Molecular Biology of the Cell
Vol. 6, 71-85, January 1995

Properties of Rab5 N-Terminal Domain Dictate Prenylation of C-Terminal Cysteines

Jack C. Sanford, Yan Pan, and Marianne Wessling-Resnick†

Department of Nutrition, Harvard School of Public Health, Boston, Massachusetts 02115

Submitted July 11, 1994; Accepted November 7, 1994
Monitoring Editor: Suzanne R. Pfeffer

Rab5 is a Ras-related GTP-binding protein that is post-translationally modified by prenylation. We report here that an N-terminal domain contained within the first 22 amino acids of Rab5 is critical for efficient geranylgeranylation of the protein's C-terminal cysteines. This domain is immediately upstream from the "phosphate binding loop" common to all GTP-binding proteins and contains a highly conserved sequence recognized among members of the Rab family, referred to here as the YXYLFK motif. A truncation mutant that lacks this domain ($Rab5^{23-215}$) fails to become prenylated. However, a chimeric peptide with the conserved motif replacing cognate Rab5 sequence ($MAYDYLFKRab5^{23-215}$) does become post-translationally modified, demonstrating that the presence of this simple six amino acid N-terminal element enables prenylation at Rab5's C-terminus. H-Ras/Rab5 chimeras that include the conserved YXYLFK motif at the N-terminus do not become prenylated, indicating that, while this element may be necessary for prenylation of Rab proteins, it alone is not sufficient to confer properties to a heterologous protein to enable substrate recognition by the Rab geranylgeranyl transferase. Deletion analysis and studies of point mutants further reveal that the lysine residue of the YXYLFK motif is an absolute requirement to enable geranylgeranylation of Rab proteins. Functional studies support the idea that this domain is not required for guanine nucleotide binding since prenylation-defective mutants still bind GDP and are protected from protease digestion in the presence of GTPγS. We conclude that the mechanism of Rab geranylgeranylation involves key elements of the protein's tertiary structure including a conserved N-terminal amino acid motif (YXYLFK) that incorporates a critical lysine residue.

INTRODUCTION

Vesicle traffic of the endocytic pathway mediates the entry of macromolecular nutrients, peptide hormones, and other extracellular constituents into the cell's interior. This process must involve a complex series of selective fusion and fission reactions between endocytic vesicles and endosomal structures but the molecular mechanisms regulating these events remain largely unknown. However, the development of cell-free systems that reconstitute elemental stages of the endocytic pathway has begun to provide insight into how the specificity of vesicle targeting is accomplished and to reveal protein components that are involved (Balch, 1989; Pfeffer, 1992; Sztul *et al.*, 1992). In vitro studies have established a role for GTP[1] hydrolysis in endosome fusion (Mayorga *et al.*, 1989; Wessling-Resnick and Braell, 1990) and evidence has been presented to support the involvement of at least three GTP-bindings proteins in early endocytic events: ADP-ribosylation factor (Lenhard *et al.*, 1992), a yet-

†Corresponding author: Dr. Marianne Wessling-Resnick, Department of Nutrition, Harvard School of Public Health, 665 Huntington Avenue, Boston, MA 02115.

[1]Abbreviations used: CHAPS, 3-([3-cholamidopropyl]dimethylammonio)-2-hydroxy-1-propanesulfonate; DTT, dithiothreitol; GDP, guanine nucleotide disphosphate; GDI, GDP dissociation inhibitor; GGPP, geranylgeranyl pyrophosphate; GTP, guanine nucleotide triphosphate; GTPγS, guanosine 5′-*O*-(thiotriphosphate); PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PIMA, pattern-induced multisequence alignment; RNase A, ribonuclease A; wt, wild type.

to-be identified heterotrimeric G protein (Colombo *et al.*, 1992), and Rab5 (Gorvel *et al.*, 1991). The latter belongs to a large and growing class of Ras-related proteins, the Rab family, that have close sequence homology with the Ypt/Sec family of factors implicated in vectorial secretory transport in yeast (Zahraoui *et al.*, 1989; Chavrier *et al.*, 1990). Key evidence in support of a role for Rab5 in endocytic vesicle traffic comes from immunocytolocalization studies identifying Rab5 on the cytosolic surface of the plasma membrane, coated vesicles and early endosomes (Chavrier *et al.*, 1990). In addition, Gorvel *et al.* (1991) have demonstrated that anti-Rab5 antibodies will block in vitro fusion of endocytic vesicles and that cytosol enriched in this specific factor promotes fusion activity. These findings have recently been complemented by in vivo studies in both Chinese hamster ovary and baby hamster kidney cells demonstrating that overexpression of Rab5 enhances receptor-mediated endocytosis of transferrin (Bucci *et al.*, 1992) and endocytosis of horseradish peroxidase (Li and Stahl, 1993). One conclusion drawn from these experiments is that Rab5 is the rate-limiting factor in endosome-endosome fusion.

A unique and significant aspect of Rab5's endocytic function centers on its mechanism of membrane association. The C-terminus of the protein is required for this purpose since deletion of the last 9 amino acids causes the truncated Rab5 molecule to partition into a soluble cellular fraction (Gorvel *et al.*, 1991; Chavrier *et al.*, 1990). Moreover, this domain is critical for correct subcellular targeting; for example, replacement of the last 34 amino acids with Rab7 sequence directs a chimeric Rab5/Rab7 molecule to late, rather than early endosomes (Chavrier *et al.*, 1991). Membrane attachment is also thought to be aided by hydrophobic modification(s) on the protein's C-terminus, since like H-Ras, Rab protein family members are prenylated at C-terminal cysteine residues to promote interaction with membrane surfaces (Kinsella and Maltese, 1991; Khosravi-Far *et al.*, 1991 Farnsworth *et al.*, 1991). Functional interaction of Rab proteins with a GDP dissociation inhibitor (GDI) also requires geranylgeranylation of the C-terminal cysteine acceptor sites (Musha *et al.*, 1992). GDI is an accessory factor that complexes with Rabs in the GDP-bound conformation and regulates the cycling of Rabs on and off membranes (Ullrich *et al.*, 1993, 1994). Interestingly, GDI shows scattered sequence identity with component A, now called Rab escort protein, of Rab geranylgeranyl transferase (Andres *et al.*, 1993).

Although members of the Rab family do have molecular domains homologous to H-Ras, these proteins do not contain the typical CAAX motif that is required for prenylation of H-Ras and many other factors (Sinensky and Lutz, 1992). Rather, known Rab proteins typically display one of three C-terminal sequences, either CXC (e.g., Rab3A), XXCC (e.g., Rab1B) or CCXX (e.g., Rab5), all of which present critical cysteine residues for geranylgeranylation. Surprisingly, the C-terminal cysteine-rich motifs of Rab3A and the yeast homolog Ypt1 are not substrates for prenylation by themselves and can not confer the capacity for prenylation when spliced to the C-terminus of a neutral protein (Moores *et al.*, 1991), properties that are manifested by the CAAX motif (Reiss *et al.*, 1990). This information, coupled with the observations that C-terminal Rab5 cognate peptides do not block prenylation of the native protein (Kinsella and Maltese, 1992; Peter *et al.*, 1992), suggested that there may be structural information exclusive of the C-terminal cysteine-rich domain that directs the prenylation of Rab5. Two lines of evidence now support this idea. Wilson and Maltese (1993) have found that point mutations in the effector domain of Rab1B perturb the ability of in vitro synthesized peptides to be recognized by Rab geranylgeranyl transferase. In addition, we have found that $Rab5^{N133I}$, a mutant defective in guanine nucleotide binding, fails to become efficiently prenylated (Sanford *et al.*, 1993). Furthermore, Rab5 prenylation is inhibited by a Rab5 C-terminal truncation mutant when it is charged with GDP but not when it is charged with GTPγS (Sanford *et al.*, 1993). Hence, elements of Rab5 tertiary structure that are exclusive of the C-terminal domain and that are promoted by GDP binding appear to establish a preferred substrate conformation for efficient prenylation by Rab geranylgeranyl transferase.

To identify structural elements exclusive of the C-terminal CCXX motif of Rab5 that mediate post-translational geranylgeranylation, we have utilized a cell-free translation system that supports protein prenylation (Sanford *et al.*, 1993). Through screening Rab5 mutants and H-Ras/Rab5 chimeras for the ability to become prenylated in vitro, our findings demonstrate that the protein's N-terminal domain is required for geranylgeranylation of C-terminal cysteines. It is of great interest that elements of Rab5's N-terminal domain include a highly conserved sequence, referred to here as YXYLFK, that is represented in all known members of the Rab family and that can effectively replace the first 22 amino acids of the protein to enable prenylation of Rab5. Amino acid changes and deletions within this motif indicate that the lysine residue (residue 22 in wild-type Rab5) is critical for geranylgeranylation of Rab5. This domain is not simply required for guanine nucleotide binding, since the prenylation-defective mutants are capable of binding GDP and are protected from protease digestion in the presence of GTPγS. Rather, the functional importance of elements of the YXYLFK motif reside in structural properties clearly necessary for efficient prenylation. The results reported here identify that mod-

ification of Rab5 relies on a tertiary-structure directed mechanism involving the protein's N-terminus and, in addition, locate the position of a lysine residue critical for this function.

MATERIALS AND METHODS

Materials

Radiochemicals, including [^{35}S]methionine (1200 Ci/mmol), R-[5–3H]mevalonolactone (35.1 Ci/mmol), and [α-^{32}P]GTP (3000 Ci/mmol) were purchased from Dupont-NEN (Boston, MA). Unlabeled geranylgeranyl pyrophosphate (GGPP) was obtained from American Radiolabeled Chemicals (St. Louis, MO). Restriction endonucleases were purchased from Boehringer Mannheim (Indianapolis, IN) and New England Biolabs (Beverly, MA); Vent DNA polymerase was from New England Biolabs; RNasin, RQI DNase, and reticulocyte lysate from Promega (Madison, WI); the Klenow fragment of DNA polymerase and T4 DNA ligase from Boehringer Mannheim; agarose from FMC BioProducts (Rockland, MD); and T7 DNA polymerase from Pharmacia LKB (Piscataway, NJ).

Construction of Chimeric Rab5 cDNAs

Standard molecular biological techniques were performed with regard to restriction digests, plasmid purification, and nucleic acid manipulations (Sambrook *et al.*, 1989). The cDNAs for human Rab5 (Sanford *et al.*, 1993) and H-Ras were previously subcloned into pAGA, a T_7 polymerase-based transcription vector (Sanford *et al.*, 1991). pAGA is derived from pGEM3Zf(−) and is engineered to contain a limited cloning cassette that is flanked on the 5′ side by the alfalfa mosaic virus RNA-4 5′-untranslated sequence and to the 3′ side by a poly(A) track of 92 nucleotides. All studies reported here were carried out with cDNAs subcloned into pAGA in such a manner that the ATG of the open reading frame encoding the alfalfa mosaic virus RNA became the ATG initiation codon of the expressed protein.

