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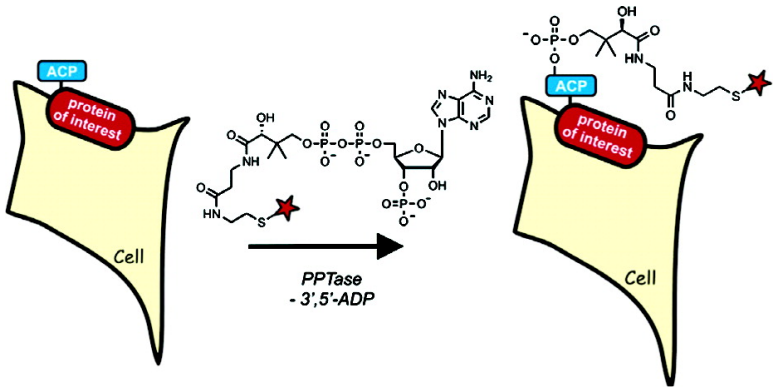
J. Am. Chem. Soc., **2004**, 126 (29), 8896-8897• DOI: 10.1021/ja048396s • Publication Date (Web): 03 July 2004



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Published on Web 07/03/2004

Specific Labeling of Cell Surface Proteins with Chemically Diverse Compounds

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Current approaches for the specific and covalent labeling of proteins with synthetic probes in live cells are mostly based on the presence of reduced cysteines, making their application toward proteins that face the extracellular space problematic.[1−4] Here we introduce a general method for the covalent labeling of fusion proteins on cell surfaces with chemically diverse compounds such as fluorophores, affinity ligands, or quantum dots. The specificity and versatility of the approach make it unique among the existing methods for protein labeling on cell surfaces and should allow it to become an important tool for cell surface engineering.

Our labeling scheme for the covalent modification of cell surface proteins makes use of the post-translational modification of acyl carrier protein (ACP) by phosphopantetheine transferase (PPTase), leading to the transfer of 4′-phosphopantetheine from coenzyme A (CoA) to a serine residue of ACP.[5] It has been previously shown that the modification of the thiol of the phosphopantetheine moiety of CoA with synthetic molecules does not interfere with the transfer reaction.[6−8] Using modified CoA derivatives as substrates and an appropriate PPTase should therefore allow for a specific labeling of ACP fusion proteins displayed on cell surfaces with a diverse range of synthetic molecules (Figure 1). The display of ACP on the cell surface separates the cell-impermeable CoA derivatives and the appropriate PPTase from host PPTase and underivatized CoA, thereby suppressing unwanted side reactions. Since the main goal of our work was to achieve specific labeling of ACP fusion proteins on the surface of eukaryotic cells, we chose the ACP/PPTase pair from *Escherichia coli*. PPTase from *E. coli* (AcpS) can be easily purified and possesses a relative narrow substrate specificity with respect to ACP.[5,9] Furthermore, bacterial ACPs are not efficient substrates of mammalian PPTases.[10] ACP from *E. coli* is a protein of only 77 residues containing no cysteine; the cofactor is attached to Ser36.[11,12] For the labeling experiments, we prepared CoA derivatives carrying either fluorescence or affinity labels (Figure 1B). The synthesis of such substrates comprises only the reaction of CoA with known maleimide derivatives.[8]

We first tested the labeling of recombinant ACP carrying an N-terminal 6×His tag (6×His-ACP) in vitro using CoA-Bt as the substrate and recombinant 6×His-PPTase (Figure 1C). The biotinylation was found to be complete after 10 min and, as indicated by a gel shift assay, nearly quantitative (Figure 1D). The amount of 6×His-ACP not biotinylated in this experiment (∼20%) corresponds to the amount of holo-6×His-ACP already present in the sample (Supporting Information). To evaluate the influence of the thiol-modification of CoA on the specificity constant k_{cat}/K_M of the reaction, we performed competition experiments by varying the concentration of unmodified CoA at fixed concentrations of either CoA-Bt or CoA-Dg and measured the extent of labeling as a function of the CoA concentration by Western blotting (Figure 1E).

