ANALYTICAL BIOCHEMISTRY 225, 60–63 (1995)

A Fluorescence Assay for Geranylgeranyl Transferase Type I

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Received October 5, 1994

A new fluorescence assay for measuring the activity of geranylgeranyl transferase (type I) is described. It does not require the use of either radiolabeled geranylgeranyl diphosphate or the purified recombinant Ras protein substrate with the carboxy terminal sequence of CVLL. Dansyl GCVLL and unlabeled geranylgeranyl diphosphate are used as substrates. The K_m for Dansyl GCVLL and for geranylgeranyl diphosphate is 5 μM and 800 nM, respectively. At equimolar concentrations, enzymatic activity is higher when Dansyl GCVLL is used as a substrate compared to Dansyl GCVII. Dansyl GCVLS, a substrate for farnesyl transferase, is inactive in this assay. CVFL is a competitive inhibitor of geranylgeranyl transferase and exhibits a K_i of 200 nM. © 1995 Academic Press, Inc.

Studies in mammalian systems have shown that farnesylation (prenylation) is an essential post-translational modification required for the association of the Ras protein (p21) with the membrane and for signaling of cell growth (1–9). Farnesyl transferase, a metalloenzyme responsible for farnesylation of Ras, catalyzes the transfer of the C-15 isoprenyl group from farnesyl diphosphate to the cysteine residue (thioether linkage) present at the carboxy terminus of p21. Structural studies have demonstrated that this enzyme recognizes the consensus sequence CAAX (C, cysteine; A, an aliphatic amino acid; and X, methionine, serine, alanine, or glutamine) present at the carboxy terminus of proteins such as nuclear lamins, transducin, rhodopsin kinase, skeletal muscle phosphorylase kinase, and the γ subunit of the yeast heterotrimeric G protein. Tetrapeptides with differing sequences have been synthesized that are either substrates or competitive inhibitors of this enzyme (10–14). When the carboxy terminal amino acid sequence CAAX is mutated to CAAL, the protein is no longer a substrate for farnesyl transferase. Instead, this sequence is recognized by a related enzyme, geranylgeranyl transferase I (15–20), and the cysteine residue is geranylgeranylated. Substrates of geranylgeranyl transferase I include rap1, CDC42, and the γ subunit of mammalian G proteins. A third member of this prenyl transferase family is geranylgeranyl transferase II. This enzyme is responsible for the addition of the geranylgeranyl group to proteins such as rab that have the consensus sequence CC or CXC at the carboxy terminus (21).

These prenyl transferases have been characterized. Farnesyl transferase and geranylgeranyl transferase I are heterodimers composed of two subunits, an α (48 kDa) and a β subunit (46 kDa). The α subunit is shared by both of these prenyl transferases, whereas the β subunits, although homologous, appears to be different (19,20,22). It is suggested that the β subunit of the enzyme contains the binding site for the protein substrate (CAAX or CAAL) and it is likely that the α subunit recognizes the prenyl diphosphate. Geranylgeranyl transferase II is a heterotrimer with one subunit sharing homology with the β subunit of farnesyl transferase or geranylgeranyl transferase I.

Since mutant Ras is oncogenic and is present in a wide variety of tumors, it has been proposed that inhibitors of Ras farnesylation will prevent membrane association and cause growth arrest due to inhibition of cellular signaling (23,24). Ideally, these inhibitors should be specific and have no effect on geranylgeranyl transferase I or II. Pampiliano *et al.* (25) have developed a fluorescence assay of farnesyl transferase that uses Dansyl GCVLS and farnesyl diphosphate as substrates. By substituting serine with leucine at the carboxy terminal se-