Several mutant Rab5 cDNAs and Rab5/H-Ras chimeric constructs were prepared in the vector pAGA (Figure 1). A truncation mutant Rab5$^{88-215}$ was amplified from Rab5^{wt} to produce a fragment blunt-ended at the 5′ end, beginning with the codon for amino acid 89, and an *Xba*I site 3′ to the stop codon as described for the subcloning of Rab5^{wt} (Sanford *et al.*, 1993). After restriction digestion with *Xba*I, the amplified product was subcloned into pAGA that had been digested with *Nco*I, treated with Klenow fragment, and post-digested with *Xba*I. A second truncation mutant Rab5$^{23-215}$ was generated by digesting with restriction enzymes *Nco*I and *Spe*I, treating with Klenow fragment, and self-ligating the plasmid. This produced an N-terminal truncation of Rab5 beginning with the methionine codon supplied by pAGA followed by the codon for amino acid 23 of Rab5.

Two PCR sense primers (5′-GCTTATGACTATCTGTTCAAGCTGGTACTGCTGGG and 5′-GCTTATGACTATCTGTTCAAACTAGTGGTGGTGGGCGC) were designed to generate clones designated MAYDYLFKRab5$^{23-215}$ and MAYDYLFKRas$^{6-189}$, respectively. The resulting PCR products were processed and subcloned according to the conditions used for Rab5$^{88-215}$. Additionally, Ras$^{1-185}$CCSN was constructed via PCR such that the codons for the last four amino acids of H-Ras (CVLS) were replaced with the codons for the last four amino acids of Rab5. The Ras$^{1-185}$CCSN PCR product was digested with *Eco*RI and *Xba*I before in-gel ligation into an *Eco*RI/*Xba*I-digested pAGA. Finally, MAYDYLFKRas$^{6-185}$CCSN was constructed such that both the N- and C-termini of H-Ras were altered via PCR to contain the conserved N-terminal Rab motif and the C-terminal four amino acids of Rab5. This PCR product was also processed and subcloned into pAGA as described above.

To construct C-terminal chimeras of H-Ras, Rab5 in pAGA was digested with *Eco*RI and *Acc*I. The resulting vector retained 48 nucleotides at the 3′ end, corresponding to the C-terminal 16 amino acids of Rab5. Additionally, PCR-generated H-Ras constructs were

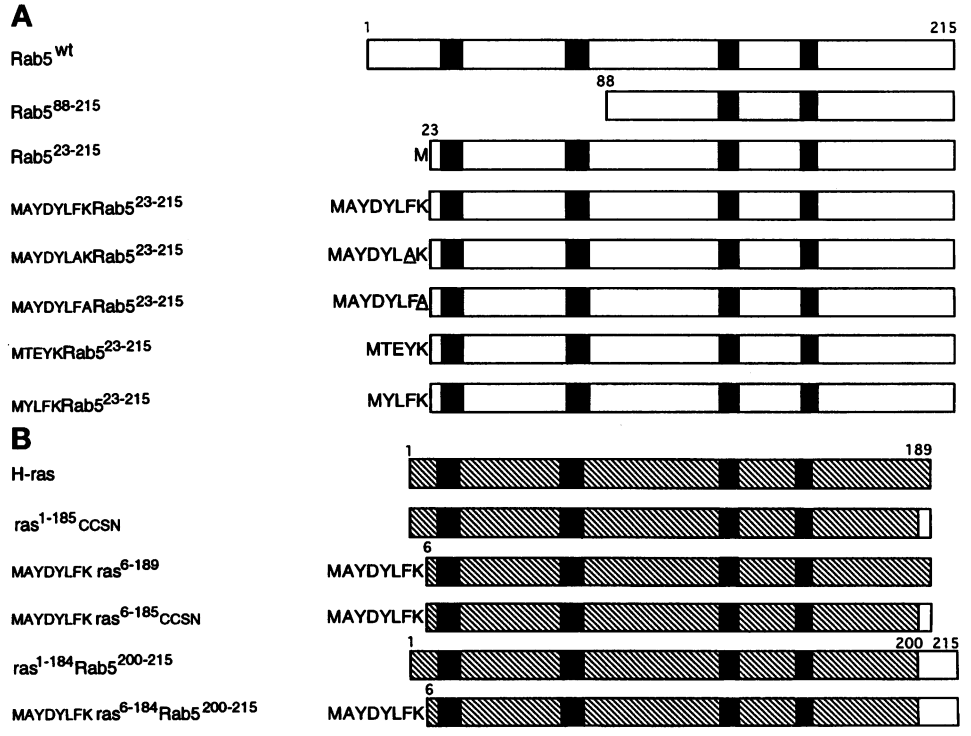

Figure 1. Rab5 and H-Ras peptides. Shown are schematic linear models based on the known primary structure of Rab5^{wt} (A) and H-Ras (B). The various chimeras of Rab5 and H-Ras that were constructed for prenylation experiments are depicted, with the conserved GTP-binding domains indicated by the filled areas. Ras protein sequence is cross-hatched, while Rab5 sequence is represented by open areas. See MATERIALS AND METHODS for details on the construction of these mutants.

created to introduce an *Acc*I site at nucleotides 546–552. Thus, identical restriction digestions of H-Ras and MAYDYLFKRas$^{6-189}$, both in pAGA, yielded fragments that could then be directionally subcloned into *Eco*RI/*Acc*I-digested Rab5 (in pAGA) to produce Ras$^{1-184}$Rab5$^{200-215}$ and MAYDYLFKRas$^{6-184}$Ras5$^{200-215}$, respectively.

Point mutants at positions 7 and 8 of MAYDYLFKRab5$^{23-215}$ were constructed with the respective amino acid substitutions, F→A and K→A. These mutants were generated by PCR using MAYDYLFKRab5$^{23-215}$ in pAGA as template and the T_7 polymerase promoter primer as the sense primer and either 5′-GACTAGTTTTGCCAGATAGTCATA (for F→A) or 5′-GACTAGTGCGAACAGATAGTCATA (for K→A) as the antisense primer. The PCR products were digested with *Eco*RI and *Spe*I and subcloned into Rab5^{wt} in pAGA that was processed with the identical enzymes. Another mutant was created by deletion of three residues to produce the construct MYLFKRab5$^{23-215}$. Briefly, the corresponding PCR product, amplified using the primer 5′-CGGGATCCATATGTATCTGTTCAAACTA and an Sp6 promoter primer, was digested with *Bam*HI and *Pst*I before subcloning into pAGA digested with the identical enzymes. Finally, a chimera MTEYKRab5$^{23-215}$ was prepared in which the first 22 amino acids of Rab5 were replaced by the first four amino acids of H-Ras. This construct was generated by PCR using Rab5^{wt} as template DNA and primers 5′-ACGGAATATAAACTAGTACTTCTG and 5′-CCTCTAGATTAGTTACTACAACACTGATTC. The entire insert cDNAs from positive recombinants were sequenced using the dideoxy chain-termination method of Sanger *et al.* (1977) to check for the absence of Vent DNA polymerase-induced mutations.

Linear models of the primary structure of the constructs discussed above are illustrated in Figure 1. Highlighted by solid areas in the linear bar models are four domains that form the GTP-binding pocket upon folding of the protein into a tertiary conformation. Panel A depicts those constructs based on the Rab5 sequence depicting N-terminal deletions and the conserved N-terminal motif MAYDYLFK– and mutants thereof spliced onto the Rab5$^{23-215}$ backbone. Panel B depicts the H-Ras constructs in which domains have been exchanged between H-Ras and Rab5. H-Ras domains are represented by hatched areas while Rab5 domains are indicated by the open areas.

In Vitro Transcription, Translation, and Processing

Procedures for in vitro transcription, translation and processing of nascent peptides with prenyl groups have been previously detailed (Sanford *et al.*, 1993). Typically, 50 μg of CsCl-purified pAGA plasmid DNA containing wild-type or mutant cDNAs was linearized with *Hin*dIII. The cDNAs were transcribed from 10 μg of linearized template with T_7 RNA polymerase. After termination of the reaction with RQ1 DNase, the transcripts were extracted successively with phenol, phenol/chloroform (1:1), and chloroform before ethanol precipitation from 2.5 M ammonium acetate.

Reticulocyte lysate (Promega) was programmed with transcripts to synthesize peptides for 20 min at 30°C in the presence of 150,000 cpm/μl of [^{35}S]methionine (1200 Ci/mmol). Synthesis was quenched by the addition of 50 μg/ml RNase A to limit the final amount of protein expressed. Aliquots from the translation mixtures were precipitated with 20% trichloroacetic acid/2% casamino acids for quantitation of expressed protein. The concentration of peptide synthesized was then calculated on the basis of the percentage of added [^{35}S]methionine incorporated into trichloroacetic acid-insoluble product and the number of methionine residues in the mature protein. To achieve complete prenylation of Rab5, a post-incubation in the presence of 500 μM mevalonate or 10 μM GGPP for 1–4 h at 37°C was required. For analysis of prenylation, [^{35}S]methionine was replaced by [^{3}H]mevalonolactone (35 Ci/mmol) at a concentration of 10 μM.

Polyacrylamide Gel Electrophoresis

Unless otherwise indicated, translation products were analyzed by electrophoresis through polyacrylamide gel slabs with an acrylamide to bis-acrylamide ratio of 36:1. In vitro prenylation of translated proteins was analyzed by mobility shift of the products on combined urea (4–8 M)/acrylamide (10–15%) gradient gel slabs. Gels were processed for fluorography to analyze for incorporation of ^{35}S or ^{3}H. For quantitative analysis of the extent of prenylation of Rab5 and mutants, films were densitometrically scanned using a Bio Image System (Millipore, Bedford, MA).

[^{32}P]GTP Overlay Blots

H-Ras and H-Ras/Rab5 chimeric peptides were immunoprecipitated from reticulocyte lysate with agarose-linked anti-v-H-Ras antibody (Ab-1; Oncogene, Uniondale, NY). The immunoprecipitates (150 fmol) were electrophoresed under denaturing conditions on a urea/acrylamide gradient gel. Determination of the mass of peptide immunoprecipitated was based on parallel immunoprecipitation of ^{35}S-labeled peptides. Proteins were transferred to Immobilon-P in the absence of SDS at 200 mA for 2 h (Towbin *et al.*, 1979). The filter membrane was soaked for 30 min in binding buffer (50 mM Tris, pH 8.0, 5mM $MgCl_2$, 2mM DTT, 0.3% bovine serum albumin, and 0.1 mM ATP). GTP binding was initiated by incubating the blot for 1 h in 25 ml fresh binding buffer containing [α-^{32}P]GTP (10^6 cpm/ml). After three washes in binding buffer minus ATP, the filter membrane was exposed to film for autoradiography.

