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The Farnesyl Group of H-Ras Facilitates the Activation of a Soluble Upstream Activator of Mitogen-activated Protein Kinase*

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To study the function of the farnesyl modification of Ras, the farnesyl group and a variety of its structural analogs, which lack one or more double bonds and/or the methyl groups, were enzymatically incorporated into recombinant H-Ras *in vitro*. These proteins were used in a cell- and membrane-free, Ras-dependent mitogen-activated protein kinase (MAP kinase) activation system derived from *Xenopus laevis* eggs to examine the contribution of the farnesyl group toward the activation of the kinase. Whereas non-farnesylated H-Ras is unable to activate MAP kinase, farnesylation of H-Ras alone, in the absence of further processing, is sufficient to cause the activation of MAP kinase in this system. All of the analogs of the farnesyl group, when incorporated into H-Ras, support the activation of the kinase to variable extents. These results suggest a direct but fairly nonspecific interaction of the farnesyl moiety of H-Ras with a soluble upstream activator of MAP kinase.

The Ras GTP-binding proteins play a pivotal role in a variety of signal transduction and differentiation processes (1, 2). Ras is also involved in the generation of a number of human cancers, and several oncogenic point mutations of Ras are known (3, 4). Ras is activated by the conversion of the GDP-bound inactive form to the GTP-bound active form in response to various extracellular signals (5). A variety of extracellular signals can activate mitogen-activated protein kinase (MAP kinase)[1] (also known as extracellular signal-regulated kinase (ERK)) through both Ras-dependent and Ras-independent mechanisms (6). A Ras-dependent pathway linking the epidermal growth factor receptor to MAP kinase, through the protein kinase Raf, has been elucidated (7, 8). A Ras-dependent, Raf-independent MAP kinase activation system has also been identified (9, 10).

Ras proteins are part of the group of proteins that are post-translationally prenylated (11). In the case of Ras, this modification involves the attachment of the farnesyl group to the protein through a thioether linkage to a cysteine located four residues from the carboxyl terminus, followed by removal of the three carboxyl-terminal amino acids and methylation of the newly exposed α-carboxyl group of the farnesyl cysteine residue (12). Additionally, H-Ras and N-Ras, but not K-Ras, undergo palmitoylation at one or more upstream cysteine residues (13). Although necessary for the normal and oncogenic functions of many proteins, including Ras (14), the specific properties imparted by these post-translational modifications have, to a large extent, remained unclear. Prenylation of proteins has been implicated in membrane binding (13, 15) and in protein-protein recognition (16, 17). Furthermore, the relative contribution of each of the processing steps is unknown.

Previously we developed a cell-free assay system, derived from *Xenopus laevis* eggs, to identify a direct target molecule for Ras. In this system, Ras promotes the activation of MAP kinase through MAP kinase kinase/ERK kinase (MEK). Using this system, we have identified a Ras-dependent MEK kinase termed REKS (**R**as-dependent **E**RK **K**inase **S**timulator) (18). Subsequently, we have highly purified this protein and have determined that it is distinct from c-Raf-1, Mos, and mSte11, all of which are known to both phosphorylate and activate MEK (19). Furthermore, c-Raf-1 partially purified by Mono-S chromatography from *X. laevis* eggs did not cause activation of MAP kinase either in the presence or the absence of Ras under these assay conditions (20). We have previously shown that fully processed K-Ras (*i.e.* farnesylated, proteolysed, and methylated) is far more active than unmodified K-Ras in the REKS-dependent activation of MAP kinase (19, 21). Similar results for the Ras-dependent activation of yeast adenylate cyclase have been reported (22, 23). In the earlier reports, we could not exclude the possibility that the unmodified Ras was denatured, nor was it possible to examine the individual processing steps of Ras (see above) to determine which of these steps provides the critical modification.

