Tetrahedron 81 (2021) 131917

Contents lists available at ScienceDirect

Tetrahedron

journal homepage: www.elsevier.com/locate/tet

Labeling of polyprenylated macromolecules using mild Ene reactions

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ARTICLE INFO

Article history:
Received 19 November 2020
Received in revised form
12 December 2020
Accepted 28 December 2020
Available online 6 January 2021

Keywords:
Prenyl
Fluorination
Ene
Labeling
Geranylated

ABSTRACT

Protein posttranslational modifications play pivotal roles in a wide range of functions such as gene expression regulation, differentiation, cell migration, various human diseases and so on. Polyprenylated group are a lipid modification only found in the Ras super family protein. It was demonstrated that farnesyl, geranylgeranyl groups are covalently attached into sulfur atom on cysteine of *Caxx* motif, playing essential roles in anchoring on plasma membrane and signal transduction etc. We first reported a straightforward chemical approach to labelling of polyprenylated macromolecules. Our method mainly relied on a novel fluorination/Ene reaction, exhibiting application potential in the study of proteome-wide mapping of protein prenylation target.

1. Introduction

Covalent modifications on histone tails have been well identified, involving in the regulation of a wide range of physiological process in living cell [1]. Protein posttranslational modifications include methylation [2a], acetylation [2b], phosphorylation [2c], sulfation [2d], *N*-myristylation [2e], S-palmitoylation [2f], prenylation [2g] and many others [3]. Two types of protein post-translational modifications by lipids have been documented, namely, palmitoylation and prenylation. The prenyl groups are covalently attached to cysteine residues of a subset of Ras superfamily proteins in eukaryotic cells (this polyprenylated modifications also found on nucleic acids termed geranyl) [9]. This polyprenylated modifications have demonstrated to be localized into a carboxyl-terminal ***Caax*** motif, whereas ***C*** represents cysteine, and ***a*** represents aliphatic amino acid and ***x*** is any amino acid. In 1978, Fujino and co-workers first reported the prenylated protein in fungi [4]. In 1988, this lipid-modification have been found in mammalian cells in Lamin B by Glomset and coworkers [5]. Recently, genome-wide analysis of protein posttranslational modifications of the human cell has revealed that there are more than 600 potentially prenylatable proteins [6]. Prenylations were facilitated by three types of eukaryotic protein prenyl transferases, namely FTase (farnesyl transferases) and GGTase (geranylgeranyl). Polyprenylated (including geranylgeranyl and farnesyl) modifications in Ras family proteins are highly conserved, playing diverse roles in varieties of fields, for instance, signaling transduction pathway are activated by prenylation via selectively anchoring cell membrane and lipid side chain [7]. It has been noted that prenylated proteins are involved in a wide range of human diseases such as precocious aging, malignant disorders osteoporosis and up to 30% of cancers are associated with protein prenylation.

While evolving technologies for global profiling of gene expression regulated by polyprenylated modification have achieved significantly, technique for selective and direct enrichment of prenylation proteomic with reduced complexity remains elusive. The efficiency of current methods is very dependent on the reaction specificity in virtue of competitive nonspecific interaction in the complicated endogenous environment. In order to form a covalent adduct based on the intrinsic property of polyprenylated group(s), unique chemical reaction with fast kinetics and high specificity deserve to be explored. Proteomic investigations of polyprenylated protein have been reported in recent years [7], in which the metabolic labelling using prenylated analogues was carried out to selectively tag prenylated proteins with a clickable handle (i.e., azide or alkyne). These modified proteins are further modified by bioorthogonal click reactions, adding either a fluorophore for gel-based proteomic or a biotin moiety for enrichment of polyprenylated proteins (route 1, Fig. 1) [7a-7b]. However, chemical modifications on prenyl functionalities may lead to the structure perturbation, critical prenylation information may be incomplete or missing. Therefore, exploration of molecular tools for selective