Proteolysis of Synthesized Peptides

Structural integrity of in vitro synthesized Rab5 and chimeric peptides was also determined by limited proteolysis. Rab5 and mutant peptides were translated in vitro as detailed above with the exception that the amount of [^{35}S]methionine was increased to 10^6 cpm/μl. The reaction mixtures were then preincubated in a total volume of 7 μl (3.5 nM final peptide concentration) with or without 10 mM GTPγS or 25 mM EDTA in a reaction buffer composed of 80 mM Tris-Cl, pH 8.0, 2 mM $MgCl_2$, 100 mM NaCl, and 1 mM DTT for 45 min at 30°C. Proteolysis was subsequently initiated by the addition of 0.022 U of trypsin (Boehringer Mannheim) in a final volume of 10 μl. After 45 min, proteolysis was terminated by the addition of 2 μl of 1 mg/ml stock of soybean trypsin inhibitor and further incubation at 25°C for 10 min. Samples were then diluted into 68 μl of Laemmli buffer and heated at 100°C for 5 min. Digestion products were analyzed on urea/acrylamide SDS gels described above.

Subcloning of Rab5 Constructs into a T7-Based Expression System

Several constructs in pAGA, including Rab5^{wt}, Rab5$^{23-215}$, MAYDYLFKRab5$^{23-215}$, MYLFKRab5$^{23-215}$, MAYDYLFARab5$^{23-215}$ and MTEYKRab5$^{23-215}$, were subcloned into pT7–7 for expression of proteins in *Escherichia coli* (Studier *et al.*, 1990). This was accomplished via PCR of the pAGA templates to engineer an *Nde*I site at the initiating ATG codon that is immediately downstream from a *Bam*HI site. The PCR products were processed with *Bam*HI and *Pst*I and first subcloned into pAGA. The Rab5 constructs were then excised from pAGA by digestion with *Nde*I and *Pst*I before subcloning into pT7–7 that was digested with the identical enzymes. To generate MAYDYLFARab5$^{23-215}$ in pT7–7, a strategy identical to that described for the assembly of this clone into pAGA was followed except that the template for PCR was MAYDYLFKRab5$^{23-215}$ in pT7–7 and the resulting product was digested with *Xba*I and *Pst*I before subcloning into pT7–7 containing Rab5^{wt} also digested with *Xba*I and *Pst*I. Construction of Rab5^{N133I} in pAGA and subcloning into pT7–7 have been previously described (Sanford *et al.*, 1993).

Expression of recombinant peptides was initiated by transformation by electroporation of plasmid into BL21(DE3) cells (Novagen), followed by induction with isopropyl-1-thio-β-D-galactopyranoside (0.8 mM) for 2 h. Cells were subsequently lysed in 50 mM Tris, pH 8.0, 2 mM EDTA, 1 mM DTT, 5 mM $MgCl_2$, 10 μM GDP, 0.1% CHAPS, 100 μg/ml lysozyme and then sonicated. The cell lysates

were centrifuged at 16,000 × *g* for 10 min and the supernatants were analyzed by electrophoresis for the extent of expression of the full length Rab5 constructs. Protein determination of Rab5 peptides was accomplished by comparing the Coomassie Blue staining of expressed peptides with known amounts of lysozyme loaded on the same gel slab. Stained gels were densitometrically scanned using a Bio Image System (Millipore).

[3H]GDP-Binding Assays

Bacterial lysate supernatant fractions containing 400 pmol of each Rab5 peptide were diluted to a final concentration of 1 μM in GDP-binding buffer (50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 8.0, 150 mM KCl, 1 mM EDTA, 0.5 mM $MgCl_2$, 1 mM DTT, and 0.1% CHAPS). Recombinant peptides were incubated with 20 μM [^{3}H]GDP (3500 cpm/pmol) at 25°C and at indicated times 20 μl of the reaction mixture were removed and filtered through nitrocellulose discs. Filters were washed twice with 2 ml and once with 1 ml of ice-cold GDP wash buffer (20 mM Tris-Cl, pH 8.0, 150 mM NaCl, and 1 mM DTT). Parallel reaction mixtures were also prepared for each peptide to measure non-specific binding in the presence of unlabeled 35 mM GDP. Duplicate measurements were made both in the presence and absence of excess cold GDP and specific binding was taken as the difference between the averaged values of counts/min collected on filters.

RESULTS

Co- and post-translational protein modifications are essential for the molecular function of several classes of proteins. Myristoylation of heterotrimeric G-protein α subunits (Linder *et al.*, 1991), palmitoylation of H-Ras (Hancock *et al.*, 1989), methylation of yeast mating factors (Anderegg *et al.*, 1988), and prenylation of Rab3A (Musha *et al.*, 1992) are just a few specific examples of proteins known to require hydrophobic modifications for their respective activities. We are investigating the mechanism of prenylation of Rab5 in order to understand the significance of this and other post-translational modifications, particularly in relation to the role of Rab5 in vesicle dynamics. Since several Rabs, including Rab5, must be geranylgeranylated prior to forming a complex with GDI (Ullrich *et al.*, 1993), prenylation of Rabs is of major significance with regards to cycling of the proteins on and off membranes. To assess structural features required for prenylation of Rab5, the experimental strategy employed here is to monitor modification of nascent peptide expressed in an in vitro translation system. Prenylation of Rab5 may thus be followed by the incorporation of radiolabel from [^{3}H]mevalonate into in vitro synthesized peptide and by observing the concomitant mobility shift of the peptide modified with geranylgeranyl upon urea-gradient SDS-PAGE.

N-Terminal Domain Required for Rab5 Prenylation

If the mechanism of geranylgeranylation of Rab5 is similar to the modification of CAAX-containing polypeptides, then N-terminal truncation mutants should be substrates for prenylation. However, as shown by the results of Figure 2, N-terminal deletion of Rab5 perturbs post-translational processing. For this experiment, $Rab5^{wt}$, $Rab5^{88-215}$ and $Rab5^{23-215}$ were synthesized in the presence of [^{35}S]methionine and the nascent peptides are post-translationally incubated with the prenyl precursor mevalonate (500 μM) for 4 h at 37°C. Parallel reactions were performed under the identical conditions except that peptide synthesis was in the absence of [^{35}S]methionine but post-translational incubation was with [^{3}H]mevalonate (10 μM). After the prenylation reaction, aliquots were removed, diluted into Laemmli buffer, and samples were subjected to urea-gradient SDS gel electrophoresis to separate the prenylated and unprocessed isoforms of Rab5 as previously described (Sanford *et al.*, 1993). The fluorograph of the results presented in Figure 2 shows ^{35}S-radiolabeled $Rab5^{wt}$ (left panel) as a single species when incubated in the absence of mevalonate; when prenylated upon incubation in the presence of mevalonate, this peptide shifts to a greater mobility isoform. That this isoform is covalently modified is demonstrated by the fact that the mobility-shifted peptide incorporates radiolabel from the isoprenoid precursor [^{3}H]mevalonate. While $Rab5^{wt}$ displays the mobility shift in the presence of mevalonate and incorporates radiolabel from [^{3}H]mevalonate into the lower isoform, $Rab5^{88-215}$ is not converted to a greater mobility isoform nor does it become radiolabeled. This is in contrast to prenylation of the CAAX motif of proteins such as H-Ras, Rap1A, and γ subunits of regulatory G-proteins, which is modified whether fused to a neutral protein or present as a tetrapeptide alone (Reiss *et al.*, 1990). This result is not surprising, however, since previous studies in our laboratory have established that prenylation of Rab5 is dependent on guanine nucleotide bind-

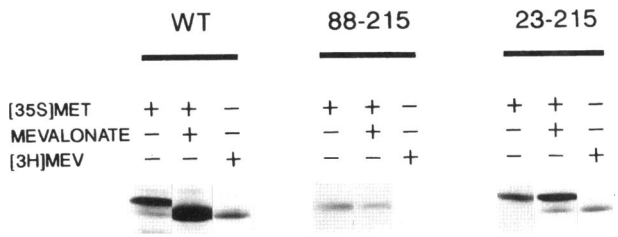

Figure 2. N-terminal truncation mutants fail to become modified. Constructs were prepared to generate transcripts of $Rab5^{wt}$ and the N-terminal truncation mutants $Rab5^{88-215}$ and $Rab5^{23-215}$. These transcripts were translated in vitro in the presence or absence of [^{35}S]methionine (150,000 cpm/μl) as detailed in the MATERIALS AND METHODS. Briefly, transcripts were incubated in reticulocyte lysate at 100–200 μg/ml for 20 min at 30°C. Reaction mixtures were then supplemented with either 500 μM mevalonate or [^{3}H]mevalonate (25,000 cpm/μl) and incubated for 4 hours at 37°C as indicated. All of the samples were electrophoresed on a 4–8 M urea/10–15% polyacrylamide SDS gel slab that separates isoforms of Rab5. Incorporation of radiolabel form [^{3}H]mevalonate confirms that the mobility shifted isoform is the prenylated species. Shown are fluorographs in which labeled peptides corresponding to $Rab5^{wt}$ (35 fmol per lane), $Rab5^{88-215}$ (20 fmol per lane), and $Rab5^{23-215}$ (30 fmol per lane) were electrophoresed.

ing, and this severe truncation eliminates domains critical for this function. Therefore, to ascertain features of the N-terminal domain that might be crucial for Rab5 processing, the post-translational modification of $Rab5^{23-215}$ was also studied. N-terminal truncation of this mutant is such that the functionally important guanine nucleotide binding domains remain intact (Figure 1). As shown by the results of Figure 2, in vitro prenylation of $Rab5^{23-215}$ is inefficient with only ~20% of the peptide converted to the greater mobility isoform (right panel). Thus, it appears that features inherent in the N-terminal domain outside of the guanine nucleotide binding pocket must contribute to the ability of Rab5 to become modified.

Conservation in the N-Terminal Domain of Rab Family Members

One interpretation of the results described above is that structural information crucial for the recognition of Rab5 by a geranylgeranyl transferase is located in the 22 amino acid N-terminal domain. This region of the molecule is adjacent to a sequence of aliphatic residues positioned immediately upstream from the glycine-rich "phosphate binding loop," a consensus sequence present in all GTP-binding proteins (Valencia *et al.*, 1991). A multiple alignment of Rab family members reveals a highly conserved domain that is coincident within the first 22 amino acids of Rab5 (Table 1). PIMA (pattern-induced multisequence alignment) analysis (Smith and Smith, 1992) of Rabs 1–4, 8, 10, and 11 identifies a pattern of highly conserved sequence elements: YXYLFK, where X is any amino acid, typically D. The general features of this pattern are also recognized in Rabs 5, 6, and 7, albeit with limited, non-conservative sequence substitutions of amino acids which do not coincide with otherwise hydrophobic elements within this motif (see Table 1). Our analysis suggested that this domain may represent a novel motif involved in initiating and/or regulating Rab protein prenylation, particularly since the data presented in Figure 2 support the idea that features of Rab5's N-terminus play an important role in post-translational modification.

