Prenyl group identification of rap2 proteins: a ras superfamily member other than ras that is farnesylated

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Rap proteins comprise a subset of the large family of ras-related proteins. They contain the C-terminal tetrapeptide sequence motif Cys-Ali-Ali-Xaa (Ali is an aliphatic amino acid and X is any amino acid), which has been found to be the site of membrane attachment via isoprenylation for ras, nuclear lamins and the γ subunits of the heterotrimeric G-proteins. To investigate the isoprenylation of rap2a and rap2b, human cDNAs coding for these proteins were expressed in COS cells incubated in the presence of [^{3}H]mevalonolactone. Both proteins incorporated a product of [^{3}H]mevalonolactone, as judged by Western blot analysis. To identify the specific isoprenoid attached to each protein, the cDNAs were transcribed *in vitro* and the rap2 specific RNA was translated in a rabbit reticulocyte lysate system in the presence of [^{3}H]mevalonolactone. The translation products were treated with methyl iodide and the released isoprenoid groups were analysed by h.p.l.c. Rap2b, which terminates in Cys-Val-Ile-Leu, is geranylgeranylated as predicted while rap2a, which terminates in Cys-Asn-Ile-Gln, incorporated farnesyl. A mutant construct generated by site-directed mutagenesis of rap2a cDNA yielding a protein terminating in leucine instead of glutamine incorporated geranylgeranyl, lending further support to the notion that isoprenoid specificity is governed by the terminal amino acid. In addition, when the CAAX motif cysteine at position 180 of rap2a was replaced by a serine residue no isoprenoid incorporation was observed. Thus rap2a and rap2b, despite showing 90% sequence identity, incorporate different isoprenoid groups. Thus glutamine is a signal for farnesylation, and rap2a is the first non-ras member of the ras superfamily that is farnesylated.

INTRODUCTION

Rap proteins are low-molecular mass GTP-binding proteins that show a high degree of sequence identity to the *ras* oncogene product [1]. The greatest sequence similarity between rap and ras occurs in the regions important for ras function, including four highly conserved nucleotide binding domains, a putative effector domain, and a tetrapeptide sequence in the C-terminus involved in membrane localization [2,3]. Currently, four rap genes subdivided into two groups by sequence similarity have been isolated [1,4,5]. Although the function of rap proteins is largely unknown, one member, rap1a (smg-21, Krev-1), has been shown to revert the transformed phenotype of a K-*ras*-transformed 3T3 cell line [6]. Rap1b is phosphorylated by both a cyclic AMP-dependent protein kinase (protein kinase A) [7] and a neuronal calcium/calmodulin-dependent kinase [8]. Rap2 (hereafter referred to as rap2a) [9] and the more recently isolated rap2b [10] have been expressed in *Escherichia coli* systems, both bind GTP and exhibit low intrinsic GTPase activity. Rap1 proteins are found in many animal tissues [11], whereas rap2 proteins are found mostly in platelets and brain tissue (F. X. Farrell and E. G. Lapetina, unpublished work). In addition, all rap proteins are usually associated with the membrane fraction of the tissues examined.

It is generally accepted that ras must exist in the GTP-bound state and be localized at the plasma membrane in order to exert transforming activity [12]. The localization to the plasma membrane occurs by sequential post-translational modifications at the C-terminus, specifically the last four amino acids. This tetrapeptide sequence found on all ras proteins, most nuclear lamins and several low-molecular-mass GTP-binding proteins, including all rap family members, has been denoted as the CAAX motif (C is cysteine, A is an aliphatic amino acid and X is any amino acid) [13]. The first step in the processing event involves the incorporation of an isoprenoid on cysteine via a thioether linkage. This step is followed by the proteolytic cleavage of the last three amino acids, AAX, and then by methyl esterification at the newly exposed cysteine residue [14]. The isoprenoid moiety associated with ras has been identified as farnesyl [15]. It has been proposed that the first step (isoprenylation) precedes membrane association, while proteolysis and methylation occur upon association. The localization of ras to the membrane is stabilized by palmitoylation at an upstream cysteine (H-ras, N-ras) or by a polybasic sequence (K-ras) [16].

In addition to the 15 isoprenoid, several CAAX motif proteins incorporate the C_{20} carbon isoprenoid geranylgeranyl. A general rule for predicting the isoprenoid attached has emerged from analysis of the CAAX amino acid sequence of the prenylated proteins characterized to date. Proteins which end in methionine or serine incorporate farnesyl, while proteins terminating in leucine incorporate geranylgeranyl. Recent evidence indicates that the attachment of the respective isoprenoid to the protein occurs by the action of two different enzymes. Surprisingly, the two enzymes have similar molecular masses on gel filtration [17]. Farnesyltransferase has been purified to homogeneity and exists as a dimer of non-identical subunits, designated α and β [17].

