Rapid Analysis of Protein Farnesyltransferase Substrate Specificity Using Peptide Libraries and Isoprenoid Diphosphate Analogues

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S *Supporting Information*

ABSTRACT: Protein farnesytransferase (PFTase) catalyzes the farnesylation of proteins with a carboxy-terminal tetrapeptide sequence denoted as a Ca_1a_2X box. To explore the specificity of this enzyme, an important therapeutic target, solid-phase peptide synthesis in concert with a peptide inversion strategy was used to prepare two libraries, each containing 380 peptides. The libraries were screened using an alkyne-containing isoprenoid analogue followed by click chemistry with biotin azide and subsequent visualization with streptavidin-AP. Screening of the CVa_2X and CCa_2X libraries with *Rattus norvegicus* PFTase revealed reaction by many known recognition sequences as well as numerous unknown ones. Some of the latter occur in the genomes of bacteria and viruses and may be important for pathogenesis, suggesting new targets for therapeutic intervention. Screening of the CVa_2X library with alkyne-functionalized isoprenoid substrates showed that those prepared from C_{10} or C_{15} precursors gave similar results, whereas the analogue synthesized from a C_5 unit gave a different pattern of reactivity. Lastly, the substrate specificities of PFTases from three organisms (*R. norvegicus*, *Saccharomyces cerevisiae*, and *Candida albicans*) were compared using CVa_2X libraries. *R. norvegicus* PFTase was found to share more peptide substrates with *S. cerevisiae* PFTase than with *C. albicans* PFTase. In general, this method is a highly efficient strategy for rapidly probing the specificity of this important enzyme.

Protein prenylation with isoprenoids has been the focus of numerous studies since its discovery in the early 1990s because of its connection to cancer.[1] Members of the Ras family of proteins are normally prenylated, and mutated forms of Ras, especially K-Ras, are involved in as many as 30% of all human cancers.[2] Protein prenylation is not only common in mammals[3,4] but is also a ubiquitous post-translational modification in all eukaryotes. For example, prenylated Ras is a more potent *in vitro* activator of *Saccharomyces cerevisiae* adenylyl cyclase than is the nonprenylated form.[5] It has also been found that prenylation of signal transduction proteins is essential for viability of *Candida albicans*, which is an opportunistic fungal pathogen.[6] Other groups have identified prenylated proteins and confirmed their significance in *Plasmodium falciparum*, which is the causative agent for malarial disease.[7] A vast array of prenylation inhibitors have been developed to combat numerous illnesses caused by cancers, protozoan pathogens, and fungal infections.[8−11] Recently, interest in prenylation has expanded to include biotechnology applications because prenyltransferases can be used to enzymatically incorporate non-natural functional groups into protein substrates.[12−14] The resulting modified polypeptides can be further transformed via bio-orthogonal reactions to produce a variety of useful species including PEGylated proteins,[15] protein multimers,[16] and protein−DNA conjugates.[17]

Members of the protein prenyltransferase class of enzymes include protein farnesyltransferase (PFTase) and protein geranylgeranyltransferase I (PGGTase I), which catalyze the transfer of farnesyl (C15) and geranylgeranyl (C20) groups, respectively, from the corresponding diphosphates. In the resulting alkylated protein, the isoprenoid group is linked to a cysteine residue within the C-terminal amino acid sequence referred to as a Ca_1a_2X motif, where C is a cysteine, a_1 and a_2 are usually aliphatic amino acids, and X is the major determinant for modification by either PFTase or PGGTase I (Figure 1).[18,19] It has been reported that farnesylation by PFTase occurs when X is alanine, serine, methionine, or glutamine, whereas geranylgeranylation by PGGTase I occurs when X is leucine or phenylalanine. Additional proteins are digeranylgeranylated at their C-terminus when they contain sequences including CC and CXC; these latter sequences are prenylated by protein geranylgeranyltransferase type II (PGGTase-II). GGTase-II, also called RabGGTase, differs both structurally and functionally from the canonical PFTase and PGGTase-I because it recognizes more extensive elements from its cognate protein substrates, Rab proteins.[20] Once prenylated, the resulting proteins move to the endoplasmic reticulum, where they are further processed by proteases that
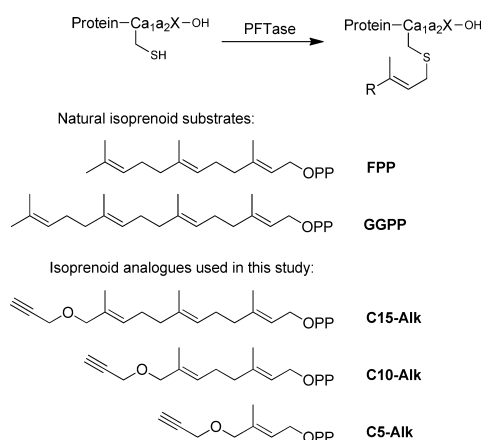
Figure 1. Post-translational prenylation reactions catalyzed by PFTase and PGGTase-I and the probes used here to investigate PFTase. The R group can be a farnesyl or geranylgeranyl group in cells.

remove the a_1a_2X tripeptide and are methylated by a SAM-dependent methyltransferase to produce proteins with a C-terminal methyl ester.[21]

Early investigations of Ca_1a_2X substrate preferences of prenyltransferases involved mutations of the C-terminal sequences of Ras proteins. Individual purified proteins were assayed using $[^3H]$-FPP or $[^3H]$GGPP as the isoprenoid substrate.[22] This method is inconvenient and labor-intensive. Subsequently, a fluorescence assay was developed to study peptide specificity of PFTase and PGGTase-I[23,24] that involves the preparation of dansylated Ca_1a_2X-box containing peptides and the measurement of dansyl-group fluorescence that

increases upon prenylation.[25] However, this method still requires the synthesis, purification, and analysis of individual peptides, which is a time-consuming process. Previously we reported the synthesis of C-terminal peptides via SPOT synthesis and the subsequent screening of a peptide library containing 340 different sequences prepared on a single membrane using *S. cerevisiae* PFTase (yPFTase) to study its Ca_1a_2X-box specificity.[26] Here, we applied the SPOT synthesis method[27] to study the specificity of *Rattus norvegicus* PFTase (rPFTase) and to investigate the interplay between peptides and isoprenoid substrates of varying length (Figure 1) and the specificity of PFTases from different organisms.

