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Identification of novel peptide substrates for protein farnesyltransferase reveals two substrate classes with distinct sequence selectivities

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Abstract

Prenylation is a post-translational modification essential for the proper localization and function of many proteins. Farnesylation, the attachment of a 15-carbon farnesyl group near the C-terminus of protein substrates, is catalyzed by protein farnesyltransferase (FTase). Farnesylation has received significant interest as a target for pharmaceutical development and farnesyltransferase inhibitors (FTIs) are in clinical trials as cancer therapeutics. However, as the total complement of prenylated proteins is unknown, the FTase substrates responsible for FTI efficacy are not yet understood. Identifying novel prenylated proteins within the human proteome constitutes an important step towards understanding prenylation-dependent cellular processes. Based on sequence preferences for FTase derived from analysis of known farnesylated proteins, we selected and screened a library of small peptides representing the C-termini of 213 human proteins for activity with FTase. We identified 77 novel FTase substrates that exhibit multiple-turnover reactivity within this library; our library also contained 85 peptides that can be farnesylated by FTase only under single-turnover conditions. Based on these results, a second library was designed that yielded an additional 29 novel multiple-turnover FTase substrates and 45 single-turnover substrates. The two classes of substrates exhibit different specificity requirements. Efficient multiple-turnover reactivity correlates with the presence of a nonpolar amino acid at the a_2 position and a Phe, Met, or Gln at the terminal X residue, consistent with the proposed Ca_1a_2X sequence model. In contrast, the sequences of the single-turnover substrates vary significantly more at both the a_2 and X residues and are not well-described by current farnesylation algorithms. These results improve the definition of prenyltransferase substrate specificity, test the efficacy of substrate algorithms, and provide valuable information about therapeutic targets. Finally, these data illuminate the potential for *in vivo* regulation of prenylation through modulation of single- versus multiple-turnover peptide reactivity with FTase.

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Keywords

protein farnesyltransferase; farnesylation; substrate specificity; prenylation; peptide library

Introduction

Protein farnesyltransferase (FTase)¹ and protein geranylgeranyltransferase type I (GGTase-I) are members of the prenyltransferase family of sulfur alkyltransferases [reviewed in^{1; 2}]. These enzymes, both heterodimers of α and β subunits, use a zinc ion to catalyze the covalent attachment of a 15-carbon farnesyl group from farnesyl diphosphate (FPP) or a 20-carbon geranylgeranyl group from geranylgeranyl diphosphate (GGPP) to the thiol side chain of a cysteine residue near the C-terminus of a protein substrate^{2; 3}. The attached lipid aids in localization of proteins to cellular membranes and enhances protein-protein interactions^{4; 5}. Prenylation is required for the proper function of many proteins, including members of the Ras and Rho superfamilies of small GTPases^{1; 6}. While many proteins have been experimentally shown to be prenylated *in vivo* (see Table S1), the total number of prenylated proteins within the cell remains unknown^{7; 8; 9; 10}.

Both FTase and GGTase-I are proposed to recognize protein or peptide substrates containing a C-terminal “Ca₁a₂X” sequence^{11; 12; 13; 14; 15; 16; 17}. In this model, “C” refers to a cysteine residue three residues upstream of the C-terminus that is prenylated at the thiol group to form a thioether, “a” refers to any aliphatic amino acid, and “X” refers to a subset of amino acids that are proposed to determine specificity for FTase (methionine, serine, glutamine, alanine) or GGTase-I (leucine, phenylalanine). Expanding upon the “Ca₁a₂X” box paradigm, bioinformatic analysis and biochemical studies of known substrates and related proteins indicate that sequences immediately upstream of the conserved cysteine residue may also play a role in substrate selectivity^{8; 9; 18}. Biochemical studies of prenyltransferase substrate specificity also indicate that recognition of peptide substrates is more complex than originally proposed. For example, a₂ selectivity is affected in a context-dependent manner by the identity of the terminal X residue¹⁹, and some substrates are modified efficiently by both FTase and GGTase-I^{11; 20}. Predicting the complete set of FTase and GGTase-I substrates requires a deeper understanding of the substrate specificity of these enzymes, posing a significant challenge to defining the extent of prenylation within the proteome.

Interest in identifying the number and identities of prenylated proteins stems in part from development of protein farnesyltransferase inhibitors (FTIs) as therapeutics to treat cancer, parasitic infection, and other medical conditions^{6; 21; 22; 23}. Initial studies of FTIs focused on blocking signaling pathways associated with Ras proteins, which requires farnesylation for membrane association and activation of signaling pathways^{6; 24; 25; 26; 27}. However, subsequent studies have shown that FTI efficacy can be transduced through non-Ras-related pathways²⁸. Determining the prenylation-dependent pathways potentially responsible for the efficacy of FTI treatment could provide valuable insights for development of novel pharmaceuticals. To characterize these pathways, the farnesylated proteins involved must first be identified. In the absence of a comprehensive predictive model for FTase specificity, defining the complete set of FTase substrates requires probing each potential FTase substrate within the proteome for the ability to be farnesylated.

¹Abbreviations: FTase, protein farnesyltransferase; GGTase-I, protein geranylgeranyltransferase type I; FPP, farnesyl diphosphate; E•FPP, enzyme-farnesyl diphosphate complex; HEPES, N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid; HEPPSO, N-2-Hydroxyethylpiperazine-N'-2-hydroxypropanesulfonic acid; TCEP, Tris(2-carboxyethyl)phosphine; HPLC, high pressure liquid chromatography; TFA, trifluoroacetic acid; MDCC, N-[2-(1-maleimidyl)ethyl]-7-(diethylamino)coumarin-3-carboxamide; PP_iase, inorganic pyrophosphatase; STO, single-turnover; MTO, multiple-turnover.

To identify potential novel prenylated proteins, we searched the human genome database for proteins that contain a cysteine four amino acids from the C-terminus as a minimal specificity requirement. From the resulting list of ~600 proteins, we selected approximately half to screen for activity with FTase. Peptides corresponding to the last four amino acids of these candidate substrates along with N-terminal Thr and Lys residues (TKCxxx) were screened for farnesylation by FTase under both $k_{cat}/K_m^{peptide}$ (multiple-turnover, $[E] \ll [S]$) and single-turnover reaction conditions ($[E] > [S]$); short peptides containing the Ca₁a₂X sequence have been shown to serve as competent substrates for FTase^{13; 29}. Out of the >300 peptides screened, FTase catalyzed multiple-turnover farnesylation of 106 peptides, consistent with the parent proteins serving as FTase substrates *in vivo*. Surprisingly, of the remaining peptides, 67% were farnesylated under single-turnover conditions, suggesting that there are two classes of substrates for FTase with distinct reactivity profiles. Analysis of the sequence preferences for these two classes of substrates illustrates a significant difference in FTase selectivity for the multiple- and single-turnover substrates, with the multiple-turnover substrates adhering closely to the current model for FTase selectivity while the single-turnover substrates show distinct sequence preferences. These different classes of FTase substrates may reflect an unanticipated mechanism for regulating farnesylation, with potential impact on the localization, trafficking, and activity of prenylated proteins within the cell.

Results

Determination of initial library members

To identify potential FTase substrates, we searched open reading frames within the human genome using the Swiss-PROT database to identify proteins containing a cysteine residue at the fourth amino acid from the C-termini (–Cxxx)³⁰. Proteins that had been previously confirmed to be prenylated were eliminated^{8; 9; 10}; several of these proteins were reintroduced into the peptide substrate library to serve as positive controls, as described below. The list of potential substrates was also edited to remove protein fragments and viral proteins. This search of the human genome and subsequent refinement yielded ~620 proteins whose prenylation status was unknown at the time of library selection. During the course of this work, the prenylation status of several of these proteins has been determined^{7; 8; 9; 27}.

To both limit the number of peptides in the library and bias the library toward potential FTase substrates, we started by predicting which of the remaining proteins are most likely to be farnesylated using a model based on the sequence of known farnesylated proteins (Table S1) and peptides^{10; 18; 20; 27; 31; 32}. We selected all of the proteins that have amino acids at each of the a₁, a₂ and X positions that were represented in known substrates. We also included the sequences from several proteins with interesting biological functions, resulting in an initial library of 213 peptides to assay for reactivity with FTase.