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https://doi.org/10.1016/j.tet.2020.131917
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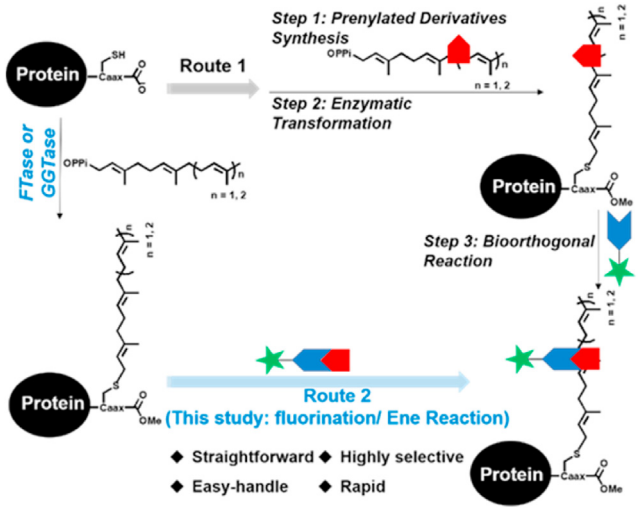

Fig. 1. Strategies for genome-wide analysis of prenylated proteins. Traditional approaches relied mainly on the derivation of prenyl functionalities with the so-called handles (i.e., azido, propargyl) for the subsequent biorthogonal reaction, biotin group thus are able to be introduced for further enrichment. Our strategy focuses on the chemical reactivity of prenylated functionality, providing straightforward route for labelling or enrichment studies. Route 1 include: Step 1, synthesis of prenylated derivative; Step 2, evaluation of the recognition efficiency by corresponding enzyme; Step 3, utilization of biorthogonal reaction to label or enrich target proteins. This study use fluorination/fluorescent PTAD to label polyprenylated proteins.

and direct detection and quantification of prenylated proteins would be great beneficial for early diagnosis study of Ras related cancers, such as lung cancer, rectal cancer and pancreatic cancer [7d,2g].

Considering the protein abundances varying across a wide dynamic range in cells and probably prenylations are sub stoichiometric (approximate 0.4% in tRNA, compared to 0.4–0.6% of methyl in proteome), sensitive enrichment tool for capturing polyprenylated group along with mass spectrometry-based proteomics profiling is in high demand. Global analysis of posttranslational polyprenyl modifications could largely be restricted by the synthesis of the polyprenyl analogues, recognition efficiency of prenyl transferases and beyond. We aim to develop a chemical probe based on polyprenylated group's functionality and usher it as a surrogate to profile polyprenylated protein in *vitro* and in *vivo*. On the basis of our preliminary results, we reported here a straightforward and chemical approach for detection of polyprenylated macromolecules via the so-called fluorination/Ene reaction (Fig. 1). In view of the unique nature of prenyl functionality, we explored and developed a practical [2,3]-rearrangement process using Selectfluor/4-phenyl-3H-1,2,4-triazole-3,5(4H)-dione (PTAD) derivatives to introduce a fluorine or fluorescence/biotinated groups to the target macromolecules under mild condition. Given the importance of polyprenylated groups in Ras relevant proteins, our approach was able to omit a complicated synthesis of prenyl analogues process and further substrate recognition by prenyl transferase.

To address these challenges, we reported herein a chemically fluorination/Ene reaction of polyprenylated macromolecules to facilitate the detection of polyprenylated protein, thus paving the way in the extensive study of protein (or nucleic acid) prenylation (route 2, Fig. 1). The process was mainly relied a fluorination/Ene reaction depending on the reaction activity of the prenyl functionality, which can be carried out in a physiological condition. Moreover, intensive studies of prenylated molecules, enabled by our Ene reaction along with the fluorine-19 nuclear magnetic resonance, would facilitate deep understanding of genome-wide protein prenylation. Application potential for the studies of genome-wide polyprenylated proteins, especially associated with various cancer disease are also underway.