■ RESULTS AND DISCUSSION

Peptide Library Design, Synthesis, and Screening. In previous work, we reported the screening of a library of peptides for catalytic activity using *S. cerevisiae* PFTase (yPFTase).[26] A similar strategy was used here for peptide synthesis and subsequent evaluation. An automated SPOT synthesizer was used to create two kinds of peptide libraries: a 19×20 CVa_2X library and a 19×20 CCa_2X library, with X being 1 of the 20 proteogenic amino acids except P and a_2 being 1 of the 20 proteogenic amino acids. Because peptides are chemically synthesized in a C- to N-terminal direction, we adopted a "peptide inversion" strategy to prepare peptide libraries with free C-termini.[28−31] In this approach, synthetic peptides are cyclized between their N-terminus, and an internal carboxyl group that is installed via a bifunctional linker followed by acidolytic global deprotection and ester cleavage to yield resin-bound peptides with free C-termini (Figure 2a). To confirm the production of the desired synthetic peptides, a
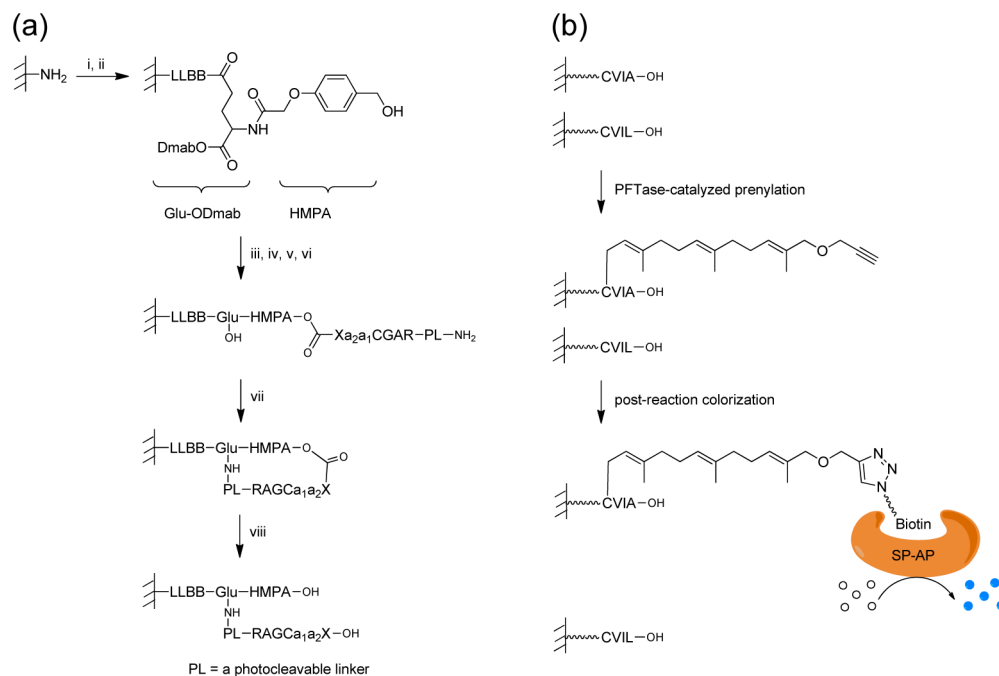

Figure 2. Strategy for the synthesis of C-terminal Ca_1a_2X-box peptide libraries and their subsequent use to explore the specificity of PFTase. (a) Synthesis of C-terminal peptides. Reagents and conditions: (i) standard DIC coupling of Fmoc-Aa (2×), then capping, then 20% piperidine; (ii) standard DIC coupling of HMPA (2×); (iii) 0.4 M Fmoc-Aa and 1.2 M CDI in DMF (4×), then capping, then 20% piperidine; (iv) standard DIC coupling of Fmoc-Aa (2×), then capping, then 20% piperidine; (v) 0.5 M photocleavable linker, 0.5 M Et$_3$N in DMF (3×); (vi) 2% N$_2$H$_4$; (vii) 0.05 M BOP, 0.05 M 6-Cl-HOBt, and 0.1 M DIEA in DMF (2×); (viii) modified Reagent K. (b) Screening and imaging strategy using CVIA (a substrate) and CVIL (nonsubstrate) as examples. Post-reaction colorization was accomplished by click reaction with biotin-azide followed by incubation with SP-AP. Colorization occurred upon the addition of BCIP.
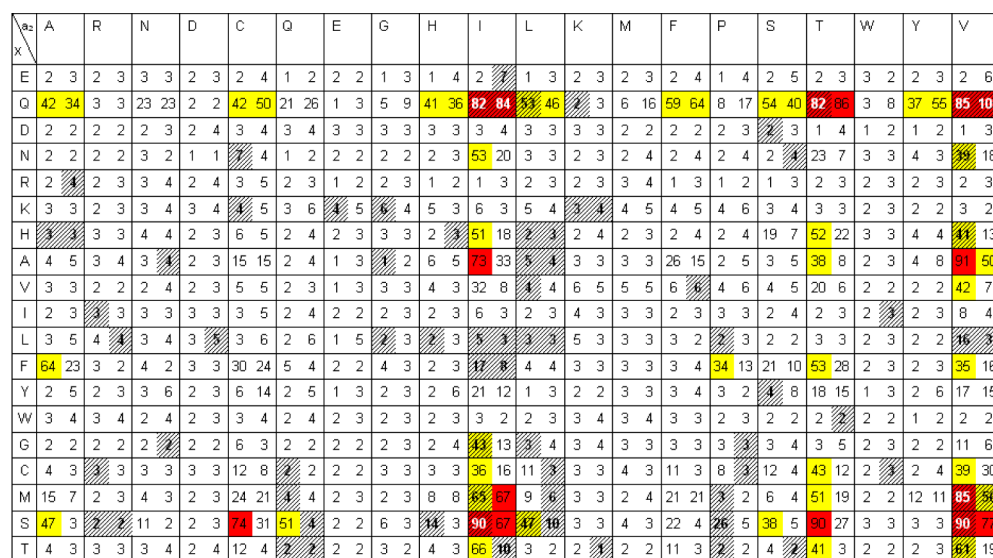
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a₂ / X	A	R	N	D	C	Q	E	G	H	I	L	K	M	F	P	S	T	W	Y	V
E	2 3	2 3	3 3	2 3	2 4	1 2	2 2	1 3	1 4	2 ▨	1 3	2 3	2 3	2 4	1 4	2 5	2 3	3 2	2 3	2 6
Q	42 34	3 3	23 23	2 2	42 50	21 26	1 3	5 9	41 36	82 84	53 46	▨ 3	6 16	59 64	8 17	54 40	82 36	3 8	37 55	85 106
D	2 2	2 2	2 3	2 4	3 4	3 4	3 3	3 3	3 4	3 3	3 3	3 3	2 2	2 2	2 3	▨ 3	1 4	1 2	1 1	1 3
N	2 2	2 2	3 2	1 1	▨ 4	1 2	2 2	2 2	2 3	53 20	3 3	2 3	2 4	2 4	2 ▨	23 7	3 3	4 3	39 18	
R	2 ▨	2 3	3 4	2 4	3 5	2 3	1 2	2 3	1 2	1 3	2 3	2 3	3 4	1 3	1 2	1 3	2 3	2 3	2 3	
K	3 3	2 3	3 4	3 4	▨ 5	3 6	4 5	▨ 4	5 3	6 3	5 4	▨ ▨	4 5	4 5	4 6	3 4	3 3	2 3	2 2	3 2
H	▨ ▨	3 3	4 4	2 3	6 5	2 4	2 3	3 3	2 ▨	51 18	▨ ▨	2 4	2 3	2 4	2 4	19 7	52 22	3 3	4 4	41 13
A	4 5	3 4	3 ▨	2 3	15 15	2 4	1 3	▨ 2	6 5	73 33	5 4	3 3	3 3	26 15	2 5	3 5	38 8	2 3	4 8	91 50
V	3 3	2 2	2 4	3 5	5 2	3 1	3 3	4 3	32 8	▨ 4	6 5	5 5	6 ▨	4 6	4 5	20 6	2 2	2 2	42 7	
I	2 3	▨ 3	3 3	3 3	3 5	2 4	2 2	2 3	6 3	2 3	4 3	3 3	2 3	3 3	2 ▨	2 3	8 4			
L	3 5	4 ▨	3 4	3 ▨	3 6	2 6	1 5	▨ 3	▨ 3	▨ ▨	5 3	3 2	2 3	3 2	3 3	2 2	16 ▨			
F	64 23	3 2	4 2	3 3	30 24	5 4	2 4	3 2	▨ 8	4 4	3 3	3 3	4	34 13	21 10	53 28	2 3	2 3	35 16	
Y	2 5	2 3	3 6	2 3	6 14	2 5	1 3	2 3	6	21 12	1 3	2 2	3 3	3 4	3 2	▨ 8	18 15	1 3	2 6	17 15
W	3 4	3 4	2 4	2 3	3 4	2 4	2 3	2 3	2 3	3 2	2 3	3 4	3 4	3 3	2 3	2 2	2 ▨	2 2	1 2	2 2
G	2 2	2 2	2 ▨	2 2	6 3	2 2	2 2	2 3	2 4	▨ 13	▨ 4	3 4	3 3	3 3	▨ 3	4 3	4 5	2 3	2 2	11 6
C	4 3	▨ 3	3 3	3 3	12 8	▨ 2	2 2	3 3	3 3	36 16	11 ▨	3 3	4 3	11 3	8 ▨	12 4	43 12	2 ▨	2 4	39 30
M	15 7	2 3	4 3	2 3	24 21	4 4	2 3	2 3	8 8	65 57	9 6	3 3	2 4	21 21	▨ 2	6 4	51 19	2 2	12 11	85 56
S	47 3	▨ ▨	11 2	2 3	74 31	51 4	2 2	6 3	14 3	90 67	47 10	3 3	4 3	22 4	26 5	38 5	90 27	3 3	3 3	90 77
T	4 3	3 3	3 4	2 4	12 4	▨ 2	2 3	2 4	3	66 10	3 2	2 ▨	2 2	11 3	▨ 2	4 ▨	41 3	2 2	2 3	61 19

