We recently identified a prenyl peptide-binding protein in microsomal membranes from bovine brain (Thissen, J. A., and Casey, P. J. (1993) J. Biol. Chem. 268, 13780–13783). Through a variety of approaches, this binding protein has been identified as the cytoskeletal protein tubulin. Prenyl peptides bind to purified tubulin with a $K_d$ of 40 nM and also bind to tubulin polymerized into microtubules. Microtubule affinity chromatography of extracts from cells in which the prenyl protein pool was metabolically labeled revealed that prenyl proteins bound to the immobilized microtubules; one, a 24-kDa protein, was tentatively identified as a GTP-binding protein. Of several prenylated GTP-binding proteins tested, including Ki-Ras4B, Ha-Ras, RhoB, RhoA, and Rap1B, only Ki-Ras was found to bind significantly to microtubules, and this was in a prenylation-dependent fashion. A potential significance of the interaction of Ki-Ras4B with microtubules was indicated from analysis of the localization of newly synthesized Ki-Ras4B and Ha-Ras, each tagged with green fluorescence protein (GFP). Treatment of NIH-3T3 cells expressing GFP-Ki-Ras with Taxol (paclitaxel) resulted in accumulation of the expressed protein in intracellular locations, whereas in control cells the protein was correctly targeted to the plasma membrane. Importantly, such treatment with paclitaxel did not affect the cellular localization of expressed GFP-Ha-Ras. These results indicate that an intact microtubule network may be directly involved in Ki-Ras processing and/or targeting and provide direct evidence for a physiological distinction between Ki-Ras and Ha-Ras in cells. Additionally, the finding that paclitaxel treatment of cells disrupts Ki-Ras trafficking suggests an additional mechanism for the anti-proliferative effects of this drug.

Post-translational addition of isoprenoid lipids via a process termed prenylation is important in the maturation of many proteins that play critical roles in signal transduction and cell growth regulation. In many cases, a requirement for the attached isoprenoid for correct cellular localization and function has been demonstrated (for reviews, see Refs. 1–3). Most prenylation events involve addition of either a 15-carbon farnesyl or 20-carbon geranylgeranyl isoprenoid to a conserved Cys residue in a C-terminal sequence termed a “CAAX motif.” The X residue of the CAAX motif generally specifies which isoprenoid is to be attached to the protein by one of two cytosolic protein prenyltransferases, designated protein farnesyltransferase and protein geranylgeranyltransferase type I (4–6). The addition of the isoprenoid lipid is followed by the proteolytic removal of the three C-terminal amino acids (the AAX) and methylation of the now C-terminal prenylated Cys residue in a process generally thought to occur in the microsomal membrane compartment of cells (7–10). In addition to this sequence of protein modifications, many prenyl proteins contain either an upstream polybasic region or an attached palmitoyl lipid near the C terminus that influences membrane binding (11, 12).

Several studies implicate prenylated proteins in regulation of cytoskeletal events. Two members of the Ras superfamily of G proteins, Rap1B and Rap1A, associate with the cytoskeleton during agonist-stimulated platelet activation (13, 14). The related proteins Rac and Rho are thought to be important regulators of actin filament organization. Evidence to support this hypothesis comes primarily from studies demonstrating that Rac and Rho, respectively, are involved in regulation of growth factor-induced membrane ruffling and assembly of focal adhesion and actin stress fibers in Swiss 3T3 cells (15, 16). Rac1 has also been found to associate with tubulin in the GTP-bound form, but the physiological significance is unknown (17). The role of prenylation in these processes and interactions is unclear; however, association of RHO1, the Saccharomyces cerevisiae equivalent of the mammalian RhoA, with cortical actin patches requires that it be prenylated for this function (18).

There is also precedence in the literature for involvement of prenylated proteins in microtubule-dependent processes. For example, morphological changes induced by oncogenic Ras transformation can be profoundly influenced by vinca alkaloids, which promote microtubule breakdown, and Taxol (paclitaxel), which promotes formation of short bundled microtubules (19–21). In one of these studies, epithelial cells transfected with oncogenic N-Ras exhibited a distinct polarized cell morphology that was abolished by treatment of the cells with either colcemid or paclitaxel (19). Additional evidence for an interaction between prenylated proteins and cellular processes involving microtubules comes from studies using hydroxymethylglutaryl-CoA reductase inhibitors. These inhibitors, such as lovastatin and compactin, ultimately inhibit the synthesis of prenylated proteins and produce distinct changes in cell morphology (22, 23). In one study, the appearance of the morphological changes in epithelial cells induced by compactin was found to coincide with the retraction of the microtubules from the submembrane regions (24). Pretreatment of the cells with colchicine to depolymerize the microtubules.
bules before treatment with compactin prevented these changes, suggesting that intact microtubules were necessary for the compactin-induced morphological changes observed (24).

It is now established that mature prenylated proteins are associated with many different membrane compartments in cells, including plasma and intracellular membranes, and the cytoskeleton (12, 25–27). However, little information is available on how newly prenylated proteins are directed from the cytosol to sites for subsequent processing and, ultimately, to their final destination, and it seems likely that additional factors must be involved in the correct targeting of these proteins. In attempts to identify factors involved in this process, we initiated a search for proteins that bind prenylated peptides encompassing the C termini of known prenylated proteins. This ligand binding approach resulted in the identification of a specific binding site in a microsomal membrane fraction that recognized both farnesyl- and geranylgeranyl-modified peptides (28). The properties and specificity of this binding site were consistent with those expected for a molecule involved in the correct targeting of these proteins. A variety of experimental approaches indicate a physiological significance for the association of a specific prenylated protein, that being Ki-Ras4B with microtubules. The relationship between these findings and specific microtubule-dependent processes and prenyl protein trafficking is discussed.

