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The COOH-Terminal Domain of the Rap1A (Krev-1) Protein Is Isoprenylated and Supports Transformation by an H-Ras:Rap1A Chimeric Protein

J. E. BUSS,[1]* L. A. QUILLIAM,[2] K. KATO,[1] P. J. CASEY,[3]† P. A. SOLSKI,[1] G. WONG,[4] R. CLARK,[4] F. McCORMICK,[4] G. M. BOKOCH,[2] AND C. J. DER[1]

La Jolla Cancer Research Foundation[1] and Department of Immunology, Scripps Clinic and Research Foundation,[2] La Jolla, California 92037; Department of Pharmacology, University of Texas Southwestern Medical School, Dallas, Texas 75235[3]; and Department of Molecular Biology, Cetus Corporation, Emeryville, California 94608[4]

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Although the Rap1A protein resembles the oncogenic Ras proteins both structurally and biochemically, Rap1A exhibits no oncogenic properties. Rather, overexpression of Rap1A can reverse Ras-induced transformation of NIH 3T3 cells. Because the greatest divergence in amino acid sequence between Ras and Rap1A occurs at the COOH terminus, the role of this domain in the opposing biological activities of these proteins was examined. COOH-terminal processing and membrane association of Rap1A were studied by constructing and expressing a chimeric protein (composed of residues 1 to 110 of an H-Ras activated by a Leu-61 mutation attached to residues 111 to 184 of Rap1A) in NIH 3T3 cells and a full-length human Rap1A protein in a baculovirus-Sf9 insect cell system. Both the chimeric protein and the full-length protein were synthesized as a 23-kDa cytosolic precursor that rapidly bound to membranes and was converted into a 22-kDa form that incorporated label derived from [^{3}H]mevalonate. The mature 22-kDa form also contained a COOH-terminal methyl group. Full-length Rap1A, expressed in insect cells, was modified by a C_{20} (geranylgeranyl) isoprenoid. In contrast, H-Ras, expressed in either Sf9 insect or NIH 3T3 mouse cells contained a C_{15} (farnesyl) group. This suggests that the Rap1A COOH terminus is modified by a prenyl transferase that is distinct from the farnesyl transferase that modifies Ras proteins. Nevertheless, in NIH 3T3 cells the chimeric Ras:Rap1A protein retained the transforming activity conferred by the NH_2-terminal Ras61L domain. This demonstrates that the modifications and localization signals of the COOH terminus of Rap1A can support the interactions between H-Ras and membranes that are required for transformation.

The Rap1A protein (also known as Krev-1 or smg p21) was isolated on the basis of strong structural (50% amino acid identity) and biochemical similarities to Ras (24, 35) and, independently, by its ability to reverse transformation caused by Ras proteins (27). Despite the many similarities between Ras and Rap1A proteins, the biochemical basis for this reversion remains unknown. It is possible that the regions of sequence identity between Rap1A and Ras allow Rap1A, when expressed at high levels (27), to act as a competitor for regulatory or target proteins of Ras. This model has recently received very strong support from studies that show that Rap1A can interfere in vitro with the activity of Ras–GTPase-activating protein (GAP) (17, 22), a protein which binds to and stimulates the GTPase activity of Ras, but which fails to affect the ability of Rap1A to hydrolyze GTP (17, 37). An alternate model suggests that Rap1A causes suppression through a mechanism independent of Ras; this would suggest that regions specific to Rap1A participate in interactions with proteins of an inhibitory pathway. This possibility is supported by evidence for a GAP-like protein that is unique to Rap1A (25). Because the greatest amino acid sequence divergence between Ras and Rap1A is in the COOH-terminal domain (25% identity in the last 74 residues), we examined whether this Rap1A-specific region abrogated transformation.

For Ras proteins, the COOH terminus is essential for localizing the protein to the plasma membrane (42, 43), in large part because of an unusual series of modifications to the last four amino acids, which have been termed a CAAX motif (C, cysteine; A, aliphatic; X, other) (8, 31, 36). These modifications include attachment of a C_{15} (farnesyl) isoprenoid to the cysteine of the CAAX motif (4, 21), removal of the final three amino acids (18, 20), and methylation of the newly exposed α-carboxyl group (8, 12, 20). In the H-Ras, N-Ras, and K-Ras4A proteins, a second lipid, palmitate, can be attached to a nearby cysteine (21). These modifications participate in as yet unknown ways to promote interactions between Ras proteins and the plasma membrane, but it is clear that such interactions are required for the biological function of both normal (11) and oncogenic (23, 40, 42) forms of Ras.

Although Rap1A has a CAAX sequence at its COOH terminus (24, 27, 35), its association with membranes, the presence of an isoprenoid, and other modifications have not been studied. It is possible that the Rap1A COOH terminus differs in some crucial modification or is bound to membranes differently from Ras. The protein might then interfere with transformation by binding a regulator or target of Ras in an inappropriate location. Competitive interference for a common effector has already been observed between membrane-bound and soluble (nonprocessed) forms of Ras (19, 33). In these cases, the soluble form is thought to inhibit the function of the membrane-bound form of Ras by trapping a Ras effector protein in a nonfunctional, cytosolic complex.

* Corresponding author.
† Present address: Section of Cell Growth, Regulation and Oncogenesis, Duke University Medical Center, Durham, NC 27710.

