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EXPLORING THE SPECIFICITY OF PRENYL PROTEIN-SPECIFIC METHYLTRANSFERASE WITH SYNTHETIC PRENYLATED RAB PEPTIDES

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Abstract: Most Rab proteins contain two C-terminal geranylgeranyl groups. Some members are C-terminally methylated, whereas others are not. Synthetic peptides bearing the sequence of the C-termini of Rab proteins and containing the C-terminal motif C(S-geranylgeranyl)AC(S-geranylgeranyl) are substrates for the membrane-bound prenyl protein-specific methyltransferase, whereas those that end in C(S-geranylgeranyl)C(S-geranylgeranyl) are not.

Rab proteins are 21 to 25-kDa GTP-binding proteins that play a role in intracellular vesicle trafficking and cellular secretion.[1] Most, and perhaps all, Rab proteins contain covalently attached geranylgeranyl groups at their C-termini, and some are methylated at their C-termini.[2-6] As a first step toward the understanding of the role of posttranslational modifications in supporting rab functions, it is necessary to fully characterize the structure of these modifications and to understand the enzymatic pathways that gives rise to them. Unlike proteins such as the γ-subunits of heterotrimeric G proteins which contain a single C-terminal geranylgeranyl group on the cysteine in the sequence CXXX,[7, 8] some Rab proteins, such as Rab 3a, contain a C-terminal sequence CAC and a geranylgeranyl group thioether-linked to both cysteine residues.[2] Other Rab proteins, such as Rab 1a, contain a C-terminal sequence CC and are known to be geranylgeranylated, but the precise number and location of the prenyl groups is not known with certainty. However, the enzyme that prenylates Rab proteins, termed Rab geranylgeranyltransferase,[9] has recently been shown to catalyze the geranylgeranylation of both cysteines of Rab 3a and Rab 1a *in vitro* suggesting that Rab 1a, like Rab 3a, contains a doubly geranylgeranylated C-terminus.[10]

The α-carboxyl group of the C-terminal prenylated cysteine of many prenylated proteins is methylated by a membrane-bound, prenyl protein-specific methyltransferase.[11-17] This enzyme methylates a variety of prenylated proteins or peptides that contain a C-terminal geranylgeranylated or farnesylated cysteine. Whereas some Rab proteins, such as Rab 3a isolated from bovine brain (with C-terminal CAC), are methylated,[2] other Rab proteins, such as Rab 1a (with C-terminal CC), are not.[5, 18, 10] Since it is likely that both Rab 3a and Rab 1a contain a C-terminal geranylgeranylated cysteine, their differential methylation states may reflect the intrinsic specificity of the methyl transferase toward different Rab proteins. This idea was tested in the present study using synthetic geranylgeranylated Rab peptides of defined structure.

EXPERIMENTAL

Peptide Synthesis. All peptides were prepared by solid-phase, machine synthesis on a scale of 0.25 mmol with an Applied Biosystems machine (ABI 430A) using the standard procedure provided by the manufacturer

and employing FMOC-protected amino acids. Additional details have been described.[20] Peptides were purified by HPLC as described[20] using 0.06 % trifluoroacetic acid in water (Milli-Q, Millipore) (solvent A) and 0.06 % trifluoroacetic acid in acetonitrile (Baker Resi-Analyzed) (solvent B). The following solvent programs were used: Program 1, 2 to 100 % B over 60 min; Program 2, 0 to 30 % B over 60 min; Program 3, 0 to 20 % B over 20 min, then to 30 % B over 30 min, program 4, 0 to 30 % B over 60 min. The crude peptides were first dissolved in 0.5 ml DMF containing 1-2 drops of water, and 20 equivalents of DTT was added. After 30 min at room temperature, the peptides were injected onto the HPLC column. After elution from the column the peptide solutions were concentrated to dryness in a Speed-Vac (Savant) and stored in tightly-capped vials under argon at -80°C. The structures of the peptides were verified by electro-spray mass spectrometry by dissolving them in 0.1 % trifluoroacetic acid in methanol and injecting them onto a Kratos Profile HV-4 mass spectrometer with a HV-R2500 electro-spray source. Peptide HPLC conditions and retention times and mass spectrometry data are: QGPQLTDQQAPPHQDCAC (25 min, program 1, 2.5 ml/min, Dynamax; +2 calc 970, obsd 971); QGPQLTDQQAPPHQDCFC (54 min, program 2, 1.5 ml/min, Vydac; +2, calc 1008, obsd 1008); QGPQLTDQQAPPHQDCC (50 min, program 4, 1.5 ml/min, Vydac; +2, calc 934, obsd 936; +3, calc 623, obsd 625); IQSTPVKQSGGGCC (19 min, program 1, 2.5 ml/min, Dynamax; +2, calc 791, obsd 719); IQSTPVKQSGGGCAC (27 min, program 3, 1.5 ml/min, Vydac; +2, calc 719, obsd 719).