Figure 3. Heat map representation of the extent of farnesylation of RAGCVa₂X and RAGCCa₂X libraries of peptides by rPFTase. Columns represent the a₂ position, and rows represent the X position. For each box, there are two numbers. The left and right values are spot intensities obtained with the CVa₂X and CCa₂X libraries, respectively. For comparison, the intensity of both libraries was normalized relative to that observed with the sequence CIIS. Each library was synthesized and screened at least two times, and the average color intensities were color-coded to produce the heat map. The intensities below 34% are shown in white, those between 34 and 66% are shown in yellow, and those above 66% are shown in red. Diagonally shaded boxes represent sequences that are found in the human or rat genome.

photocleavable linker was incorporated N-terminal to the Ca₁a₂X sequence so that at the end of the synthesis, peptides from individual spots could be released from the membrane by UV irradiation and analyzed by MALDI. Following synthesis, each membrane was subjected to PFTase-catalyzed prenylation with an alkyne-containing FPP analogue followed by derivatization with biotin-azide via copper-catalyzed azide–alkyne cycloaddition (CuAAC). Peptides that were prenylated by PFTase were conjugated to biotin at this step. The membrane was then subjected to an enzyme-linked assay involving streptavidin-alkaline phosphatase (SA-AP) and the chromogenic substrate, 5-bromo-4-chloro-3-indolyl phosphate (BCIP). Spots containing prenylated peptides appear turquoise colored, whereas spots where the prenylation reaction was inefficient remain colorless (Figure 2b).

Library Screening Using _R. norvegicus_ PFTase. In previously reported work, Fierke and co-workers studied the specificity of rPFTase by synthesizing and assaying 213 Ca₁a₂X-box containing peptide sequences found in the human proteome.[25] That sparse sampling of the total sequence space available from varying three residues (8000 possibilities) was quite useful for understanding prenylation specificity in the context of human biochemistry. However, such a study cannot reveal the complete specifity profile of the enzyme given the limited sampling. To complement that work, we chose to systematically assess the substrate specificity of mammalian PFTase at the a₂ and X positions by preparing and screening two peptide libraries (19 × 20 CVa₂X library and 19 × 20 CCa₂X library) using _R. norvegicus_ PFTase (rPFTase). These libraries densely sample the possible range of sequence space from the last two positions. Inspection of the data provided in Figure 3 confirms that many of the sequences found in the human genome including CVIQ, CVIM, CVIS, CVTQ, CVVQ, CVVM, and CVVS are efficiently prenylated, as was previously reported by Fierke and co-workers and references therein.[25] However, a number of additional sequences not present in the human genome were prenylated with high efficiency including

CVIA, CVIT, CVTS, CVVA, CVCS, and others. This general observation is consistent with previous reports that rPFTase can prenylate peptide sequences not present in the genome, and most of these have been demonstrated to be substrates using the _in vitro_ fluorescence assay.[32,33] Some of these sequences are found in the genomes of other organisms including _Schizosaccharomyces pombe_ (CVIA), _Schizophyllum commune_ (CVVA), _Drosophila menanogaster_ (CVSS), _Arabidopsis thaliana_ (CVTA), and _Mus musculus_ (CVAS), whose prenylated proteins have not been studied in detail. Of potential greater significance are sequences found in human pathogens including CVFQ (_Shigella dysenteriae_) and CVTH (_Bacillus circulans_) as well as various herpesviruses (CVSS and CVPF). Recent reports suggest that, in some cases, bacterially and virally encoded proteins must be prenylated by endogenous human prenyltransferases for pathogenesis.[34−36] These results highlight the utility of this library approach as a tool for rapidly discovering/confirming new substrates.