The NH_2-terminal domain of Rap1A is quite homologous (70% identity in the first 110 residues) to the NH_2 terminus of H-Ras and, of particular interest, has a threonine residue at position 61. Mutation of residue 61 in H-Ras to threonine causes oncogenic activation of the protein (9). This raises the possibility that the NH_2 terminus of Rap1A has the potential to be transforming, but is prevented from expressing this potential by Rap1A-specific sequences, perhaps those at its COOH terminus. An example of this type of inhibition by a COOH-terminal domain exists, although the mechanism is unknown: substitution of amino acids 138 to 218 from the COOH terminus of R-Ras (another Ras-related protein) is reported to inactivate the transforming activity of H-Ras in an H-Ras:R-Ras chimeric protein (28).

To test these ideas we first examined in detail the COOH-terminal modifications and membrane association of Rap1A. We then determined the ability of the NH_2 terminus of Rap1A to cause transformation, or the ability of the COOH terminus to interfere with transformation, when coupled to the H-Ras protein. The results indicate that the COOH terminus of Rap1A is modified by a geranylgeranyl isoprenoid that differs from the farnesyl attached to Ras. Despite this difference, the processing and membrane localization signals of Rap1A protein are sufficiently similar to those of Ras that the COOH-terminal Rap1A-specific domain can support transforming activity when fused to the Ras NH_2 terminus.

MATERIALS AND METHODS

Cell culture. *Spodoptera frugiperda* cells (Sf9 cells) were grown in Grace insect medium (GIBCO) supplemented with 3.33 mg of yeastolate and lactalbumin hydrolysate per ml and 10% heat-inactivated fetal calf serum (41). NIH 3T3 cells were grown in Dulbecco modified Eagle medium supplemented with 10% calf serum. Rat-1 cells were maintained in DMEM-F12 (1:1) supplemented with 10% fetal calf serum.

Construction of chimeric genes. For construction of chimeric genes, a 1.3-kb *Bam*HI fragment containing a cDNA sequence for the human *rap*1A gene (37) was used. Oligonucleotide-directed mutagenesis was performed to change *rap*1A codon 181 to create a cysteine (TGT)-to-serine (AGT) substitution in the CAAX motif of Rap1A [designated Rap1A(181S)]. Additionally, a 20-base synthetic oligonucleotide (5′-GATGTTCCCATGGTTTTGGT-3′) was used to introduce single-base substitutions into *rap*1A codons 110 (CCA to CCC) and 112 (GTT to GTG) to generate an *Nco*I site at codon 111 of the human *rap*1A sequence. Mutagenesis was performed by using either the double-primer method of Zoller and Smith (46) or the DNA polymerase III Mutator protocol (Stratagene). This introduced *Nco*I site corresponds to an *Nco*I site present in the homologous sequence in human H-*ras*. To construct the Ras61L:Rap chimera, we ligated a 0.4-kb *Bam*HI-*Nco*I fragment containing the coding sequence for H-Ras residues 1 to 110 to a 0.9-kb *Nco*I-*Bam*HI fragment containing the coding sequence for Rap1A residues 111 to 184. To construct the Rap1A:Ras chimera, we ligated a 0.4-kb *Bam*HI-*Nco*I fragment containing the coding sequence for Rap1A residues 1 to 110 to a 0.8-kb *Nco*I-*Bam*HI fragment containing the coding sequence for H-Ras residues 111 to 189. A myristylated form of Rap1A (designated Gag-Rap1A) was generated by ligation of a 0.4-kbp fragment, which encodes an 11-amino-acid Gag-derived myristylation signal sequence (2) and the first five residues of human H-Ras, to a 0.7-kb *Hin*dIII-*Bgl*II fragment that codes for Rap1A residues 6 to 184. The resulting chimeric genes were introduced into the *Bam*HI site of the pZIP-NeoSV(X)1 retrovirus vector plasmid (5).

Construction of recombinant plasmids and recombinant baculoviruses. Molecular constructs of *rap*1A were generated from either the human cDNA clone (37) or a synthetically generated *rap*1A sequence (44). Recombinant baculovirus vectors containing either the human H-*ras* or *rap*1A sequences were constructed in the pAcC12 vector plasmid (37). The pAcRAS vector was constructed by ligating a 0.7-kb *Hin*dIII-*Bam*HI fragment containing the coding sequence for residues 6 to 189 of normal human H-ras (from pBW 1699; a gift of Berthe M. Willumsen) plus the oligonucleotides CATGACAGAATACA and AGCTTGTATTCTGT (an *Nco*I-*Hin*dIII linker coding for residues 1 to 5) into the *Nco*I and *Bam*HI sites of the transfer vector pAcC5 (30). The *rap*1A vector (pAcRP1) was constructed by using either a 610-bp *Hin*dIII-*Eco*RI fragment containing a partial (residues 5 to 184) *rap*1A synthetic coding sequence, or a 0.7-kbp *Hin*dIII-*Bgl*II fragment of Ser-181-encoding Rap1A containing codons 6 to 184 as described previously (37, 45). Sf9 cells were transfected by using the calcium phosphate procedure, and recombinant viruses were isolated by procedures described by Summers and Smith (41).

DNA transfection of rodent fibroblasts. NIH 3T3 and Rat-1 cells were transfected by the calcium phosphate precipitation technique (10). Transfected cells were maintained in growth medium, and transformed foci were quantitated after 14 (NIH 3T3) or 24 (Rat-1) days. For protein analysis, transfected NIH 3T3 cells were isolated and established in growth medium containing 400 μg of G418 (geneticin; GIBCO) per ml.