EXPERIMENTAL PROCEDURES

Materials—[3H]Farnesyldiphosphate ([3H]FPP), 22 Ci/mmole, [3H]Geranylgeranyldiphosphate ([3H]GGPP, 15 Ci/mmole) and [3-32P]GTP (3000 Ci/mmole) were purchased from NEN Life Science Products. [3H]Mevalonolactone ([3H]MVA, 35 Ci/mmole) and unlabeled FPP and GGPP were obtained from American Radiolabeled Chemicals (St. Louis, MO). Peptides were synthesized on a Synergy peptide synthesizer (Applied Biosystems Inc.) and purified by high performance liquid chromatography before use. Activated CH-Sepharose 4B and glutathione-Sepharose 4B were purchased from Pharmacia Biotech Inc. Ni-NTA resin was from Qiagen (Chatsworth, CA). The BS2 cross-linking reagent was from Pierce (Rockford, IL). Trifluoroacetic acid and iodoacetamide were from Aldrich. Affi-Gel 10 resin was from Bio-Rad. Protgel™was from National Diagnostics (Atlanta, GA). Ham’s F-12 medium was from Bio Whittaker (Walkersville, MD), and all other cell culture reagents were from Life Technologies, Inc. Lovastatin was a generous gift from Al Alberts (Merck, Rahway, NJ). Bacterial expression plasmids for Ha-Ras and Ki-Ras4B were gifts from Channing Der (University of North Carolina, Chapel Hill, NC (UNC-Chapel Hill)) and Ana Maria Garcia (Eisai Research Institute, Andover, MA), respectively, and the bacterial expression plasmid for Rap1B was from Guy James (University of Texas Southwestern Medical Center, Dallas, TX). RhöB was provided as a glutathione S-transferase fusion protein by Charles Minkoff (Duke University Medical Center, Durham, NC), and RhöB was provided by Peter Lewowitz (The Wistar Institute, Philadelphia, PA). NIH-3T3 cells stably transfected with Ha-Ras was provided by Adrienne Cox (UNC-Chapel Hill).

Preparation of Bovine Brain Cytosolic and Membrane Fractions—Bovine brains were fractionated into cytosolic microsomal membrane and plasma membrane-enriched fractions by differential centrifugation. Homogenates, prepared as described (28), were centrifuged first at 100,000 × g for 60 min at 4 °C to collect plasma membranes and then at 16,900 × g for 60 min at 4 °C to obtain cytosolic (supernatant) and microsomal membrane (pellet) fractions. Membrane fractions were resuspended at protein concentrations of 1–2 mg/ml in 20 mM Hepes, pH 7.0, 1 mM EDTA and 1 mM dithiothreitol (DTT) (Buffer A) containing a mixture of protease inhibitors (29). Both the cytosolic and microsomal membrane fractions were flash-frozen in liquid nitrogen and stored at −80 °C until use (28).

Enzymatic Prenylation of Peptides and Proteins—The peptide corresponding essentially to the C terminus of a major G protein subunit (sequence: REKKKKA1M) was enzymatically prenylated using [3H]FPP (15–22.5 Ci/mmole) and purified protein farnesyltransferase (28). A typical reaction mixture contained 400 pmole of the peptide, 200 pmole of [3H]FPP, and 200 units of enzyme in 30 μl of 50 mM Tris-HCl, pH 8.0, 20 mM KCl, 5 mM MgCl2, 5 μM CaCl2, and 2 mM DTT (4). The reaction was initiated with enzyme and incubated 20 min at 37 °C, whereupon octylglucoside was added to a final concentration of 0.1% (to stabilize the prenylated peptide) and the reaction allowed to continue for an additional 60 min. The [3H]farnesylated peptide product was purified on a C18 reverse-phase HPLC column (Phenomenex), lyophilized, and stored at −20 °C until use. All GTP-binding proteins were produced in Escherichia coli. Ha-Ras and RhöB were purified as described (4), and glutathione S-transferase-RhoA was purified using glutathione-Sepharose 4B (Pharmacia Biotech Inc.) according to the manufacturer’s instructions, and purified using Ni-NTA resin (Qiagen) according to the manufacturer. Each GTP-binding protein was enzymatically prenylated by the appropriate isoprenoid as described previously (4).

Chemical Prenylation of Peptides—Two different methods were used to chemically prenylate peptides used in this study. The first method provides a yield of ∼30–50% of the prenyl peptide product and was used in the initial part of this study, whereas the second method results in >90% product formation and was used in the latter part of this work. In the first method, peptides were chemically prenylated essentially as described (30), as modified by Robert Deschenes (University of Iowa). Briefly, 0.9 mg of peptide was dissolved in 1.26 ml of H2O; acetonitrile (1:5) on ice, followed by the addition of 210 μl of 0.5 M NaHCO3. The solution was placed under argon, and the prenylation reaction initiated by addition of farnesyl bromide in Me2SO (1.2 mol of farnesyl bromide/ mol of peptide). The reaction was stirred at 4 °C for 3–4 h, and the product was then purified by reverse-phase HPLC, lyophilized, and stored at −20 °C. The second method involved dissolving the peptide and farnesyl bromide at a 1:1 molar ratio in methanol containing 4 M ammonia (31). After a 3-h incubation at 4 °C, the reaction was dried under vacuum and the product purified by HPLC.