Identification and characterization of novel FTase substrates in the initial library

A library of peptides of the form dansyl-TKCxxx was purchased and screened for multiple-turnover reactivity with FTase by monitoring the change in the fluorescence of the dansyl group upon farnesylation of the cysteine residue or by a radioactivity-based assay using ³H-FPP and autoradiography. FTase catalyzes multiple-turnover farnesylation of 77 peptides, constituting 36% of the library (Table 1). We determined $k_{cat}/K_m^{peptide}$ values for a subset of these multiple-turnover (MTO) peptides for comparison to known FTase substrates (Table 2). The specificity constant ($k_{cat}/K_m^{peptide}$) is the most important parameter for consideration of reactivity within a biological context, as it most accurately reflects whether a substrate protein is likely to be modified when in competition with other substrates³³. The peptide

substrates characterized from the library exhibit a range of $k_{\text{cat}}/K_{\text{m}}^{\text{peptide}}$ values. The best substrate (TKCAVQ) exhibits a $k_{\text{cat}}/K_{\text{m}}^{\text{peptide}}$ value of $44 \text{ mM}^{-1}\text{s}^{-1}$, comparable to the reactivity of known FTase substrates^{34; 35; 36}. Roughly half of the characterized peptides are modified with $k_{\text{cat}}/K_{\text{m}}^{\text{peptide}}$ values above $1 \text{ mM}^{-1}\text{s}^{-1}$, while the rest of the peptides have $k_{\text{cat}}/K_{\text{m}}^{\text{peptide}}$ values in the range of $0.09\text{--}0.9 \text{ mM}^{-1}\text{s}^{-1}$ (Table 2). The positive control peptides exhibit a comparable range of $k_{\text{cat}}/K_{\text{m}}^{\text{peptide}}$ values, from $14 \text{ mM}^{-1}\text{s}^{-1}$ (TKCSIM, Rho6) to $0.092 \text{ mM}^{-1}\text{s}^{-1}$ (TKCTII, 2',3'-cyclic nucleotide 3-phosphodiesterase) (Table 1), indicating that the new substrates identified in this screen display reactivities consistent with farnesylation *in vivo*.

Proceeding from the proposed catalytic cycle for FTase (Scheme 1,^{35; 37; 38; 39; 40; 41; 42}), we sought to determine which reaction step(s) are involved in substrate specificity under $k_{\text{cat}}/K_{\text{m}}^{\text{peptide}}$ reaction conditions. The kinetic parameter $k_{\text{cat}}/K_{\text{m}}^{\text{peptide}}$ only reflects reaction steps up to the first irreversible step³³, which in the case of FTase is either farnesylated peptide formation or the rapid dissociation of pyrophosphate^{35; 43; 44}. Therefore,

$k_{\text{cat}}/K_{\text{m}}^{\text{peptide}}$ includes the rate constants for peptide binding to E•FPP through the farnesylation step (k_{farn}), which is composed of a conformational change step followed by the chemical step of farnesylation (Scheme 1)^{40; 43; 44; 45}. To assess the contributions of these steps to substrate selectivity, we measured binding affinities and single-turnover rate constants for a subset of the newly identified substrate peptides (Table 2). The affinity of FTase for these peptides varies approximately 9-fold, compared to a range of ~500-fold in $k_{\text{cat}}/K_{\text{m}}^{\text{peptide}}$ values, suggesting that binding affinity is not the primary determinant of FTase substrate specificity.

In contrast, the rate constants for peptide farnesylation exhibit large variation, approximately 700-fold, consistent with the observed range of $k_{\text{cat}}/K_{\text{m}}^{\text{peptide}}$ values. Furthermore, there is a rough correspondence between catalytic efficiency ($k_{\text{cat}}/K_{\text{m}}^{\text{peptide}}$) and the farnesylation rate constant (k_{farn}); in general, peptides with the highest $k_{\text{cat}}/K_{\text{m}}^{\text{peptide}}$ values also have high farnesylation rate constants. This correlation suggests that substrate specificity under $k_{\text{cat}}/K_{\text{m}}^{\text{peptide}}$ conditions predominantly results from changes in the rate constants of the conformational change and chemistry steps monitored by k_{farn} (Scheme 1), a model consistent with findings from targeted studies of substrate specificity at the α_2 and X positions^{19; 20}. However, several peptides do not fit this correlation; for example, TKCIVA has a $k_{\text{cat}}/K_{\text{m}}^{\text{peptide}}$ value of $0.44 \text{ mM}^{-1}\text{s}^{-1}$ but one of the largest farnesylation rate constants ($k_{\text{farn}} = 4.7 \text{ s}^{-1}$). One possible explanation for this result is that the peptide association rate constant for TKCIVA and similar peptides is decreased to a value comparable to $k_{\text{cat}}/K_{\text{m}}^{\text{peptide}}$. Therefore, it is likely that FTase substrate selectivity under $k_{\text{cat}}/K_{\text{m}}^{\text{peptide}}$ conditions arises from modulation of all of the reaction steps up to and including chemistry.

Novel FTase substrates that undergo farnesylation but not turnover

In a previous study, we observed that FTase catalyzes farnesylation of some peptides readily under single-turnover conditions even though multiple-turnover farnesylation is not observed, presumably due to slow dissociation of the prenylated peptide under steady state reaction conditions²⁰. To determine whether any of the peptides in this library exhibit similar behavior, we used an endpoint assay to screen the peptides that are “inactive” in the initial multiple-turnover reactivity screen for single-turnover farnesylation catalyzed by FTase. This secondary screen revealed that 85 of the library peptides are farnesylated under single-turnover conditions without turning over efficiently (Table 3). These 85 peptides

correspond to 40% of the initial library peptides, compared to the 36% of the library peptides capable of multiple-turnover activity. Taken in combination, FTase catalyzes farnesylation of 76% of the library peptides. The remaining non-substrate peptides display no detectable activity with FTase in any assay, and do not inhibit FTase (data not shown).

To further investigate the kinetic behavior of the STO peptides, we measured single-turnover rate constants (k_{farn}) for a subset of these peptides (Table 4). One possible explanation for the STO behavior is that the farnesylation rate constants for these peptides are fast enough to observe farnesylation in the endpoint single-turnover assay but not sufficient to measure multiple-turnover activity. However, with the exception of TKCYSN (0.0025 s^{-1}), the STO peptides display a range of k_{farn} values ($0.019 - 10 \text{ s}^{-1}$) comparable to those measured for the multiple-turnover substrates (Tables 2 and 4). Therefore, the inefficient turnover of the STO peptides likely results from sequence-dependent effects on other reaction steps, such as FPP binding to the E•farnesylated peptide complex and/or product dissociation.

Amino acid distribution in the $\text{Ca}_1\text{a}_2\text{X}$ sequences of novel FTase substrates

The current model for FTase substrate specificity consists primarily of amino acid preferences at the a_2 and X positions within the $\text{Ca}_1\text{a}_2\text{X}$ sequence^{11; 12; 13; 14; 15; 17; 20; 46; 47}. To ascertain whether the substrates identified in this screen conform to the $\text{Ca}_1\text{a}_2\text{X}$ model, we compared the amino acid composition of the two substrate classes, the MTO and STO peptides, and the pool of non-substrate peptides to the composition of the initial library. Analysis of the a_2 amino acid distribution in the MTO peptides indicated that it significantly differed from the initial library ($p < 0.02$). To further examine FTase sequence preferences at the a_2 position, we divided the peptides into two groups, those containing a “canonical” residue (V, I, L, M, T) at the a_2 position and those with “non-canonical” a_2 residues. The initial library is composed of roughly equal numbers of peptides with canonical and non-canonical a_2 residues (Figure 1a). The MTO substrate peptides are significantly enriched in canonical residues ($p < 0.02$), with 84% of these substrates containing V, I, L, M, or T at the a_2 position. This preference for nonpolar amino acids at the a_2 position is consistent with the current model for FTase selectivity based on biochemical and crystallographic data^{9; 10; 17; 19; 38}. Unexpectedly, the amino acid distribution at the a_2 position in the STO peptides shows no statistically significant deviation compared to the initial library. This lack of a_2 selectivity observed in the STO peptides indicates that the steps monitored in the STO assay have a differential dependence on substrate structure, particularly at the a_2 position; this possibility is explored in further detail in the Discussion section. The non-substrate peptides exhibit changes in composition opposite to those seen in the MTO peptides, with significant depletion of canonical residues and enrichment of non-canonical residues (Figure 1a).

