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Analogs of farnesyl diphosphate alter CaaX substrate specificity and reactions rates of protein farnesyltransferase

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ABSTRACT

Attempts to identify the prenyl-proteome of cells or changes in prenylation following drug treatment have used 'clickable' alkyne-modified analogs of the lipid substrates farnesyl- and geranylgeranyl-diphosphate (FPP and GGPP). We characterized the reactivity of four alkyne-containing analogs of FPP with purified protein farnesyltransferase and a small library of dansylated peptides using an in vitro continuous spectrofluorimetric assay. These analogs alter prenylation specificity and reactivity suggesting that in vivo results obtained using these FPP analogs should be interpreted cautiously.

Protein farnesyltransferase (FTase) is a heterodimeric zinc metalloenzyme that catalyzes the covalent attachment of a 15-carbon isoprenoid unit from farnesyl diphosphate (FPP) to a cysteine four residues from the C-terminus of a protein substrate.[1] Farnesylation is required for the proper localization of many proteins involved in signal transduction, including Ras.[2] Mutated Ras has been found in over 16% of all human cancers,[3] and the initial step of Ras farnesylation is sufficient for conferring transforming potential.[4] Therefore, FTase inhibitors (FTIs) were developed, which displayed anti-cancer properties and have been tested in clinical trials.[5] However, in FTI-treated cells, Ras can be alternatively prenylated by a related enzyme, GGTase-I (geranylgeranyltransferase-I).[6] This finding suggests that FTIs may inhibit the farnesylation of other proteins involved in oncogenesis and proliferation of cancer cells. Identifying these proteins would aid the development of novel strategies for treating cancer.

Previous studies have shown that protein substrates of FTase share a common Ca_1a_2X motif at their C-termini where 'C' is a cysteine residue, 'a_1' and 'a_2' are frequently aliphatic amino acids and 'X', in the case of an FTase substrate, is generally a serine, methionine, glutamine, or alanine.[7,8] While this paradigm has been useful for predicting some of the endogenous targets of farnesylation,[9] it does not fully encompass the range of potential protein substrates. For example, structure-based modeling predicted a novel class of substrates with negatively charged C-terminal residues (CxxD and CxxE) that was experimentally verified.[10] Additionally, several studies have indicated that amino acid residues upstream of the cysteine residue may also contribute to substrate selectivity.[11] Due to the limitations of the CaaX paradigm and the large number of proteins in a typical mammalian cell that could potentially be farnesylated, all of the endogenous targets of FTase have yet to be identified. A strategy for the global discovery of proteins modified by FTase would be beneficial for determining the targets of FTIs and for characterizing the full complement of farnesylated proteins.

One strategy for the *in cellulo* identification of post-translationally modified proteins takes advantage of bio-orthogonal 'click' chemistry between an azide and an alkyne and has been applied to the study of farnesylation.[12] As FPP analogs can be easily synthesized, several groups have developed alkyne- or azide-modified FPP derivatives.[13–18] FTase uses these derivatives as a substrate in place of endogenous FPP *in cellulo*, resulting in the covalent modification of native protein substrates. Tagged proteins are then

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captured and identified by mass spectrometry. Similar methods based on either a biotin-modified isoprenoid and mutant transferases[19] or antigenic isoprenoid derivatives that can be detected via western blotting techniques[20] have also been employed for this purpose. Several of these FPP derivatives have been used to identify FTase protein substrates from cells.[13,16,17,21,22] Some of the results from these initial experiments have been surprising. For example, several studies using different analogs have identified annexin proteins (a class of proteins that do not contain a CaaX motif, rather a –CGGDD C-terminus) as potential FTase substrates.[13] Additionally, several of the analogs tested have not detected known farnesylated proteins such as transducin γ (CVIS).[13,16] Finally, different analogs have identified partially overlapping but distinct sets of prenylated proteins. Some variability is expected from the complex processing during click-conjugation, purification, and mass spectrometry; from differences in protein abundance, concentration, and location with different cell types; and from differences in the reactivity of prenyltransferases with each analog. However, it is also possible that the location of the alkyne modification in the analog may influence which proteins each analog can modify.

This information led to the hypothesis that the structures of the various analogs affect the substrate selectivity of FTase. Consistent with this, the crystal structure of FPP-bound FTase shows that the second isoprenoid unit of FPP interacts with the amino acid residue at the a_2 position of the bound peptide substrate.[23] Similarly, biochemical studies demonstrate that the steady-state rate constants of farnesylation depend on both the size of the residue at the a_2 position and modifications on the isoprenoid units of FPP analogs.[24–26] Based on these data, we hypothesized that different FPP analogs would alter the peptide selectivity of FTase based on the size of the residue in the a_2 position. To test this hypothesis, four alkyne-modified FPP analogs, analog **1**,[27] **2** and **3**,[28] and **4**[14] (Fig. 1), were synthesized, as previously reported. The affect of these analogs on FTase peptide sequence specificity was then determined by comparing their in vitro kinetic activity to that of FPP for catalyzing farnesylation of a library of peptides with varying CaaX sequences.[29]

Initially, we assayed a peptide library of the form dansyl-GCVa_2S where non-ionizable side chains were varied at the a_2 position. Previous work using FPP demonstrated that when neutral residues were varied in the a_2 position, there was a correlation between the value of k_{cat}/K_M and the volume of the amino acid side chain; the most efficient reactivity was observed with medium-sized residues at the a_2 position (residue volume = 140 Å^3).[24] Here, we compared the value of k_{cat}/K_M with FPP to those obtained using analogs **1**–**4** to determine if the modified structures alter substrate selectivity. These data demonstrate that the structure of the FPP analog alters the dependence of the FTase activity on the size of the residue at the a_2 position (Fig. 2).

