The C-terminal Polylsine Region and Methylation of K-Ras Are Critical for the Interaction between K-Ras and Microtubules

Received for publication, July 26, 2000, and in revised form, September 12, 2000
Published, JBC Papers in Press, September 27, 2000, DOI 10.1074/jbc.M006687200

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After synthesis in the cytosol, Ras proteins must be targeted to the inner leaflet of the plasma membrane for biological activity. This targeting requires a series of C-terminal posttranslational modifications initiated by the addition of an isoprenoid lipid in a process termed prenylation. A search for factors involved in the intracellular trafficking of Ras has identified a specific and prenylation-dependent interaction between tubulin/microtubules and K-Ras. In this study, we examined the structural requirements for this interaction between K-Ras and microtubules. By using a series of chimeras in which regions of the C terminus of K-Ras were replaced with those of Ha-Ras and vice versa, we found that the polylysine region of K-Ras located immediately upstream of the prenylation site is required for binding of K-Ras to microtubules. Studies in intact cells confirmed the importance of the K-Ras polylysine region for microtubule binding, as deletion or replacement of this region resulted in loss of paclitaxel-induced mislocalization of a fluorescent K-Ras fusion protein. The additional modifications in the prenyl protein processing pathway also affected the interaction of K-Ras with microtubules. Removal of the three C-terminal amino acids of farnesylated K-Ras with the specific endoprotease Rce1p abolished its binding to microtubules. Interestingly, however, methylation of the C-terminal prenylcysteine restored binding. Consistent with these results, localization of the fluorescent K-Ras fusion protein remained paclitaxel-sensitive in cells lacking Rce1, whereas no paclitaxel effect was observed in cells lacking the methyltransferase. These studies show that the polylysine region of K-Ras is critical for its interaction with microtubules and provide the first evidence for a functional consequence of Ras C-terminal proteolysis and methylation.

Ras GTPases operate as molecular switches in signal transduction cascades controlling cell proliferation, differentiation, and apoptosis (1). The biological activity of the Ras proteins, like that of other G proteins, is controlled by a regulated GTP/GDP cycle. Upstream regulators, generally responding to tyrosine phosphorylation events, induce inactive, GDP-bound Ras to exchange bound GDP for GTP, resulting in activation, whereas GTPase-activating proteins enhance the intrinsic GTPase activity of Ras and restore Ras to its inactive form (2, 3). Mutations at codons 12, 13, or 61 of Ras lead to inactivation of the GTPase activity and/or its sensitivity to GTPase-activating proteins, resulting in Ras proteins that are constitutively active and able to transform mammalian cells (1). Such mutually activated forms of Ras are found in a large number of human cancers (1, 4).

Three Ras genes have been identified that encode closely related 21-kDa proteins: Ha-Ras, N-Ras, K-Ras4A, and K-Ras4B (4). K-Ras4B is the predominant form of K-Ras in nature, and the term K-Ras has frequently been used to denote K-Ras4B. All the Ras proteins share high degrees of homology in their N termini but diverge in a hypervariable region in their C termini. The differences in this hypervariable region are thought to be associated with different cellular functions for the proteins (5–7). Specific Ras isoforms are mutated in different tumor types, with mutations in K-Ras appearing most frequently (3, 8). Recent studies with knockout mice have revealed that K-Ras (but not Ha-Ras or N-Ras) is essential in mouse embryonic development (9). However, the precise nature of the functional differences among Ras isoforms remains largely unclear.