Radiolabeling of NIH 3T3 mouse and baculovirus-infected Sf9 insect cells. G418-selected NIH 3T3 cells transfected with the appropriate plasmid DNA were labeled overnight with the appropriate radioisotope as described previously (4). Sf9 cells were plated at 6×10^5 cells per well and infected with baculovirus stocks at a multiplicity of infection of 10. After 48 h, the growth medium was removed and replaced with growth medium containing the specified radioisotope. Both NIH 3T3 and Sf9 cultures were incubated with 100 μCi of *R,S*-[5-^{3}H]mevalonolactone (Dupont, NEN) per ml in the presence of the indicated concentrations of compactin (a gift of M. S. Brown and J. L. Goldstein), or 100 μCi of [^{35}S]methionine/cysteine (Tran^{35}S-label; ICN) per ml, or 1 μCi of [^{3}H]palmitic acid (Dupont, NEN) per ml. After 16 h, cells were washed in phosphate-buffered saline (PBS) and solubilized in Hi-SDS RIPA buffer (0.5 M NaCl, 50 mM Tris [pH 7.0], 1% Trasylol, 1% sodium deoxycholate, 1% Nonidet P-40 NP-40, 0.5% sodium dodecyl sulfate [SDS], 0.1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol) for immunoprecipitation analysis. Analysis of carboxyl methylation was performed as described previously (6, 8). Briefly, infected Sf9 cultures were incubated overnight in growth medium containing 200 μCi of L-[*methyl*-^{3}H]methionine per ml (80 Ci/mmol; Dupont, NEN), and the resulting labeled cells were solubilized, immunoprecipitated, and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). After fluorography, the labeled protein bands were excised as described in Table 1 and incubated with 1 M NaOH for 12 h at 37°C, and the released volatile [^{3}H]CH_3OH was captured in Liquiscint and counted. Radioactivity remaining with the gel slice was determined after neutralization of the NaOH.

Immunoprecipitation and subcellular fractionation. The mouse anti-Ras monoclonal antibody 142-24E5 (7) recognizes a domain (H-Ras residues 96 to 118) that is conserved between H-Ras and Rap1A with only four amino acid

TABLE 1. Methylation of mature but not precursor forms of H-Ras and Rap1A in insect cells

Protein	Gel slice[a]	Amt of $[^3H]CH_3OH$[b] (cpm)	Amt of [^{3}H]Methionine (cpm)
H-Ras			
22 kDa	A	52	836
21 kDa	B	163	146
	C[d]	6	26
Rap1A			
23 kDa	A	87	1,422
22 kDa	B	272	240
	C[d]	14	46

[a] The fluorogram was aligned with the salicylate-impregnated gel, and 3-mm slices were removed from immediately below (C) the images of the precursor (A) or mature (B) proteins.
[b] Radioactivity released by NaOH.
[c] [^{3}H]Methionine remaining in the gel slice after alkali treatment.
[d] Radioactivity in slice C has been subtracted from that in slices A and B.

differences and also recognizes insect cell-expressed recombinant Rap1A protein (37). Detergent lysates of radiolabeled cells were boiled for 1 min, subjected to immunoprecipitation with the mouse monoclonal antibody 142-24E5 (4), and analyzed by SDS-PAGE and fluorography (4). For fractionation analysis, radiolabeled cells were lysed in hypotonic buffer and separated into crude membrane- and cytosol-containing fractions by centrifugation (100,000 × *g* for 30 min) as described previously (4). The cytosolic (S100) and membrane-containing (P100) fractions were then immunoprecipitated and analyzed as described above.

Chemical analysis of isoprenoids. The method for chemical analysis of isoprenoids is a modification of that previously reported (4) in which trypsin digestion is used to prepare samples for analysis. G418-selected NIH 3T3 cells that were transfected with retrovirus constructs encoding Ras61L:Rap, Ras(61L), or no insert [pZIP-NeoSV(X)1] were labeled with [^{3}H]mevalonic acid and immunoprecipitated as described above, and proteins were precipitated from the resulting preparation (50 μl) by the addition of 0.5 ml of 15% trichloroacetic acid. The precipitated protein was delipidated by three washes with acetone (>6 h per wash) at 0 to 2°C. The protein pellets were air dried and suspended in 200 μl of Tris-HCl (pH 7.7) containing 5% acetonitrile. Trypsin (8 μg in 10 μl of 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 8.0], 1 mM EDTA, 1 mM dithiothreitol, and 10 mM $MgSO_4$) was added to each sample. After an overnight incubation at 37°C, 200 μl of 25 mM Tris-HCl [pH 7.7] containing 80% acetonitrile was added. After a 4-h incubation at 20°C, 0.8 ml of 3% formic acid was added and the cleavage reaction initiated by the addition of 100 μl of methyl iodide. After an overnight stirred incubation in the dark, the methyl iodide was removed by evaporation under reduced pressure. The reaction was quenched by the addition of 150 μl of 35% Na_2CO_3, and the preparation was incubated in the dark for an additional 12 h. The resulting mixture was extracted twice with 1.2 ml of $CHCl_3$-CH_3OH (9:1), and the organic phases were combined and dried under a stream of N_2. The sample was dissolved in 400 μl of 50% CH_3CN–25 mM H_3PO_4 (solvent A) and spiked with 6 nmol each of geraniol, farnesol, and geranylgeraniol, and a sample (60% of the total) was injected onto a C_{18} reverse-phase high-pressure liquid chromatography (HPLC) column (0.5 by 25 cm; Phenomenex). The column was developed with a 40-ml linear gradient of solvent A to 100% CH_3CN–25 mM H_3PO_4 (solvent B), followed by 20 ml of solvent B, at a flow rate of 1 ml/min. Fractions of 1 ml were collected and suspended in Liquiscint, and their radioactivity was determined by scintillation counting. Control samples were processed identically except that the methyl iodide was omitted from the reactions. *trans*-Geraniol and *trans,trans*-farnesol were obtained from Aldrich Chemical. All-*trans* geranylgeraniol was a gift of R. Coates, University of Illinois.