Analog **1** does not have an additional bulky group incorporated within the second isoprenoid unit. As predicted, this analog displayed the characteristic 'pyramid' reactivity pattern with FTase as observed for FPP, although the maximal activity (a_2 = valine) is decreased (Fig. 2A). When using analogs **2** and **3**, both of which have the alkyne moiety appended to the second isoprenoid unit as a branch, a different selectivity pattern emerged. We found the reactivity of FTase with **3**, which contains a propargyl group, is more promiscuous than with FPP, with modest discrimination (⩽4-fold) based on the size of the a_2 residue (Fig. 2C). This indicates that using analog **3** in endogenous studies may lead to capturing a wider variety of potential protein substrates of farnesylation. Analog **2**, which incorporates a homopropargyl substituent, displayed a unique pattern where the value of k_{cat}/K_M varied little for peptide substrates with a_2 position side chain volumes between 80 and 170 Å^3. Analog **2**, however, was ineffective at modifying substrates with a_2 residue side chain volumes either smaller than 80 Å^3 or larger than 170 Å^3. Substrates with a_2 residues in these size ranges exhibited more than a 10-fold reduction in rate relative to peptide substrates with midsized a_2 side chain volumes (Fig. 2C). Analog **4**, structurally similar to **1** but one isoprenoid unit shorter, displayed selectivity similar to **1** for residue volumes less than 170 Å^3 (Fig. 2D). However, the reactivity with peptides with bulky a_2 sides chains (Phe, Tyr, and Trp) was decreased.

In light of this evidence that alkyne modifications affect the peptide selectivity of FTase at the a_2 position, we examined if the differences in structure of the analogs affected reactivity with sequences from protein substrates that were previously identified from cells using analogs. To this end, a library of the form dansyl-TKCxxx was designed with sequences from proteins that were previously identified with sequences that either fit or do not fit the CaaX paradigm as well as sequences from prenylated proteins that have not been identified using analogs. Since this peptide library represents biological substrates, there is significant variation in sequence (Table 1) relative to Figure 2. Correspondingly, there is significant variation in observed reactivity for differences in the upstream sequence (TKCaaX vs GCaaX)[11] and in the identity of the terminal a_2X residue pairs, as previously observed.[24]

The first set of substrates tested were those that were identified *in cellulo* using one or more of the analogs, but that were not predicted as FTase multiple turnover substrates. Annexin proteins were reported as FTase substrate from cells in studies using either analog **4**[16] or an FPP-azido analog.[13] The C-terminus of annexin A2 and A3 is –CGGDD; thus these proteins are not predicted as FTase substrates since the cysteine at the C-terminus is 5 amino acids from the end. We demonstrated a lack of modification of annexin peptides catalyzed by FTase in the presence of either FPP or analogs **1**–**4** (Table 1) using the in vitro farnesylation assay. This lack of modification suggests the following possibilities in vivo: an enzyme other than FTase prenylates annexin; more sequence than the mimetic CaaX peptide is necessary for prenylation; and/or detection was an artifact. Two other protein substrates identified as FTase substrates *in cellulo* using analog **1** have CaaX sequences of CVLL[21,22] and CSVL[21] and are likely modified by GGTase-I rather than FTase. In vitro these two peptides (TKCVLL and TKCSVL) showed low multiple turnover activity ($k_{cat}/K_M < 0.2$ mM^{-1} s^{-1}) catalyzed by FTase in the presence of FPP, suggesting that the comparable proteins are not readily farnesylated in cells. A similar minimal activity ($k_{cat}/K_M < 0.1$ mM^{-1} s^{-1}) was also observed with all four analogs (Table 1). Analog **1** has previously been shown to be an in vitro substrate for GGTase-I[27] and this may explain the observed *in cellulo* activity for this and the other analogs.

The results from the in vitro farnesylation assay using expected substrates (TKCCIQ and TKCVLS)[13,21] demonstrated that the peptide substrate selectivity depends on the structure of both the FPP analog and the peptide. Following the same trend as the previous library, analog **4** displayed a similar reactivity pattern as FPP (Table 1). In contrast the other analogs alter substrate selectivity. For example, FTase did not catalyze modification of the TKCCIQ peptide with analogs **2** and **3** while TKCVLS reacted slowly with analogs **1** and **2**. These results indicate that reactivity is affected by both the size of the a_2 residue (167 Å^3 for both isoleucine and leucine) and the identity of residues at other positions. This is not unexpected as it has been previously demonstrated that the rates of farnesylation are also dependent on the identity of the amino acid in the 'X' position.[24]

The last set of substrates tested were those that have been previously shown by radiolabeling[32] or selective inhibition[33] to be farnesylated in vivo but were not detected in large prenyl proteomic studies using analogs. Peptides representing these substrates (TKCVIS and TKCTIQ) were both modified by FPP in the in vitro assay, verifying the previous findings that they are readily farnesylated.[32,33] These substrates were also both modified by analogs **1** and **4** in the presence of FTase (Fig. 3). FTase catalyzed the reactions