Ubiquitin-RAS peptide extensions as substrates for farnesyl-protein transferase and carboxymethyltransferase

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Using oligonucleotide-mediated 'loop-in' mutagenesis strategies in M13, a heat-inducible ubiquitin (Ub) gene was extended by sequences coding for the C-terminal 11 amino acids of Ha-RAS. The resulting gene was transformed into AR13 and production of the Ub-peptide extension was induced by heat treatment. After one-step purification, the fusion protein (Ub-cRAS) was used as a substrate for farnesyl-protein transferase. Ub-cRAS was farnesylated on incubation in *Xenopus* egg extract or rabbit reticulocyte lysate. In contrast, when serine was substituted for the last cysteine in the RAS extension, transfer of the [³H]farnesyl group from [³H] farnesyl pyrophosphate to the modified Ub-cRAS was not observed. Farnesylation of Ub-cRAS permitted us to develop an easy membrane-binding assay for farnesyl-protein transferase enzyme activity. Using this assay, we partially purified the enzyme from rabbit reticulocyte lysate. We also detected methylation of the farnesylated Ub-cRAS terminus in *Xenopus* egg extract.

INTRODUCTION

Among the enzymes left to be discovered and characterized, many catalyse reactions in which proteins are the substrate. This class of enzymes includes the ever-expanding families of protein kinases [1,2], enzymes that add lipids to proteins [3,4] and a wide variety of enzymes that modify specific amino acid side chains on cellular and secreted proteins [5–7]. Analysis of such enzymes requires peptides, either as potential substrates for measuring kinetic features of these catalysts or in the preparation of affinity matrices for purifying them. Recently, we described a method for synthesizing peptides as ubiquitin (Ub) extensions [8]. We have also demonstrated that Ub-peptide extensions can be used as substrates for a specific protein kinase [9]. In this regard, they offer distinct advantages over smaller peptide substrates commonly used to assay phosphotransferases.

The RAS proteins, members of a large family of small guanine nucleotide-binding proteins, are present in all eukaryotic organisms. In order to function, they have to be localized at the plasma membrane [10]. This localization requires a series of post-translational modifications which include the farnesylation of cysteine (the fourth residue from the C-terminus), proteolytic cleavage of the C-terminal three amino acids and the methylation of exposed COOH of cysteine [3,11–12]. These modifications seem to require only the C-terminal consensus motif Cys-A-A-X (A, aliphatic amino acid; X, any amino acid) which is referred to as a CAAX box [13].

Exploring further the utility of Ub-peptides as enzyme substrates, we have constructed a ubiquitin molecule extended by 11 amino acids from the C-terminus of Ha-RAS (PGCMSCKCVLS in the one-letter code). Here we describe the preparation and use of the Ub-peptide extension (Ub-cRAS) as a substrate for farnesyl-protein transferase (FPT) and carboxymethyltransferase. We also report on the partial purification and characterization of FPT from rabbit reticulocyte lysate.

MATERIALS AND METHODS

Materials

[³H]Farnesyl pyrophosphate (FPP; 20 Ci/mmol), [³H-methyl]-adenosylmethionine (10 Ci/mmol) and En³Hance spray were

purchased from DuPont-New England Nuclear (Boston, MA, U.S.A.). Muta-Gene M13 *in vitro* mutagenesis kit and Sequanase Version 2.0 were obtained from Bio-Rad and United States Biochemical (Cleveland, OH, U.S.A.). Amplify was purchased from Amersham. Unlabelled FPP was generously provided by Dr. Dale Poulter (University of Utah). Immobilon-P poly(vinylidene difluoride) (PVDF) membrane was purchased from Millipore.

Construction of expression vectors by oligonucleotide-mediated 'loop-in' mutagenesis