To explore the feasibility of the approach, the dynamic property of a model reaction with polyprenylated functionality has been implemented. The reaction of geranyl acetate with Selectfluor reagent was performed in a mixture of PBS/acetonitrile at ambient temperature. The reaction proceeded smoothly, forming the desired products (1a and 1x, which means several fluorine atoms attached) in 5 min (Fig. 2A, top). It has been proposed that this process was enabled by the so-called Ene-reaction [8]. Notably, several point(s) in allylic positions of geranyl acetate have been identified to be attached with fluorine atom(s) (Supplementary Material, S1–S6). Crude or purified products have been proven to be attached with several fluorine atoms, which was confirmed by high-resolution liquid chromatography-mass spectrometry and NMR studies (S1–S6, Supplementary Material). The results hinted that polyprenylated substrate was able to be selectively modified with fluorine atom(s) under the mild reaction condition by use of commercially available Selectfluor reagent. The fluorination proceeded in a superfast manner, exhibiting significant application potential in the study of labeling and detection of polyprenylated proteins. As mentioned, method used for distinguishing farnesyl and geranylgeranyl substrates by use of chemical tool remain

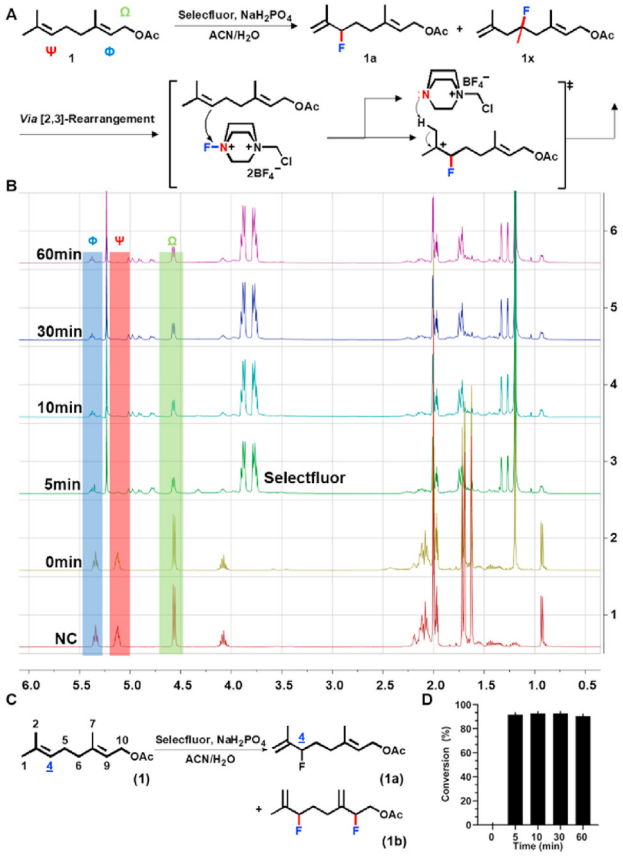

Fig. 2. Investigations of model reaction of fluorination of geranyl acetate. (A) Proposed reaction mechanism of the fluorination of geranyl acetate (**1**) with Selectfluor. (B) Proton NMR monitoring of the model reaction. The conversion of the reaction was calculated by use of internal standard tertbutyl alcohol. The conversion reached up to 90% in as soon as 5 min (Ψ, red color). (C) Isolated products (**1a** and **1b**) and the calculated conversion results. Over 90% conversion in 5 min was observed under the physiological condition. NC represents negative control. Three duplicates were run.

elusive (Chart 3, SI) [7a]. To explore our method's practicality, we performed the competition reaction of geranyl acetate/geranylgeranyl alcohol with Selectfluor in a mixture of acetonitrile and PBS buffer. We noticed that the former substrate seemed to proceed in a more rapid manner. (Fig. 1).