Chemical Cross-linking—Chemical cross-linking analysis was performed essentially as described (32). Briefly, bovine brain microsomal membranes or cytosols (each at 12 μg of protein) were incubated with 120–133 nmol of [3H]labeled prenyl peptide in 150 μl of Buffer A plus 0.05% Triton X-100. After a 20-min incubation at 24 °C, BS2 cross-linking reagent was added to the indicated concentration, the incubation continued for 2 min, and the reaction quenched by the addition of 50 mM Tris-Cl, pH 8.0. Protein was precipitated with trichloroacetic acid at 15% final concentration, and the precipitated protein containing the chemically cross-linked prenylated peptide-protein complex was collected by microcentrifugation and washed with ice-cold acetone. Precipitated proteins were then suspended in Laemmli sample buffer and subjected to SDS-PAGE (33), and the resulting gels processed for fluorographic analysis as described (32).

Preparation of Peptide Affinity Columns—A 50-nmol amount of either the farnesylated peptide or the corresponding unprenylated peptide was dissolved 20 mM Hepes, pH 8.0, containing 50% dimethylformamide. To this was added 0.3 mg of activated CH-Sepharose 4B, which had been prepared as per the manufacturer’s instructions. The mixture was incubated at room temperature for 1 h, followed by an overnight incubation with mixing at 4 °C. The resin was then poured into a 2-ml column and extensively washed using alternating pH buffers as described by the manufacturer. The column was stored in 50 mM sodium acetate, pH 4.0, containing 500 mM NaCl, 0.05% Triton X-100, and 0.025% NaN3 at 4 °C until use.

Preparation of Porcine Brain Cytosol and Tubulin—Tubulin was purified from extracts of freshly isolated porcine brain essentially as described (34). Briefly, a porcine brain (220 g) was homogenized at a ratio of 0.5 ml of buffer/g of brain tissue in 100 mM Pipes, pH 6.9, containing 2 mM EGTA and 1 mM MgSO4 (PES buffer), which also contained 1 mM ATP.

1 The abbreviations used are: FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; MVA, mevalonolactone; BS2, bis(sulfosuccinimidyldimaleate); DTT, dithiothreitol; CHO, Chinese hamster ovary; Pipes, 1,4-piperazinediethanesulfonic acid; GFP, green fluorescent protein; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; MAP kinase, mitogen-activated protein kinase.

2 R. Deschenes, personal communication.
and the protease inhibitors noted above. The homogenate was centrifuged at 100,000 × g for 1 h at 4 °C to remove membranes and organelles, and the supernatant diluted 1:1 with PEM buffer containing 60% glycerol and 0.2 mM GTP. After a 45-min incubation at 37 °C to polymerize tubulin, microtubules were collected by centrifugation at 100,000 × g for 45 min at 29 °C. The pellet containing the microtubules was then processed through a second depolymerization-polymerization process to regrow tubulin, microtubules were collected by centrifugation at 100,000 × g for 45 min at 37 °C. The two-cycle purified tubulin was then purified to >99% homogeneity using phosphocellulose chromatography as described (35). Purified tubulin was stored in aliquots at −80 °C until use.

Prenyl Peptide-Tubulin Binding Assays—Purified tubulin (50 ng) was incubated with [3H]-farnesylated peptide (8–600 nM) in 10 mM Hepes, pH 7.0, containing 1 mM EGTA, 0.5 mM MgCl2, 50 mM NaCl, and 0.01% octylglucoside (Buffer B) in a volume of 50 µl. After a 20-min incubation at room temperature, samples were applied to a column containing 1 ml of Sephadex G-50 and washed with 0.5-ml aliquots of Buffer B. Tubulin-bound [3H]-farnesylated peptide eluted in the void fraction and was detected by liquid scintillation spectroscopy. Specific binding was determined by the difference between [3H]-farnesylated peptide bound to tubulin in the absence and presence of 2 µM unlabeled prenyl peptide.

Preparation of Microtubule Affinity Column—Microtubule affinity columns were constructed essentially as described (36). Briefly, tubulin (2–3 mg/ml) in 80 mM Pipes, pH 6.8, 1 mM MgCl2, 1 mM Na2EGTA was assembled into microtubules by adding 1 mM GTP, followed by the stepwise addition of paclitaxel. Immediately before applying the sample to the activated resin, the pH of the solution containing the paclitaxel-stabilized microtubules was adjusted to 7.6 with 3 M KOH and a volume of the paclitaxel-stabilized microtubule solution equivalent to one-half of the resin volume added to the resin. The mixture was left undisturbed overnight at 4 °C to allow coupling, and then washed several column volumes of 50 mM Hepes, pH 7.5, 1 mM EGTA, and 1 mM MgCl2 (Buffer C), containing 10 mM ethanolicamine to block unreacted groups. The resin was then washed exhaustively with Buffer C containing 1 mM DTT and 500 mM KCl. Typical tubulin concentration obtained on the column was 6–9 µM. The column was stored at 4 °C in the assembly buffer containing 10% glycerol, 1 mM DTT, 5 µM paclitaxel, 0.02% sodium azide, and protease inhibitors.

