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Recent Advances in Protein Prenyltransferases: Substrate Identification, Regulation, and Disease Interventions

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Abstract

Protein post-translational modifications increase the functional diversity of the proteome by covalently adding chemical moieties onto proteins thereby changing their activation state, cellular localization, interacting partners, and life cycle. Lipidation is one such modification that enables membrane association of naturally cytosolic proteins. Protein prenyltransferases irreversibly install isoprenoid units of varying length via a thioether linkage onto proteins that exert their cellular activity at membranes. Substrates of prenyltransferases are involved in countless signaling pathways and processes within the cell. Identification of new prenylation substrates, prenylation pathway regulators, and dynamic trafficking of prenylated proteins are all avenues of intense, ongoing research that are challenging, exciting, and have the potential to significantly advance the field in the near future.

Introduction

Protein prenyltransferases catalyze attachment of lipid moieties onto the cysteine residue of the C-terminus of a protein substrate to enable normally hydrophilic proteins to localize to cell membranes via a hydrophobic lipid modification [1]. Protein farnesyltransferase (FTase) catalyzes the transfer of a 15-carbon farnesyl group from farnesyl diphosphate (FPP) to a substrate protein, and protein geranylgeranyltransferase (GGTase-I) performs the same reaction using a 20-carbon geranylgeranyl diphosphate (GGPP) donor group. The substrate proteins are proposed to contain a "Ca_1a_2X" motif, where `C' is the cysteine residue, `a_1' and `$a_2$' are aliphatic amino acids, and `X' determines whether a protein is modified by FTase or GGTase-I. This lipidation step is followed by cleavage of the -aaX residues catalyzed by an endoplasmic reticulum (ER)-bound protease (Rce1 or ZMPSTE24) and C-terminal carboxymethylation catalyzed by the integral membrane enzyme, isoprenylcysteine carboxylmethyltransferase (Icmt) [2].

Although the "CaaX" prenylation paradigm has served fairly well in describing many of the prenyltransferase substrates, recent studies have shown that this motif is too narrowly defined and does not adequately predict all potential FTase and GGTase-I substrates.

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Advances in understanding prenyltransferase substrate recognition have significantly expanded the pool of potential substrates and thus enabled elucidation of new roles for lipidated proteins in cell signaling pathways. Furthermore, several bacteria were recently discovered to use the mammalian prenylation machinery to lipidate bacterial proteins to enhance membrane localization. Although prenylation is irreversible, new modes of regulation have been identified in mammalian cells that modulate progression of proteins through the prenylation pathway. This review will focus on the most significant findings in the field of prenylation published since May 2010.

Substrate Identification

Identification of the substrates of FTase and GGTase-I has been an important goal for understanding the biological function of these enzymes. Traditional radiological methods where cells are treated with a radiolabeled analog of FPP, GGPP or a precursor of these molecules have not been generally useful for identifying prenyltransferase substrates mainly due to the low signal of the radiolabel [3]. Despite these challenges, great progress has been made in identifying potential and substantiated *in vivo* substrates of the prenyltransferases using peptide library studies, computational techniques, and lipid analogs.

Analysis of the reactivity of prenyltransferases with diverse libraries of peptides has provided significant insight into the substrate selectivity of FTase and GGTase-I, as these enzymes react efficiently with short peptides. Using this approach, Krzysiak and colleagues demonstrated that FTase readily catalyzes farnesylation of a large number of peptides terminating in Leu (-CaaL), contrary to the CaaX paradigm which describes these as canonical GGTase-I substrate sequences [4]. This result expands the pool of FTase substrates and demonstrates additional cross-reactivity with GGTase-I. Hougland and colleagues showed that the identity of the amino acid at the X position affects the selectivity of the amino acid at the a_2 position [5], suggesting that FTase recognizes the entire Ca_1a_2X sequence cooperatively rather than each amino acid individually. Building on this result, a large scale study of the reactivity of a library of 300 dansyl-TKCxxx peptide sequences based on the human proteome [6] revealed two classes of peptide reactivity with FTase: 1) peptides farnesylated under multiple turnover (MTO) conditions; and 2) peptides farnesylated only under single turnover (STO) (excess enzyme) conditions. For the STO substrates, FTase binds the substrate and catalyzes farnesylation but the product dissociates from the enzyme very slowly. Statistical analysis of these two classes of substrates revealed significantly different sequence preferences (Figure 1). The MTO peptides typically display canonical Ca_1a_2X sequences, including Ile and Leu at the a_2 position and Phe, Met, and Gln at the X position. STO FTase substrates are more diverse in sequence, often containing a Ser at the a_2 position and little sequence preference at the X position. The biological role of the STO substrates is not yet clear. Together, these studies show that FTase catalyzes farnesylation of a wide range of substrates, suggesting that a large cohort of proteins is farnesylated *in vivo*.