We next analyzed amino acid selectivity at the X position, which also had a statistically significant change in amino acid distribution for MTO peptides, by dividing the peptides into three groups based on previous reports of the FTase preferences at the X group^{13; 17; 20; 47}: “canonical” (A, M, S, Q, F); “reactive” (C, N, T); and “non-canonical” (I, L, R, Y, D, V, E, G, H, K, P, W) (Figure 1b). Compared to the initial library, the MTO peptide substrates are significantly enriched in the canonical amino acids at the X position (61% vs. 36% in the initial library) and depleted in the non-canonical amino acids ($p < 0.02$). In contrast, the distribution of the amino acids at the X position in the STO peptide substrates exhibits significant depletion of canonical residues and enrichment of non-canonical amino acids ($p < 0.02$) in a pattern similar to that seen for the non-substrate peptides. These comparisons illustrate that the sequence of most of the MTO peptide substrates correspond to predictions from the current model for FTase selectivity, whereas the STO peptide substrates exhibit a distinctly different sequence composition.

Statistical analysis of individual amino acid distribution in novel substrates

Given the observed enrichments in types of amino acids among the newly discovered FTase substrates, we analyzed MTO substrates, STO substrates, and non-substrate peptides using hypergeometric probabilities to determine if any individual amino acids are enriched (preferred) or depleted (selected against) at the a_1 , a_2 , and X positions in a statistically significant manner (see *Materials and Methods*). In this analysis, we interpret enrichments and depletions occurring with $p < 0.02$ as statistically significant and enrichments / depletions occurring with $0.02 < p < 0.05$ as suggestive. Contrary to current models of FTase substrate selectivity suggesting little discrimination at the a_1 position^{10; 17}, we observe statistically significant enrichments and depletions at the a_1 position (Table 5). The STO substrates are enriched in C and depleted in L ($p < 0.02$) at a_1 ; in contrast, the MTO substrates display suggestive enrichment of L and depletion of C at a_1 . This anticorrelation in amino acid preference at the a_1 position suggests that recognition of this residue may affect step(s) in the FTase reaction cycle impacting product release. Further characterization of the a_1 dependence of peptide substrate reactivity may yield insights into the discrimination between MTO and STO reactivity with FTase.

At the a_2 position, the MTO peptide substrates are enriched in I and L and depleted in C and K ($p < 0.02$), with the suggestion of enrichment of V and depletion of D. These enrichments and depletions are consistent with a preference for moderately sized, nonpolar a_2 residues^{10; 17; 19}. The STO peptides are enriched in S and depleted in I and K ($p < 0.02$) and show a suggested enrichment of A, possibly reflecting a preference within the STO substrates for small amino acids at the a_2 position. Finally, the non-substrate peptides are enriched in charged amino acids (D, K, R) and depleted in moderately sized hydrophobic amino acids (V, I, L, T) in a pattern consistent with the canonical Ca_1a_2X model.

Similar to the a_1 position, comparison of amino acid enrichment and depletions at the X position reveals a possible correlation between peptide sequence and MTO versus STO peptide reactivity (Table 5). At the X position, the MTO peptides are significantly enriched in F, M, and Q, as predicted from previous studies^{10; 20; 47}. In contrast, the STO peptides are significantly depleted in M and exhibit a suggestive depletion of Q. Comparing the MTO and STO substrates, the anti-correlation at the X position of the MTO (enriched in M and Q) and STO (depleted in M and potentially Q) peptides suggests that interactions between FTase and the X position of the peptide may regulate the magnitude of the kinetic barrier, likely product dissociation, leading to either efficient or inefficient turnover. The non-substrate peptides exhibit suggestive depletions of F and Q at the X position, providing further evidence that the presence of these amino acids at the X position enhances peptide reactivity and turnover with FTase.

Design and analysis of a targeted library for identifying FTase substrates

Based on analysis of the initial library, we designed a second library consisting of 88 peptides selected from additional uncharacterized Ca_1a_2X sequences observed in human proteins and mammalian homologues. For this library we constrained the sequence at the a_2 position to V, I, L, or T in a majority of the peptides (76 of 88 total peptides) with the remainder containing mainly nonpolar amino acids (A, G, P, Y or H). We then screened the peptides in the targeted library for activity per the assays used for the initial library.

As shown in Table 6, 29 peptides from the targeted library (33% of library) exhibit significant MTO activity, with $k_{cat}/K_m^{peptide}$ values ranging from 0.09 to 12 $mM^{-1}s^{-1}$. Additionally, a majority of the peptides that lack efficient multiple-turnover activity can be farnesylated by FTase under single-turnover conditions (45 out of 59, or 51% of the total targeted library) (Table 3). While analysis of selectivity at the a_2 position is limited by the

constrained sequence in the targeted library, statistical analysis of the amino acid composition at the X position yields similar results to those from the initial library (Figure 2). The targeted library exhibits significant enrichment in canonical X residues (52% vs. 31% in the initial library) and depletion of “non-canonical” X residues in the MTO peptides; hypergeometric probability analysis indicates that these effects are statistically significant ($p < 0.02$; Table S6). Distribution of the amino acids at the X position in the STO peptide substrates is comparable to that of the initial library. Thus, amino acid composition at the X position in both the MTO and STO peptide substrates from the targeted library mirrors that observed in the initial peptide library.

Discussion

Combined results from both libraries

By analyzing the reactivity of FTase with libraries of peptides, we have identified 106 novel $\text{Ca}_1\text{a}_2\text{X}$ sequences that exhibit multiple-turnover reactivity. We also identified 130 peptides that can be farnesylated under single-turnover conditions, leading to a total of 236 peptide sequences that can serve as substrates for FTase. In the initial library, the total substrates (MTO + STO) composed 76% of the library (162 out of 213). The hit efficiency increased for the targeted library, wherein 83% of the library peptides (74 out of 88) can be farnesylated by FTase. The percentage of MTO substrates is similar in the two libraries, with 36% in the initial library (77 out of 213) and 33% in the targeted library (29 out of 88).

The identification of ~240 potential novel farnesylated proteins would roughly double the number of known FTase substrates^{8; 9; 10}. However, the use of short peptide substrates as proxies for full-length proteins entails a number of caveats. Previous studies have shown that peptides are able to compete with full-length proteins during the farnesylation reaction and have similar binding constants and kinetic parameters^{13; 48}. However, it is possible that structural elements present in full-length proteins might interfere with FTase activity, for example, by making the cysteine inaccessible. Furthermore, as the region upstream of the $\text{Ca}_1\text{a}_2\text{X}$ box can modestly enhance peptide binding to FTase, the lack of native upstream sequences in our library peptides could also result in peptide reactivity distinct from that of the parent protein^{9; 18}. Thus, the peptide reactivity reported herein provides a list of likely FTase substrates that should be further analyzed to determine their farnesylation status *in vivo*.

Insights into the $\text{Ca}_1\text{a}_2\text{X}$ box paradigm

The results from the library screens for FTase substrates provide an opportunity to functionally evaluate the current model for FTase specificity. The $\text{Ca}_1\text{a}_2\text{X}$ box paradigm describing FTase selectivity, first proposed almost 20 years ago^{14; 49; 50; 51; 52}, has been refined using biochemical, structural, and bioinformatics analyses to arrive at a more sophisticated model describing FTase selectivity^{8; 9; 10; 19; 20; 38}. In this refined model, selectivity at the a_1 position is relaxed to accept most amino acids, including positively charged residues. At the a_2 position, FTase more efficiently catalyzes farnesylation of peptides containing moderately sized nonpolar amino acids, with a recent study indicating that a_2 selectivity is affected by the identity of the adjacent X residue¹⁹. Finally, FTase prefers moderately sized, moderately nonpolar amino acids such as A, S, M, F, and Q at the X position although it will accept other amino acids as well²⁰.

