Replacement of the H-Ras Farnesyl Group by Lipid Analogues: Implications for Downstream Processing and Effector Activation in *Xenopus* Oocytes†

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ABSTRACT: Ras proteins must undergo a series of posttranslational lipidation steps before they become biologically functional. While the fact that farnesylation is required for subsequent processing steps and indispensable for Ras function has been established, the significance of the isoprenoid structure *per se* in the context of fully processed Ras is unknown. Here, we describe a novel approach for studying the isoprenoid structure−function relationship *in vivo* by replacing the H-Ras farnesyl group with synthetic analogues and analyzing their biological functions following microinjection into *Xenopus* oocytes. We show that the H-Ras farnesyl group can be stripped of most of its isoprenoid features that distinguish it from a fatty acid without any apparent effect on its ability to induce oocyte maturation and activation of mitogen-activated protein kinase. In contrast, replacement by the less hydrophobic isoprenoid geranyl causes severely delayed oocyte activation. Analysis of posttranslational processing reveals a striking correlation between the kinetics of processing, membrane binding, and the onset of biological activity regardless of lipid structure and suggests that slow C-terminal proteolysis and/or methylation can become rate-limiting for H-Ras function. Thus, while our results suggest no stringent requirement for the H-Ras farnesyl structure for effector activation in *Xenopus* oocytes, they reveal an important role for the lipid present at the farnesylation site in promoting efficient proteolysis and/or methylation which allows rapid palmitoylation, membrane localization, and biological activity. *Xenopus* oocytes provide a useful *in vivo* system for the kinetic analysis of the function of the protein of interest present at the physiological dose, which is required for accurate determination of structure−function relationships.

Ras proteins act as molecular switches in the transduction of many extracellular signals from cell surface receptors to the nucleus and are involved in a variety of cellular processes from mitosis and differentiation to apoptosis (Hersowitz, 1995; Casey, 1995). Ras proteins are synthesized as cytosolic precursors and must undergo posttranslational modifications at their C termini before they can translocate to the inner leaflet of the plasma membrane and become biologically functional. These modifications include farnesylation at a cysteine residue located four residues from the C terminus, followed by removal of the C-terminal tripeptide and methylation of the newly exposed C terminus (Schafer & Rine, 1992; Clarke, 1992; Gelb, 1997). Some Ras proteins (H-Ras, N-Ras, and Ras2) are further lipidated by palmitoylation at one or two cysteines near the farnesylated C terminus (Hancock et al., 1989; Bhattacharya et al., 1995).

The contribution of individual lipids to the overall biology of Ras is incompletely understood. Palmitoylation anchors Ras firmly to membranes (Hancock et al., 1989, 1990; Bhattacharya et al., 1995; Dudler & Gelb, 1996; Willumsen et al., 1996) and strongly potentiates its function at least in some cellular systems (Bhattacharya et al., 1995; Dudler & Gelb, 1996; Willumsen et al., 1996). Farnesylation is indispensable for all biological functions of H-Ras *in vivo*, but since prevention of this initial modification blocks all subsequent processing steps, the role of the farnesyl group in the context of the fully matured H-Ras protein is unknown. Studies in transfected cell lines indicate that geranylgeranylated H-Ras is as effective as the farnesylated form in supporting cell transformation, suggesting that, at least when overexpressed, other isoprenoids might be able to support biological functions (Hancock et al., 1991).

Several models for the function of the isoprenoid group have been proposed. Since the fusion of a farnesylation site together with appropriate flanking sequences onto the C terminus of normally cytosolic proteins results in their predominant membrane localization, the farnesyl group might act as a simple hydrophobic membrane-targeting signal (Hancock et al., 1989, 1991; Stokoe et al., 1994; Levers et al., 1994; Hall, 1994). Alternatively, isoprenylation of proteins has been invoked in protein−protein recognition (Marshall, 1993; Casey, 1995).

Studies using transfected cell lines mostly favor a membrane binding model since the ability of Ras to activate effector cascades and induce changes in cell physiology correlates with its membrane localization. In intact cells, however, a clear distinction between a role for the Ras prenyl group in membrane binding versus specific interaction(s) with effector molecules has not been possible so far since genetic tools only permit mutations at the farnesylation site which lead to modification with another isoprenoid or abrogate posttranslational processing and membrane binding altogether.

In the Ras−MAPK[1] cascade, a major signaling pathway activated by Ras (de Vries-Smith et al., 1992; Marshall,

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[1] Abbreviations: F, farnesyl; G, geranyl; MD, (*E*)-3-methyl-2-dodecenyl; THF, 6,7,10,11-tetrahydrofarnesyl; GVBD, germinal vesicle breakdown; MAPK, mitogen-activated protein kinase; MPF, maturation-promoting factor.

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1995), considerable evidence for a specific role of the H-Ras farnesyl group in protein−protein interaction has accumulated from *in vitro* experiments. Reconstitution of the Ras−MAPK pathway in a cell-free system revealed a strict dependence on H-Ras prenylation without the need for membrane components (Itoh et al., 1993a; Yamamori et al., 1995). In this *in vitro* system derived from *Xenopus* oocytes, farnesylation is necessary and sufficient to convert inactive unprocessed H-Ras into its fully active form (McGeady et al., 1995). Studies in this system using Ras modified with farnesyl analogues further indicated some degree of specificity based on the prenyl structure as well as on the hydrophobicity of the lipid (McGeady et al., 1995). While these results clearly suggest a specific role for the H-Ras farnesyl group in interactions with effector molecules in the MAPK pathway, the *in vivo* significance of such observations is unknown.