RESULTS

Prenylation and membrane localization of Rap1A in mammalian cells. A chimeric protein, consisting of residues 111 to 184 of Rap1A attached to residues 1 to 110 of a transforming version (Leu-61) of the H-Ras protein, was used to study processing of the COOH terminus of Rap1A in mammalian cells (Fig. 1). When NIH 3T3 cells transfected with DNA encoding the Ras61L:Rap protein were labeled overnight with [^{3}H]mevalonolactone ([^{3}H]MVA), a 22-kDa protein that was ca. eightfold more abundant than the endogenous Ras and Ras-related proteins could be detected by immunoprecipitation with the 142-24E5 antibody (Fig. 2A). Examination of cytosolic and particulate fractions revealed that the prenylated 22-kDa chimeric Ras61L:Rap protein was associated primarily with cellular membranes (Fig. 2A). Pulse-chase analysis of Ras61L:Rap labeled with [^{35}S]methionine/cysteine showed that the chimeric protein was synthesized as a ca. 23-kDa cytosolic precursor that rapidly associated with membranes and was converted to a mature form that comigrated with the prenylated 22-kDa protein (Fig. 2B). All three forms (the soluble 23-kDa form, the particulate 23-kDa form, and the particulate 22-kDa form) could be observed in proteins synthesized during an overnight incubation with [^{35}S]methionine/cysteine (Fig. 2C). Both the 23-kDa soluble and 23-kDa particulate forms accumulated in cells labeled during overnight treatment with 20 μM compactin, an inhibitor of mevalonate and therefore isoprenoid biosynthesis (Fig. 2C). At higher concentrations of compactin, only the soluble 23-kDa form was observed (data not shown). The 23-kDa particulate form migrates just ahead of the 23-kDa soluble form, suggesting that it has undergone some subtle modification. The mechanism(s) by which the 23-kDa form associates with membranes is being studied, but appears not to involve direct prenylation of the Rap1A protein or modifications that grossly alter the mobility of the protein on gels.

Isoprenylation of H-Ras and Rap1A in insect cells. The full-length Rap1A protein was introduced into NIH 3T3 cells, but was not expressed at levels sufficient for biochemical analysis. High-level expression of Rap1A in NIH 3T3 cells does not appear to be tolerated, except in the presence of an oncogenic Ras protein (unpublished observation). Modifications of full-length Rap1A were therefore studied by using the baculovirus-insect cell system to produce larger amounts of protein. In Sf9 cells infected with the virus encoding Rap1A, two proteins, of ca. 22 and 23 kDa, were immunoprecipitated specifically (Fig. 3, lane 1). As with the chimeric Ras61L:Rap protein in NIH 3T3 cells, the 23-kDa form accumulated in compactin-treated cells (Fig. 3, lane 2). Although only a small amount of Rap1A was processed to the 22-kDa form under these conditions, incorporation of a product of [^{3}H]MVA into Rap1A could be detected (lane 3). Rap1A could also be detected in lysates of [^{3}H]MVA-labeled Sf9 cells (lane 4) without the need for immunoprecipitation and was at least 10-fold more abundant than endogenous isoprenylated proteins in these infected cells. Subsequent experiments showed that larger amounts of [^{3}H]MVA were