Each Ras protein terminates in a so-called CAAX sequence, where C is a cysteine; the A is usually an aliphatic amino acid, and X can be almost any amino acid. This C-terminal CAAX motif undergoes a trio of posttranslational modifications (10, 11) that are important for the activity of normal and oncogenic Ras proteins (12). The initial processing step, farnesylation, is catalyzed by the enzyme protein farnesyltransferase (FTase)3 and involves the addition of a 15-carbon farnesyl isoprenoid to the thiol group of the cysteine residue (13, 14). In the second step, the CAAX residues are removed by a specific endoprotease, Rce1 (15, 16). Finally, the carboxyl group of the newly exposed prenylcysteine at the C terminus is methylated by a specific methyltransferase, isoprenylcysteine methyltransferase (termed Ste14p in yeast and ICMT in mammals) (17, 18). Although these modifications, particularly prenylation, are essential for the association of Ras proteins with membranes, a second membrane localization signal in the hypervariable region is required for targeting of this article were delayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported by a fellowship from the Leukemia Society of America.
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1 The abbreviations used are: FTase, farnesyltransferase; FPP, farnesyl diphosphate; DTT, dithiothreitol; GFP, green fluorescent protein; Rce1, a CAAX-specific prenyl-protein endoprotease; ICMT, isoprenylcysteine methyltransferase; ER, endoplasmic reticulum; AdoMet, S-adenosylmethionine; PCR, polymerase chain reaction; Pipes, 1,4-piperazinediethanesulfonic acid.
region is required for optimal membrane affinity. In Ha-Ras and N-Ras, this second signal is the palmitoylation of cysteine residues located near the C-terminal prenylcysteine (12). In the case of K-Ras, the second signal is a stretch of basic (lysine) residues located just upstream of the CAAX motif (19). The additional membrane stability provided by palmitoylation and the polybasic domains appears to be important for Ras function, as their elimination has been shown to influence the cellular activity and transforming ability of the Ras proteins (5, 12).

Although the protein prenyltransferases are cytosolic, both the endoprotease and the methyltransferase are located in the endoplasmic reticulum (ER) (17, 20, 21). Thus, the current view is that Ras proteins are prenylated in the cytosol, translocated to the ER for further processing, and finally targeted to the plasma membrane (12, 22, 23). At this time, the mechanism(s) of intracellular Ras trafficking is incompletely understood.

To identify factors involved in trafficking of Ras and other CAAX-type proteins, we began a search for proteins that could associate with prenylated CAAX peptides. One such protein was identified as the cytoskeletal protein tubulin, which binds (24). A closer analysis revealed that microtubules bind to a small subset of farnesylated proteins, including K-Ras (25). The binding of K-Ras to tubulin and microtubules was specific; Ha-Ras and a variety of other prenylated proteins did not bind. Additionally, prenylation of K-Ras was essential for this interaction. A direct link between K-Ras and microtubules was confirmed in living cells with experiments that showed that a K-Ras-green fluorescent protein fusion (GFP-K-Ras) did not reach the plasma membrane when the microtubule network was disrupted with paclitaxel, whereas GFP-Ha-Ras localization was unaffected (25). These results suggested that an intact microtubule network is required for correct K-Ras targeting in cells and hinted that some K-Ras-dependent signaling processes might be dependent on the microtubule network (25). In this study, we sought to determine the structural requirements for the interaction between K-Ras and microtubules.

Experimental Procedures

Materials—[1-^3H]Farnesylidophosphate ([^3H]FPP, 22 Ci/mmol) was purchased from PerkinElmer Life Sciences. Unlabeled FPP was obtained from American Radiolabeled Chemicals (St. Louis, MO). Paclitaxel (taxol) was obtained from Sigma. Glutathione-Sepharose 4B was obtained from Amersham Pharmacia Biotech. Nickel-nitrilotriacetic acid resin was from Qiagen (Chatsworth, CA). Affi-Gel 10 resin was from Bio-Rad. Cell culture reagents were from Life Technologies, Inc. S-Adenosylmethionine (AdoMet) was purchased from Research Biochemicals International (Natick, MA). S-Adenosyl-l-[methyl-^3H]Homethionine ([^3H]AdoMet) was purchased from PerkinElmer Life Sciences.