Although the Ene reactions of nitroso derivatives with prenylated (Fig. 3B)substrates have been reported [9], there is no such fluorination/Ene reaction carried out in aqueous phase or utilized in genome-wide analysis of protein prenylation. With these in mind, we next sought to investigate whether our method can be utilized in the studies of detection of macromolecules, i.e., peptide or proteins. Bovine serum albumin (BSA) was selected as a model. First, the 4-nitrophenyl geranyl formate (**4**, Fig. 3A) was synthesized in linear two-steps, which was identified by NMR and mass spectrometry. To testify the viability of our method, this intermediate (**4**) was used to in *situ* modify the BSA (fraction V, **5**). The geranylated BSA was shown to be a very narrow band (Fig. 3B, Lane 3) after the attachments of this hydrophobic group into the various lysines (and other amino acids) of BSA at different sites. Next, the fluorination with Selectfluor reagent was performed, which was analyzed by the gel electrophoresis assays. Upon observation of tiny newly-generated band in the gel electrophoresis assay (Fig. 3B, Lane 4), we aimed to verify whether our fluoridation can be utilized in the study of detection of polyprenylated BSA via ^{19}F NMR experiment. Delightedly, a fluorine NMR signal at −179.99 ppm (Fig. 3C) was indeed observed, which was consistent with reported data [8b]. To further illustrate our hypothesis, we also sought to run the mass spectrometry of this crude samples (Table S1 and S2). It was demonstrated that K187 of BSA was successfully attached with the geranyl group, followed by the selective attachment with one and two fluoride atoms (*m*/*z* 198 and *m*/*z* 216, red color in Fig. 3D). Moreover, three additional sites (K273, K275 and K413 in red color, respectively) have further been identified to be anchored with geranyl groups and further attachment with fluorine atom(s). It was suggested that our fluorination was a rather random process based on the crystal structure of BSA (Fig. 3D and Fig. S10), modifications were identified at varied sites of lysines in BSA (Fig. 3D, red parts),

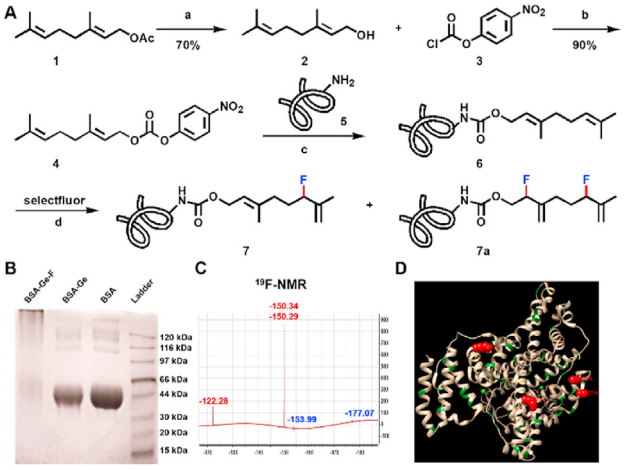

Fig. 3. Selective detection of geranyl-modified BSA by fluorination. (A) Reaction conditions: (a) potassium carbonate (3 eq.), MeOH, r. t., 12 h, 70%. (b) DIPEA, CH_2Cl_2, r. t., 12 h, 90%. (c) PBS/CH_3CN, r. t., 30 min. (d) Selectfluor (100 eq.), PBS/CH_3CN, r. t., 30 min. Identify of the fluoride BSA by SDS-PAGE and ^{19}F Nuclear Magnetic Resonance. (B) Gel electrophoresis analysis: BSA lane is the sample (**5**) used herein. BSA-Ge is the sample (**5**) treated with excess of geranylated reagent (**4**). BSA-Ge-F is the sample (**6**) treated with excess of Selectfluor reagent. Only tiny band (55 kDa) are observed. (C) The ^{19}F NMR of samples (**7**) shown −177.07 ppm fluorinated peak. (D) Structural analysis of BSA (chain A form, PDB 3v03), Red color residues indicated the fluorinated geranyl-modified sites and sequence were also depicted (Supplementary Material, Fig. S3).

although light modifications may be incomplete and ignored due to the sensitive of the NMR and mass spectrums. In a word, we proven herein a simple addition of the Selectfluor reagent into BSA in *vitro* would facilitate the detection of the polyprenylated protein by ^{19}F NMR spectroscopy and mass spectrometry.