EXPERIMENTAL PROCEDURES

Materials—Farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate are from American Radiolabeled Chemicals. Geranyl pyrophosphate, (7*S*)-6,7-dihydrofarnesol, and 6,7–10,11-tetrahydrofarnesol were gifts from R. B. Croteau (Washington State University), D. Cane (Brown University), and Hoffman La Roche (Basel), respectively. 10,11-Dihydrofarnesol and (*Z*)-3-methyl-2-dodecenol were synthesized by reducing the corresponding methyl esters with $LiAlH_4$ (24). The alcohols were converted to the pyrophosphates (25), and the products were purified by TLC using 1-propanol:concentrated NH_4OH:water (6:3:1). The pyrophosphate esters were eluted from the TLC plates using 10 mM tetrabutylammonium hydroxide containing approximately 0.5% concentrated ammonium hydroxide. The tetrabutylammonium hydroxide was removed by loading the sample on a Sep-Pak C_{18} cartridge (Waters) washing with water and eluting with MeOH containing approximately 1% concentrated ammonium hy-

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[1] The abbreviations used are: MAP kinase, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, MAP kinase kinase/ERK kinase; REKS, Ras-dependent ERK kinase stimulator; FPP, farnesyl pyrophosphate; PFT, protein-farnesyl transferase; MBP, myelin basic protein; GST, glutathione *S*-transferase; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); GDPβS, guanosine 5′-*O*-(2-thiodiphosphate); CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

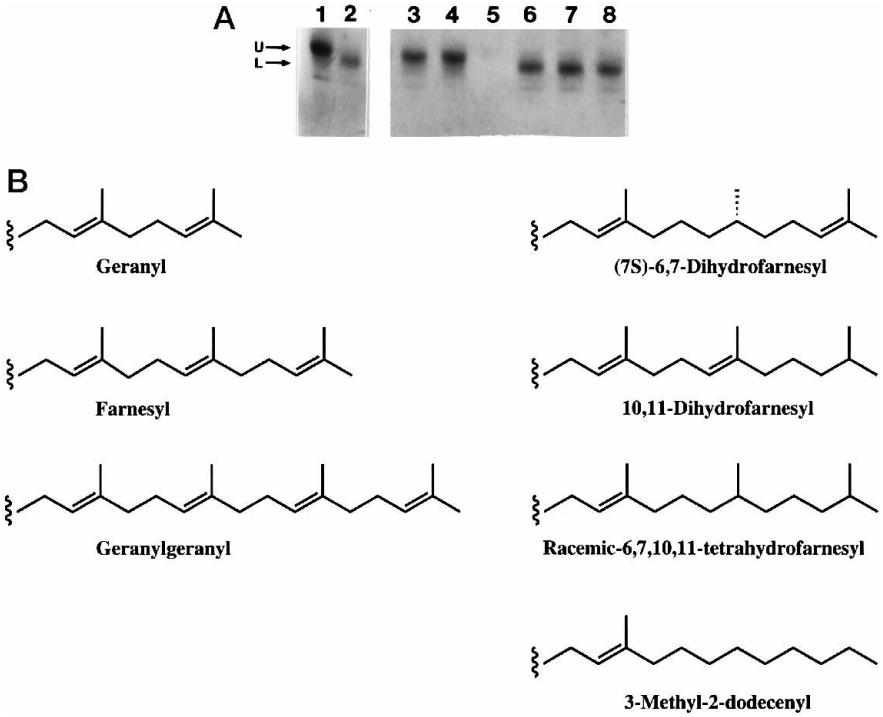

FIG. 1. *A*, incorporation of the farnesyl group and its analogs into H-Ras as monitored by SDS-polyacrylamide gel electrophoresis (15% acrylamide, 0.087% bisacrylamide) (28). *Arrows* indicate the positions of unmodified (*U*) and lipidated (*L*) H-Ras. *Lane 1*, unmodified H-Ras produced in *E. coli*; *lane 2*, complete reaction mixture containing H-Ras, FPP, and PFT; control farnesylation reactions containing all ingredients except FPP (*lane 3*), PFT (*lane 4*), or H-Ras (*lane 5*); lipidation reactions containing all ingredients except that FPP is replaced with (*Z*)-3-methyl-2-dodecenyl pyrophosphate (*lane 6*), (7*S*)-6,7-dihydrofarnesyl pyrophosphate (*lane 7*), or 6,7,10,11-tetrahydrofarnesyl pyrophosphate (*lane 8*). The incorporation of the other analogs into H-Ras was also quantitative (not shown). *B*, structures of the farnesyl group and its analogs.