Bacterial expression plasmids for Ha-Ras and K-Ras were gifts from Dr. Guy James (University of Texas Health Sciences Center, San Antonio), Dr. Channing Der (University of North Carolina, Chapel Hill, NC) and Dr. Michael Der (University of California, San Diego), respectively. The QE31-N-Ras expression plasmid was a gift from Dr. Robert Bishop (Schering-Plough Research Institute). Oligonucleotide primers were synthesized at the Duke University DNA Core Facility. Construction of Ras Chimeras—Plasmids encoding chimeric Ras proteins were constructed by polymerase chain reaction (PCR) techniques. The primers were generated containing either a BgII or a KpnI restriction endonuclease site at 5' and a KpnI site at 3'. The 3' PCR primers also encoded the desired C-terminal amino acids, as well as a stop codon. Each of the BgII-KpnI or KpnI-BglII PCR products was digested with the appropriate restriction endonuclease and inserted into pEGFP-C1 (CLONTECH, Palo Alto, CA) or pRSETB vectors (Invitrogen, Carlsbad, CA), respectively. The integrity of each plasmid was confirmed by restriction mapping and DNA sequencing.

Production of Farnesylated Proteins—Unprenylated K-Ras, N-Ras, and Ras chimeras, all containing N-terminal hexahistidine tags, were expressed in Escherichia coli and purified as described previously (26). Ha-Ras was also expressed in E. coli and purified as described (14). Ras proteins were farnesylated by incubation of 2 μM protein farnesyltransferase and 6 μM farnesyl dipiphosphate in 5 mM MgCl₂, 5 μM ZnCl₂, 2 mM DTT, 5 μM GDP, 50 mM Tris-HCl (pH 7.7) for 1 h at 37 °C (25). Farnesylated K-Ras, Ha-Ras, and N-Ras were resolved from the unprenylated precursor and modifying enzyme by applying the farnesylated proteins to a heptyl-Sepharose column (for K-Ras) or a phenyl-Sepharose column (for Ha-Ras and N-Ras) in Buffer A (50 mM Hepes, 3 mM MgCl₂, 1 mM DTT, 5 μM GDP) for K-Ras or Buffer A containing 500 mM NaCl (for Ha-Ras) or 750 mM NaCl (for N-Ras). Columns were washed with the loading buffer containing 0.1% Lubrol, which eluted unprenylated Ras and the protein prenyltransferase. The purified farnesyl-Ras was eluted with Buffer A containing either 2% sodium cholate (for K-Ras), 0.5% Tween 20 (for Ha-Ras), or 1% Tween 20 (for N-Ras). The farnesyl-Ras preparations were diluted 20-fold in buffer A and applied to a column of S-Sepharose (for K-Ras) or DEAE-Sepharose (for Ha-Ras and N-Ras). The column was washed with Buffer A, and the farnesyl-K-Ras was eluted with Buffer A containing 0.1% octyl glucoside and 750 mM NaCl (for K-Ras) or 400 mM NaCl (for Ha-Ras and N-Ras). Purified prenylated Ras proteins were flash-frozen in liquid nitrogen and stored at −80 °C.

Enzymatic Endoproteolysis and Methylation of Farnesyl-K-Ras—The C-terminal endoproteolysis of farnesyl-Ras proteins was initiated by the addition of 80 μg of membranes containing yeast Rce1p and 80 μg of membranes containing yeast Ste14p (15) to a mixture containing prenylated Ras in 100 mM Hepes (pH 7.4), 5 mM MgCl₂, and 20 μM AdoMet, either unlabeled or the ^3H form (at 0.5 Ci/mmol) where indicated, in a total volume of 150 μl. Reactions were conducted at 37 °C for 30 min. The reaction mixture was then centrifuged at 100,000 × g for 1 h at 4 °C to remove the membranes, and the resulting supernatant was diluted 1:5 and used in binding assays described below.