Comparison of the $\text{Ca}_1\text{a}_2\text{X}$ model to the two substrate classes identified in this work reveals two distinct substrate specificity patterns: MTO peptides are reasonably described by the $\text{Ca}_1\text{a}_2\text{X}$ model, while STO peptides display a much wider sequence tolerance than predicted by the $\text{Ca}_1\text{a}_2\text{X}$ model. At the a_1 residue, the lack of sequence preference in the MTO

substrates is consistent with the predicted relaxed specificity at this position,^{9; 10} whereas the preference for C and discrimination against L at a₁ in the STO substrates suggests that recognition of the a₁ position may play a role in modulating product release. At the a₂ position, the two substrate classes exhibit significantly different specificities. The MTO peptides demonstrate a strong preference towards aliphatic residues, including V, I, L, M, or T, consistent with preferences predicted from structural analysis of the a₂ binding pocket^{10; 38}. In contrast, the STO peptides appear to favor small amino acids at the a₂ position, although when analyzed in pools of canonical versus non-canonical a₂ residues no significant differences are observed compared to the starting library. These results suggest that recognition of the a₂ residue may be important during a reaction step that is suppressed or not monitored in the single-turnover reaction, such as peptide association or product release.

Finally, at the X position, the MTO and STO peptide substrates again exhibit differing preferences. The MTO peptides correspond well with the Ca₁a₂X model, with a majority of the peptides containing canonical residues at the X position and statistically significant enrichment of F, M, and Q relative to the library; peptides terminating in M and F have shown potential as dual substrates for both FTase and GGTase-I.^{20; 47; 53; 54; 55; 56; 57; 58} The STO peptides do not exhibit preference for any particular amino acid at the X position, and are depleted in peptides terminating in M. This depletion suggests that certain residues at the X position can impact the ability of a peptide to turn over efficiently, leading to a suppression of STO activity in favor of MTO reactivity. Another possible distinction between the STO and MTO peptides could arise at the level of di- or tripeptides, as cross-talk between the a₂ and X positions has recently been shown¹⁹; however, the libraries described here are insufficiently random and have sample sizes that are too small to conclude anything definitive.

Implications of MTO versus STO reactivity

The two substrate classes observed in our peptide libraries indicate that FTase uses a multi-step selectivity mechanism and expands the potential pool of FTase substrates *in vivo*. The observed specificity in FTase arises from sequence-dependent effects on steps up to and including the rate-determining step. For peptides that are unreactive under either STO or MTO conditions, selectivity occurs at either the peptide binding or farnesylation steps. For MTO peptides under $k_{cat}/K_m^{peptide}$ reaction conditions, specificity arises in steps at or before chemistry, the first irreversible step in the FTase reaction cycle^{19; 33}. Thermodynamic and kinetic analyses of the MTO peptides indicate that specificity is mainly determined by effects on the farnesylation rate constant rather than the peptide affinity (Table 2), consistent with previous data^{19; 20}. In contrast, the rate-limiting step for the STO peptides appears to be release of the prenylated peptide, even at low peptide concentrations. For the STO peptides, the variability and value of the farnesylation rate constant is similar to that observed for the MTO peptides (Tables 2 and 4), indicating that some selectivity is retained in this step. However, the product dissociation step has an even larger dependence on peptide sequence, leading to a change in the rate-limiting step from farnesylation to product dissociation. This change in rate-determining step results in a more restrictive sequence dependence for peptides undergoing multiple turnovers compared to single turnovers. The substrate selectivity of the farnesylation step is indicated by the sequences of both the STO and MTO peptides, while the sequence dependence of the product dissociation step is demonstrated by the MTO peptides.

The existence of the STO peptide substrate class suggests that FTase may be able to prenylate many more peptides than it turns over efficiently during *in vitro* assays. If peptide reactivity were unaltered in the cell, the presence of proteins bearing slow-releasing STO C-

terminal sequences could result in severe inhibition of farnesylation through formation of long-lived FTase-STO protein complexes. The simplest model to address the *in vivo* impact of STO peptide reactivity would be that these substrates are not biologically relevant, with proteins bearing STO sequences not appreciably farnesylated *in vivo*. However, the Ca₁a₂X sequence of one peptide in our library that displays single-turnover only activity, CAVL, corresponds to a protein that has been shown to be farnesylated *in vivo*⁷, indicating that this substrate exhibits multiple-turnover activity *in vivo*. This result suggests that dissociation of these farnesylated proteins (and perhaps others) from FTase *in vivo* is likely accelerated, switching the reactivity from single- to multiple-turnover.

One possible explanation for the difference in the mode of reactivity between peptides and full-length proteins is that the MTO activity is enhanced with proteins as compared to the short peptide substrates used herein. Studies of 15-mer peptides bearing STO Ca₁a₂X sequences indicate that the STO reactivity remains in these longer substrates (data not shown); however, these peptides may not incorporate the required portions of the parent proteins needed to enhance MTO behavior. Alternatively, dissociation of farnesylated substrates bound to FTase could be accelerated by the presence of a release factor within the cell, such as a small molecule or auxiliary protein. There is evidence that peptide substrates can catalyze product release from FTase via an alternative pathway to FPP-catalyzed product release^{20; 42; 59}, providing support for this model. The involvement of a release factor could provide the basis for targeting prenylated STO proteins to specific cellular locations. Regardless of the specific mechanism, conversion of STO reactivity to biologically relevant MTO reactivity provides a potential regulation point for a subset of putative FTase substrates within the cell. Furthermore, the biological relevance of at least a subset of the STO peptides indicates that FTase has wider selectivity than implied by the canonical Ca₁a₂X model.

Comparison of results to predictions from the PrePS algorithm

A number of algorithms have been developed to predict protein farnesylation based on the sequence of putative substrate proteins^{8; 9; 10}. The most recently developed of these algorithms, PrePS, analyzes the last 15 amino acids of a protein, including the Ca₁a₂X sequence, to calculate a predicted likelihood for prenylation of the submitted protein⁹. We submitted the 213 proteins from the initial library to the PrePS web interface to compare our peptide reactivity measurements to the predictions from analysis of the full-length parent protein (Figure 3); a table of protein sequence and corresponding PrePS scores is included in the supplemental data (Table S10). The PrePS algorithm predicted farnesylation (ranked ++ or +) for 57 of the 213 initial library peptides (27%). Of the 77 peptides for which we observed multiple-turnover farnesylation, 44 (57%) were predicted to be farnesylated by PrePS. In contrast, only 12 of the 85 (14%) peptides that exhibit single-turnover reactivity were predicted as FTase substrates. Additionally, only one peptide corresponding to a protein scored as likely to be farnesylated was not farnesylated under either single- or multiple-turnover conditions (2% of non-substrates). In summary, the PrePS algorithm yields a very low number of false positive results while predicting a majority of the proteins that exhibit multiple-turnover reactivity with FTase. This is consistent with the observed adherence of the multiple-turnover peptides to the canonical Ca₁a₂X sequence model. However, the large number of false negative predictions (31 of 77 MTO peptides, 40%) suggests that the PrePS algorithm may not identify a significant number of proteins farnesylated by FTase. The lack of accuracy in predicting farnesylation of peptides that exhibit single-turnover reactivity presumably reflects the altered sequence preferences observed in these peptides, as described above. Disagreements between our reactivity data and the algorithm predictions may arise from the lack of upstream sequence in our peptides, a region that PrePS uses in calculating the likelihood of protein prenylation⁹, or from cross-

talk between amino acids at different positions in the Ca₁a₂X sequence that is currently difficult to predict¹⁹. The results from this study should be useful in refining prediction algorithms to increase accuracy in evaluating protein farnesylation.