droxide. The methanolic solutions were concentrated to dryness and taken up in 5 mM NH_4HCO_3 containing approximately 0.8% added concentrated ammonium hydroxide.

Preparation of Lipidated H-Ras Proteins—Bacterial H-Ras was prepared by a modification of the published procedure (26). Frozen cells (27 g) were thawed on ice and lysed by sonication in 35 ml of buffer A (20 mM Tris-HCl, pH 7.6, 0.1 mM EDTA, 0.2 mM dithiothreitol). All steps were carried out at 4 °C. The 100,000 × *g* supernatant was diluted to 70 ml with buffer A and applied to a column of DEAE CL-6B (2.5 × 18 cm, Pharmacia Biotech Inc.). After washing with 100 ml of buffer A, the protein was eluted with a linear gradient (500 ml, 0–0.4 M NaCl in buffer A). H-Ras was located by protein-farnesyl transferase (PFT)-catalyzed incorporation of [1-^{3}H]FPP (27). The pool of active fractions (~0.20–0.24 M NaCl) was ultrafiltered (Amicon, YM-10) and applied to an S-200 gel filtration column (1.6 × 95 cm, Pharmacia). After elution with buffer A, the pool of active fractions was applied to a Mono-Q column (10/10, Pharmacia), which was eluted with a gradient (80 ml, 0–0.4 M NaCl in buffer A). The fraction having the highest activity in the PFT assay and by GTP binding was used in subsequent experiments. SDS-polyacrylamide gel electrophoresis (28) indicated that the protein was >95% pure. The protein was diluted with an equal volume of glycerol and stored at −20 °C. GTP binding for location of H-Ras was done using a nitrocellulose filter binding assay with [*guanosine*-8-^{3}H]GTP. Sample (5 μl) was incubated in buffer (100 μl) (20 mM Tris-HCl, pH 7.4, 2 mM EDTA) containing 10 μM [^{3}H]GTP (406 mCi/mmol) for 20 min at 30 °C. The assays were quenched with ice-cold $MgCl_2$ buffer (200 μl) (20 mM Tris-HCl, pH 7.4, 20 mM $MgCl_2$) on ice and filtered over nitrocellulose (0.45 μm, Schleicher & Schuell) and washed extensively with $MgCl_2$ buffer. The nitrocellulose was dissolved in ethylene glycol dimethyl ether, and the radioactivity bound was quantitated by liquid scintillation counting.

H-Ras protein (1 nmol) was incubated with recombinant PFT (0.25 nmol), obtained as described (29), and 20 μM FPP or FPP analogs in 100 μl of buffer (16 mM Tris-HCl, pH 7.5, 0.34 M NaCl, 10 mM $MgCl_2$, 0.65 mM dithiothreitol, 12.6 μM $ZnCl_2$, 12.5 mM EDTA) for 3 h at 30 °C. Buffer exchange and removal of excess pyrophosphate ester were performed using a spin column of P-6 gel (Bio-Rad) equilibrated in column buffer (20 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol). The eluant was diluted with an equal volume of column buffer containing 1.2% CHAPS. Geranylgeranylated H-Ras was produced by incubation of geranylgeranyl pyrophosphate and recombinant protein-geranylgeranyl transferase-I (obtained from P. Casey, Duke University) with bacterial H-Ras containing a modified carboxyl terminus (CVLL, obtained from P. Casey, Duke University).