In order to test this hypothesis, a Rab5 cDNA clone was constructed to encode elements of the conserved motif at the N-terminus of $Rab5^{23-215}$, referred to as $MAYDYLFKRab5^{23-215}$. As shown by the results of Figure 3, the presence of this motif enables the new construct to become efficiently prenylated—in striking contrast to the truncation mutant lacking the N-terminal domain. In order to judge whether the N-terminally modified Rab5 construct is prenylated as efficiently as $Rab5^{wt}$, the rates of geranylgeranylation of $Rab5^{wt}$, $Rab5^{23-215}$, and $MAYDYLFKRab5^{23-215}$ were compared. Following synthesis, each peptide was adjusted to 20 fmol/μl lysate prior to incubation at 37°C in the presence of mevalonate. Aliquots of each reaction mixture were removed at the indicated times and diluted into Laemmli buffer for electrophoresis on urea-gradient SDS-polyacrylamide gels as described in Figure 2. Quantitation of the extent of modification is facilitated by densitometric scanning of appropriate bands representing the nascent and prenylated isoforms of Rab5. The percentage of peptide modified measured in this manner is plotted as a function of time (panel A). Parallel reaction mixtures (prepared in the absence of [^{35}S]methionine) were shifted to 37°C and incubated in the presence of [^{3}H]mevalonate for 4 h in order to verify prenylation of the second isoform (right lanes, panel B). Our results show that greater than 95% of both $Rab5^{wt}$ (■) and $MAYDYLFKRab5^{23-215}$ (●) peptides become prenylated within 2 h of incubation. The initial rates of conversion calculated for these peptides are similar, 14.6 and 15.3 fmol/min, respectively. In contrast, the $Rab5^{23-215}$ truncation mutant fails to be completely converted to the greater mobility form even after 4 h of incubation (panel B). The apparent rate of prenylation of the truncated Rab5 (based on the 4-h time point), 2.3 fmol/min, is ~6-fold less than that measured for the other peptides (panel A). Having shown that the rate of prenylation for $MAYDYLFKRab5^{23-215}$ is nearly identical to that of $Rab5^{wt}$, we conclude that the N-terminal domain is essential for efficient prenylation and that a conserved six-amino acid motif (YXYLFK) can effectively replace the first 22 amino acids of Rab5 to enable post-translational modification of the peptide.

Table 1. Rab protein structure: analysis of N-terminal domains

Generic Rab:	(M A)**Y D Y L F K** - - -
Rab11	M G T R D D E **Y D Y L F K** - - -
Rab2	M A **Y A Y L F K** - - -
Rab1	... M N P E **Y D Y L F K** - - -
Rab8	M A K T **Y D Y L F K** - - -
Rab4	M(A/S)E **T Y D F L F K** - - -
Rab10	M A K K **T Y D L L F K** - - -
Rab3	... S D Q N **F D Y M F K** - - -
Rab5	... N T G N **K I C Q F K** - - -
Rab7	M T S R **K K V L L K** - - -
Rab6	... D F G N **P L R K F K** - - -

A computer program called PIMA (Smith and Smith, 1992) was used to evaluate Rab family protein sequences. Two methods of evaluation (Sequential Branching and Multiple Linking) were employed with both leading to the recognition of 6-amino acid motif YXYLFK represented in sequences available for dRab11, rRab11, hRab2, dRab2, rRab1B, hRab1, dRab1, dRab8, dRab4B, hRab4, and dRab10. This particular pattern is not as well defined in hRab3A, hRab3B, dRab5, hRab5, dRab7, and hRab6, but elements can be immediately recognized. Underlined amino acids are non-hydrophobic elements which do not coincide with otherwise hydrophobic elements within the motif. The top line is the "generic" Rab sequence employed to construct Rab5 and H-Ras variants used in this study.

YXYLFK Motif Necessary but not Sufficient to Confer Specificity of Prenylation

The ultimate test in evaluating the ability of a putative motif to promote geranylgeranylation is to generate a hybrid peptide incorporating the domain in question and to investigate whether or not the motif confers the ability for modification to the hybrid species. Therefore, a series of chimeric peptides were constructed to incorporate a "generic Rab" YDYLFK motif and spliced domains of Rab5 and H-Ras. The chimeras were then examined for their ability to become prenylated in vitro. Figure 1 illustrates the following constructs that were translated and the corresponding synthesized peptides that were screened as substrates for prenylation by their capacity to become mobility-shifted in the presence of mevalonate: $Rab5^{wt}$; MAYDYLFK$Rab5^{23-215}$; H-Ras; Ras^{1-185}CCSN; MAYDYLFKRas^{6-189}; MAYDYLFKRas^{6-185}CCSN; $Ras^{1-184}Rab5^{200-215}$; and MAYDYLFK$Ras^{6-184}Rab5^{200-215}$. As shown by the results of Figure 4, $Rab5^{wt}$, MAYDYLFK$Rab5^{23-215}$, H-Ras and MAYDYLFKRas^{6-189} all become prenylated, exhibiting second isoforms due to modification supported by the presence of mevalonate. The appearance of greater mobility isoforms of $Rab5^{wt}$ and MAYDYLFK$Rab5^{23-215}$ as a consequence of the prenylation of these peptides is consistent with the results of Figures 2 and 3. Other studies have demonstrated that H-Ras peptides with an unmodified, intact C-terminus (CAAX motif) will be shifted to a greater mobility (Sanford *et al.*, 1991; Kato *et al.*, 1992), due to the autonomous properties of the motif to support farnesylation where X is Ser in H-Ras. Therefore, the observations that both H-Ras and MAYDYLFKRas^{6-189} are shifted to a second isoform in the presence of mevalonate are consistent with the post-translational modification of these peptides in vitro. For each of these peptides, control experiments demonstrated the incorporation of radiolabel in the presence of [^{3}H]mevalonate, confirming that $Rab5^{wt}$, MAYDYLFK$Rab5^{23-215}$, H-Ras, and MAYDYLFKRas^{6-189} all become prenylated (our unpublished results).

Studies involving Ypt1 and Rab3A have indicated structural determinants for prenylation in addition to the C-terminal modification sites (Moores *et al.*, 1991; Khosravi-Far *et al.*, 1991). For example, a H-Ras/Ypt1 chimera with six C-terminal residues of Ypt1 fails to be modified by a prenyl transferase that recognizes wild-type Ypt1 as a substrate. Hence, it might be expected that a hybrid construct encoding a H-Ras/Rab5 peptide would not be a suitable substrate for prenylation in our in vitro system. Correspondingly, Ras^{1-184}CCSN, which contains the C-terminal four amino acids of Rab5 spliced to the C-terminus of H-Ras, is not converted to a greater mobility isoform in the presence of mevalonate (Figure 4). Thus, unlike the CAAX motif, the C-terminal Rab5 CCSN tetrapeptide fails to support prenylation of a neutral protein. This result is compatible with the inability of Rab5 C-terminal peptides to block prenylation of full-length Rab5 molecules (Kinsella and Maltese, 1992; Peter *et al.*, 1992), in contrast to CAAX-containing peptides that interfere with Ras prenylation (Reiss *et al.*, 1990).

To critically determine if the conserved Rab motif has structural information sufficient to promote prenylation of Ras^{1-184}CCSN, a construct was engineered to encode the peptide MAYDYLFKRas^{6-185}CCSN. This chimera is also a poor substrate for prenylation, as indicated by its failure to be converted to a greater mobility form upon incubation with mevalonate (Figure 4) or to incorporate radiolabel in the presence of [^{3}H]mevalonate (data not shown). Thus, although the conserved N-terminal motif

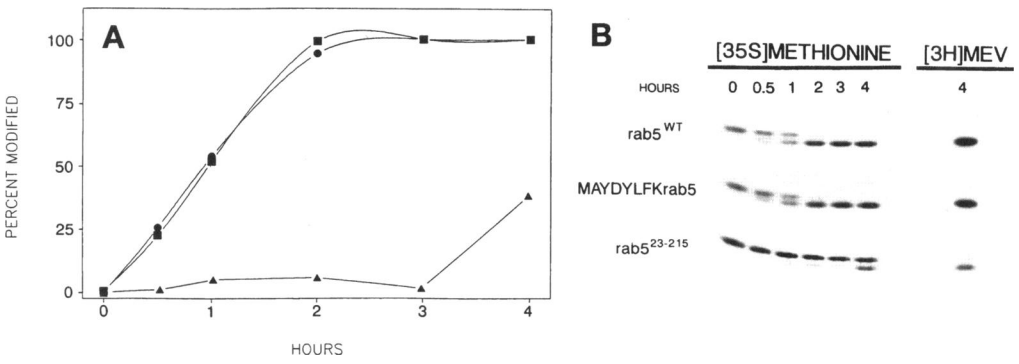

Figure 3. Time course of modification demonstrates that YXYLFK motif enables N-terminal truncation mutant to become efficiently prenylated. Translation reactions were carried out as described for Figure 2 to prepare [^{35}S]methionine-labeled peptides generated from $Rab5^{wt}$ (■), MAYDYLFK$Rab5^{23-215}$ (●), and $Rab5^{23-215}$ (▲) transcripts. Each translation mixture was adjusted to contain 20 nM peptide in stop buffer: 12 mM Tris-HCl, pH 8.0, 3 mM $MgCl_2$, 0.6 mM DTT, and 100 μg/ml RNase A; the final volume was 20 μl with reticulocyte lysate at 40% (v/v). Prenylation reactions were performed in the presence of 500 μM mevalonate at 37°C and aliquots (20 fmol) were removed at indicated times and diluted into Laemmli buffer. Samples were electrophoresed on urea-gradient gels and processed for fluorography. Films were densitometrically scanned and bands were quantified using a Bio Image whole band analyzer (Millipore). (A) The percentage of peptide converted to the greater mobility isoform was calculated based on integrated optical densities measured for the two isoforms. Shown is the percentage of nascent peptide modified as a function of time. (B) The actual fluorographs of ^{35}S-labeled peptides that were scanned to generate the plot in panel A are presented. Also shown are equivalent samples prepared in parallel except that [^{35}S]methionine was omitted from the translation reaction and [^{3}H]mevalonate was added during the post-incubation prenylation reaction to confirm that the second isoform is modified.

is important for Rab5 prenylation, it alone is not sufficient to promote prenylation of chimeric molecules with the CCSN C-terminal motif. Since a 10 residue sequence immediately upstream of Cys^{212} has been shown to be required for the partitioning of Rab5 peptides into a membrane fraction (Chavrier *et al.*, 1991) and deletion of this region of the molecule prevents its prenylation in vitro (Peter *et al.*, 1992), it is possible that modification of Rab5 is also dependent on this 10 amino acid C-terminal domain (Leu^{201}–Gln^{211}). In order to investigate this idea, two clones were constructed to encode H-Ras/Rab5 chimeric peptides wherein the last 16 amino acids of Rab5 are fused to the C-terminus of H-Ras at amino acid 184 with one containing the N-terminal motif in addition to this domain (see Figure 1). Both $Ras^{1-184}Rab5^{200-215}$ and $MAYDYLFKRas^{6-184}Rab5^{200-215}$ fail to become modified in vitro since processed isoforms are not generated upon incubation in the presence of mevalonate (Figure 4).