The ubiquitin coding gene in pNMHUb (generously given by David Echer of SmithKline French, Philadelphia, PA, U.S.A.) was digested with *Xba*I and *Kpn*I and the resulting fragment was subcloned into the polylinker site of M13mp18 using standard recombinant DNA techniques [14]. This construct (M13mp18Ub) was used to prepare the single-stranded uracil-containing template DNA. A synthetic oligonucleotide (AGA-CTAAGAGGTGGTCCCGGATGCATGTCTTGTAATGTGTACTATCATGAGGTACCGAGCTC) was designed to add 11 amino acids [the residues (PGCMSCKCVLS) in the one-letter code] to the C-terminus of ubiquitin, and oligonucleotide-mediated 'loop-in' mutagenesis was performed according to the methods of Kunkel *et al.* [15] using the Bio-Rad mutagenesis kit. Detection of the desired mutation (M13mp18Ub-p) was performed by sequencing the resulting phage DNAs via dideoxy chain termination [16]. The mutated fragment was removed by *Xba*I and *Kpn*I from the replicative form of the recombinant phage DNA, ligated into pNMHUb, and then transformed into the wild-type lysogen, MM294CI⁺. After the correct transformants had been confirmed by double-stranded DNA sequencing, the mutated plasmid was transformed into the expression strain, AR13, which contains a temperature-sensitive λ repressor. Another single-stranded uracil-containing template DNA was prepared from M13mp18Ub-p, and site-directed mutagenesis was performed to replace the last cysteine in the Ha-RAS extension with serine using the mutagenic primer (TCTTGTAATCAGTACTATCAT). The construction of Ub-cRAS extensions in M13 is shown diagrammatically in Fig. 1.

Expression and isolation of Ub-peptide extensions

Ub-peptide extensions were expressed as described previously

Abbreviations used: DTT, dithiothreitol; FPP, farnesyl pyrophosphate; FPT, farnesyl-protein transferase; Ub, ubiquitin; PVDF, poly(vinylidene difluoride).

Fig. 1. Strategy for cloning the recombinant plasmid (pNMHUb-p) of ubiquitin-cRAS peptide extension protein using oligonucleotide-mediated 'loop-in' mutagenesis

[8]. Cells were harvested by centrifugation and washed once with ice-cold water. The cell pellet was resuspended in 25 mM-sodium citrate (pH 3.5) after a freeze-thaw, allowed to stand at room temperature for 1 h with occasional stirring, and centrifuged at 20000 *g* for 30 min. Protein in the supernatant was concentrated by Amicon filtration using a YM5 membrane. Purity of the Ub-cRAS extension protein was generally greater than 90 % (see Fig. 2).

Farnesylation of ub-peptide *in vitro*

The standard reaction mixture for FPT, adapted from the procedure of Schaber *et al.* [17], contained the following components in a final volume of 20 μ l: 50 mM-Hepes (sodium salt; pH 7.5), 5 mM-MgCl₂, 5 mM-dithiothreitol (DTT), 12.5 pmol of [³H]FPP (20 Ci/mmol) and 10 μ g of Ub-cRAS. The reaction was initiated by adding various amounts of rabbit reticulocyte lysate, *Xenopus* egg extract, or partially purified FPT. After incubation at 25 °C for 30 min, reactions were quenched by adding 5 μ l of 5 $\times$ SDS-sample buffer [0.3 M-Tris/HCl, pH 6.8, containing 10 % (w/v) SDS, 10 % (w/v) β -mercaptoethanol, 40 % glycerol and 0.02 % Bromophenol Blue]. After the sample had been boiled for 3 min, proteins were separated by 10–20 % gradient

SDS/PAGE [18], and incorporated ³H was visualized by autoradiography after fluorographic enhancement with Amplify.

For quantification, FPT activity was determined by measuring the amount of [³H]farnesyl transferred from [³H]FPP to the substrate, Ub-cRAS, using a PVDF membrane. After reactions had been performed as described above, the reaction mixture was transferred to a PVDF membrane using a VacuSlot-VS (American Biogenek, San Ramon, CA, U.S.A.). The membrane was stained with Coomassie Brilliant Blue R-250 and destained in 40 % (v/v) methanol/10 % (v/v) acetic acid, before the radioactivity in each band was measured by liquid-scintillation spectroscopy. Incorporation of ³H was also visualized by fluorography of the membrane after enhancement with En³Hance spray.

Carboxymethylation of ub-peptide *in vitro*

Xenopus egg extract (0.5 μ l) was incubated with Ub-cRAS and [³H-methyl]adenosylmethionine in the presence or absence of 1 nmol of unlabelled FPP at 25 °C. Reactions were stopped by adding SDS-sample buffer and analysed by 10–20 % gradient SDS/PAGE followed by fluorography.