Previously, Fierke and co-workers reported that peptides containing cysteine as the a₁ position tend to have lower multiple turnover reactivity.[25] That inspired us to study CCa₂X peptides more thoroughly using a CCa₂X library. Because we had not previously prepared libraries containing adjacent Cys residues, a number of spots were selected, and the peptides were cleaved from the membrane via UV irradiation and analyzed by MALDI MS. The spots gave the expected _m/z_ values for the corresponding target peptides with an acceptable signal-to-noise ratio except when X was C, G, or W. In screening the CCa₂X library, fewer positive spots were detected (compared to our observations with the CVa₂X library), indicating that, generally, CCa₂X peptides have lower reactivity. It was previously reported that the a₁ position of the Ca₁a₂X motif is more solvent-exposed than the a₂ and X positions and that there are no apparent restrictions at this position.[37] However, our screening results show that the a₁ position does have an effect on PFTase specificity and that PFTase discriminates against sequences with Cys as the a₁ residue
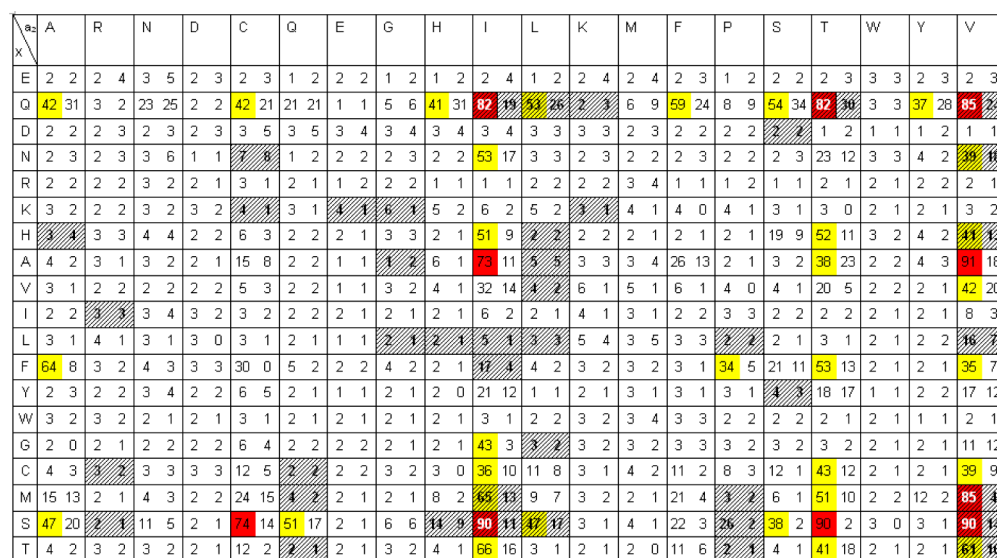
X \ a₂	A	R	N	D	C	Q	E	G	H	I	L	K	M	F	P	S	T	W	Y	V
E	2 2	2 4	2 3	3 5	2 3	2 3	1 2	2 2	1 2	1 2	2 4	1 2	2 4	2 4	2 3	1 2	2 3	3 3	2 3	2 3
Q	42 31	3 2	23 25	2 2	42 21	21 21	1 1	5 6	41 31	82 19	53 26	2 3	6 9	59 24	8 9	54 34	82 30	3 3	37 28	85 24
D	2 2	2 3	2 3	2 3	3 5	3 5	3 4	3 4	3 4	3 4	3 3	3 3	2 3	2 2	2 2	2 1	1 2	1 1	1 1	1 1
N	2 3	2 3	3 6	1 1	7 8	1 2	2 2	2 3	2 2	53 17	3 3	2 3	2 2	2 3	2 2	2 3	23 12	3 3	4 2	39 18
R	2 2	2 2	2 2	3 2	2 1	3 1	2 1	1 2	2 2	1 1	1 1	2 2	2 2	3 4	1 1	1 2	1 1	2 1	2 1	2 1
K	3 2	2 2	3 2	3 2	—	3 1	—	16 1	5 2	6 2	5 2	—	4 1	4 0	4 1	3 1	3 0	2 1	2 1	3 2
H	— 4	3 3	4 4	2 2	6 3	2 2	2 1	3 3	2 1	51 9	2 2	2 2	2 1	2 1	19 9	52 11	3 2	4 2	11 13	44 13
A	4 2	3 1	3 2	2 1	15 8	2 2	1 1	1 2	6 1	73 11	5 5	3 3	26 13	2 1	3 2	38 23	2 2	4 3	91 18	18
V	3 1	2 2	2 2	2 2	5 3	2 2	1 1	3 2	4 1	32 14	4 2	6 1	5 1	6 1	4 0	4 1	20 5	2 2	2 1	42 20
I	2 2	3 3	3 4	3 2	3 2	2 2	2 1	2 1	2 1	6 2	2 1	4 1	3 1	2 2	3 3	2 2	2 2	2 1	2 1	8 3
L	3 1	4 1	4 1	3 1	3 0	3 1	2 1	1 1	1 1	—	—	5 4	3 5	3 3	—	2 2	1 2	1 2	2 2	16 7
F	64 8	3 2	4 3	3 3	30 0	5 2	2 2	4 2	2 1	—	4 2	3 2	3 2	3 1	34 5	21 11	53 13	2 1	2 1	35 7
Y	2 3	2 2	3 2	3 4	2 2	6 5	2 1	1 1	1 2	21 12	1 1	2 1	3 1	3 1	3 1	—	2 2	1 1	2 2	17 12
W	3 2	3 2	2 1	2 1	3 1	2 1	2 1	2 1	2 1	3 1	2 2	3 1	2 1	2 1	3 1	2 1	2 1	1 1	2 1	2 1
G	2 0	2 1	2 2	2 2	6 4	2 2	2 2	2 1	2 1	43 3	3 —	3 2	3 2	3 3	3 2	3 2	3 2	2 1	2 1	11 12
C	4 3	3 2	3 3	3 3	12 5	2 2	2 2	3 2	3 0	36 10	10 11	8 3	4 2	11 2	8 3	12 1	43 12	2 1	2 1	39 9
M	15 13	2 1	4 3	2 2	24 15	—	2 1	2 1	8 2	65 13	9 7	3 2	2 1	21 4	—	6 1	51 10	2 2	12 2	85 4
S	47 20	2 2	11 5	2 1	74 14	51 17	2 1	6 6	—	90 11	47 11	3 1	4 1	22 3	26 —	38 2	90 —	2 3	3 1	90 14
T	4 2	3 2	3 2	2 1	12 2	—	2 1	3 2	4 1	66 16	3 1	2 1	2 0	11 6	—	4 1	41 18	2 1	2 1	61 16