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0003-2697/95 $6.00

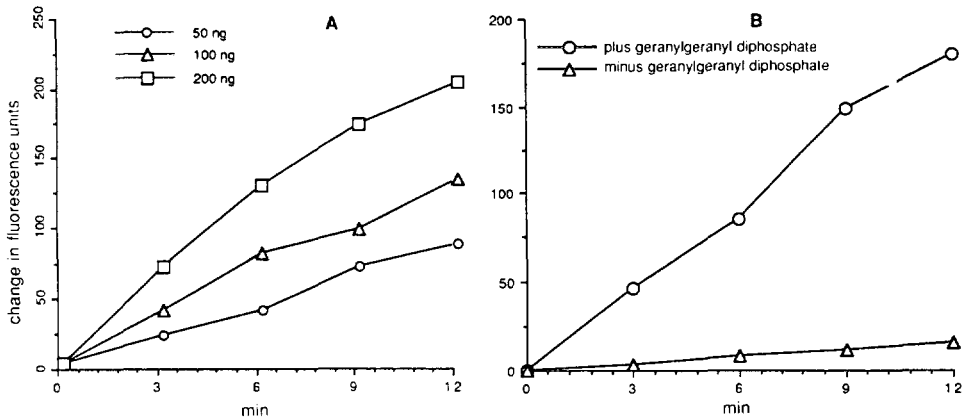

FIG. 1. (A) The effect of varying concentrations of geranylgeranyl transferase I on geranylgeranylation of Dansyl GCVLL. (B) Dependence of enzymatic activity on geranylgeranyl diphosphate.

quence, we have developed a fluorescence assay for geranylgeranyl transferase I. Using Dansyl GCVLL and geranylgeranyl diphosphate as substrates, the present studies describe this assay. The assay is conducted in microtiter plates and has been validated. By conducting both assays in parallel, it is possible to ascertain the specificity of agents that inhibit the activity of farnesyl transferase.

MATERIALS AND METHODS

Materials. Pure recombinant geranylgeranyl transferase I expressed in baculovirus (26,27) was used in all the assays described below. The tetrapeptides GCVLL, GCVII, and GCVLL were purchased from AnaSpec Inc. (San Jose, CA). These peptides were dansylated and purified by procedures described by Pampiliano *et al.* (25). Geranylgeranyl diphosphate was obtained from BioMol Research Laboratories (Plymouth Meeting, PA). ^{3}H-labeled geranylgeranyl diphosphate (specific activity 15 Ci/mmol) and geranylgeraniol were purchased from ARC (St. Louis, MO).

Assays. Geranylgeranyl transferase I assays were conducted in 96-well microtiter plates. The reaction mixture (100 μl) contained 50 mM Tris chloride, pH 7.5, 50 μM $ZnCl_2$, 5 mM $MgCl_2$, 1 mM dithiothreitol, 20 mM KCl, 0.2% octyl-β-D-glucoside, 10 μM Dansyl GCVLL, and 10 μM geranylgeranyl diphosphate. The reaction was initiated by the addition of 50–200 ng of geranylgeranyl transferase I and the catalysis was followed by measuring the increase in fluorescence (excitation at 360 nm and emission at 460 nm) using a Cytofluor 2350 fluorescence measurement system (Millipore Corp., Bedford, MA). In some assays, the Dansyl GCVLL was replaced with either Dansyl GCVLS or Dansyl GCVII. The same buffer system was used for measuring farnesyl transferase activity with Dansyl GCVLS and farnesyl diphosphate as substrates.

Characterization of the geranylgeranylated Dansyl GCVLL. ^{3}H-labeled geranylgeranyl diphosphate (1 μCi; 0.06 nmol) was used instead of cold geranylgeranyl diphosphate in the reaction mixture. A reaction mixture that did not contain geranylgeranyl transferase was used as control. After a 30-min incubation, 0.5 ml of water was added followed by formic acid to 0.5%. The thioether-linked [^{3}H]geranylgeranyl group was cleaved to [^{3}H]geranylgeraniol by the addition of 100 μl of methyl iodide. After 48 h at room temperature in the dark, carrier geranylgeraniol was added and the reaction mixture was processed by methods described by Casey *et al.* (6). The mixture was subjected to reverse-phase HPLC and the eluting fractions were monitored for absorbance (220 nm) and for radioactivity.

Studies with CVFL. Using radiolabeled Ras-CVLL as one of the substrates, Zhang *et al.* (27) have demonstrated that CVFL is a competitive inhibitor of geranylgeranyl transferase I. The effect of CVFL on the activity of the enzyme was ascertained. Varying concentrations of CVFL were added to the assay system described above and the activity of geranylgeranyl transferase I was determined at two different concentrations of Dansyl GCVLL. The K_i for CVFL was calculated by the graphical method of Dixon (28).

