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Targeted Reengineering of Protein Geranylgeranyltransferase Type I Selectivity Functionally Implicates Active-Site Residues in Protein-Substrate Recognition

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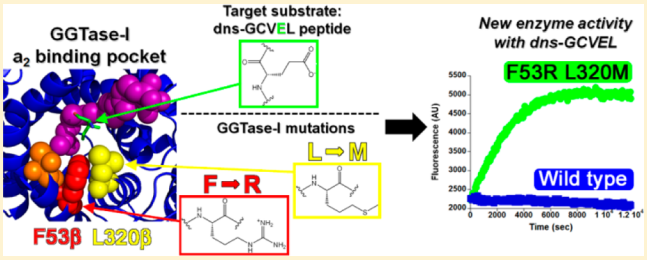
Supporting Information

ABSTRACT: Posttranslational modifications are vital for the function of many proteins. Prenylation is one such modification, wherein protein geranylgeranyltransferase type I (GGTase-I) or protein farnesyltransferase (FTase) modify proteins by attaching a 20- or 15-carbon isoprenoid group, respectively, to a cysteine residue near the C-terminus of a target protein. These enzymes require a C-terminal Ca_1a_2X sequence on their substrates, with the a_1, a_2, and X residues serving as substrate-recognition elements for FTase and/or GGTase-I. While crystallographic structures of rat GGTase-I show a tightly packed and hydrophobic a_2 residue binding pocket, consistent with a preference for moderately sized a_2 residues in GGTase-I substrates, the functional impact of enzyme−substrate contacts within this active site remains to be determined. Using site-directed mutagenesis and peptide substrate structure−activity studies, we have identified specific active-site residues within rat GGTase-I involved in substrate recognition and developed novel GGTase-I variants with expanded/altered substrate selectivity. The ability to drastically alter GGTase-I selectivity mirrors similar behavior observed in FTase but employs mutation of a distinct set of structurally homologous active-site residues. Our work demonstrates that tunable selectivity may be a general phenomenon among multispecific enzymes involved in posttranslational modification and raises the possibility of variable substrate selectivity among GGTase-I orthologues from different organisms. Furthermore, the GGTase-I variants developed herein can serve as tools for studying GGTase-I substrate selectivity and the effects of prenylation pathway modifications on specific proteins.



Posttranslational modifications are essential for the proper function of many proteins. These modifications increase the functional and chemical complexity of the proteome beyond that encoded within the genome.[1] The enzymes that catalyze these modifications must often selectively recognize and modify a subset of target proteins among a complex mixture of other potential substrates. This type of "multispecific" reactivity presents a formidable challenge in molecular recognition,[2,3] with many biological catalysts providing examples for studying how nature has addressed this problem. Furthermore, defining how enzymes involved in posttranslational modification achieve this type of multisubstrate recognition is essential for predicting the extent of posttranslational modification within the human proteome.

The prenyltransferases protein geranylgeranyltransferase type I (GGTase-I) and protein farnesyltransferase (FTase) have emerged as model systems of multispecific enzymes involved in posttranslational modification. GGTase-I and FTase catalyze the transfer of a 20- and 15-carbon isoprenoid groups, respectively, from geranylgeranyl diphosphate (GGPP) or farnesyl diphosphate (FPP) to the cysteine side-chain thiol near the C-terminus of a target protein.[4−6] This modification increases the hydrophobicity of the substrate protein and aids its localization to the plasma membrane, an essential step for the biological function of most prenylated proteins.[5,7,8] Both GGTase-I and FTase have been proposed to recognize a Ca_1a_2X motif at the C-terminus of a target protein (reviewed in refs 6 and 9−11). This sequence consists of a cysteine residue whose side-chain thiol is alkylated with the isoprenoid group, two aliphatic amino acids (a_1 and a_2), and the C-terminal X residue that plays a central role in determining whether a target protein is a substrate for FTase and/or GGTase-I.[12−14] In the case of FTase, the classical Ca_1a_2X model predicts only a subset of potential substrate sequences as functional and computational studies have revealed that FTase accepts a much wider range of peptide substrates than had been previously thought.[15−18] Bioinformatics and biochemical studies indicate that the sequence upstream of the cysteine residue to be prenylated also affects prenyltransferase substrate selectivity.[19−22]

Defining GGTase-I substrate selectivity also remains an area of intense interest, as many geranylgeranylated proteins play important roles in signaling pathways and cellular functions.[7,23−29] GGTase-I has largely been considered to exhibit

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similar substrate selectivity as FTase at the a_1 and a_2 positions. FTase and GGTase-I both exist as heterodimers composed of α and β subunits, with identical α subunits and distinct β subunits for each enzyme.[6,30,31] Crystallographic structures of rat GGTase-I complexed with peptide substrates and isoprenoid mimetic inhibitors suggest that the a_2 residue binding site in rat GGTase-I is composed of three amino acids in the GGTase-I β subunit, T49β, F53β, and L320β, and the fourth isoprene unit of GGPP (Figure 1).[14] This arrangement yields a closely

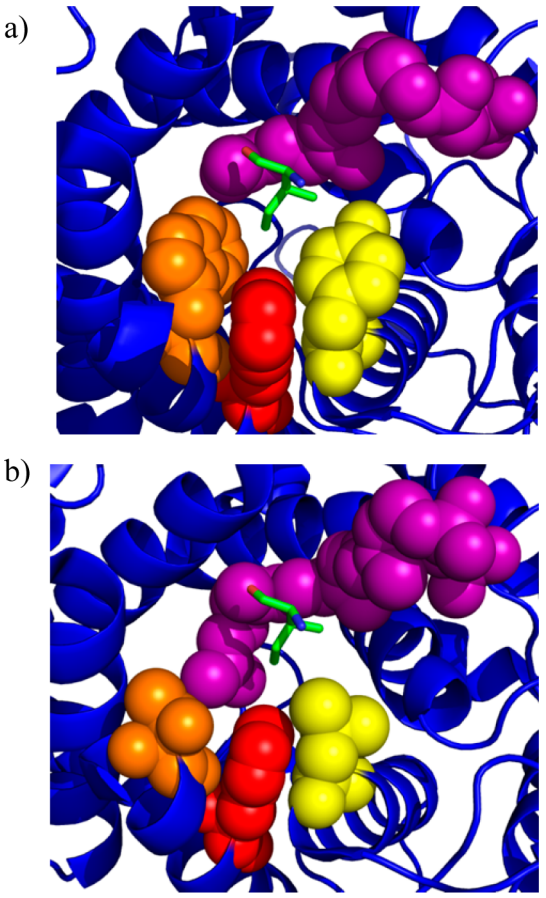

Figure 1. Binding sites of the a_2 residue in FTase and GGTase-I. Structure of a peptide substrate bound to FTase and GGTase-I reveals active-site amino acids in close contact to the a_2 residue of the Ca_1a_2X motif. (a) FTase; the a_2 residue is shown in green, W102β, in orange, W106β in red, Y361β in yellow, and the FPP analogue in purple. (b) GGTase-I; the a_2 residue is shown in green, T49β in orange, F53β in red, L320β in yellow, and the GGPP analogue in purple. Images were generated from PDB 1D8D and PDB 1TNB.[14,66]

packed hydrophobic binding site consistent with the preference for moderately sized hydrophobic a_2 residues and bears a striking resemblance to the a_2 binding site within FTase composed of W102β, W106β, Y361β, and the isoprenoid substrate.

These structural studies highlight interactions that may engender a_2 selectivity, providing a guide for biochemical investigation to ascertain the role of and requirement for these interactions in a_2 residue recognition within the Ca_1a_2X sequence. In FTase, enzyme selectivity at the a_2 position can be drastically altered by mutation of two amino acids predicted to contact the a_2 residue.[32] FTase recognizes the a_2 residue based on both side-chain size and polarity,[33] and mutation of W102β and/or W106β is sufficient to significantly increase FTase reactivity with substrates bearing both large and polar/charged a_2 residues. The tunability of these interactions between the peptide substrate a_2 residue and FTase site residues suggests that FTase utilizes negative discrimination against nonsubstrates to achieve substrate selectivity. This molecular-recognition strategy may aid in fulfilling the need for FTase to recognize and react with a broad range of potential substrate sequences in its role as a multispecific enzyme.