In this report we identify the lipid moiety attached to rap2a and rap2b. The two proteins are 90% similar, with the greatest divergence in the C-terminus. Interestingly, the two proteins possess different CAAX motifs. Rap2b terminates in a leucine, suggesting that the protein is geranylgeranylated. In contrast, rap2a terminates in glutamine. We present evidence that rap2a incorporates farnesyl.

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol.

MATERIALS AND METHODS

Materials

[^{3}H]Mevalonolactone (27.8 Ci/mmol) was purchased from New England Nuclear. pSVL and pGEM-3Z were purchased from Pharmacia Molecular Biologicals and Promega respectively. All transcription and translation reagents were purchased from Promega. Methyl iodide and lipid standards *trans*-geranol (C_{10}), all-*trans*-farnesol (C_{15}) were purchased from Aldrich. Geranylgeranol was provided by Dr. Pat Casey, Duke University. Nitrocellulose was purchased from Schleicher and Schuell. PCR reagents were obtained from GeneAmp (Perkin–Elmer Cetus). All tissue culture reagents were purchased from Gibco/BRL

Subcloning of rap2 cDNAs into the eukaryotic expression vector pSVL

Two pairs of oligonucleotide primers were synthesized corresponding to the flanking DNA sequence of each cDNA, with a *Bgl*II restriction site added 5′ to the coding sequence. The isolation and sequencing of rap2a and rap2b are described elsewhere [1,5]. The sequences of the respective primer pairs were as follows: rap2a: 5′-CCCGGGAGATCTCCACCATGCGCGAGTACAAAGTG-3′ and 5′-CCCGGGAGATCTTTACTATTGTATGTTACATGC-3′; rap2b: 5′-CCCGGGAGATCTCCACCATGAGAGAGTACAAAGTG-3′ and 5′-CCCGGGAGATCTTTATCAGAGGATCACGCAGGC-3′. The PCR reaction was performed as described by the manufacturer. The elongated products were purified and digested with *Bgl*II overnight and cloned into a *Bam*HI-digested pSVL. The orientation and integrity of the insert was confirmed by the double-stranded sequencing protocol of Sequenase (U.S. Biochemical).

Transient transfection and labelling of COS cells

COS cells were plated at a density of 5×10^5 cells per 75 cm^2 tissue culture flask on the day before transfection. Plasmid DNA (15 μg) was transfected using Lipofectin (Gibco/BRL). Transfection was carried out according to the manufacturer's directions with minor modifications. DNA and Lipofectin were diluted separately in OptiMEM (Gibco). The DNA and Lipofectin were gently mixed together and added to cells dropwise. The DNA–Lipofectin complex was incubated on the cells for 24 h, followed by the addition of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. The incubation was continued for an additional 24 h. At 36 h post-transfection the medium was removed from each flask and replaced by DMEM supplemented with 10% fetal bovine serum containing 100 μCi/ml of [^{3}H]mevalonolactone. At 4 h prior to the addition of [^{3}H]mevalonolactone, lovastatin (25 μM) was added to the medium to enhance labelling. The cells were incubated overnight and harvested as described.

Antibody

Antibody 9264 is a polyclonal rabbit antibody directed against a bacterially expressed recombinant rap2b protein. Antibody specificity and characterization are described elsewhere [18]. The antibody recognizes both rap2a and rap2b equally well (results not shown).

Electrophoresis and immunoblotting

SDS/PAGE was performed as described by Laemmli [19] using 12.5% acrylamide minigels. COS cell lysates (control and transfected) were obtained by repeated freeze–thawing in 0.25 mM Tris, pH 7.8. Cellular proteins (50 μg) were solubilized in SDS/PAGE sample buffer [60 mM Tris, pH 7.4, 10% (v/v) glycerol, 3% (w/v) SDS; 1 mM dithiothreitol (DTT)] before loading on to the gel. The gels were electrophoretically transferred to nitrocellulose using the Bio-Rad Mini Protean Blotter. Blots were incubated with antibody 9264 (diluted 1:100) for 2 h and developed using alkaline phosphatase-linked goat anti-rabbit IgG. The developed blots were soaked in Autofluor reagent, dried and exposed to X-Omat AR film at −80 °C with an intensifying screen for 3 weeks.

