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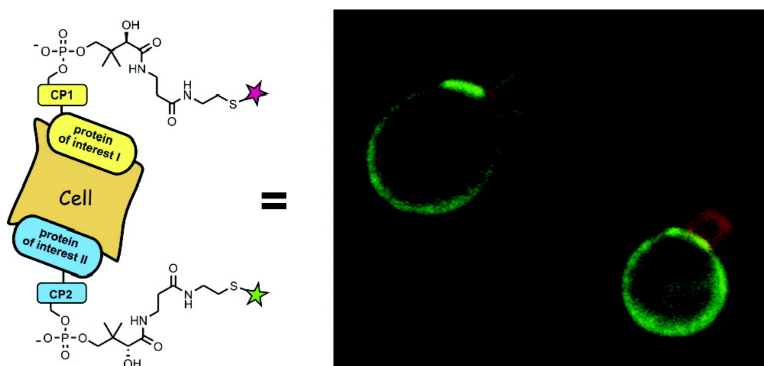

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Multicolor Imaging of Cell Surface Proteins

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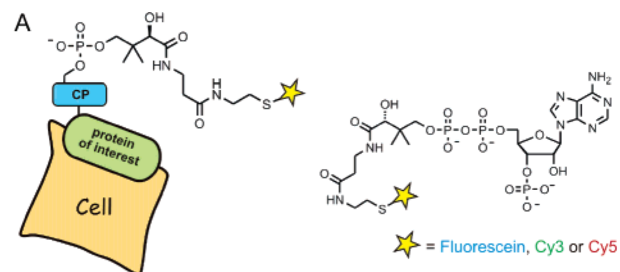
We report on methods for the multicolor imaging of cell surface proteins based on the covalent modification of carrier proteins (CP). We show how subsequent generations of a CP−cell surface fusion protein can be sequentially labeled with different fluorophores and how different proteins can be selectively labeled via different CPs. Both methods should become important tools for the in vivo characterization of cell surface proteins.

CPs are integral components of diverse metabolic pathways. They are small proteins (generally <85 residues) that harbor a phosphopantetheine (Ppant) as a covalently attached prosthetic group.[1] The Ppant serves as the attachment site for various intermediates in metabolic pathways, and depending on the structure of the bound substrate, CPs are named acyl carrier proteins (ACPs) or peptidyl carrier proteins (PCPs). Specific phosphopantetheine transferases (PPTases) catalyze the transfer of Ppant from CoA to an invariant serine residue of CPs.[1] Taking advantage of the fact that PPTases generally accept thiol-modified derivatives of CoA,[2] we have previously labeled ACP fusion proteins on cell surfaces with fluorophores, quantum dots, and affinity probes.[3,4] We therefore reasoned that the approach would be ideally suited for the multicolor imaging of cell surface proteins, either through sequential labeling of different protein generations (Figure 1A) or through the selective labeling of different cell surface proteins (Figure 2A).

To demonstrate the utility of sequential labeling of CP fusion proteins for the characterization of dynamic processes, such as protein secretion, we studied the localization of the cell wall protein Sag1p of the yeast *Saccharomyces cerevisiae*. Sag1p is transported to the sites of active cell growth and fixed to the surface by a covalent linkage to the β1,6-glucan of the cell wall.[5] This feature immobilizes Sag1p at its site of secretion and physically separates the newly synthesized molecules from their predecessors. To render the secretion of Sag1p amenable to multicolor imaging, we expressed Sag1p as a fusion protein with *Escherichia coli* ACP. Treating cells expressing ACP−Sag1p with the PPTase AcpS from *E. coli* and CoA-Cy3 resulted in a homogeneous surface labeling (Figure 1B). However, when cells were first incubated with AcpS and CoA-Cy3, washed, reintroduced into growth medium, and, after defined intervals, labeled again with AcpS and CoA-Cy5, a clear separation of the differently colored fusion proteins emerged. Relabeling the cells with CoA-Cy5 after 15 min revealed small spots of Cy5 at the incipient buds (Figure 1C). When the second labeling was performed after longer intervals, the proportion of the Cy5-stained regions of the cell wall gradually increased. While Cy5 became visible in newly formed buds, Cy3 was mainly restricted to the mother cells (Figure 1D). The resolution of this method is best documented in those cells where a Cy3-stained mother is capped by a small stripe of Cy5-labeled protein at its neck followed by a region of Cy3- and finally Cy5-labeled protein (Figure 1D).