In this study, we investigated whether the substrate selectivity of rat GGTase-I can be reengineered using insights derived from FTase. A combination of site-directed mutagenesis of residues within the GGTase-I active site and structure–function studies of peptide substrate reactivity has identified interactions used by GGTase-I to generate a_2 selectivity. Randomization of the active-site residues potentially involved in recognizing the a_2 residue and subsequent enzyme-activity screening using target peptide substrates has identified a small group of GGTase-I variants capable of prenylating peptides with charged residues at the a_2 position. The increase in reactivity with substrates bearing polar amino acids does not necessitate loss of reactivity with natural substrate sequences with nonpolar residues. Comparison of the engineered GGTase-I variants in this work to the previously identified FTase variants highlights qualitative similarities in the molecular-recognition mechanisms employed by these two enzymes while also revealing functional distinctions in the active-site residues playing key roles in determining substrate selectivity. These findings provide additional support for a model wherein enzymes that must act upon multiple substrates employ discrimination against nonsubstrates to achieve selectivity. Our findings suggest that substrate selectivity potentially differs between GGTase-I orthologues in different organisms on the basis of the low conservation of certain active-site residues involved in recognizing the a_2 residue. The GGTase-I variants developed in this work can also serve as novel reagents for probing the effects of prenylation pathway modifications on proteins involved in signaling pathways and other cellular processes.

MATERIALS AND METHODS

Miscellaneous Methods. All assays were performed at 25 °C. All curve fitting was performed with KaleidaGraph (Synergy Software, Reading, PA). Geranylgeranyl diphosphate (GGPP) was purchased from Isoprenoids (Tampa, FL). Dansylated peptides were synthesized by Sigma-Genosys (The Woodlands, TX) in the Pepscreen format. Peptide purities were ~75%, with the majority of peptides examined exhibiting >90% purity, as determined by HPLC.[33] Major contaminants consist of smaller peptide fragments that are not efficient substrates for FTase or GGTase-I.[34,35] Peptides were solubilized in absolute ethanol containing 10% (v/v) DMSO and stored at −20 °C. Peptide concentrations were determined spectrophotometrically using Ellman's reagent.[36]

Preparation and Purification of GGTase-I and Single-Site GGTase-I Variants. Wild-type (WT) rat GGTase-I and GGTase-I variants were expressed in BL21(DE3) *Escherichia coli* using a previously described pET23a GGTase-I vector.[37] The bacterial cells were grown in either autoinduction media or rich induction media (20 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl, 1% glucose, and 100 μg/mL ampicillin, with expression induced by adding 0.5 mM $ZnSO_4$ and 0.4 mM IPTG) and purified as described previously, with dialysis steps

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replaced by desalting using a HiTrap desalting column (GE Healthcare).[12,37−39] Briefly, expressed GGTase-I was purified by FPLC (AktaPrime) with sequential DEAE Sephadex and Q Sepharose anion-exchange columns (GE Biosciences). Enzyme concentrations were measured by active-site titration using dansylated peptides, as described previously.[12] The concentration of the F53A L320D GGTase-I variant was estimated using UV absorbance at 280 nm (ε =134,000 $M^{-1}cm^{-1}$),[37] as the active-site titration with this enzyme variant using the dansyl-GCVKL peptide was unsuccessful, potentially because of low peptide-binding affinity. Mutations at F53β and L320β were introduced into the pET23a GGTase-I plasmid using PCR mutagenesis and confirmed by DNA sequencing (Genscript, Piscataway, NJ).

Construction of GGTase-I Libraries with Randomized Codons. Randomized codons (NNK, where N = equal mixture of A, T, G, and C and K = G or T) were introduced at positions T49β, F53β, and L320β in GGTase-I using a modified PCR mutagenesis protocol.[32] Five duplicate PCR reactions were performed using the primers encoding the randomized codons (NNK) at position F53β, L320β, or T49β followed by *Dpn*I digestion for 1 h at 37 °C. The PCR reactions were combined and purified using the BioBasic EZ-10 spin column PCR purification kit, with the purified PCR products then transformed into DH5α *E. coli* cells and plated onto 3−5 prewarmed LB plates containing 100 μg/mL ampicillin. Following overnight growth, colonies were collected from the plates by scraping, and the pool of mutated GGTase-I plasmids were purified from the resulting cell pellets using the BioBasic DNA purification kit per the manufacturer's protocol.

Expression of GGTase-I Codon-Randomized Libraries. Plasmid DNA encoding a library containing a randomized codon for one of the three target residues (T49β, F53β, or L320β) was transformed into chemically competent BL21-(DE3) *E. coli* followed by selection on LB agar plates containing 100 μg/mL ampicillin. Single colonies (94 total) were inoculated into single wells of a 96-deep-well (2.2 mL volume) plate containing 1 mL of autoinduction media with 100 μg/mL ampicillin.[39] Each 96-well plate was inoculated with a colony transformed with WT GGTase (well H12, positive control), and well H11 was not inoculated (negative control; no background/endogenous prenylation activity in *E. coli*). Plates were sealed with gas-permeable seals (Abgene) and shaken for 24 h (400 rpm) at 28 °C. Following growth, glycerol stocks were prepared and stored at −80 °C.

Cell-Lysate-Based GGTase-I Activity Assay. Cell lysates were prepared by addition of 100 μL of lysis solution [BugBuster bacterial lysis reagent (Millipore) supplemented with 4 mg/mL lysozyme, 12.5 U/mL benzonase (Sigma-Aldrich), and 200 μg/mL phenylmethylsulfonyl fluoride] to each 1 mL of cell culture in the 96-well plate followed by shaking (400 rpm) at 28 °C for 20 min. Lysates were stored on ice until assayed for GGTase-I activity.

GGTase-I activity in the cell lysates was measured under steady-state conditions by measuring the time-dependent increase in fluorescence (λ_{ex} 340 nm, λ_{em} 520 nm) upon geranylgeranylation of the dansylated peptide.[12,32] Initial screens were performed with 3 μM dansylated peptide, 10 μL cell lysate, and 10 μM GGPP in reaction buffer at 25 °C in a 96-well plate (Corning, model 3650). Peptides were incubated in reaction buffer for 20 min before initiation of the assay reactions by addition of the peptide solution to a solution containing the cell lysate. Fluorescence was measured at time points (1 min, 5 min, 10 min, 20 min, 30 min, 1 h, 2 h, 3 h, 4 h, and 5 h) in a Synergy H1 multimode plate reader (Biotek, Winooski, VT). To be considered active with a given target peptide, variants were required to exhibit the following reaction parameters: dns-GCVLL, minimum increase of 3000 fluorescence units and a plateau in fluorescence within 1.4 h (indicating reaction completion); dns-GCVEL, minimum increase of 3000 fluorescence units and a plateau in fluorescence within 5 h; dns-GCVDL, minimum increase of 2000 fluorescence units and a plateau in fluorescence within 5 h; and dns-GCVKL, minimum increase of 800 fluorescence units and a plateau in fluorescence within 2.1 h. Variants active with dns-GCVEL, dns-GCVDL, and dns-GCVKL were subjected to secondary screening wherein reaction fluorescence was measured at intervals of 40 s over a period of 8 h under the same reaction conditions and activity criteria described above.

