involved in inducing the mitogenic response are not identical to those leading to cellular transformation.

To test the role of the cell morphology pathway in RAS-induced mitogenesis, we tested the effect of the H-RAS$^{V12C40}$ mutant on BrdU incorporation. This mutant was also inefficient in stimulating DNA synthesis when microinjected into quiescent REF-52 cells (Fig. 3). Thus, RAS-induced activation of the cell morphology pathway is by itself not sufficient to promote a mitogenic response. However, coinjection of expression vectors encoding the H-RAS$^{V12S35}$ and H-RAS$^{V12C40}$ mutants stimulated DNA synthesis to nearly to the same level as that induced by H-RAS$^{V12}$. Significantly, co-expression of H-RAS$^{V12S35}$ and H-RAS$^{V12C40}$ did not have a synergistic effect on MAP kinase activation (Fig. 2). These results suggest that the mitogenic activity of RAS proteins requires inputs from at least two distinct RAS effector pathways. Because the morphological effects produced by H-RAS$^{V12C40}$ are RAC-dependent, the induction of cell proliferation by RAS may result from complementary signals contributed by the MAP kinase pathway and the RAC pathway. Consistent with this interpretation, RAC and MAP kinase pathways cooperate to cause cell transformation (*18*).

Our results provide evidence that RAS activates the cell morphology and MAP kinase pathways through distinct effector systems. The MAP kinase pathway is critical for the transmission of signals from RAS to the nucleus (*19*). The RAC pathway mediates the effects of RAS on actin cytoskeleton (*14*) and links RAS activation to nuclear events (*20*). Identification of the mechanisms by which RAS exerts dual control over the RAC and MAP kinase pathways should provide insights into the respective contribution of these pathways to the biological effects of RAS on cell proliferation and differentiation.

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6. REF-52 cells were plated onto gridded glass cover slips and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal bovine serum (FBS, 10%). The cells were grown to confluency, then placed in starvation medium (DMEM with 0.5% FBS) 24 hours before microinjection. A mixture containing the specified plasmids in microinjection buffer [50 mM Hepes (pH 7.2), 100 mM KCl, and 5 mM $NaHPO_4$] was injected into the nuclei of all the cells present in a chosen square of the cover slip. H-RAS$^{V12}$, H-RAS$^{V12C40}$, and H-RAS$^{V12S35}$ were cloned into the pDCR expression vector encoding the hemagglutinin (HA) nonapeptide epitope [I. Wilson *et al.*, *Cell* **37**, 767 (1984)], and RAF-CAAX was cloned into the pCDNA vector (Invitrogen). The 5XSRE-CAT expression plasmid was constructed as described [R. Graham and M. Gilman, *Science* **251**, 189 (1991)].
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9. For monitoring membrane ruffling, cells on cover slips were fixed in 3.7% formaldehyde in phosphate-buffered saline (PBS) for 1 hour at room temperature. The cells were then stained for 1 hour at room temperature in a humidified chamber with rhodamine-labeled phalloidin (0.1 μg/ml) dissolved in a solution of 90% PBS and 10% methanol. The cells were photographed with a Zeiss Axiophot fluorescence microscope.
10. T. Joneson *et al.*, unpublished data.
11. M. A. White and M. H. Wigler, unpublished data.
12. REF-52 cells were cultured in DMEM supplemented with 10% FBS. Cells were transfected by the standard calcium phosphate ($CaPO_4$) method with 4 μg of HA-tagged p42$^{MAPK}$ and 4 μg of either vector pDCR, pDCR-H-RAS$^{V12}$, pDCR-H-RAS$^{V12C40}$, or H-RAS$^{V12S35}$ and supplemented with vector pDCR to a total of 16 μg of DNA. After a 12-hour incubation with DNA-$CaPO_4$ precipitates, cells were incubated in serum-free DMEM for 48 hours. Cells were lysed in lysis buffer [10 mM tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10% glycerol, 1 mM $Na_3VO_4$, 50 mM NaF, 1 mM okadaic acid, 1 mM benzamidine, 1 mM phenylethylsulfonyl fluoride, leupeptin (10 μg/ml), and aprotinin (10 μg/ml)], and lysates were clarified by centrifugation. HA epitope-tagged p42$^{MAPK}$ was immunoprecipitated with the HA monoclonal antibody 12CA5. Immune complexes were collected by binding to protein A–Sepharose, washed extensively in lysis buffer, then assayed for 30 min at 37°C in kinase assay buffer [20 mM tris (pH 7.4), 20 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM $Na_3VO_4$, 20 mM ATP (adenosine triphosphate), and 10 μCi of [γ-$^{32}$P]ATP with 0.2 mg/ml of myelin basic protein]. Reaction products were analyzed by SDS–polyacrylamide gel electrophoresis and developed with the Fuji imaging system.
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16. For monitoring DNA synthesis, BrdU was added to the cell culture medium at a concentration of 10 μM, 2 hours after injection. After 30 hours, cells were fixed and permeabilized in acid alcohol (ethanol:water:acetic acid, 90:5:5) for 1 hour at −20°C. Then, cover slips were incubated with mouse antibody to BrdU and then with horseradish peroxidase–conjugated goat antibody to mouse immunoglobulin G (IgG). Immunocytochemical color development was done according to the manufacturer's instructions (Cell Proliferation Kit, Amersham). At least 100 injected cells were scored in each assay for quantitation.
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21. For immunofluorescence, injected cells were fixed at the indicated times after injection in 3.7% formaldehyde in PBS for 1 hour at room temperature, then permeabilized with 0.1% Triton X-100 in PBS for 3 min at room temperature. The cover slips were incubated for 1 hour at 37°C with a mixture of either rat antibody to RAS (Y13-259) or mouse antibody to RAF (Transduction Laboratories) and rabbit antibody to CAT (5′→3′ Co.) in PBS containing bovine serum albumin (2 mg/ml) and then with a mixture of either fluorescein-conjugated goat antibody to rat IgG or goat antibody to mouse IgG and rhodamine-conjugated goat antibody to rabbit IgG (Cappel).


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