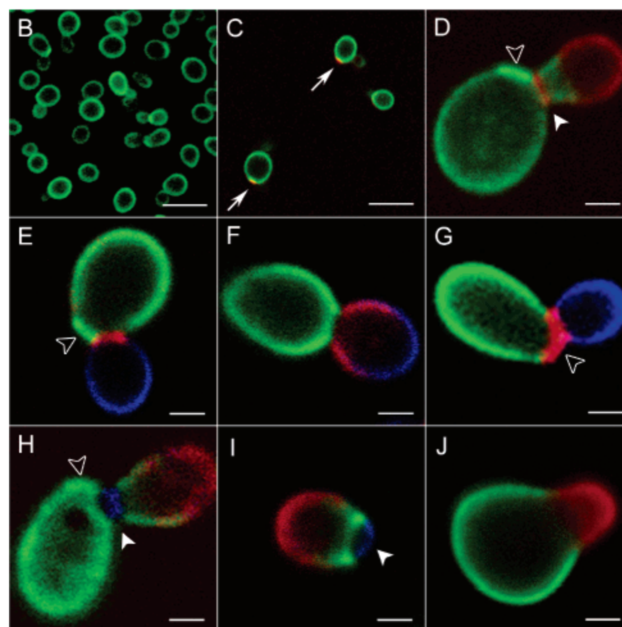


Figure 1. Multicolor imaging of CP fusion proteins. (A) Cells displaying a CP fusion protein can be labeled with up to three different fluorophores by sequential incubation with AcpS and CoA-Cy3, CoA-Cy5, or CoA-fluorescein (represented as a star). (B−J) Fluorescence micrographs of ACP−Sag1p expressing yeast cells. Cells were first labeled with CoA-Cy3 (green) and after 15 (C) or 150 min (D) with CoA-Cy5 (red). Arrows indicate the incipient buds, arrowheads the septum, and open arrowheads indicate the sites where a daughter cell was recently detached (bud scar). (E−I) Cy3-stained cells were labeled after 60 min with CoA-Cy5 and again after 60 min with CoA-fluorescein (CoA-F, blue). (J) Cy3-stained cells were treated with α-factor for 120 min before labeling the cells with CoA-Cy5. Labeling was performed by incubating cells with 2 μM PPTase and 10 μM of CoA derivative for 30 min. The bars correspond to 10 μm (B and C) and to 2 μm (D−J).

This pattern is a direct consequence of the polarized secretion in yeast: new cell wall proteins are directed to the tip of young buds, but are later redirected to the neck to build two new cell walls between the mother and daughter cells. In the case of the cell shown in Figure 1D, the first labeling apparently took place when the bud had already started to grow, whereas the second labeling occurred after the septum was completed. Taking advantage

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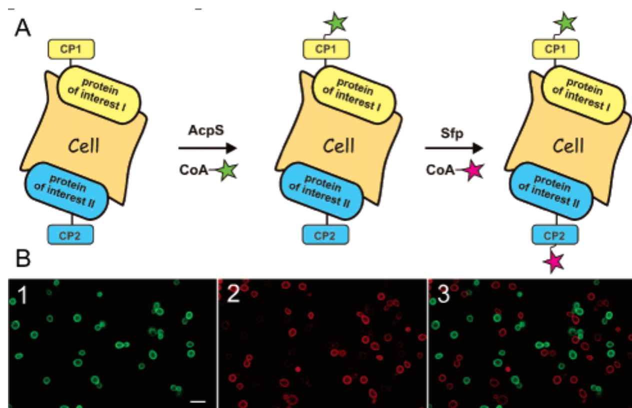

Figure 2. Selective labeling of different CP fusion proteins with different fluorophores. (A) Cells expressing fusions to two different CPs are incubated first with AcpS and a CoA derivative to label all copies of CP1 and, subsequently, with Sfp and a second CoA derivative to label the unmodified copies of CP2. ACP was chosen as CP1 and PCP as CP2. (B) Labeling of ACP−Sag1p and PCP−Sag1p on yeast cells. A mixture of yeast cells expressing either ACP−Sag1p or PCP−Sag1p was sequentially incubated with AcpS/CoA-Cy3 and Sfp/CoA-Cy5 and analyzed for fluorescence: (1) detection of Cy3 (green); (2) detection of Cy5 (red) in the same sample; (3) overlay of (1) and (2). The bar in (B) corresponds to 10 μm.