Steady-State Kinetic Measurements with Purified GGTase-I and GGTase-I Variants. Steady-state kinetics were determined for GGTase-I from a time-dependent increase in fluorescence (λ_{ex} 340 nm, λ_{em} 520 nm) upon geranylgeranylation of the dansylated peptide.[12] Assays were performed with 0.2−10 μM dansylated peptide, 20−100 nM purified GGTase-I or GGTase-I variants, 10 μM GGPP, 50 mM HEPPSO, pH 7.8, and 5 mM tris(2-carboxyethyl)phosphine (TCEP) at 25 °C in a 96-well plate (Corning). Peptides were incubated in reaction buffer for 20 min prior to initiation by addition of GGTase-I and GGPP, with the GGTase-I concentration at least 5-fold lower than the peptide concentration. Fluorescence was measured as a function of time in a Synergy H1 multimode plate reader (Biotek, Winooski, VT) to define both the initial linear velocity as well as the reaction end point. The total fluorescence change observed upon reaction completion was divided by the initial concentration of the peptide substrate in a given reaction to yield a conversion from fluorescence units to product concentration; these values were averaged over several peptide concentrations to produce an amplitude conversion (Amp_{Conv}). The linear initial velocity, in fluorescence intensity per second, was then converted to a velocity (micromolar product produced per second) using eq 1, where V is velocity in micromolar per second, R is the velocity of the reaction in fluorescence units per second, and Amp_{Conv} refers to the ratio described above in fluorescence units per micromolar product.

$$V = \frac{R}{\mathrm{Amp_{Conv}}} \tag{1}$$

To confirm geranylgeranylation of the target peptides, HPLC analysis was performed on a representative set of peptide reactions. Reactions containing 3 μM dansyl-GCVa_2L peptide were monitored as described until reaction completion was attained, as indicated by a plateau in the observed fluorescence. The reactions were then analyzed by HPLC (Zorbax Eclipse XDB-C18 column) with a gradient from 30% acetonitrile in 25 mM ammonium acetate to 100% acetonitrile flowing at 1 mL/min over 30 min; peptides and products were detected by fluorescence (λ_{ex} 340 nm, λ_{em} 496 nm). In all cases, HPLC analysis indicates that the peak for the dansyl-GCVa_2L peptide shifts to a longer retention time while exhibiting an increase in observed fluorescence, consistent with quantitative geranylgeranylation, whereas parallel reactions performed without GGPP showed no change in peptide retention time. Representative HPLC traces are included in the Supporting Information.

Table 1. Steady-State Parameters for Geranylgeranylation of dns-GCVLL and dns-GCVEL Peptides Catalyzed by WT GGTase-I and Single-Mutation GGTase-I Variants

	peptide substrate			
	dns-GCVLL			dns-GCVEL
GGTase-I variant	k_{cat} (10^{-2} s^{-1})[a]	K_M (μM)[a]	k_{cat}/K_M (mM^{-1} s^{-1})[a]	k_{cat}/K_M (mM^{-1} s^{-1})[a]
WT (FL)	8 ± 2	0.7 ± 0.1	120 ± 20	b.d.[b]
F53A	4 ± 1	1.3 ± 0.1	28 ± 6	b.d.
L320A	1.2 ± 0.1	1.5 ± 0.2	8 ± 1	b.d.
F53R	2.3 ± 0.4	3.8 ± 0.6	6 ± 1	1.06 ± 0.05

[a]Steady-state parameters were determined at saturating GGPP (10 μM) and varying peptide concentrations under conditions described in the Materials and Methods. [b]b.d., reactivity below detection limit.

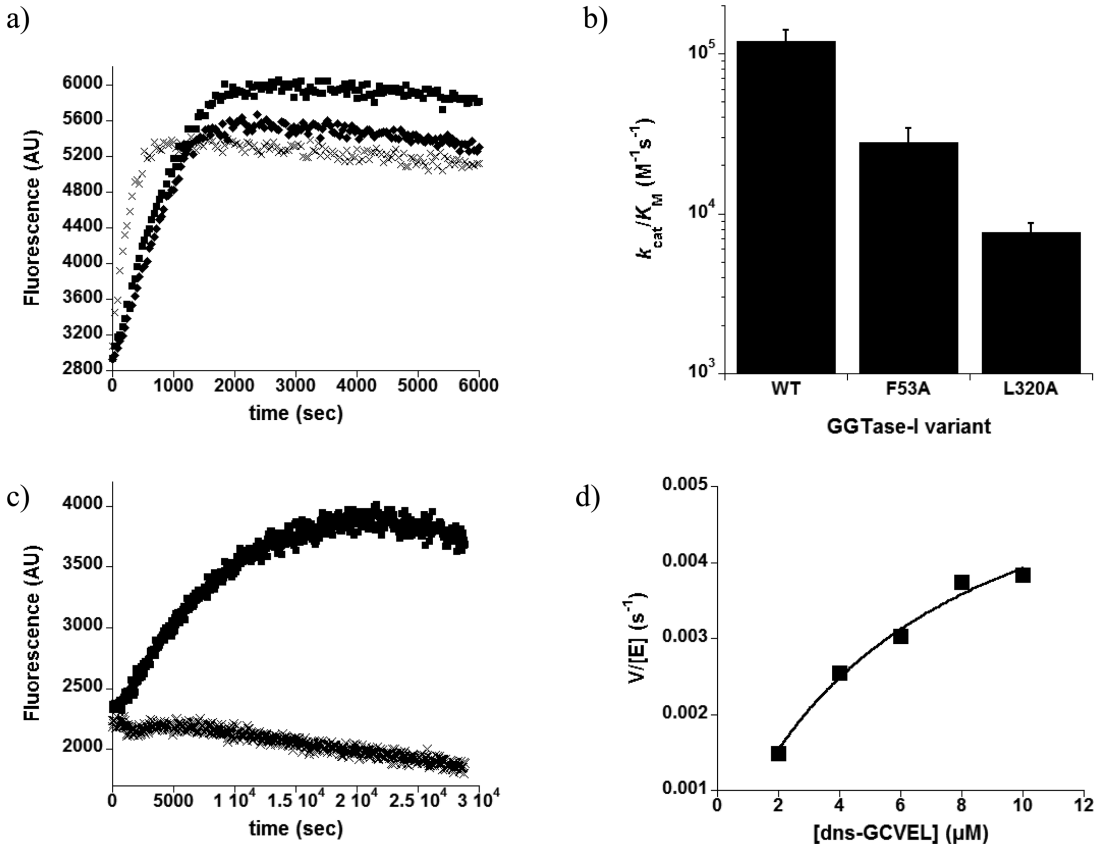

Figure 2. Effects of single-site mutation at F53β and L320β on GGTase-I activity and selectivity. (a) Geranylgeranylation of the dns-GCVLL peptide substrate catalyzed by WT GGTase-I (×), F53A GGTase-I (♦), and L320A GGTase-I (■) in the cell-lysate-based prenylation assay. (b) Reactivity of WT, F53A, and L320A GGTase-I with dns-GCVLL, as reflected by $k_{cat}/K_M^{dns-GCVLL}$. Error bars represent the standard deviation from a minimum of three replicates. (c) Geranylgeranylation of the dns-GCVEL target peptide substrate catalyzed by WT GGTase-I (×) and F53R GGTase-I (■) in the cell-lysate-based prenylation assay. (d) Dependence of prenylation activity on the dns-GCVEL peptide substrate concentration catalyzed by F53R GGTase-I. All assays were performed as described in the Materials and Methods.

Steady-state kinetic parameters (k_{cat}, K_M, and $k_{cat}/K_M^{peptide}$) were determined from a fit of the Michaelis–Menten equation to the dependence of initial velocity divided by enzyme concentration (V/E) on the peptide concentration in the presence of saturating GGPP (10 μM).

Detection of Dansylated Peptide Geranylgeranylation under Single-Turnover and Multiple-Turnover Conditions by HPLC. Assays to detect dansylated peptide geranylgeranylation under single-turnover conditions ([E] > [S]) were performed with 2 μM dansylated peptide, 3 μM purified WT GGTase-I or GGTase-I variant, 10 μM GGPP, 50 mM HEPPSO, pH 7.8, and 5 mM tris(2-carboxyethyl)-phosphine (TCEP) in a total volume of 100 μL at 25 °C in low-adhesion polypropylene microcentrifuge tubes. Peptides were incubated in reaction buffer for 20 min prior to initiation by addition of GGTase-I and GGPP. Reactions were incubated for 8 h at 25 °C followed by reaction termination by addition of an equal volume of 20% acetic acid in isopropanol. Reactions were then analyzed by HPLC (Zorbax Eclipse XDB-C18 column) with a gradient from 30% acetonitrile in 25 mM ammonium acetate to 100% acetonitrile flowing at 1 mL/min over 30 min. Peptide substrates and geranylgeranylated peptide products were detected by fluorescence (λ_{ex} 340 nm, λ_{em} 496 nm). Chromatogram analysis and peak integration was performed using Chemstation for LC (Agilent Technologies),