Eggs were obtained from fully mature *X. laevis* females and activated by electric shock (30) to drive them into interphase and inactivate endogenous MAP kinase and MEK activities (31, 32). The cytosol of activated eggs was obtained by centrifugation as described (20). REKS was partially purified by Mono-Q chromatography (19).

REKS Assay—REKS activity was assayed by measuring the phosphorylation of myelin basic protein (MBP) by recombinant glutathione *S*-transferase (GST)-MAP kinase in the presence of recombinant GST-MEK as described (20). Mono-Q purified REKS was incubated for 10 min at 30 °C in a final volume of 50 μl containing 20 mM Tris-HCl, pH 8.0, 120 μM ATP, 10 mM $MgCl_2$, 6 mM EGTA, 80 nM GST-MEK, and the indicated amounts of GTPγS·Ras or GDPβS·Ras (loading with nucleotide was as described (20)). 10 μl of 3 μM GST-MAP kinase was added, and the incubation was continued for 20 min. 20 μl of a mixture containing 20 mM Tris-HCl, pH 8.0, 100 μM [γ-^{32}P]ATP (150–460 cpm/pmol), 220 μM MBP (Sigma), 10 mM $MgCl_2$, and 6 mM EGTA was added, and the incubation was continued for 10 min, after which 30 μl of the reaction mixture was spotted onto phosphocellulose paper. The paper was washed with 75 mM phosphoric acid, and the radioactivity was measured by liquid scintillation counting. Recombinant GST-MEK and GST-MAP kinase were obtained as described (33). Fully modified K-Ras and H-Ras were obtained as described (34).

Fatty Acid Analysis—Analysis for fatty acids was carried out on the various components of the system by negative ion, chemical ionization mass spectrometry after conversion to pentafluorobenzyl esters (35, 36) following saponification of the fractions with 1 N KOH in 90% ethanol and extraction with ether:pentane 1:1 (37). Heptadecanoic acid was added as an internal standard, and octadeutereo-arachadonic acid or 1-stearoyl-2-arachidonyl phosphatidylcholine was added to some samples as an internal control.

RESULTS

It has been proposed that the role of the farnesyl group is primarily to bind proteins to membranes (13). In the Ras- and Raf-dependent system, the primary function of Ras appears to be the recruitment of Raf to the plasma membrane, where this kinase is somehow activated through phosphorylation by an unknown kinase. Raf, in turn, activates the protein kinase MEK, which subsequently activates MAP kinase (7, 8). Although Raf has been identified as one of the downstream targets of Ras, recent evidence indicates that other targets exist (9, 10, 38, 39). In fact, Zheng *et al.* (40) have presented evidence that the Raf pathway plays only a relatively minor role in the growth factor-induced activation of MEK and, subsequently, of MAP kinase. Hence, an important aspect of the present system is that it is membrane free. To confirm