Biological relevance of newly identified farnesylated substrates

Many previously identified farnesylated proteins are associated with a range of biological functions; for example, some farnesylated proteins are classified as tyrosine phosphatases, and many GTP binding proteins are prenylated, including members of the Rho and Ras families^{1; 2; 8; 10}. These proteins are involved in cellular growth and differentiation, protein transport, vision, and glycogen metabolism²⁷. The potentially farnesylated proteins identified from data in this study also have diverse biological functions, with more than 60% of the newly identified farnesylated proteins annotated with a proposed function in the Swiss-Prot database. Some of the novel FTase substrate sequences identified herein may correspond to proteins that serve as downstream FTI targets, as blocking prenylation of these proteins could interfere with their cellular function. For example, Ras-related C3 botulinum substrate 3 (Rac3) (peptide sequence: CTVF) is thought to inhibit neurotransmitter release at presynaptic nerve terminals and stimulate the c-Jun amino-terminal kinase signaling pathway⁶⁰. Overexpression of another novel substrate, RhoD (peptide sequence: CVVT), inhibits cytokinesis, antagonizes RhoA function, and is proposed to cause the formation of multinucleated cells⁶¹. While far from an exhaustive list of potential downstream targets for FTIs, these examples highlight the range of new FTase substrates identified in this study for further investigations into FTI efficacy.

Implications for in vivo prenylation

This study, when combined with previous reports of substrates for FTase (summarized in^{8; 9; 10}), sheds light on the potential farnesylation status of approximately half of the possible FTase substrates within the human genome. The resulting set of substrates provides important insights into substrate recognition by FTase and suggests the existence of multiple FTase substrate classes, with the potential for regulation and targeting of the prenylation of specific proteins within the cell based on modulation of FTase reactivity and product release. While the results presented here support previous observations in the literature that FTase substrates often function in G-protein-mediated signal transduction, the identification of substrate sequences involved in diverse cellular processes unrelated to G-proteins adds to the functional breadth observed for farnesylated proteins. Through characterization of FTase substrates within the human proteome, we can better understand the importance of farnesylation in cellular function as well as advance development of inhibitors and therapies targeting farnesylation-dependent pathways for disease treatment and prevention.

Materials and Methods

Miscellaneous methods

All assays were performed at 25 °C. All curve fitting was performed with either Kaleidagraph (Synergy Software, Reading, PA) or Graphpad Prism (Graphpad Software, San Diego, CA). Farnesyl protein transferase inhibitor II (I2) was purchased from Calbiochem-Novabiochem Corporation (San Diego, CA). The custom peptide library was ordered from Sigma Genosys (The Woodland, TX) in PEPscreen™ 96 well plate format. Purity of the peptides was >70%, with the majority of the peptides exhibiting >90% purity. Purity was determined by HPLC (Alltech Nucleosil C-18 column) with a gradient from water containing 0.1% trifluoroacetic acid (TFA) to 45% acetonitrile in water containing 0.1% TFA. HPLC runs were performed at 1 mL per minute over 25 minutes; peptides were detected by UV absorption at 220 nm. Major contaminants were mainly smaller peptide fragments, as shown by mass spectrometry, which are not efficient FTase substrates^{19; 62}.

⁶³. Peptides were dissolved in ethanol and the concentration of peptide was determined spectroscopically at 412 nm by reaction of the cysteine thiol with 5,5'-dithio-bis(2-nitrobenzoic acid), using an extinction coefficient of $14150 \text{ M}^{-1}\text{cm}^{-1}$ ⁶⁴. Inorganic pyrophosphatase (PPiase) from Bakers' Yeast, 7-methylguanosine (MEG), and purine nucleoside phosphorylase (PNPase) were all purchased from Sigma (St. Louis, MO). All other chemicals were reagent grade.

Determination of potential library members within the human genome

Peptide library members were chosen based on scans of the human genome using the Swiss-Prot/TrEMBL Scan ProSite server to identify all human proteins containing a cysteine four amino acids from the C-termini (Cxxx> in Swiss-Prot syntax)³⁰. All viral proteins, known prenyltransferase substrates, and protein fragments were removed from the list.

Peptide design

All library peptides were of the form dansyl-TKCxxx, where x indicates any amino acid. The dansyl fluorophore appended to the N-terminus of the peptide allows use of a previously developed fluorescence-based assay of prenylation activity^{29; 65}. The lysine residue upstream of the Cxxx sequence increases the solubility of the peptides and prevents deleterious interactions between the N-terminus and FPP³⁸; the presence of an upstream polybasic sequence has limited effect on the prenylation reaction¹⁸.

Preparation of FTase

Wild type FTase was expressed in BL21(DE3) pET23aFPT *Escherichia coli* and purified as described previously^{37; 66}. FTase concentration was determined by active site titration as described previously⁶⁶. The purified FTase was determined by SDS-PAGE to be >90% pure. The protein was dialyzed against HT buffer [50 mM HEPES, pH 7.8, 1 mM TCEP], concentrated to 225 μM , and stored at -80°C .

Steady state assays

The steady state kinetic constant $k_{\text{cat}}/K_{\text{m}}^{\text{peptide}}$ was measured for the dansylated peptides using a continuous spectrofluorometric assay^{29; 65}. Activity was measured by an increase in fluorescence intensity of the dansyl group ($\lambda_{\text{ex}} = 340 \text{ nm}$, $\lambda_{\text{em}} = 520 \text{ nm}$) upon prenylation of the dansylated peptides in a POLARstar Galaxy plate reader (BMG Labtechnologies, Durham, NC).

To determine if peptides displayed multiple-turnover activity with FTase greater than the detection limit of the assay ($>100 \text{ M}^{-1}\text{s}^{-1}$), an initial scan was done with 3 μM dansylated peptide and 10 μM FPP in 50 mM HEPPSO buffer, pH 7.8, 5 mM TCEP, 5 mM MgCl_2 , and 10 μM ZnCl_2 . Reactions were initiated by the addition of FTase (200 nM). The fluorescence intensity was measured discontinuously for one hour, with an increase in fluorescence of $\geq 10\%$ suggesting that this peptide was prenylated. For these peptides, the steady state activity was measured at varying dansylated peptide concentrations (0.4–10 μM) and constant, saturating FPP concentration (10 μM). Reactions were initiated by adding FTase (25–300 nM) and fluorescence was monitored until the endpoint was reached (~2–6 hours).

In order to calculate multiple-turnover rate constants, the rate of increase in fluorescence intensity per second was converted to the rate of increase in the product concentration per second using equation 1, where V refers to the velocity of the reaction in $\mu\text{M s}^{-1}$, R refers to the velocity of the reaction in fluorescence units, P refers to the concentration of the limiting substrate, and F_{max} refers to the maximal fluorescence intensity at the endpoint⁶⁷.

$$V = \frac{R * P}{F_{max}} \quad (1)$$

The value of $k_{cat}/K_m^{peptide}$ was determined either by fitting a line or the Michaelis-Menten equation to the initial rate of product formation per second divided by the enzyme concentration ($V / [E]$) as a function of peptide concentration. For peptides that exhibit substrate inhibition, $k_{cat}/K_m^{peptide}$ was determined by a linear fit to the initial rate of product formation per second divided by the enzyme concentration ($V_{init}/[E]$) at peptide concentrations below the onset of inhibition. Lower limits for k_{cat} were estimated based on the Michaelis-Menten fit or the velocity values at high peptide concentration (10 μ M).

Endpoint assays to detect farnesylation under multiple-turnover conditions

Peptides that did not exhibit multiple-turnover activity catalyzed by FTase using the fluorescence-based assays were re-screened for their ability to be farnesylated by FTase under multiple-turnover conditions with a radioactive endpoint assay using 3H -FPP under the following conditions: 50 mM HEPPSO buffer pH 7.8, 1 mM TCEP, 5 mM $MgCl_2$, 0.8 μ M 3H -FPP, 3.2 μ M dansylated peptide, and 0.05 μ M FTase. FTase and 3H -FPP were incubated at 25 $^{\circ}C$ for 15 minutes to form the $E \cdot ^3H$ -FPP complex. The peptide was then added to the $E \cdot ^3H$ -FPP complex and incubated for 2 hours followed by quenching with 80:20 isopropanol:acetic acid. Prenylated peptide was separated from FPP by TLC (8:1:1 isopropanol: NH_4OH : H_2O on silica plates) and detected by autoradiography, excision of substrate and product bands, and scintillation counting. Peptides that were prenylated under these conditions were defined as MTO peptides.

