Journal of Cellular Biochemistry 97:412–422 (2006)

Prenylation is Not Necessary for Endogenous Ras Activation in Non-Malignant Cells

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Abstract Ras monomeric GTPases are pivotal to many core cellular processes such as proliferation and differentiation. The post-translational prenylation of Ras with a farnesyl or a geranylgeranyl moiety is thought to be critical for its membrane binding and consequent signaling activity. Inhibitors of Ras prenylation have an anti-proliferative effect in some Ras-transformed cells. We present a study of the effects of prenylation inhibitors on endogenous, wild-type Ras in three renal cell types, namely primary adult human renal fibroblasts, primary adult human mesangial cells, and a primate renal fibroblast cell line (Vero cells). We have previously demonstrated that Ras is necessary for normal proliferation in these cells. Here we show that Ras is farnesylated and not geranylgeranylated in all three cell types. Furthermore, inhibiting Ras farnesylation has no effect on cell proliferation or Ras activation. Although inhibiting geranylgeranylation in these cells does inhibit proliferation, this is through an Ras-independent mechanism. Non-prenylated Ras is able to localize to the plasma membrane, bind Raf when cells are stimulated by epidermal growth factor or platelet-derived growth factor, and activate the Ras downstream effectors mitogen-activated protein kinase and phosphotidylinositol 3-kinase. We conclude that in wild-type cells, endogenous Ras does not need to be prenylated to be active. J. Cell. Biochem. 97: 412–422, 2006.

Key words: prenylation; Ras monomeric GTPases; Ras isoforms; endogenous; cell proliferation

Ras monomeric GTPases are a family of guanine-nucleotide binding proteins that play a critical role in regulating cell proliferation and differentiation [Barbacid, 1987]. Ras proteins function as molecular switches and when activated, Ras cycles from an inactive GDP-bound form to an active GTP-bound form. *Ras* genes were first identified as the transforming element of the Harvey and Kirsten strains of the rat sarcoma viruses in the 1970's and in man Ras exists as three closely related isoforms known as Harvey (Ha)-, Kirsten (Ki)-, and Neural (N)-Ras. Mutated Ras is now the most frequently detected oncogene in human cancers [Bos, 1988].

Ras molecules require three or four steps of post-translational modification to create the hydrophobic domains, which enable membrane binding, a step thought to be necessary for Ras to function as a signaling molecule. The first step, prenylation, involves the addition of a farnesyl group in the cytosol and is catalyzed by farnesyl transferase (FTase). The following three stages of terminal amino acid cleavage, methylation, and palmitoylation occur in the endoplasmic reticulum and Golgi apparatus [Apolloni et al., 2000]. Ki(4B)-Ras alone does not undergo this last stage but has, instead, a polybasic-domain consisting of a chain of six lysine residues, which act as a membrane targeting sequence.

Given the importance of *ras* oncogenes in human carcinogenesis, several approaches have been developed to target Ras-signaling aiming to reduce the altered gene product or to eliminate its biological function [Hill et al.,

Grant sponsor: Department of Health Clinician Scientist; Grant number: DHCS/G121/33; Grant sponsor: National Kidney research Fund; Grant number: TF5/99; Grant sponsor: Medical Research Council; Grant number: G84/5721.

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Received 14 June 2005; Accepted 2 August 2005
DOI 10.1002/jcb.20641

2000; Scharovsky et al., 2000]. Much of this work over the last 10 years has focused on the use of inhibitors of farnesylation of oncogenic Ras [Gibbs et al., 1994; Hill et al., 2000; Prendergast, 2000] and many of these compounds are in late clinical trials for the treatment of human cancers [Downward, 2003]. These farnesyl transferase inhibitors (FTI) have been shown to have potent effects on malignant transformation in a number of murine models and human tumor cell lines, in particular those transformed by oncogenic Ha-Ras [Sinensky, 2000]. However, an FTI can block tumor growth even in the absence of an activating Ras mutation [Nagasu et al., 1995; Sepp-Lorenzino et al., 1995]. Furthermore, despite the fact that oncogenic Ki(4B)-Ras can be geranylgeranylated, FTI could still inhibit cell proliferation in vitro and in vivo without suppressing Ki(4B)-Ras prenylation [Lerner et al., 1997; Sun et al., 1998]. Data from clinical trials have shown that FTIs appear to show little activity in a range of solid tumors, whilst demonstrating efficacy in a number of hematological malignancies [Downward, 2003]. Again the sensitivity of a particular tumor cannot be predicted by the presence or absence of a *ras* oncogene suggesting that a protein(s) other than Ras is the target of these drugs [Sebti and Adjei, 2004].

Non-malignant cells appear to be relatively resistant to the effects of farnesyl transferase inhibition and this has been assumed to be due to the 'alternative' geranylgeranylation of Ras [Suzuki et al., 1998; Prendergast, 2000]. However, little work has been done to study why potent inhibitors of Ras prenylation fail to disrupt endogenous, wild-type Ras-dependent pathways in normal, non-malignant cells. We and others have previously demonstrated that Ras, in particular Ha- and Ki(4B)-Ras expression is necessary for the stimulated proliferation of normal human fibroblasts [Chen et al., 1996; Sharpe et al., 1999, 2000]. Here, we present the first evidence to show that in a number of non-malignant, renal cell types, Ras does not require prenylation to be able to bind GTP and hence activate downstream effector pathways.