Preparation of Microtubule Affinity Resin—Tubulin was purified from extracts of freshly isolated porcine brain, and microtubule affinity columns were constructed as described (25). Briefly, tubulin (2–3 mg/ml) in 80 mM Pipes, 1 mM MgCl₂, 1 mM Na₂EGTA (pH 6.8) was assembled into microtubules by adding 1 μM GTP followed by paclitaxel. The pH of the solution containing the paclitaxel-stabilized microtubules was adjusted to pH 7.6 with 3 M KOH, and the microtubules were immobilized by addition to activated resin. The mixture was left undisturbed overnight at 4 °C to allow coupling and washed with several column...
cells were transfected with 0.5 μg of pEGFP-Ras DNA or pEGFP-C1 vector with LipofectAMINE according to the manufacturer’s protocol (Life Technologies, Inc.). After an additional 5 h, the transfection medium was replaced with fresh medium containing 10% calf serum; the paclitaxel concentration was maintained at 3 μM throughout the process. Microscopy was performed after 24 h with a Zeiss Axiovert 100 confocal microscope. Cells were imaged with a ×63 oil immersion lens at 488 nm.

Miscellaneous Methods—Protein concentrations were determined with the Bio-Rad protein assay reagent. Western blot analysis was performed with an anti-rabbit alkaline phosphatase conjugate (Promega, Madison, WI).

RESULTS

The direct link between K-Ras and the microtubule network in cells identified in previous work suggested a role for this interaction for the localization and function of specific prenylated proteins (25). In those studies, the cytoskeletal protein tubulin was found to specifically bind K-Ras in a fashion that required prenylation; farnesyl-Ha-Ras was not capable of binding to tubulin. To determine if N-Ras could associate with microtubules, we first analyzed the binding of N-Ras to microtubules in vitro. Recombinant N-Ras was produced in E. coli, enzymatically farnesylated with [3H]FPP, and loaded onto microtubule columns. The columns were washed at low ionic strength, and bound proteins were eluted with a buffer of high ionic strength. Fluorographic analysis of [3H]farnesyl-N-Ras in vitro.

Interaction of chimeric Ras proteins with microtubules in vitro—Equal amounts of purified recombinant chimeric Ras proteins (see “Experimental Procedures” and Table I) were subjected to in vitro farnesylation with purified recombinant FTase and [3H]FPP. The radiolabeled farnesylated Ras chimeras were applied to microtubule affinity columns, which were then washed with buffer and eluted as described in the legend to Fig. 1. Protein in the eluted fraction was collected, and [3H]farnesyl-Ras bound to the filters was quantitated by liquid scintillation spectroscopy; see “Experimental Procedures” for details. Amounts of Ras in the eluted fraction were plotted as percentages of the total radiolabeled protein applied to the column.

In the previous study, we examined the effects of paclitaxel disruption of the microtubule network on the localization of GTP-Ras fusions, and we found that the microtubule disruption abolished targeting of K-Ras, but not Ha-Ras, to the plasma membrane (25). In this study, we employed a similar approach to determine the effect of disrupting the microtubule network on the localization of a GTP-N-Ras fusion protein. Cells were treated with paclitaxel before transfection with plasmids encoding GTP-Ras. After 24 h, GTP-Ras localization was assessed with confocal microscopy. GTP-N-Ras remained on plasma membrane both before and after paclitaxel treatment (Fig. 2). Although the isoforms of Ras proteins are highly homologous, they can be distinguished by a hypervariable region encompassing ~25 residues of the C-terminal sequence (4). K-Ras

FIG. 2. Comparison of the effect of paclitaxel on plasma membrane targeting of GFP-tagged K-Ras and N-Ras. Plasmids encoding GFP-N-Ras (left panels) or GFP-K-Ras (right panels) were introduced by transfection into NIH-3T3 cells that had been treated with vehicle (upper panels) or paclitaxel (lower panels). The localization of the fluorescent proteins was assessed by confocal microscopy 24 h after transfection.

FIG. 3. Interaction of chimeric Ras proteins with microtubules in vitro—Equal amounts of purified recombinant chimeric Ras proteins (see “Experimental Procedures” and Table I) were subjected to in vitro farnesylation with purified recombinant FTase and [3H]FPP. The radiolabeled farnesylated Ras chimeras were applied to microtubule affinity columns, which were then washed with buffer and eluted as described in the legend to Fig. 1. Protein in the eluted fraction was collected, and [3H]farnesyl-Ras bound to the filters was quantitated by liquid scintillation spectroscopy; see “Experimental Procedures” for details. Amounts of Ras in the eluted fraction were plotted as percentages of the total radiolabeled protein applied to the column.