Computational techniques have also been developed to probe and predict the molecular recognition of the prenyltransferases. London and coworkers developed FlexPepBind to predict FTase substrates based on a calculated binding energy using the Hougland peptide library as a training set [7]. Analysis of the binding energy of all 8,000 possible CaaX peptide sequences identified potential novel FTase substrates. In a test of the validity of these predictions, FTase catalyzed prenylation of 26 out of 29 of the proposed peptides, including a subgroup containing Asp or Glu at the X residue. These data confirm that FTase catalyzes prenylation of a variety of diverse sequences, a significant portion of which do not fit into the traditional “CaaX” motif. Finally, Yang and colleagues used quantum mechanical molecular mechanical studies (QM/MM) to demonstrate a correlation between peptide

structure and transition state structure for FTase [8]. These computational studies indicated that the transition state for farnesylation catalyzed by FTase changes from S_N2-like for CVLS to S_N1-like for CVIM, consistent with previously determined alterations in secondary kinetic isotope effects [9]. This change in transition state structure is likely due to steric interactions between the peptide and FTase.

Lipid donor analogs with reactive moieties have been used to identify *in vivo* prenyltransferase substrates. Upon addition of these analogs to cells, the prenyltransferases incorporate the modified lipids into substrate proteins. The modified lipid is used to enrich the labeled proteins which are then identified using mass spectrometric methods. One approach uses geranylgeranyl-azide analogs that are labeled with alkyne tetra-methylrhodamine using Cu(I)-catalyzed “click” reaction. Using this technique, many known GGTase-I substrates were identified, including members of the Rab protein family, as well as Rap2c, a novel member of the Ras family [10]. Using a similar method, a C10-alkyne analog was used to identify seven *in vivo* FTase substrates, including GNBP, Lamin B1, Rab 1B, Rab 2A, Rab 6A, Rab 7, and Annexin X3 [11]. Alternatively, an immunogenic analog, anilinogeraniol, was incorporated into cellular proteins to identify a number of substrate proteins, including Ras, Rho, and Rac proteins [12]. Finally, a biotin-GGPP analog was used to identify prenylatable proteins in cell lysates using engineered FTase and GGTase-I enzymes [13]. Although these various analogs have successfully identified prenylated proteins *in vivo*, each analog identifies a somewhat different set of substrates, suggesting that the analog structure may alter the prenyltransferase substrate selectivity. This is not surprising as *in vitro* and structural studies demonstrate that FPP and GGPP directly contact the peptide, contributing to molecular recognition of the peptide substrates [14,15].

Regulation of prenylation pathway

Although the importance of prenylation for biological activity of many proteins has been extensively studied, regulatory mechanisms of this pathway are only now being identified. Presence of a polybasic region (PBR) upstream of the CaaX sequence dictates that a prenylated protein is trafficked directly from the endoplasmic reticulum (ER) to the plasma membrane (PM); absence of such a region dictates that the protein undergoes additional processing via the Golgi compartment (GC) such as attachment of palmitoyl groups (Figure 2A) [16].

Williams and coworkers demonstrated that prenylation and trafficking of PBR-containing small GTPases is regulated by their interactions with SmgGDS and by GDP/GTP exchange [17] (Figure 2B). The longer SmgGDS-607 variant interacts with non-prenylated small GTPases and regulates their entry into the prenylation pathway, while a shorter SmgGDS-558 splice variant specifically associates with prenylated small GTPases and regulates their trafficking to the PM. SmgGDS-607 captures newly synthesized small GTPases and prevents prenylation until GDP/GTP exchange occurs leading to dissociation of the SmgGDS-607 complex. For Rho GTPases, SmgGDS-607 may function as a guanine nucleotide exchange factor (GEF), while other GTPases require a separate GEF where SmgGDS-607 may act as a scaffold [17,18]. An electronegative surface patch and substrate binding groove in SmgGDS-607 interact with the C-terminal PBR of RhoA and impart its biological activity.