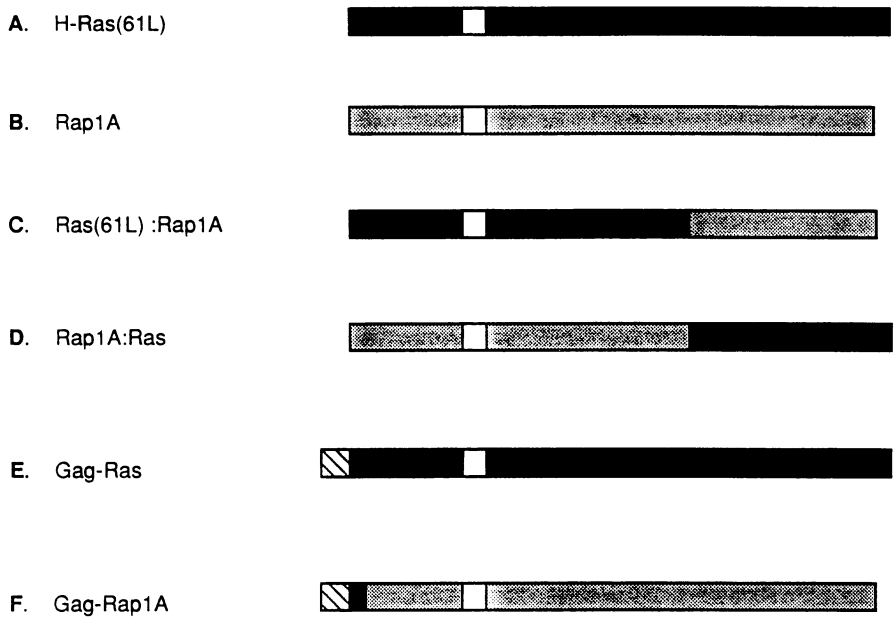

FIG. 1. Structure of chimeric Rap1A proteins. Proteins encoded by molecular constructs containing H-Ras (■), Rap1A (▩), and Gag-derived myristylation signal sequences (▨) are shown; the identical sequences of the putative effector domains of H-Ras and Rap1A (residues 32 to 40) are also indicated (□). (A) H-Ras(61L), which contains the full-length, 189-amino-acid sequence of the human H-Ras protein with a Leu-61 activating mutation. (B) Rap1A, which contains the full-length, 184-amino-acid sequence of human Rap1A. (C) Ras61L:Rap1A, which contains H-Ras(61L) amino acids 1 to 110 followed by Rap1A amino acids 111 to 184. (D) Rap1A:Ras, which contains Rap1A residues 1 to 110 followed by H-Ras amino acids 111 to 189. (E) Gag-Ras, which contains the first 6 amino acids of the rat leukemia virus p15 Gag linked to 5 amino acids encoded by the pAT-H-ras vector sequence and the full 189-amino-acid sequence of normal human H-Ras. (F) Gag-Rap1A, which contains the first 6 amino acids of the rat leukemia virus p15 Gag linked to 5 vector-derived amino acids, the first 5 amino acids of H-Ras, and Rap1A amino acids 6 to 184.

incorporated if compactin was omitted and processing was allowed to take place at the normal rate (data not shown). Even without compactin, the abundance of the 23-kDa precursor form suggested that, as had been observed with the H-Ras protein (29), processing of Rap1A was inefficient in insect cells, possibly because of the large amounts (1 to 5% of total protein) of protein which were expressed. In Sf9 cells, as in NIH 3T3 cells, the 23-kDa form of the Rap1A protein was found in both the cytosolic and the membrane-containing (P100) fractions, whereas the mature, prenylated 22-kDa form was detected only in the membrane fraction (lanes 5 and 6). A mutant Rap1A protein (Rap181S), in which the cysteine of the CAAX motif had been mutated to a serine, was neither labeled detectably with [^{3}H]MVA (data not shown) nor processed to the mature 22-kDa form (lane 7). This makes it quite likely that, as with Ras proteins, the CAAX cysteine of Rap1A is the site at which the isoprenoid is attached and demonstrates that Sf9 cells contain prenyl transferase(s) capable of attaching isoprenoids to both Rap1A and Ras. These data confirm the observations made with the chimeric protein that the COOH terminus of Rap1A is prenylated.

The identity of the isoprenoid attached to the Rap1A protein was examined by direct chemical analysis. Hydrolysis of [^{3}H]MVA-labeled Rap1A with methyl iodide released radioactivity, indicating that the isoprenoid was attached through a thioether linkage to a cysteine residue. The isoprenoid attached to the full-length Rap1A protein, expressed in Sf9 cells, was identified as a C_{20} (geranylgeranyl) group (Fig. 4A), whereas the H-Ras protein was modified by a C_{15} (farnesyl) isoprenoid (Fig. 4B). The oncogenic H-Ras protein from Harvey murine sarcoma virus, expressed in NIH 3T3 cells, was also modified by a farnesyl group (Fig. 4C). In all three cases, more than 80% of the isoprenoid was of a single size, an indication that the isoprenoids were not altered or interconverted during sample preparation.

Methylation of Rap1A. In mammalian cells, the mature, 21-kDa form of the H-Ras protein is methylated, apparently on the α-carboxyl group of the cysteine of the CAAX motif, which becomes exposed after the final three (AAX) residues are removed (8). In Sf9 cells, the 21-kDa form (but not the 22-kDa precursor) of H-Ras incorporated radioactivity derived from [^{3}H]methylmethionine that could be released as volatile [^{3}H]CH_3OH by NaOH and captured in liquid scintillant for analysis (Table 1). Such lability is a characteristic of methylated α-carboxyl groups (8). The 22-kDa mature form of the Rap1A protein (but not the 23-kDa presursor) was also methylated (Table 1), demonstrating that modification of Rap1A resembles that of H-Ras in this respect. The 22-kDa form of the chimeric Ras61L:Rap protein synthesized in NIH 3T3 cells also contained base-labile methyl groups (data not shown). The observations that the α-COOH group of the Rap1A precursor is not methylated and that the electrophoretic mobility differs between the precursor and methylated form suggest that carboxy-terminal trimming of amino acid residues occurs in Rap1A processing. By analogy with Ras and mating factors, it is likely that all three of the AAX residues are trimmed. This indicates that both trimming and methylation occur in insect cells.

Lack of palmitate in Rap1A. The COOH terminus of the H-Ras protein is modified by a second type of lipid, palmitate, which is proposed to be attached to cysteine residues (residues 181 and/or 184) near cysteine 186 of the CAAX motif (21). The Rap1A protein has no cysteine near the