Similar results for polyprenylated peptides have also been identified (Supplementary Material, Schemes S2 and S3, S10). Either by employing organic synthesis (peptide **I**, Gly-Cys (SStBu)-Met-Gly-Leu-Pro-Cys (geranyl), Supplementary Material, Scheme S2, Fig. S13), or from other source (peptide **II**, Phe-Gly-Gly-Gly-Lys-Lys-Lys-Ser-Lys-Thr-Lys-Cys (farnesyl), Supplementary Material, S15), we performed the fluorination reaction based on the established method. The prenylated intermediate peptide **I** and peptide **II** were both isolated and identified by fluorine-19 NMR. Mass identification of the fluoride peptide **II** remains elusive, since it has reported that the geranyl/farnesyl motifs are liable to decompose [10] (unreported results). Nevertheless, the fluorine NMR along with mass spectrum results indicated that the target fluoride peptides were proven to be formed, indicating the application potential by use of our fluorination.

Eventually, the application of the polyprenylated group involving Ene reaction in the study of live cell labelling was investigated. In addition to fluorination process, we adopted the Ene reaction using PTAD derivatives considering that fluorination is not feasible with conventional fluorescent labeling [11]. In order to introduce the polyprenylated (geranylgeranyl, gege) functionality onto live cells, the antibody cetuximab was chosen as a substrate for the incorporation of geranylgeranyl functionality and further installation gege-cetuximab for specifically targeting antigens on HeLa cells through the specific binding to EGFR on the HeLa cells surface (Fig. 4B). Upon addition of our well-designed FITC-PTAD reagent (Fig. 4B, dash frame), we observed that the cells with the gege-cetuximab on the cell surface was able to be labelled with green fluorescence by using FITC. It has been demonstrated that intense green fluorescence was observed on the cell surface upon addition of FITC-PTAD, although the difference was minimal (in the presence or absence of gege-treatment, Supplementary Material, Fig. S22), partly due to the geranylgeranyl functionality bearing no fluorescence and HeLa cells itself bind extensively with this FITC-PTAD reagent in a nonspecific manner (Supplementary Material, Fig. S22). In addition, control experiment using FITC-PTAD to incubate with HeLa cells without gege-cetuximab treatment showed tiny fluorescence signal (Supplementary Material, Fig. S22, right lane). Taken together, our HeLa cells labelling assays confirmed the successful of the Ene reaction of geranylgeranyl functionality with our well-designed FITC-PTAD reagent. The capability of control chemical ligation in living systems with temporal resolution will pave the way to various biological applications of this polyprenylated-involving bioorthogonal click reaction.

2. Conclusions

In summary, we reported here a method using the fluoridation/Ene reaction to introduce a fluorine atom or fluorescent/biotinated functionalities into the macromolecules (proteins and peptides). The reactions have been shown to proceed smoothly in a superfast manner (5 min). We demonstrated that the polyprenylated BSA or peptides (I and II) can be further modified by fluorination following our established protocol. In regard for application in living cell, we modified the cetuximab with geranyl functionality, which was facilitated by the cetuximab-EGFR selective binding, the fluorescent labelling of HeLa cells was demonstrated to be efficient. Method applied to investigate the genome-wide protein prenylation remain elusive and tedious, our approach exhibits potential in the genome-wide study of protein prenylation. While evolving applications of