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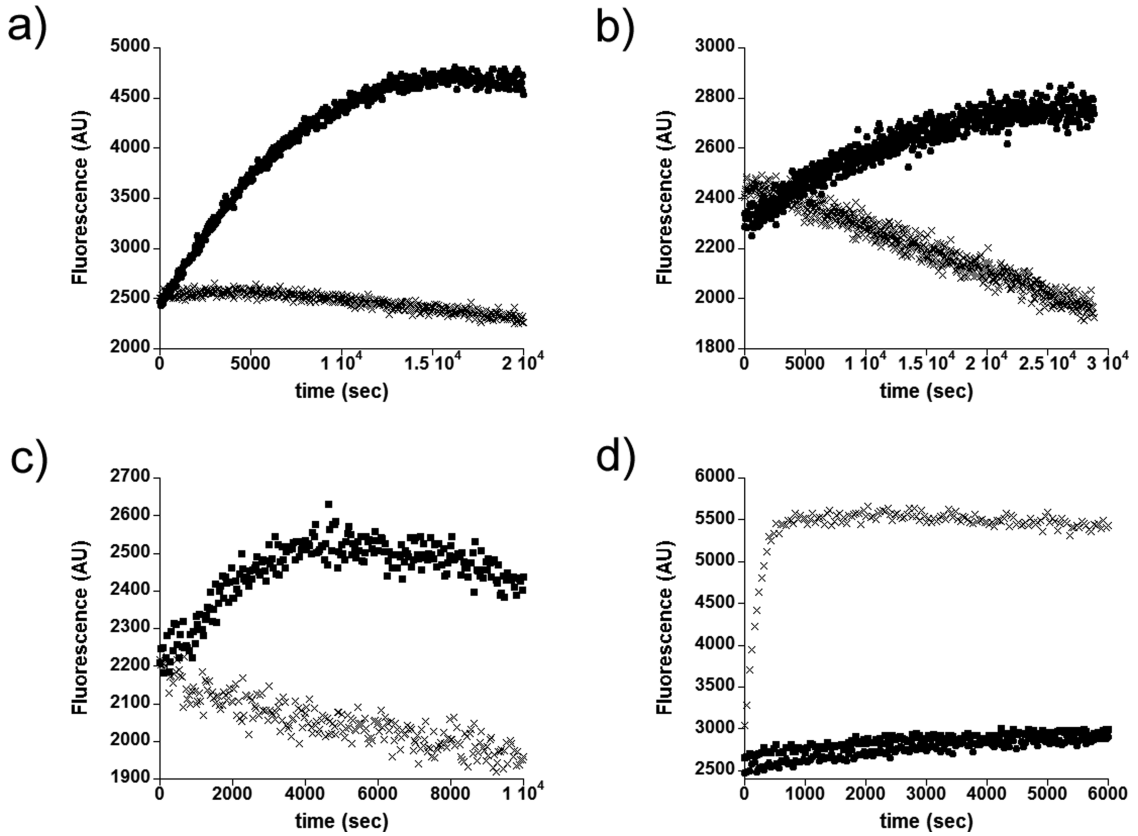

Figure 3. Simultaneous mutation of F53β and L320β leads to GGTase-I-catalyzed geranylgeranylation of substrates bearing charged a_2 residues. (a) Geranylgeranylation of the dns-GCVEL target peptide substrate catalyzed by F53R L320A GGTase-I (●) in the cell-lysate-based prenylation assay, with negligible activity exhibited by WT GGTase-I (×).(b) Geranylgeranylation of the dns-GCVDL target peptide substrate catalyzed by F53R L320A GGTase-I (●) in the cell-lysate-based prenylation assay, with negligible activity exhibited by WT GGTase-I (×). The loss of fluorescence signal in the reaction with WT GGTase-I, as indicated by a downward slope at longer time points, is due to fluorescence inactivation/photobleaching of the dns-GCVDL substrate upon extended irradiation. (c) Geranylgeranylation of the dns-GCVKL target peptide substrate catalyzed by F53A L320D GGTase-I (■) and WT GGTase-I (×) in the cell-lysate-based prenylation assay. The loss of fluorescence signal at long time points, as indicated by a downward slope in the reaction with WT GGTase-I, is due to fluorescence inactivation/photobleaching of the dns-GCVKL substrate upon extended irradiation. (d) Geranylgeranylation of the dns-GCVLL peptide substrate is not efficiently catalyzed by F53R L320A GGTase-I (●) and F53A L320D GGTase-I (■) when compared to WT GGTase-I (×) in the cell-lysate-based prenylation assay. All assays were performed as described in the Materials and Methods.

with geranylgeranylated peptide product peak integrations reported in Table S2 in the Supporting Information.

Assays to detect dansylated peptide geranylgeranylation under multiple-turnover conditions ([E] ≪ [S]) were performed under similar conditions as single-turnover experiments except with the concentration of WT GGTase-I or GGTase-I variants held at 100 nM. Other than the reduction in enzyme concentration, multiple-turnover assays were performed and analyzed as described above for the single-turnover reactions.

RESULTS

Design of Target Peptides. As a template for our target peptides, we chose a CVLL peptide sequence present in several human proteins known to be geranylgeranylated (e.g., CDC42, R-Ras, Rab8, GBG8, and GBP5)[5,14,19] as well as in rat orthologues of CDC42 and R-Ras. The CVLL sequence has also been well-characterized as a substrate for in vitro GGTase-I activity assays, and our steady-state characterization of WT rat GGTase-I with the dns-GCVLL substrate agrees with data from previous reports (Table 1).[12,37,40] To design peptides as targets for generating novel GGTase-I variants, we mutated the penultimate leucine residue of the CVLL sequence to aspartate (CVDL), glutamate (CVEL), and lysine (CVKL). These three target sequences are not found within the predicted human or rat proteomes based on a search of the ProSite database,[41] and the introduction of charged amino acids at the a_2 position is predicted to block reactivity with GGTase-I.[14,19,20] We verified that these three target peptides are not substrates for WT rat GGTase-I, with reactivity below the detection limits of our fluorescence-based assay under conditions that result in robust prenylation of the parent dns-GCVLL peptide substrate (Table 1 and Supporting Information).

Mutations at F53β and L320β to Introduce Charge Complementarity. Three amino acids within the GGTase-I active site (T49β, F53β, and L320β) are predicted to contact the a_2 residue of the peptide substrate based on structural studies.[14] To attempt to avoid perturbations of GGPP cosubstrate binding, we chose to leave T49β unchanged in the initial round of mutagenesis.[14,40] Inspired by mutations that alter FTase substrate selectivity,[32] we introduced single mutations at F53β and L320β that can potentially lead to charge pairing with negatively charged (CVDL and CVEL) and positively charged (CVKL) target peptides. Both F53β and L320β were mutated to alanine (A), arginine (R), histidine (H), glutamate (E), and aspartate (D). Alanine mutations were

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included to assess the functional requirement of the phenylalanine and leucine side chains at these position for GGTase-I activity. Similar studies in FTase revealed that highly conserved tryptophan residues within the FTase active site can be mutated to alanine without loss of enzyme activity, indicating that the tryptophan indole side chains were not essential for enzyme function.[32]

Single-mutation GGTase-I variants were first tested for prenylation activity with the parent dns-GCVLL substrate to ascertain if the mutations led to loss of enzyme activity. In bacterial cell lysate-based activity assays, all single-mutation GGTase-I variants generated by site-directed mutagenesis were capable of prenylating dns-GCVLL except for L320βR, with L320βE and L320βD exhibiting reduced but detectible activity (see Supporting Information Figure S2b). Changes in enzyme activity for GGTase-I variants do not appear to arise from different expression levels, as reflected by gel electrophoresis and Coomassie staining of crude bacterial lysates (see Supporting Information Figure S3). The F53βA and L320βA variants displayed activity comparable to WT GGTase-I in the cell lysate-based assay, indicating the amino acid side chains at these two positions are not essential for enzyme function (Figure 2a). Steady-state characterization of purified F53βA and L320βA GGTase-I variants indicates alanine mutation at these residues reduces $k_{cat}/K_M^{dns-GCVLL}$ by ~4- and ~15-fold, respectively (Figure 2b and Table 1), suggesting that F53β and L320β contribute to GGTase-I function but are not essential.

When the single-mutation GGTase-I variants were screened against the dns-GCVEL, dns-GCVDL, and dns-GCVKL target peptides, the F53βR mutant exhibited activity with dns-GCVEL and also exhibited reduced but detectable activity with dns-GCVDL (Figure 2c and Supporting Information Figure S3). Steady-state analysis of the purified F53βR variant indicates that this enzyme catalyzes geranylgeranylation of the dns-GCVEL peptide ~100-fold less efficiently than WT GGTase-I modifies dns-GCVLL, as reflected by relative $k_{cat}/K_M^{peptide}$ values (Table 1). The F53βR variant retains prenylation activity with the natural dns-GCVLL substrates, exhibiting a 20-fold drop in $k_{cat}/K_M^{dns-GCVLL}$ relative to WT GGTase-I and a ~5-fold reduction in activity compared to alanine substitution at this position. In contrast to the activity of the F53βR variant with dns-GCVEL and dns-GCVDL target peptides bearing negatively charged a_2 residues, none of the single-mutation GGTase-I variants exhibited detectable prenylation activity with the dns-GCVKL substrate.