In vitro transcription and translation

The full-length cDNAs of rap2a and rap2b isolated from a human platelet cDNA library were subcloned into the *Eco*RI site of pGEM-3Z. Rap2a constructs containing C-terminal mutations were generated via PCR using oligonucleotide primers as described above. The sequences of the primers generated to replace the terminal glutamine with leucine or cysteine at position 180 with a serine residue were as follows. (1) N-Terminus: 5′-CCCGGGAGATCTCCACCATGCGCGAGTACAAAGTG-3′; (2) rap2a-CNIL: 5′-CCCGGGAGATCTTTACTATAGTATGTTACATGC-3′; (3) rap2a-SNIQ: 5′-CCCGGGAGATCTTTACTATTGTATGTTACTTGCAG-3′.

The PCR-generated fragments were digested with *Bgl*II and subcloned into the *Bam*HI site of pGEM-3Z. Orientation of the inserts was established by sequencing and/or restriction digest analysis. Run-off transcripts were prepared by digesting the plasmids at the unique *Nar*I site located 3′ to the insert. The linearized plasmids were transcribed with SP6 polymerase according to the manufacturer's instructions (Promega). RNA transcripts were translated in a methionine-deficient rabbit reticulocyte lysate system according to manufacturer's instructions (Promega). A standard 50 μl reaction containing 2 μg of RNA and 4 μl of [^{35}S]methionine (1232 Ci/mmol) at 10 mCi/ml was incubated for 1 h at 30 °C. A 10 μl aliquot of the reaction was subjected to SDS/PAGE and exposed to Kodak X-AR film overnight. Prestained low-molecular-mass markers (Bio-Rad) were used to orient the autoradiograph to the dried gel.

In vitro isoprenylation of translation products

The *in vitro* isoprenylation of rap2 proteins was carried out in a standard reaction mixture [35 μl of rabbit reticulocyte lysate (nuclease-treated), 5 μl of water, 1 μl of RNasin ribonuclease inhibitor (40 units/μl), 1 μl of a 1 mM amino acid mixture and 2 μl of RNA (1 μg/μl)] as described above with the following modifications. All components were added to a microcentrifuge tube containing 25 μCi of [^{3}H]mevalonolactone dried under a stream of nitrogen. The reaction mixture was incubated at 30 °C for 1 h followed by incubation at 37 °C for an additional 1 h. A 25 μl sample of the reaction mixture was subjected to SDS/PAGE. The area of the gel containing the translation product was excised from the gel and the radiolabelled protein was eluted from the gel slice in a buffer containing 25 mM Tris, pH 7.5, 190 mM glycine and 0.1% SDS. The sample was concentrated in a 200 μl volume and subjected to precipitation as described below.

Lipid analysis of labelled rap2 proteins

Prenyl group identification was by the method of Farnsworth et al. [20] with minor modifications. The electroeluted translation

product was subjected to protein precipitation by the addition of trichloroacetic acid to a final concentration of 15%. The sample was incubated on ice for 15 min in a 1.5 ml Microfuge tube, followed by centrifugation in a refrigerated microcentrifuge for 15 min. The protein precipitate was suspended in 0.5 ml of ice-cold acetone and placed on ice for 4 h. The protein precipitate was centrifuged in a refrigerated Microfuge for 15 min and the acetone wash of the protein precipitate was repeated twice. After the last wash the pellet was air-dried and suspended in 200 μl of 100 mM Tris, pH 7.7, containing 5% (v/v) acetonitrile. A proteinase K (Boehringer Mannheim) solution was prepared (8 mg/ml in 50 mM Hepes, pH 8.0, 1 mM EDTA, 1 mM DTT, 10 mM $MgSO_4$) and 10 μl was added to the sample which was then incubated for 2 h at 37 °C. The sample was then transferred to an amber vial with a Teflon-coated cap containing a small stir bar, and 200 μl of 25 mM Tris, pH 7.7, containing 80% acetonitrile was added. The sample was prepared for thioether cleavage by the addition of 800 μl of 3% formic acid followed by the addition of 100 μl of methyl iodide (Aldrich). The methyl iodide was added in low light and the reaction was allowed to proceed overnight with gentle stirring in the dark. The methyl iodide was removed from the vial by first removing the aqueous layer (top) and subjecting the remaining methyl iodide to evaporation under reduced pressure for 1 h. The aqueous layer was then added back to the original vial and the evaporation was allowed to continue for an additional 1 h. The solution was neutralized by the addition of 30% sodium carbonate (100 μl added in 20 μl aliquots) The solution was stirred for > 6 h. Lipids were extracted by the addition of 1.2 ml of chloroform/methanol (9:1, v/v). The lower (organic) phase was removed and the solution was subjected to a second extraction. The two extractions were combined and dried under a gentle stream of nitrogen. The sample was then ready for h.p.l.c. analysis.