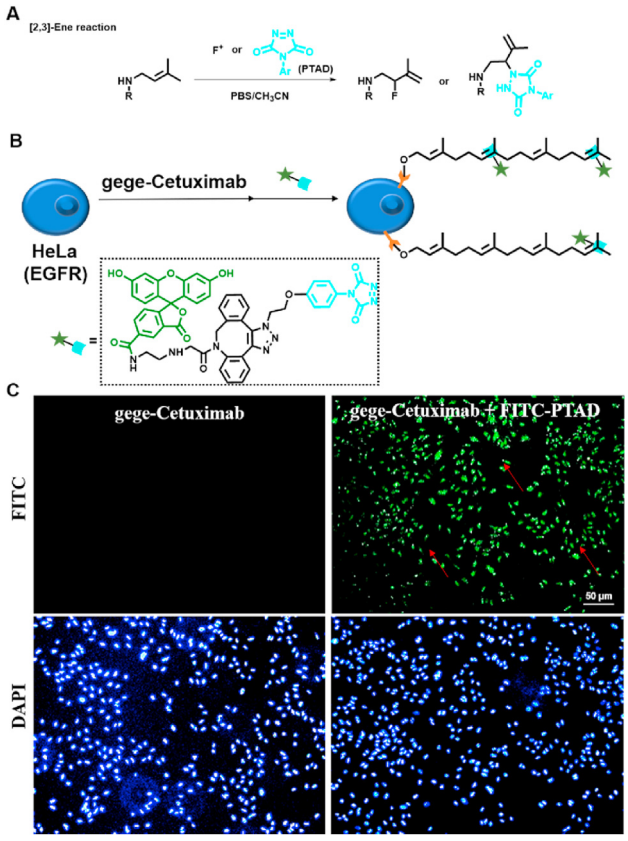

Fig. 4. The mechanism of prenyl-involved Ene reaction and its labeling of HeLa cells in *vitro*. (A) [2,3]-Ene reaction using fluorinating reagent or PTAD derivative under PBS/CH_3CN condition at ambient temperature. (B) Overall strategy of geranylgeranyl modified cetuximab targeting EGFR of HeLa cells, and subsequently fluorescent labeling using our well-designed FITC-PTAD reagent. (C) Confocal fluorescent labeling of the Geranylgeranyl-modified HeLa cells. Blank control; GeGe-cetuximab (400 nM), Cetuximab (400 nM); GeGe-cetuximab (400 nM) + FITC-PTAD (6.70 μM), Cetuximab (400 nM) + FITC-PTAD (6.70 μM); FITC-PTAD (6.70 μM). Addition of FITC-PTAD on the geranylgeranyl modified HeLa cells shown an obvious green fluorescence, although the fluorescence on the nuclear staining in non-obvious. Scale bar: 50 μm. GeGe is short for geranylgeranyl. FITC represents fluorescein isothiocyanate, as indicated in green color. PTAD means 4-phenyl-3H-1,2,4-triazole-3,5(4H)-dione, as marked in cyan color.

bioorthogonal reaction have been reported in recent years, our future work will focus on optimizing tools such as Ene reaction displayed herein for direct and easy capturing of prenylated protein, so that those techniques are applicable to decipher molecular mechanism of specific prenylated modification to be further evaluated in clinical contexts. It has been revealed that PTAD reagent is also able to be attached into tyrosine [11], the fluorination maybe a good choice for selective labeling of polyprenylated molecules. Nevertheless, application of our strategy to tracking and labeling of proteins associated with Ras-related clinical bio-marker or polyprenylated oligonucleoside is underway in my lab and will be reported in due course.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

R. Wang thanks the starting fund from Huazhong University of Science and Technology for financial support. One-Hundred-Talents Youth Program of Hubei Province is appreciated. NSFC (21904044) is appreciated for financial support. We thank Prof. Chaomei Xiong for NMR experiment and Prof. Lingkui Meng for mass spectrum.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tet.2020.131917.

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