Preparation of extracts

Rabbit reticulocyte lysate was prepared as described previously

Fig. 2. Expression and purification of Ub-cRAS

Escherichia coli AR13 cells transformed with pNMHUb-cRAS were grown at 32 °C to an $A_{600} = 0.8$, and rapidly shifted to 42 °C. After 3 h, cells were harvested and washed with ice-cold water. Proteins were extracted from the cells in 25 mM-sodium citrate (pH 3.5) after a freeze-thaw and concentrated by Amicon filtration using a YM5 membrane. Samples were then analysed on a 10–20% SDS/polyacrylamide gel and visualized by staining with Coomassie Brilliant Blue. WE, whole extract; CVLS, ubiquitin-PGCMSCCKVLS; SVLS, ubiquitin-PGCMSCCKSVLS.

[19] and was chromatographed on a Q-Sepharose column (1 cm × 10 cm) using a 0–0.5 M-NaCl gradient in 20 mM-Tris/HCl (pH 7.6)/5 mM-MgCl₂/1 mM-DTT. A single peak of FPT activity was detected among those proteins eluted between 0.3 and 0.34 M-NaCl. Fractions containing FPT activity were pooled, concentrated through PM10 membrane and subjected to a Sephacryl S-300 column (1 cm × 90 cm) equilibrated with 20 mM-Tris/HCl (pH 7.6)/5 mM-MgCl₂/1 mM-DTT/0.1 M-NaCl. *Xenopus* egg extract was prepared as described by Murray & Kirschner [20].

RESULTS

The Ub-cRAS extension proved to be an excellent substrate for FPT activities in *Xenopus* egg extract and rabbit reticulocyte lysate. Extracts prepared from these sources were incubated with either ubiquitin or Ub-cRAS in the presence of [³H]FPP. After termination of the reactions with SDS-sample buffer, the samples were electrophoresed on a 10–20% polyacrylamide gel, and X-ray film was exposed to the fluor-impregnated gel. It is clear from the resulting autoradiogram shown in Fig. 3(a) that an activity in both extracts transferred ³H from [³H]FPP to Ub-cRAS but not to ubiquitin. The Cys → Ser variant of Ub-cRAS was also inactive as a substrate for FPT (results not shown). Moreover, the extent of Ub-cRAS farnesylation was proportional to enzyme concentration (Fig. 3b), thereby providing a quantitative assay for FPT.

Farnesylation is just the first of several modifications to which the C-terminus of RAS is subjected. Several groups [21–24] have presented evidence for subsequent removal of the last three amino acids from RAS or yeast a factor followed by methylation of the carboxy group at the farnesylated cysteine terminus. Some RAS termini are palmitoylated as well [13]. Clarke and colleagues have examined the carboxymethylation reaction using farnesylated peptides as substrate [25]. Although their assay is rigorous and quantitative, it is somewhat inconvenient because of the necessity of capturing gaseous methanol after methyl ester hydrolysis. For this reason, we asked whether farnesylated Ub-cRAS could also serve as a substrate for the carboxymethyl-transferase. Accordingly, we incubated Ub-cRAS in *Xenopus* egg extracts with or without unlabelled FPP (1 nmol) and added *S*-adenosylmethionine as the labelled methyl donor. The reaction mixture was analysed by SDS/PAGE followed by fluorography.

The resulting autoradiogram (Fig. 4) shows that Ub-cRAS was labelled by [³H-methyl]adenosylmethionine only when excess FPP was also added to the reaction mixture. The Cys → Ser variant of Ub-cRAS did not accept methyl groups, presumably because it cannot be farnesylated. In contrast, an endogenous *Xenopus* protein was labelled in an FPP-stimulated manner in the presence of either ubiquitin extension peptide.

Fig. 3. Farnesylation of Ub-cRAS *in vitro*

(a). Ub-cRAS fusion protein or ubiquitin (1 nmol) was incubated with rabbit reticulocyte lysate (1.0 μl) or *Xenopus* egg extract (1.0 μl) in a final volume of 20 μl containing 50 mM-Hepes (pH 7.5), 5 mM-MgCl₂, 5 mM-DTT and 12.5 pmol (0.25 μCi) of [³H]FPP. After 30 min, SDS/PAGE sample buffer was added, and the reaction mixes were analysed by 10–20% gradient SDS/PAGE and visualized by autoradiography after fluorographic enhancement. (b) Ub-cRAS (1 nmol) was also incubated with various amounts of reticulocyte lysate, and the reaction mixtures were analysed as described above.