Simultaneous Mutation at F53β and L320β Synergistically Expands GGTase-I Selectivity. In an earlier study, we found that altering FTase substrate selectivity to allow for charged residues within the peptide substrate usually required two active-site mutations: one providing charge complementarity to the target substrate and another that reduced steric bulk and hydrophobicity within the active site.[32] To investigate if GGTase-I was also susceptible to a two-mutation mechanism for altering enzyme selectivity, we created double-mutation variants wherein either F53β or L320β were mutated to alanine to provide space within the active site and the other residue was mutated to A, R, H, E, and D as in the single-mutation variants. Two of these double mutants, F53βR L320βA and F53βA L320βD, exhibited activity with target peptides in cell lysate screening. The F53βR L320βA variant exhibited prenylation activity with the dns-GCVEL substrate similar to that observed with the F53βR single mutant and also exhibited reduced but detectable activity with the dns-GCVDL substrate (Figure 3a,b). In contrast to the failure of the single-mutation GGTase-I variants to catalyze prenylation of the dns-GCVKL substrate, the F53βA L320βD variant was able to geranylgeranylate this peptide (Figure 3c). These two double-mutation variants (F53βR L320βA and F53βA L320βD) also exhibited dramatically reduced activity with the dns-GCVLL substrate as compared to wild-type GGTase-I (Figure 3d), indicating that the novel reactivity observed for these variants arises from a switch in substrate selectivity as opposed to expanded tolerance for non-natural substrate sequences.

Randomization of T49β, F53β, and L320β. Given the success of our targeted mutations within the GGTase-I active site in altering GGTase-I substrate selectivity, we sought to assess the full potential for engineering GGTase-I substrate selectivity through saturation mutagenesis of three amino acids predicted to interact with the a_2 residue: T49β, F53β, and L320β. We generated a series of variant libraries wherein one of these active-site residues is randomized through incorporation of an NNK codon. On the basis of the reduced codon redundancy resulting from use of the NNK codon, screening 94 variants from each single-site randomization library provides a 95% probability of complete coverage of the 21 unique enzyme variants possible in each library (WT enzyme, 19 point mutations, and a stop codon), as indicated by statistical analysis.[42,43] Therefore, each single-site variant library was screened in the context of a 96-well plate consisting of 94 library variants, WT GGTase-I as a positive control, and a negative control well without GGTase-I.

We constructed a total of eight variant libraries, subdivided into three classes (Table 2). The first set of three libraries involved randomization of each of the targeted active-site residues to yield the T49X, F53X, and L320X libraries. The next set of three libraries randomized these same three amino acids in the context of alanine mutations at either F53β or L320β (T49X F53A, F53A L320X, and F53X L320A). Finally, we generated libraries incorporating either an arginine mutation at F53β (F53R L320X) or aspartate mutation at L320β (F53X L320D) to determine if mutations other than alanine could also act in synergy with the F53βR and L320βD mutations described earlier.

Table 2. GGTase-I Randomization Libraries Yield Variants Capable of Catalyzing Geranylgeranylation of dns-GCVa_2L Target Peptides

randomization library[a]	active with dns-GCVLL	active with dns-GCVEL	active with dns-GCVDL	active with dns-GCVKL
T49X	68	0	0	0
F53X	32	5	0	0
L320X	60	0	0	0
T49X F53A	70	0	0	0
F53X L320A	63	8	0	0
F53A L320X	56	0	0	1
F53R L320X	34	35	20	0
F53X L320D	0	0	0	7

[a]Each randomization library consisted of 94 variants, with prenylation activity screened as described in the Materials and Methods.

Each of these libraries, consisting of a total of 94 individual variants, was screened for geranylgeranylation activity with dns-GCVLL and the three target peptides: dns-GCVEL, dns-GCVDL, and dns-GCVKL (Table 2). None of the variants in

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the two libraries wherein T49 was randomized were active with the target peptides, whereas the majority of variants in both T49 randomized libraries retained activity with the dns-GCVLL peptide substrate. This suggests that T49β does not play a significant functional role in recognizing the a_2 residue within GGTase-I substrates. The F53X, F53X L320A, and F53R L320X libraries produced variants active with dns-GCVEL and/or dns-GCVDL, whereas the F53A L320X and F53X L320D libraries produced variants active with dns-GCVKL. After primary and secondary screening to identify and confirm active variants from libraries, a subset of variants active with dns-GCVEL, dns-GCVDL, and dns-GCVKL were isolated and sequenced.

Sequencing of the variants active with dns-GCVEL and/or dns-GCVDL revealed that F53β is mutated to arginine (R) in each case, with L320β either retained or mutated to alanine (A), glycine (G), methionine (M), or tyrosine (Y) (Table 3).

Table 3. F53β/L320β Variants Identified from Randomization Libraries That Catalyze Geranylgeranylation of dns-GCVEL, dns-GCVDL, or dns-GCVKL with Enhanced Efficiency Relative to WT GGTase-I

GGTase-I variants		peptide substrate reactivity[a]		
F53β	L320β	dns- GCVEL	dns- GCVDL	dns- GCVKL
R	L	+	+	−
R	M	+	+	−
R	G	+	+	−
R	A	+	−	−
R	Y	−	+	−
A	D	−	−	+

[a]A plus sign (+) indicates that a given GGTase-I variant exhibited sufficient activity with a given peptide substrate to be considered active per the criteria outline in the Materials and Methods; a minus sign (−) indicates the absence of sufficient activity to be considered active.

In the only variant to catalyze prenylation of the dns-GCVKL substrate, L320β is mutated to aspartic acid (D) and F53β is replaced by alanine (A). These results suggest that GGTase-I requires both introduction of charge complementarity and reduction in steric bulk within the active site to gain activity with dns-GCVKL. However, the activity of the F53βR L320βM and F53βR L320βY variants with dns-GCVEL and dns-GCVDL indicates that reduction in steric bulk is less essential for activity with substrates bearing anionic a_2 residues, as leucine and methionine are comparable in size (166.7 and 162.9 Å^3, respectively) while tyrosine is ∼16% larger than leucine at 193.6 Å^3.[44] The larger number of rotamers available to methionine compared to leucine could possibly indicate that either decreased steric bulk (F53βR L320βG) or increased side-chain flexibility (F53βR L320βM) at position 320β increases reactivity with a substrate bearing glutamate at the a_2 position.[45,46]

Steady-State Characterization of GGTase-I Double-Mutation Variants. We selected several of the most active double-mutation GGTase-I variants for steady-state characterization to determine if the increase in activity with the target peptides arose from an increase in k_{cat}, decrease in K_M, or effects on both parameters (Table 4 and Figure 4). The two variants that exhibited the highest reactivity with dns-GCVEL, F53βR L320βG and F53βR L320βM, appear to increase enzyme activity with this substrate using different approaches. For the F53βR L320βG variant, the K_M for prenylation of dns-GCVEL is ∼4-fold higher than that for WT GGTase-I with dns-GCVLL, whereas k_{cat} is ∼13-fold lower (Table 4). In contrast, the F53βR L320βM variant does not exhibit saturation within the experimentally accessible concentrations of the dns-GCVEL substrate, while the observed reaction velocity approaches 35% of k_{cat} for WT GGTase-catalyzed prenylation of dns-GCVLL (Table 4 and Figure 4a). Thus, the enhancement of activity with dns-GCVEL appears to be due to a decrease in K_M in the case of the F53βR L320βG mutant, whereas the increased catalysis of dns-GCVEL prenylation by the F53βR L320βM variant may rely more on an increase in k_{cat}. We note that definitive conclusions cannot be drawn because of the lack of information regarding steady-state parameters for WT GGTase-I-catalyzed prenylation of dns-GCVEL resulting from the lack of observable prenylation activity for this enzyme−substrate pair in the fluorescence-based assay. However, examination of the reactivity of the F53βR L320βG and F53βR L320βM variants with dns-GCVLL supports our assertions. Both F53βR L320βG and F53βR L320βM are ∼24-fold less active with dns-GCVLL compared to WT GGTase-I, but this loss in activity arises primarily in k_{cat} (37-fold decrease) for F53βR L320βG and in both k_{cat} (4-fold decrease) and K_M (7-fold increase) for F53βR L320βM.