Endpoint assays to detect farnesylation under single-turnover conditions

Peptides that were not farnesylated by FTase under multiple-turnover conditions were screened for farnesylation catalyzed by FTase under single-turnover conditions with a modification of the radioactive endpoint assay described above. In the STO endpoint assay, the FTase concentration was increased to equal the FPP concentration (0.8 μ M) and the peptide was reacted with the $E \cdot ^3H$ -FPP complex for 1 hour. Peptides that were prenylated under these conditions were defined as STO peptides.

Transient kinetics

Single-turnover rate constants for farnesylation of peptides catalyzed by FTase were determined using a stopped-flow fluorescent assay as described⁴⁰. "Phosphate mop", composed of 0.5 units mL^{-1} purine nucleoside phosphorylase (PNPase) and 15 mM 7-methylguanosine (MEG), was added to remove any contaminating phosphate present in the reaction mixture. The assays were done in a total reaction volume of 120 μ L containing 0.8–1.6 μ M enzyme, 0.2–0.4 μ M FPP, 50 mM HEPPSO, pH 7.8, 5 mM $MgCl_2$, 2 mM TCEP, and the "phosphate mop". These reagents were mixed in a stopped flow apparatus (KinTek Corporation, Austin, TX) with an equal volume of dansylated peptide (100 or 200 μ M) in 5–10 μ M MDCC-labeled A197C PBP, 33 units mL^{-1} inorganic pyrophosphatase, 50 mM HEPPSO, 1–2 mM TCEP, and the "phosphate mop". Equation 2 was fit to the resulting data to calculate k_{farn} values, where FI refers to the fluorescence emission at 450 nm, Amp represents the amplitude of the fluorescence change at 450 nm, k_{farn} represents the rate constant for formation of monophosphate (which equals the prenylation rate constant) and IF is the initial fluorescence.

$$F1 = \text{Amp} \left(1 - e^{-k_{\text{fluor}} * t} \right) + IF \quad (2)$$

Peptide binding affinity

The binding of dansylated peptides to FTase was observed by fluorescence anisotropy, as previously described^{68; 69}. In this method, the dansyl group of the peptide is excited at 340 nm and fluorescence anisotropy is monitored at 496 nm using an Aminco-Bowman series 2 (AB2) spectrofluorometer. Dansylated peptide (1–2 nM) in 50 mM HEPPSO, pH 7.8, 2 mM TCEP, 1 mM MgCl₂, 10 nM EDTA was titrated with a stoichiometric FTase-I2 complex (0–500 nM) in the same buffer. Following a 15 minute incubation at 25 °C, the fluorescence anisotropy was measured after each addition. A weighted fit of equation 3 to the data yields the apparent dissociation constants, where ΔA corresponds to the observed fluorescence anisotropy at 496 nm, EP is the fluorescence anisotropy endpoint, IF is the initial fluorescence anisotropy, [enzyme] is the concentration of FTase, and K_D^{peptide} is the dissociation constant for the dansylated peptide.

$$\Delta A = \frac{EP}{1 + K_D^{\text{peptide}} / [\text{enzyme}]} + IF \quad (3)$$

Statistical analysis of FTase Ca₁a₂X sequence preferences

Chi-square tests were used to determine whether MTO, STO, and non-substrate pools had different distributions of amino acids at each position (a₁, a₂, and X). To determine the nature of the differences suggested by chi-square analysis, a hypergeometric model was used. The statistical significance of enrichment or depletion of a given amino acid with the pool of identified FTase substrates, relative to the starting library of potential substrates, was calculated using a hypergeometric distribution model with a null hypothesis that the observed pool arose randomly. The derivation to justify selection of the hypergeometric distribution is shown in the supplemental data; other frequency-based probability models, such as the binomial distribution, yield similar overall outcomes. For a particular position in the peptide sequence (a₁, a₂, and X), the hypergeometric probability is the probability that the distribution of amino acids seen in the substrates at that position would arise by chance, given the distribution of amino acids at that position in the starting library (i.e., what is probability that the substrate pool sequences could arise randomly). Low probabilities suggest that there is a non-random factor causing enrichment or depletion in the substrates (i.e., selective pressure to favor or disfavor a particular amino acid at a particular position). At each position (a₁, a₂, and X), the probability (*p*) of a given amino acid occurring with the observed frequency at the position of interest in the substrate peptide pool by random chance was calculated using equation 4, where *N* is the total library size, *P* is the number of peptides in the total library with a given amino acid at the position (a₁, a₂, or X), *S* is the total number of peptide substrates, and *R* is the number of peptides in the substrate pool with a given amino acid at the position of interest:

$$p = \frac{\frac{P!(N-P)!}{(R!(P-R)!(S-R)!(N-P-S+R)!)}}{\frac{N!}{(S!(N-S)!)}} \quad (4)$$

The reported probabilities for enrichment at a particular position in the substrate pool are the probability of a given amino acid occurring by chance at that position *at least* the observed

number of times (i.e., the actual frequency or more often). Similarly, the reported probabilities for depletion at a particular position are the probability of a given amino acid occurring by chance at that position *at most* the observed number of times (i.e., the actual frequency or less often). Enrichments and depletions with $p < 0.02$ are reported in bold in Table 5; this stringent threshold was chosen to allow for possible artifacts from the small sample size and the discrete sampling method. Additional enrichments and depletions observed with the more relaxed threshold of $p < 0.05$ are noted in italics. The statistical analysis of the data presented in Figures 1 and 2 were calculated in the same manner, but using the groups of amino acids rather than single amino acids at each position.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Amino acid compositions at the a₂ and X positions of the Ca₁a₂X sequence in the initial library. (a) Amino acid compositions at the a₂ position of the Ca₁a₂X sequence. The percentages of amino acids at the a₂ position, grouped into either canonical (V, I, L, M, T) or non-canonical residues, are plotted for the initial library, the multiple-turnover substrates, the single-turnover substrates, and the non-substrate peptides. Wedges are labeled with the percentage representation of the classes of amino acids within each pool of peptides. An asterisk (*) denotes those percentages that are significantly different in the substrate pools as compared to the library ($p < 0.02$). (b) Amino acid compositions at the X position of the Ca₁a₂X sequence. The percentages of amino acids at the X position, grouped into canonical (A, S, M, Q, F), reactive (C, N, T), and non-canonical residues, are plotted for the initial library, the multiple-turnover substrates, the single-turnover substrates, and the non-substrate peptides. Wedges are labeled with the percentage representation of the classes of amino acids within each pool of peptides; percentages may not add to 100% due to rounding. An asterisk (*) denotes those percentages that are significantly different in the substrate pools as compared to the library ($p < 0.02$).

Figure 2.

Amino acid compositions at the X position of the Ca₁a₂X sequence in the targeted library. The percentages of amino acids at the X position, grouped into canonical (A, S, M, Q, F), reactive (C, N, T), and non-canonical residues, are plotted for the targeted library, the multiple-turnover substrates, and the single-turnover substrates. Wedges are labeled with the percentage representation of the classes of amino acids within each pool of peptides; percentages may not add to 100% due to rounding. An asterisk (*) denotes those percentages that are significantly different in the substrate pools as compared to the library ($p < 0.02$).

Figure 3. Predicted farnesylation of initial library peptides. Sequences for proteins terminating in $\text{Ca}_1\alpha_2\text{X}$ sequences in the initial library were analyzed for likelihood of farnesylation using the PrePS prediction algorithm⁹. The resulting predictions are plotted as histograms, with peptides binned according to the nomenclature output by the PrePS algorithm from --- (low) to ++ (high) likelihood of farnesylation. The vertical dotted line denotes the border between peptides predicted to be farnesylated and those predicted not to be FTase substrates. The plots show an analysis of the initial library including: (a) All of the peptides; (b) Multiple-turnover substrate peptides; (c) Single-turnover substrate peptides; and (d) Non-substrate peptides.

Scheme 1.