Fig. 4. Methylation of Ub-cRAS *in vitro*

Ub-cRAS (1 nmol), *Xenopus* egg extract (0.5 μl) and *S*-[methyl-³H]adenosylmethionine (5.5 μCi) were incubated in the presence or absence of FPP (1 nmol) for 30 min at 25 °C. The resulting products were analysed by SDS/PAGE and autoradiography. CVLS, Ub-PGCMSCCKVLS; SVLS, Ub-PGCMSCCKSVLS.

Fig. 5. Assay of FPT activity using a PVDF membrane

FPT activity was determined by measuring the amount of [^3H]farnesyl transferred from [^3H]FPP to substrate, Ub-cRAS, using a PVDF membrane. (a) None, no substrate added; Ub-CVLS, ubiquitin-PGCMSCCKVLS; Ub-SVLS, ubiquitin-PGCMSCCKSVLS; Ub, ubiquitin. (b) Ub-cRAS was incubated with *Xenopus* egg extract under standard conditions. The reaction was stopped by adding 2× SDS/PAGE sample buffer at the times shown above each lane. Half of the reaction mixture was transferred to the PVDF membrane above and the other half was subjected to 10–20% gradient SDS/PAGE. Autoradiograms of the gel (▲, upper panel) and the membrane (○, lower panel) were obtained after fluorographic enhancement. (c) The radioactivity in each band from the gel (▲) or the PVDF membrane (○) was measured with a liquid-scintillation counter and plotted against incubation time.

It is cumbersome to use gel electrophoresis in enzyme assays. For this reason, we examined the use of PVDF membranes as a way to capture the farnesylated product. Ub-cRAS was incubated with 0.5 μl of *Xenopus* egg extract for 30 min at 25 °C, and the reaction was terminated by adding 40 μl of 40% methanol plus 1% SDS. The reaction mixture was transferred to a PVDF membrane (see the Materials and methods section) and radioactivity in each slot was measured by liquid-scintillation spectroscopy. The data in Fig. 5(a) show that adsorption of farnesylated Ub-cRAS to PVDF membrane provides a highly discriminating assay for FPT. To determine whether the membrane-binding assay is as quantitative as SDS/PAGE analysis of reaction products, we directly compared the two methods. According to the results in Fig. 5(b) and 5(c), the much easier membrane-binding assay is equally as sensitive as the electrophoretic procedure, and both methods provide quantitative estimates of product formation.

With this assay in hand, 4 ml of reticulocyte lysate was chromatographed on Q-Sepharose to obtain a partially purified preparation of FPT. A single region of FPT activity eluted at approximately 300–340 mM-NaCl was detected (Fig. 6). Fractions containing FPT activity were pooled, concentrated through PM10 membrane, and then subjected to gel filtration on Sephacryl S-300. Again, a single peak of activity with an apparent molecular mass of approx. 180 kDa was observed (Fig. 7). Since the pig brain FPT has been purified to homogeneity by affinity chromatography [26], we did not pursue complete purification of the rabbit reticulocyte enzyme.

DISCUSSION

The studies presented above indicate that Ub-cRAS extensions provide suitable substrates for assaying three enzymes that modify the C-terminus of the Ha-RAS protein. Data in Figs. 3 and 4 show that Ub-cRAS (CVLS) can accept [^3H]farnesyl from [^3H]FPP and, in the presence of excess unlabelled FPP, [^3H]methyl groups from S-adenosylmethionine. Since there is substantial evidence that removal of the three terminal residues is needed for carboxymethylation [21–24], the data in Fig. 4 indicate that the

Ub-cRAS extension is also a suitable substrate for the processing proteinase. Moreover, we have observed that Ub-cRAS can accept [^{14}C]palmitate from palmitic acid in *Xenopus* egg extract (results not shown), so it seems that Ub-cRAS extensions are subjected to all known modifications of the C-terminus of RAS.

