Protein Chemistry on the Surface of Living Cells

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The interplay between carbohydrates, lipids, and proteins determines the stability and flexibility as well as the adhesive and responsive features of the surfaces of all cells. The molecular understanding of the interactions among and between the different classes of these biomolecules is rudimentary at best, a lack of suitable experimental methods being the major reason. Here we discuss a new approach for the specific labeling of fusion proteins of carrier proteins with synthetic compounds on cell surfaces and describe how this approach can be used to investigate the properties of the labeled molecules.

Introduction

The cell surface plays a key role in a variety of complex biological processes ranging from signal transduction to cell–cell and host–pathogen interactions. Proteins that act as receptors, channels, transporters, or enzymes that build and remodel the extracellular matrix play the most prominent role in these activities. The detailed in vivo characterization of its proteins is therefore an important prerequisite for understanding the biology of the cell surface in molecular terms.

As the surfaces of cultured cells are freely accessible to chemical treatment, the labeling of their proteins with synthetic molecules appears as an attractive strategy to equip them with probes that allow for their functional characterization. However, to guarantee specificity in the labeling, the protein of interest must harbor a unique reactivity that distinguishes it from all other biomolecules present on the cell surface. Such a unique reactivity can arise in a very few cases from the intrinsic properties of the protein itself, but in the majority of the cases one has to resort to molecular tricks to provide individual proteins with unique reactivities. One such approach is the so-called metabolic oligosaccharide engineering, which was pioneered by the group of Carolyn Bertozzi.[1,2] Here, cells are fed with unnatural azide-containing monosaccharides that are incorporated by the cellular biosynthetic machinery into glycoconjugates and subsequently transported to the cell surface. By using phosphine derivatives, the azido-glycoproteins can then be modified by Staudinger ligation on the cell surface with a large variety of different probes. The azide can be considered as a bio-orthogonal handle that can be selectively derivatized even in living organisms without affecting other cellular components.[3] However, a drawback of the approach for the study of individual proteins is that the incorporation of the unnatural monosaccharide is relatively unspecific, leading to the promiscuous labeling of different glycoconjugates and glycoproteins.[4] One ingenious way to restrict the incorporation of bio-orthogonal functional groups to individual (cell-surface) proteins in living cells is the incorporation of unnatural amino acids by using nonsense codon suppression; this was introduced as a general approach by the group of Peter G. Schultz.[5,6] To incorporate unnatural amino acids into cell-surface proteins in living cells, the suppressor tRNA charged with the unnatural amino acid has to be introduced into cells through microinjection, electroporation, or the use of transfection reagents.[7–9] Recently, the Schultz group has created unique tRNA/aminoacyl-tRNA synthetase pairs that expand the number of genetically encoded amino acids in *Escherichia coli* and *Saccharomyces cerevisiae*, thereby allowing for the specific incorporation of selected unnatural amino acids in vivo.[10,11] For example, an amino acid with a keto group was site-specifically incorporated into the outer-membrane protein LamB of *E. coli* and then subsequently labeled with fluorophores by incubating the cells with the corresponding hydrazide derivatives.[12] Clearly, the possibility to endow proteins with unnatural amino acids in living cells opens up exciting possibilities for functional studies of cell-surface proteins. However, more general applications of the technology will depend on its establishment in different cell types and multicellular organisms.

An alternative approach to labeling proteins with specific probes is by expressing them as a fusion to a peptide or protein tag that equips the protein of interest with a new functionality.[13] The tetracysteine tag and the protein O^6-alkylguanine-DNA alkyltransferase (AGT) are two promising examples that were designed for the covalent modification of intracellular proteins.[14–17] Consequently, these protein tags are not necessarily suitable for applications in the oxidizing environment of the cell surface. The tetracysteine tag is a short peptide containing four neighboring cysteines that can be specifically labeled with biarsenical derivatives inside living cells.[14,15] Its application on cell surfaces requires the reduction of the otherwise oxidized and unreactive cysteines of the tag by using membrane-impermeable reductants such as 2-mercaptoethanesulfonate and tris(carboxyethyl)phosphine.[15] Since this