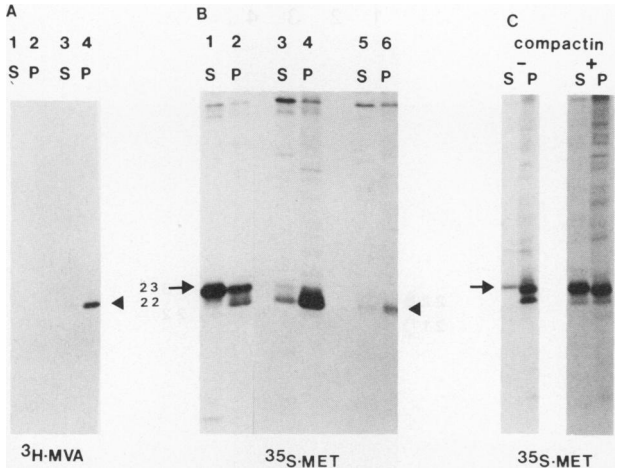

FIG. 2. Isoprenylation and membrane binding of chimeric Ras61L:Rap protein. (A) Incorporation of [^{3}H]MVA into Ras61L:Rap protein. NIH 3T3 cells (lanes 1 and 2) or cells expressing the Ras61L:Rap protein (lanes 3 and 4) were labeled overnight with [^{3}H]MVA, and soluble (S) or particulate (P) fractions were prepared. Immunoprecipitates were formed with antibody 142-24E5 and analyzed by SDS-PAGE with a 21-day fluorographic exposure. Symbols: →, 23-kDa precursor; ◀, mature 22-kDa Ras61L:Rap protein. (B) Cells expressing the Ras61L:Rap protein were labeled for 30 min with [^{35}S]methionine/cysteine, and soluble (S) or particulate (P) fractions were prepared immediately (lanes 1 and 2), or after a 3-h (lanes 3 and 4) or 14-h (lanes 5 and 6) chase in nonradioactive medium. Immunoprecipitates were analyzed as in panel A, with a 14-day fluorographic exposure. (C) Cells expressing the Ras61L:Rap protein were labeled overnight with [^{35}S]methionine/cysteine in the absence (−) or presence (+) of 20 μM compactin. Soluble (S) and particulate (P) fractions were prepared, and immunoprecipitates were analyzed as in panel A, with a 7-day fluorographic exposure.

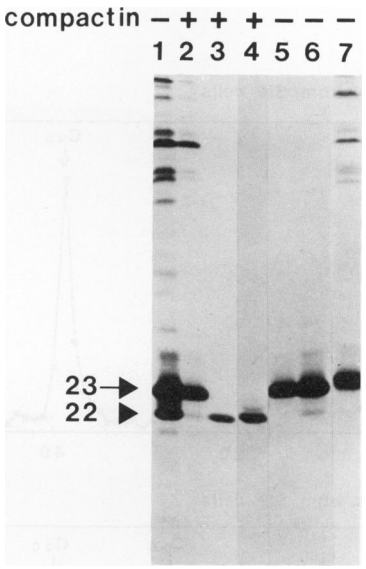

FIG. 3. Prenylation and membrane association of Rap1A in insect cells. Sf9 cells were infected with virus encoding Rap1A or Rap1A(181S) (lane 7). Cells were labeled overnight with [^{35}S]methionine/cysteine (lanes 1, 2, 5, 6, and 7) or [^{3}H]MVA (lanes 3 and 4) in the absence or presence of 10 μM compactin as indicated. Soluble (lane 5) and particulate (lane 6) fractions were also prepared. Immunoprecipitates formed with antibody 142-24E5, or total cellular lysates (lane 4), were analyzed by SDS-PAGE followed by a 5-day fluorographic exposure. Symbols: →, position of the 23-kDa precursor; ▶, position of the mature 22-kDa form of Rap1A.

COOH terminus other than that of its CAAX sequence (24, 27, 35). However, because little is known about sites of palmitoylation, it was considered possible that the CAAX cysteine of Rap1A was modified alternatively by either one lipid or the other. This possibility was tested by comparing the incorporation of [^{3}H]palmitic acid into the full-length Rap1A protein with that of H-Ras expressed in Sf9 cells. Labeling of the mature, 21-kDa form of H-Ras could be observed easily (Fig. 5, lane 2), whereas the Rap1A protein, expressed at equivalent levels, failed to incorporate detectable amounts of [^{3}H]palmitate (lane 3), although the cells infected with the Rap1A virus incorporated [^{3}H]palmitic acid into proteins efficiently (data not shown). This indicates that Rap1A is not palmitoylated and, in this respect, resembles the nonpalmitylated K-Ras4B protein (21, 23) more closely than it resembles H-Ras.

Ability of the COOH-terminus of Rap1A to affect transformation. We examined the ability of the NH_2- and COOH-terminal domains of Rap1A to participate in or prevent transformation in two rodent fibroblast cell lines, NIH 3T3 and Rat-1 cells, which differ in sensitivity to transformation by Ras proteins. Full-length Rap1A was not transforming, nor was a chimera between a normal cellular Ras NH_2 terminus and the COOH terminus of Rap1A (Table 2). However, the chimera possessing the NH_2 terminus of activated H-Ras(61L) and the COOH-terminal third of Rap1A caused transformation of both NIH 3T3 and Rat-1 cells. Transformation appeared to be dependent upon prenylation of the chimeric protein, as mutation of the CAAX cysteine 181 to serine abolished the transforming activity of the chimera (Table 2). The lack of palmitoylation of the Rap1A COOH terminus (Fig. 5), which is present in the Ras61L:Rap chimera, indicates that H-Ras does not require a palmitylated COOH terminus to cause transformation, but may explain in part the observed decrease in transforming potency from full-length H-Ras (21). A reciprocal chimera, between the NH_2 terminus of Rap1A and the COOH terminus of Ras, failed to cause transformation even in the very sensitive NIH 3T3 cell assay. A myristoylation signal sequence derived from a retroviral Gag protein has also been shown to activate the transforming potential of normal, cellular H-Ras (2). However, full-length, normal Rap1A protein bearing a Gag myristoylation signal sequence did not cause transformation.