EXPERIMENTAL PROCEDURES

Cell Culture

Primary culture of adult human renal fibroblasts (HRF) has been described previously [Sharpe et al., 2000]. Briefly, tumor-free renal cortex was obtained from native kidneys all removed for renal cell carcinoma. After removing the capsule, the cortex was minced and digested in a 1 mg/ml solution of collagenase type IV (Sigma), passed through a 70 μm mesh and separated on a 50% percoll density gradient (containing 0.84 g mannitol and 100 μl 1 M HCl in 30 ml) by centrifugation at 30,000*g*. The top band (F1) was seeded onto 10 cm culture dishes (90 mg per dish) in DMEM/Hams F12 Nut-mix with 10% FCS, allowed to grow to confluence then passaged twice in the presence of serum to select for fibroblast growth. Cryopreserved normal adult human mesangial cells (HMC) were obtained from Clonetics at passage 3 (CC-2559) and stored in liquid nitrogen. Prior to first use, the cells were rapidly thawed at 37°C, suspended in medium, and plated into 75cm^2 tissue culture flasks. The standard growth medium was RPMI 1640 medium (Gibco) supplemented with insulin-transferrin-sodium selenite (5 μg/ml), penicillin (100 IU/ml), streptomycin (100 μg/ml), amphotericin (2.5 μg/ml), and with 10% fetal calf serum (FCS). Vero cells (primate renal fibroblasts) were obtained from ECACC (no. 84113001). For routine subculture, the cells were split 1:20 when confluent. The standard growth medium was DMEM (Gibco) supplemented with 10% FCS, penicillin (100 IU/ml), and streptomycin (100 μg/ml).

Immunoprecipitation and Western Blotting

Cells were grown to 50–80% confluence in 35 or 100 mm dishes in 10% FCS or stimulated as described in figure legends. Cells were then lysed in PBSTDS (1 × phosphate buffered saline, 1% Triton-X 100, 0.5% deoxycholate, 0.1% SDS, leupeptin 0.5 μg/ml, pepstatin 1.0 μg/ml, EDTA 1.0 mM, PMSF 0.2 mM). Cell lysates were assayed for protein content and equal amounts of protein were made up to a uniform volume. Immunoprecipitation and Western blotting for Ras was performed as described previously [Sharpe et al., 1999]. To assess downstream effector activation HMC were serum-starved then lysed or stimulated with PDGF 200 nM for 5 min prior to lysis. Ten micrograms of cell lysate was then analyzed by SDS–PAGE and Western blotting using either phospho-specific antibodies for P-Akt and P-MAPK (p42/44) or antibodies specific for total Akt or MAPK (p42/44) (all from New England Biolabs).

Triton X-114 Partitioning

Treated cells were washed twice in phosphate buffered saline (PBS) then lysed in 1 ml of an ice cold solution of 1% Triton X-114 in tris buffered saline (TBS) containing protease inhibitors. Cells were scraped from the culture dish and the lysate was transferred to a cold microcentrifuge tube, vortexed, and incubated on ice for 15 min. The lysate was then clarified by centrifugation at 14,000*g* at 4°C for 5 min to remove insoluble debris. The supernatant was harvested to a fresh tube and incubated at 37°C for 2 min then centrifuged for 2 min at room temperature to separate the detergent from the aqueous phase. The top, aqueous phase was removed and collected leaving a thin interface between the two phases. The interface was then removed leaving a pure detergent phase. The aqueous portion was made up to 1 ml with 100 μl of 10% triton X-114 and the detergent portion was made up to 1 ml with TBS. Both samples were then immunoprecipitated for total Ras and analyzed with SDS–PAGE and Western blotting. For experiments in which Triton X-114 partitioning was followed by the Ras/Raf affinity, pull-down assay, the Triton X-114 lysis buffer was made up in magnesium containing lysis buffer (1% Triton X-114, 25 mM HEPES pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 25 mM Na Fluoride, 1 mM EDTA, 1 mM Na Vanadate, 10 μg/ml Leupeptin, 10 μg/ml Aprotonin) rather than TBS.

Inhibition of Ras Prenylation

Enzyme specific inhibitors of farnesyl transferase (FTI 277 and A197574) and geranylgeranyl transferase I (GGTI 298) were used. FTI 277 and GGTI 298 were a kind gift from S.M. Sebti, University of Florida, and A197574 was a kind gift from Abbott Pharmaceuticals. All inhibitors were dissolved in 10-mM dithiothreitol in DMSO in a stock concentration of 10 mM which was further diluted directly in culture medium.

Membrane and Cytosol Preparations

Treated cells were grown to confluence in 75-cm^2 tissue culture flasks, trypsinized and pelleted by centrifugation at 300*g* at 4°C. The pellet was then resuspended in 1 ml of ice-cold hypotonic buffer (10 mM Tris pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF), which was then sonicated for two 15 s cycles. The cell suspension was then centrifuged at 800*g* to remove cell debris after which the supernatant was spun at 100,000*g* for 60 min at 4°C to separate membrane (pellet) and cytosol (supernatant) fractions. The membrane pellet was resuspended in 1 ml of ice-cold hypotonic buffer and both fractions were made up to 2 ml with 1 ml of 2 × PBSTDS lysis buffer and put on ice for 30 min. Total Ras was immunoprecipitated from equal volumes of paired samples and analyzed by SDS–PAGE and Western blotting.