Because small peptides can also be used in assays for lipid modification [26–30], one might question potential complications due to the N-terminal ubiquitin moiety. As enzyme substrates, Ub-peptide extensions offer several distinct advantages over short peptides. First, they are extremely inexpensive to synthesize in large quantities. The single-stranded procedure outlined in Fig. 1 lowers the initial cost for peptides to about \$8 (~£4.60) per residue. Expenses for subsequent rounds of synthesis are decreased almost to the price of bacterial growth medium. Second, Ub-peptides can, in certain cases, be purified from bacterial extracts by simple acid extraction (see Fig. 2). Third, some assays may require electrophoretic analysis for accurate quantification. For example, the methyl-transferase activity detected in Fig. 4 is not specific to the Ub-cRAS extension. The fact that Ub-peptides migrate on SDS/polyacrylamide gels in a region (approx. 9 kDa) virtually devoid of other proteins decreases greatly any possible confusion from endogenous proteins that may also become modified. Fourth, the presence of ubiquitin at the N-terminus of the peptide substrate is probably crucial for product capture by PVDF membranes. We did not directly compare Ub-cRAS peptides with cRAS peptide alone in membrane binding, but it seems very likely that farnesylated short peptides will adsorb to PVDF membranes much less well than Ub-peptide extensions. We believe these advantages far outweigh any potential complications resulting from the presence of ubiquitin at the peptide's N-terminus.

Although the thrust of these studies was to assess further the suitability of Ub-peptides as enzyme substrates, we also made several observations relevant to the biochemistry of RAS modification. In Fig. 4 it is shown that the Ub-cRAS becomes methylated in a FPP-stimulated reaction whereas the Cys → Ser variant does not. In the light of previous studies, we interpret these results as indicating that in *Xenopus* egg extract Ub-cRAS is farnesylated, processed by proteolysis and the C-terminal

Fig. 6. Chromatography of rabbit reticulocyte FPT on Q-Sepharose

Rabbit reticulocyte lysate (4 ml) was chromatographed on Q-Sepharose. Every other fraction (10 μ l each out of 8 ml fraction) was incubated with 1 nmol of Ub-cRAS under standard assay conditions. FPT activity was measured using PVDF membrane as described in Fig. 5 and the products were also analysed by SDS/PAGE. (a) Profile of protein (—) and FPT activity (●) from Q-Sepharose. (b) Autoradiogram showing incorporation of [3 H]farnesyl into Ub-cRAS in fractions 19 and 23.

carboxy group is methylated. Both the failure of the serine variant to become carboxymethylated and the absence of Ub-cRAS methylation when excess FPP is omitted would indicate that farnesylation precedes methylation, an inference for which there is already substantial evidence (see ref. [12] for a review). It should be noted that carboxymethylation was not observed when reticulocyte lysate was substituted for *Xenopus* egg extract under identical reaction conditions (results not shown). This observation is in accord with studies that place the carboxymethyl-transferase on the intracellular surface of the plasma membrane [24–25].

We also partially purified and sized rabbit reticulocyte FPT. Several groups have characterized FPT from various tissues, and there are substantial differences in reported molecular mass. Reiss *et al.* [26] found that the rat brain FPT has an apparent size of about 100 kDa. Manne *et al.* [31] observed two peaks of pig kidney FPT activity with apparent molecular masses of approx. 300 kDa and approx. 100 kDa, whereas Schaber *et al.* [17] reported a molecular mass of 190 kDa for bovine brain FPT. Our estimate for the molecular mass of rabbit reticulocyte FPT, 180 kDa, is in close agreement with that reported for the bovine brain enzyme. FPT from rat brain consists of two non-identical subunits of about 50 kDa [32]. Assuming that reticulocyte FPT

Fig. 7. Gel filtration of partially purified FPT

Fractions containing FPT activity from Q-Sepharose were pooled, concentrated, and then subjected to a Sephacryl S-300 column (1 cm $\times$ 90 cm) equilibrated with 20 mM-Tris (pH 7.6)/5 mM-MgCl₂/1 mM-DTT/0.1 M-NaCl. Fractions of 1 ml were collected at a flow rate of 0.1 ml/min. A 10 μ l sample of each fraction was assayed for FPT activity (●) as described in Fig. 5. The column was calibrated with thyroglobulin (669 kDa), β -amylase (200 kDa), alcohol dehydrogenase (150 kDa), BSA (66 kDa), carbonic anhydrase (29 kDa) and cytochrome *c* (12.4 kDa). The inset below the lines is an autoradiogram showing bound radiolabelled product from every other fraction bound to the PVDF membrane.

is composed of similar subunits, the holoenzyme is probably a tetramer.