We also characterized variants that demonstrated the highest geranylgeranylation activity with dns-GCVDL (F53βR L320βY) and dns-GCVKL (F53βA L320βD) in the cell lysate-based assay. Similar to the F53βR L320βM variant, steady-state characterization of these variants following purification indicates that neither of these variants achieves saturation with their cognate substrates below 10 μM, with these variants demonstrating reaction efficiencies ∼200-fold

Table 4. Steady-State Parameters for Geranylgeranylation of dns-GCVa_2L Target Peptides Catalyzed by WT GGTase-I and Double-Mutation GGTase-I Variants[a]

	peptide substrate (dns-GCVa_2L)							
	a_2 = L			a_2 = E			a_2 = D	a_2 = K
GGTase-I variant	k_{cat} (10^{-2} s^{-1})	K_M (μM)	k_{cat}/K_M (mM^{-1} s^{-1})	k_{cat} (10^{-2} s^{-1})	K_M (μM)	k_{cat}/K_M (mM^{-1} s^{-1})	k_{cat}/K_M (mM^{-1} s^{-1})	k_{cat}/K_M (mM^{-1} s^{-1})
WT (FL)	8 ± 2	0.7 ± 0.1	120 ± 20	b.d.[b]	b.d.	b.d.	b.d.	b.d.
RG	0.27 ± 0.07	0.5 ± 0.2	5 ± 1	0.6 ± 0. 2	3 ± 1	2.1 ± 0.1	n.e.[c]	n.e.
RM	2.3 ± 0.4	5 ± 1	4.7 ± 0.4	>5	>10	4.2 ± 0.8	n.e.	n.e.
RY	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	0.49 ± 0.05	n.e.
AD	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	0.32 ± 0.06

[a]Steady-state parameters were determined at saturating GGPP (10 μM) and varying peptide concentrations under conditions described in the Materials and Methods. [b]b.d, reactivity below detection limit. [c]n.e., reactions were not evaluated.

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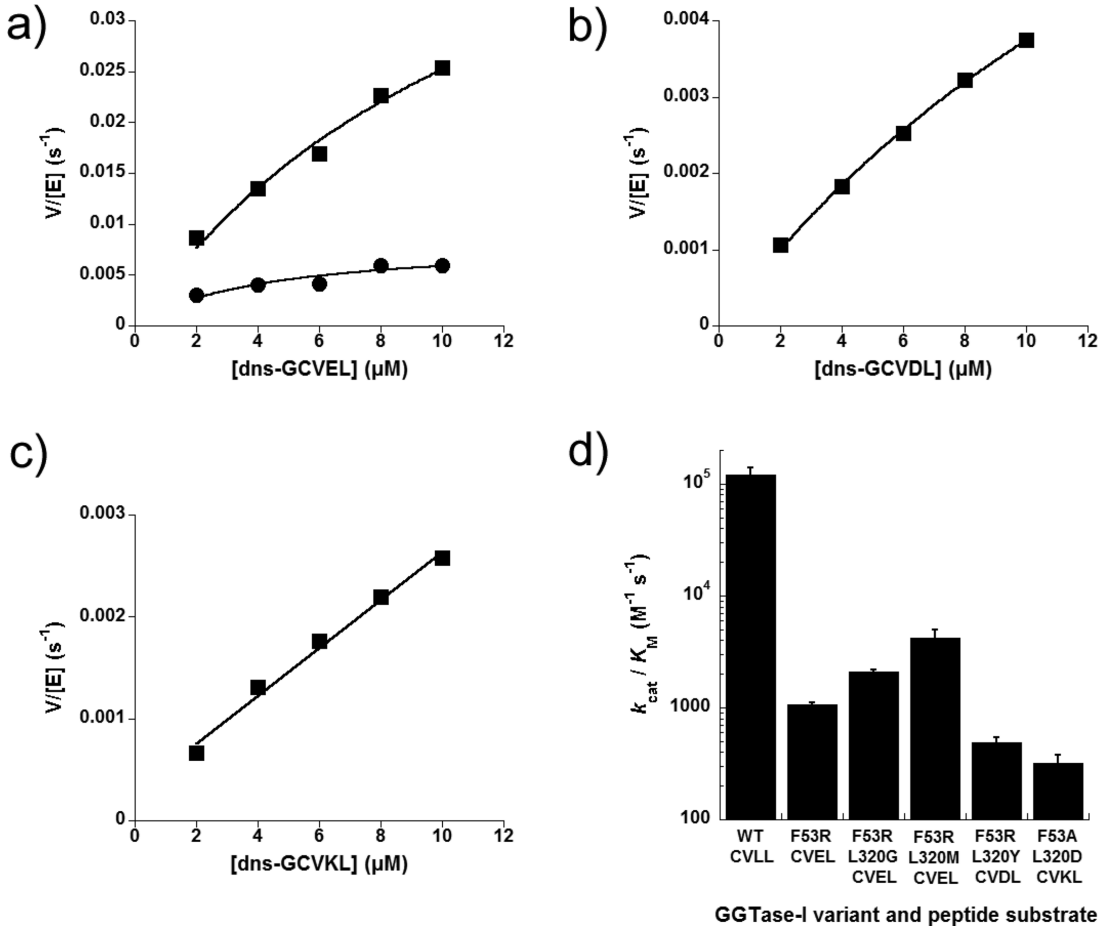


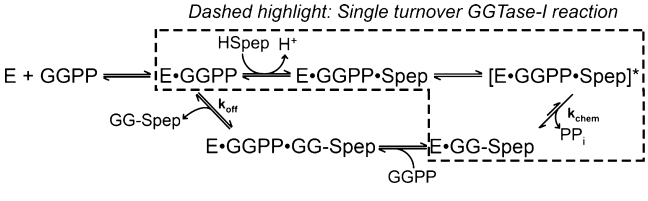
Figure 4. Steady-state characterization of the reactivity of F53β/L320β double-mutant GGTase-I variants with target peptides. (a) Dependence of prenylation activity on the dns-GCVEL peptide substrate concentration catalyzed by F53R L320G GGTase-I (●) and F53R L320 M GGTase-I (■). (b) Dependence of prenylation activity on the dns-GCVDL peptide substrate concentration catalyzed by F53R L320Y GGTase-I. (c) Dependence of prenylation activity on the dns-GCVKL peptide substrate concentration catalyzed by F53A L320D GGTase-I. Reactivity was measured using purified enzymes, and all assays were performed and analyzed as described in the Materials and Methods. (d) Reactivity of WT GGTase-I and GGTase-I variants with different peptide substrates, as reflected by $k_{cat}/K_M^{dns\text{-}GCVa2L}$: WT with dns-GCVLL; F53R, F53R L320G, and F53R L320M with dns-GCVEL; F53R L320Y with dns-GCVDL; and F53A L320D with dns-GCVKL. Error bars represent the standard deviation from a minimum of three replicates.

lower than WT GGTase-I with dns-GCVLL, as reflected by relative $k_{cat}/K_M^{peptide}$ values (Table 4).