Cell Culture—Stock cultures of the met18b-2 variant of Chinese hamster ovary (CHO) cells (37) were grown in Ham’s F-12 medium containing 100 units/ml penicillin, 100 units/ml streptomycin, and 2 mM glutamine and supplemented with 5% (v/v) fetal calf serum (supplemented Ham’s F-12 medium) at 37 °C in 5% CO2. Cells were seeded at 3 × 106 cells/100-mm dish and grown to about 70% confluence. In preparation for [3H]mevalonic acid labeling, cells were incubated in 5 ml of supplemented F-12 medium containing 30 µM lovastatin for 60–90 min at 37 °C. After this treatment, the medium was replaced by 5 ml of supplemented F-12 medium containing 250 µCi of [3H]mevalonolactone and 20 µM lovastatin and incubated overnight at 37 °C. Cell
from ICN, a polyclonal antibody directed against residues 428–437 of
the β subunit (a gift from M. Rasenick (University of Illinois, Chicago, IL),
and two polyclonal antisera directed against C-terminal sequences
(both gifts from G. Gundersen, Columbia University, New
York, NY; Ref. 39). Antibody binding was detected using the alkaline
phosphatase method (Promega). Immunoblot analysis of Ras proteins
was performed using monoclonal antibody Y13-259 (Santa Cruz
Biotechnology, Inc.).

**GTP-binding Overlay Assay**—GTP binding was determined by an
overlay assay on nitrocellulose essentially as described (40). Proteins
were separated by SDS-PAGE and transferred to nitrocellulose.
The nitrocellulose blot was first washed in 50 mM Tris-Cl, pH 7.5, containing
50 mM MgCl₂ and 0.3% Tween 20 (Buffer D) for 5 min. The washed blot
was then incubated in Buffer D containing 1 μCi/ml [3H-GTP and 10 μM
ATP for 2–3 h. After the incubation, the blot was washed six times for
10 min each in Buffer D containing 5 mM MgCl₂, dried, and exposed to
Fuji RX film with an intensifying screen.

**Isoprenoid Analysis**—Isoprenoids attached to proteins were deter-
mined by HPLC analysis of the methyl iodide cleavage product essen-
tially as described (41, 42). Briefly, samples containing [3H]-labeled pre-
nyl proteins were precipitated with 15% trichloroacetic acid, and the
precipitated proteins were washed extensively with ice-cold acetone.
The acetone-washed pellets were then subjected to trypsin digestion,
followed by methyl iodide cleavage. Released [3H]-labeled isoprenoids
were then extracted and resolved by C₁₈ reverse-phase HPLC.

**Production of mRNA Encoding GFP Ki-Ras Fusion Protein**—Ki-
Ras4B was cloned into vector pHROI by BamHI sites to obtain the
resulting GFP-Ki-Ras fusion construct (43). Ha-Ras was cloned into
vector pHIRO2 by BamHI and XbaI cloning sites. pHIRO2 was con-
structed using pHROI as a template by introducing a S65T mutation
by polymerase chain reaction mutagenesis. The orientation of Ki-Ras and
Ha-Ras and the integrity of the reading frame were verified by
restriction analysis and sequencing of the GFP-Ras fusion constructs.
**In vitro** transcription and RNA processing of these fusion constructs
were performed according to the procedure described in Ref. 43.

**Electroporation and Confocal Imaging of Adherent NIH-3T3 Cells**—
NIH-3T3 cells were grown as described above. Cells were harvested and
plated on glass coverslips at least 5 h before each experiment. Cover-
slips were washed three times with an extracellular buffer (5 mM KCl,
125 mM NaCl, 20 mM Hepes, 1.5 mM CaCl₂, 1.5 mM MgCl₂, pH 7.4, and
10 mM glucose) and treated with 3 μl maclactaxel or 0.1% Me₂SO for 2 h.
The GFP, GFP-Ki-Ras, or GFP-Ha-Ras mRNAs were then electropor-
ted into these cells 3 h before imaging using a small volume (1 μl)
electroporation device. Electroporation was performed at 350 V/cm
using three rectangular voltage pulses, each 40 ms long and 20 s apart.
After electroporation, the cells were replaced with Dulbecco’s minimum
essential medium supplemented with either paclitaxel or carrier
(Me₂SO) and incubated at 37 °C and 5% CO₂ in the incubator.
Fluorescence images were taken in a confocal microscope (Zeiss LSM;
Carl Zeiss, Inc., Thornwood, NY) using a 63× objective lens. In all images,
the noise levels were reduced by line scan averaging.

**RESULTS**

**Detection of Proteins in Bovine Brain That Bind a Farnesyl-
ated Peptide**—Prenylated peptides corresponding to the C ter-
mini of known prenylated proteins were shown recently to
interact specifically with a protein in microsomal membranes
from bovine brain (28). To identify the protein(s) involved in
this interaction, a cross-linking study was initiated. Several
different chemical cross-linking reagents were initially tested
for their ability to cross-link a labeled farnesylated decapeptide
(REKKFCS|S|3H|C₁₅)AlM; 3H-farnesylated peptide) to proteins
present in the microsomal membrane fraction. Most of the
reagents tested, which included glutaraldehyde, BS², N-hy-
droxysuccinimidyld-4-azidosalicic acid, and dimethylsuberimi-
date, cross-linked the 3H-farnesylated peptide to a protein
with an apparent molecular mass of about 56 kDa, whereas a second
protein with an apparent molecular mass of ~43 kDa was
detected using BS² (32).