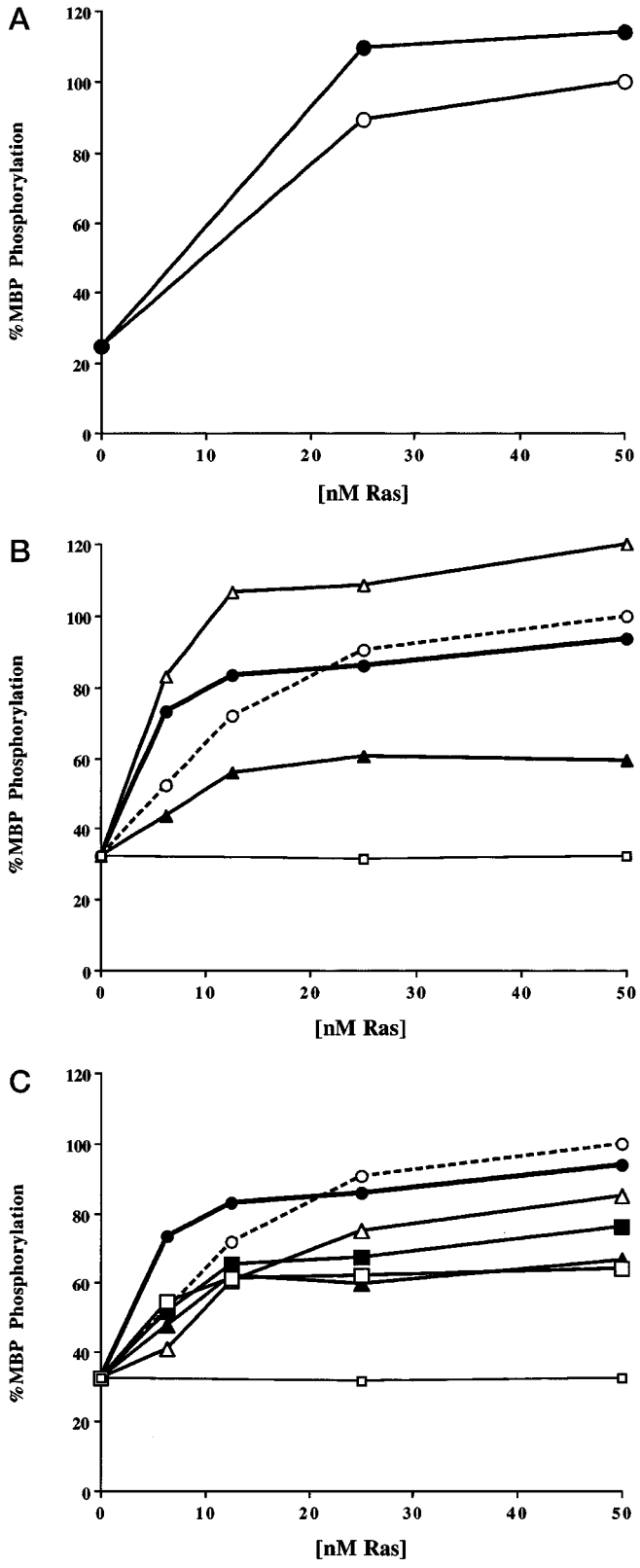

FIG. 2. *A*, activation of MAP kinase, as measured by incorporation of ^{32}P into MBP, by the indicated concentrations fully processed GTPγS·K-Ras (○) or fully processed GTPγS·H-Ras (●). 100% MBP phosphorylation is the activation caused by 50 nM fully processed GTPγS·K-Ras. *B*, activation of MAP kinase, as measured by incorporation of ^{32}P into MBP, by the indicated concentrations of GTPγS·Ras containing prenyl chains of different length. ○, fully processed K-Ras; ●, farnesylated H-Ras; , geranylgeranylated H-Ras; , geranylated H-Ras; □, non-prenylated H-Ras, prepared by *in vitro* prenylation with all of the reaction components except FPP. Control prenylations with H-Ras in the absence of PFT or H-Ras (CVLL) and protein-geranylgeranyl transferase-I in the absence of geranylgeranyl pyrophosphate also caused no activation. 100% MBP phosphorylation is the activation caused by 50 nM fully processed GTPγS·K-Ras; in this case, 100% = 43.3 pmol. *C*, activation of MAP kinase, as measured by the incorporation of ^{32}P into MBP by the indicated concentrations of GTPγS·Ras modified with farnesyl analogs. ○, fully processed K-Ras; ●, farnesylated H-Ras; , 10,11-dihydrofarnesylated H-Ras; ■, racemic-6,7,10,11-tetrahydrofarnesylated H-Ras; , (7*S*)-6,7-dihydrofarnesylated H-Ras; □, 3-methyl-2-dodecenylated H-Ras; □, non-prenylated H-Ras prepared by *in vitro* prenylation with all of the reaction components except FPP). 100% MBP phosphorylation is the activation caused by 50 nM fully processed GTPγS·K-Ras; in this case, 100% = 43.3 pmol. The results shown are representative of three independent experiments. The error for each individual point is less than ±10%.

this, the various components of the cell-free system were analyzed for their membrane content. This was done by searching for the common fatty acid linolenic acid by negative chemical ionization mass spectrometry. Only a trace amount of this fatty acid was found, representing 1 nM or less in the final assay mixture, which is well below the concentration of H-Ras added to the assays, indicating that the assay mixture was virtually membrane free.