Finally, although all of the H-Ras/Rab5 chimeras were constructed without alterations in conserved GTP-binding domains, failure of the chimeric peptides to become prenylated could be explained by a defect in the ability of the molecules to bind guanine nucleotides, since the GDP-bound state of Rab5 is the preferred substrate conformation for recognition by the Rab geranylgeranyl transferase (Sanford *et al.*, 1993). In order to assess the structural integrity of these peptides, the Ras chimeras were immunoprecipitated and electrophoresed for Western blotting to Immobilon P membrane. The blot was then probed with [α-^{32}P]GTP following the overlay protocol detailed in the MATERIALS AND METHODS. All of the Ras chimeras, $Ras^{1-185}CCSN$, $MAYDYLFKRas^{6-189}$, $MAYDYLFKRas^{6-185}CCSN$, $Ras^{1-184}Rab5^{200-215}$, and $MAYDYLFKRas^{6-184}Rab5^{200-215}$, were found to bind [$\alpha$-^{32}P]GTP on blots. Thus, the structural integrity of the H-Ras/Rab5 chimeras appears to be maintained despite the alterations at the extreme N- and C-termini. This indicates that other elements inherent in Rab5's tertiary structure that are lacking in H-Ras are required for geranylgeranylation. It should be noted, however, that although the YXYLFK motif and elements of Rab5's C-terminal domains do not confer the capacity for modification to the H-Ras/Rab5 chimeras, we can not exclude the possibility that their interaction is a prerequisite for Rab5 post-translational modification. Combined, our observations indicate the strong likelihood that geranylgeranylation of Rab5 is dictated by tertiary structure that is dependent on both N- and C-terminal domains of the molecule.

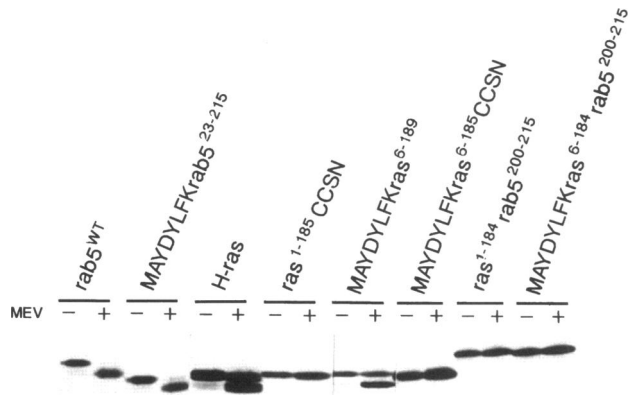

Figure 4. Analysis of the capacity of H-Ras/Rab5 chimeras to be modified. Chimeric constructs containing N- and C-terminal domains of Rab5 and H-Ras were constructed as illustrated in Figure 1. Translation reactions with [^{35}S]methionine supported by transcripts indicated in the Figure were performed. The nascent peptides were post-incubated at 37°C for 2 hours as detailed in Figures 2 and 3 in the presence or absence of mevalonate as indicated. Shown is fluorography of samples analyzed on urea-gradient gels for the ability of the corresponding peptide to become mobility-shifted upon prenylation: $Rab5^{wt}$ (15 fmol per lane); $MAYDYLFKRab5^{23-215}$ (15 fmol per lane); H-Ras (80 fmol per lane); Ras^{1-185} CCSN (20 fmol per lane); $MAYDYLFKRas^{6-189}$ (20 fmol per lane); $MAYDYLFKRas^{6-189}$CCSN (25 fmol per lane); $Ras^{1-184}Rab5^{200-215}$ (25 fmol per lane); and $MAYDYLFKRas^{6-184}Rab5^{200-215}$ (25 fmol per lane).

Deletion Mutants of YXYLFK Motif Are Geranylgeranylated

To further delineate structural elements present in the YXYLFK motif that appear to be required for Rab5 geranylgeranylation, a construct was prepared corresponding to a deletion of three amino acids to produce the peptide $MYLFKRab5^{23-215}$. This particular mutant retains the amino acid residues most conserved within the YXYLFK motif of the Rabs evaluated (see Table 1) and our results demonstrate that the "YLFK" sequence also confers to the truncated $Rab5^{23-215}$ peptide the ability to be fully processed when incubated with geranylgeranyl pyrophosphate. Figure 5 compares the extent of processing of ^{35}S-labeled $MAYDYLFKRab5^{23-215}$ and $MYLFKRab5^{23-215}$ when incubated at 37°C in the presence of geranylgeranyl pyrophosphate (+). Both peptides are fully converted to the prenylated isoform within 4 h. Surprisingly, when the YLFK sequence was replaced with the first four amino acids of H-Ras to create an N-terminal H-Ras/Rab5 chimera, the corresponding peptide was also found to be processed in a manner similar to wild-type. That is, $MTEYKRab5^{23-215}$ was found to become geranylgeranylated to the same extent as both $MYLFKRab5^{23-215}$ and $MAYDYLFKRab5^{23-215}$ (Figure 5). The time course of prenylation for $MTEYKRab5^{23-215}$, $MYLFKRab5^{23-215}$, and $MAYDYLFKRab5^{23-215}$ was also identical. These results suggest that within the YXYLFK motif, the last two residues are of greatest functional importance. In particular, the finding that the H-Ras motif "TEYK" also supports geranylgeranylation when spliced to the N-terminal truncation mutant $Rab5^{23-215}$ raises the idea that the lysine resi-

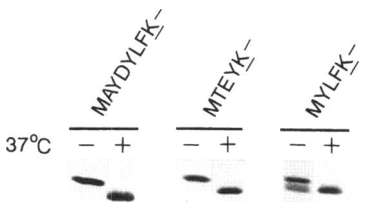

Figure 5. Deletion analysis of the YXYLFK motif identifies vital primary structure required for geranylgeranylation. Chimeric constructs were assembled on the Rab5$^{23-215}$ backbone, as indicated in the Figure, with the amino acid lysine (underlined) common to all three constructs. MAYDYLFKRab5$^{23-215}$, METYKRab5$^{23-215}$, and MYLFKRab5$^{23-215}$ were translated in vitro as detailed in MATERIALS AND METHODS. Each synthesized peptide was adjusted to 5 nM by dilution in stop buffer as described in Figure 3, except that mevalonate is replaced by 10 μM geranylgeranyl pyrophosphate, and incubated at 37°C where indicated (+). After 4 h, 15 fmol was diluted into Laemmli buffer and 5 fmol was applied to urea-gradient gels. Shown is fluorography of samples demonstrating that these peptides are fully processed with geranylgeranyl and convert to the lower isoform.

due and/or neighboring aromatic residues (either F or Y) are critical within the Rab YDYLFK domain. The lysine residue is immediately adjacent to a stretch of four aliphatic amino acids N-terminal with respect to the phosphate binding loop domain and is highly-conserved in both Rab and Ras family members (Valencia *et al.*, 1991). This lysine residue is typically preceded by an aromatic amino acid (Y/F). Thus, based on the results shown in Figure 5, we reasoned that one or both of these conserved amino acids may be critically positioned for recognition by the Rab geranylgeranyl transferase.

Lys→Ala Mutation in MAYDYLFKRab5$^{23-215}$ Blocks Geranylgeranylation

The putative role of both the F and K in the YDYLFK sequence as structural determinants necessary for Rab5 post-translational modification was further examined using point mutations created at these sites. Both MAYDYLAKRab5$^{23-215}$ (F→A) and MAYDYLFARab5$^{23-215}$ (K→A) were assayed for the ability to become geranylgeranylated. Figure 6 compares the time course of post-translational modification of these mutants with the control peptide, MAYDYLFKRab5$^{23-215}$. Conditions for translation and in vitro geranylgeranylation were as described for Figure 3, except that the final substrate concentration was 12 nM. The percent conversion due to modification of each peptide with geranylgeranyl is plotted over time (Figure 6, panel A). Panel B presents the actual fluorograph scanned to generate the plot in panel A. The point mutation MAYDYLAKRab5$^{23-215}$ was modified to the same extent and rate as MAYDYLFKRab5$^{23-215}$ (Figure 6, panel A). However, the K→A mutant, MAYDYLFARab5$^{23-215}$, was a poor substrate with a greatly reduced rate of modification (~15-fold slower than the other two peptides). As shown by the results in Figure 6, less than 15% of the mutant peptide becomes geranylgeranylated. Therefore, within the context of the YXYLFK motif this lysine residue appears to be critical for efficient Rab5 prenylation.

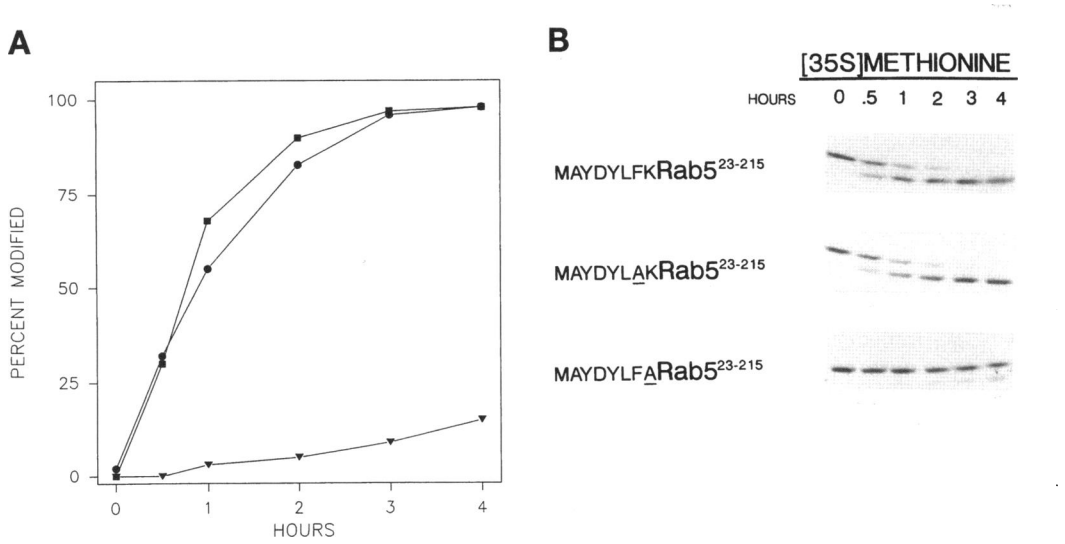

Figure 6. MAYDYLFARab5$^{23-215}$ fails to be geranylgeranylated. The timecourse of geranylgeranylation of MAYDYLFKRab5$^{23-215}$ (■) and two point mutants, MAYDYLAKRab5$^{23-215}$ (●) and MAYDYLFARab5$^{23-215}$ (▼), was determined. The translated, ^{35}S-labeled peptides were diluted to 12 nM in stop buffer and incubated in the presence of 10 μM geranylgeranyl pyrophosphate at 37°C. At the indicated times, 3 μl were removed (36 fmol of peptide) and diluted into Laemmli buffer. Samples were applied to a urea-gradient gel which was subsequently processed for fluorography. (A) Densitometric scans of the processed and unprocessed isoforms were obtained as in Figure 3 and the percentage of peptide converted to the greater mobility isoform was plotted versus time of incubation for the prenylation reaction. (B) The actual fluorographs scanned to generate the data presented in panel A are shown.