Figure 4. Heat map representation of the extent of farnesylation of a RAGCVa₂X library by rPFTase. Columns represent specific a₂ positions and rows represent specific X positions. For each box, there are two numbers. The left and right values are spot intensities and standard deviations obtained from three screening experiments, respectively. For comparison, the intensities of both libraries were normalized relative to that observed with the sequence CIIS. The intensities below 34% are shown in white, those between 34 and 66% are shown in yellow, and those above 66% are shown in red. Diagonally shaded boxes indicate sequences that are found in the human or rat genome.

unless the a₂ position is Ile or Val and the X position is Gln, Met, or Ser. In vitro enzymatic assay of selected peptides also confirmed these screening results (Tables S1 and S4). The observations described here are consistent with previously reported in vivo farnesylated protein sequences. For example, it has been reported that the tyrosine phosphatases PRL-1, -2, and -3 associate with the cellular membrane or endosome in a farnesylation-dependent manner.[38] Their C-terminal sequences are CCIQ, CCVQ, and CCVM, respectively, which also showed high reactivity in our screening analysis. As was noted above for the CVa₂X library, the CCa₂X library also contains numerous sequences that do not exist in the human genome but are still modified with high efficiency by rPFTase, including CCIM, CCIS, CCTQ, and CCVS. The sequence CCSQ is particularly interesting because it occurs in the genome of the pathogenic bacteria *Neorickettsia sennetsu* that resides within human cells; as noted above, prenylation of that protein by endogenous farnesyltransferase may be essential for pathogenesis. Finally, it should be noted that some proteins with C-terminal CCa₂X sequences are farnesylated or geranylgeranylated on the first Cys and then palmitoylated at the second Cys (the a₂ position) in lieu of proteolytic removal of the a₁a₂X tripeptide.[39] Thus, this study using a CCa₂X library paves the way for the use of such libraries to probe palmitoylation specificity. For this, a CCa₂X library could be prenylated chemically using orthogonally protected Cys[40,41] or enzymatically with PFTase or PGGTase-I and then screened for enzymatic palmitoylation using an alkyne-containing palmitoyl-CoA analogue.[42]

Overall, the results from screening CVa₂X and CCa₂X libraries confirm many of the previously reported observations concerning rPFTase specificity. However, importantly, the use of these peptide libraries has allowed us to identify numerous sequences not present in the human genome that are efficient substrates for rPFTase. The discovery of such sequences may be useful for biotechnology applications in which Ca₁a₂X box sequences are appended onto proteins for site-specific labeling and builds upon results reported by the Fierke[43] and

Hougland[44] groups on altering peptide specificity and work from Nguyen et al.[45] on modulating isoprenoid substrate recognition. The ability of rPFTase to recognize sequences not found in the genome could serve as an important step in the development of orthogonal PFTase variants. Additionally, given that many of the PFTase inhibitors created to date are peptidomimetic molecules based on Ca₁a₂X box sequences,[46,47] the insights obtained from the peptide libraries described here should be useful for inhibitor design. Finally, the discovery of substrate sequences present in the genomes of bacteria and viruses may shed light on their mechanisms of pathogenesis and suggest new approaches for therapeutic intervention.

Statistical Analysis of Library Screening Method and Enzyme Concentration Dependence. In interpreting the data from the studies described above, we focused on the medium (yellow) and high (red) intensity spots. To assess the validity of those measurements, statistical analysis was performed using the data from the CVa₂X library. Figure 4 shows the average spot intensity (from three separate experiments) at each position along with the standard deviation. Comparison of the percent standard deviation from the mean for each of the three categories (low, medium, and high) gives values of 73, 35, and 18%, respectively. Further calculation for the 95% confidence interval for the same three categories yields values of 186, 88, and 44%, suggesting that there is insufficient certainty (the uncertainty is larger than the values measured) in the data for the low-intensity spots. Thus, this analysis suggests that spots that appear as yellow (medium intensity) or red (high intensity) are highly likely to be *bona fide* substrates. In contrast, it is not possible to differentiate with certainty whether spots that appear as white in the heat maps are poor substrates or are not substrates at all.

The statistical analysis described above suggests that it is not possible to conclude anything concerning the low intensity substrates. To examine this in greater detail, the effect of using a higher enzyme concentration was explored. Thus, the 380-member CCa₂X library was screened using an enzyme concentration 3-fold higher than that used for the earlier