To overcome the limitations of a genetic approach, we have developed an experimental system combining *in vitro* modification of H-Ras with structurally altered synthetic lipids with microinjection into *Xenopus laevis* oocytes to study the specific function(s) of the H-Ras farnesyl group for signal transduction in intact cells. An additional advantage of the *Xenopus* oocyte system is the ability to analyze the function of the protein of interest microinjected at a physiological dose, which is required for accurate studies of structure−function relationships.

Oocytes are naturally arrested at the G2-M boundary of the first meiotic cell division. When microinjected with oncogenic H-Ras, oocytes resume meiotic maturation (Birchmeier et al., 1985) in a process which is accompanied by activation of the mitogen-activated protein kinase (MAPK) cascade (Hattori et al., 1992; Pomerance et al., 1992; Fabian et al., 1993; Barrett et al., 1990; Nebreda et al., 1993; Fukoto et al., 1994) and an increase in the $p34^{cdc2}$ kinase activity of maturation-promoting factor (MPF) (Barrett et al., 1990). Here, we report that in *Xenopus* oocytes the H-Ras farnesyl group can tolerate substantial structural changes without loss of function, while its role in promoting further posttranslational processing appears to be pivotal for biological activity.

EXPERIMENTAL PROCEDURES

Proteins. Oncogenic H-Ras carrying the Val-12 mutation was expressed in *Escherichia coli* using the pMG27/T24 plasmid (kindly provided by Prof. Tamanoi, UCLA) and purified as previously described (McGeady et al., 1995). Ras protein concentrations were determined by GTP binding using nitrocellulose filter binding assays or by incorporation of [^{3}H]farnesyl pyrophosphate catalyzed by recombinant farnesyltransferase following the established procedure (McGeady et al., 1995; Dudler & Gelb, 1996). The synthesis of pyrophosphates of the farnesol analogues geraniol (G), tetrahydrofarnesol (THF), and (*E*)-3-methyl-2-dodecenol (MD) was described previously (McGeady et al., 1995). Bacterial H-Ras was lipidated *in vitro* with farnesyl or its analogues by incubating H-Ras samples (1 nmol) with 50 pmol of farnesyltransferase for 4 h at 30 °C in 200 μL of buffer containing 30 mM Tris (pH 7.4), 50 mM NaCl, 5 mM $MgCl_2$, 10 μM $ZnCl_2$, 0.5 mM DTT, and the respective lipid pyrophosphate at 50 μM. In all cases, stoichiometric lipidation was verified by observing the shift to a higher electrophoretic mobility on SDS−PAGE as well as by measuring the radioactivity incorporated into H-Ras when using tritiated lipid pyrophosphates with known specific activities (McGeady et al., 1995). Both methods confirmed that Ras was essentially fully lipidated with farnesyl and all of its analogues that were used. After lipid-modified H-Ras samples were loaded with GTP (McGeady et al., 1995), free nucleotides and lipid pyrophosphates were removed by passing samples through a 1 mL spin column of P-6 gel (Bio-Rad) that had been previously equilibrated with Ras buffer [20 mM Tris (pH 7.4), 100 mM NaCl, 4 mM $MgCl_2$, 0.1 mM DTT, and 0.1% octyl glucoside]. The presence of detergent was necessary to allow microinjection of lipidated H-Ras without a significant loss of protein and did not affect cell viability. The lipid-modified Ras samples were finally concentrated to about 20 μM using a Microcon-10 device (Amicon), snap-frozen in liquid nitrogen in small aliquots, and stored at −80 °C. GTP-binding assays used to determine Ras protein concentrations showed that nucleotide binding was not affected by the *in vitro* lipidation procedure (McGeady et al., 1995).

The relative hydrophobicity of H-Ras modified with farnesyl or its analogues was assessed by subjecting H-Ras samples prenylated with ^{3}H-labeled lipids to Triton X-114 phase partitioning as described (Gutierrez et al., 1989; Dudler & Gelb, 1996). Proteins from the aqueous and detergent phase were then precipitated with 10 volumes of ice-cold acetone, solubilized in SDS−PAGE buffer, and resolved on 15% polyacrylamide gels. After the gels were stained with Coomassie Blue, the amount of lipidated Ras in the detergent and aqueous phase was quantified by liquid scintillation counting of excised H-Ras bands (Celis, 1994).

Oocyte Microinjection. Oocytes were isolated from *X. laevis* females and cultured as described previously (Dudler & Gelb, 1996). Unless specified otherwise, all culture media were supplemented with 50 μM mevinolin (an inhibitor of isoprenoid biosynthesis) to prevent lipid modification of microinjected H-Ras with endogenous farnesyl pyrophosphate. Ras proteins were diluted in Ras buffer, and 50 nL was microinjected into the cytoplasm. Oocytes were kept in culture medium and scored for germinal vesicle breakdown (GVBD) by the appearance of a white spot on the animal hemisphere. For biochemical analysis, five oocytes per group were randomly harvested and homogenized in 50 μL of lysis buffer [60 mM β-glycerophosphate (pH 7.4), 20 mM EGTA, 15 mM $MgCl_2$, 1 mM Na_3VO_4, 1 mM NaF, 1 mM benzamidine, 5 μg/mL leupeptin, and 1 mM PMSF]. The crude lysates were either processed immediately or flash-frozen in liquid nitrogen and stored at −80 °C.