To examine the specificity of the cross-linking of the 3H-
farnesylated peptide to these two proteins, competition analy-
sis was performed. The unlabeled farnesylated peptide com-

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of the column flow-through, lane 3 mM KCl elution from chromatography of extracts from 3H-labeled CHO prenyl proteins eluted from the microtubule affinity column. The 500 Lane 1 presence of 20°C20 C20 (ferred to nitrocellulose, and probed using [H]farnesyl peptide affinity chromatography as described in Fig. 1). Additionally, neither the unprenylated peptide nor FPP competed significantly for binding level of this protein in the column flow-through fraction (Fig. 2). Most notably, the 56-kDa proteins of 56 kDa and 43 kDa were preferentially retained in proteins involved in prenylation, most notably the two brain was passed over the farnesylated peptide affinity column, coupled to Sepharose. When the cytosolic fraction of bovine protein prenyltransferases (5, 44). We therefore pre- peted for binding to both the 56-kDa and 43-kDa proteins, and no significant competition was observed in the presence of either the farnesylated peptide missing the three C-terminal residues (farnesylated-REKKFFC) or a farnesylated tetrapeptide (farnesyl-CAIM) (Fig. 1A). Additionally, neither the unprenylated peptide nor FPP competed significantly for binding (data not shown). These results indicated that the binding of the farnesylated peptide to these two proteins was specific, and that this interaction was dependent on the farnesyl group as well as both the upstream region containing the basic residues and the three C-terminal amino acids.

The initial binding assay relied on membrane isolation to assess binding; hence, it had not been possible to examine the cytosolic fraction (28). Subjecting the cytosolic fraction to cross-linking analysis, however, revealed two proteins with the same electrophoretic mobilities as those identified in the microsomal membranes (Fig. 1B). Using equivalent amounts of protein from microsomal membrane and soluble fractions, both proteins were found to be substantially higher in abundance in the cytosolic than in the microsomal membrane fraction (results not shown). We elected to focus on the cytosol fraction to determine the identities of these proteins and to assess their potential involvement in cellular processes involving prenyl proteins.

Prenyl Peptide Affinity Chromatography—Peptide affinity chromatography has been utilized as an efficient tool in isolating proteins involved in prenylation, most notably the two CAAX protein prenyltransferases (5, 44). We therefore prepared an affinity resin containing the farnesylated peptide coupled to Sepharose. When the cytosolic fraction of bovine brain was passed over the farnesylated peptide affinity column, proteins of 56 kDa and 43 kDa were preferentially retained in a ratio similar to that seen for the 56-kDa and 43-kDa proteins observed by cross-linking (Fig. 2). Most notably, the 56-kDa protein was significantly depleted from the cytosolic proteins applied to the column, as revealed by a distinct reduction in the level of this protein in the column flow-through fraction (Fig. 2A, compare lanes 1 and 2). A small amount of both the 56-kDa and 43-kDa proteins could be released from the affinity resin with 500 mM NaCl (Fig. 2A, lane 5), and the remainder stayed

Fig. 4. Detection of farnesylated proteins that bind to a microtubule affinity column. The met18b-2 variant of CHO cells was metabolically labeled with [3H]mevalonolactone (35 Ci/mmol) in the presence of 20 μM lovastatin. Labeled cells were extracted with 1% Triton X-100 to solubilize prenylated proteins. Insoluble material was removed by centrifugation at 100,000 x g for 1 h, and the soluble fraction diluted 5-fold and applied to a microtubule affinity column containing ~8 μM tubulin. A, elution profile of [3H]prenyl proteins from the microtubule column. Lane 1, 10% of the applied sample; lane 2, 10% of the column flow-through, lane 3, 50 mM KCl wash; lane 4, 60 μM farnesylated peptide wash, lane 5, 100 mM KCl wash; lane 6, 500 mM KCl elution. Lanes 3–6 contain 100% of each fraction. Lane 7 contains 25% of the column resin that had been extracted with hot SDS after chromatography. B, GTP overlay analysis of fractions from microtubule affinity chromatography of CHO cell extracts. Unlabeled CHO cells were harvested, extracted, and the detergent extract processed on the microtubule affinity column as described in A. Aliquots of each fraction were electrophoresed through 12% SDS-polyacrylamide gels, transferred to nitrocellulose, and probed using [α-32P]GTP. The samples in each lane correspond directly to those in A. C, isoprenoid analysis of prenyl proteins eluted from the microtubule affinity column. The 500 mM KCl elution from chromatography of extracts from 3H-labeled CHO cells (see lane 6 in A) was subjected to methyl iodide cleavage after trypsin digestion and released isoprenoids analyzed by reverse-phase HPLC as described under “Experimental Procedures.” The arrows indicate the elution time for authentic farnesyl (C15) and geranylgeranyl (C20) isoprenoid standards chromatographed with the samples. Exposure time of fluorogram was 5 days.

Fig. 5. Association of purified Ras proteins with microtubule affinity column. Purified recombinant Ha-Ras (H-Ras) and Ki-Ras (K-Ras) (~5 μg of protein) were chromatographed through the microtubule affinity column either as a mixture of unprenylated and farnesylated proteins produced by enzymatic farnesylation with radiolabeled isoprenoid carrier (A, B, D, and E) or as unprenylated proteins (C and F). Ten percent of each applied sample (LD) and resulting flow-through fraction (FT), as well as 50% of the 500 mM KCl elute (KCI) were processed by SDS-FAGE. Farnesylated Ras proteins were visualized by fluorographic detection of the attached [3H]farnesyl group (A and D), whereas total Ras protein was detected by immunoblot analysis using the monoclonal Ab Y13-259 (B, C, E, and F).
Figure 6. Association of prenylated G proteins with microtubules. A, the C-terminal sequences, NH₂-fusion, and isoprenoid attached to the proteins tested for their ability to bind microtubule affinity resin (MT binding). Symbols and abbreviations used are as follows: ++ +, strong microtubule binding; –, no microtubule binding; NT, not tested; H-Ras, Ha-Ras; K-Ras, Ki-Ras; Farn, farnesylated; GST, glutathione S-transferase. B, microtubule binding data. Each of the proteins listed in A were enzymatically prenylated with either [³H]FPP (Farn-) or [³H]GGPP (GG-) and incubated with microtubule affinity resin (100 µl bed volume) as described under “Experimental Procedures.” Reaction samples were incubated with continuous rocking for 30 min at room temperature, centrifuged at 14,000 × g in a microcentrifuge, washed twice, and suspended in SDS-PAGE sample buffer for electrophoresis. An aliquot of the resin from each binding reaction (B) and an equivalent amount of the total [³H]prenyl protein (T) were analyzed by SDS-PAGE/fluorography. The exposure time for each panel was 14 h.