of the ease with which CoA derivatives can be synthesized, we expanded our repertoire of CoA derivatives and used the approach to simultaneously visualize three different generations of ACP−Sag1p. Cells were first labeled with CoA-Cy3 and, after different time intervals of growth, subsequently treated with CoA-Cy5 followed by CoA-fluorescein (CoA-F). The triple-colored cells document the sequential appearance of the different cell wall structures and prove the aptness of the approach for a multicolor imaging of surface proteins (Figures 1E−I). The labeling unambiguously shows that the wall between the cells is indeed the latest structure formed before they separate (Figure 1H, I).

Yeast cells respond to mating hormones by forming a projection through polarized cell growth.[6] In a further application of our method, we followed the dynamics of formation of this structure by labeling all accessible copies of ACP−Sag1p with CoA-Cy3 before exposing a-cells to 0.02 mM α-factor. After 120 min of α-factor treatment, the cells were incubated with AcpS and CoA-Cy5. Cy5 uniformly stains the newly formed cell projections, whereas Cy3 is restricted to the rest of the cell body. This pattern directly reflects the dynamic response of the cell to mating hormone (Figure 1J).

The multicolor imaging of two different CP fusion proteins in one sample requires PPTases with distinct substrate specificities (Figure 2A). Two different PPTases, the PPTase AcpS from *E. coli* and the PPTase Sfp from *Bacillus subtilis*, have already been employed for the labeling of CP fusion proteins and should be well suited for this purpose.[2a,7] AcpS possesses a very narrow substrate specificity and modifies only ACPs, whereas Sfp modifies both PCPs and ACPs. To obtain CP-specific labeling, the sample must be incubated first with AcpS and a CoA derivative to label all accessible ACP fusions and, subsequently, with Sfp and a different CoA derivative to label the remaining PCP fusions. To evaluate the feasibility of this strategy, we expressed ACP−Sag1p and PCP−Sag1p in two different yeast strains and mixed equal amounts of the cells shortly before the labeling experiment. In these experiments, we used TycC3-PCP, a PCP domain of the NRPS tyrocidine synthetase from *Bacillus brevis*.[8] The expression of the two CP fusion proteins on separate cells rather than on a single cell served to facilitate the analysis of the specificity of the labeling. Treatment of cells with AcpS and CoA-Cy3 resulted in the labeling of roughly half of all cells, whereas incubation with Sfp and CoA-Cy5 led to a uniform Cy5 labeling of all cells (Supporting Information). If, however, the cells were incubated first with AcpS and CoA-Cy3 and, after a brief washing step, with Sfp and CoA-Cy5, two nonoverlapping populations of Cy3- and Cy5-labeled cells were observed (Figure 2B). To further confirm the validity of the approach, we expressed fusion proteins of ACP and PCP with O^6-alkylguanine−DNA alkyltransferase (AGT). AGT fusion proteins can be immobilized on surfaces displaying the AGT substrate O^6-benzylguanine (BG).[9] Using this procedure, we created a small protein microarray displaying ACP−AGT and PCP−AGT. The microarray was incubated first with AcpS and CoA-Cy3 and then with Sfp and CoA-Cy5, resulting in specific labeling of ACP-AGT with Cy3, and PCP-AGT with Cy5 (Supporting Information).

In summary, we have introduced methods for the multicolor imaging of cell surface proteins that are expressed as fusions to CPs. The methods can be used on the surfaces of different (mammalian) cell types and as fusions to a variety of different proteins.[2a,3,4,7] We envision future applications in the study of macromolecular structure formation on the cell surface, protein−protein interactions between differently labeled cell surface proteins, and the observation of the individual fates of different proteins in one sample.

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Supporting Information Available: Procedures for construction of fusion proteins, synthesis of CoA-F, and all assays (pdf). This material is available free of charge via the Internet at http://pubs.acs.org.

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