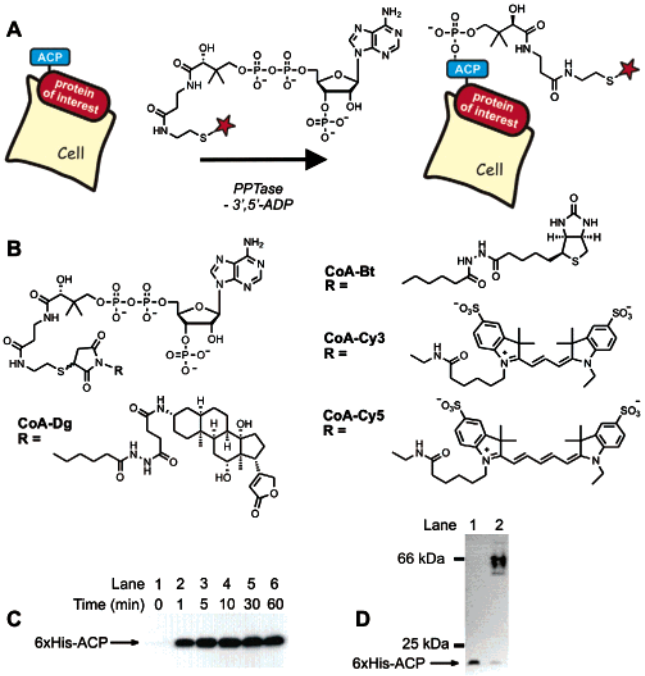

Figure 1. (A) Labeling of ACP fusion proteins on cell surfaces. (B) CoA derivatives used for labeling with biotin (CoA-Bt), digoxigenin (CoA-Dg), Cy3 (CoA-Cy3), and Cy5 (CoA-Cy5). (C) Analysis of the reaction of 6×His-ACP (1 μM), 6×His-PPTase (0.2 μM), and CoA-Bt (5 μM). At indicated times, aliquots were removed from the reaction mixture, and biotinylation of 6×His-ACP was probed by Western blotting using a streptavidin−peroxidase conjugate. (D) Quantification of biotinylation of 6×His-ACP. 6×His-ACP (3 μM), 6×His-PPTase (5 μM), and CoA-Bt (10 μM) were incubated for 30 min and dialyzed, and aliquots of the sample were incubated with streptavidin and applied to SDS-PAGE (lane 2). The formation of a stable biotin−streptavidin complex leads to a gel shift of biotinylated protein. The amount of biotinylation was estimated by comparing the band intensity of 6×His-ACP in lane 2 with that of a sample containing identical concentrations of 6×His-ACP but no streptavidin (lane 1).

These experiments yielded ratios of the specificity constants of 0.48 for $(k_{cat}/K_M)_{CoA}/(k_{cat}/K_M)_{CoA-Bt}$ and of 0.35 for $(k_{cat}/K_M)_{CoA}/(k_{cat}/K_M)_{CoA-Dg}$, revealing no significant discrimination between free and derivatized CoA.

To demonstrate the use of ACP to selectively label proteins on cell surfaces, we expressed ACP C-terminally attached to the a-agglutinin receptor Aga2p (Aga2p-ACP) in the yeast *Saccharomyces cerevisiae*. Aga2p is exposed at the cell surface of yeast cells via its binding to Aga1p. The complex has already been exploited to display heterologous proteins as fusions to Aga2p on yeast cells.[13] Incubation of yeast cells coexpressing Aga1p and Aga2p-ACP with CoA-Cy3 and 6×His-PPTase led to a clear fluorescent signal around the rim of the cells (Figure 2A). No significant fluorescence staining was observed when the experiment

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10.1021/ja048396s CCC: $27.50