The peptides (typically 2-3 mg) were prenylated as described previously.[20] Reaction mixtures were directly injected onto the HPLC column, and fractions were screened for the presence of peptide with Pauli reagent.[20] The structures of the peptides were confirmed by electro-spray mass spectrometry on a VG Trio 2000 instrument. All peptides gave a negative test with Ellman's reagent indicating that both cysteines were prenylated. The retention times and mass spectrometry data are as follows (cysteine residues with a thioether-linked geranylgeranyl group are abbreviated as C(gg): QGPQLTDQQAPPHQDC(gg)AC(gg) (124 min, program 4: 0 to 30 % B over 60 min, to 60 % B over 30 min, to 80 % B over 15 min, kept at 80 % B for 30 min, 0.5 ml/min, Vydac 218TP54, 5 μ; +2, calc 1242, obsd 1242); IQSTPVKQSGGGC(gg)C(gg) (128 min, program 4, 1.5 ml/min, Vydac 218TP1010; +2, calc 955, obsd 956); QGPQLTDQQAPPHQDC(gg)C(gg) (158 min; program 4, 1.5 ml/min; Vydac 218TP1010; +2, calc 1206, obsd 1209; +3, calc 804, obsd 806); QGPQLTDQQAPPHQDC(gg)FC(gg) (155 min, program 4, 1.5 ml/min, Vydac 218TP1010; +2, calc 1280, obsd 1280; +3, calc 853, obsd 853); IQSTPVKQSGGGC(gg)AC(gg) (149 min, program 4, 1.5 ml/min, Vydac 218TP1010; +2, calc 991, obsd 991). The peptides were stored in vials under argon at -80°C to prevent oxidation of the C(gg) thioether to the sulfoxide and sulfone. Typical yields for the double geranylgeranylation were 10-30 %.

Peptide amounts were determined by weighing and also from the relative peak area integrals in the 500 MHz 1H-NMR spectra of the peptides in CD_3OD containing a known amount of the internal standard benzene (the numbers obtained by the two methods differed by ≤ 20 %). Peptide stock solutions were prepared in DMF and stored at -80°C under argon.

Methyltransferase studies. Crude membranes from rat cerebellum were used as the source of methyltransferase.[21] Cerrebella from 40 freshly sacrificed rats (male, Charles River, 24-30 days old) were dissected from brains and homogenized with 10 volumes (w/v) of ice-cold buffer (50 mM Tris, 3 mM EDTA, 0.32 M sucrose, pH 7.5) in a teflon-pestle homogenizer. All subsequent steps were carried out at 4°C. The

homogenate was centrifuged at 800 g for 10 min, the supernatant was centrifuged again at 800 g for 10 min, and the supernatant was centrifuged at 20,000 g for 20 min. The pellet was resuspended in homogenation buffer and stored at -80°C.