Metabolic Labeling. To assess Ras methylation, oocytes were cultured in mevinolin-containing medium supplemented with 0.4 mCi/mL of [*methyl*-^{3}H]methionine (NEN, 70 Ci/mmol) for 4 h prior to injection with 0.5 pmol of lipid-modified H-Ras. After continued incubation in labeling medium for the indicated time periods, oocytes were homogenized in lysis buffer containing 1% Triton X-100, and insoluble debris was removed by centrifugation at 10000*g* for 10 min at 4 °C. Ras was immunoprecipitated by incubating 20 μL of cleared extract with 1 μg of monoclonal anti-Ras antibody Y13-259 (Oncogene Sciences) in 0.5 mL of immunoprecipitation buffer [10 mM Tris (pH 7.5), 100 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, and 0.5% NP-40] overnight at 4 °C followed by addition of 15 μL of a Protein G−Sepharose suspension

(Sigma). After another 1 h of incubation at 4 °C on a rocking platform, immunoprecipitates were collected by centrifugation and washed four times with immunoprecipitation buffer. Immunoprecipitates were resolved by SDS−PAGE (15%), and radiolabeled proteins were visualized by fluorography (Magee et al., 1995).

To assess palmitoylation, oocytes microinjected with lipid-modified H-Ras were cultured in "cold" medium for 2−20 h before healthy oocytes were transferred to medium containing 2 mCi/mL of [^{3}H]palmitate (NEN, 60 Ci/mmol). After the indicated labeling period, groups of oocytes were harvested and homogenized in lysis buffer containing 1% Triton X-100. Ras proteins were immunoprecipitated, and radioactivity was detected as described above.

Oocyte Fractionation. Oocytes were fractionated into cytosolic and membrane components using standard procedures (Celis, 1994). The membrane fraction was extracted with buffer containing 1% Triton X-100, and equal portions of cytosolic and membrane fractions were analyzed by immunoblotting.

Immunoblotting and Kinase Assays. Immunoblotting studies were carried out as described using anti-Ras or anti-MAPK (anti-ERK2) mAb (both from transduction laboratories in Lexington, KY) and the ECL detection system (Amersham) (Dudler & Gelb, 1996). Kinase activities were measured in cleared extracts after insoluble debris was removed by centrifugation at 12000*g* for 15 min at 4 °C. MAPK activity was determined using myelin basic protein (Sigma) as substrate (Ahn et al., 1990; Dudler & Gelb, 1996). MPF activity was measured by histone H1 phosphorylation following previously described procedures (Dudler & Gelb, 1996). Fluorographs, autoradiographs, and immunoblots were quantified by scanning the exposed films with a Bio-Rad GS-670 densitometer according to the manufacturer's instructions.

RESULTS

We have analyzed the structure−function relationship of the H-Ras farnesyl group for signal transduction *in vivo* using *X. laevis* oocytes as an experimental system. Bacterially expressed activated H-Ras has been shown to activate the MAPK cascade (Fabian et al., 1993; Pomerance et al., 1992) and to induce meiotic maturation (Birchmeier et al., 1985) when microinjected into *Xenopus* oocytes. These biological effects are strictly dependent on *in vivo* farnesylation of the injected H-Ras protein since mutation of the farnesylation site, blocking of isoprenoid biosynthesis, and inhibition of farnesyltransferase in oocytes all suppress these activities (Gibbs et al., 1989; Kim et al., 1990; Zhao et al., 1994). We therefore studied the role of the isoprenoid structure for H-Ras function *in vivo* by testing the ability of the structural analogues of the farnesyl group to rescue H-Ras biological functions in isoprenoid-depleted oocytes.

Overnight treatment of oocytes with 50 μM mevinolin (an inhibitor of mevalonate biosynthesis) effectively depletes the endogenous pool of farnesyl pyrophosphate; bacterially expressed oncogenic H-Ras (up to 5 pmol) was unable to induce detectable maturation for at least 48 h after microinjection in these oocytes, while 0.5 pmol of the same H-Ras preparation typically induced 50% maturation after 4−6 h in untreated cells (data not shown). However, *in vitro* farnesylation prior to microinjection rescues the ability of

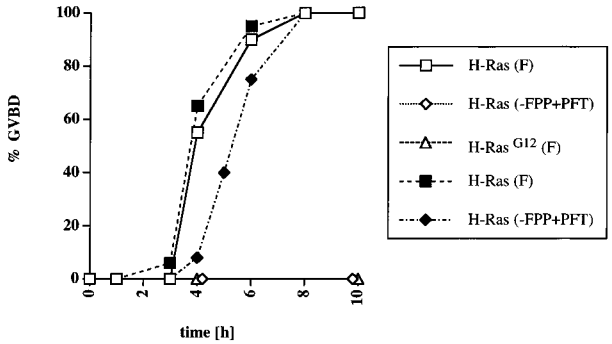

FIGURE 1: *In vitro* farnesylation specifically rescues the biological activity of oncogenic H-Ras in isoprenoid-depleted oocytes. Oocytes were isoprenoid-depleted by overnight treatment with 50 μM mevinolin (open symbols) and then microinjected with 0.5 pmol of *in vitro*-farnesylated oncogenic H-Ras (squares), nonfarnesylated oncogenic H-Ras [treated with PFT without FPP (diamonds); identical results are obtained for oncogenic H-Ras treated with FPP in the absence of PFT (not shown)], or farnesylated non-oncogenic H-Ras Gly-12 (triangles). After incubation in mevinolin-containing medium at 18−20 °C for the indicated time periods, oocytes were scored for GVBD. Results are expressed as a percentage of the number of injected oocytes (25−30 per group). No change in the maturation pattern was observed during an additional 36 h incubation period. The maturation kinetics observed in untreated cells (all media without mevinolin) injected with 0.5 pmol of *in vitro*-farnesylated oncogenic H-Ras (filled squares) and nonfarnesylated oncogenic H-Ras (treated with PFT without FPP; filled diamonds) are shown for comparison.

