Nonfarnesylated Tetrapeptide Inhibitors of Protein Farnesyltransferase*

(Received for publication, June 6, 1991)

Joseph L. Goldstein†, Michael S. Brown‡, Sarah J. Stradley§, Yuval Reiss¶, and Lila M. Giersch¶

From the Departments of †Molecular Genetics and §Pharmacology, University of Texas Southwestern Medical Center, Dallas, Texas 75235

The protein farnesyltransferase from rat brain was previously shown to be inhibited competitively by tetrapeptides that conform to the consensus Cys-A1-A2-X, where A1 and A2 are aliphatic amino acids and X is methionine, serine, or phenylalanine. In the current studies we use a thin layer chromatography assay to show that most of these tetrapeptides are themselves farnesylated by the purified enzyme. Two classes of tetrapeptides are not farnesylated and therefore act as true inhibitors: 1) those that contain an aromatic residue at the A2 position and 2) those that contain penicillamine (β,β-dimethylcysteine) in place of cysteine. The most potent of these pure inhibitors was Cys-Val-Phe-Met, which inhibited farnesyltransferase activity by 50% at <0.1 μM. These data indicate that the inclusion of bulky aromatic or methyl residues in a tetrapeptide can abolish prenyl group transfer without blocking binding to the enzyme. This information should be useful in the design of peptides or peptidomimetics that inhibit farnesylation and thus block the action of p21\textsuperscript{G1S} proteins in animal cells.

A protein farnesyltransferase purified from rat brain (1) attaches farnesyl to cysteines in p21\textsuperscript{G1S} proteins (2), nuclear lamins (3), and the γ-subunit of retinal transducin (4, 5). All of these proteins terminate in the consensus sequence Cys-A1-A2-X, where A1 and A2 are aliphatic amino acids and X is methionine, serine, or phenylalanine with a free carboxyl group (6). The enzyme is competitively inhibited by peptides as short as four amino acids in length that conform to this consensus, suggesting that all of the recognition determinants of a protein substrate reside in these four residues (1, 6).

In a previous study we explored the amino acid requirements at each of these positions by making systematic substitutions in the sequence Cys-Val-Ile-Met (CVIM), which corresponds to the COOH terminus of p21\textsuperscript{G1S}, and testing the ability of each of the peptides to compete with p21\textsuperscript{G1S} in a farnesyltransferase assay (6). The experiments revealed that the requirements at the A2 and X positions are stringent. The A2 residue must be uncharged. Aromatic and aliphatic residues are preferred over more polar residues. The X position strongly favors Met, Phe, or Ser. The A1 position is somewhat less stringent in that it accommodates uncharged hydrophilic residues without a marked loss of affinity for the enzyme. The most potent inhibitory tetrapeptide, CVFM, gave 50% inhibition at a concentration of 25 nM, which was 6-fold lower than that of the natural sequence, CVIM (6).

Inasmuch as farnesylation of cellular and oncogenic mutant p21\textsuperscript{G1S} proteins is obligatory for stimulation of cell growth (7–10), it is possible that an inhibitory tetrapeptide or an analogue that penetrates cells would block the farnesylation of ras and would thus be useful in studies of the role of ras proteins in cellular physiology. Such an inhibitor might also be useful in helping to slow the growth of malignant cells that are driven by mutated ras proteins.

A drawback in the use of peptide inhibitors was disclosed by the observation that the peptides serve as alternate substrates for the enzyme and are thereby farnesylated (6, 11). This farnesylation was demonstrated directly with a biotinylated heptapeptide, KTSCVIM (6, 11). Schaber et al. (12) showed that a farnesylated peptide corresponding to the COOH terminus of p21\textsuperscript{G1S} (CVLS) was about 13-fold less potent than CVLS itself as an inhibitor of partially purified bovine brain farnesyltransferase activity. A nonfarnesylated peptide inhibitor would be desirable since it would not be rendered less active by the enzyme.

In the current paper we establish a quantitative thin layer chromatography assay to follow farnesylation of tetrapeptides by a highly purified rat brain farnesyltransferase. We use this assay to show that substitution of aromatic residues at the A2 position of CVIM creates pure inhibitory peptides that abolish farnesyltransferase activity at concentrations in which they themselves are not farnesylated. Interestingly, a similar result is obtained when penicillamine (β,β-dimethylcysteine) is substituted for cysteine in CVIM. These results establish the feasibility of designing pure peptide inhibitors of the farnesyltransferase and suggest that the insertion of bulky methyl or aromatic groups into the peptide blocks its ability to accept a farnesyl without impairing its ability to bind to the active site of the enzyme. The ability of such peptides to bind but not to serve as substrates should also provide insight into the mechanism of the farnesyl transfer reaction.