Methyltransferase assays were carried out in polypropylene microfuge tubes in a final volume of 25 µl containing buffer (50 mM Tris, pH 7.5), 15 µM [methyl-^{3}H]S-adenosyl-L-methionine [270 Ci/mmol, prepared from labeled material (American Radiolabeled Chemicals) and unlabeled material (Sigma)], 75-125 µg of membrane protein (determined with the Bradford assay from BioRad using immunoglobulin as a standard), and the desired amount of peptide (added from a stock solution in DMF such that the DMF concentration in the assay did not exceed 0.2 %). Reactions were incubated at 37°C for 30 min and stopped by the addition of 250 µl water and 500 µl of methanol:chloroform (1:1). The tubes were vortexed for 1 min and microfuged for 5 min to separate the phases. The upper phase was removed by aspiration, and 125 µl of the lower phases were transferred to new microfuge tubes. The caps were removed from the tubes, and the solvent was evaporated by placing the tubes in a 50°C water bath. Fifty µl of 1 N NaOH, 1 % SDS was added, and the tubes were placed inside of scintillation vialsthat contained 1 ml of scintillation fluid. The vials were immediately capped and left overnight before analysis in a scintillation counter.

RESULTS AND DISCUSSION

The doubly geranylgeranylated Rab peptides were prepared by treating the fully deprotected peptides with excess geranylgeranyl chloride. Although this reaction generated the desired products in moderate yield, it is probably the simplest and most efficient route to these lipidated peptides.

The synthesized Rab peptides were tested as substrates for the prenyl protein specific methyltransferase present in rat cerebellum membranes; the latter is a rich source of the enzyme.[21] The structures of the various geranylgeranylated peptides synthesized for this study along with the names of the corresponding Rab proteins are listed in the legend to Figure 1.

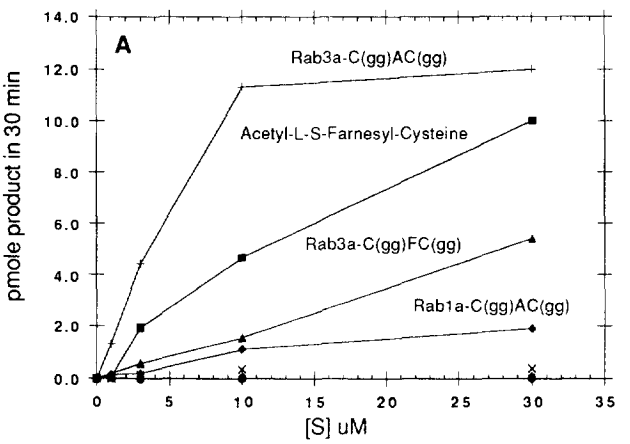

Figure 1. *In vitro* methylation of synthetic Rab peptides by rat cerebellum membranes: crosses, bovine brain

Rab 3a peptide QGPQLTDQQAPPHQDC(gg)AC(gg); squares, Rab 3a mutant peptide QGPQLTDQQAPPHQDC(gg)C(gg); filled triangles, Rab 3a mutant peptide QGPQLTDQQAPPHQDC(gg)FC(gg); open triangles, human Rab 1a peptide IQSTPVKQSGGGC(gg)C(gg); diamonds, Rab 1a mutant peptide IQSTPVKQSGGGC(gg)AC(gg).