Once prenylated, Rab and Rho proteins are shuttled between different membrane compartments by guanine nucleotide dissociation inhibitors (GDIs) that sequester and bury the hydrophobic lipid moiety. When the lipidated cargo molecules reach their destination, GDI displacement factors (GDFs) unload these proteins from GDIs. The search for

analogous chaperones for the Ras protein family identified several candidates, including PDEδ, PRA1, and galectin [19]. PDEδ solubilizing factor was shown to modulate the dynamic shuttling of H-Ras and K-Ras, and it is required for the membrane localization of Ras family proteins by facilitating their diffusion in the cytoplasm [20]. In the structure of PDEδ complexed with farnesylated Rheb-GDP, the prenyl group is buried deeply in a hydrophobic pocket of PDEδ [21]. This interaction occurs primarily with the farnesylated and carboxymethylated C-terminus of Rheb and helps to explain the observed lack of specificity of PDEδ for GTPases or their guanine-nucleotide binding states. Dissociation of the Rheb-PDEδ complex is accelerated by formation of a transient ternary complex with Arl2/3-GTP, and since binding of farnesylated Rheb and Arl2/3-GTP to PDEδ is mutually exclusive, Arl2/3 is presumed to be a GDF.

Palmitoylation of prenylated proteins provides additional regulation of membrane localization [22]. While palmitoylation occurs exclusively in the Golgi, depalmitoylation has been observed in all cellular compartments, thus providing directionality to the membrane targeting process. Depalmitoylation can either be non-enzymatic modulated by prolyl isomerase [23] or catalyzed by enzymes such as acyl protein thioesterase 1 (APT1) [24]. Depalmitoylation weakens the interaction of Ras with the PM, allowing its diffusion into the cytoplasm.

Upon farnesylation, the –aaX group of the C-terminus is proteolyzed and the terminal carboxyl group is methylated, catalyzed by Rce1 or ZMPSTE24 and Icmt enzymes, respectively. These modifications are required for proper function and/or localization of many farnesylated proteins such as Rheb proteins [25], but can be circumvented with geranylgeranylated proteins, such as Rho GTPases [26]. Rce1 deficiency has been implicated in hematological malignancies and is essential for photoreceptor cell survival [27]. Icmt inhibition induces tumor cell death and reduces tumor growth *in vivo* [28]; thus, Rce1 and Icmt inhibitors are being investigated as potential cancer therapeutics [29–31].

Role of prenylation in bacterial infection

Introduction of macromolecules, such as DNA and protein, into host cells via a secretion system is a common mechanism used by intracellular pathogenic bacteria. Some of these secreted proteins are effectors that modulate host cell processes to favor bacterial growth and survival. Furthermore, recent experiments demonstrate that effector proteins from *Salmonella enterica typhimurium* and *Legionella pneumophila* can be post-translationally modified by eukaryotic enzymes, including prenyltransferases.

Secreted effector proteins of *Legionella pneumophila*, an aquatic gram-negative bacterium that can cause pneumonia, are prenylated [32,33]. Several *Legionella* effector proteins contain C-terminal CaaX motifs and a number of these effectors exhibit localization to membranes when overexpressed in mammalian cells. Mutation of the CaaX cysteine or FTI treatment disrupts localization, suggesting that these bacterial effectors are modified by host protein prenyltransferases. Ankyrin B, a *Legionella* effector, both interacts with the host ubiquitin machinery and contains a C-terminal CaaX motif (CVLC). The host FTase catalyzes farnesylation of ankyrin B which anchors this protein to the *Legionella*-containing vacuole during infection (Figure 3). This modification is required for sustained infection; *L. pneumophila* expressing ankyrin B with a Cys to Ala mutation in the CaaX box cannot sustain infection in mice [32]. This is the first example where host prenylation of a bacterial effector is required for survival of an intracellular pathogen.