The dried radiolabelled lipids were dissolved in 900 μl of 50% acetonitrile containing 25 mM phosphoric acid. All three standards, geranol, farnesol and geranylgeranol, were added (0.5 μl of each) to the sample before injecting on to a 0.5 cm × 25 cm ODS reverse-phase h.p.l.c. column (Phenomenex). The column was developed with a 40 ml linear gradient of 50% acetonitrile containing 25 mM phosphoric acid to pure acetonitrile containing 25 mM phosphoric acid, followed by 20 ml of pure acetonitrile containing 25 mM phosphoric acid. A flow rate of 1 ml/min was used and 1 ml fractions were collected. Scintillation fluid (10 ml) was added to each fraction and radioactivity was determined by scintillation counting. The entire h.p.l.c. analysis was monitored by absorbance at 205 nm to record the elution times of the standards.

Purification of recombinant rap2a and protein prenyltransferase assays

Rap2a coding sequence was subcloned into *Bam*HI/*Eco*RI-digested pGEX-2T vector (Pharmacia) and expressed as a fusion protein. Expression and purification of the glutathione S-transferase/rap2a protein was as described in [21]. Thrombin (1% w/w fusion protein) was incubated with the purified fusion protein for 1 h at 25 °C to liberate recombinant rap2a. Protein prenyltransferase assays were carried out as previously described [22]. Briefly, a high-speed supernatant fraction obtained from bovine brain was subjected to sequential chromatography on DEAE-Sephacel AcA34 gel filtration and Mono-Q. At this stage of purification, two distinct peaks of prenyltransferase activity corresponding to geranylgeranyltransferase and farnesyltransferase were obtained. Protein prenyltransferase activity was determined by quantifying the amount of radioactivity transferred from [^{3}H]geranylgeranyl pyrophosphate or [^{3}H]-farnesyl pyrophosphate on to CAAX motif proteins. Purified recombinant proteins of known prenyl specificity (ras-CVLL for geranylgeranyl and H-ras for farnesyl) [22] were used as standards.

RESULTS

Expression of rap proteins in COS cells and incorporation of [^{3}H]mevalonolactone (mevalonic acid)

The rap2 cDNA sequences subcloned into pSVL were transfected into COS cells by the Lipofectin method. Cells were incubated in the presence of [^{3}H]mevalonolactone at 36 h post-transfection as described in the Materials and methods section and harvested 12 h later. Cell lysates were prepared and analysed for rap2 expression via Western blotting. As shown in Figure 1(a), antibody 9264 recognized a protein of approx. 21 kDa, indicative of rap2 expression in both rap2a- and rap2b-transfected cells. The specificity of the antibody was previously shown by its inability to recognize recombinant rap1a and rap1b [18]. In contrast to the rap2 expression seen in transfected COS cells was the absence of endogenous rap2 expression in untransfected cells. This same result was seen when increasing amounts (> 100 μg) of COS cell lysates were analysed (results not shown).

The nitrocellulose blot shown in Figure 1(a) was exposed to film for 3 weeks to assess incorporation of radiolabelled mevalonolactone into proteins. When the fluorograph generated by the nitrocellulose blot was analysed, we observed that COS cells incorporated mevalonic acid into proteins mostly in the 20–30 kDa range (Figure 1b). When the labelling pattern of transfected cells was examined, we observed a band of approx. 21 kDa which was not present in the control (Figure 1b, arrow). When the fluorograph was aligned with the nitrocellulose, these unique bands seen only in the transfected cells aligned exactly with the immunoreactive bands. These results indicate that rap2a and rap2b incorporate mevalonic acid *in vivo*, most likely as an isoprenoid.

Transcription/translation and isoprenylation of rap2a and rap2b *in vitro*

Since we were able to show that both rap2a and rap2b are isoprenylated *in vivo*, we sought to determine if the proteins are also isoprenylated in an *in vitro* system. Given that COS cells contain several proteins in the 20–30 kDa range that are isoprenylated, an *in vitro* system allows one to unambiguously identify the isoprenoid attached to the protein in question without interference from these other prenylated proteins in this molecular mass range. We chose the rabbit reticulocyte system for our studies because it has been shown to faithfully translate mRNA into mature protein [23]. In addition, others have shown that it contains all the components necessary for isoprenylation of proteins, including the enzymes which convert mevalonic acid into isoprenoids and both prenyltransferases identified to date [24]. To assess whether our cDNA sequences produced proteins of the predicted molecular mass, RNA species generated from the cDNAs in a run-off transcription reaction were incubated in the presence of radiolabelled methionine. Radiolabelled products of ~ 21 kDa were observed in the reactions containing rap2a, rap2b or rap2a mutant RNA (results not shown). In contrast, no radiolabelled products of endogenous RNA were observed in the control lysate reaction. To assess isoprenoid incorporation into the translation products, the translational reactions were repeated in the presence of unlabelled methionine and radio-