RESULTS

The effect of varying concentrations of geranylgeranyl transferase I on the geranylgeranlyation of Dansyl GCVLL is shown in Fig. 1A. The reaction was linear with respect to the time of incubation and with enzyme concentration. The reaction was also dependent on geranylgeranyl diphosphate (Fig. 1B). The K_m for Dansyl GCVLL was 5 μM and for geranylgeranyl diphosphate it was 800 nM. Using Ras-CVLL and [^{3}H]geranylgeranyl diphosphate as substrates, earlier studies by Zhang *et al.* (27) have shown that the K_m for Ras-CVLL and for ger-

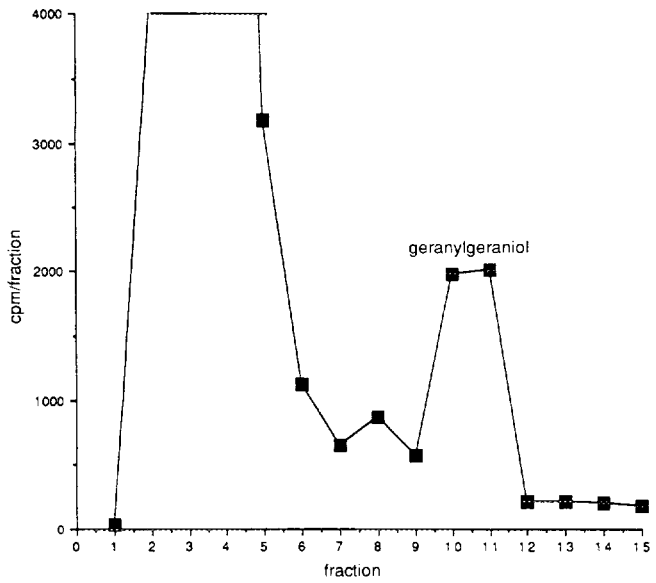

FIG. 2. Reverse-phase HPLC analysis of the reaction mixtures and identification of the reaction product as geranylgeraniol.

anylgeranyl diphosphate is 1.2 μM and 3 nM, respectively. Hence, in the fluorescence assay described here, the K_m for geranylgeranyl diphosphate is approximately 300-fold higher than that ascertained with the radiolabeled assay (27). The K_m either for the CVLL Ras protein or for Dansyl GCVLL is essentially similar.

CVFL is a competitive inhibitor of this enzyme with a K_i of 50 nM (27). Using the fluorescence assay described here, the effect of CVFL on geranylgeranyl transferase I was ascertained. Similar to results published by Zhang *et al.* (27), CVFL is a competitive inhibitor of the enzyme with a K_i of 200 nM.

To demonstrate that the geranylgeranyl group was transferred to the cysteine residue of Dansyl GCVLL, the incubation mixture was treated with methyl iodide. The thioether linkage resulting from this transfer is cleaved, resulting in the formation of the corresponding alcohol. When the complete reaction mixture (plus 200 ng of enzyme) containing radiolabeled geranylgeranyl diphosphate was subjected to methyl iodide and the products were analyzed by HPLC, radioactivity was detected in fractions that cochromatographed with carrier geranylgeraniol (Fig. 2). If geranylgeranyl transferase was omitted from the incubation, then no radioactivity was detected in these fractions. The identity of the radioactive peaks that did not coelute with geranylgeraniol is not known. It is likely that the first peak detected in both reaction mixtures represents residual geranylgeranyl diphosphate. The identity of the second peak (in the incubation mixture that did not contain the enzyme) is not known.

Additional studies to validate this fluorescence assay were undertaken by examining the substrate specificity. In results not shown, farnesyl diphosphate was not a substrate for geranylgeranyl transferase. Among the dansyl peptides used, Dansyl GCVLL was a better substrate than Dansyl GCVII. Dansyl GCVLS, a substrate used for measuring farnesyl transferase activity, was completely inactive (Fig. 3).