Figure 7. Effect of paclitaxel (taxol) on the targeting of GFP-Ki-Ras and GFP-Ha-Ras to the plasma membrane in NIH-3T3 cells. RNA constructs directing the expression of GFP alone (A and B), GFP-Ki-Ras fusion protein (GFP-K-Ras; C and D), or GFP-Ha-Ras fusion protein (GFP-H-Ras; E and F) were introduced into NIH 3T3 cells as described under “Experimental Procedures.” After 3 h, cells were subjected to fluorescence imaging. A and B, fluorescence images of control cells expressing GFP alone (A) or of cells treated with 3 µM paclitaxel before expressing GFP (B); C and D, fluorescence images of control cells expressing GFP-Ki-Ras (C) or of cells treated with 3 µM paclitaxel before expressing GFP-Ki-Ras (D); E and F, fluorescence images of control cells expressing GFP-Ha-Ras (E) or of cells treated with 3 µM paclitaxel before expressing GFP-Ha-Ras (F). The bar in F represents 10 µm.

Labeled peptide to purified tubulin. Nonspecific binding was determined by performing the binding assay in the presence of an excess of unlabeled farnesylated peptide. The results of this analysis revealed that tubulin contains a saturable binding site for the [³H]-farnesylated peptide with an apparent Kd of 40 nm (Fig. 3A). Similar binding is observed when the [³H]-farnesylated peptide is incubated with bovine brain cytosol containing an equivalent amount of tubulin (Fig. 3A), indicating that the presence of microtubule-associated proteins in bovine brain cytosol does not interfere with the prenyl peptide-tubulin interaction.

Inasmuch as the tubulin isolated by the prenyl peptide affinity column contained α and β subunits, we sought to determine whether the [³H]-farnesylated peptide bound to both of these subunits. Results from cross-linking analysis revealed that both the α and β subunits of tubulin cross-linked to the prenyl peptide as visualized by fluorographic analysis of proteins separated under conditions designed to resolve the two tubulin subunits (Fig. 3B). It was also of interest to determine whether the farnesylated peptide also binds to microtubules or if the binding required the free form of tubulin. We therefore prepared microtubules from purified tubulin through use of the microtubule-stabilizing drug, paclitaxel. Cross-linking analysis revealed that the [³H]-farnesylated peptide also binds to such paclitaxel-stabilized microtubules (data not shown), indicating that the prenyl peptide binding site on tubulin remains accessible after polymerization.
Detection of Farnesylated Microtubule-associated Proteins—
The results from the in vitro studies described above suggested the possibility of the presence of prenylated proteins in the cell that bind to tubulin and/or microtubules. To attempt to identify such proteins, a microtubule affinity column was prepared; this approach has been used to isolate a number of proteins that associate with microtubules (36, 45). To test for the presence of putative microtubule-associated prenylated proteins in cells, we labeled the met18b-2 variant of CHO cells, which exhibit high-efficiency uptake of the isoprenoid precursor MVA, with \[^3H\]MVA to generate \[^3H\]labeled prenylated proteins (37). Labeled cells were extracted with detergent to solubilize prenylated proteins, centrifuged at 100,000 × g to remove insoluble material, and the extracts diluted and passed through a microtubule affinity column. The vast majority (>95%) of the \[^3H\]labeled proteins passed through the affinity column and were detected in the void fraction (Fig. 5A, compare lanes 1 and 2). However, some of the \[^3H\]labeled protein was retained by the microtubule affinity column even after several washing steps, and these proteins could be eluted with 500 mM KCl (Fig. 5, lane 6). Analysis of these fractions revealed that three prenylated proteins of about 20, 24, and 50 kDa were present in the eluate.

We next determined which of the two isoprenoids, i.e. farnesyl or geranylgeranyl, was attached to the eluted proteins. The fractions containing the three \[^3H\]labeled prenylated proteins were subjected to isoprenoid analysis after methyl iodide cleavage. Surprisingly, only the 15-carbon farnesyl isoprenoid was detected (Fig. 5C), even though greater than 50% of the prenylated proteins passed over the column contain the geranylgeranyl isoprenoid, suggesting specificity in the binding of these proteins to microtubules. These data do not, however, exclude the possibility that there are geranylgeranylated proteins that bind to microtubules, inasmuch as such proteins may be in low abundance or not prenylated under the conditions used to label cells.