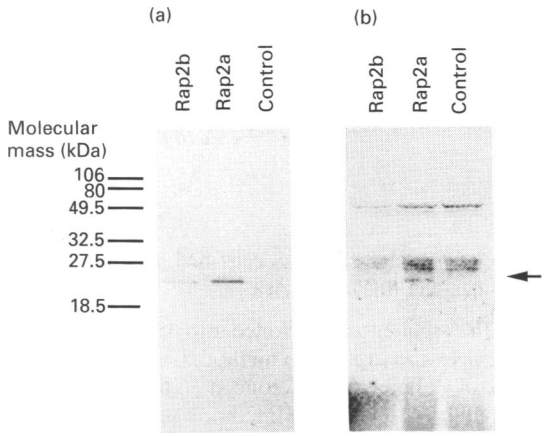

Figure 1 (a) Western blot analysis of rap2a and rap2b expression in COS cells and (b) fluorograph of radiolabelled COS cells transfected with rap2 cDNAs

(**a**) COS cells were transfected with rap2 cDNAs subcloned into pSVL as described in the Materials and methods section. Cells were incubated with [^{3}H]mevalonolactone at 36 h post-transfection and harvested 12 h later. Cell lysates were obtained by repeated freeze/thawing in 0.25 mM Tris, pH 7.5. Proteins (50 μg) were separated by SDS/12.5%-PAGE followed by Western blotting with anti-rap2 antiserum 9264 (1:100 dilution). Cell lysates from control, rap2a, or rap2b transfected COS cells are as indicated. (**b**) The nitrocellulose blot generated in (**a**) was placed on film for 21 days. The arrow indicates the radiolabelled species corresponding to rap2a and rap2b. Molecular masses, obtained from prestained SDS/PAGE standards, low range (Bio-Rad), are as indicated.

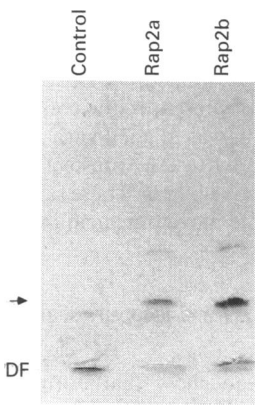

Figure 2 Fluorograph of radiolabelled rap2 proteins generated in a rabbit reticulocyte lysate

cRNA species corresponding to rap2a and rap2b were translated in a rabbit reticulocyte lysate system in the presence of [^{3}H]mevalonolactone as described in the Materials and methods section. A 20 μl portion of the reaction mixture was subjected to SDS/PAGE. The gel was placed in Autofluor, dried and exposed to X-Omat AR film at −80 °C with an intensifying screen for 4 days. Reaction mixtures from control, rap2a and rap2b are as indicated. The arrow denotes the 21 kDa radiolabelled proteins that were subjected to lipid analysis. DF corresponds to non-specific radioactivity observed at the dye front of the gel.

labelled mevalonate. As shown in Figure 2, both rap2a and rap2b incorporated mevalonate, as evidenced by the strong labelling observed at 21 kDa. To further assess C-terminal processing in the rabbit reticulocyte system, the banding pattern obtained from [^{35}S]methionine incorporation was compared with [^{3}H]mevalonate incorporation for rap2a and two rap2a mutants generated as described in the Materials and methods section. As shown in Figure 3(a), we observed the presence of two radioactive bands of similar molecular mass in the 21 kDa region in each lane, with the exception of the lane containing the

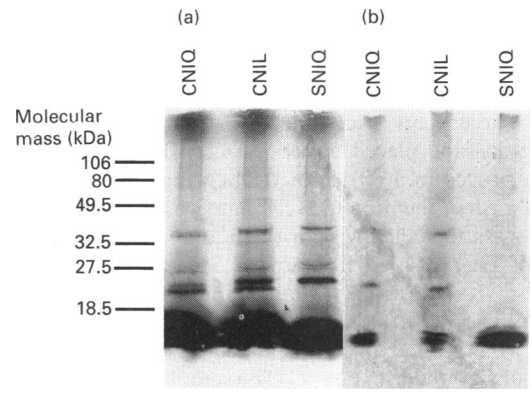

Figure 3 [^{35}S]Methionine (a) and [^{3}H]mevalonate (b) labelling of rap2a and rap2a mutants in a rabbit reticulocyte system

cRNA species generated from rap2a or rap2a mutant plasmids as described in the Materials and methods section were translated as in Figure 2 in the presence of [^{35}S]methionine (**a**) or [^{3}H]mevalonate (**b**) as indicated. Letters indicate the C-terminal tetrapeptide amino acid sequence. Molecular masses obtained from prestained SDS/PAGE standards, low range (Bio-Rad), are indicated.