Cell Proliferation Assays

Cell numbers were determined by MTS assay (Promega, Cell Titre 96^{TM}) measuring absorbance at 490 nm. This is a tetrazolium salt-based assay of viable cell number and the correlation between cell number and absorbance at 490 nm is linear [Cory et al., 1991]. For this assay, HFB were seeded at 5,000 cells per well in either epidermal growth factor (1 μg/ml) or 10% FCS in a 96-well plate and treated with increasing concentrations of FTI 277. Cell number was assessed daily for 5 days (0–96 h). Additionally, HMC were treated with either 10% FCS or platelet derived growth factor (PDGF) at 200 nM in 100 mm dishes plus A197574 at 0, 50, or 100 nM for 24 h. Cells were then incubated with 10 μM bromodeoxyuridine (BrdU) and incubated for a further 16 h. Cells were then fixed in a solution containing three parts 50 mM glycine pH 2.0 and seven parts absolute alcohol for 45 min, washed in PBS 3 times, then incubated in 4M HCl for 10 min, washed 5 times in PBS, and blocked in a solution containing 5% goat serum and 0.05% Tween 20 in PBS for 15 min. Primary anti-BrdU antibody (Sigma) was then added at a dilution of 1:100 in blocking solution and the cells were incubated at 4°C overnight. After washing, cells were incubated with the TRITC-conjugated anti-mouse IgG secondary antibody (Sigma) at a dilution of 1:50 for 30 min. BrdU + ve cells were compared to total cell number in four low power fields for each condition.

Ras/Raf Affinity Pull-Down Assay

Cells were grown to 50–70% confluence then serum-starved for 24 h with or without an FTI. Cells were then lysed or stimulated with either EGF (0.5 μg/ml or PDGF 200 nM) for 5 min prior to lysis in the appropriate lysis buffer. Activated, GTP-bound Ras was precipitated

using the Raf-RBD-GST reagent (Upstate, UK), which contains the Ras binding domain of Raf linked to sepharose beads. The beads were pelleted, washed and the protein analyzed by SDS–PAGE and Western blotting.

RESULTS

Ras Exists in Both a Prenylated and Non-Prenylated Form in Normal Cells

Ras proteins are small, soluble molecules that become hydrophobic with the addition of the lipid prenyl moiety thus allowing them to associate with the plasma membrane. Not all Ras within a cell is in the modified state as can be demonstrated by Western blotting. The non-prenylated Ras runs slower on SDS–PAGE and can be distinguished from the faster running prenylated form (Fig. 1). Figure 1 demonstrates separation of the hydrophobic, prenylated Ras protein from the hydrophilic non-prenylated Ras protein using the temperature-dependent triton X-114 detergent by separating the aqueous from detergent phase in (A) Vero cells and (B) mesangial cells.

In Renal Fibroblasts and Mesangial Cells Ras is Farnesylated and Not Geranylgeranylated

The most effective and specific anti-prenylation drugs are peptidomimetic analogs of the specific CAAX box motifs [James et al., 1993]. They act as substrates for farnesyl transferase (FTase) or geranylgeranyl transferase I (GGTase I) and competitively inhibit these enzymes. For this study, FTI 277 and A197574 were used as farnesyl transferase inhibitors (FTI) and GGTI 298 as a geranylgeranyl transferase I inhibitor (GGTI). These are potent and highly specific for their target enzymes and are effective in both cells and cell-free biochemical systems [Hill et al., 2000]. When cells are treated with an FTI, Ras is shifted from the prenylated form to the non-prenylated form. This does not occur with GGTI 298 (Fig. 2). Separation of hydrophobic Ras from hydrophilic Ras in cells pre-treated with the prenylation inhibitors (Fig. 2D) also confirms that only an inhibitor of farnesylation can shift Ras to the aqueous phase, whereas inhibiting geranylgeranylation leaves Ras almost exclusively in the hydrophobic state. GGTI 298 was confirmed to effectively inhibit geranylgeranylation in HMC at 10 and 20 μM by shifting Rap1A from the prenylated form to the non-prenylated form in SDS–PAGE, a protein known to be exclusively geranylgeranylated (Fig. 2E). No effect on Rap1A processing was noted using the farnesyltransferase inhibitor (data not shown).

Inhibiting Ras Farnesylation Results in Incomplete Shift of Ras From the Membrane Fraction to the Cytosol Fraction

Ras localization to the plasma membrane is thought to be fundamental to its ability to receive upstream activating signals and to transduce these to downstream effectors. The prenylation of Ras assists in this membrane targeting by making the molecule hydrophobic. In Figure 3, we demonstrate that inhibiting the farnesylation of Ras in HMC shifts the majority of Ras from the membrane fraction to the cytosolic fraction. However, the membrane fraction in untreated cells contains both prenylated and non-prenylated Ras although the prenylated band predominates. When the FTI was added, the ratio of non-prenylated to prenylated Ras in the membrane fraction increased so that there was more non-prenylated Ras, though total Ras in the membrane was reduced.