Recently, the FTase inhibitor, FTI-277, has been investigated as treatment of bacterial infection. In septic mice, treatment with FTI-277 significantly increased phagocytosis by peritoneal macrophages in comparison to controls leading to enhanced bacterial clearance

and survival [34]. Additionally, FTI-277 treatment of LPS-injected mice increased survival rates and decreased processes associated with LPS-induced apoptosis, such as caspase-3 cleavage and JNK phosphorylation [35]. These data suggest that FTI treatment may be a viable approach to treating some bacterial infections and septicemia.

Numerous infectious bacteria, such as *Mycobacterium tuberculosis* and *Francisella tularensis*, are predicted to express secreted effectors containing CaaX motifs that may be prenylated by host protein prenyltransferases. Further exploration of this modification would be beneficial in two ways: 1) FTIs and GGTIs currently approved for clinical use could be added to treatment regimens for bacterial infections, and 2) substrate specificity of FTase and GGTase-I can be further defined, as many of the CaaX sequences observed in bacterial genomes are not observed in the human genome. Further delineation of the human and bacterial prenylome will provide insight into the exploitation of prenylation during bacterial infection.

Clinical Investigations

Inhibition of protein prenylation has been pursued as a strategy for cancer therapy since oncogenic Ras proteins require farnesylation for their biological function [36]. Although FTIs have low toxicity, achieving clinical efficacy has been challenging and the most advanced candidates, tipifarnib and lonafarnib, failed to demonstrate efficacy in Phase III clinical trials. One potential reason for the lack of clinical efficacy of FTIs is that GGTase-I can compensate for the inhibited FTase and catalyze prenylation of key proteins, thereby allowing cancer cells to proliferate. Genetic knockouts of both FTase and GGTase-I in a mouse model of K-Ras-induced lung cancer significantly reduced tumor sizes and improved overall survival, suggesting that dual FTase/GGTase-I inhibitors may prove to be a more effective therapeutic approach [37]. This strategy is further supported by two studies: (1) GGTase-I deficiency decreased the severity of K-Ras-induced myeloid leukemia in a mouse model [38] and (2) a dual FTase/GGTase-I inhibitor was more effective than an FTI at inducing apoptosis of myeloid leukemia cells via inhibition of K-Ras prenylation [39].

A second complication in prenyltransferase-targeted therapeutics is the lack of validated biomarkers for patient selection. Currently the most promising cancer indications for FTIs are in hematological malignancies, specifically for patients with leukemia, lymphoma and myelodysplastic syndrome [40–42], and gene and protein expression signatures of tumors from these patients are being studied to help identify response predictors. A high gene expression ratio of Ras guanyl releasing protein 1 and DNA excision repair protein aprataxin (*RASGRP1/APTX*) correlated with a positive response to tipifarnib therapy in patients with leukemia [43] and lymphoma [44] and could potentially be used to predict responsiveness to FTIs. Pro-apoptotic Bim and Bcl-2 proteins could also serve as potential FTI sensitivity determinants in patients with lymphoma; tipifarnib inhibits the Raf/MEK/ERK signaling pathway through abrogation of prenylation of H-Ras or N-Ras GTPases, which leads to Bim and Bcl-2 up-regulation [45]. Given this mechanism of FTI-induced apoptosis, combinations of MEK and Akt kinase inhibitors with FTIs showed synergistic effects and may show clinical benefit [46,47].

Glioblastoma tumors have highly overexpressed epidermal growth factor receptor (EGFR) that leads to increased Ras activity. Kieran and coworkers showed that inhibition of H-Ras farnesylation by lonafarnib potentiated both temozolomide, a DNA alkylating agent currently approved for the treatment of astrocytoma, and radiation treatments in a murine model of glioblastoma [48]. Subsequent clinical evaluation of combinations of lonafarnib with temozolomide [49] and tipifarnib with radiation [50] showed encouraging results.