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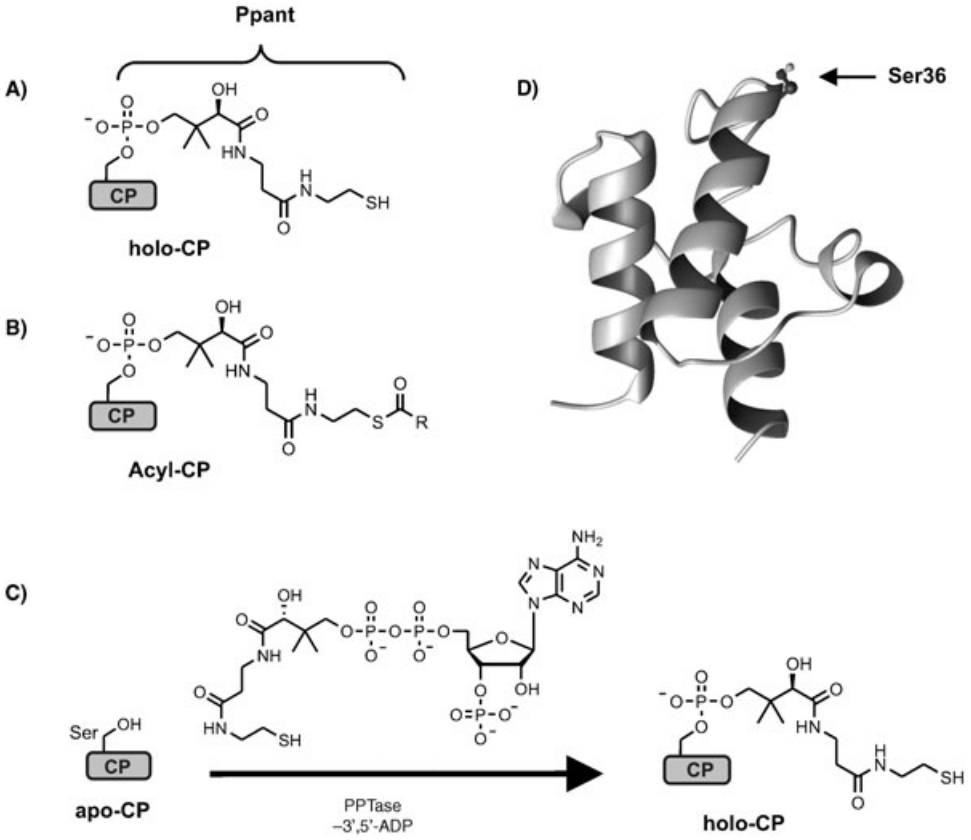
treatment will also reduce the disulfide bridges of most cell-surface proteins, it will automatically perturb many of their activities. The labeling of AGT fusion proteins on the other hand relies on the alkylation of a cysteine of AGT with O^6-benzylguanine derivatives.[16] While we have previously shown that AGT fusion proteins can principally be displayed in an active form on cell surfaces or viral particles, the requirement for a reactive cysteine makes AGT fusion proteins also sensitive to the oxidative environment of cell surfaces.[17,18] Specific cell-surface labeling can also be achieved by expressing the protein of interest as a fusion protein with a polypeptide that is recognized by a biotin ligase such as BirA.[19] Addition or coexpression of biotin ligase results in the selective biotinylation of the fusion proteins. However, the attachment of fluorophores or other useful probes requires the incubation of the biotinylated proteins with the correspondingly tagged avidins or streptavidins; this makes the labeling less direct and noncovalent. The noncovalent labeling of cell-surface proteins can alternatively be achieved by expressing them with an oligohistidine tag and incubating the corresponding cells with probes comprising a chromophore together with a metal-ion-chelating nitrilotriacetate (NTA) moiety.[20] This moiety binds reversibly to the oligohistidine sequences that are displayed by the fusion proteins. The feasibility of the approach has been demonstrated by binding NTA–chromophore conjugates to oligohistidine fusion proteins of a ligand-gated ion channel and a G protein-coupled receptor.[20] Possible drawbacks of the approach are the modest stability of the complex and unspecific binding of the NTA derivate to other proteins.