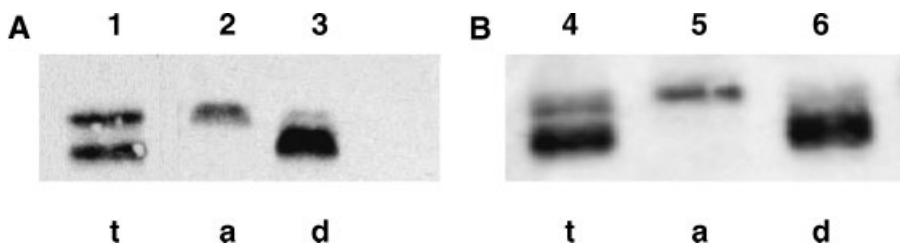

Fig. 1. Triton X-114 extraction of Ras. Vero cells (**A**) lane 1 and mesangial cells (**B**) lane 4 were lysed in normal PBSTDS lysis buffer and total cell Ras was immunoprecipitated (t) demonstrating the characteristic double band on Western blotting. In lanes (A) 2 and 3 and (B) 5 and 6, cells were lysed in Triton X-114 and the aqueous (a) and detergent (d) phases were separated prior to immunoprecipitation and Western blot analysis. The upper, non-prenylated band appeared in the aqueous phase and the lower, prenylated band appeared only in the detergent phase. This experiment is representative of three similar experiments.

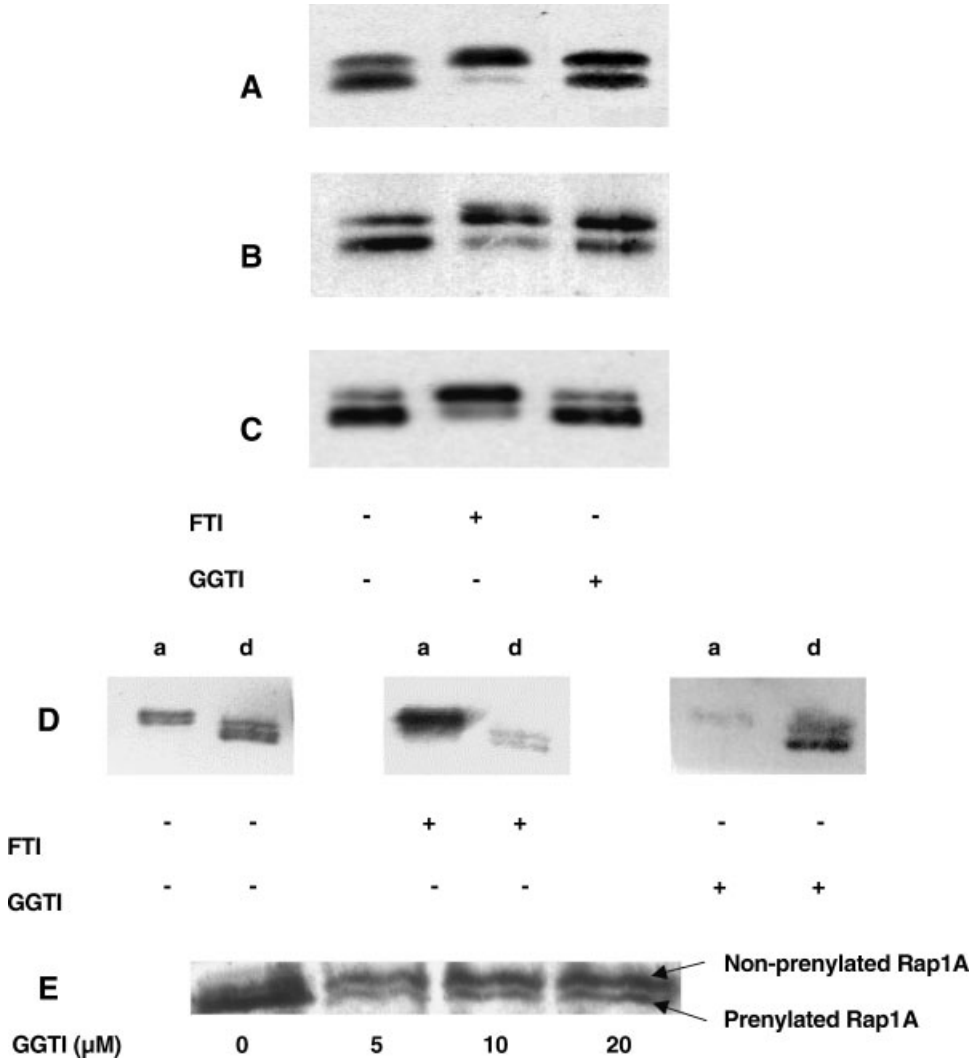

Fig. 2. Effects of prenylation inhibitors on Ras modification. **A**: Vero cells, (**B**) human renal fibroblasts, and (**C**) mesangial cells were grown to approximately 70% confluence and incubated with the prenylation inhibitors FTI (FTI 277 5 μM (A and B), A197547 100nM (C)) or GGTI 298 (20 μM) for 24 h. The cells were then lysed and total Ras was immunoprecipitated from equalized amounts of total protein. This was analyzed by SDS–PAGE and Western blotting. These blots are representative of at least three similar experiments for each cell type. **D**: Vero cells were incubated with FTI 277 (10 μM) or GGTI 298 (20 μM) then lysed in triton X-114. The aqueous (a) and detergent (d) phases were separated. **E**: Mesangial cells were grown to approximately 70% confluence and incubated with GGTI 298 (at the concentrations indicated) for 24 h. The cells were then lysed and equal amounts of total protein were analyzed for Rap1A expression by SDS–PAGE and Western blotting. Prenylated and non-prenylated forms are indicated.

