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Site-specific sortase-catalyzed lipidation of proteins

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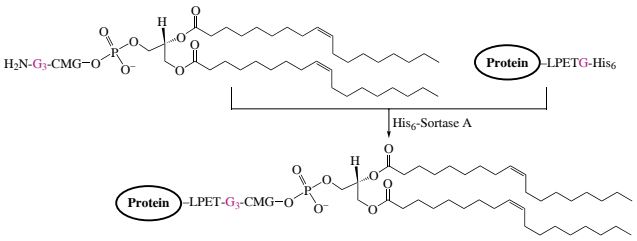
A methodology for the enzymatic ligation of proteins with a lipid is proposed, a feature of which is the use of a hydrophilized [thanks to a CMG(2) spacer] and therefore highly water-soluble lipid. Its H_2N-Gly_5 terminus and the recombinant protein amino acid sequence LPETG are substrates of the enzyme sortase A. Two lipid-ligated proteins, red fluorescent mCherry and Protein A, are shown to be inserted into the cell membrane.



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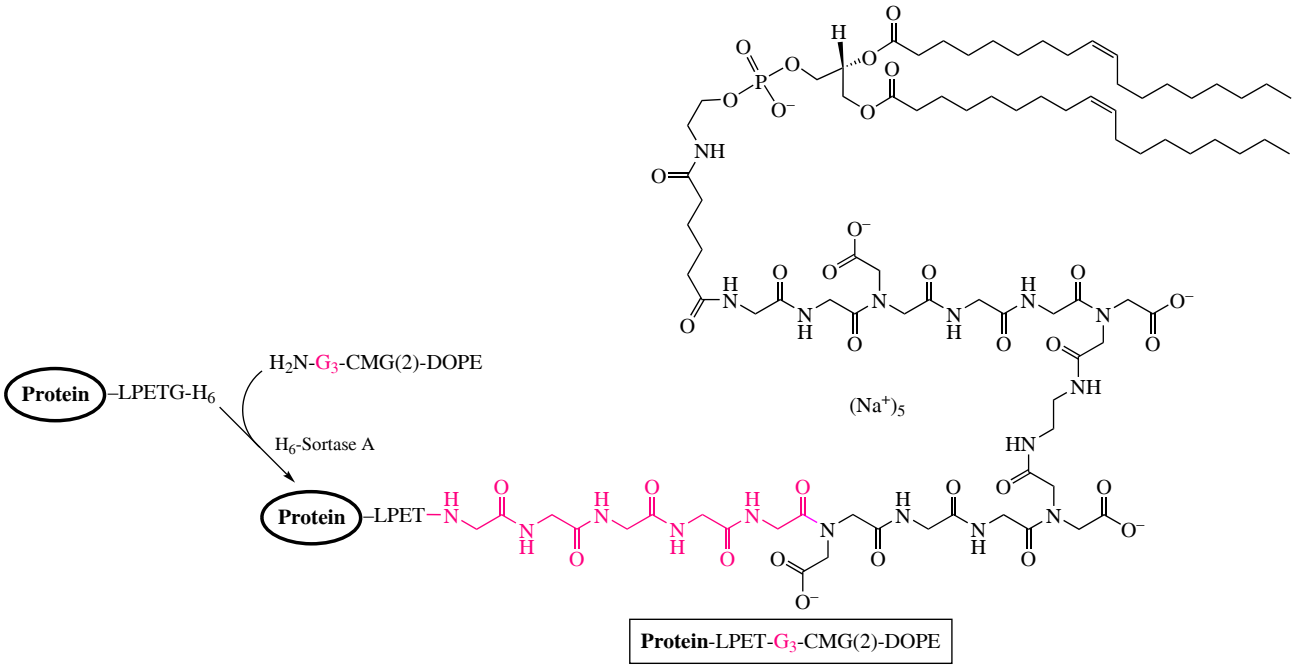
Modification of the membrane of a living cell is achieved using genetic,[1,2] chemical,[3–8] or enzymatic[9–14] strategies, which are discussed in detail in the review.[15] One more, apparently the most controllable modification method, is the insertion into the membrane of a protein monosubstituted with a lipophilic tail, ideally, if the substitution is site-specific, *i.e.*, only one certain amino acid is lipidated. For specific lipidation, the enzymatic process is the most promising, and sortase A (SrtA)[15,16] is well suited for this purpose. Enzyme SrtA transfers any chemical fragment in the form of an oligoglycine derivative to a protein that has an additional short peptide LPXTG (Leu-Pro-X-Thr-Gly, X is any amino acid).[17]