Structural Analysis of Rab Peptides

One potential explanation of the finding that the truncated Rab5$^{23–215}$ (Figure 2) and the K→A Rab5 mutant (Figure 6) both fail to become geranylgeranylated is that they are unable to fold appropriately and/or their interactions with guanine nucleotides may be impaired. Previous studies in our laboratory have shown that post-translational modification of the Rab protein is conformationally sensitive (Sanford *et al.*, 1993). Because of this concern, these and other Rab5 prenylation substrates were analyzed for the ability to bind guanine nucleotides.

As a functional criteria of the Rab5 molecules' structural integrity, the protection from proteolytic digestion afforded by the presence of bound nucleotides was assessed (Figure 7). For these experiments, the Rab5 peptides were translated in the presence of [^{35}S]methionine, then preincubated in the presence or absence of 10 mM GTPγS, or with 25 mM EDTA, followed by limited proteolytic digestion with 0.022 unit of trypsin for 45 min at 30°C. When Rab5 is trypsinized in the absence of GTPγS or EDTA (*panel A*), a single radiolabeled band is observed of 14 kDa molecular weight. The inclusion of EDTA in the reaction mixture promotes guanine nucleotide dissociation due to chelation of Mg^{2+}, thus destabilizing the protein's structure, and in fact under these conditions the amount of intact 14-kDa fragment is reduced. Preincubation in the presence of GTPγS, however, interferes with the limited digestion of Rab5^{wt} by trypsin, and an additional 19-kDa radiolabeled species is observed. We interpret the appearance of the "core" fragment to reflect a conformational change in the activated Rab protein in the GTP-bound state such that when GTPγS is bound, Rab5^{wt} adopts a conformation resistant to tryptic cleavage.

The protease protection observed in the presence of GTPγS can be utilized to identify the guanine nucleotide binding capacity of other Rab5 mutants. This assay is validated by the tryptic pattern observed for Rab5^{N133I} which is sensitive to the protease under all conditions tested (panel A). Since guanine nucleotides are thought to rapidly dissociate from the latter mutant (Walter *et al.*, 1986), it is not surprising that this peptide is completely digested. Only a minor amount of the 19-kDa fragment is observed to be protected in the presence of GTPγS. In contrast, MAYDYLFKRab5$^{23–215}$ displays a pattern of sensitivity to tryptic proteolysis that is identical to that of Rab5^{wt} (panel A). Here, a 18-kDa fragment is observed in the presence but not in the absence of GTPγS. It is likely that the protected 19-kDa tryptic fragment of Rab5^{wt} contains an N-terminal portion of the protein, thus accounting for the difference in mobility of the protected "core" species due to the reduced length of this domain in the mutant. Given that both of these peptides are efficiently prenylated in the in vitro assay (Figure 3), both would be expected to maintain native tertiary structure to bind guanine nucleotides (Sanford

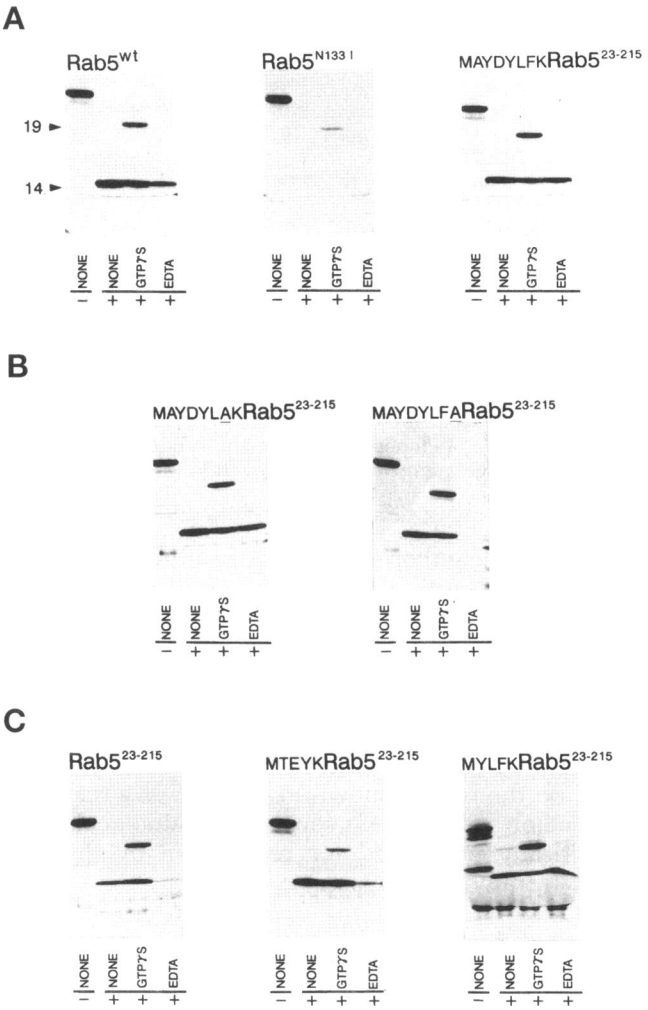

Figure 7. N-terminal mutants Rab5$^{23–215}$ and MAYDYLFARab5$^{23–215}$ exhibit native structural stability. Transcripts programmed a reticulocyte lysate to synthesize the following peptides to be analyzed for GTPγS protection of limited proteolysis: for (A) Rab5^{wt}, Rab5^{N133I}, and MAYDYLFKRab5$^{23–215}$; for (B), MAYDYLAKRab5$^{23–215}$ and MAYDYLFARab5$^{23–215}$; for (C), Rab5$^{23–215}$, MYLFKRab5$^{23–215}$ and MTEYKRab5$^{23–215}$. Peptides were diluted to 3.5 nM in a reaction buffer containing 80 mM Tris-Cl, pH 8.0, 2 mM $MgCl_2$, 100 mM NaCl, and 1 mM DTT and precincubated in the presence of the indicated reagents (10 mM GTPγS or 25 mM EDTA) for 45 min at 30°C. As detailed in the MATERIALS AND METHODS, reaction mixtures were then incubated for an additional 45 min at 30°C with (+) or without (−) trypsin as indicted (final volume, 10 μl). Control reactions (NONE) were performed such that no other reagents were added during either the preincubation or trypsin incubation steps. After the termination of proteolysis upon addition of soybean trypsin inhibitor, samples were diluted into Laemmli buffer, boiled for 5 min and electrophoresed on a urea-gradient gel. Shown is the proteolytic pattern of ^{35}S-labeled peptides obtained by fluorography. The arrowheads indicate molecular weight in kilodaltons.

et al., 1993), a prediction that is born out by the results of Figure 7.

To study the structural integrity of the two point mutants, both MAYDYLAKRab5$^{23-215}$ and MAYDYLFARab5$^{23-215}$ were synthesized in vitro and digested with trypsin as described above (Figure 7, panel B). In the presence of GTPγS, both of the point mutants were protected from proteolytic cleavage and a 18-kDa fragment is observed, similar in size to the core species observed for MAYDYLFKRab5$^{23-215}$. While it was anticipated that MAYDYLAKRab5$^{23-215}$ would be protected from digestion based on the peptide's ability to serve as a substrate for prenylation (Figure 6), this result provides strong evidence that the failure of MAYDYLFARab5$^{23-215}$ to become geranylgeranylated is not simply explained by a lack of tertiary structure stability. Moreover, the N-terminal truncation mutant Rab5$^{23-215}$ is also protected from proteolytic cleavage in the presence of GTPγS (panel C). This confirms the idea that the N-terminal domain is not required for proper protein folding and underscores the critical importance of the lysine residue for functional interactions with the Rab geranylgeranyl transferase. MTEYKRab5$^{23-215}$ and MYLFKRab5$^{23-215}$, which have the critical lysine, serve as substrates for prenylation and are protected from tryptic digestion in the presence of GTPγS as well (panel C).

Based on the results of the protease protection experiments, we conclude that all of the Rab5 mutants studied here fold in an appropriate manner to maintain the GTP-binding pocket. It should be noted that the key advantage of this method is that is permits a direct comparison of the ability of the nascent peptides to become geranylgeranylated with their capacity to functionally interact with guanine nucleotides in the reticulocyte lysate. However, since the lysate contains mM levels of GTP, it was necessary to conduct these experiments using a relatively high concentration of GTPγS (10 mM). Thus, it is possible that a significantly impaired affinity for guanine nucleotide might be obscured under the conditions of our assay. Due to the limited amounts of Rab protein expressed and the presence of high concentrations of endogenous GTP-binding proteins in the reticulocyte lysate, it is not possible to directly examine the GDP-binding properties of the newly synthesized proteins. Therefore to assess the ability of these mutants to bind GDP, the following peptides were expressed in *E. coli* under the control of the T_7 promoter: Rab5^{wt}, MAYDYLFKRab5$^{23-215}$, MAYDYLFARab5$^{23-215}$, MTEYKRab5$^{23-215}$, MYLFKRab5$^{23-215}$, Rab5$^{23-215}$ and Rab5^{N133I}. All of the peptides except for MAYDYLFARab5$^{23-215}$ were found in soluble fractions of *E. coli* lysate which were then employed in [^{3}H]GDP binding studies (Figure 8). Briefly, bacterial lysate supernatants containing Rab proteins (1 μM final concentration) were incubated with [^{3}H]GDP (20 μM) and specific binding of the guanine nucleotide was determined in a filter binding assay described in MATERIALS AND METHODS. As a control, GDP-binding of Rab5^{N133I} was examined and found to be less than 10%; this is the anticipated result based on the idea that nucleotides rapidly dissociate from this mutant (Walter *et al.*, 1986) and it demonstrates that background binding of GDP to other bacterial proteins is insignificant.