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REFERENCES

- Edelman, A. M., Blumenthal, D. K. & Krebs, E. G., (1987) *Annu. Rev. Biochem.* **56**, 567–614
- Hunter, T. (1989) *Curr. Opin. Cell Biol.* **1**, 1168–1181
- Glomset, J. A., Gelb, M. H. & Farnsworth, C. C. (1990) *Trends Biochem. Sci.* **15**, 139–142
- Gordon, J. I., Duronio, R. J., Rudnick, D. A., Adams, S. P. & Gokel, G. W. (1991) *J. Biol. Chem.* **266**, 8647–8650
- Clark, S. (1985) *Annu. Rev. Biochem.* **54**, 479–506
- Hart, G. W., Haltiwanger, R. S., Holt, G. D. & Kelly, W. G. (1989) *Annu. Rev. Biochem.* **58**, 841–874
- Rechsteiner, M. (1989) *Ubiquitin*, Plenum Press, New York
- Yoo, Y., Rote, K. V. & Rechsteiner, M. (1989) *J. Biol. Chem.* **264**, 17078–17083
- Yoo, Y. & Rechsteiner, M. (1990) *Anal. Biochem.* **191**, 35–40
- Willumensen, B. M., Norris, K., Papageorge, A. G., Hubbert, N. L. & Lowy, D. R. (1984) *EMBO J.* **3**, 2581–2585
- Maltese, W. A. (1990) *FASEB J.* **4**, 3319–3328
- Gibbs, J. B. (1991) *Cell* **65**, 1–4
- Hancock, J. F., Magee, A. I., Childs, J. E. & Marshall, C. J. (1989) *Cell* **57**, 1167–1177
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- Kunkel, T. A., Roberts, J. D. & Zakour, R. A. (1987) *Methods Enzymol.* **154**, 367–382
- Sanger, F., Dicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463–5467
- Schaber, M. D., O'Hara, M. B., Garsky, V. M., Mosser, S. D., Bergstrom, J. D., Moores, S. L., Marshall, M. S., Friedman, P. A., Dixon, R. A. F. & Gibbs, J. B. (1990) *J. Biol. Chem.* **265**, 14701–14704

18. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
19. Hough, R., Pratt, G. & Rechsteiner, M. (1986) *J. Biol. Chem.* **261**, 2400–2408
20. Murray, A. & Kirschner, M. (1989) *Nature (London)* **339**, 275–280
21. Gutierrez, L., Magee, A. I., Marshall, C. J. & Hancock, J. F. (1989) *EMBO J.* **8**, 1093–1098
22. Marcus, S., Caldwell, G. A., Xue, G. B., Naider, F. & Becker, J. M. (1990) *Biochem. Biophys. Res. Commun.* **172**, 1310–1316
23. Stimmel, J. B., Deschenes, R. J., Volker, C., Stock, J. & Clarke, S. (1990) *Biochemistry* **29**, 9651–9659
24. Hancock, J. F., Cadwallader, K. & Marshall, C. J. (1991) *EMBO J.* **10**, 641–646
25. Stephenson, R. C. & Clarke, S. (1990) *J. Biol. Chem.* **265**, 16248–16254
26. Reiss, Y., Goldstein, J. L., Seabra, M. C., Casey, P. J. & Brown, M. S. (1990) *Cell* **62**, 81–88
27. Goldstein, J. L., Brown, M. S., Stradley, S. J., Reiss, Y. & Gierasch, L. M. (1991) *J. Biol. Chem.* **266**, 15575–15578
28. Kim, R., Rine, J. & Kim, S. H. (1990) *Mol. Cell. Biol.* **10**, 5945–5949
29. Reiss, Y., Stradley, S. J., Gierasch, L. M., Brown, M. S. and Goldstein, J. L. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 732–736
30. Tan, E. W., Perez-Sala, D., Canada, F. J. & Rando, R. R. (1991) *J. Biol. Chem.* **266**, 10719–10722
31. Manne, V., Roberts, D., Tobin, A., O'Rourke, E., De Virgilio, M., Meyers, C., Ahmed, N., Kurz, B., Resh, M., Kung, H. F. & Barbacid, M. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 7541–7545
32. Reiss, Y., Seabra, M. C., Armstrong, S. A., Slaughter, C. A., Goldstein, J. L. & Brown, M. S. (1991) *J. Biol. Chem.* **266**, 10672–10677

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