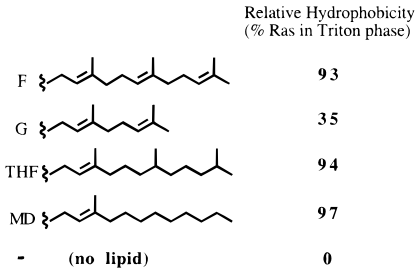

FIGURE 2: Structures of farnesyl analogues and properties of H-Ras lipid variants *in vitro*. H-Ras was stoichiometrically lipidated with the following lipids: F, farnesyl; G, geranyl; MD, 3-methyl-2-dodecenyl; and THF, 6,7,10,11-tetrahydrofarnesyl. The relative hydrophobicity was assessed by subjecting H-Ras samples prenylated with tritiated lipids to Triton X-114 phase partitioning (see Experimental Procedures). The values represent [counts per minute in the detergent phase/total counts per minute] × 100 for each sample. Nonlipidated H-Ras could not be detected in the detergent phase.

H-Ras to induce oocyte responses in mevinolin-treated cells using as little as 0.25 pmol/cell. This effect is specific since neither oncogenic H-Ras treated with farnesyltransferase or farnesyl pyrophosphate alone nor farnesylated non-oncogenic H-Ras (Gly-12) displayed any biological effect (Figure 1). In untreated cells, both the farnesylated and nonfarnesylated forms of activated H-Ras induce meiotic maturation and activation of MAPK, with the farnesylated form exhibiting accelerated kinetics with a comparable dose (Figure 1). Thus, microinjection of *in vitro*-lipidated H-Ras into isoprenoid-depleted *Xenopus* oocytes provides a means of specifically studying the biological role of the H-Ras farnesyl group in signal transduction in intact cells.

In a first set of experiments, H-Ras was lipidated *in vitro* with the farnesyl group or its structural analogues shown in Figure 2, and 0.5 pmol was microinjected into isoprenoid-depleted oocytes. This dose was found to induce suboptimal oocyte stimulation; if 0.25 pmol was microinjected, the time

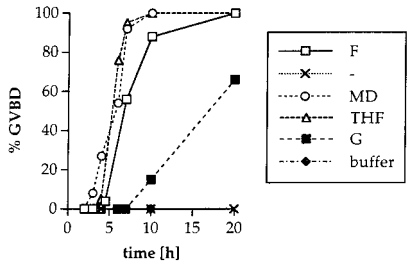

FIGURE 3: Induction of meiosis by *in vitro*-lipidated H-Ras in isoprenoid-depleted *X. laevis* oocytes. Mevinolin-treated oocytes were microinjected with 0.5 pmol of H-Ras modified with the lipids shown in Figure 2, nonmodified H-Ras treated with farnesyltransferase in the absence of lipid pyrophosphate (−), or buffer alone and incubated in mevinolin-containing medium at 18−20 °C. After the indicated time periods, oocytes were monitored for GVBD. Results are expressed as a percentage of the number of injected oocytes (30−40 per group). A representative experiment is shown.

required for 50% of the oocytes to mature was 10−12 h compared to 5−6 h typically observed for 0.5 pmol, while at doses of 2 pmol, 50% maturation could be observed after 4 h. Under these suboptimal conditions, the oocyte system is most sensitive to structural alterations of Ras which affect its biological activity. Furthermore, 0.5 pmol of H-Ras results in an intracellular Ras concentration (∼0.5 μM) comparable to the endogenous Ras levels found in NIH 3T3 cells (∼6 μg/mL) (Caruso et al., 1986). The time course of oocyte maturation following microinjection (Figure 3) reveals that H-Ras biological function is supported by the THF and MD groups in a manner indistinguishable from that of the F group, while G modification of H-Ras results in severely compromised biological activity. The maturation kinetics of oocytes stimulated with G-modified H-Ras are slow, with a time necessary to achieve 50% maturation of typically 15−20 h, whereas all other modified H-Ras proteins induce rapid oocyte maturation which is essentially complete after 6 h. After extended periods of culturing (30−48 h), oocytes injected with G-modified H-Ras also undergo complete maturation, whereas unmodified H-Ras elicits no detectable response.

In a second set of experiments where isoprenoid-depleted oocytes were co-injected with unmodified H-Ras and lipid pyrophosphates (0.5 pmol and 25 nmol, respectively), all of the lipid pyrophosphates were found to rescue the biological activity of unmodified H-Ras. The time course of maturation was virtually identical with that shown in Figure 3, and a similar delay in the cellular response to G-modified H-Ras was also evident under these experimental conditions. These data demonstrate that lipidation with prenyl analogues also occurs *in situ* and that the biological differences observed are not the result of the *in vitro* lipidation procedure.