In summary, despite the recent progresses in the specific labeling of cell-surface proteins there is still a generally acknowledged need for robust procedures that allow for a specific and covalent labeling of cell-surface proteins with chemically diverse compounds. In this article we present a very recently developed labeling strategy that promises to overcome some of the limitations of the current approaches. Here, the protein of interest is fused to an acyl carrier protein (ACP), and the fusion protein is then specifically labeled with CoA derivatives through a post-translational modification catalyzed by phosphopantetheine transferase.

Carrier Proteins and Phosphopantetheine Transferases

Carrier proteins (CPs) are integral components of various primary and secondary metabolic pathways. These pathways include fatty acid synthesis, nonribosomal peptide synthesis, polyketide synthesis, and lysine biosynthesis.[21,22] All CPs harbor a phosphopantetheine (Ppant, Scheme 1) as a covalently attached prosthetic group. The Ppant serves as the attachment site for the building blocks and intermediates (acetate, propionate, butyrate, amino acid) of different pathways. The different substrates are coupled as acyl thioesters to the free SH group of Ppant (Scheme 1 B). Depending on the structure of the bound substrate, CPs are named acyl carrier proteins (ACPs), peptidyl carrier proteins (PCPs), or aryl carrier proteins (ArCPs). The covalent attachment of Ppant to the CP is catalyzed by a group of enzymes named phosphopantetheine transferases (PPTases, Scheme 1 C).[23] PPTases use CoA as the source for Ppant and attach it as a phosphodiester to an invariant serine residue of the CP. Based on sequence identities and substrate specificities, PPTases can be divided into three different groups.[23] The first group comprises PPTases of about 120 amino acids in length that modify the ACPs of type II fatty acid synthetase (FAS) and polyketide synthetase (PKS). PPTases from