Inhibiting Ras Farnesylation Does not Inhibit Cell Proliferation

We have previously demonstrated that Ki(4B)-Ras is the predominantly expressed isoform in both Vero cells and human renal fibroblasts [Sharpe et al., 1999, 2000]. Silencing Ki(4B)-Ras in Vero cells and either Ki(4B)-Ras or Ha-Ras in human renal fibroblasts significantly inhibited proliferation demonstrating that Ras is necessary for stimulated proliferation in these cells. Here we demonstrate however that in marked contrast to the Western blot data, inhibiting farnesylation of Ras has no effect on cell proliferation in either HRF, Vero cells or HMC as assessed by a viable cell number assay and BrdU incorporation (Fig. 4A, C, and E and 5). GGTI 298, however, caused a dose-dependent reduction in cell number in both HRF and Vero cells using the MTS assay (Fig. 4B, D, and F) and in BrdU incorporation in HMCs (data not shown). This anti-proliferative

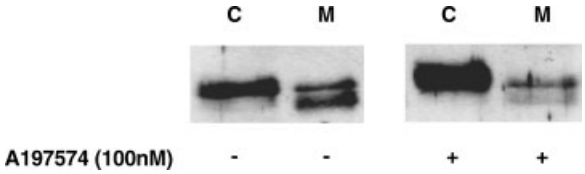

Fig. 3. Effects of prenylation inhibitors on Ras sub-cellular localization. Cells were grown to 80% confluence then either treated with 10% FCS alone or A197547 100nM in the presence of 10% FCS for 24 h. The cells were lysed and separated into membrane (M) and cytosolic (C) fractions as described in experimental procedures. Total Ras was immunoprecipitated from equal volumes of paired samples and analyzed by SDS–PAGE and Western blotting.

effect is apparent at doses shown to have no effect on Ras prenylation.

Both Prenylated and Non-Prenylated Ras Can Bind Raf

In keeping with the above finding, we have demonstrated that activated, GTP-bound Ras can exist in the cell in both the prenylated and non-prenylated forms. Figure 6A shows a Western blot of fibroblasts that have been serum-starved for 24 h then stimulated for 5 min with EGF. The resultant activated GTP-bound fraction of Ras was then "pulled down" using the Ras binding domain of Raf linked to sepharose beads. The GTP-bound Ras can clearly be seen as a double band that is in both its prenylated and non-prenylated states. This was confirmed in human mesangial cells by pre-treating them with an FTI and shifting the activated Ras from the lower, prenylated band to the upper non-prenylated band. Pre-treatment with GGTI 298 did not cause this shift in the activated Ras band nor did it alter the result when used in addition to the FTI (Fig. 6B). In order to further demonstrate this phenomenon, primary human fibroblasts were serum-starved for 24 h then either left unstimulated or stimulated with EGF for 5 min. These cells were then lysed in 1% triton X-114/magnesium containing lysis buffer and the aqueous and detergent phases were separated. Only the active, GTP-bound Ras was then recovered by the RBD-sepharose compound from each of these phases. Figure 7 illustrates that GTP-bound Ras can be pulled down from both the aqueous and detergent phases again suggesting that Ras does not need to be prenylated to be activated.

Inhibiting Ras Farnesylation Does not Inhibit Activation of Downstream Effectors

To assess whether this activation was transferred to downstream effectors, human mesangial cells were serum-starved and pre-treated with an FTI for 24 h. The cells were then either lysed or treated with PDGF prior to lysis and immunoblotted for phospho-Akt/total Akt and phospho-MAPK 41/42/total MAPK 41/42. Figure 8 demonstrates that inhibition of farnesylation has no effect on the activation of these two Ras downstream effectors.

DISCUSSION

These results demonstrate that in primary human renal mesangial cells and fibroblasts and in Vero cells, Ras undergoes farnesylation rather than geranylgeranylation. Moreover, it is apparent from our findings that in these non-malignant renal cells, inhibition of Ras farnesylation does not inhibit Ras-dependent processes or signaling. In neither HRF, Vero cells, nor HMC did an FTI affect cell proliferation at doses known to inhibit Ras farnesylation. Additionally, Ras can be shown to bind the Ras binding domain of Raf and therefore be activated whether prenylated or not. Equally, activation of Ras downstream effector molecules was not influenced by inhibition of Ras farnesylation. In contrast, inhibition of geranylgeranylation had no effect on Ras processing or activation but it did inhibit cell proliferation in all cell types in a dose-dependent manner. Although different cell types have been illustrated in different experiments, no discernable difference in results was noted between any of the cells types described for any of the different experimental procedures.

We have previously shown the Ki(4B)-Ras isoform is the predominantly expressed isoform in HRF, Vero cells [Sharpe et al., 1999, 2000] and HMC (unpublished data). Therefore, the inhibition of prenylation of total cellular Ras by an FTI but not by a GGTI as shown in Figure 1, suggests that Ki(4B)-Ras undergoes farnesylation in all three cell types. Previous studies have shown that over-expressed wild-type or oncogenic Ki(4B)-Ras can be geranylgeranylated if farnesyl transferase is inhibited [Lerner et al., 1995; Osman et al., 1997; Fiordalisi et al., 2003] and this has been used to explain the lack of effect of FTIs on Ras-dependent signaling in some non-malignant cells. Whilst this may also