Activated H-Ras stimulates the MAPK cascade in *Xenopus* oocytes (Hattori et al., 1992; Pomerance et al., 1992), and MAPK activity is necessary to mediate Ras-induced GVBD (Kosado et al., 1994; Fukoto et al., 1994; Carnero et al., 1994). Thus, we compared the ability of the different lipid-modified H-Ras proteins to activate MAPK. G-modified H-Ras elicits no detectable MAPK activation 6 h after injection and induces 40−75% of the maximal MAPK activity only after a 24 h incubation period, while MAPK is near-maximally induced with all other lipid-modified H-Ras proteins after 6 h (Figure 4). In all cases, MAPK activation correlates with ERK2 phosphorylation as shown by gel shift analysis (Figure 4, bottom).

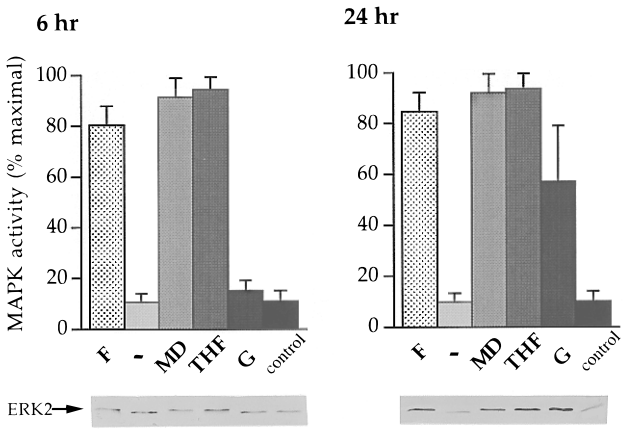

FIGURE 4: MAPK activation. Oocytes stimulated as in Figure 3 were lysed 6 or 24 h after injection. (Top) The MAPK activity in these extracts was measured by incorporation of ^{32}P into myelin basic protein (Ahn et al., 1990). The activity measured in individual extracts was standardized relative to the maximal MAPK activity within each batch of oocytes (highest MAPK levels measured in any of the Ras-stimulated extracts). The mean ± standard deviation (SD) from four experiments using oocytes from different frogs is shown. (Bottom) Cleared extracts (0.1 oocyte equivalent per lane) were separated by SDS−PAGE (10%), transferred onto nitrocellulose, and probed with antibody against MAPK (ERK2).

Analysis of the time course of MAPK activation reveals that the delay in the onset of maturation observed with G-modified H-Ras is paralleled by a similar delay in the kinetics of MAPK activation (Figure 5A). Ras-induced meiotic maturation is also associated with activation of MPF (Barrett et al., 1990; Nebreda et al., 1993), a cell cycle regulatory kinase that controls the G2-M transition in eukaryotic cells and in *Xenopus* oocytes (Gautier et al., 1988, 1989). This kinase is equally activated by all lipid-modified H-Ras proteins but shows markedly delayed kinetics when stimulated with G-modified H-Ras (Figure 5B). After extended periods of culturing (up to 48 h), MAPK and MPF are activated to a comparable degree by all lipid-modified H-Ras proteins, whereas kinase activities in cells injected with nonlipidated H-Ras remained at control levels (not shown). Taken together, the results suggest that while lipid modification at the farnesylation site is necessary for H-Ras function *in vivo*, there is no stringent structural requirement for a farnesyl group to activate the MAPK cascade and induce cell cycle progression in *Xenopus* oocytes.

Following isoprenylation, H-Ras undergoes further processing by C-terminal proteolysis, methylation, and palmitoylation (Hancock et al., 1989, 1990), and it has been recently shown that palmitoylation is of paramount importance for activation of the MAPK cascade and induction of meiosis in *Xenopus* oocytes (Dudler & Gelb, 1996). Since structural alterations at the farnesylation site might affect further processing, we analyzed the kinetics of methylation, palmitoylation, and membrane binding for the differently lipidated H-Ras proteins. Labeling of oocytes with [*methyl*-^{3}H]methionine reveals that, while all lipidated Ras proteins are equally labeled 24 h after microinjection, G-modified H-Ras is poorly methylated 4 h after microinjection (Figure 6). A time course analysis shows that methylation for F-, MD-, or THF-modified H-Ras is rapid, with a $t_{1/2}$ of 1−2 h. In contrast, methylation of H-Ras−G is slow and appears to progress linearly over a 24 h period. Cell fractionation studies 24 h after injection reveal that all farnesyl analogues

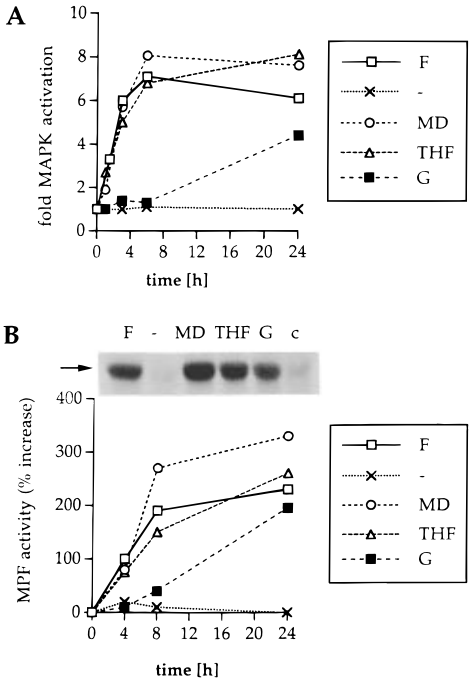

FIGURE 5: Kinetics of MAPK and MPF activation. Isoprenoid-depleted oocytes were stimulated as in Figure 3. After the indicated incubation periods, groups of five oocytes were lysed and kinase activity was measured in cleared lysates. (A) The fold MAPK activation represents the (counts per minute of Ras injected)/(counts per minute of buffer-injected oocytes), after subtracting the minus substrate control from each data point. (B) MPF activity was measured by incorporation of ^{32}P into histone H1 substrate. (Top) Reaction mixtures using 24 h extracts were separated by SDS−PAGE and subjected to autoradiography. The arrow indicates the position of the H1 band. (Bottom) The time course of MPF activation was evaluated by densitometric analysis of H1 phosphorylation in autoradiographs. Lane c is buffer-injected cells.