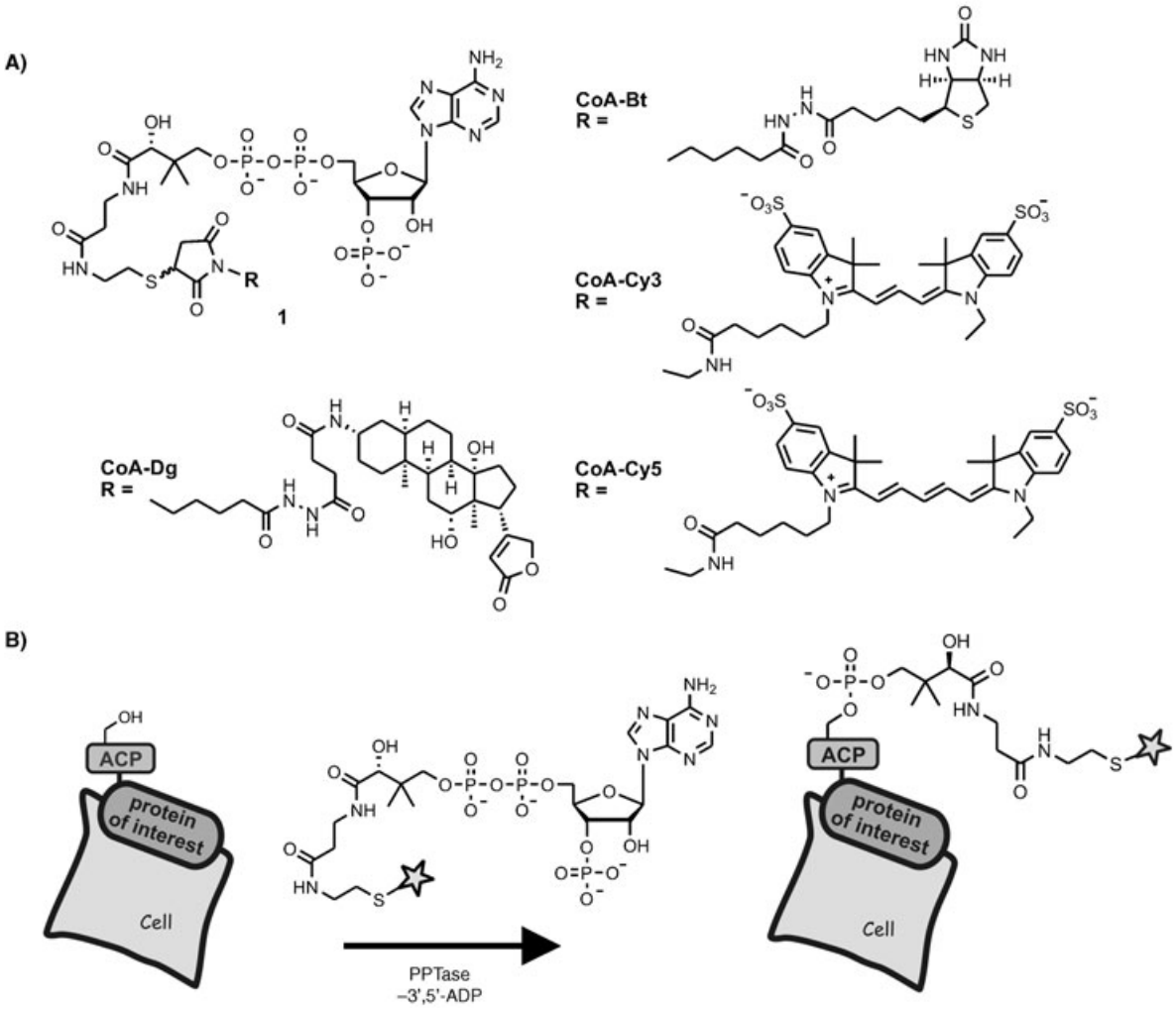


Scheme 1. *Carrier proteins and phosphopantetheine transferases. A) CP with bound phosphopantetheine; B) Acylated CP; C) Modification of CPs by PPTases; D) Structure of ACP from E. coli.*[33]

this class possess relatively narrow substrate specificities, a representative example being the PPTase AcpS, which modifies ACP, from *E. coli*.[24] The second group of PPTases is characterized by their larger size, usually more than 240 amino acids, and by the fact that their genes are usually associated with the gene clusters of the nonribosomal peptide synthetases (NRPS). The prototype of this class is the PPTase Sfp from *Bacillus subtilis*, which accepts as substrates not only PCPs from NRPS but also ACPs of FAS and PKS.[23,25] The overlapping substrate specificity of Sfp is in contrast to that of AcpS from *E. coli*, which does not transfer the Ppant to the PCPs of the enterobactin synthetase EntF from *E. coli* or other PCPs. PPTases of the third group are part of the multidomain type I FAS, which transfers Ppant to an ACP located in the same polypeptide as the enzyme.

ACPs and PPTases as Tools in Cell-Surface Engineering

Structural and biochemical studies have revealed that the β-mercaptoethylamine group of CoA does not participate in the recognition of CoA by PPTases or in the transfer of Ppant to CPs.[26–29] For example, both the crystal structures of AcpS from *Bacillus subtilis* and of Sfp have revealed that the β-mercaptoethylamine end of the phosphopantetheine arm of CoA does not make any significant contacts with the synthase.[26,27] Furthermore, there is ample biochemical evidence that the β-mercaptoethylamine group of CoA can be derivatized without affecting the activity of CoA in its reaction with PPTases.[28,29] This lack of sensitivity with respect to the modification of the β-mercaptoethylamine of CoA has been exploited by the group of Michael Burkart to modify CPs of PKSs and NRPSs with a variety of reporter groups using CoA derivatives of the type 1 and the PPTase Sfp (Scheme 2 A).[30] The goal of these experiments was the detection and purification of recombinant PKSs and NRPSs in order to track and quantify them. We applied the same rational to use the derivatization of ACPs by PPTase as a tool to selectively modify cell-surface proteins (Scheme 2 B).[31] Since the main goal of our approach was to achieve specific labeling of ACP fusion proteins on the surface of eukaryotic cells, we first chose the ACP/PPTase pair from *E. coli*. As stated above, the PPTase AcpS from *E. coli* possesses a relatively