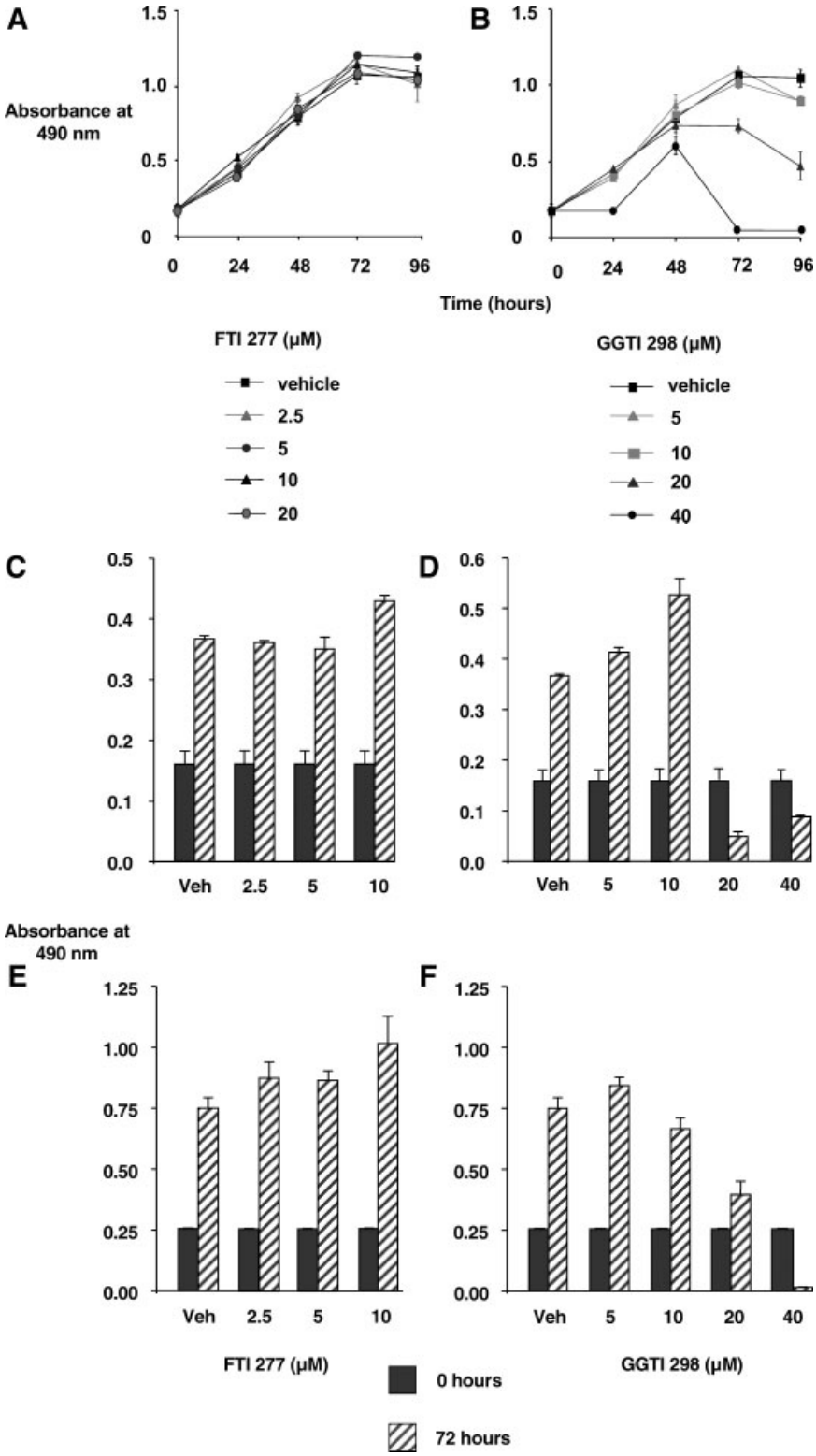

Fig. 4. Effects of inhibiting prenylation on cell proliferation. HRF (A and B) were grown in the presence of 10% FCS and either vehicle or increasing doses of FTI 277 (**A**) or GGTI 298 (**B**). Proliferation of viable cells against time is directly correlated with absorbance at 490 nm using the MTS assay. HRF (**C** and **D**) and Vero cells (**E** and **F**) were grown for 72 h in the presence of EGF (1 μg/ml) and either FTI 277 or GGTI 298 in the concentrations indicated. Cell number was assessed using the MTS assay at time 0 and 72 h (n = 3).

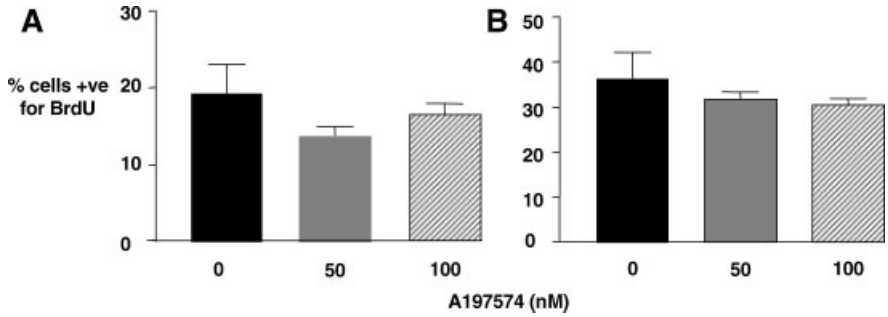

Fig. 5. Effects of inhibiting farnesylation on BrdU incorporation. HMC were grown in the presence of increasing doses of A197547 for 40 h and either PDGF (200ng/ml) (**A**) or 10% FCS (**B**). Cell proliferation was assessed by BrdU incorporation (n = 3–6).

be the case in our cell types, it is clear that after FTI treatment Ki(4B)-Ras predominantly exists in its non-prenylated form. Were Ki(4B)-Ras to undergo significant geranylgeranylation one would still expect to see a 'double-band' on SDS–PAGE as geranylgeranylated Ras has greater electrophoretic mobility than non-prenylated Ras [Cox et al., 1995]. Furthermore, the addition of a GGTI to an FTI does not appear to further increase the ratio of non-prenylated Ras to prenylated Ras in the activated Ras pull-down assay in Figure 6B. The studies that suggest that Ki(4B)-Ras can be geranylgeranylated have demonstrated this either in cells over-expressing Ki(4B)-Ras or in cell-free systems and so this may in fact be an artifact of experimental design and not truly reflect what occurs with endogenous Ras in the wild-type cell.