DISCUSSION

In the present studies a new assay of geranylgeranyl transferase I is described. This fluorescence-based assay has been validated by demonstrating that the geranylgeranyl group from geranylgeranyl diphosphate is transferred to the cysteine residue of the peptide substrate Dansyl GCVLL. Additional validation has been provided by examining the substrate specificity and the effect of CVFL, a known inhibitor of geranylgeranyl transferase I. Using this fluorescence assay, the K_m for Dansyl GCVLL is similar to the value obtained for Ras-CVLL by Zhang *et al.* (27). However, the K_m for geranylgeranyl diphosphate was approximately 300-fold higher than that obtained with the protein substrate. Using a phosphocellulose absorption assay, recent studies by Roskoski *et al.* (29) have shown that the K_m for geranylgeranyl diphosphate using KKCAIL as a substrate is 55 nM and the K_m for the peptide substrate can vary from 7 to 50 μM depending upon the amino acids coupled to the cysteine residue. Thus, residues on the amino terminus of the peptide substrate can alter the steady-state kinetic values of geranylgeranyl transferase I. The fluorescence assay described in this communication has used a bulky dansyl group at the amino terminus of GCVLL and while this group has no major effect

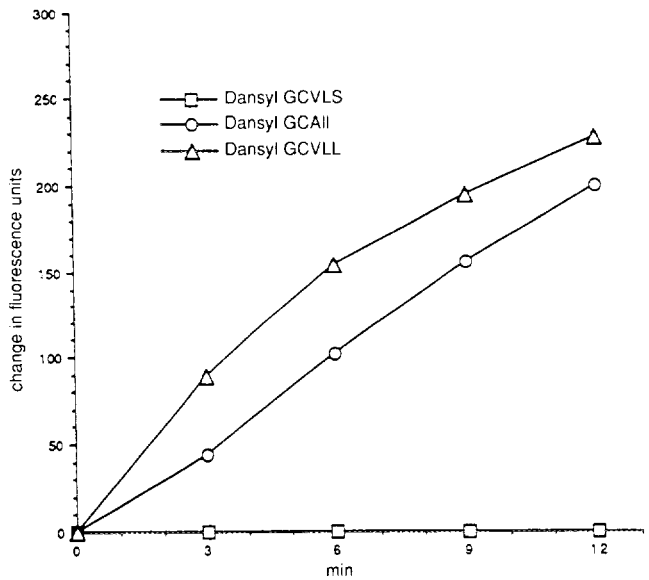

FIG. 3. The effect of various dansyl pentapeptides on the enzymatic activity of geranylgeranyl transferase.

on the K_m of the peptide substrate, it may alter the interaction of the two subunits of the enzyme, resulting in an increase in the K_m value for geranylgeranyl diphosphate. It should be noted that this explanation is only speculative and without supportive data.

Three major advantages of this assay are that it does not use radiolabeled geranylgeranyl diphosphate, it does not require purified recombinant Ras-CVLL, and it is conducted in 96-well microtiter plates and is therefore adaptable to robotics. Studies conducted with CVFL suggest that the fluorescence assay is as sensitive as the radiolabeled assay. It is not known whether this paradigm can be used to measure the activity of geranylgeranyl transferase II with Dansyl GCC or GCXC and geranylgeranyl diphosphate as substrates.

Since mutant Ras has been detected in a variety of human tumors and since farnesyl transferase plays a pivotal role in Ras-dependent signaling and cell growth, this enzyme represents a new antitumor target. Several inhibitors (30–32) of this enzyme have been identified. Addition of these inhibitors to Ras-transformed cells converts their phenotype to that of normal cells. In addition, these inhibitors delay the maturation of frog oocytes that have been microinjected with mutant Ras.

A fluorescence assay that uses Dansyl GCVLS and farnesyl diphosphate has already been described by Pampiliano *et al.* (25). In the search for specific inhibitors of farnesyl transferase, a parallel assay for geranylgeranyl transferase I such as the one described here can be conducted to ascertain the specificity. The buffer system and the concentration of salts are similar for both assays. Specific inhibitors of farnesyl transferase that do not affect the activity of geranylgeranyl transferase I can then be evaluated in tumor models.

ACKNOWLEDGMENTS

We thank Dr. Pat Casey for his interest in this study and for many valuable discussions on prenyl transferases. Studies at Duke University were supported by research grants from the National Science Foundation, American Cancer Society, and the March of Dimes. We thank Ms. Annette Schwendimann for her assistance in the preparation of the manuscript.

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