TABLE I

C-terminal sequences of wild-type and chimeric Ras proteins used in the study

| Protein | C-terminal sequence |
|---------|---------------------|
| Ha-Ras  | DESGPCCMSKCVIM      |
| K-Ras   | KKKKKKSKTCVIM       |
| H-CVLS  | DESGPCCMSKCVIM      |
| H-(K)CVLS | KKKKKKSKTCVIM   |
| H-(K)CVIM | KKKKKKSKTCVIM     |
| K-(Q)CVLS | QQQQQQSKTCVIM     |
| K-(H)CVLS | Desgpccmskcvim    |

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volumes of Buffer B (50 mM Hepes, 1 mM EGTA, and 1 mM MgCl₂ (pH 7.5)) containing 10 mM ethanolamine to block unreacted amino groups. The resin was washed exhaustively with Buffer B containing 1 mM DTT and 500 mM KCl. Typical tubulin densities achieved on the column were 6–9 nmol/ml of gel. 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 the protease inhibitors.

Ras Protein Binding to Immobilized Microtubules—Proteins were bound to immobilized microtubules as described previously (25). Briefly, Ras proteins with various states of C-terminal processing (see above) were applied to microtubule affinity columns (25 μl of immobilized microtubule resin) equilibrated in Buffer B. Columns were then washed with 1 ml of 50 mM KCl and eluted with 1 ml of 500 mM KCl.

Cell Culture and Fluorescence Microscopy—Plasmids encoding GFP-N-Ras (left panels) or GFP-K-Ras (right panels) were introduced by transfection into NIH-3T3 cells that had been treated with vehicle (upper panels) or paclitaxel (lower panels). The localization of the fluorescent proteins was assessed by confocal microscopy 24 h after transfection.
is unique in that its hypervariable region contains a CAAAX sequence of CVIM and a polylysine stretch just upstream from the CAAAX motif. To explore whether these unique structural elements are required for K-Ras to interact with microtubules, we created a series of chimeric proteins in which regions of the C-terminal sequence of Ha-Ras were replaced with the corresponding regions of K-Ras and vice versa (see Table I). The exchanges included the following: (i) replacing the polylysine domain just upstream from the farnesylated cysteine in K-Ras with the corresponding region from Ha-Ras; (ii) replacing the polylysine stretch of K-Ras with a polyglutamine sequence, thereby abolishing the positive charged sequence; and (iii) replacing the C-terminal three residues of the CAAAX motif of K-Ras with that found in Ha-Ras (i.e. -CVLS for -CVIM). The binding of these chimeric Ras proteins to microtubules was initially analyzed in the in vitro microtubule assay. The three Ras proteins lacked the polylysine domain upstream from the farnesylcysteine, (Ha-CVIM, K-(Q)CVIM, and K-(H)CVLS), failed to bind to the immobilized microtubules (Fig. 3). However, the three chimeras that retained the polylysine sequence (i.e. Ha-(K)CVLS, Ha-(K)CVIM, and K-CVLS) showed significant microtubule binding. In control experiments, farnesylated K-Ras clearly bound to microtubules, whereas farnesylated Ha-Ras did not (data not shown). Immunoblot analysis of total Ras protein confirmed these results (data not shown). These findings demonstrate that the polylysine domain of K-Ras, in addition to the prenyl group, is important for the interaction of K-Ras with microtubules, whereas the specific CAAAX sequence (i.e. CVIM versus CVLS) is not.