Whilst Ki(4B)-Ras may act as a substrate for GGTase I, Ha-Ras does not [James et al., 1995]. Furthermore, Ki(4B)-Ras also exhibits a 50-fold higher affinity for FTase than Ha-Ras and, therefore, Ha-Ras is much more sensitive to the effects of an FTI than Ki(4B)-Ras [James et al., 1995]. Thus, if the farnesylation of Ki(4B)-Ras is effectively inhibited then the farnesylation of any Ha-Ras present in these cells will also have been inhibited. Using antisense oligonucleotides, we have previously demonstrated that Ha-Ras is necessary for normal cell proliferation in HRF [Sharpe et al., 2000] and HMC (unpublished data). Taking these findings together one would

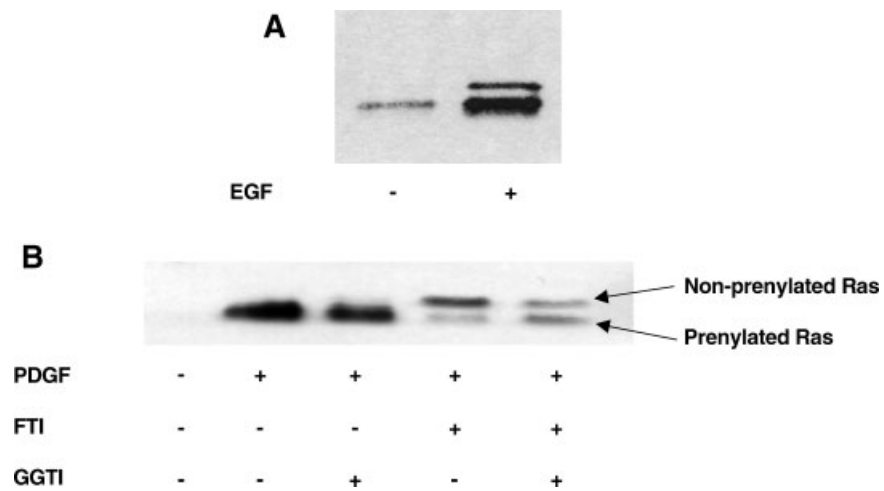

Fig. 6. Ras activation assay on stimulated cells. **A**: Vero cells were grown to 50% confluence then serum-starved for 24 h. They were then either lysed or stimulated with EGF (1 μg/ml) for 5 min prior to lysis. GTP bound Ras was immediately precipitated with 15 μl of a 50% slurry of the Ras binding domain of Raf (RBD)-GST-sepharose. The precipitated protein was analyzed by SDS–PAGE and Western blotting. This is representative of four similar experiments. **B**: HMC were grown to 70% confluence then serum-starved for 24 h. Cells were then incubated with A197547 (100 nM), GGTI 298 (20 μM) or both compounds in serum-free media for a further 24 h then either lysed or stimulated with PDGF (200 ng/ml) for 5 min prior to lysis. Active GTP-bound Ras was recovered using RBD-GST-sepharose, prior to analysis by SDS–PAGE and Western blotting.

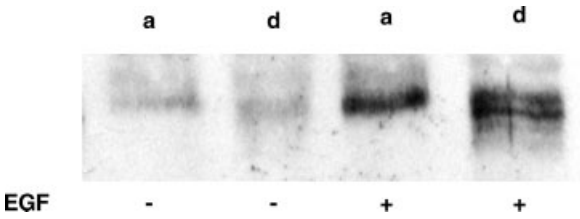

Fig. 7. GTP-Ras Pull-down on T-X114 extracted cell lysates. HFB were grown to 70% confluence, serum-starved for 24 h, and either unstimulated or stimulated with EGF 0.5 μg/ml for 5 min. Cells were then lysed in Triton X-114 and the aqueous (a) and detergent (d) phases were separated prior to affinity precipitation with 15 μl of a 50% slurry of the RBD-GST-sepharose. The results were analyzed by Western blotting. This is representative of three similar experiments.

predict that were prenylation necessary for Ras to function, then inhibiting the farnesylation of Ha-Ras with an FTI should inhibit cell proliferation.

The observation that inhibiting geranylgeranylation in cells inhibits proliferation is not new and has previously been attributed to the inhibition of Rho proteins. Vogt et al. [1996, 1997] have previously shown that GGTI 298 inhibits cell proliferation in both NIH3T3 mouse fibroblasts and numerous human tumor cell lines. This inhibition of proliferation was associated with blocking of the cell cycle in G1, thereby, preventing transition to S phase. A similar effect was not found with FTI 277. Hirai et al. noticed a similar effect when using pravastatin, a HMG CoA reductase inhibitor, on rat thyroid (FRTL-5) cells. This drug caused cell cycle arrest in the G1 phase and prevented the Rho-dependent elimination of the cyclin-dependent kinase inhibitor, $p27^{Kip1}$ normally associated with the transition to S phase. This effect was reversed with geranylgeranylpyrophosphate but not farnesylpyrophosphate. The reversal was prevented by the addition of Rho inhibitor, C3 transferase [Hirai et al., 1997].