In this work, we performed sortase-promoted modification of recombinant protein A (ProtA)[18] and red fluorescent protein mCherry using 1,2-di-*O*-oleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE). ProtA is capable of binding immunoglobulins,[19,20] which opens up the possibility of loading any immunoglobulin onto the cell surface, and the lipophilized version of the mCherry protein[21] opens up the possibility of gently fluorescent labeling living cells. Modification of cells by lipidated proteins has been described previously,[22] the advantage of our construct is a highly hydrophilic spacer between DOPE (and, therefore, the lipid bilayer of the cell) and the protein, which, firstly, makes the inserted protein more accessible for further interaction and, secondly, makes the lipid attached to the protein water-soluble. We used a flexible-rigid spacer CMG(2)[23,24] [the designation CMG was introduced for the fragment with repeating *N*-(CarboxyMethyl)Glycine motif] with a length of ~7 nm (Scheme 1), consisting of repeating glycyl-glycyl-(*N*-carboxymethyl)glycyl motifs. The oligoglycyl region of the spacer serves as an excellent substrate for SrtA. In addition to the LPETG motif, the recombinant protein contains an auxiliary six-histidine fragment (see Scheme 1, His tag), which is cleaved off as a result of the enzymatic reaction; its role is to facilitate the isolation of the original protein during its production, as well as the separation of the target lipidated protein from the original protein and SrtA. Substrate H_2N-Gly_3-CMG(2)-DOPE (hereinafter referred to as lipid) was synthesized as follows: CMG(2)-DOPE amine[25] was condensed with Fmoc-Gly_3 NHS ester in DMSO/DMF followed by deprotection with piperidine, yield 86%. The enzymatic reaction was carried out in aqueous buffer solution at a molar ratio of 3 : 30 : 1 both for mCherry-LPETG-His_6/lipid/SrtA and ProtA-LPETG-His_6/lipid/SrtA. The reaction time was optimized by sampling the reaction mixture over 24 h and analyzing its composition using HPLC; the maximum yield was achieved after 3 h in both cases. The experimental details are given in Online Supplementary Materials.

N-(Carboxymethyl)glycines (CMGs) represent a family of molecules built from the repeating glycyl-glycyl-(*N*-carboxymethyl)glycyl motifs; the number of repeats can be 4, as in CMG(2) used here, or 8, as in the 11-nm long CMG(4), or can take another value.[23] The presence of His tag in both modified proteins, as well as in SrtA, caused the key stage in the isolation procedure of the aimed conjugates, namely, binding the tag-containing residual proteins and the already unnecessary SrtA to a nickel affinity resin. Note that the tag contained in mCherry and ProtA is cleaved off during the enzymatic conjugation (see Scheme 1), that is, it is absent in the target condensation products; final purification was carried out on TOYOPEARL Butyl resin to remove lipophilic impurities and side peptides.

We attribute a good yield of 64% for the condensation of ProtA to the fact that it is a natural substrate of SrtA in *Staphylococcus*.[17] On the contrary, mCherry turned out to be a suboptimal, ‘non-native’ substrate for this type of sortase (the yield in the condensation reaction was 19%) and, in addition, the construct of the original mCherry with the tag apparently turned out to be suboptimal. However, a practical isolation procedure made it possible to obtain its lipophilic conjugate in an individual state (as shown by HPLC and SDS-PAGE).