X \ a₂	A	R	N	D	C	Q	E	G	H	I	L	K	M	F	P	S	T	W	Y	V
E	3 4	3 6	3 6	3 4	4 3	2 3	2 3	3 5	4 5	7 6	3 4	3 5	3 6	4 5	4 4	5 4	3 6	2 7	3 7	6 6
Q	34 24	3 10	23 11	2 4	50 26	26 11	3 3	9 4	36 23	84 56	46 29	3 8	16 8	64 55	17 18	40 41	86 76	8 7	55 44	106 82
D	2 5	2 5	3 6	4 6	4 5	4 6	3 7	3 7	3 7	4 7	3 5	3 6	2 3	2 4	3 3	3 4	4 4	2 4	2 4	3 4
N	2 5	2 7	2 4	1 3	4 7	2 5	2 5	3 5	20 36	3 4	3 9	4 8	4 8	4 7	4 7	7 16	3 7	3 8	18 25	
R	4 10	3 6	4 8	4 7	5 10	3 8	2 5	3 11	2 9	3 8	3 7	3 5	4 7	3 8	2 6	3 7	3 10	3 8	3 10	3 7
K	3 7	3 5	4 11	4 8	5 10	6 10	5 9	4 13	3 10	3 11	4 9	4 8	5 13	5 13	6 13	4 8	3 9	3 10	2 10	2 7
H	3 4	3 6	4 5	3 4	5 7	4 6	3 5	3 6	3 6	18 37	3 8	4 10	3 11	4 9	4 7	7 14	22 36	3 7	4 9	13 22
A	5 9	4 8	4 7	3 4	15 12	4 7	3 3	2 6	5 12	33 52	4 4	3 7	3 5	15 29	5 6	5 7	8 26	3 5	8 17	50 64
V	3 9	2 8	4 10	3 7	5 11	3 7	3 6	3 7	3 9	8 21	4 7	5 13	5 7	6 10	6 6	5 8	6 19	2 5	2 7	7 30
I	3 6	3 7	3 5	3 4	5 7	4 7	2 5	3 6	3 6	3 15	3 5	3 9	3 9	3 7	3 6	4 6	3 8	3 6	3 6	4 10
L	5 7	4 9	4 7	5 6	6 9	6 6	5 5	3 6	3 7	3 12	3 4	3 5	3 5	2 4	3 6	2 5	3 6	3 5	2 6	3 23
F	23 56	2 8	2 13	3 6	24 33	4 11	2 7	3 12	3 8	8 25	4 10	3 10	3 9	4 10	13 39	10 29	28 68	3 7	3 8	16 35
Y	5 8	3 7	6 12	3 4	14 18	5 13	3 5	3 11	6 24	12 59	3 13	2 7	3 18	4 25	2 12	8 24	15 39	3 6	6 23	15 36
W	4 7	4 8	4 6	3 6	4 6	4 6	3 5	3 6	3 6	3 9	2 7	3 4	9 2	7 3	4 2	4 6	2 5	5 2	2 6	
G	2 6	2 8	2 8	2 8	3 9	2 8	2 6	3 8	4 8	13 33	4 6	4 9	3 7	3 8	4 6	4 5	5 11	3 7	2 4	6 23
C	3 6	3 7	3 5	3 3	8 22	2 5	2 4	3 5	3 8	16 37	4 7	3 6	3 9	3 20	4 10	4 18	12 39	4 7	4 9	30 34
M	7 20	3 5	3 11	3 4	21 50	4 13	3 5	3 8	8 21	67 87	6 12	3 6	4 7	21 48	2 7	4 10	19 56	2 6	11 50	56 102
S	3 28	2 6	2 10	3 4	31 54	4 28	2 5	3 9	3 15	67 81	10 25	3 8	3 8	4 23	5 41	5 25	27 77	3 6	3 10	77 87
T	3 6	3 6	4 7	4 5	4 12	2 6	2 4	2 5	3 8	10 48	2 6	4 7	2 7	3 16	2 5	2 8	3 32	2 6	3 7	19 48

Figure 5. Evaluation of the extent of farnesylation of a RAGCCa₂X library of peptides using different concentrations of rPFTase. Columns represent specific a₂ positions, and rows represent specific X positions. For each box, there are two values. The left and right numbers indicate [rPFTase] = 55 and 182 μg/mL, respectively. Color intensities were quantified by ImageJ software. For comparison, the intensities in the CCa₂X library were normalized relative to that observed with the CIIS sequence. The library was synthesized and screened at least two times, and the average color intensities were color-coded. The intensity below 34% is shown in white. The intensity between 34 and 66% is shown in yellow. The intensity above 66% is shown in red. Shaded boxes indicate the peptide sequences that are found in the human or rat genome.

experiments. Figure 5 summarizes the results obtained from screening the CCa₂X library at the two different concentrations. Inspection of that data reveals several interesting observations. Most importantly, numerous spots that fell into the low intensity category at low enzyme concentration moved into the medium or high category when the higher enzyme concentration was used including several whose intensity increased more than 2-fold. In some cases, sequences that were in the low intensity category increased significantly (more than 3-fold) but that increase was not sufficient to move them into the medium class given their low initial intensity. Of those, it should be noted that CCQS, CCIL, CCIF, CCGY, CCKT, CCPC, and CCVL are present in the human genome. These results suggest that those sequences are real but poor substrates for the rPFTase. That conclusion is supported by data from Hougland et al.[25] who did observe catalytic activity with the sequences CCIF and CCIL using *in vitro* fluorescence assays although it was 15- and 140-fold lower, respectively, compared with that for optimal substrates. In contrast, for most of the white spots, no significant increase in spot intensity was observed when the enzyme concentration was increased 3-fold, indicating that those sequences are either extremely poor substrates or not substrates at all in terms of multiple-turnover reactions. An alternative explanation is that some of these sequences may be single-turnover substrates under these *in vitro* conditions.[25] Efforts to explore the screening of these libraries using stoichiometric quantities of enzyme to reveal single-turnover substrates are in progress. Overall, this experiment demonstrates how the dynamic range of this screening method can be increased by raising the enzyme concentration and be used to detect activities that are more than 100-fold lower than that manifested by the fastest substrates.

Comparison with Direct Kinetic Analysis and Bioinformatics Methods. Before using the libraries reported here in additional experiments, the results obtained with them in the experiments described above were compared with data obtained using two other methodologies. First, the intensity data obtained from the CVa₂X library screening was compared with data obtained via direct kinetic analysis using individually synthesized peptides. Hougland et al.[33] reported such data for a series of peptides (n = 73) based on CVa₂X, where X was restricted to A, M, Q, and S. A plot of the library screening intensity versus k_{cat}/K_M gave a statistically significant correlation (Figure S1, $p < 0.001$), although there is significant deviation for some sequences. Comparison of data from individual sequences after binning the k_{cat}/K_M data into low, medium, and high values (Figure S2) allows the correlation to be easily visualized. Inspection of that data reveals that methionine-containing sequences (with M at the a₂ or X position) correlate particularly poorly, which may be due to oxidation to the sulfoxide during the synthesis and visualization process. In the MS analysis of selected methionine-containing peptide sequences, the sulfoxide is either the only detectable species or the major product present (see MS data for CCIM, Figure S15); similar results were noted in our earlier library work with yeast PFTase (with the sequence CVIM)[26] and in other libraries currently being prepared including the sequences CKIM, CDIM, CTIM, CFIM, and CGIM (data not shown). The library screening data obtained here was also compared with predictions made using PrePS, a web-based bioinformatics program that scores sequences for their potential to be PFTase substrates.[48] A plot of the library screening intensity versus PrePS score gave a statistically significant correlation (Figure S3, $p < 0.005$). Comparison of data from individual sequences after binning the PrePS scores into low, medium, and high values (Figure S4) shows that many sequences observed as positives in the library screening are predicted to be PFTase substrates by PrePS. As noted for the k_{cat}/K_M data, methionine-containing sequences also correlate poorly. Conversely, the library screening reveals a number of sequences that are enzyme substrates that are not predicted by PrePS, including CVFQ, CVSQ, and CVYQ. Those sequences are particularly noteworthy because they were also identified as substrates in assays with individual peptides by Fierke and co-workers.[33] In

Figure 6. Heat map representation of the extent of farnesylation of a RAGCVa₂X library of peptides with different isoprenoid substrates. For each sequence, the left value shows the results using C5-Alk, the middle value, C10-Alk, and the right value, C15-Alk. For comparison, the intensity was normalized relative to that observed with CVIS. Color coding and shading was performed as described for Figure 3.

general, the comparisons noted above show that the data obtained with the peptide libraries described herein reproduces many of the features of substrate recognition previously reported for PFTase and thus underscores the validity of using these libraries for rapidly assessing aspects of PFTase substrate specificity.