Many prenylated proteins are small GTP-binding proteins with molecular masses in the 20–30-kDa range. Because most of these proteins are important regulators of cellular events, it was of interest to determine whether any of the prenylated microtubule-binding proteins detected was a GTP-binding protein. To test this, unlabeled met18b-2 CHO cells were extracted as above and the extracts processed by microtubule affinity chromatography and fractions analyzed for GTP binding using an overlay assay with \[^32P\]GTP. Although this type of analysis detects only those G proteins that can reature their GTP-binding site after SDS-PAGE, it is still a quite useful tool in identifying such proteins. This analysis revealed the presence of a ~24-kDa GTP-binding protein that eluted from the microtubule affinity column under the same conditions as the prenylated proteins noted above (Fig. 5B, lane 6). The majority of the GTP-binding proteins, however, did not associate with the microtubules as indicated by the essentially identical patterns of GTP-binding capacity in the column’s load and flow-through fractions (Fig. 5B, compare lanes 1 and 2), suggesting selective retention of a specific GTP-binding protein(s) by the immobilized microtubules. This evidence was supported by the finding that several anti-Ras antibodies tested detected a protein of 24 kDa in the 500 mM NaCl eluate (data not shown). This finding was not sufficient to establish the identity of the Ras-like protein detected, however, as in our hands all of these antibodies cross-react with several Ras family members (data not shown).

Specific Association of Ki-Ras with Microtubules—The results from the above analysis revealed the existence of at least three farnesylated proteins, one of which was apparently a Ras-like GTP-binding protein, that bind to microtubules. The nature of the interaction, however, was not yet clear, in particular whether the binding of the proteins to microtubules was prenylation-dependent or whether this interaction was with specific proteins or a family of related GTP-binding proteins. In an attempt to examine this, we elected to explore the ability of specific prenylated G proteins to interact with microtubules. Initially, we chose Ki-Ras4B, which contains a polybasic sequence near the C-terminal prenyl Cys, and Ha-Ras, which has no such polybasic region (46). Recombinant Ki-Ras and Ha-Ras were produced in E. coli and enzymatically farnesylated producing a product mixture of about 20% farnesylated protein in the case of Ha-Ras, and about 5% of the Ki-Ras was farnesylated. These two mixtures of \[^3H\]farnesylated and unfarnesylated Ras proteins were then chromatographed through the microtubule affinity column as described above for the CHO cell extracts. \[^3H\]Farnesylated Ras proteins were detected by fluorography after SDS-PAGE of elution fractions, whereas total Ras protein was detected by immunoblot analysis. Ki-Ras, but not Ha-Ras, was found to associate with the microtubule affinity resin in a farnesylation-dependent fashion, and the bound farnesylated proteins could be eluted with 500 mM KCl (Fig. 5, A, B, D, and E). The requirement for the farnesyl lipid was verified by performing the affinity chromatography with completely unprenylated Ki-Ras and Ha-Ras; neither of these proteins associated with the microtubule resin (Fig. 5, C and F).

The failure of Ha-Ras to bind to microtubules was not apparently due to an additional requirement that this protein also be palmitoylated, as Ha-Ras produced in NIH-3T3 cells, which would be both farnesylated and palmitoylated, also did not bind to the microtubule affinity resin under similar conditions (data not shown). The conclusion drawn from these studies was that microtubules could bind, in a prenylation-dependent fashion, a farnesylated protein that contained a polybasic C-terminal domain.

To determine whether the farnesylation-dependent association of Ki-Ras with microtubules was specific or if other prenylated GTP-binding proteins with similar charge properties could also bind microtubules, several additional proteins were tested. Additionally, because other GTP-binding proteins that contain a polybasic sequence upstream from their prenyl Cys residue are primarily geranylgeranylated, and because Ki-Ras can also be geranylgeranylated (47, 48), the question concerning the specificity of the microtubule binding for proteins containing the farnesyl versus geranylgeranyl isoprenoid could be addressed using these proteins, which included RhoA, Rap1B, and Ki-Ras (see Fig. 6A for C-terminal sequence comparison and isoprenoid specificity). Each of the GTP-binding proteins listed in Fig. 6A was enzymatically prenylated with the appropriate \[^3H\]isoprenoid and incubated with an aliquot of microtubule affinity resin as described under “Experimental Procedures.” The binding of the \[^3H\]prenylated proteins to the microtubule resin was determined by comparing its presence in the initial sample to that bound to the resin by SDS-PAGE and fluorographic analysis of an equivalent amount of each sample.