FTIs are also being explored for treatment of other diseases. Hutchinson-Gilford progeria syndrome (HGPS), a premature aging disease, is caused by the accumulation of prelamin A due to lack of proteolysis of the farnesylated C-terminus in the nucleus [51]. FTIs have been shown to improve this disease in a mouse model [52], and based on these data, lonafarnib entered clinical evaluation for HGPS and is currently being evaluated in Phase II clinical trials in combination with zoledronic acid and pravastatin [53]. Additionally, recent studies have provided evidence that alterations in the lamin A processing pathway may play a role in the aging process in the general population [54,55], and could thus ameliorate some of the age-related vascular degeneration. Farnesylation of ubiquitin C-terminal hydrolase-L1 has been linked to progression of neurodegenerative disorders leading FTIs to be evaluated in patients with Alzheimer's disease [56].

Conclusions

Investigation of protein lipidation remains an exciting field that has progressed significantly in the last few years. *In vitro* peptide library studies with FTase along with computational studies have demonstrated that the CaaX paradigm of substrate recognition does not sufficiently describe the molecular recognition of this enzyme. Expansion of the long-held “CaaX” paradigm heralds a new era of identification of noncanonical prenylated proteins. These studies in combination with multiple novel approaches to identify proteins that are prenylated *in vivo* suggest that complete identification of the human “prenylome” could be realized within a decade.

Furthermore, the assumption that newly synthesized small GTPases enter the prenylation pathway without regulation is no longer plausible. SmgGDS binds to newly synthesized small GTPases and regulates their entry into the prenylation pathway; this protein sequesters unprenylated small GTPases and releases them for prenylation upon receipt of a specific cellular signal. So far SmgGDS has been demonstrated to interact with only a select group of small GTPases, but it is reasonable to speculate that other prenylated proteins associate with SmgGDS-like chaperones that regulate their prenylation status.

Finally, although FTIs have not yet translated into the clinic as a solid tumor cancer therapeutic, there is still potential for using them to treat hematological malignancies and potentially other diseases as well. Excitingly, lonafarnib has shown promise in treating children with HGPS, a devastating premature aging disease. Treatment with FTIs may both halt disease progression and reverse clinical features of the disease in humans, as phenotypic improvement was observed both on cellular and organismal levels in animal models. FTIs also have potential as antibacterial agents as mammalian FTase was found to be involved in intracellular proliferation of *Legionella* bacterium.

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Highlights

- Expansion of the CaaX paradigm by biochemical and computational methodologies
- Improvement of chemical biology tools to define mammalian prenylome
- Identification of proteins that regulate entry into prenylation pathway
- Involvement of mammalian prenyltransferases in bacterial pathogenesis
- Clinical evaluation of inhibitors for cancer and premature aging diseases

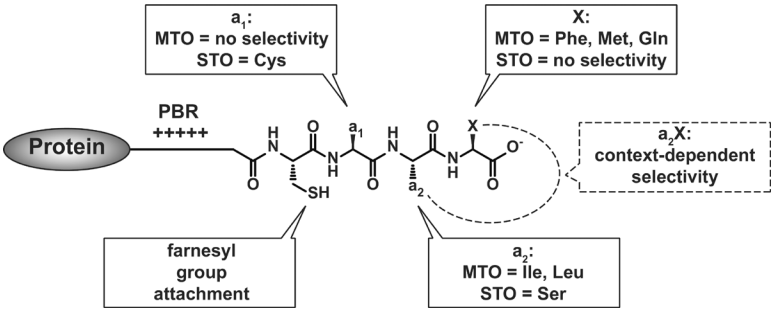

Figure 1.

The substrate selectivity of FTase for multiple turnover (MTO) and single turnover (STO) substrates. The region upstream of the CaaX sequence may contain a polybasic region (PBR). The selectivity at the a_2 and X positions is context-dependent.