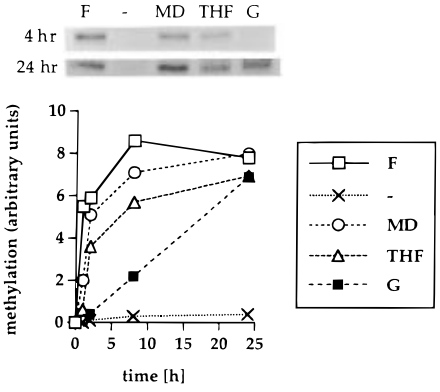

FIGURE 6: Effect of H-Ras lipid structure on methylation. Oocytes were pulsed with [*methyl*-^{3}H]methionine for 4 h before microinjection. At indicated times after injection, oocytes were lysed and Ras was immunoprecipitated, loaded onto polyacrylamide gels (15%), and visualized by fluorography (top). Immunoblot analysis showed that comparable amounts were immunoprecipitated (not shown). The time course of methylation was evaluated by densitometric analysis of fluorographs.

restore the ability of H-Ras to bind to membranes in isoprenoid-depleted oocytes, although the G group is less effective. Assessment of the time course of membrane binding (Figure 7) shows that G-modified H-Ras exhibits slow kinetics with approximately 60% membrane binding achieved after 24 h, while MD-, THF-, and F-modified H-Ras translocate rapidly to the membrane and reach steady state (about 80% membrane bound) after 6 h. Consistent with

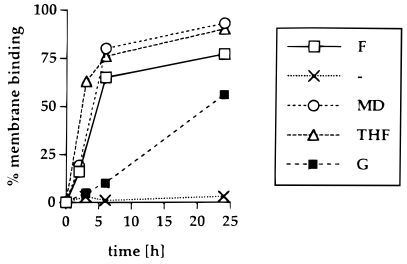

FIGURE 7: Effect of lipid structure on the kinetics of H-Ras membrane translocation. Isoprenoid-depleted oocytes were injected as in Figure 3. After the indicated incubation periods, oocytes were lysed and fractionated into cytosolic and membrane fractions. Equivalent portions were resolved by 15% SDS−PAGE and analyzed by immunoblotting with anti-Ras mAb as described (Dudler & Gelb, 1996). Densitometric analysis of immunoblots was used to determine the percentage of Ras in each lysate present in the membrane pellet.

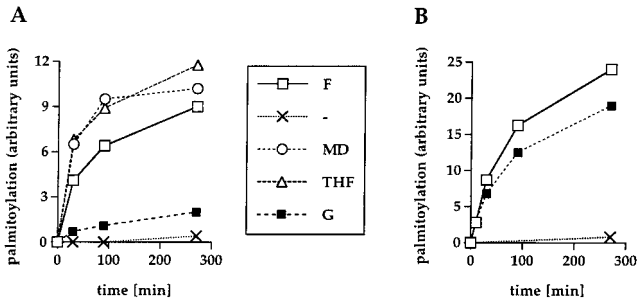

FIGURE 8: Effect of H-Ras lipid structure on palmitoylation. Oocytes microinjected as in Figure 3 were cultured in "cold" medium for 2 h (A) or 20 h (B) before they were transferred to medium containing 2 mCi/mL of [^{3}H]palmitic acid. After the indicated pulse periods in labeling medium, oocytes were lysed and Ras was immunoprecipitated, loaded onto polyacrylamide gels (15%), and visualized by fluorography. Immunoblot analysis was used to verify that comparable amounts were immunoprecipitated. The kinetics of palmitoylation were evaluated by densitometric analysis of fluorographs.

the slower membrane binding kinetics, incorporation of the palmitoyl group into G-modified H-Ras was strongly reduced if oocytes were labeled with [^{3}H]palmitate 2 h after protein microinjection, while the kinetics and extent of palmitate labeling are comparable for the MD-, THF-, and F-modified forms (Figure 8A). If [^{3}H]palmitate labeling is performed 20 h after protein injection, however, H-Ras−F and H-Ras−G are labeled with comparable efficiency (Figure 8B). These data suggest that the delayed biological activity observed for H-Ras−G is a consequence of its slow kinetics of proteolysis and/or methylation resulting in a delay in palmitoylation, cytosol-to-membrane translocation, and hence the onset of biological activity in *Xenopus* oocytes. Our results also indicate that H-Ras can tolerate substantial changes in the lipid structure at the farnesylation site without an apparent loss of biological function, provided that the lipid allows further C-terminal processing and membrane binding. The nature of the lipid, however, can affect further processing (particularly proteolysis and/or methylation) which can become limiting for biological activity.