The results of these analyses revealed that only Ki-Ras, either in a farnesylated or geranylgeranylated state, associated with the microtubule (Fig. 6B). This study revealed that both the farnesyl and geranylgeranyl isoprenoid is recognized by microtubules if presented by the appropriate protein, i.e. Ki-Ras. The observation that other proteins containing a prenylated, polybasic C terminus (i.e. RhoA and Rap1B) do not bind to the microtubule resin suggests that the interaction between Ki-Ras and microtubules is a specific one and that epitopes in addition to the prenylated C terminus are probably involved in this interaction.
Ki-Ras, but Not Ha-Ras, Is Mislocalized When Expressed in Paclitaxel-treated NIH-3T3 Cells—Several possibilities exist for a functional significance of the specific interaction between Ki-Ras with microtubules. One possibility is that the microtubule network may play a role in the processing and cellular targeting of Ki-Ras. It is not yet clear how the many prenyl proteins in cells are directed to their correct cellular compartments, and it is likely that components yet to be identified are involved in this targeting. Cell fractionation and immunolocalization studies have shown that Ki-Ras is mostly localized to the plasma membrane and that its prenylated C terminus, and the polybasic region just upstream, is required for this localization (12). Recently, an approach was developed in which expression of a fusion protein between a green fluorescent protein and Ki-Ras (GFP-Ki-Ras) in mammalian cells clearly demonstrated localization to the plasma membrane in living cells (43). We employed this approach to determine if the plasma membrane targeting of Ki-Ras would be affected by perturbation of the cell’s microtubule network. NIH-3T3 cells were treated with paclitaxel, which disrupts the cells microtubule network by bundling the microtubules, or with carrier alone before introduction of an mRNA encoding either GFP alone, the GFP-Ki-Ras4B protein or GFP-Ha-Ras. The localization of the newly synthesized GFP and GFP-Ras proteins were determined 2–3 h after introduction by fluorescence confocal microscopy. In control cells expressing GFP alone, paclitaxel treatment had no effect on the localization of GFP (Fig. 7, A and B). As expected, in cells not treated with paclitaxel, the majority of both GFP-Ki-Ras and GFP-Ha-Ras were localized to the plasma membrane (Fig. 7, C and E). In contrast, GFP-Ki-Ras synthesized in paclitaxel-treated cells was found almost exclusively in intracellular locations, indicating that it could not target to the plasma membrane (Fig. 7D), whereas paclitaxel treatment had no significant affect on the localization of GFP-Ha-Ras (Fig. 7F). These results suggest that an intact microtubule network is required for the correct localization of Ki-Ras, but not Ha-Ras, in cells.

DISCUSSION

Following up on a study in which a prenyl peptide-binding protein was detected in bovine brain (28), we used a chemical cross-linking approach to visualize a 56-kDa protein as the predominant protein in this tissue that specifically binds the farnesylated peptide ligand. Isolation of this protein by prenyl peptide affinity chromatography led to its identification as tubulin, a major cytoskeletal protein. Extension of these studies revealed that a small subset of cellular prenylated proteins could be retained on immobilized microtubules, suggesting the involvement of microtubules in cellular events controlled by specific prenyl proteins. The findings that at least one of the cellular prenyl proteins that bind microtubules was a farnesylated GTP-binding protein of 24 kDa, and that several anti-Ras antibodies also recognized a 24-kDa protein that eluted in the same fraction, prompted us to examine the association of Ras proteins, and a variety of other prenylated GTP-binding proteins, with microtubules. The finding that Ki-Ras, but not Ha-Ras or a number of related proteins, binds to microtubules in a fashion dependent on its prenylation provided evidence both for specificity of this interaction and for the involvement of determinants in addition to the prenylated C terminus for the association of Ki-Ras with microtubules.

A direct connection between Ki-Ras4B and microtubules in living cells was made by the finding that GFP-tagged Ki-Ras4B is not correctly targeted to the plasma membrane in cells in which the microtubular network is disrupted by treatment with paclitaxel. By contrast, a similar paclitaxel treatment had no effect on the cellular localization of GFP-tagged Ha-Ras. Although the precise significance of this finding is not yet clear, there are several quite intriguing possibilities. One explanation for Ki-Ras mislocalization in paclitaxel-treated cells is that an intact microtubular network is required to deliver newly prenylated Ki-Ras to the plasma membrane. In this scenario, the bundling of the microtubule network by paclitaxel may create a new, stable set of binding sites for Ki-Ras that prevent the protein from continuing down its normal processing and targeting pathway. This seems possible, since members of the prenylated protein family are targeted to many different membranes in cells, that several different mechanisms are involved in directing the members of this group of proteins through their post-prenylation processing and to their final destination in cells. An alternative explanation is that in normal cells, Ki-Ras that is newly prenylated but not yet fully processed is first targeted to the microtubules, where it performs a yet undetected function before completing its processing and being targeted to the plasma membrane. In this case, the effect of paclitaxel on Ki-Ras localization may be to stabilize this microtubule-associated state.

Whatever the exact process is, the results from the present study reveal a direct interaction between Ki-Ras and the microtubule network in cells. In addition, they point to a previously undetected connection between Ras-induced cell transformation and microtubule-based drug treatments in that they demonstrate a clear physiological effect of paclitaxel on Ki-Ras targeting to the plasma membrane. Several studies have in fact hinted at the involvement of prenylated proteins, including oncogenic Ras, in microtubule-dependent processes. In one such study, morphological changes induced by oncogenic Ras transformation were profoundly influenced by both paclitaxel, which promotes formation of short bundled microtubules, and vinca alkaloids, which promote microtubule breakdown (19). Paclitaxel has also been shown to down-regulate Ki-Ras in human leukemia and ovarian cells in culture (21). Additional studies that have indicated a relationship between Ras and microtubules include the findings that paclitaxel can affect the activity state of MAP kinase pathways; paclitaxel-induced activation of Raf-1 and MAP kinase was observed in macrophages, whereas paclitaxel-induced inhibition of MAP kinase has been reported in lung cancer cells (49, 50). Although the basis for the differing effects of paclitaxel in the two systems has not been addressed, these studies do provide evidence for a possible link between microtubules and Ki-Ras, which signals through the MAP kinase pathway.

In summary, the data presented here provide strong evidence for involvement of the microtubular network in the trafficking and/or cellular function of Ki-Ras. In addition, this study also provides direct evidence for a physiological distinction between Ha-Ras and Ki-Ras in cells. Studies are currently under way to elucidate the mechanism(s) by which paclitaxel treatment of cells prevents targeting of Ki-Ras to the plasma membrane and/or for a potential signaling function of microtubule-associated Ki-Ras. Results from these studies may provide additional insight as to how anti-microtubule drugs function as anti-cancer agents.

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