Table 1

Peptides from the initial library that exhibit multiple-turnover activity with FTase.^{a,b}

CGIA	CSLG	CGIM	CKQQ	CNTS
CTLA	CFMG	CNIM	CITQ	CSVS
CVLA	CVLH	CSIM	CQTQ	CLIT
CQTA	CIII	CLLM	CVTQ	CLLT
CIVA	CSII	CNLM	CAVQ	CVTT
CSLC	CTII	CSLM	CSVQ	CNVT
CYPD	CAIL	CTLM	CVHS	CSVT
CSHF	CCIL	CVQM	CKIS	CVVT
CCIF	CIIL	CLVM	CRIS	CIGW
CKIF	CLIL	CYVM	CSIS	CRVW
CTKF	CNIL	CLLN	CFLS	CSVW
CGLF	CSIL	CVVN	CQLS	CGLY
CLLF	CILL	CSGQ	CTMS	CTVY
CNLF	CLML	CNLQ	CFPS	
CTVF	CSFM	CTLQ	CSSS	
CFLG	CLHM	CVLQ	CLTS	

^aPeptides are of the form dansyl-TKCxxx, where x represents any amino acid

^bPeptides exhibited multiple-turnover reactivity in initial screens using either fluorescence or radioactive detection, as described in *Materials and Methods*.

Kinetic and binding constants of selected novel farnesylated substrates from the initial peptide library that exhibit multiple-turnover reactivity with FTase.^a

Table 2

Peptide ^b	Protein Name(s)	k_{cat}/K_m (mM ⁻¹ s ⁻¹)	k_{farn} (s ⁻¹)	K_d (nM)	Pred ^c
CAVQ	Rab28	44 ± 5	11 ± 1	13 ± 3	Yes
CSIM ^d	Rho6 and Lamin A/C	14 ± 1	9.7 ± 0.9	6 ± 2	Yes
CLVM	Rho-related BTB domain containing protein	6 ± 1	8.2 ± 0.8	6 ± 2	Yes
CTKF	Neuronal membrane glycoprotein M6-b	4.4 ± 0.4	0.24 ± 0.02	6 ± 1	No
CRIS	Phosphatase 1 regulatory subunit	3.8 ± 0.4	2.5 ± 0.5	12 ± 2	Yes
CCIF	Cell Division Cycle 42 (GTP Binding)	3.0 ± 0.3	0.59 ± 0.06 ^f	270 ± 70	Yes
CKIS	Rab-40(A/B/C)	2.8 ± 0.6	0.64 ± 0.06	4.0 ± 0.8	No
CSIS	Hypothetical protein	2.7 ± 0.3	3.8 ± 0.3	3.4 ± 0.8	Yes
CYVM	Lamin B2	2.5 ± 0.6	1.1 ± 0.1	12 ± 4	Yes
CLIT ^c	RhoQ	2.5 ± 0.3	0.74 ± 0.07	4.1 ± 0.8	Yes
CNIM	Hypothetical protein	2.1 ± 0.2	3.5 ± 0.3	5 ± 1	No
CTLQ	Hypothetical protein	1.9 ± 0.1	1.2 ± 0.1	7 ± 1	Yes
CSVS	Phosphatidylinositol polyphosphate 5-phosphatase	1.8 ± 0.2	0.97 ± 0.09	2.1 ± 0.9	Yes
CSLM	Ras-like protein RRP22	1.8 ± 0.4	7.0 ± 0.7	8 ± 2	Yes
CLLM ^c	Phosphatase 1 regulatory subunit	1.7 ± 0.3	4.9 ± 0.5	4.7 ± 0.9	Yes
CTVF	Ras-related C3 botulinum toxin substrate 3	1.3 ± 0.1	0.40 ± 0.04	ND ^e	Yes
CSVQ	GTP binding protein Rab28 (splice variant)	1.2 ± 0.4	4.4 ± 0.5	ND ^e	Yes
CGIM	Glutathione transferase	1.1 ± 0.3	4.0 ± 0.4	3.6 ± 0.7	Yes
CTLM ^c	GTP-binding tumor suppressor 1	1.1 ± 0.2	ND ^e	5.2 ± 0.8	Yes
CGLY	Guanine nucleotide binding protein G(O) alpha subunits 1&2	1.0 ± 0.1	0.13 ± 0.01	ND ^e	No
CNLM	Rho-related GTP binding protein RhoN	1.0 ± 0.3	3.1 ± 0.3	ND ^e	Yes
CYPD	Immunoglobulin J chain	0.9 ± 0.1	0.56 ± 0.05	3.3 ± 0.9	No
CVLQ ^c	Hypothetical protein/Ubiquitin-specific processing protease 32	0.78 ± 0.03	ND ^e	ND ^e	Yes
CITQ	Stoned B-like factor SBLF	0.71 ± 0.05	1.1 ± 0.1	3.6 ± 0.8	Yes
CFLS ^c	SCAMPER	0.6 ± 0.1	0.46 ± 0.05	19 ± 6	Yes
CNLQ	Tetraspanin 1/lens derived growth factor	0.67 ± 0.04	1.6 ± 0.2	8 ± 1	No

Peptide ^b	Protein Name(s)	k_{cat}/K_m (mM ⁻¹ s ⁻¹)	k_{farn} (s ⁻¹)	K_d (nM)	Pred ^c
CTMS	TRIP protein	0.53 ± 0.03	0.91 ± 0.09	6 ± 2	Yes
CQTA	DNA J homolog subfamily A member 4	0.53 ± 0.02	0.23 ± 0.02	ND ^e	Yes
CVLA ^c	CAAX box protein 1 (Cerebral protein-5)	0.5 ± 0.1	0.51 ± 0.05	4.6 ± 0.9	Yes
CGLF	Guanine nucleotide binding protein alpha-1 subunit	0.45 ± 0.06	0.051 ± 0.005	ND ^e	No
CIVA	LIM-only protein 6 AND LIM domain only 6	0.44 ± 0.07	4.7 ± 0.5	5 ± 2	Yes
CSFM	Ras-related protein Rab-37	0.39 ± 0.04	0.44 ± 0.04	10 ± 3	No
CTLA	Hypothetical protein	0.36 ± 0.04	0.015 ± 0.002	4.4 ± 0.7	No
CCIL ^c	Rod cGMP specific 3',5' cyclic phosphodiesterase beta subunit	0.32 ± 0.07	0.13 ± 0.02 ^f	300 ± 40	Yes
CVQM	Cystatin M precursor	0.27 ± 0.03	1.7 ± 0.2	ND ^e	No
CVVT	RhoD	0.27 ± 0.02	0.33 ± 0.03	7 ± 3	No
CSLG	Ras-related protein Rab-13	0.24 ± 0.03	0.059 ± 0.006	3.4 ± 0.7	No
CSLC ^c	LIM-only protein 6	0.24 ± 0.05	4.7 ± 0.5	5 ± 2	Yes
CLTS	Hypothetical protein	0.22 ± 0.03	0.35 ± 0.03	5 ± 1	No
CKIF	Rho-related GTP binding protein RhoH	0.17 ± 0.04	0.74 ± 0.07	2.5 ± 0.5	Yes
CNLF	Hypothetical protein	0.17 ± 0.03	0.18 ± 0.02	3.7 ± 0.7	No
CGIA	Hypothetical protein	0.16 ± 0.05	0.75 ± 0.07	ND ^e	Yes
CVLH	Hypothetical protein	0.15 ± 0.03	0.32 ± 0.03	14 ± 3	No
CLML	Cone cGMP specific 3',5' cyclic phosphodiesterase alpha subunit	0.15 ± 0.02	0.018 ± 0.002	ND ^e	Yes
CTH ^c	2',3'-cyclic nucleotide 3'-phosphodiesterase	0.092 ± 0.002	0.25 ± 0.03	4.2 ± 0.9	Yes

^a Assays include: 10 μM FPP, 0.4–10 μM dansylated peptide, 25–300 nM FTase, 50 mM HEPPO buffer, pH 7.8, 5 mM TCEP, 5 mM MgCl₂, 10 μM ZnCl₂, 25 °C

^b Peptides are of the form dansyl-TKCxxx, where x represents any amino acid.