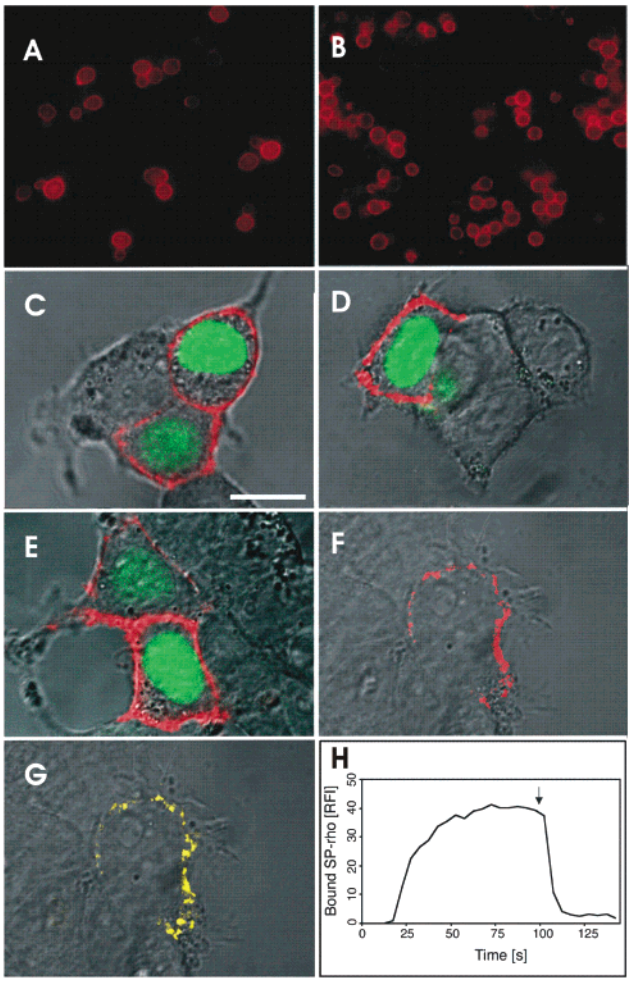

Figure 2. Labeling of ACP fusion proteins on cell surfaces. (A, B) Fluorescence micrographs of yeast cells expressing Aga2p-ACP. (A) Cells were labeled with either Cy3 or (B) biotin followed by SA-coated quantum dots. (C−H) Labeling of HEK293 cells transiently coexpressing ACP-NK_1 and EGFP-NLS_3. Confocal micrographs show overlays of fluorescence and transmission channels. (C) Labeling with Cy3. (D) Labeling with Cy5. (E) Labeling with biotin and subsequent incubation with SA-Cy5. (F) Labeling with Cy5. (G) Incubation of same cells as in (F) with SP-rho. (H) Time-resolved binding of SP-rho (100 nM) to HEK293 cell displaying ACP-NK_1 and its replacement through the addition of unlabeled ligand (3 μM) at the indicated time (black arrow).

was repeated with yeast cells not expressing Aga2p-ACP (Supporting Information). Aga2p-ACP was also biotinylated with CoA-Bt and subsequently incubated with commercially available CdSe quantum dots conjugated to streptavidin. The resulting bright fluorescence of yeast displaying Aga2p-ACP was highly specific (Figure 2B).

Next, we investigated the labeling of ACP on the surface of mammalian cells. ACP was attached to the exoplasmic N-terminus of the human G protein-coupled receptor neurokinin-1 (NK_1).[14] G protein-coupled receptors (GPCRs) represent one the most important class of therapeutic targets, and the specific labeling of these proteins with spectroscopic probes on live cells should prove important for various functional assays.[15] For the labeling experiments, HEK293 cells were transiently cotransfected with plasmids encoding ACP-NK_1 and an enhanced green fluorescent protein with a nuclear localization sequence (EGFP-NLS_3). The coexpression of ACP-NK_1 and EGFP-NLS_3 allows distinguishing between transfected and nontransfected cells. Cells were incubated with 6×His-PPTase and either CoA-Cy3, CoA-Cy5, or CoA-Bt for 10 min and washed with buffer to remove excess label. In the case of CoA-Bt, cells were subsequently also incubated with Cy5-labeled streptavidin (SA-Cy5). Cells were then analyzed by laser confocal scanning microscopy. The obtained images clearly show that only transfected cells are efficiently labeled, indicating a specific labeling of ACP-NK_1 with Cy3, Cy5, and biotin, respectively, (Figure 2C−E). The co-staining of the ACP-NK_1 expressing cells with tetramethylrhodamine-labeled substance P (SP-rho), the natural ligand of NK_1, and the reversal of this staining by an excess of unlabeled ligand furthermore prove the specificity of the labeling and also the functionality of ACP-NK_1 with respect to ligand binding (Figure 2F−H). We then studied the kinetics of the labeling reaction on the surface of HEK293 cells displaying ACP-NK_1 by incubating with 5 μM CoA-Cy3 and 1 μM 6His-PPTase and measuring the increase in fluorescence on the cell surface as a function of time (Supporting Information). In these measurements, the fluorescence signal was saturated after 20 min, indicating that the kinetics of the labeling on cell surfaces are comparable to those measured in vitro. It should be noted that the restriction of the labeling reaction to the surface of the cells allows for discrimination between copies of a receptor that are already displayed on the surface and those that are either retained in the secretory pathway or internalized. The labeling with CoA derivatives can also be precisely timed, enabling studies of internalization and recycling events as well as the time-dependent appearance of new molecules at the surface.

In conclusion, we present here a general method for the covalent labeling of cell surface fusion proteins with chemically diverse molecules. Importantly, ACP is a very small protein, and its modification by 6×His-PPTase is highly specific with respect to the fusion protein but promiscuous with respect to the nature of the label. These features will prove important for studying cell surface proteins and for complementing existing approaches in cell surface engineering.[16,17]

Acknowledgment. We thank Stefan Pitsch, Philipp Wenter, Alain Razaname, Diana Plaumann, and Henri Saleh for technical support. N.J. was supported by a grant from the BMBF.

Supporting Information Available: Procedures for syntheses, construction of fusion proteins, and all assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA048396S