The role of Ras farnesylation on membrane targeting has also been examined here (Fig. 3). Whilst it is apparent that inhibiting farnesylation prevents the bulk of intracellular Ras localizing to the membrane fraction, some non-prenylated Ras is able to associate with the membrane. This could occur through a number of mechanisms. It can be seen that the inhibition of farnesylation with an FTI is incomplete in as much as a small amount of Ras remains in the prenylated state. It is possible that this membrane bound Ras is able to sequester non-prenylated Ras to the membrane via a process of dimerization, a phenomenon which has previously been described [Inouye et al., 2000]. However, in our results, when farnesylation is inhibited there appears to be more non-prenylated than prenylated Ras in the plasma membrane, a situation that cannot be explained by dimerization alone. Furthermore, using the pull-down assay for Ras-GTP after FTI treatment, the prenylated and non-prenylated forms of Ras were not of equal intensity, again suggesting that Ras dimerization is not the only mechanism by which non-prenylated Ras can become activated.

Alternatively, the mechanism of the interaction of non-prenylated Ras with the plasma membrane may differ for the different isoforms. Ha-Ras has been shown by a number of groups to bind to the plasma membrane via interaction of its 'linker' region in the hypervariable domain with caveolin-1; even when not prenylated

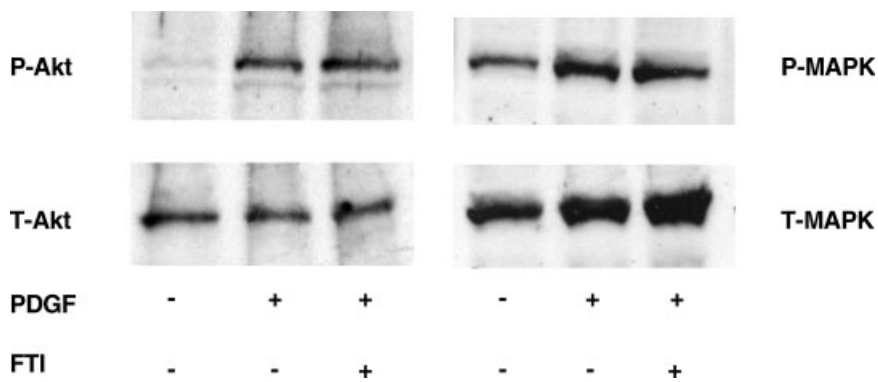

Fig. 8. Effects of an FTI on Ras downstream effector activation. HMC were grown to 70% confluence and serum-starved for 48 h ± A197547 (100 nM). Cells were then lysed or stimulated with PDGF (200 nM) for 5 min prior to lysis. Western blot was then performed for phospho-Akt (P-Akt) and total Akt (T-Akt) and phospho-MAPK (P-MAPK) and total MAPK (T-MAPK).

[Song et al., 1996; Roy et al., 1998; Prior et al., 2001]. Prior et al. also found Ki(4B)-Ras to localize to different regions of the disordered plasma membrane by virtue of its polybasic domain [Prior and Hancock, 2001].

One can also speculate that Ras can localize to different intracellular 'compartments' and that prenylation may not be necessary for Ras to signal from these compartments. Recent evidence to support such a model of Ras signaling comes from work showing that a sub-fraction of Ras, which is localized to the Golgi as opposed to the plasma membrane can be activated by translocation of Ras GRP1 in a phospholipase Cγ1-dependent manner [Bivona et al., 2003]. Alternatively, Rocks et al. have elegantly demonstrated recently that Ha- and N-Ras localization to the plasma membrane is in fact mediated by acylation [Rocks et al., 2005]. They have shown that the addition of one or two palmitoyl groups directs Ha- and N-Ras from the Golgi to the plasma membrane. Once in the plasma membrane however, these Ras isoforms undergo depalmitoylation, which rapidly dislodges Ras from the plasma membrane despite the presence of a farnesyl group. The farnesylated but deacylated Ras quickly reaches equilibrium between the cytosol and cell membranes. That fraction of deacylated Ras, which returns to the Golgi becomes palmitoylated again and is redirected back to the plasma membrane. In this way Ras cycles between the plasma membrane and the Golgi allowing the activated, GTP bound form to translocate to the Golgi and signal from there. Although these studies were not undertaken with Ras lacking a prenylation site, they do suggest that palmitoylation rather than prenylation targets Ha and N-Ras to the plasma membrane and may indeed be sufficient to do so in the absence of prenylation. This may also hold true for the polybasic domain in Ki(4B)-Ras which, being a permanent feature, will ensure that a certain fraction of Ki(4B)-Ras will remain in the plasma membrane allowing activation to take place even when prenylation is inhibited.

Taking these findings together, it can be concluded that endogenous, wild-type Ras is able to function in its prenylated or non-prenylated form. This may not be the case in cells that are immortalized by over-expression of constitutively active Ras whose cellular biology differs greatly from the wild-type phenotype. This phenomenon should be taken into consideration when extrapolations are made from results obtained from malignant, over-expressing cells to explain normal cell signaling in non-malignant primary cells and indeed from one species to another.

ACKNOWLEDGMENTS

We thank the Department of Urology at King's College Hospital for their assistance in providing nephrectomy specimens. We further thank the National Kidney Research Fund and the Department of Health for funding CCS and the Medical Research Council for funding AK.

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