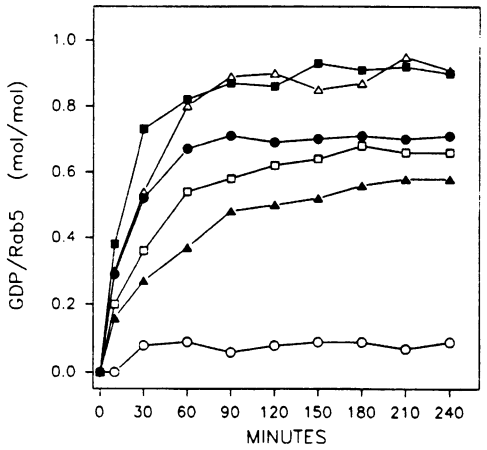

Figure 8. [^{3}H]GDP-binding of Rab5 peptides. Recombinant wild-type and mutant Rab5 peptides were incubated in GDP-binding buffer with [^{3}H]GDP (3500 cpm/pmol) for 4 h at 25°C. At the indicated times, 20-μl aliquots (20 pmol peptide) were filtered in duplicate through nitrocellulose discs. The filters were washed twice with 2 ml and once with 1 ml of GDP-wash buffer and the trapped [^{3}H]GDP-Rab5 complex was measured by liquid scintillation counting. Non-specific binding was measured in parallel for each recombinant peptide by including 35 mM unlabeled GDP. Specific [^{3}H]GDP binding reflects the difference in cpm measured in the absence versus the presence of unlabeled GDP. Shown are the results obtained for Rab5^{wt} (●), Rab5^{N133I} (○), MAYDYLFKRab5$^{23-215}$ (■), Rab5$^{23-215}$ (□), MTEYKRab5$^{23-215}$ (▲), and MYLFKRab5$^{23-215}$ (△).

With this approach, MYLFKRab5$^{23-215}$ and MAYDYLFKRab5$^{23-215}$ were found to bind [^{3}H]GDP rapidly and with a stoichiometry of ~0.9 mol GDP per mol protein. In contrast, although the binding of [^{3}H]GDP by Rab5^{wt} was as rapid, a final stoichiometry of 0.71:1 was observed. This difference may be due to the presence of a small amount of inactive protein expressed in the bacterial lysate. Similarly, the truncation mutant Rab5$^{23-215}$ also bound [^{3}H]GDP at a final stoichiometry of 0.66:1, although the rate of binding is slightly lower than that displayed by wild-type Rab protein (0.0008 s^{-1} versus 0.0012 s^{-1}, respectively). These data suggest that the interactions of the mutant peptide with GDP may be altered relative to wild-type Rab5, potentially accounting for the failure of this mutant to become prenylated. However, the kinetics of [^{3}H]GDP binding to MTEYKRab5$^{23-215}$ is also slower than wild type (0.0004 s^{-1}) with a final stoichiometry of 0.58 mol GDP per mol peptide. Because the latter mutant contains the critical lysine

residue and is a good substrate for prenylation, it appears unlikely that the inability of $Rab5^{23-215}$ to become efficiently geranylgeranylated is due to functionally significant changes in GDP binding by the truncation mutant.

Another argument that GDP-binding is not significantly impaired in the mutant is that the kinetics of post-translational modification of $Rab5^{23-215}$ suggest that it is a poorer substrate for processing relative to $Rab5^{N133I}$ expressed in the reticulocyte lysate (Figure 3; Sanford *et al.*, 1993). The latter mutant clearly has altered interactions with guanine nucleotides and fails to bind significant levels of [^{3}H]GDP in our assay while $Rab5^{23-215}$ binds GDP to the same extent observed for $Rab5^{wt}$ (Figure 8). The kinetics of binding also demonstrate that over the time course of the prenylation reaction a significant fraction of $Rab5^{23-215}$ should bind GDP; maximal GDP-binding of the recombinant peptide occurs within 2 h and yet less than 25% of the mutant is processed within 4 h of post-translational modification in the reticulocyte lysate.

One caveat to our conclusion is that these experiments do not rigorously identify the state of guanine nucleotide binding of the mutant proteins in the prenylation reaction supported by mammalian lysate; instead we can only infer the conformational state of the protein from studies using the recombinant peptides expressed in *E. coli*. However, the protease protection results obtained in the reticulocyte lysate on in vitro expressed proteins (Figure 7) do support the notion that the truncation mutant and the K→A point mutant both interact with guanine nucleotides in a functional manner. $Rab5^{N133I}$ in the absence of GTPγS (i.e., GDP bound form) is completely digested, while under these conditions $Rab5^{23-215}$ and MAYDYLFARab5^{23-215} both display a 14-kDa fragment similar to $Rab5^{wt}$. If GDP were to rapidly dissociate from the mutants, the 14-kDa peptide should become completely proteolyzed. Thus, within the limits of our analysis, Rab5's N-terminal domain appears to be dispensible for functional aspects of protein folding and GDP binding, while features of the conserved YXYLFK domain, in particular the critical lysine residue, are more important for recognition of the protein by the Rab geranylgeranyl transferase.

DISCUSSION

The Rab protein family is thought to participate in the signaling process that maintains the integrity of vectorial transport within cells. Initial events of endocytosis have been established to involve at least one member of this family Rab5, which is cytolocalized to the plasma membrane, coated vesicles, and endosomal structures (Chavrier *et al.*, 1990). A current hypothesis is that membrane localization of Rab proteins is mediated, in part, by C-terminal structural elements that are post-translationally incorporated via prenylation. Our studies on the covalent modification of Rab5 by the 20-carbon isoprenoid geranylgeranyl are focused on understanding structure-function relationships between this hydrophobic modification and Rab5's mechanism of action. To facilitate these studies, we have established conditions that allow the prenylation of nascent Rab5 peptide to be assessed in a quantitative manner (Sanford *et al.*, 1993). Post-translational processing of cell-free synthesized Rab5 with geranylgeranyl is monitored by the appearance of a greater mobility isoform on urea-gradient SDS-PAGE gels. The observation that this second isoform will also incorporate radiolabel in the presence of either [^{3}H]geranylgeranyl pyrophosphate or [^{3}H]mevalonate confirms our ability to study post-translational processing by prenylation (Sanford *et al.*, 1993).

Using this approach, we have identified that N-terminal regions of the molecule are important to support post-translational geranylgeranylation in vitro. The results obtained with the N-terminal truncation mutants $Rab5^{88-215}$ and $Rab5^{23-215}$ indicate that domains present in the first 22 amino acids of the protein provide critical elements for the efficient processing of the GTP-binding protein. Prompted by this observation, a computer analysis of Rab protein N-terminal domains was performed and revealed a highly conserved motif, YXYLFK, that was represented in all of the known Rab family members and that was included in the first 22 amino acids required for Rab5 modification (Table 1). This motif is immediately adjacent to a stretch of aliphatic residues upstream from the glycine-rich "phosphate binding loop" present in all GTP-binding proteins (Valencia *et al.*, 1991). Moreover, when the GenBank database was searched for proteins containing the pattern YXYLFK followed by several aliphatic residues, *only Rab proteins were identified*. This distinguishes the six-amino acid domain as unique to the Rab family of GTP-binding proteins and suggests the idea that this motif may play a key role in initiating and/or regulating the modification of C-terminal cysteines of Rab proteins. Strong evidence to support this hypothesis is given by the fact that the addition of a "generic Rab" sequence MAYDYLFK to the $Rab5^{23-215}$ N-terminal truncation mutant enabled the chimera to become modified to the same extent and at the same apparent rate as wild-type Rab5 (Figure 3).

Results from other studies also support the importance of the N-terminal domain in Rab protein prenylation. For example, the use of oligopeptides as inhibitors of prenylation has been of great utility in defining the role of C-terminal motifs in directing either farnesylation or geranylgeranylation of CAAX-containing proteins (Reiss *et al.*, 1990). Nonetheless, a six-amino acid C-terminal Ypt1-cognate peptide fails to block the geranylgeranylation of full-length Ypt1, unlike the

ability of CAAX-containing peptides to inhibit modification of Ras and other proteins (Moores *et al.*, 1991). Short (4 ~ 15-amino acid) cognate peptides corresponding to the C-terminus of Rab5 also do not interfere with the geranylgeranylation of the full-length molecule (Kinsella and Maltese, 1992; Peter *et al.*, 1992). Although one Rab5 peptide corresponding to the last 34 amino acids of the protein does display the ability to block modification (Peter *et al.*, 1992), its inhibitory effects are only partial (~50%) and are only observed at high peptide concentrations (1 mM). Furthermore, chimeric peptides including the last seven C-terminal amino acids of Rab3A or the last six C-terminal residues of Ypt1 fused to H-Ras fail to be substrates for the prenyl transferase that modifies authentic Rab proteins (Moores *et al.*, 1991). Finally, the ability of purified Rab prenyl transferase to geranylgeranylate Rab3A or Rab1A is blocked by the presence of a mutant form of Rab1A that has serine residues replacing the C-terminal cysteines of the molecule (Seabra *et al.*, 1992). All of this information points to the fact that regions of Rab proteins outside of the immediate C-terminal cysteine-rich domain must be critical in directing prenylation at these sites. Our results indicate that an N-terminal region, containing the highly conserved element YXYLFK with the critical lysine residue, confers properties necessary to efficiently prenylate Rab5. This is in sharp contrast to modification of CAAX-containing proteins by farnesyl transferase or geranylgeranyl transferase I, which is dictated solely by the immediate C-terminal domain of the substrate (Moores *et al.*, 1991; Reiss *et al.*, 1990).

Although the transfer of the conserved YXYLFK sequence to the truncated $Rab5^{23-215}$ enabled recovery of post-translational modification, it alone was not sufficient to confer the specificity of prenylation to an H-Ras chimera containing the last 4 residues of Rab5, including the C-terminal cysteines as acceptor sites of geranylgeranylation. This finding agrees with the results of Zerial and co-workers, who have previously defined that deletion of 10 amino acids upstream of the C-terminal cysteines of Rab5 is sufficient to eliminate prenylation of the molecule (Peter *et al.*, 1992). It is interesting to note that the latter domain is included in the hypervariable region of Rab proteins that appears to confer the specificity of cytolocalization to family members (Chavrier *et al.*, 1991). However, a more extensive H-Ras/Rab5 chimera incorporating both the YXYLFK sequence and the last C-terminal amino acids of Rab5 also fails to become modified in vitro. Thus, although the highly conserved N-terminal domain and the hypervariable C-terminal region appear to be necessary for Rab5 geranylgeranylation, neither are sufficient to confer the specificity of prenylation to a neutral protein. One conclusion drawn from these experiments is that the tertiary structure of Rab proteins is likely to be a fundamental element in the geranylgeranylation of this class of GTP-binding proteins.

A tertiary structure-directed mechanism of Rab protein prenylation is supported by several observations. Studies by Maltese and co-workers indicate that point mutations in the conformationally sensitive effector domain of Rab1B interfere with appropriate processing (Wilson and Maltese, 1993). We have recently defined that the GDP-bound conformation of Rab5 is the preferred substrate recognized by Rab geranylgeranyl transferase (Sanford *et al.*, 1993). This is an intriguing finding particularly since component A of the transferase, called REP, has homology with GDI, a factor that regulates the interactions of Rab proteins with guanine nucleotides (Musha *et al.*, 1992). It is clear that association with GDI is dependent on the state of prenylation of the Rab proteins (Musha *et al.*, 1992; Ullrich *et al.*, 1993), suggesting a relationship between these two factors. Complex interactions between the Rab proteins and their escorts REP and GDI most likely involve GTP/GDP-sensitive alterations in tertiary structure as well as recognition of the C-terminal domain containing the geranylgeranyl sites. The experiments presented here suggest that in addition, the YXYLFK motif may play a role in promoting interactions between Rabs and these regulatory proteins.