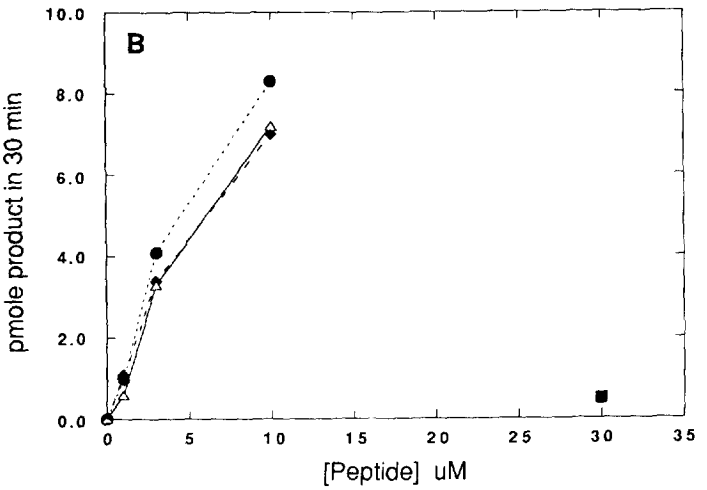

Figure 2. Effect of Rab 3a mutant peptide QGPQLTDQQAPPHQDC(gg)C(gg) (0 μM, triangles; 10 μM, diamonds; 30 μM, circles) on the methylation of Rab 3a peptide QGPQLTDQQAPPHQDC(gg)AC(gg). The square is the methylation of the Rab 3a mutant peptide QGPQLTDQQAPPHQDC(gg)C(gg) alone. All data were obtained in duplicate or triplicate, and the standard deviations were ≤ 20 % for all points.

As can be seen from Figure 1, the Rab 3a-C(gg)AC(gg) peptide is the best substrate. The data were fit to the Michaelis-Menten equation using non-linear regression, and K_M = 5.8 ± 1.7 μM and V_{max} = 0.51 ± 07 pmole/min were obtained for this peptide. The Rab 3a peptide in which the C-terminus is changed from C(gg)AC(gg) to C(gg)C(gg) is not detectably methylated even when tested at 30 μM. Likewise the Rab 1a-C(gg)C(gg) peptide is methylated only 2 % as well as the Rab 3a-C(gg)AC(gg) peptide (both at 10 μM). When the C-terminus of the Rab 1a peptide is changed to C(gg)AC(gg), a significant increase in the rate of methylation is observed (K_M = 25 μM ±, V_{max} = 0.12 ± 0.04 pmole/min). The data clearly show that when the C-terminal C(gg) residue is juxtapose to another C(gg) residue, methylation does not occur, and the insertion of a single A residue between the two prenylated residues converts the peptide to a substrate for the methyltransferase. In addition, the results with the Rab 3a-C(gg)FC(gg) peptide show that replacement of the A residue with the more bulky F residue results in a substrate that interacts considerably weaker with the methyltransferase (K_M >> 30 μM). Thus, the results indicate that the methyltransferase discriminates against

species that have a bulky residue juxtapose to the C-terminal C(gg). The results of this study also suggest that the observed lack of C-terminal methylation of CC-containing Rab proteins[5, 18, 19] is a result of their intrinsic low reactivity with the methyltransferase. None of the peptides in non-prenylated form were methylated (data not shown).

Although the cysteine-containing C-terminus of the peptide is by far the major determinant for methylation, the fact that the Rab 1a-C(gg)AC(gg) peptide is significantly poorer as a substrate compared to the Rab 3a-C(gg)AC(gg) peptide indicates that other factors contribute to the specificity. Comparison of specificity should be based on the ratio of kinetic parameters, V_{max}/K_M, and based on this the Rab 3a-C(gg)AC(gg) peptide is 18-fold preferred over the Rab 1a-C(gg)C(gg) peptide. This indicates that amino acids upstream of the C-terminus partially dictate the preferences of the methyltransferase. It is not clear if these upstream residues contact the methyltransferase directly or whether they influence the conformation of that portion of the peptide that contacts the enzyme.

Since crude membranes are the source of the methyltransferase, it is possible that peptides with the C-terminal sequence C(gg)C(gg) are methylated but then rapidly enzymatically demethylated. However, this possibility seems most unlikely since the data in Figure 2 shows that addition of the C(gg)C(gg)-containing Rab 3a peptide does not inhibit the methylation of the C(gg)AC(gg)-containing Rab 3a peptide even when the former peptide is present at 30-fold higher concentration than the latter peptide. Thus it is clear that the C(gg)C(gg)-containing Rab 3a peptide is not methylated because it does not bind to the methyltransferase.

After the completion of this study, Smeland and co-workers reported studies on the *in vitro* methylation of recombinant geranylgeranylated Rab proteins.[22] The conclusions from both studies are consistent.