For the present studies, we used REKS that was partially purified from *X. laevis* eggs by ion exchange chromatography to a completely soluble form to study the role of the farnesyl group of Ras in promoting the activation of MAP kinase. We have been able to quantitatively farnesylate recombinant H-Ras produced in *Escherichia coli* by incubation with FPP and recombinant PFT (Fig. 1). The unmodified H-Ras was prepared in such a way as to produce an intact carboxyl-terminal CVIS sequence, necessary for farnesylation. Lower than maximal incorporation of the farnesyl group into H-Ras is observed when using other procedures for isolating this protein, probably due to proteolytic damage at the carboxyl terminus.

Using Mono-Q purified REKS, the GTPγS-bound form of non-prenylated H-Ras, even at high concentrations, does not detectably activate MAP kinase (Fig. 2, *B* and *C*). In marked contrast, farnesylated but otherwise unprocessed GTPγS·H-Ras, produced by the *in vitro* farnesylation of recombinant H-Ras, is as active as fully processed H-Ras or K-Ras in activating MAP kinase. Fully processed K-Ras was used as a standard to compare the activity of the modified H-Ras proteins due to the difficulty in purifying and handling fully processed H-Ras. However, fully processed H-Ras supports activation of MAP kinase, through REKS, to roughly the same extent as fully processed K-Ras (Fig. 2*A*).

This result clearly shows that inactive unmodified H-Ras can be converted to a fully active form solely by farnesylation, and thus further processing (carboxyl-terminal proteolysis and methylation and palmitoylation) are not required.

To determine if the precise structure of the farnesyl group is necessary for REKS-dependent activation of MAP kinase, a variety of farnesyl analogs (Fig. 1*B*) were also incorporated enzymatically into recombinant H-Ras (Fig. 1*A*). Using this method, the activity of these differently modified proteins toward the activation of MAP kinase in the cell-free system described above was examined (Fig. 2, *B* and *C*). H-Ras proteins containing the farnesyl analogs were able to support the activation of MAP kinase to variable extents. A significant dependence on the size of the prenyl group was observed (Fig. 2*B*); H-Ras bearing the longer 20-carbon geranylgeranyl group was the best activator, while H-Ras containing the shorter 10-carbon geranyl group was the least potent activator. With regard to the structurally modified farnesyl analogs, saturation of either of the more distal double bonds of

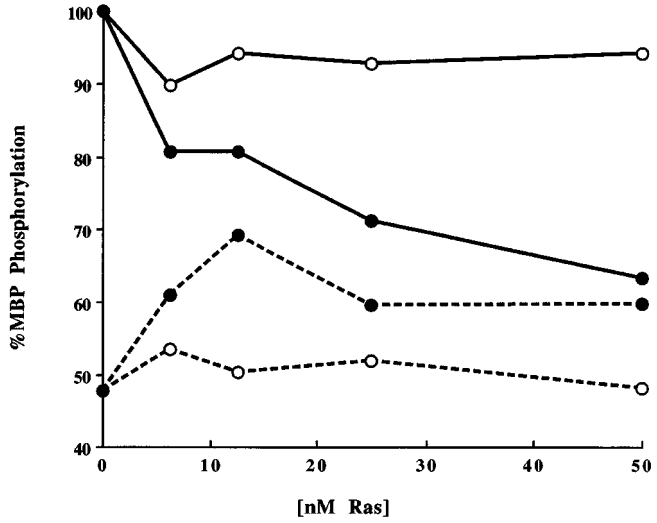

FIG. 3. **Competition of the farnesylated GTPγS·H-Ras-dependent activation of MAP kinase by farnesylated GDPβS·H-Ras or non-farnesylated GTPγS·H-Ras.** Effect of the indicated concentrations of farnesylated GDPβS·H-Ras (●) or non-farnesylated GTPγS·H-Ras (○) on the activation of MAP kinase in the presence (*solid line*) or absence (*dashed line*) of 6.25 nM farnesylated GTPγS·H-Ras. 100% MBP phosphorylation is the activation caused by 6.25 nM farnesylated GTPγS·H-Ras alone. The results shown are representative of three independent experiments. The error for each individual point is less than ±10%.