The results of deletion analysis and point mutagenesis identify the essential elements of the YXYLFK motif to reside in the latter half of this sequence and define that the lysine (residue 22 in Rab5) is critical for prenylation of Rab5. The N-terminal domain itself, and in particular, the critical lysine residue, do not appear to be essential for guanine nucleotide binding based on our protease protection data (Figure 7) and the ability of recombinant $Rab5^{23-215}$ to bind GDP (Figure 8). The data are consistent with the results of Li and Stahl (1993) indicating that, although a point mutant at this site $Rab5^{K22A}$ functionally binds guanine nucleotides, it has significantly less activity than $Rab5^{wt}$ in the stimulation of HRP uptake in BHK cells. Based on our results, it is possible that the post-translational modification of this particular mutant may be inefficient, potentially accounting for its impaired function. Li and Stahl (1993) have also studied the function of an N-terminal truncation mutant, $MFKRab5^{23-215}$; this mutant retains the highly conserved and important residues identified by our study to reside in the YXYLFK motif. The fact that this truncation mutant is post-translationally prenylated but fails to significantly stimulate HRP uptake by BHK cells compared to $Rab5^{wt}$ suggests that elements of the conserved motif besides the critical lysine may be essential for appropriate Rab protein

function (Li and Stahl, 1993). Since structural and functional features appear to be conferred through the hypervariable C-terminal region of Rab proteins, the ability of different escort proteins, such as REP and GDI, to discriminate between individual family members may rest in the interactions promoted by tertiary structure determinants residing in both C- and N-terminal domains. Although all of our data are compatible with this idea, the relative contributions of this unique N-terminal domain to Rab protein function, and the exact role of the lysine in protein geranylgeranylation, remain to be defined in future studies.

ACKNOWLEDGMENTS

We thank the Molecular Biology Computer Research Resource (MBCRR) of the Dana Farber Cancer Institute, and in particular, Tom Graf, for assistance in sequence analysis using the PIMA program. J.C.S. is a fellow of the American Heart Association, Massachusetts Affiliate, Inc. M.W.-R. is the recipient of a Junior Faculty Award from the American Cancer Society. This work was supported in part by a grant from the Council for Tobacco Research-USA, Inc.

REFERENCES

Anderegg, R.J., Betz R., Carr, S.A., Crabb, J.W., and Duntze, W. (1988). Structure of *Saccharomyces cerevisiae* mating hormone a-factor: identification of *S*-farnesyl cysteine as a structural component. J. Biol Chem. *263*, 18236–18240.

Andres, D.A., Seabra, M.C., Brown, M.S., Armstrong, S.A., Smeland, T.E., Cremers, F.P.M., and Goldstein, J.L. (1993). cDNA cloning of component A of rab geranylgeranyl transferase and demonstration of its role as a rab escort protein. Cell *73*, 1091–1099.

Balch, W.E. (1989). Biochemistry of interorganelle transport: a new frontier in enzymology emerges from versatile *in vitro* model systems. J Biol. Chem. *264*, 16965–16968.

Bucci, C., Parton, R.G., Mather, I.H., Stunnenberg, H., Simmons, K., Hoflack, B., and Zerial, M. (1992). The small GTPase rab5 functions as a regulatory factor in the early endocytic pathway. Cell *70*, 715–728.

Chavrier, P., Gorvel, J.-P., Stelzer, E., Simons, K., Gruenberg, J., and Zerial, M. (1991). Hypervariable C-terminal domain of rab proteins acts as a targeting signal. Nature *353*, 769–772.

Chavrier, P., Parton, R.G., Hauri, H.P., Simons, K., and Zerial, M. (1990). Localization of low molecular weight GTP binding proteins to exocytic and endocytic compartments. Cell *62*, 317–329.

Colombo, M.I., Mayorga, L.S., Casey, P.J., and Stahl, P.D. (1992). Evidence of a role for heterotrimeric GTP-binding proteins in endosome fusion. Science *255*, 1695–1697.

Farnsworth, C.C., Kawata, M., Yoshida, Y., Takai, Y., Gelb, M., and Glomset, J.A. (1991). C terminus of the small GTP-binding protein smg p25A contains two geranylgeranylated cysteine residues and a methyl ester. Proc. Natl. Acad. Sci. USA *88*, 6196–6200.

Gorvel, J.-P., Chavrier, P., Zerial, M., and Gruenberg, J. (1991). Rab5 controls early endosome fusion in vitro. Cell *64*, 915–925.

Hancock, J.F., Magee, A.I., Childs, J.E., and Marshall, C.J. (1989). All ras proteins are polyisoprenylated but only some are palmitoylated. Cell *57*, 1167–1177.

Kato, K., Cox, A.D., Hiaka, M.M., Graham, S.M., Buss, J.E., and Der, C.J. (1992). Isoprenoid addition to Ras protein is the critical modification for its membrane association and transforming activity. Proc. Natl. Acad. Sci. USA *89*, 6403–6407.

Khosravi-Far, R., Lutz, R.J., Cox, A.D., Conroy, L., Bourne, J.R., Sinensky, M., Balch, W.E., Buss, J.E., and Der, C.J. (1991). Isoprenoid modification of rab proteins terminating in CC or CXC motifs. Proc. Natl. Acad. Sci. USA *88*, 6264–6268.

Kinsella, B.T., and Maltese, W.A. (1991). Rab GTP-binding proteins implicated in vesicular transport are isoprenylated *in vitro* at cysteines within a novel carboxyl-terminal motif. J. Biol. Chem. *266*, 8540–8544.

Kinsella, B.T., and Maltese, W.A. (1992). Rab GTP-binding proteins with three different carboxyl-terminal cysteine motifs are modified in vivo by 20-carbon isoprenoids. J. Biol. Chem. *267*, 3940–3945.

Lenhard, J.M., Kahn, R.A., and Stahl, P.D. (1992). Evidence for ADP-ribosylation factor (ARF) as a regulator of in vitro endosome-endosome fusion. J. Biol. Chem. *267*, 13047–13052.

Li, G., and Stahl, P.D. (1993). Structure-function relationship of the small GTPase *rab5*. J. Biol. Chem. *268*, 24475–24480.

Linder, M.E., Pang, I., Duronio, R.J., Gordon, J.I., Sternweiss, P.C., and Gilman, A.G. (1991). Lipid modifications of G protein subunits. J. Biol. Chem. *266*, 4654–4659.

Mayorga, L.S., Diaz, R., and Stahl, P.D. (1989). Regulatory role for GTP-binding proteins in endocytosis. Science *244*, 1475–1477.

Moores, S.L., Schaber, M.D., Mosser, S.D., Rands, E., O'Hara, M.B., Garsky, V.M., Marshall, M.S., Pompliano, D.L., and Gibbs, J.B. (1991). Sequence dependence of protein isoprenylation. J. Biol. Chem. *266*, 14603–14610.

Musha, T., Kawata, M., and Takai, Y. (1992). The geranylgeranyl moiety but not the methyl moiety of the *smg*-25A/rab3A protein is essential for the interactions with membrane and its inhibitory GDP/GTP exchange protein. J. Biol. Chem. *267*, 9821–9825.

Peter, M., Chavrier, P., Nigg, E.A., and Zerial, M. (1992). Isoprenylation of rab proteins on structurally distinct cysteine motifs. J. Cell Sci. *102*, 857–865.

Pfeffer, S.R. (1992). GTP-binding proteins in intracellular transport. Trends Cell Biol. *2*, 41–45.

Reiss, Y., Goldstein, J.L., Seabra, M.C., Casey, P.J., and Brown, M.S. (1990). Inhibition of purified $p21^{ras}$ farnesyl:protein transferase by Cys-AAX tetrapeptides. Cell *62*, 81–88.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Sanford, J., Codina, J., and Birnbaumer, L. (1991). γ-Subunits of G proteins, but not their α- or β-subunits, are polyisoprenylated. J. Biol Chem. *266*, 9570–9579.

Sanford, J.C., Pan, Y., and Wessling-Resnick, M. (1993). Prenylation of Rab5 is dependent on guanine nucleotide binding. J. Biol. Chem. *268*, 23773–23776.

Sanger, F., Nicklen, S., and Coulson, A.B. (1977). DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA *74*, 5463–5467.

Seabra, M.C., Brown, M.S., Slaughter, C.A., Sudhof, T.C., and Goldstein, J.L. (1992). Purification of component A of rab geranylgeranyl transferase: possibly identity with the choroideremia gene product. Cell *70*, 1049–1057.

Sinensky, M., and Lutz, R.J. (1992). The prenylation of proteins. BioEssays *14*, 25–31.

Smith, R.F., and Smith, T.F. (1992). Pattern-induced multi-sequence alignment (PIMA) algorithm employing secondary structure-dependent gap penalties for use in comparative protein modeling. Protein Eng. *5*, 35–41.

Studier, F.W., Rosenberg, A.H., Dunn, J.J., and Dubendorff, J.W. (1990). Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol. *185*, 60–89.

Sztul, E.S., Melançon, P., and Howell, K.E. (1992). Targeting and fusion in vesicular transport. Trends Cell Biol. *2*, 381–386.

Towbin, H., Staehelin, T., and Gordon, J. (1979). Electrophoretic transfer to proteins from polyacrylamide gels to nitrocellulose sheets. Procedure and some applications. Proc. Natl. Acad. Sci. USA *76*, 4350–4354.

Ullrich, O., Stenmark, H., Alexandrov, K., Huber, L.A., Kaibuchi, K., Sasaki, T., Takai, Y., and Zerial, M. (1993). Rab GDP dissociation inhibitor as a general regulator for the membrane association of rab proteins. J. Biol. Chem. *268*, 18143–18150.

Ullrich, O., Horiuchi, H., Bucci, C., and Zerial, M. (1994). Membrane association of Rab5 mediated by GDP-dissociation inhibitor and accompanied by GDP/GTP exchange. Nature *368*, 157–160.

Valencia, A., Chardin, P., Wittinghofer, A., and Sander, C. (1991). The ras protein family: evolutionary tree and role of conserved amino acids. Biochemistry *30*, 4648–4654.

Walter, M., Clark, S.G., and Levinson, A.D. (1986). The oncogenic activation of human $p21^{ras}$ by a novel mechanism. Science *233*, 649–652.

Wessling-Resnick, M., and Braell, W.A. (1990). Characterization of the mechanism of endocytic vesicle fusion in vitro. J. Biol Chem. *265*, 16751–16759.

Wilson, A.L., and Maltese, W.A. (1993). Isoprenylation of Rab1B is impaired by mutations in its effector domain. J. Biol. Chem. *269*, 14561–14564.

Zahraoui, A., Touchot, N., Chardin, P., and Tavitian, A. (1989). The human *Rab* genes encode a family of GTP-binding proteins related to yeast YPT1 and SEC4 products involved in secretion. J. Biol. Chem. *264*, 12394–12401.