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References

1. Zerial, M., Stenmark, H. *Curr. Opin. Cell. Biol.* **1993,** *5*, 613-620.

2. Farnsworth, C. C., Kawata, M., Yoshida, Y., Takai, Y., Gelb, M. H., Glomset, J. A. *Proc. Natl. Acad. Sci. USA* **1991,** *88*., 6196-6200.

3. Khosravi-Far, R., Clark, G. J., Abe, K., Cox, A. D., McLain, T., Lutz, R. J., Sinensky, M., Der, C. J. *J. Biol. Chem.* **1992,** *267*, 24363-24368.

4. Kinsella, B. T., Maltese, W. A. *J. Biol. Chem.* **1991,** *266*, 8540-8544.

5. Newman, C. M., Giannakouros, T., Hancock, J. F., Fawell, E. H., Armstrong, J., Magee, A. I. *J Biol. Chem* **1992,** *267*, 11329-36.

6. Khosravi-Far, R., Lutz, R. J., Cox, A. D., Conroy, L., Bourne, J. R., Sinensky, M., Balch, W. E., Buss, J. E., Der, C. J. *Proc. Natl. Acad. Sci. U.S.A.* **1991,** *88*, 6264-6268.

7. Yamane, H. K., Farnsworth, C. C., Xie, H., Howald, W., Fung, B. K.-K., Clarke, S., Gelb, M. H., Glomset, J. A. *Proc. Nat. Acad. Sci. USA* **1990,** *87*, 5868-5872.

8. Mumby, S. M., Casey, P. J., Gilman, A. G., Gutowski, S., Sternweis, P. C. *Proc Natl Acad Sci USA* **1990,** *87*, 5873-7.

9. Seabra, M. C., Goldstein, J. L., Sudhof, T. C., Brown, M. S. *J Biol Chem* **1992,** *267*, 14497-503.

10. Farnsworth, C. C., Seabra, M. C., Ericsson, L. H., Gelb, M. H., Glomset, J. A. *Proc. Natl. Acad. Sci. USA* **1994,** *91*, 11963-11967.

11. Clarke, S. *Annu. Rev. Biochem.* **1992,** *61*, 355-386.

12. Stephenson, R. C., Clarke, S. *J. Biol. Chem.* **1992,** *267*, 13314-13319.

13. Hrycyna, C., Clarke, S. *Molec. Cell. Biol.* **1990,** *10*, 5071-5076.

14. Marr, R. S., Blair, L. C., Thorner, J. *J. Biol. Chem.* **1990,** *265*, 20057-20060.

15. Perez-Sala, D., Tan, E. W., Canada, F. J., Rando, R. R. *Proc. Natl. Acad. Sci. USA* **1991,** *88*, 3043-3046.

16. Philips, M. R., Pillinger, M. H., Staud, R., Volker, C., Rosenfeld, M. G., Weissmann, G., Stock, J. B. *Science* **1993,** *259*, 977-80.

17. Stephenson, R. C., Clarke, S. *J. Biol. Chem.* **1990,** *265*, 16248-16254.

18. Li, G., Stahl, P. D. *Arch. Biochem. Biophys.* **1993,** *304*, 471-478.

19. Wei, C., Lutz, R., Sinensky, M., Macara, I. G. *Oncogene* **1992,** *7*, 467-473.

20. Liu, L., Jang, G.-F., Yokoyama, K., Ghomashchi, F., Farnsworth, C. C., Glomset, J. A., Gelb, M. H. *Methods Enzymol.* **1994,** *in press*,

21. Paz, A., Ben-Baruch, G., Marciano, D., Egozi, Y., Halkai, R., Kloog, Y. *FEBS Lett.* **1993,** *332*, 215-217.

22. Smeland, T., Seabra, M. C., Goldstein, J. L., Brown, M. S. *Proc. Natl. Acad. Sci. USA* **1994,** *91*, 10712-10716.

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