^c Predicted to be farnesylated based on^{9,10}.

^d Sequences identified as farnesylated before or during this study.

^e ND = not determined

^f The peptide concentration in this assay was increased to 50 μM.

Table 3

Peptides from the initial and targeted peptide libraries that only exhibit single-turnover activity with FTase.^{a,b}

Initial Library					Targeted Library				
CKAA	CPLG	CHVI	CISL	CGHS	CRLW	CPLA	CFVI	CTLR	
CGCA	CSSG	CSVI	CVSL	CAKS	CGTW	CVPA	CDTK	CFTR	
CQDA	CATG	CWHK	CTTL	CFSS	CEVW	CAAF	CKGL	CGVR	
CSEA	CCTG	CIVK	CAVL	CVSS	CQVW	CFAF	CMIL	CTFS	
CTKA	CCAH	CSFL	CCVL	CEYS	CWDY	CHIF	CPLL	CRPS	
CCLA	CVAH	CIHL	CNVL	CALT	CDIY	CDLF	CPVL	CEVS	
CCNA	CGCH	CLHL	CSVL	CHLT	CVLY	CFTF	CCLM	CVLT	
CCTA	CCHH	CGIL	CNFN	CSPT	CNSY	CFVF	CYPN	CPTT	
CNWA	CCLH	CPIL	CCSN	CKTT	CSVY	CLAG	CRVN	CGLV	
CSAC	CACI	CALL	CYSN	CACV	CIIY	CNFG	CLIP	CKLW	
CTGC	CHH	CCLL	CVKQ	CCFV		CHLG	CHLP	CAAY	
CSVD	CGII	CSLL	CACR	CTHV		CPVG	CFLR	CRLY	
CIHF	CQNI	CTLL	CPAS	CWQV		CHTH	CGLR	CYTY	
CSKF	CSPI	CVLL	CGCS	CPFW		CHII	CPLR	CAVY	
CGQF	CNTI	CSQL	CAES	CQLW		CCLI	CSLR	CYVY	

^aPeptides are of the form dansyl-TKCxxx, where x represents any amino acid

^bPeptides did not exhibit detectable multiple-turnover reactivity in initial screens, but were prenylated in a single-turnover endpoint assay, as described in *Materials and Methods*.

Table 4

Reactivity of selected peptides from the initial library that exhibit single-turnover activity with FTase^a.

Peptide ^b	Protein Name	k_{farn}^c (s ⁻¹)
CVLL	Related RAS viral (R-Ras) oncogene homolog	0.83 ± 0.08
CAVL	Tectorin beta	0.91 ± 0.09
CAKS	Rab 38	2.3 ± 0.2
CYSN	Ubiquitin-conjugating enzyme E2 variant 1	0.0025 ± 0.0002
CPLG	Ras association domain family 1	0.019 ± 0.001
CSEA	Cystatin S Precursor	0.020 ± 0.002
CVSS	Cervical cancer suppressor gene 5	0.097 ± 0.009

^a All peptides classified as single-turnover only (STO) exhibit $k_{\text{cat}}/K_{\text{M}}^{\text{peptide}}$ values $<10^2 \text{ M}^{-1} \text{ s}^{-1}$.

^b Peptides are of the form dansyl-TKCxxx, where x represents any amino acid.

^c Assays contain 0.4–0.8 μM FTase, 50–100 μM dansylated peptide, 0.1–0.2 μM FPP, 50 mM HEPPSO, pH 7.8, 5 mM MgCl_2 , 2 mM TCEP, 2.5–5 μM MDCC-labeled A197C PBP, 16.5 units mL^{-1} inorganic pyrophosphatase, 0.25 units mL^{-1} PNPase and 7.5 mM CEG, 25 °C.

FTase substrate preferences within the Ca₁a₂X sequence as reflected by enrichment or depletions within MTO, STO, and non-substrate pools relative to the initial library.^a

Table 5

Position	a ₁	a ₂	X			
	Enriched ^b (overrepresented)	Depleted ^c (underrepresented)	Enriched ^b (overrepresented)	Depleted ^c (underrepresented)	Enriched ^b (overrepresented)	Depleted ^c (underrepresented)
MTO	L	C	I, L, V	C, D, K	F, M, Q	none
STO	A, C	L	A, S	I, K	none	M, P, Q
Non-substrate	K	I	D, E, K, R	V, I, L, T	P, R	F, Q

^a *p*-values were calculated for enrichment or depletion within a given pool of substrates using a hypergeometric distribution model as described in *Materials and Methods*. All enrichments or depletions noted in bold occur with $p \leq 0.02$; enrichments or depletions occurring with $0.02 \leq p \leq 0.05$ are noted in italics.

^b Enrichment of a specified amino acid at a given position is defined as the probability (*p*) of an amino acid occurring as often as or more frequently than observed in the substrate pool based on random selection from the composition of the initial peptide library.

^c Depletion of a given amino acid at a given position is defined as the probability (*p*) of an amino acid occurring as often as or less frequently than observed in the substrate pool based on random selection from the composition of the initial peptide library.

Table 6Kinetic constants of novel farnesylated substrates from the targeted library^a

Peptide ^b	Protein Name	$k_{\text{cat}}/K_{\text{M}}^{\text{peptide}}$ (mM ⁻¹ s ⁻¹)	Pred ^c
CALQ	MKIAA0903 protein fragment (<i>Mus musculus</i>)	12 ± 2	Yes
CYIS	Hypothetical protein FLJ32421	12 ± 5	Yes
CHVM	Hypothetical protein FLJ13993	10 ± 8	Yes
CTIV	Novel protein	7.2 ± 0.8	Yes
CNLK	Tetraspanin 1 (<i>Rattus norvegicus</i>)	6.3 ± 1.6	No
CTVS	72 kDa inositol polyphosphate 5-phosphatase (<i>Mus musculus</i>)	5.8 ± 1.2	Yes
CFPS	Cyclin G2	4.7 ± 0.5	No
CYLA	Hypothetical protein PP1057	4.7 ± 0.3	No
CHVQ	N/A ^d	4.5 ± 0.8	N/A ^d
CDVK	Ciliary dynein heavy chain 5	3.6 ± 0.8	No
CHIM	Complexin III (<i>Mus musculus</i>)	3.3 ± 0.4	Yes
CEIQ	PRO3077	3.1 ± 0.6	No
CSLA	RAB13, member RAS oncogene family (<i>Bos taurus</i>)	2.5 ± 0.3	Yes
CTV	CUB and sushi multiple domains protein 1	2.3 ± 0.1	No
CHIQ	Chromosome 9 open reading frame 25	2.2 ± 0.6	No
CFVN	Splice isoform 2	2.1 ± 0.7	No
CHLM	Ras homolog enriched in brain like 1	2.0 ± 0.3	No
CFTN	Seven transmembrane helix receptor	0.97 ± 0.07	No
CKIV	Hypothetical protein FLJ30834	0.87 ± 0.07	No
CHLS	CLN3 protein (<i>Canis familiaris</i>)	0.46 ± 0.02	No
CVHF	Lipophilin CS (<i>Oryctolagus cuniculus</i>)	0.46 ± 0.04	No
CSLH	Hypothetical protein DKFZp469H1712 (<i>Pongo pygmaeus</i>)	0.21 ± 0.01	Yes
CLLV	Hypothetical protein FLJ41429	0.10 ± 0.06	No
CALV	Hypothetical protein DKFZp761K0511	0.10 ± 0.02	Yes
CGVV	Integral membrane protein 2C	0.09 ± 0.03	No

^aFour other Ca_v2X sequences (CHLH, CKTV, CSLK, and CYLM) exhibited multiple-turnover reactivity in initial screens, but $k_{\text{cat}}/K_{\text{M}}^{\text{peptide}}$ values were not measured due to interference in the fluorescence-based assay.

^bPeptides are in the form dansyl-TKCxxx, where x represents any amino acid

^cPredicted to be farnesylated based on^{9, 10}.

^dN/A = not applicable; CHVQ is a non-human-derived sequence designed by substitution at the a1 position of a substrate (CAVQ) identified in the initial library.