translation product generated from the rap2a-SNIQ RNA, where only one band was observed. These two bands most likely represent the unprocessed and processed forms of each rap protein, since the single band generated by the rap2a-SNIQ RNA migrated with the slower-migrating species. Further evidence for this conclusion is given by the labelling pattern obtained in the presence of [^{3}H]mevalonate. As shown in Figure 3(b), a single radioactive band migrating at the position of the faster-migrating form in the presence of labelled methionine was observed in the rap2a and rap2a-CNIL lanes. In addition, an absence of mevalonate labelling in this region was observed in the lane containing the rap2a mutant in which the CAAX cysteine has been replaced by a serine. This result further supports the conclusion that the rap2 translation products undergo C-terminal processing in the reticulocyte system. Interestingly, the lysate does not contain endogenous proteins which incorporate mevalonate, since we observed an absence of labelling in the control (−RNA) lane. This result suggests that the reticulocyte lysate is a good model system to assess prenyl identification of uncharacterized CAAX-terminating cDNAs (proteins).

Isoprenoid identification on rap2a and rap2b

Once we established that rap2 proteins incorporate mevalonic acid in both *in vitro* and *in vivo* systems, we proceeded to identify the specific group attached to each protein. By CAAX motif sequence it would appear they may incorporate different lipid moieties (see Figure 5). The band corresponding to rap2a and rap2b from the translation reactions in the presence of radiolabelled mevalonate was excised from the gel and the protein eluted as described in the Materials and methods section. An area of the gel at the same position as the rap2 translation products was excised from the control lane (−RNA) as a control for background radioactivity. This sample was processed in an identical way to the radiolabelled rap2 protein samples as described in the Materials and methods section, and served as a background control for the h.p.l.c. analysis.

Upon h.p.l.c. analysis, the control gel slice yielded low radioactivity overall and an absence of radioactivity corresponding to the elution positions of the unlabelled standards geranol, farnesol and geranylgeranol (results not shown). When rap2b was analysed, a single radioactive peak co-migrating with the C_{20}