**EXPERIMENTAL PROCEDURES**

**Peptides**—Peptides were prepared by established procedures of solid-phase synthesis (13). Peptides were synthesized on the Milligen 9050 synthesizer using Fmoc (9-fluorenylmethyloxycarbonyl) chemistry and purified as described (6). L-penicillamine-Val-Phe-Met, L-penicillamine-Val-Ile-Met, Cys-Val-pCl-Phe-Met (where pCl is pChloro), mercaptopropionic acid-Val-Phe-Met (mpaVFM), and the N-octyl-modified version of CVIM were synthesized on an Applied Biosystems model 430A synthesizer using t-butoxycarbonyl chemistry and purified as described (6). Identity was confirmed for all peptides by quantitative amino acid analysis and for the NH\textsubscript{2}-terminally modified peptides by fast atom bombardment mass spectrometry (6). Just prior to use, each peptide was dissolved at a concentration of 0.8 mM in 10 mM dithiothreitol (DTT) in water except for several of the most hydrophobic peptides, which were dissolved at a concentration of 1 mM in dimethyl sulfoxide, 10 mM DTT. All dilutions were made in water containing 10 mM DTT.

*This research was supported by National Institutes of Health Grants HL20948 and GM37616 and by grants from the Lucille P. Markey Charitable Trust and the Perot Family Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†Recipient of a Chaim Weizmann postdoctoral fellowship award.

1 The abbreviations used are: mpaVFM, mercaptopropionic acid-Val-Phe-Met; DTT, dithiothreitol, FPP, farnesyl pyrophosphate.
Purified Protein Farnesyltransferase—Protein farnesyltransferase was purified to apparent homogeneity from rat brain homogenates by sequential ammonium sulfate fractionation, Mono Q ion-exchange chromatography, and peptide affinity chromatography as previously described (1, 11).

Transfer of [3H]Farnesyl from [3H]FPP to Tetrapeptides—Each reaction mixture contained the following components in a final volume of 25 μl: 50 mM Tris chloride (pH 7.5), 50 μM ZnCl₂, 3 mM MgCl₂, 20 mM KCl, 1 mM DTT, 0.2% (v/v) octyl-β-D-glucoside, 17 pmol of [3H]FPP (44,000 dpm/pmol; Du Pont-New England Nuclear), 90 pmol of peptide (3.6 μM), and 5 ng of affinity-purified protein farnesyltransferase. After incubation at 37 °C for 15 or 30 min, the entire reaction mixture was spotted onto a plastic-backed Silica Gel G thin layer sheet (20 × 20 cm, Brinkmann Instruments) and placed in a tank containing n-propyl alcohol/ammonium hydroxide/water (6:3:1, v/v/v). The chromatogram was run for 3 h, after which it was either subjected to autoradiography or quantified by scintillation counting. Peptide spots were visualized with chlorine-o-tolidine reagent (14). For scintillation counting, the origin (2-cm strip) and 14 sequential 1-cm fractions of the chromatogram were cut and individually counted in 10 ml of 3a7OB scintillation mixture (Research Products International). The amount of 3H-labeled farnesylated tetrapeptide was calculated by summing the radioactivity in fractions 10–12.

RESULTS
To monitor the attachment of [3H]farnesyl to tetrapeptides, we used a thin layer chromatography system in which the substrate [3H]FPP remains near the origin whereas farnesylated tetrapeptides migrate near the solvent front (Fig. 1). Peptides at a saturating concentration of 3.6 μM were incubated with [3H]FPP in the presence of purified rat brain farnesyltransferase. In the absence of acceptor peptide, all recovered radioactivity remained at the origin (lane 1). As expected, the addition of CVIM led to the production of radiolabeled peptide (lane 3). In experiments not shown, we demonstrated that authentic S-[3H]farnesyl acetyl-CVIM (custom synthesized by Du Pont-New England Nuclear) had the same mobility as did enzymatically prepared S-[3H]farnesyl acetyl-CVIM (R₂ identical to that of [3H]farnesyl CVIM in Fig. 1).

The S-[3H]farnesyl CVIM had a mobility similar to unlabeled farnesol in this system (data not shown). To be certain that the radiolabeled band represented farnesylated peptide and not farnesol, we performed an experiment with a hexapeptide, TKCVIM, which has a slower mobility than the tetrapeptide. The hexapeptide was also farnesylated in this assay (Fig. 1, lane 5). The control peptide SVIM, which lacks a cysteine, was not farnesylated (lane 2), confirming the specificity of this assay for the farnesylation of cysteine. Unexpectedly, the tetrapeptide CVFM, which was the most potent inhibitor of farnesyltransferase previously tested (6), was not an acceptor of [3H]farnesyl in this assay (lane 4). In separate experiments not shown, we confirmed that CVFM had the same mobility in this thin layer system as did CVIM (R₂ = 0.5), and thus we should have observed a radiolabeled spot at about the same position as farnesylated CVIM if the CVFM had been farnesylated.