We also examined the effect of the disruption of microtubule network on the localization of GFP-tagged Ras chimeras. Consistent with the in vitro data on microtubule binding, K-Ras proteins that were unable to bind microtubules in vitro (i.e. those lacking the polylysine domain) were resistant to paclitaxel-induced redistribution in cells (Fig. 4A). On the other hand, Ha-Ras chimeras containing the polylysine sequence acquired sensitivity to paclitaxel-induced mislocalization (Fig. 4B). As previously demonstrated, switching of the polylysine to polyglutamine sequence alone resulted in mislocalization of most of the K-Ras to cytosolic locations even in untreated cells (19), but the paclitaxel insensitivity of the distribution of this protein (i.e. K-Q, CVIM) still confirmed the in vitro result that it fails to interact with microtubules. The presence of the CVIM sequence (i.e. the K-Ras CAAAX sequence) did not affect the cellular localization of Ras after paclitaxel treatment. These findings confirm that the polylysine stretch in K-Ras is critical for the interaction of K-Ras with microtubules and support the notion that an intact microtubule network is important for correct localization of K-Ras in cells.

Next, we examined the influence of endoproteolysis and carbamyl methylation on the association between K-Ras and microtubules. [3H]Farnesyl-K-Ras was subjected to in vitro endoproteolysis or to both endoproteolysis and methylation and assessed for microtubule binding. Surprisingly, endoproteolytic removal of these residues abolished the binding of K-Ras to microtubules. However, methylation of the C-terminal farnesylcysteine restored binding activity essentially to the original level (Fig. 5A). To exclude the possibility that the loss of binding in the endoprotease-treated K-Ras was due to artifactual changes unrelated to endoproteolysis, the proteolyzed species that flowed through the microtubule columns were methylated in vitro and reapplied to the microtubule column. Consistent with the in vitro binding studies, the K-Ras proteins in these fractions regained their ability to bind microtubules (Fig. 5B). Further confirmation of the requirement for methylation came from studies in which unlabeled farnesyl-K-Ras was proteo-

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**Fig. 4. Effect of paclitaxel treatment on plasma membrane targeting of GFP-tagged Ras chimeras.** Plasmids encoding GFP-tagged Ras chimeric proteins in which the parental form was K-Ras (A) or Ha-Ras (B) (also see Table I) were introduced by transfection into NIH-3T3 cells that had been treated with vehicle (left panels) or paclitaxel (right panels). The localization of the expressed fluorescent proteins were then assessed by confocal microscopy 24 h after transfection.
the methyltransferase Icmt have been generated recently (27, 28). Expression of GFP-K-Ras proteins in Rce1−/− embryonic fibroblasts and Icmt−/− embryonic stem cells showed partial mislocalization of the protein in the cytosol, although some of the GFP-K-Ras fusion remained on the plasma membrane (27, 28). To confirm the in vitro result on the effect of endoproteolysis and methylation on the interaction between K-Ras with microtubules, we examined the effect of paclitaxel treatment on the localization of GFP-K-Ras in both Rce1−/− and Icmt−/− cells. Consistent with the in vitro results, paclitaxel had a marked effect on localization of GFP-K-Ras in Rce1−/− cells but no effect on the localization of GFP-Ha-Ras (Fig. 6). In Icmt−/− cells, however, localization of GFP-K-Ras was paclitaxel-insensitive (Fig. 7). These results provide a striking demonstration that endoproteolysis and methylation of K-Ras can modulate the ability of the protein to associate with microtubules.

**DISCUSSION**

Analysis of chimeras between Ha-Ras and K-Ras revealed that the polylysine sequence from the C-terminal hypervariable region of K-Ras is essential for its interaction with microtubules. Perhaps this was not too surprising given that the major feature of the hypervariable region of K-Ras is the polylysine region; however, it is noteworthy that the Ras-related proteins Rap1B and RhoA contain stretches of basic residues near their prenylated C termini but do not bind microtubules (25). The most surprising findings of the current study were that removal of the -AAX sequence from the C terminus of K-Ras eliminated microtubule binding and that the binding could be restored by methylation of the C-terminal prenylcysteine. This is an exciting finding as it is the first demonstration of a functional consequence of Ras C-terminal proteolysis and methylation. Given that partially processed Ras that is farnesylated but retained the -AAX residues does bind microtubules, these findings suggest that the presence of a carboxylate ion on the C-terminal prenylcysteine blocks Ras interaction with microtubules, and eliminating this negative charge with the formation of an α-carboxyl methyl ester restores microtubule binding.