Investigating the Role of the Product-Release Step in the GGTase-I Reaction Pathway in Altering GGTase-I Substrate Selectivity upon Mutation of F53β and L320β. Under steady-state reaction conditions, enzyme substrate selectivity can be affected by any reaction step in the enzyme catalytic cycle. In GGTase-I, this cycle includes GGPP and peptide substrate binding, the chemical step of protein geranylgeranylation, subsequent binding of another molecule of GGPP to the enzyme-prenylated peptide complex, and release of the prenylated protein (Scheme 1).[21,47] In previous studies of FTase, mutation of a tyrosine residue near the peptide substrate binding site in either the rat or yeast orthologue led to an apparent change in substrate selectivity, with the Y361L mutant FTase exhibiting a significant loss in activity with a CVLS peptide substrate normally preferred by FTase.[48,49] Subsequent studies of this Y361L FTase mutant under both steady-state and pre-steady-state conditions revealed that the apparent change in substrate selectivity arose from severely impaired product dissociation with the CVLS peptide substrate sequence preferred by WT FTase in the presence of the Y361L mutation.[50] A number of naturally occurring peptide substrates also exhibit single-turnover-only reactivity with WT FTase, proving that enzyme mutations are not required to induce slow product release.[12,16] The phenomenon of efficient peptide prenylation but inefficient product release, leading to single-turnover reactivity, has also been observed for some peptide substrates with rat GGTase-I.[12]

In light of these findings, we can propose two models for how the mutations at F53β and L320β within GGTase-I lead to changes in enzyme activity with our target peptides under steady-state (multiple-turnover) reaction conditions. In one model, WT GGTase-I cannot bind and catalyze the prenylation of our target peptides, and the mutations introduced herein

Scheme 1



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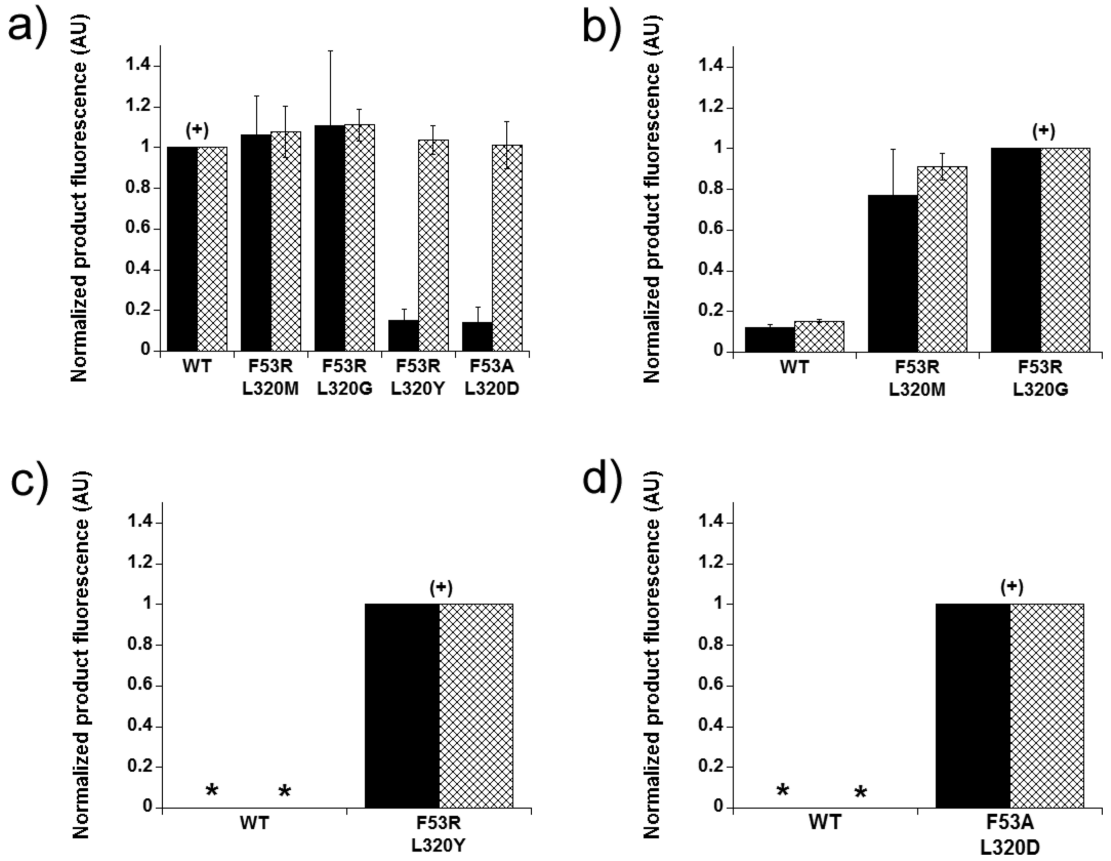

Figure 5. Comparison of geranylgeranylation activity of WT GGTase-I and GGTase-I variants with dns-GCVa_2L peptide substrates under multiple-turnover (MTO) and single-turnover (STO) reaction conditions. For reactions with each peptide substrate (dns-GCVLL, dns-GCVEL, dns-GCVDL, and dns-GCVKL), the amount of geranylgeranylated peptide product for each enzyme−peptide combination is normalized to the enzyme−peptide combination (denoted with +) that exhibited the largest amount of product formation, as reflected by fluorescence integration of the product peak as described in the Materials and Methods. Reactions that did not yield observable prenylated peptide product are marked with an asterisk (*). (a) WT GGTase-I and GGTase-I double mutation variant-catalyzed prenylation of dns-GCVLL under MTO (solid bar) and STO (crosshatched bar) conditions; product fluorescence normalized to WT GGTase-I reaction with dns-GCVLL. (b) WT GGTase-I, F53R L320M GGTase-I, and F53R L320G GGTase-I-catalyzed prenylation of dns-GCVEL under MTO (solid bar) and STO (crosshatched bar) conditions; product fluorescence normalized to F53R L320G GGTase-I reaction with dns-GCVEL. (c) WT GGTase-I and F53R L320Y GGTase-I-catalyzed prenylation of dns-GCVDL under MTO (solid bar) and STO (crosshatched bar) conditions; product fluorescence normalized to F53R L320Y GGTase-I reaction with dns-GCVDL. (d) WT GGTase-I and F53A L320D GGTase-I-catalyzed prenylation of dns-GCVKL under MTO (solid bar) and STO (crosshatched bar) conditions; product fluorescence normalized to F53A L320D GGTase-I reaction with dns-GCVKL. Error bars represent the standard deviation from a minimum of three replicates.

allow the GGTase-I variants to bind and prenylate the non-natural peptide sequences. Alternatively, WT GGTase-I could bind and prenylate the target peptides but cannot efficiently turn over because of slow product release, similar to the behavior observed with the Y361L mutant FTase described earlier. In this second model, mutation of F53β and L320β leads to faster product release and increased enzyme activity under steady-state conditions.

To distinguish between these two models, we determined the ability of WT GGTase-I to geranylgeranylate the dns-GCVLL, dns-GCVEL, dns-GCVDL, and dns-GCVKL peptide substrates under both single-turnover (STO, [E] > S) and multiple-turnover (MTO, [E] ≪ [S]) conditions. If the defect in prenylation of the target peptides by WT GGTase-I arises from slow product release, then robust prenylation would be observed under STO conditions but not under MTO conditions. In parallel, we analyzed the prenylation of dns-GCVLL and the target peptides by our most active GGTase-I variants under STO and MTO conditions: F53βR L320βG and F53βR L320βM, dns-GCVLL and dns-GCVEL; F53βR L320βY, dns-GCVLL and dns-GCVDL; and F53βA L320βD, dns-GCVLL and dns-GCVKL.

WT GGTase-I exhibits robust geranylgeranylation of the dns-GCVLL peptide under both STO and MTO conditions, as expected (Figure 5 and Table S2 in Supporting Information). The four GGTase-I double mutation variants also efficiently prenylated dns-GCVLL under STO conditions. However, the F53βR L320βY and F53βA L320βD variants exhibited reduced prenylation activity under MTO conditions, indicating that the reduced steady-state reactivity of these variants with dns-GCVLL may arise from a defect in product release in a fashion similar to that observed with the FTase Y361L variant.[50] In contrast to dns-GCVLL, the WT enzyme catalyzed minimal modification of dns-GCVEL under both reaction conditions and no detectible modification of dns-GCVDL or dns-GCVKL under either STO or MTO conditions (Figure 5b−d). These data indicate the lack of reactivity of these peptides with WT GGTase-I does not result from a defect in the product-release step (Scheme 1). Furthermore, the prenylation of the target peptides by their cognate GGTase-I variants under both STO

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and MTO conditions supports the model wherein mutation of F53β and L320β increases the ability of GGTase-I to bind and catalyze geranylgeranylation of peptide substrate bearing charged residues at the a_2 position.

DISCUSSION

The prenyltransferases FTase and GGTase-I are multispecific enzymes that must act on large pool of potential protein substrates while maintaining substrate selectivity. This type of enzyme activity presents a formidable challenge in molecular recognition, and these enzymes provide an ideal opportunity to understand how nature has addressed this problem. We have demonstrated that GGTase-I substrate selectivity can be rationally reengineered through targeted mutagenesis of active-site residues. We have identified a small cohort of GGTase-I variants containing two active-site mutations capable of accepting non-natural peptide substrate sequences, where the term non-natural refers to protein C-terminal sequences not found in the human or rat proteomes.