DISCUSSION

Our results provide direct evidence that Rap1A is both prenylated and membrane bound. Rap1A thus joins the H-Ras, N-Ras, K-Ras4A, and K-Ras4B proteins (4, 21), as well as the ras-related proteins G25K (32) and rac 1 and rac 2 (13), the nuclear lamins A and B (15) and the gamma subunits of heterotrimeric G proteins (16, 34, 44) as a member of the growing family of identified prenylated proteins. The CAAX motif of the Rap1A protein (Cys-Leu-Leu-Leu) is therefore able to function as a signal for prenylation.

The finding that insect cells can produce all of the known modifications of Ras (29) (and, as shown here, Rap1A) is significant because none of these processing steps occur in bacterial cells. Production of these proteins in insect cells therefore represents an important method of obtaining both

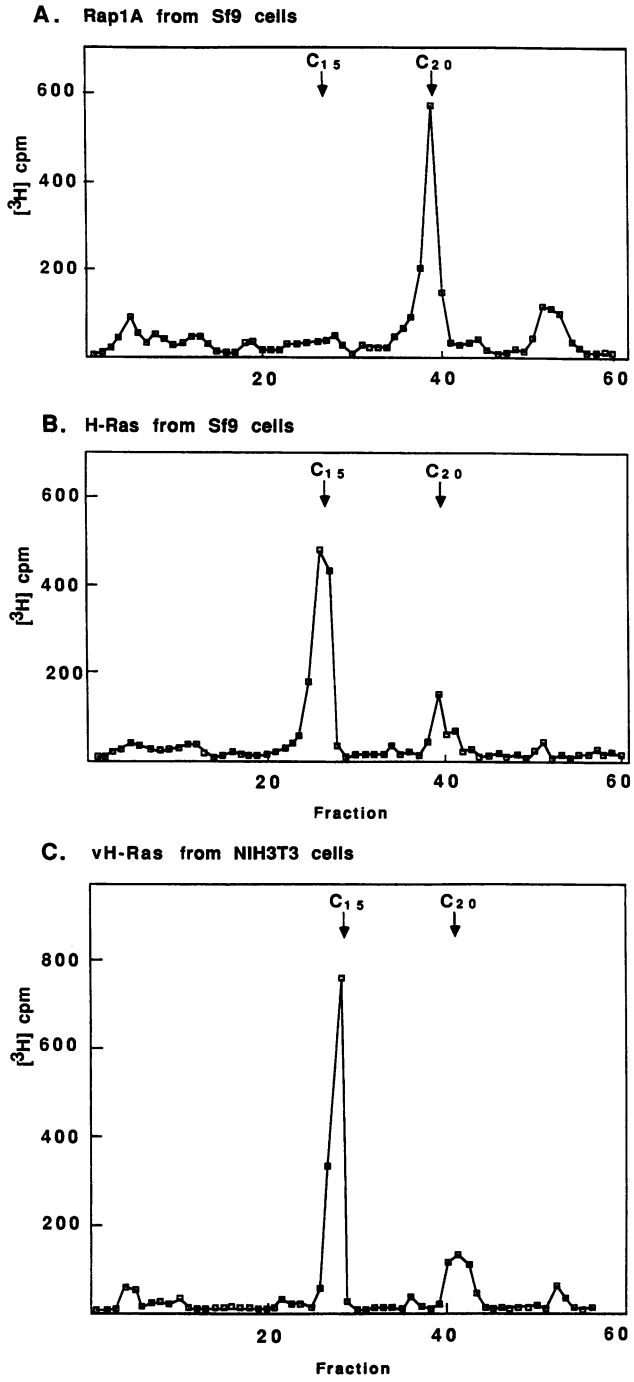

FIG. 4. HPLC analysis of isoprenoid released from [^{3}H]MVA-labeled Rap1A and H-Ras expressed in insect cells or from H-ras expressed in NIH 3T3 cells. (A) Rap1A protein was labeled overnight with [^{3}H]MVA without compactin, isolated by immunoprecipitation with antibody 142-24E5, washed, and hydrolyzed with methyl iodide as described in Materials and Methods. Elution positions of authentic isoprenoids are indicated by arrows. (B) H-Ras protein labeled and analyzed as for panel A. (C) H-Ras protein of Harvey sarcoma virus in NIH 3T3 cells, labeled overnight with [^{3}H]MVA and 20 μm compactin, and analyzed as for panel A.

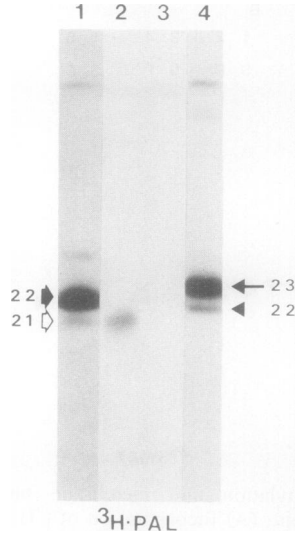

FIG. 5. Palmitylation of H-Ras but not Rap1A. Sf9 cells were infected with virus encoding H-Ras (lanes 1 and 2) or Rap1A (lanes 3 and 4) and labeled overnight with [^{3}H]palmitic acid (^{3}H-PAL) (lanes 2 and 3) or [^{35}S]methionine/cysteine (lanes 1 and 4). Immunoprecipitated proteins were analyzed as for Fig. 1 and detected by fluorographic exposure for 4 days. Arrows indicate the positions of the precursor and mature forms of the proteins.

unprocessed and fully modified Ras and Ras-related proteins. A comparison of Rap1 proteins purified from Sf9 cells and human neutrophils showed that proteins derived from either source displayed essentially identical properties (37). However, in insect cells the amount of Rap1A or Ras that is processed completely is small. We have found that supplementation of growth medium with mevalonate does not increase greatly the amount of processed protein, indicating that availability of this precursor of isoprenoids does not limit processing (23a).