Methylation by ICMT is the only potentially reversible step among the three C-terminal posttranslational modifications and thus could be exploited for dynamic regulation of protein function (20, 29, 30). The finding that carboxyl methylation is required for K-Ras to bind to microtubules suggests that the microtubule-K-Ras interaction and its related functions could be regulated. Hence, the current study not only demonstrates a novel function for methylation distinct from that of enhanced membrane association of prenyl proteins (31, 32), but provides...
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FIG. 6. Effect of paclitaxel on the targeting of GFP-tagged Ras to the plasma membrane in Rec1−/− cells. Plasmids encoding GFP-K-Ras (left panels) and GFP-Ha-Ras (right panels) were introduced by transfection into Rec1−/− embryonic fibroblasts cells that had been treated with vehicle (upper panels) or paclitaxel (lower panels). The localization of the fluorescent proteins was assessed by confocal microscopy 24 h after transfection.

FIG. 7. Effect of paclitaxel on the targeting of GFP-tagged K-Ras to the plasma membrane in Icmt−/− cells. Plasmids encoding GFP-K-Ras were introduced by transfection into Icmt+/+ (left panels) or Icmt−/− (right panels) cells that had had been treated with vehicle (upper panels) or paclitaxel (lower panels). The localization of the fluorescent proteins was assessed by confocal microscopy 24 h after transfection.

The first clear example of a mammalian CAAX protein function that is affected by the postprenylation processing events.

Two possible functions can be envisioned for the specific interaction between K-Ras and microtubules. First, the interaction might be important for subcellular trafficking, since disruption of microtubular network with paclitaxel prevents proper localization of K-Ras at the plasma membrane. A variety of studies have provided evidence that, after synthesis and prenylation, CAAX proteins are first directed to the ER/Golgi compartment for processing by Rec1 and Icmt and are then targeted to their final destination (i.e. the plasma membrane in the case of Ras proteins) (22–24). However, distinct forms of Ras may use different mechanisms in this final trafficking step. Recent studies have suggested that Ha-Ras and N-Ras traffic to the plasma membrane through a brefeldin A-sensitive pathway (i.e. vesicular transport), whereas K-Ras trafficking is brefeldin A-insensitive and requires an intact polylysine domain (22, 23). However, while our results suggest that the microtubule network may serve a role in K-Ras trafficking, this role cannot be absolute as a significant fraction of the K-Ras expressed in the Icmt−/− cells still localizes to the plasma membrane (see Fig. 7).

The second potential role for the specific interaction between K-Ras and microtubules is that an association with microtubules may provide K-Ras with certain functions distinct from those it possesses when localized to the plasma membrane. This hypothesis is supported by several reports that have linked Ras-dependent signaling cascades to microtubule-dependent processes. For instance, microtubule destabilizing agents affect a number of Ras-dependent signaling events (33–36). Several signaling proteins that function in Ras pathways, including the related protein Rac and several members of mitogen-activated protein kinase signaling cascades, have also been shown to interact with microtubules (37). Since other Ras-related proteins (e.g. Rap1, Rap2, and Rho) have long been linked to the regulation of cytoskeletal as well as nuclear events (38–40), it seems likely that some Ras-dependent signaling processes may require (i.e. may actually occur on) the microtubule network in cells. These findings may also in part explain the reported synergy between paclitaxel and inhibitors of protein farnesyltransferase, termed farnesyltransferase inhibitors, in blocking tumor cell growth (41).

Finally, the selective capacity of K-Ras for microtubule binding provides a potential explanation for reported functional differences between K-Ras and other forms of Ras noted in the Introduction, since specific functions may be associated with the microtubule-bound pool of K-Ras. Elucidation of precise physiological importance of the interaction between K-Ras and microtubules may provide not only insights into the underlying mechanisms of the distinct biological functions of K-Ras but also provide novel targets for pharmacological intervention of Ras-dependent signaling cascades.

Acknowledgments—We thank Carolyn Weinbaum for purifying Ha-Ras and Julia Thiessen for helpful advice.

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