Comparison of the GGTase-I variants generated in this work to FTase variants developed in a previous study reveals several similarities in the requirements for reengineering substrate selectivity in these two enzymes.[32] In both enzymes, mutation of only two active-site residues is sufficient to alter drastically the substrate selectivity at the a_2 position of the peptide substrate. Efficient prenylation of peptide substrates bearing charged residues at the a_2 position requires introduction of charge complementarity within the proposed a_2 residue binding pocket. In most cases, accompanying introduction of a charged residue into the active site with a second mutation that removes steric bulk aids in increasing enzyme activity with the non-natural substrate sequences. These similarities support the conclusion that FTase and GGTase-I recognize the a_2 residue within the Ca_1a_2X using similar arrays of interactions, as has been suggested by structural studies.[14] Furthermore, many of the mutations in GGTase-I that enhance reactivity with substrates bearing charged a_2 residues do not abrogate reactivity with the natural dns-GCVLL substrate, presenting another potential example of negative discrimination by a multispecific enzyme wherein the enzyme selects against reacting with nonsubstrates rather than selecting for substrates.[32]

Although this study highlights qualitative similarities between substrate selectivity reengineering in FTase and GGTase-I, we note several important distinctions between these two closely related enzymes. In FTase, the W102β residue plays a key role in defining substrate selectivity at the a_2 residue based on both polarity and steric volume, and mutation of this tryptophan can drastically alter FTase substrate selectivity.[32] In GGTase-I, mutation of the structurally homologous residue T49β was not observed to relax GGTase-I selectivity at the a_2 position. In GGTase-I, T49β also contacts the fourth isoprenoid unit of the GGPP cosubstrate, and mutation of W102β in FTase to threonine allows FTase to accept GGPP and larger FPP analogues.[14,40,51] Taken together, these data suggest that the role played by the residue at the W102/T49 position shifts from participation in both peptide and isoprenyl substrate recognition in FTase to primarily recognition of GGPP in GGTase-I. To determine whether the mutations at F53β and L320β may also affect prenyl donor selectivity, all variants were assayed for activity with FPP, but all variants exhibited similar higher activity with GGPP as is observed for WT GGTase-I (data not shown).[40,52,53]

The utility of mutating L320β in GGTase-I to alter a_2 selectivity suggests that this residue plays a key role in defining GGTase-I substrate selectivity. A ClustalW alignment of 38 GGTase-I β subunits from a range of organisms indicates L320β is not strongly conserved (53% identity), with mutations described in this work such as L320βY and L320βM represented among the aligned sequences (Figure 6 and Supporting Information). This suggests GGTase-I orthologues from different species may exhibit significantly different substrate selectivity at the a_2 position of the Ca_1a_2X sequence and supports the possibility of developing species-specific GGTase-I inhibitors as potential antiparasitic and antifungal therapeutics.[54−59] The role (if any) of the related residue Y361β in determining FTase selectivity has yet to be fully characterized, but studies indicate that Y361β may play roles in substrate recognition, enzyme activity, and resistance to prenyltransferase inhibitors.[48−50,60]

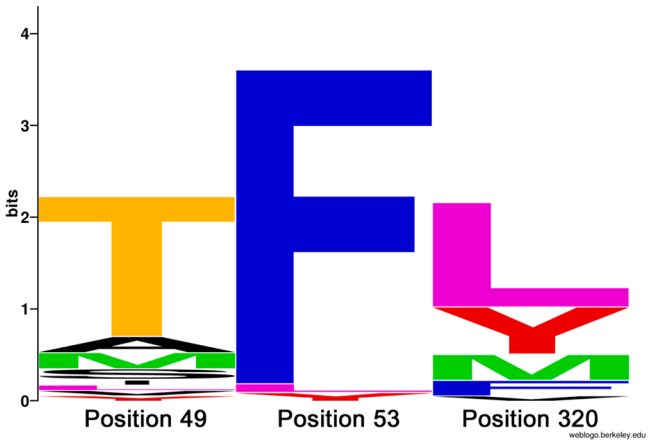

Figure 6. Conservation analysis of T49β, F53β, and L320β among GGTase-I orthologues. A ClustalW alignment of GGTase-I β subunit sequences indicates that F53β is highly conserved (95%) across different organisms, whereas L320β (53% conservation) is more variable, with both tyrosine and methionine represented at this position in different GGTase-I orthologues. Sequence conservation at these positions is presented using WebLogo, with the height of the amino acid stack proportional to the degree of conservation at a given position and the height of each individual amino acid in a stack reflecting its frequency at that position.[67]

Looking beyond the roles of specific active-site residues in controlling substrate selectivity in FTase and GGTase-I, the composition of the pools of selectivity-altered variants in the two enzymes also indicate distinctions between the two enzymes. Selection for FTase variants active with peptide substrates bearing charged residues returned a large number of distinct sequences,[32] reflecting the existence of multiple molecular solutions to the problem of recognizing a substrate bearing an aspartate or lysine at the a_2 position. In contrast, our studies of GGTase-I returned a small number of variants active with dns-GCVEL and dns-GCVDL and only one variant active with dns-GCVKL even in the presence of saturation mutagenesis at F53β and L320β (Table 3). The reduced sequence diversity in the GGTase-I variant pool may reflect the experimental difference between the FTase and GGTase-I selections, wherein both W102 and W106 were simultaneously randomized in FTase, whereas F53β and L320β were randomized sequentially in GGTase-I. However, the lower diversity observed in the GGTase-I variants could also indicate

that GGTase-I substrate selectivity is less "tunable" than FTase because of a more rigid or organized active site. Different degrees of functional plasticity within the peptide binding sites of FTase and GGTase-I could be an important consideration for the design of novel specific inhibitors of these two prenyltransferases.

Modifications catalyzed by enzymes in the protein prenylation pathway are proposed to be required for the proper function of many proteins on the basis of inhibition studies and gene knockouts of pathway enzymes.[28,29,61−65] The increasing number of peptide sequences shown to serve as efficient prenyltransferase substrates suggests the potential for broad sequence diversity in the pool of in vivo prenylated proteins.[15−18] Consequently, development of tools for studying the trafficking, activity, and fate of proteins at each step along the prenylation pathway is essential for understanding the effects of individual prenylation pathway modifications on protein structure and function. The GGTase-I variants reported in this work, in combination with previously reported FTase variants, constitute a set of novel tools for defining the role of and requirement for protein prenylation within the cell at the level of a single protein. By facilitating investigation of specific prenylated proteins and associated pathways without perturbing modifications throughout the entire prenylation proteome, these engineered enzymes will aid in identifying and characterizing prenylation-dependent pathways involved in diseases such as cancer.[25]

■ ASSOCIATED CONTENT

Supporting Information

Analysis of conservation of GGTase-I residues at positions analogous to T49β, F53β, and L320β in rat GGTase-I, as determined by ClustalW alignment; quantitation of peptide substrate geranylgeranylation by WT GGTase-I and GGTase-I variants under MTO and STO reaction conditions; cell-lysate-based activity analysis of WT GGTase-I reaction with dns-GCVEL, dns-GCVDL, and dns-GCVKL; reactivity of F53β and L320β single-site mutation GGTase-I variants with dns-GCVLL; gel analysis of GGTase-I variant expression; reactivity of F53R GGTase-I with dns-GCVDL; HPLC chromatograms of dns-GCVEL, dns-GCVDL, and dns-GCVKL peptide geranylgeranylation by GGTase-I variants. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS USED

GGTase-I, protein geranylgeranyltransferase type I; FTase, protein farnesyltransferase; GGPP, geranylgeranyl diphosphate; FPP, farnesyl diphosphate; WT, wild type; HEPPSO, *N*-2-hydroxyethylpiperazine-*N*′-2-hydroxypropanesulfonic acid; TCEP, tris(2-carboxyethyl)phosphine; HPLC, high-pressure liquid chromatography; dns, dansyl; IPTG, isopropyl β-D-1-thiogalactopyranoside; DMSO, dimethyl sulfoxide; PCR, polymerase chain reaction; STO, single turnover; MTO, multiple turnover; dns-GCVa_2L; dansyl−glycine−cysteine−valine-a_2 residue−leucine

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