DISCUSSION

In this study, we describe a whole cell system for carrying out a structure−function study of the farnesyl group of H-Ras. A critical feature of the *Xenopus* oocyte system is the fact that the lipidated protein can be tested at variable

doses near the physiological level. In contrast, transfection studies in which structurally altered Ras is expressed in mammalian cells greatly suffer from the fact that the levels of expressed Ras are usually much higher than the physiological dose, and thus, no structure−function conclusions can be reached unless the requirement for a structural feature is absolute. Consideration of these issues may explain why the importance of H-Ras palmitoylation in supporting Ras-dependent function was missed in earlier transfection studies (Hancock et al., 1989) but found in recent studies with oocytes (Dudler & Gelb, 1996) and mammalian cells (Willumsen et al., 1996). A further advantage of the *Xenopus* oocyte system is the fact that the kinetics of cellular processes can be followed by analyzing oocyte extracts as a function of time postinjection. This issue is important because structural changes in the isoprenoid moiety may affect biological activity by altering the kinetics of subsequent processing steps which are difficult to analyze in transfected cells.

We have shown that, under conditions where prenylation *in situ* is prevented, *in vitro* farnesylation is necessary and sufficient to allow bacterially expressed H-Ras to induce oocyte maturation and activation of the MAPK cascade (Figure 1). Since these oocyte responses are dependent on palmitoylation and membrane binding of H-Ras (Dudler & Gelb, 1996), this result suggests that exogenously farnesylated H-Ras is fully competent to undergo further processing *in situ*. Thus, this experimental system potentially allows one to elucidate specific functions of the H-Ras farnesyl group in the context of fully mature protein in intact cells by introducing structural changes confined to the prenyl moiety without changing the amino acid sequence of the protein. Oocytes are particularly tractable tools for this type of study since they can tolerate a small amount of detergent which is necessary to keep lipidated H-Ras in solution.

Replacing the farnesyl group of H-Ras with related structures, we show that the lipid side chain at the farnesylation site can be stripped of most of its isoprenoid features that distinguish it from a fatty acid (double bonds and methyl branches) without any apparent effect on the ability of H-Ras to induce MAPK and MPF activation (Figures 4 and 5) or promote meiotic maturation (Figure 3). Notably, the MD group is biologically equivalent to the farnesyl group for all the activities tested in this study. On the other hand, the G group, a natural farnesyl analogue shortened by one isoprenoid unit, is a poor functional farnesyl replacement. Since G-modified H-Ras is considerably less hydrophobic than all other lipidated H-Ras proteins analyzed in this study (Figure 2), this can be interpreted to mean that the hydophobicity of the side chain at the H-Ras prenylation site is functionally more important than its isoprenoid nature for biological activity. Strictly speaking, one could argue that G-modified H-Ras shows poor function not because of its loss of hydrophobicity but because of its reduced length. One could study farnesyl analogues that retain the length of the farnesyl group and contain more polar atoms such as ether oxygens to reduce hydrophobicity. Gordon, Englund, and co-workers have used this approach in their structure−function studies of protein myristoyl groups (Doering et al., 1994). However, in the case of protein prenylation, additional information gained by the use of heteroatom-substituted prenyl groups is likely to be clouded by the inability to deconvolute the loss of protein function due to poor membrane anchoring versus poor prenyl−protein interaction. For this reason, with the exception of the geranyl group, we have focused on farnesyl analogues that are expected to partition into membranes to similar extents (which seems to be the case on the basis of Triton X-114 partitioning data given above) but have altered structure which could affect prenyl−protein binding.

Does the poor activity of G-modified H-Ras reflect a specific defect in Ras−effector interaction, or is it a consequence of impaired downstream processing and membrane binding? Analysis of further processing of the *in vitro*-lipidated H-Ras proteins reveals that methylation of geranylated H-Ras is substantially delayed compared to that of the farnesylated form (a $t_{1/2}$ of 12−16 h compared to a $t_{1/2}$ of less than 1 h). In contrast, the isoprenoid nature of the lipid itself has little impact on methylation since MD- and THF-modified proteins are labeled almost as effectively as farnesylated H-Ras (Figure 6). Thus, while the two cellular enzymes involved in this process [a C-terminal tripeptidase and methyltransferase (Clarke, 1992)] have a rather broad substrate specificity with regard to the lipid structure present at the prenylation site, at least one of these integral membrane enzymes shows a strong preference for more hydrophobic lipids. The slow kinetics of G-modified H-Ras could reflect poor substrate recognition by the relevant enzyme(s) or a decrease in the effective substrate concentration in the membrane due to reduced hydrophobicity.

Palmitoylation is equally affected inasmuch as G-modified H-Ras labels poorly if [^{3}H]palmitate incorporation is measured 2 h after microinjection (Figure 8A). This inefficient palmitoylation, however, is most likely a consequence of the low degree of methylation of G-modified H-Ras soon after microinjection since no significant difference in the extent or kinetics of [^{3}H]palmitate incorporation was apparent when the labeling was done 20 h after injection (Figure 8B). Thus, the *Xenopus* homologue of protein-palmitoyltransferase, an enzyme which catalyzes the palmitoylation of H-Ras in mammals (Liu et al., 1996), appears to be rather permissive with regard to the lipid structure at the farnesylation site and efficiently palmitoylates all lipidated H-Ras proteins studied here, provided that they have previously been proteolyzed and/or methylated. Slow palmitoylation is further corroborated by membrane binding studies which show that G-modified H-Ras also becomes membrane-bound but only after extended culture periods. The time course of membrane translocation for G-modified H-Ras (Figure 7) compares favorably with the time course of methylation (Figure 6), suggesting that proteolysis and/or methylation is the rate-limiting processing step which is followed by rapid palmitoylation and membrane binding. Strictly speaking, we cannot rule out the possibility that G-modified H-Ras undergoes rate-limiting palmitoylation followed by rapid proteolysis and methylation. This possibility, however, seems unlikely because of the fact that a mutant of H-Ras in which the palmitoylation sites have been changed to serine is methylated with kinetics comparable to those of wild type H-Ras (Dudler & Gelb, 1996). Taken together, the correlation between the kinetics of methylation, membrane binding, and the time course of oocyte activation suggests that the delay in the onset of biological activity observed with G-modified H-Ras most likely reflects its inefficiency to undergo proteolysis and/or methylation rather than a reduced ability of the G group to allow productive interaction with

downstream effectors of the Ras signaling pathway.