Examining the Interplay between Peptide Specificity and Isoprenoid Length. Inspection of the crystal structure of the ternary complex of PFTase bound to a peptide substrate and isoprenoid analogue reveals extensive contacts between the isoprenoid and the peptide.[37] Previously, Gibbs and co-workers observed in studies using a series of modified isoprenoid diphosphates that peptide substrate specificity varied depending on which particular isoprenoid was used.[49] That is, while some isoprenoids worked well for certain peptide sequences, other isoprenoids worked best with different peptide substrates. Because a number of isoprenoid analogues are currently being used to probe protein prenylation in living cells,[50−56,46−52] it would be useful to know whether modifications in the isoprenoid structure show systematic differences in peptide specificity. Similarly, isoprenoid analogues are being developed to disrupt interactions with prenylated proteins.[57] In those cases, it will be important to know whether such analogues are incorporated into prenylated proteins with a specificity profile similar to farnesyl groups. While in vitro assays with individual peptides do allow the catalytic efficiencies of different isoprenoid substrates to be compared, such experiments do not allow global variations in peptide substrate efficiency to be studied. To address this question for alkyne probes currently being used for proteomic applications, we screened a 380-member CVa₂X library using three different isoprenoid analogues of varying length (C5-Alk, C10-Alk, and C15-Alk, Figure 1). The screening data is summarized in Figure 6. From the results, it can be seen that the specificity profiles obtained using the C10-Alk and C15-Alk substrates are similar. For example, CVCS, CVHQ, CVIQ, CVIM, CVIS, CVTQ, CVTS, CVVQ, CVVM, and other sequences manifest comparable reactivity with C10-Alk and C15-Alk. In some cases, C10-Alk reacts preferentially compared with C15-Alk (CVCQ, CVMQ,

CVLM, and CVLS), whereas in other cases, C15-Alk reacts preferentially compared with C10-Alk (CVAF, CVIA, CVIG, and CVTF). In most cases, the observed differences are on the order of 2-fold, making them relatively minor. However, in a few cases, more significant differences exist. Those include cases where C15-Alk exhibits preferential reactivity (CVAF, CVTF, and CVVV) as well as a few instances where C10-Alk displays greater reactivity (CVQQ and CVMQ). In aggregate, similar results were obtained with these two different isoprenoids, suggesting that probe choice does not skew results in proteomic investigations, although care should be taken in quantitative proteomic experiments because significant variations in labeling do occur in a handful of cases.

In contrast to the above observations, experiments with C5-Alk gave different results. First, it is clear that C5-Alk is not an efficient substrate for PFTase because fewer positive spots were observed in the screening experiment. A comparison of the kinetic constants measured with an *in vitro* enzyme assay using FPP, C10-Alk, and C15-Alk (Table S2) shows that the catalytic efficiencies of C10-Alk and C15-Alk are comparable but lower than FPP. C5-Alk is a much poorer substrate, with K_M 78-fold higher and k_{cat} 400-fold lower compared with those of FPP (Table S3); attenuated catalytic activity was previously noted with that analogue using yPFTase.[58] More importantly, the specificity profile with C5-Alk was significantly different from that of the other probes. For example, CVIQ, CVIS, CVVQ, and CVVS all showed high activity regardless of which isoprenoid probe was used. In contrast, CVCQ, CVHQ, CVTQ, CVAS, CVCS, and CVTS were highly reactive when using C10-Alk and C15-Alk but not in the presence of C5-Alk. These results demonstrate that the peptide substrate selectivity obtained using C5-Alk differs significantly from that observed with C10-Alk and C15-Alk and suggests that this shorter probe is not suitable for proteomic analysis of farnesylated proteins because its peptide specificity profile is likely to be different than that for FPP. However, the different pattern of reactivity observed with C5-Alk may be useful for designing orthogonal prenyltransferases that recognize novel sequences. Overall, these experiments illustrate how C-terminal peptide libraries
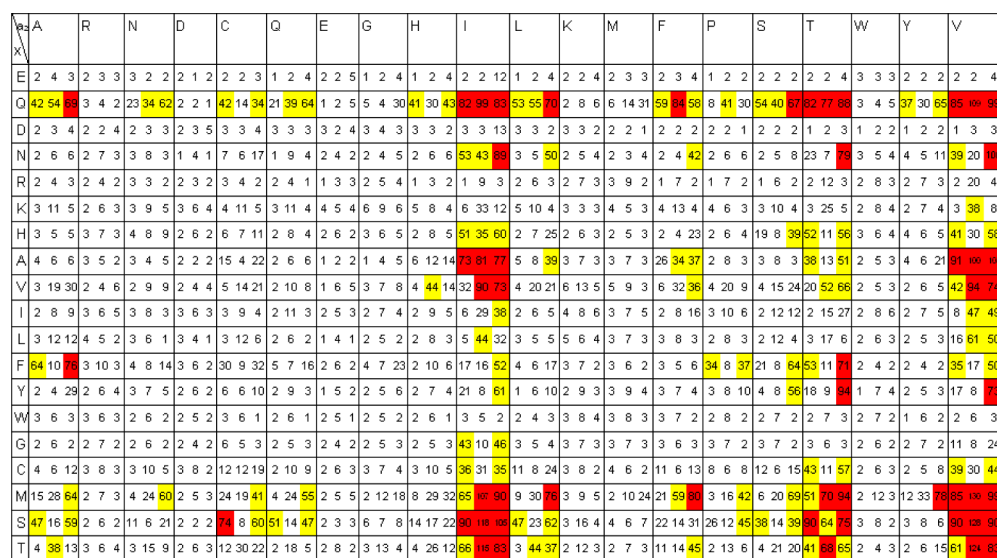
Figure 7. Heat map representation of the extent of farnesylation of a RAGCVa$_2$X library of peptides by three different PFTases. Rows represent the a$_2$ position, and columns represent the X position. For each sequence (each box), there are three sections. The left, middle, and right values indicate results obtained with *R. norvegicus*, *C. albicans*, and *S. cerevisiae* PFTases, respectively. For comparison, the intensity was normalized relative to that observed with CVIS. Color coding was performed as described for Figure 3.

can be used to rapidly examine the effect of changes in isoprenoid structure on the prenyltransferase specificity.