Scheme 2. *The ACP fusion technology. A) Structure of CoA derivatives used for the labeling of ACP fusion proteins on cell surfaces. The substrates can be used for labeling with digoxigenin (CoA-Dg), biotin (CoA-Bt), Cy3 (CoA-Cy3), and Cy5 (CoA-Cy5); B) Mechanism of labeling ACP fusion proteins on cell surfaces. The star represents the different labels.*

narrow substrate specificity with respect to ACPs. The enzyme can be readily overexpressed and purified with a high activity toward ACPs.[32] ACP from *E. coli* is a protein of only 77 residues that folds into a compact structure of four α-helices (Scheme 1 D).[33] The Ppant derivative is attached to Ser36 of ACP. The protein contains no cysteines, thus avoiding a potential misfolding of secreted ACP fusion proteins due to unwanted oxidations. When tested in vitro, ACP from *E. coli* is readily modified by CoA derivatives, and the rate of the reaction is independent of the nature of the label.[31] At concentrations of 0.2 μM of AcpS, 1 μM ACP and 5 μM of the CoA derivative, a typical labeling experiment is complete within 10 min, and the reaction is nearly quantitative. The ACP-Sag1p fusion protein serves as a representative example for the modification of a protein on the surface of the yeast *Saccharomyces cerevisiae*. Sag1p is the α agglutinin of yeast cells and is covalently attached to the β-1,6-glucan of the cell wall via its modified glycosyl phoshatidylinositol anchor. During the mating of yeast cells of opposing mating types Sag1p of the α-cells binds to the a-agglutinin Aga2p from a-cells.[34] For the construction of the fusion protein, we replaced the natural signal sequence of Sag1p with the signal sequence of the α-factor followed by the coding sequence of the bacterial ACP. The combined addition of CoA derivatives of type 1 and ACPs resulted in the specific labeling of yeast cells expressing ACP-Sag1p (Figure 1 A). The observed specificity and efficiency of labeling can be rationalized by two properties of the system. First, the cell surface separates the cell-impermeable CoA derivatives and the appropriate PPTase from host PPTase, host ACPs, and underivatized CoA, thereby suppressing unwanted side reactions, such as the labeling of internal ACPs and CPs. Second, bacterial ACPs are not efficient substrates of eukaryotic PPTases.[35] This feature minimizes unwanted phosphopantetheinylation of the fusion protein before it escapes from the cytosol into the secretory pathway. In addition to ACP-Sag1p, we have previously shown that ACP attached C-terminally to the a-agglutinin receptor Aga2p (Aga2p-ACP) can be effectively labeled on the surface of yeast.[31] Together, these experiments demonstrate the flexibility of the ACP tag with respect to different orientations in the fusion protein. As the N and C termini of ACP reside on the same side of the protein and are proximal to each other, it is likely that ACP can also be inserted into the loops of cell-surface proteins without dramatically perturbing the structures of the host and the guest protein. ACP fusion proteins can also be specifically labeled on the surfaces of mammalian cells. In a first example, ACP was attached to the exoplasmic N terminus of the human G protein-coupled receptor neurokinin-1 (NK_1).[31] G protein-coupled receptors (GPCRs) represent an important class of therapeutic targets, and the specific labeling of these proteins with spectroscopic probes on live cells makes the technique an interesting starting point for the development of functional cell-based assays.[36] As observed for yeast, HEK293 cells transiently expressing ACP–NK_1 could be marked with different fluorophores or affinity labels whereas nontransfected cells were not labeled to any significant extent (Figure 1 B). Very recently, it has also been shown that PCP fusion proteins can be labeled specifically on the surface of bacteriophage M13, further extending the number of display hosts.[37]