Since palmitoylation of H-Ras strongly enhances its biological activity in intact cells (Dudler & Gelb, 1996; Willumsen et al., 1996), an important implication of the possible requirement for proteolysis/methylation for palmitoylation of geranylated H-Ras is the fact that prenyl protein-specific protease and/or methyltransferase inhibitors may be therapeutically useful as anti-cancer drugs, provided that this requirement also holds true for farnesylated H-Ras. While our *in vivo* study does not allow us to demonstrate this for the farnesylated form, the *in vitro* properties of purified mammalian protein-palmitoyltransferase would suggest that this requirement also applies to farnesylated H-Ras (Liu et al., 1996).

Does prenylation of H-Ras mediate protein−protein interaction in Ras effector pathways *in vivo*? *In vitro* reconstitution of the Ras−MAPK pathway has identified a protein termed REKS as a Ras downstream effector molecule (Itoh et al., 1993b). REKS is distinct from c Raf-1 (Kuroda et al., 1995), and by analogy to the bovine system, REKS is a complex of B-Raf and 14-3-3 proteins (Yamamori et al., 1995). In this cell- and membrane-free system, the ability of Ras to activate REKS is strictly dependent on prenylation (Itoh et al., 1993a). Moreover, farnesylation of H-Ras by itself is sufficient to confer full biological activity, and the isoprenoid structure was found to be moderately important for function (McGeady et al., 1995). While these *in vitro* data clearly argue in favor of a specific role for the isoprenoid group in Ras−effector interaction, the situation *in vivo* is distinctly different. In intact oocytes, Raf-1 appears to be an important downstream effector of Ras (Galaktionov et al., 1995; Fabian et al., 1993). Unlike REKS, Raf-1 activation is thought to involve components of the plasma membrane (Stokoe et al., 1994; Levers et al., 1994; Dent et al., 1995; Marais et al., 1995). Consistent with a predominant role for Raf-1 in the Ras−MAPK pathway in oocytes, the data presented here showing that lipidated H-Ras proteins which are fully capable of activating REKS *in vitro* require further processing before activation of the MAPK cascade can occur *in vivo* support the view that Raf-1 and not REKS is the main MAPK stimulator in *Xenopus* oocytes.

Although Ras−Raf interaction is generally believed to be independent of posttranslational processing, recent studies have suggested a contribution of the farnesyl group in the interaction of the H-Ras "activator domain" with the "cysteine-rich region" of Raf-1, and have provided evidence that this interaction might be critical for Raf activation in cells (Hu et al., 1995). Assuming that this property of mammalian Raf-1 is shared by the *Xenopus* enzyme, our results would suggest a fairly permissive role for the farnesyl group in this interaction.

Our conclusion that the precise structure of the farnesyl group of H-Ras is not critical for the function of this protein is consistent with earlier studies in which H-Ras engineered to bear an N-terminal myristoyl group and lacking a farnesyl group is functional when overexpressed by transfection in mammalian cells (Buss et al., 1989; Willumsen et al., 1996; Cadwallader et al., 1994). Furthermore, myristoylation in the absence of farnesylation allows Ras to bind to membranes. While these studies have been interpreted to mean that the primary function of lipidation is to bring Ras to the plasma membrane, interpretation of such data is complicated by the fact that the addition of an N-myrisoylation sequence to nononcogenic farnesylated or nonfarnesylated Ras leads to cell transformation (Buss et al., 1989). Thus, the addition of an N-myrisoylation sequence probably fundamentally alters the biology of Ras by an effect unrelated to membrane binding. Another factor that compromises the interpreation of the studies with N-myristoylated Ras is the fact that the protein is overexpressed in cells, and thus, one cannot correlate structure and function. Such complications are circumvented in the present study where the role of lipid structure is studied with near-physiological concentrations of H-Ras and without altering the primary structure of the polypeptide.

While our observations favor a model where the farnesyl group of the fully matured H-Ras protein simply serves as a hydrophobic membrane-binding element, it is also conceivable that the lipid at the farnesylation site plays a structural role for Ras itself without requiring the precise farnesyl structure. A more detailed understanding of these interactions will require structural studies of the mature H-Ras protein at the water−phospholipid interface as well as crystallographic analysis of prenylated H-Ras itself and of its complexes with effector molecules.

This is the first report using unnatural lipids to probe the functional role of protein-linked isoprenoids *in vivo*, and the approach described here should become a useful tool for studying structure−function relationships of protein-linked lipids in intact cells. The data presented here suggest that the H-Ras farnesyl group primarily serves as a hydrophobic element rendering Ras accessible to downstream processing enzymes in a process which ultimately culminates in H-Ras membrane localization. While the nature of the lipid at the farnesylation site can affect further processing, its precise structure appears to have little effect on the ability of H-Ras to activate effector pathways once it is membrane-localized, which argues against a specific recognition of the farnesyl group by effector molecules at a rate-limiting step of the Ras−MAPK cascade in oocytes.

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