The data from the thin layer chromatography experiments were quantified by cutting the plastic-backed sheets into 1-cm strips and measuring the radioactivity in a liquid scintillation counter (Fig. 2). The total radioactivity in fractions 10–12 was measured and used as an index of the total amount of 3H-labeled farnesylated peptide formed. As shown in Fig. 2, in the absence of peptide or in the presence of SVIM all recovered radioactivity migrated with the [3H]FPP peak at the origin, whereas in the presence of CVIM nearly all of the radioactivity ran with the peptide.

Table I summarizes the results of five experiments (A–E) in which we compared the farnesylation of various tetrapeptides using the thin layer chromatography assay. In general, these peptides were modifications of the sequence CVIM, which we have used as a base line for most of our studies. For comparative purposes, Table I also includes estimates of the concentration of each peptide required for 50% inhibition of farnesyltransferase activity using p21H-cmt as an acceptor under defined conditions (6).

All of the peptides corresponding to known farnesylated proteins (indicated within the parentheses following the sequences in Table I) were farnesylated in the in vitro assay. Derivatization of the amino group of cysteine (i.e. octyl-CVIM) did not affect the ability of CVIM to be farnesylated (Experiment B). Substitution of any aromatic residue (F, Y, or W) into the A2 position of CVIM essentially abolished farnesyltransferase activity without affecting recognition by the enzyme as indicated in the inhibition assay (Experiment A). A similar result was obtained when penicillamine (β,β-dimethylcysteine) was substituted for cysteine (t-penicillamine-Val-Ile-Met) (Experiment C). When both substitutions were made (t-penicillamine-Val-Phe-Met), farnesylation also was not...
The highest affinity inhibitor was CVFM, which gave 50% inhibition at <0.1 \mu M (Ref. 6 and Table I). In data not shown, we found that the kinetics of the inhibition were complex and suggested that these inhibitors affected the maximal velocity as well as the affinity for the substrate. The inhibition was reversible when the CVFM was removed by dilution (data not shown).

The peptide recognition site of the farnesyltransferase resides on the \( \beta \) subunit of the \( \alpha \beta \) dimeric enzyme (15). The function of the \( \alpha \) subunit is not known. Another protein prenyltransferase from rat brain, which transfers geranylgeranyl residues, appears to contain the same \( \alpha \) subunit as the farnesyltransferase, but it does not share the \( \beta \) subunit (16). This geranylgeranyltransferase also recognizes proteins in which cysteine is fourth from the COOH terminus, but it strongly prefers peptides that terminate in leucine (16, 17). Thus, the unique \( \beta \) subunit on this geranylgeranyltransferase appears to confer on it a different specificity for peptide acceptors as well as prenyl donors.

The predicted amino acid sequence of the cloned \( \beta \) subunit of the rat brain farnesyltransferase (18) resembles that of the yeast protein DPR1/RAM1, which was previously suspected to be a component of the farnesyltransferase based on genetic arguments (8, 19). It also resembles the sequence of a suspected subunit of another yeast prenyltransferase designated Bet2 (20). Mutants in Bet2 can farnesylate proteins such as ras proteins, but they are unable to attach prenyl groups to the sec4 and YPT1 proteins. The latter are low molecular weight GTP-binding proteins that are involved in vesicular transport and are suspected to be geranylgeranylated (20). These data lend support to the suggestion that a family of \( \beta \) subunits determines the peptide and prenyl specificities of the prenyltransferases (16).

The current data on nonprenylated inhibitors, coupled with the evidence for different \( \beta \) subunits, suggest that it may be possible to produce inhibitors that are specific for individual members of this family. Selective inhibition of specific prenyltransferases should allow the resolution of the function of each of these enzymes in animal cells.

Acknowledgments—Debra Noble and Richard Gibson provided excellent technical assistance. Nathan Lewis and Candace Millhouse provided invaluable help with the synthesis and purification of peptides.