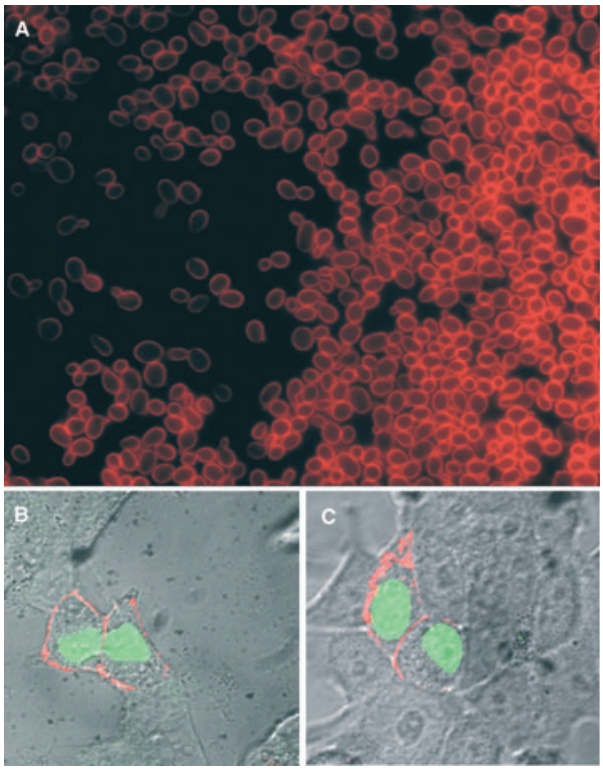


Figure 1. *Fluorescence labeling of ACP fusion proteins on cell surfaces. A) Fluorescence micrographs of yeast cells expressing ACP-Sag1p. Cells were labeled with biotin followed by incubation with commercially available CdSe quantum dots conjugated to streptavidin (www.qdots.com). B), C) Labeling of HEK293 cells transiently coexpressing ACP-NK_1 and enhanced green fluorescent protein fused to a nuclear localization sequence (EGFP-NLS_3). The nuclear green fluorescence identifies the transfected cells. The confocal micrographs show overlays of fluorescence and transmission channels. B) Labeling with Cy3 by using CoA-Cy3. C) Labeling with Cy5 by using CoA-Cy5.*

Depending on the nature of the CoA derivative, a single fusion protein can be used for a variety of different assays. Fluorophores and affinity labels have been an obvious first choice as ACP substrates, but crosslinkers, quantum dots, caged compounds, or environmentally sensitive fluorophores are exciting and straightforward extensions. Crucial for the broad applicability of the approach is, therefore, the ease with which the different CoA derivatives can be synthesized. For the substrates listed in Scheme 2, the syntheses comprised only reactions of CoA with commercially available maleimide derivatives followed by HPLC purification. If needed, more complex CoA derivatives are accessible via synthetic routes developed by the group of Drueckhammer.[38]

Beside their promiscuity toward different labels, ACP fusions of cell-surface proteins can be used for studying the dynamics of their distribution on and in the cell. Specifically, the membrane impermeability of PPTases and CoA derivatives limits the labeling to proteins that are already displayed on the cell surface during incubation and leaves unlabeled those proteins that are either still in the secretory pathway or already internalized. This feature allows monitoring of the subsequent move-

ment of the fusion protein from the plasma membrane to other cellular locations. Furthermore, the controlled addition of enzyme and substrate and their rapid removal permits a precise timing of the labeling. Thus, labeling reactions with different fluorophores at different times can discriminate between different generations of ACP fusion proteins in individual cells. Of course, speed and a high efficiency of labeling are important prerequisites for these applications. Our previous measurements have indicated that the kinetics of the labeling of ACP fusion proteins on cell surfaces are comparable to those measured for the purified ACP.[31] Consequently, labeling can be quantitative within a period of about 10 min, providing that sufficiently high substrate and PPTase concentrations are used.