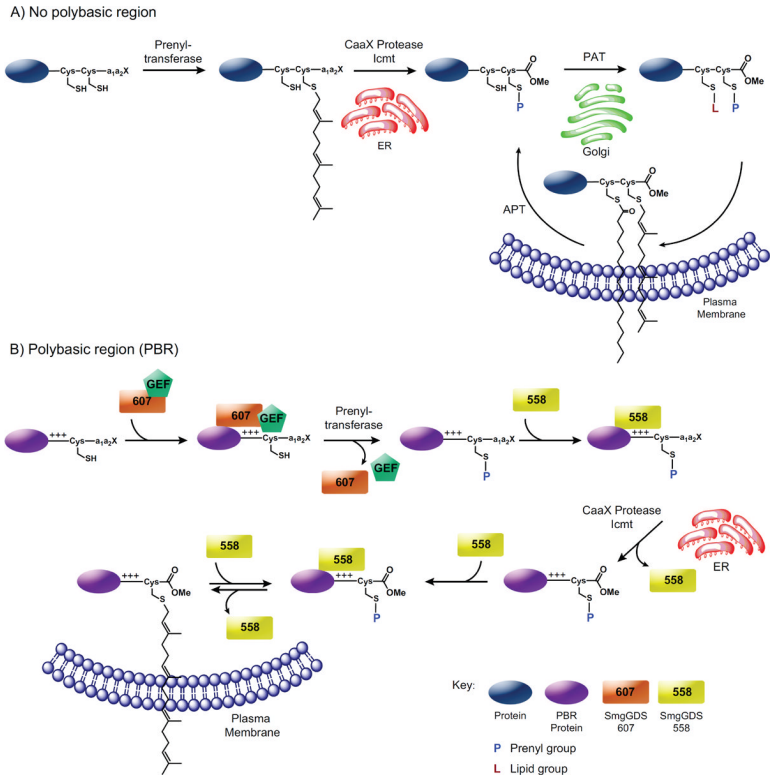

Figure 2.

Models for the trafficking and regulation of prenylated proteins. The farnesyl group is depicted here to represent prenylation. A) The prenylation of a Caax-containing protein is catalyzed by FTase or GGTase-I in the cytsosol. Next it is trafficked to the ER where it is proteoylzed by a CaaX protease such as Rce1 and carboxymethylated by isoprenylcysteine methyltransferase (ICMT). If the protein contains another cysteine near the C-terminus, a protein acyltransferase (PAT) can catalyze the palmitoylation of the protein at the Golgi. Then, the modified protein is trafficked to the plasma membrane. A protein acylthioesterase (APT) can catalyze the removal of the palmitoyl group, allowing for regulation of proteins between different cellular compartments. B) The SmgGDS-607 isoform and possibly a GEF-like protein may interact with an unmodified CaaX protein containing a polybasic region (PBR), promote GDP/GTP exchange, and allow the CaaX protein to enter the prenylation pathway. Once prenylated by GGTase-I or FTase, SmgGDS-558 may facilitate the release of the protein from the prenyltransferase and/or traffic the protein to the ER where it is subsequently proteolyzed and methylated. SmgGDS-558 may also transport the fully processed prenylated protein to and from the plasma membrane.

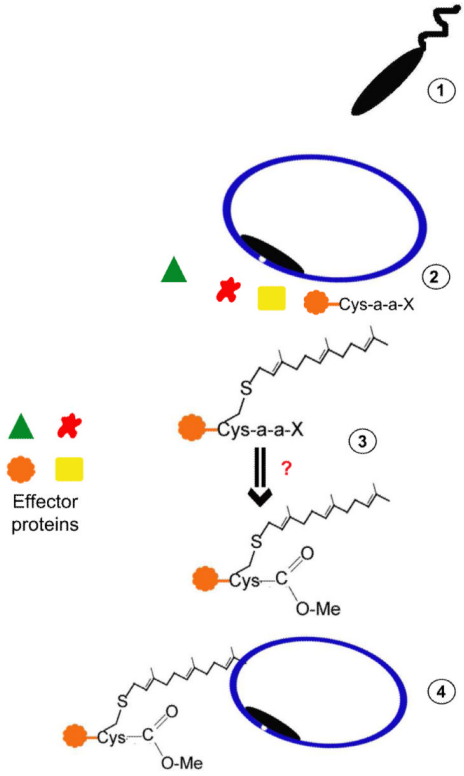

Figure 3.
Bacteria hijack host prenylation machinery during infection. Upon bacterial entry via phagocytosis (1), the infectious bacterium is surrounded by a vacuole and immediately secretes effectors into the host cytosol (2). Effectors containing a CaaX box may be prenylated and further modified by other enzymes implicated in the prenylation pathway (3). These modifications may help target bacterial effectors to the cytosolic face of the bacteria-containing vacuole or elsewhere in the host cell (4).