REFERENCES
1. Reiss, Y., Goldstein, J. L., Seabra, M. D., Casey, P. J., and Brown, M. S. (1990) Cell 62, 81-88
2. Casey, P. J., Solaki, P. A., Der, C. J., and Buss, J. E. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 8323-8327
3. Farnsworth, C. C., Wolda, S. L., Gelb, M. H., and Glomset, J. A. (1989) J. Biol. Chem. 264, 20422-20429
4. Lai, R. K., Perez-Sala, D., Canada, F. J., and Rando, R. R. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 7673-7677
5. Fukada, Y., Takao, T., Ohguro, H., Yoshizawa, T., Akino, T., and Shimonishi, T. (1990) Nature 346, 658-660
6. Reiss, Y., Stradley, S. J., Giersch, L. M., Brown, M. S., and Goldstein, J. L. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 752-736
7. Hancock, J. F., Magee, A. I., Childs, J. E., and Marshall, C. J. (1989) Cell 57, 1167-1177
8. Schafer, W. R., Trubec, C. E., Yang, C.-C., Mayer, M. P., Rosenberg, S., Poultier, C. D., Kim, S.-H., and Rine, J. (1990) Science 249, 1133-1139

Inhibitors of Protein Farnesyltransferase 15577

| Tetrapeptide   | [3H]Farnesyl formed | Concentration for 50% inhibition |
|---------------|---------------------|---------------------------------|
| Exp. A        |                     |                                 |
| CVIM (p21K-ra"B) | 15.4               | 0.15*                           |
| CVFM          | 0.14                | <0.10*                          |
| CVYM          | 0.10                |                                 |
| CVWM          | 1.5                 | 0.55*                           |
| CVIF          | 5.0                 | 0.55*                           |
| Exp. B        |                     |                                 |
| CVIM (p21K-ra"B) | 15.5               | 0.15*                           |
| CVIM (p21K-ra"B) | 12.3               | 0.15*                           |
| CVLS (p21H-ra"B) | 6.8                | 3.0*                            |
| CAIM (lamin B) | 11.6                | 0.15*                           |
| CVIS (transducin, \( \gamma \) subunit) | 15.4 | 1.0* |
| Octyl-CVIM    | 17.6                | 0.18                            |
| PenVFM        | 0.70                |                                 |
| SVIM          | 0.06                | >100                            |
| Exp. C        |                     |                                 |
| CVIM (p21K-ra"B) | 16.5               | 0.15*                           |
| PenVFM        | 0.10                |                                 |
| Exp. D        |                     |                                 |
| CVIM (p21K-ra"B) | 17.4               | 0.15*                           |
| CCIF          | 0.73                | 0.38                            |
| CIFM          | 0.26                |                                 |
| CCIF          | 1.2                 | 2.8                             |
| CV (pCl-F)M   | 0.11                |                                 |
| mpaVFM        | 12.5                | 0.09                            |
| SVIM          | 0.0                 | >100                            |
| Exp. E        |                     |                                 |
| CVIM (p21K-ra"B) | 7.4                | 0.07*                           |
| CVFM          | 0.07                | <0.10*                          |
| mpaVFM        | 7.7                 | 0.69                            |
9. Glomset, J. A., Gelb, M. H., and Farnsworth, C. C. (1990) Trends Biochem. Sci. 15, 139–142
10. Gibbs, J. B. (1991) Cell 65, 1–4
11. Reiss, Y., Seabra, M. C., Goldstein, J. L., and Brown, M. S. (1990) Methods 1, 241–245
12. Schaber, M. D., O’Hara, M. B., Garsky, V. M., Mosser, S. C., Bergstrom, J. D., Moores, S. L., Marshall, M. S., Friedman, P. A., Dixon, R. A. F., and Gibbs, J. B. (1990) J. Biol. Chem. 265, 14701–14704
13. Stewart, J. M., and Young, J. D. (1984) Solid Phase Peptide Synthesis, Pierce Chemical Co., Rockford, IL.
14. Pataki, G. (1965) J. Chromatogr. 12, 541–548
15. Reiss, Y., Seabra, M. C., Armstrong, S. A., Slaughter, C. A., Goldstein, J. L., and Brown, M. S. (1991) J. Biol. Chem. 266, 10672–10677
16. Seabra, M. C., Reiss, Y., Casey, P. J., Brown, M. S., and Goldstein, J. L. (1991) Cell 65, 429–434
17. Finegold, A. A., Johnson, D. I., Farnsworth, C. C., Gelb, M. H., Judd, S. R., Glomset, J. A., and Tamanoi, F. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 4448–4452
18. Chen, W-J., Andres, D. A., Goldstein, J. L., Russell, D. W., and Brown, M. S. (1991) Cell, in press
19. Goodman, L. E., Perou, C. M., Fujiyama, A., and Tamano, F. (1988) Yeast 4, 271–281
20. Rossi, G., Jiang, Y., Newman, A. P., and Ferro-Novick, S. (1991) Nature 351, 158–161