the farnesyl group caused a small decrease in activity (Fig. 2*C*). H-Ras containing the (*Z*)-3-methyl-2-dodecenyl side chain was one of the least potent activators; this group, while the same length as the farnesyl group, structurally bears the least resemblance to the native lipid. The trend observed indicates that there is a component of specificity based on the prenyl structure of the side chain as well as a component based on hydrophobicity. Therefore, it appears that H-Ras, in this system, is interacting at least in part through its prenyl group with some soluble component or components of the MAP kinase activation system.

As shown in Fig. 3, the GDPβS-bound form of farnesylated H-Ras is weakly stimulatory but does inhibit the stimulation of MAP kinase by farnesylated GTPγS·H-Ras, giving 50% inhibition at approximately 30 nM. Non-farnesylated GTPγS·H-Ras neither activates MAP kinase nor inhibits the activation of MAP kinase by farnesylated GTPγS·H-Ras. This result also suggests that the farnesyl group is needed for the binding of H-Ras to some protein component of the MAP kinase activation system.

DISCUSSION

Based on the current studies it is concluded that inactive bacterially produced H-Ras can be converted to an active form solely by farnesylation using FPP and recombinant PFT. Our results indicate that farnesylation of Ras is the critical modification needed for the Ras- and REKS-dependent activation of MAP kinase and that further processing (proteolysis, methylation, and palmitoylation) is not necessary for this aspect of Ras function. This is demonstrated by the results showing that the farnesylated but otherwise unprocessed H-Ras is comparable to fully processed K-Ras or H-Ras in its ability to stimulate MAP kinase in this system. This is further illustrated by the inability of non-farnesylated H-Ras to either stimulate the activity or to inhibit the activity caused by the farnesylated H-Ras. It appears that the dependence of REKS-mediated stimulation of MAP kinase by Ras on the farnesyl group arises from a fairly nonspecific hydrophobic interaction of the prenyl group with some soluble component of the system. The various analogs of the farnesyl group, when incorporated into H-Ras, cause only relatively small changes in the extent of activation.

We have recently purified a REKS activity from bovine brain cytosol. The bovine REKS was found to be a complex of three proteins, one of which was B-Raf (41). While the *Xenopus* REKS did not cross-react with anti-B-Raf antibody (18), we cannot rule out that *Xenopus* REKS is an isoform of Raf. Clearly, the interaction of this protein with Ras is different than that previously described for Raf (7, 8). Unlike the membrane binding function of Ras described previously, the effects of farnesylation in this system are not the result of membrane binding, as no membranes are present in the assay. Moreover, if the role of the farnesyl group in the H-Ras- and REKS-dependent activation of MAP kinase is only to bind H-Ras to membranes or other possible interfaces in this *in vitro* system, the level of maximal activation caused by all of the lipidated H-Ras proteins should be the same; the results in Fig. 2 clearly show that this is not the case. Taken together, the data strongly suggest that there is a direct interaction of the farnesyl group of H-Ras either with REKS, or with an as yet undetermined soluble component of the system, and that the activation of MAP kinase, in this system, is not dependent on the binding of Ras to membranes. Alternatively, prenylation may cause a structural change in Ras, which allows it to interact with its target protein. In any case, these experiments demonstrate a role for the prenyl group of Ras, which is distinct from that of a simple membrane anchor and is more similar to the prenyl protein-protein interaction model described for heterotrimeric G proteins in which the prenyl group appears to play a crucial role in subunit interaction (16).

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