TABLE 2. Transforming activity of chimeras between H-Ras and Rap1A

Protein	No. of transformed foci/μg of DNA[a] in:	
	NIH 3T3 cells[a]	Rat-1 cells
Rap1A	0	0
Ras:Rap1A	0	0
Ras61L:Rap1A	427	222
Ras61L:Rap1A(181S)	0	0
Rap1A:Ras	0	0
Gag-Rap1A	0	0
Gag-Ras	927	ND[c]
H-Ras	0	0
H-Ras(61L)	4,950	661

[a] Cells were transfected with 10 ng or 1 μg of DNA per 60-mm dish for transforming (Leu-61) or nontransforming constructs, respectively. The number of transformed foci was determined after 14 days. The numbers are the average of three independent assays.

[b] Cells were transfected with 2 μg of DNA per 60-mm dish and subcultured into 100-mm dishes after 3 days, and the number of transformed foci was determined after an additional 21 days. The numbers are the averages of two to four plates.

[c] ND, Not determined.

Very recently a new class of prenylated proteins, modified by the unusual C_{20} isoprenoid, geranylgeranyl, has been described (14, 39). Proteins modified by the C_{20} isoprenoid appear to account for the majority of prenylated proteins (3a, 14, 39). The first of these C_{20}-modified proteins to be identified include the gamma subunit of heterotrimeric G proteins (34, 44), a Ras-related protein known as G25K (32), and now the Rap1A protein. K-Ras4B (4), the lamin B protein (15), and, as shown here, H-Ras, belong to a smaller family of proteins which are modified by the C_{15} isoprenoid, farnesyl. From the few examples available, it appears that only one isoprenoid is linked to a given protein. The modification of each protein by a single isoprenoid suggests that there may be two different and selective prenyl transferases, a farnesyl transferase and a geranylgeranyl transferase, which recognize some aspect (quite possibly the CAAX motif itself [38]) of only the appropriate proteins. As with Ras proteins, prenylation is apparently important for Rap1A function. Thus, replacement of the CAAX cysteine residue by a serine, which prevents prenylation, abolishes the transforming activity of the chimeric Ras61L:Rap protein and also reduces the ability of Rap1A to cause suppression (26). The indication that the prenyl group attached to Ras proteins differs from that of most cellular prenylated proteins provides hope that agents can be developed to interfere with farnesylation without compromising geranylgeranyl modification of the larger group of cellular proteins. However, the opposing biological activities of Ras proteins and Rap1A, despite their similar dependence on prenylation for activity, imply that gross disturbances of isoprenoid synthesis such as those produced by lovastatin or compactin will probably upset the balance between the promotion or inhibition of growth caused by these proteins in ways that may not be predictable or easy to interpret.

Ras appears to be modified by a C_{15} isoprenoid when expressed in either NIH 3T3 cells or Sf9 insect cells. Thus, insect cells appear to be capable of modifying prenylated proteins in the same manner as mammalian cells are. In addition, we have shown recently that Rap1A can be modified by geranylgeranyl in vitro (3a). Confirmation of the isoprenoid type attached to Rap1A or the chimeric Ras61L:Rap protein in NIH 3T3 cells awaits the development of cell lines that express larger amounts of these proteins. However, if these proteins are modified by geranylgeranyl in mammalian cells, the transforming activity of the chimeric Ras61L:Rap protein suggests that the somewhat longer C_{20} prenyl group is capable of supporting functional and transforming interactions of Ras with membranes.

The Rap1A COOH terminus does not appear to lack any crucial modifications or interfere with binding of Ras to membranes. The failure of the COOH terminus of Rap1A to suppress the transforming activity of the NH_2 terminus of H-Ras(61L) indicates that the many Rap1A-specific residues in this domain do not impose a biological response on the (chimeric) protein. This implies that it is the NH_2-terminal domains of Ras and Rap1A that determine the biological activities of the respective proteins. This model is supported by our observation and that of Zhang et. al. (45) that the NH_2 terminus of Rap1A (despite the presence of a potentially "activating" threonine residue at position 61) does not gain transforming activity when coupled to the H-Ras COOH terminus.

Recent analysis of a series of Ras:Rap1A chimeras by Zhang et al. (45) has defined a remarkably small number of Rap1A NH_2-terminal amino acids that not only are required for suppressor activity but also, when mutated to resemble Ras, convert Rap1A into a transforming protein. These residues border a region of what is called an effector domain (residues 32 to 40), which is identical between Ras and Rap1A (24, 27, 35) and is important for interaction of Ras proteins with the GAP (1, 3). Rap1A, although having a GAP of its own (25), which appears to interact with the same domain (37), may also bind Ras-GAP, because competition of Rap1A with Ras for Ras-GAP has been detected in vitro (17, 22). These results raise the interesting possibility that Rap1A acts as a competitor or buffer for Ras-GAP. Although the isoprenoid attached to Ras appears to differ from that on Rap1A, it appears to be functionally interchangeable. Thus, it is likely that the opposing biological activity of these two proteins is regulated primarily by the competition for effectors, rather than the balance of their interactions with membranes.

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