Since lipidated proteins are intended to be inserted into the plasma membrane, DOPE was chosen as the lipid moiety, which is much better inserted into the cell than the similar distearoyl



Scheme 1 Sortase-promoted conjugation of a protein with H_2N-G_3-CMG(2)-DOPE the detailed formula of which is depicted on the right (the *N*-end marked as G3 actually has five glycines, two of them belong to CMG motif); G is glycine, H_6 is His tag. *Reagents and conditions*: 65 μM protein (mCherry-LPETG-His_6 or ProtA-LPETG-His_6), 650 μM lipid H_2N-G_3-CMG(2)-DOPE, 21.5 μM SrtA in 50 mM Tris·HCl (pH 8.0) buffer, 37 °C, 3 h. To isolate the target conjugate, Ni-IMAC SepFast metal chelate and TOYOPEARL Butyl resins were used.

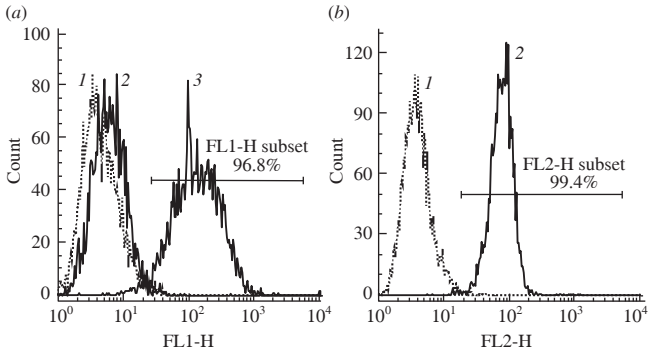


Figure 1 (*a*) Flow cytometry analysis of lipidated ProtA insertion into cells. EA.hy926 cells were washed with DMEM-F12-0.3% FCS (Dulbecco's Modified Eagle Medium containing 0.3% fetal calf serum), and DOPE derivative of ProtA (5 μM) was inserted into the monolayer and incubated at 37 °C for 1 h. The cells were removed from the plastic with Versene solution, triple-washed with PBA (PBS containing 0.2% BSA), incubated with an IgG-FITC conjugate (anti-mouse, diluted 1:50 in PBA) at 4 °C for 30 min, then triple-washed with PBA. Dotted line *1*, binding of the construct-free cells with IgG-FITC (negative control); line *2*, unmodified ProtA + IgG-FITC (second negative control); line *3*, binding of lipidated ProtA followed by IgG-FITC. The number indicates the percentage of bound cells. (*b*) Insertion of lipidated mCherry (line *2*) in EA.hy926 cells, the cells were treated as above. Flow cytometry data, 99.4% of cells are red fluorescence positive; the dotted line *1* corresponds to non-lipidated protein mCherry (negative control). On the fluorograms, the log of fluorescence intensity FL1-H, or FL2-H X-axis were plotted against cell number Y-axis.

derivative DSPE, or oleoyl version with a single fatty residue instead of two ones (OPE),[26] or a small alkyl residue, as described in literature[22] for the sortase reaction. The significant insertion of DOPE-derived ProtA into endothelial cells was demonstrated by flow cytometry [Figure 1(*a*)], the fluorescently labeled immunoglobulin capable of binding to ProtA with its Fc fragment was used for visualization. Insertion of DOPE-derived mCherry was verified directly by flow cytometry [Figure 1(*b*)].

To conclude, the hydrophilized lipid reagent proposed here for sortase-catalyzed lipidation allows the synthesis of DOPE-modified proteins in an aqueous buffer without the addition of an organic solvent or detergent.

This article does not contain any research involving humans and animals as research objects.

Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi: 10.71267/mencom.7613.

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