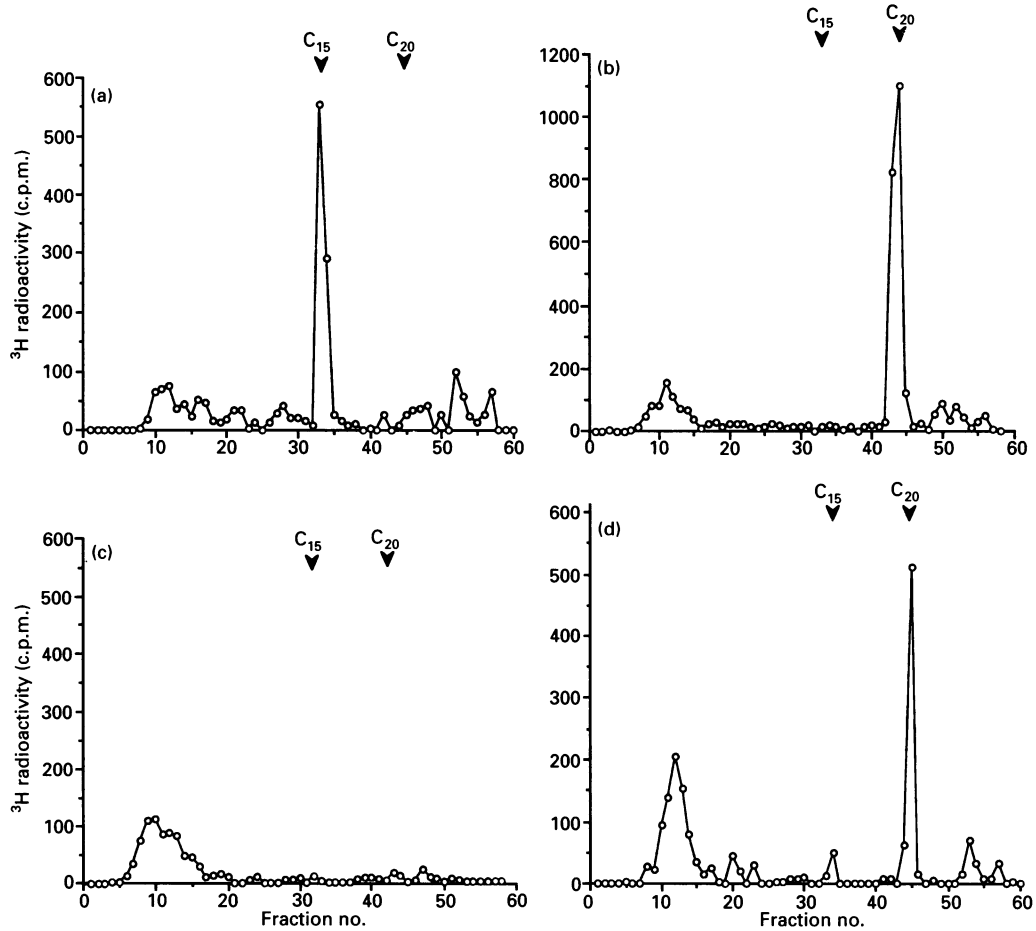

Figure 4 H.p.l.c. analysis of methyl iodide-treated proteins

Radiolabelled rap2a (**a**), rap2a mutant proteins with C-terminal sequences CNIL (**b**) and SNIQ (**c**), and rap2b (**d**) were excised and eluted from SDS/PAGE, precipitated with trichloroacetic acid, proteinase K digested and subjected to methyl iodide cleavage as described in the Materials and methods section. The methyl iodide liberated lipids were subjected to h.p.l.c. analysis as described, and 60 × 1 ml fractions were collected. The lipid moiety generated by each protein is as indicated. Ready Safe (Beckman) was added (10 ml) and each sample was counted for radioactivity by liquid scintillation for 1 or 5 min. The elution positions of the isoprenoid standards C_{15} and C_{20} are as marked by arrowheads.

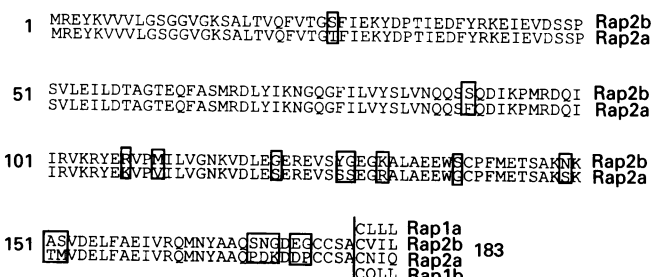

Figure 5 Amino acid alignment of rap2a and rap2b protein sequences

Boxed amino acids indicate where rap2a and rap2b differ in sequence. The CAAX motif sequences of rap1a and rap1b are also shown.

geranylgeranol standard was observed (Figure 4d). This result is in agreement with others who have observed geranylgeranyl on CAAX motif proteins terminating in leucine [25]. Rap2a, on the other hand, yielded a radioactive peak co-migrating with C_{15} farnesol (Figure 4a). This result implicates glutamine as a signal for farnesylation. Given that rap2a and rap2b share ~90% sequence identity, yet show the greatest divergence in the C-terminus, we sought to determine if the difference in isoprenoid incorporation of the two proteins is due solely to the terminal amino acid. To investigate this question, we changed the terminal glutamine of rap2a to a leucine residue by site-directed mutagenesis. As shown in Figure 4(b), this single amino acid change converted rap2a to a substrate for geranylgeranylation. An additional mutation was constructed in which the CAAX motif cysteine was converted to a serine residue. As shown in Figure 4(c), we observed a complete absence of isoprenoid incorporation into rap2a. This result reconfirms the previously established conclusion that the cysteine residue is the site of isoprenoid attachment.

Prenyl specificity of rap2a using purified prenyltransferases

To further assess the prenyl specificity of rap2a, bacterially expressed rap2a was assayed using [^{3}H]farnesyl pyrophosphate or [^{3}H]geranylgeranyl pyrophosphate with the appropriate partially purified prenyltransferase. The specificity of each enzyme

Table 1 Prenyl specificity of rap2a using purified prenyltransferases

Rap2a was assayed for prenyl specificity by incubation with [^{3}H]geranylgeranyl pyrophosphate/protein geranylgeranyl transferase (PGGT) or [^{3}H]farnesyl pyrophosphate/protein farnesyltransferase (PFT) as previously described [22]. Incorporation of labelled moiety is expressed as c.p.m. Ras terminating in CVLL and H-ras were used as geranylgeranylation and farnesylation standards respectively.