Protein–protein interactions are as important for the function of extracellular proteins or domains as they are for intracellular proteins. A logical extension of the ACP approach is the specific labeling of two simultaneously expressed but different CP fusion proteins each with a different fluorophore. Fluorescence resonance energy transfer could then be used to detect the interaction between the two labeled fusion proteins.[39] To achieve such a simultaneous but specific labeling of two CP fusion proteins, PPTases with nonoverlapping substrate specificities have to be employed. AcpS and EntD from *E. coli* are examples of two PPTases with such specificities.[23] EntD accepts the PCP domains of EntF as substrate but does not modify ACP, whereas AcpS efficiently modifies ACP but does not show any reactivity towards PCPs. As the two PPTases do not discriminate between different CoA derivatives, the two labeling reactions would have to be performed sequentially.

Although we believe that the labeling of ACP and PCP fusion proteins is in particular attractive for applications on cell surfaces, selectively modifying fusion proteins in cell extracts might facilitate the biochemical analysis of complex processes in vitro.[40] It was therefore instructive to evaluate the specificity of the labeling of ACP fusion proteins in cell extracts of eukaryotic cells. To this end, ACP from *E. coli* was C-terminally attached to AGT and expressed as a cytosolic AGT–ACP fusion protein in yeast. The expression level of the fusion was estimated to be below 1% of the total protein. After lysis, cell extracts were incubated with AcpS and CoA-Bt or CoA-Dg (Figure 2). Western blotting of the extracts and visualization by using either anti-Dg antibodies or streptavidin confirmed that only a single protein of the size of AGT–ACP was labeled by this procedure (Figure 2). Along these lines, Walsh's group has biotinylated PCP fusion proteins using the PPTase Sfp in cell extracts of *E. coli* and immobilized these proteins on streptavidin-coated glass slides.[40] These experiments further demonstrate the specificity of the labeling of ACP or PCP fusion proteins and point to interesting in vitro applications.

In summary, the PPTase-dependent modification of ACP fusion proteins on cell surfaces of living cells with chemically diverse compounds ideally complements existing approaches for labeling proteins with synthetic molecules. Its main advantages are the flexibility with respect to the nature of the probe as well as the efficiency and specificity of the labeling. These features should allow the approach to become a welcome addition in the toolbox of chemical biologists and cell-surface engineers.

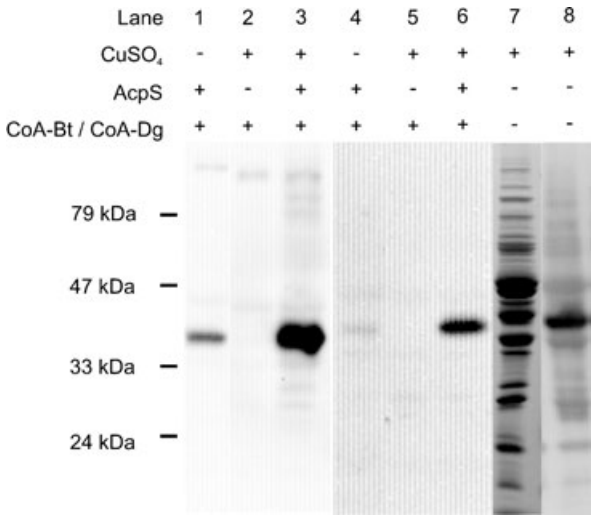


Figure 2. *Labeling of AGT–ACP in yeast extracts. AGT–ACP was expressed in yeast EGY48 cells under the control of the (leaky) copper promoter. Protein extracts of cells grown either in the absence (lanes 1, 4) or presence of 100 μM $CuSO_4$ (lanes 2, 3 and 5–8) were passed over streptavidin-coated beads (lanes 1–3) or anti-digoxigenin antibody-coated beads (lanes 4–6) and then incubated with 1 μM (lanes 1, 3, 4, 6) or without AcpS (lanes 2, 5, 7, 8), CoA-Bt (20 μM, lanes 1–3) and CoA-Dg (20 μM, lanes 4–6). Labeled proteins were visualized by Western blotting by using a neutravidin–peroxidase conjugate (lanes 1–3) or an anti-digoxigenin antibody–horseradish peroxidase conjugate (lanes 4–6). Lane 7 shows the SDS polyacrylamide gel of a crude cell extract stained with Coomassie Blue. Lane 8 shows the Western blot of the same extract probed with an anti-AGT antibody.*

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