Probing the Specificity of PFTases from Different Species. Given the efficiency of the method described here for systematically and rapidly probing a large number of peptide sequences, we envisioned that it would be an effective strategy for examining the specificity of PFTase enzymes from different species. Thus, C-terminal peptide libraries were used to compare the specificity between PFTases from three different species (*R. norvegicus*, *C. albicans*, and *S. cerevisiae*). Accordingly, SPOT synthesis was employed to prepare the same 19 × 20 CVa$_2$X library described above followed by screening and visualization using C15-Alk as a substrate. The screening results are summarized in Figure 7. A random 60-member library with a$_1$ residues different from valine was also synthesized, screened, and visualized as noted above (see Figure S5). For these experiments, screening was performed using an equivalent amount of enzymatic activity by normalizing for differences in reaction rate using DsGCVIA (2 μM) and saturating concentrations of C15-Alk (100 μM).

In general, it can be seen that the three enzymes have similar peptide specificities (Figure 7). Thus, they all display a preference for Ile and Val at the a$_2$ position and Gln, Ala, Met, Ser, and Thr at the X position. This is not surprising, given their sequence similarity. BLAST analysis of the PFTase β-subunit shows that the residues contacting the peptide substrate are mostly conserved (Figure S6). From the numbers of active peptides recognized by the three enzymes (Figure 8), several conclusions can be made. First, yPFTase prenylates more sequences (84, 19%) than the other two enzymes, making it the most promiscuous of the three. In contrast, CaPFTase is the most selective, efficiently prenylating only 47 (11%) out of the 440 examined; rPFTase exhibits similar overall specificity, prenylating 51 of the sequences (12%). Next, despite those differences in selectivity, rPFTase shares more substrates with yPFTase (51) than with CaPFTase (41). This observation is consistent with sequence alignment of the different genes. The β-subunit of rPFTase has higher similarity with yPFTase than with CaPFTase (58 vs 47%). Finally, there are significant
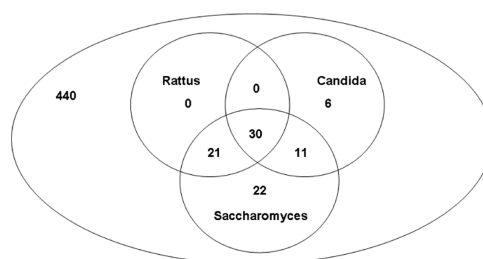
Figure 8. Number of peptide sequences that are recognized by three different PFTases. Peptides that showed high and medium intensities in the screening are grouped together. Low-intensity spots were not considered in this analysis.

differences between how the three different enzymes process identical substrates. Numerous examples occur where the *C. albicans* enzyme manifests higher relative reactivity with a given sequence. Those include CVHV, CVIV, CVLT, CVFM, CVPQ, CVTV, CVVV, CVVI, CVVL, and CVVT, which all show at least a 2-fold higher intensity with CaPFTase relative to with rPFTase (see Figure S7a for data presented in graphical form). As an example, *in vitro* analysis using the peptide Ds-GCVVV reveals that CaPFTase exhibits a 6.2-fold greater k_{cat}/K_M value when compared with rPFTase (Table S4). Similar evidence for greater preference for yPFTase compared with rPFTase was also observed in this data. The sequences CVAM, CVNM, CVIV, CVLN, CVLA, CVLM, CVLT, CVFM, CVFT, CVPM, CVSF, CVSM, CVTN, CVTV, CVTY, CVYM, CVVN, CVVI, CVVL, and CVVY all show at least a 2-fold higher intensity with yPFTase relative to rPFTase (see Figure S7b for data presented in graphical form). *In vitro* analysis using the peptide Ds-GCVTN reveals that yPFTase exhibits an 11-fold greater k_{cat}/K_M value when compared with rPFTase (Table S4). Although these differences reflect deviations in catalytic activity, they suggest that significant variations in sequence specificity occur between these enzymes. Such differences may be useful for the design of PFTase inhibitors that are selective for different species. Such inhibitors could be useful as drugs for

the treatment of a number of diseases beyond cancer including malaria, leshmania, and hepatitis.

CONCLUSIONS

In summary, two 380-member peptide libraries (CVa$_2$X and CCa$_2$X libraries) were evaluated as substrates for *R. norvegicus* PFTase. That screening revealed a number of both known and unknown substrate sequences. Some of the latter occur in the genomes of bacteria and viruses and may be important for pathogenesis, suggesting new potential targets for therapeutic intervention. Screening of the CVa$_2$X library with alkyne-functionalized isoprenoid substrates showed that those prepared from C$_{10}$ or C$_{15}$ precursors gave similar results, whereas the analogue synthesized from a C$_5$ unit gave a different pattern of reactivity. Lastly, the substrate specificities of PFTases from three organisms (*S. cerevisiae*, *R. norvegicus*, and *C. albicans*) were compared using CVa$_2$X libraries. *R. norvegicus* PFTase was found to share more peptide substrates with *S. cerevisiae* PFTase than with *C. albicans* PFTase. Overall, the 380-member CVa$_2$X library was screened using three different isoprenoid substrates and three different enzymes, whereas the CCa$_2$X library was screened using two different enzyme concentrations; thus, over 3000 different combinations were evaluated. In general, this is a highly efficient strategy for rapidly probing the specificity of this important enzyme and should be useful for a variety of future studies on PFTase and related enzymes.

ASSOCIATED CONTENT

ⓢ Supporting Information

Experimental methods; comparison of intensity values obtained via library screening with k_{cat}/K_M values obtained from kinetic analysis performed with individual synthetic peptides reported previously; comparison of screening intensities obtained using the RAGCVa$_2$X library of peptides with k_{cat}/K_M values reported previously; comparison of PrePS scores with intensity values obtained via library screening or k_{cat}/K_M values obtained from kinetic analysis performed with individual synthetic peptides reported previously; comparison of screening intensities from a RAGCVa$_2$X library of peptides with PrePS predictions; comparison of previously reported k_{cat}/K_M values with PrePS predictions; evaluation of the extent of farnesylation of a random library of peptides by PFTases from three different species; structural comparison among β-subunits of rPFTase, CaPFTase, and yPFTase; comparison of the intensities of selected peptide sequences; evaluation of Ds-GCaaX peptides for rFTase; kinetic constants for the rPFTase-catalyzed reaction of FPP and analogues with DsGCVLS peptides; evaluation of OrG-RTRCVIS peptides for *R. norvegicus* PFTase; evaluation of Ds-GCaaX peptides for *R. norvegicus* or *C. albicans* PFTase; plot of spot intensity versus k_{cat}, K_M, and k_{cat}/K_M from Table S4; screening of enzymatic prenylation of an RAGCVa$_2$X library; screening of enzymatic prenylation of an RAGCCa$_2$X library; and MALDI analysis of peptides produced by SPOT synthesis. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.

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