		^{3}H incorporation (c.p.m.)	
Enzyme	Protein (μg)...	5	20
PGGT	Ras-CVLL	44000	–
PGGT	Rap2a	1100	1500
PFT	H-Ras	70000	–
PFT	Rap2a	49000	79000

preparation was shown by the ability of a specific prenyltransferase to modify the correct substrate (Table 1). Rap2a was a poor substrate for geranylgeranylation by geranylgeranyltransferase. The specificity of the reaction was shown by the absence of increased incorporation of radioactivity from [^{3}H]geranylgeranyl pyrophosphate into rap2a at 20 μg of protein. On the other hand, rap2a was a good substrate for farnesylation by farnesyltransferase. This incorporation was concentration-dependent, as shown by the increased radioactivity associated with the protein at increased protein concentration (Table 1). This result firmly established glutamine as a signal for farnesylation and provides further evidence that the terminal amino acid of CAAX establishes prenyl specificity.

DISCUSSION

In this paper we provide evidence that rap2a and rap2b are isoprenylated. These two proteins join a growing list of CAAX motif proteins whose prenyl group has been identified (including ras, nuclear lamins and other low-molecular-mass GTP-binding proteins). In agreement with current models of isoprenylation, rap2b, which ends in leucine, is geranylgeranylated. Other geranylgeranylated proteins identified to date include the γ subunits of some heterotrimeric G-proteins [26], G25K, a G-protein recently shown to be a substrate for tyrosine phosphorylation by epidermal growth factor [27], and rapla, a suppressor of ras function [28]. It is believed that a cell will contain more proteins that are geranylgeranylated than ones that are farnesylated. Estimates suggest that geranylgeranylation is 5–10 times more common than farnesylation [29]. The significance of farnesylation versus geranylgeranylation, with respect to cell function or localization, is currently unknown. Buss et al. [28] recently showed that a chimaeric H-ras/rapla protein retains transformation activity. This construct is interesting in that the isoprenoid of ras C_{15} (farnesyl) is replaced by the isoprenoid C_{20} (geranylgeranyl) of rapla. This result suggests that either isoprenoid is sufficient for proper membrane localization of ras.

In contrast to rap2b, rap2a, which contains a glutamine at the C-terminus, incorporates farnesyl. To date, the presence of a glutamine at the C-terminus has been found for only one other CAAX motif protein, cyclic GMP phosphodiesterase α subunit, which has recently been shown to incorporate farnesyl [30]. This is in agreement with the result of Reiss et al. [31], who showed that the tetrapeptide CCVQ, corresponding to the cyclic GMP phosphodiesterase CAAX motif, is a fairly good inhibitor of farnesyltransferase activity. Only a 2-fold increase in tetrapeptide concentration was required, compared with known farnesylated ras tetrapeptide sequences, to yield the same inhibition (50%).

Seabra et al. [17] recently showed that the transfer of farnesyl or geranylgeranyl to proteins occurs by the action of two different prenyltransferases. Interestingly, the two transferases are similar in molecular mass as assessed by gel filtration [17]. Moreover, they share a common subunit, designated α. They differ by at least one subunit, designated β, as indicated by the fact that antibodies directed against this farnesyltransferase subunit do not recognize purified geranylgeranyltransferase. The specificity of the two transferases for isoprenoid attachment to proteins is governed by the C-terminal amino acid [17]. Farnesyltransferase prefers methionine or serine at the C-terminus, while geranylgeranyltransferase prefers leucine. Surprisingly, when a list of vertebrate proteins containing CAAX motifs at their C-terminus is scanned, one immediately notices that most CAAX motif proteins have methionine or leucine at their C-terminus [13]. For example, all nuclear lamins and ras members, with the exception of H-ras, contain a methionine at this position. Most ras-related low-molecular-mass GTP-binding proteins contain leucine. This observation leads one to believe that as other non-methionine/non-leucine CAAX motif proteins are discovered, the rules for predicting isoprenoid attachment will be broadened.

That rap2a and rap2b show strong sequence identity yet incorporate different lipid moieties is intriguing. Our evidence strongly suggests that this difference in isoprenoid incorporation is due solely to the terminal amino acid residue. All rap proteins discovered to date possess leucine at their C-terminus, with the exception of rap2a (Figure 5). Moreover, rap2a is the only member of the ras superfamily, with the exception of ras itself, to be farnesylated. This result is interesting, as rapla, a geranylgeranylated protein, is capable of reverting the transformed phenotype of a K-ras-transfected NIH 3T3 cell line.

The relative amount of rap2a with respect to rap2b in tissues is unknown. It is interesting to speculate that the two molecules may partition into different membrane compartments according to their isoprenoid attachment. Equally intriguing is the possibility that the isoprenoid may direct the protein to its proper compartment, allowing it to exert its function within a cell.

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