An eight residue fragment of an acyl carrier protein suffices for post-translational introduction of fluorescent pantetheinyl arms in protein modification *in vitro* and *in vivo*

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Abstract

Genetically encoded tags for tracking a given protein continue to be of great interest in a multitude of *in vitro* and *in vivo* contexts. Acyl carrier proteins, both free standing and as embedded 80-100 residue domains, contain a specific serine side chain that undergoes post-translational pantetheinylation from CoASH as donor substrate. We have previously used phage display methods to select a 12 residue fragment that retains recognition for modification by the *E. coli* phosphopantetheinyltransferase (PPTase) AcpS. In this work we have used ^{15}N-HSQC based NMR titration experiments of a 12-residue peptide substrate with AcpS to identify six specifically interacting residues (S_3, L_4, D_5, M_6, W_9 and L_{11}) without the formation of any notable secondary structure. Synthesis of a corresponding octapeptide containing 5 of the 6 interacting residues generated a minimal fragment capable of efficient post-translational phosphopantetheinylation. Genetic insertion of this eight residue coding sequence into the proteins sonic hedgehog and transferrin receptor enabled good *in vitro* and *in vivo* PPTase-mediated modification by a series of fluorescent CoAs, leading to a set of fluorescent proteins with a peptide tag minimally perturbant to protein folds.

1. Introduction

Green fluorescent protein and its family members have revolutionized the ability to track particular proteins in cells with regard to both localization and dynamics[1]. Yet, there remains interest in creating genetically encoded tags for proteins that are much smaller than the 27 kDa (circa 250 residues) mass of GFP and congeners. To this end, two sets of enzymes, the biotin ligases and the phosphopantetheinyl transferases, that carry out sequence-specific post-translational modifications of proteins have been investigated as general protein tagging reagents[2-5]. Biotin ligase can introduce fluorescent biotin analogs in amide linkage to a Lys

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side chain while PPTases can add a wide variety of substituted pantetheinyl arms from the corresponding CoASH cosubstrates (Figure 1).

In previous work we have noted orthogonal selectivity of the Sfp subfamily and the AcpS subfamily of PPTases from phage selection studies, suggesting useful orthogonal pair labeling[6]. Also, phage selection led to a significant reduction in size of peptide fragment recognized by PPTases from the native 80-100 residue (8-10 kDa) carrier protein domains to a 12 residue fragment containing a conserved sequence around the serine side chain to be phosphopantetheinylated. To evaluate a minimal size of peptide that could be post-translationally modified on its own, or embedded in other protein contexts, by PPTases, we have turned to NMR spectroscopy. And we used that information to synthesize an octapeptide that was indeed well recognized, and then shown that the octamer can be embedded in a foreign protein context and be post-translationally modified to yield a series of fluorescent forms of the protein sonic hedgehog and transferrin receptor.

2. Results

NMR studies of A1 peptide recognition by AcpS

The 12-mer peptide substrate of AcpS, A1[6], was produced in *E. coli* grown in minimal media with [^{15}N]-NH$_4$Cl to introduce ^{15}N. ^{15}N-edited 2D-Noesy spectra were recorded for the ^{15}N labeled A1 peptide (data not shown), and no α-helical structure or any other formation of a rigid secondary structure could be observed. To reveal the interaction and the site-specific protein-protein recognition between A1 peptide and AcpS, a series of ^{15}N-HSQC spectra of A1 with increasing concentrations of AcpS were recorded. In this study, authentic AcpS without any purification tags at N- or C-termini was the preferred form. The A1 peptide was titrated with AcpS in four steps from the free A1 peptide to a final molecular ratio of 1:1.1 in the absence of coenzyme A as the cofactor for the post-translational modification. A further increase of the AcpS concentration in this titration to a ratio of 1:2 did not result in further chemical shift changes and suggests a specific and rapid interaction between A1-peptide and AcpS. The superposition of the four ^{15}N-HSQC spectra of A1 peptide, reflecting the titration from the free form to the 1:1.1 complex with AcpS, are shown in Figure 2a. The sequential assignment of the backbone amides was based on ^{15}N-edited 2D-Noesy spectra recorded for A1 peptide. The amine correlation of the indole ring of W_9 could be identified at 129.1 ppm in the ^{15}N dimension and at 10.05 ppm in the proton dimension. The combined chemical shift changes (Δppm) of the nitrogen and proton dimension from the free A1 to the A1/AcpS 1:1.1 complex were calculated and plotted in Figure 3. Six residues show chemical shift differences (Δppm values) greater than 0.4 ppm. The active serine (S_3) has, as expected, the strongest chemical shift change of Δppm = 0.89, since the modification with a phosphoester on SerO$_\gamma$ will affect the back bone amide. L_4, D_5 and M_6, sequentially adjacent to S_3, as well as W_9 and L_{11}, show clear chemical shift changes in the A1/AcpS complex, corresponding to a specific recognition of the A1 peptide by AcpS. The Δppm values of G_1, D_2, L_7, E_8, S_{10} and M_{12} were lower than 0.25 ppm and the residues are considered to be less relevant for substrate recognition. According to the HSQC spectra, we noticed that no secondary structure of the A1 peptide was induced in the complex when titrated by AcpS, thus an α-helical conformation is not necessary for the substrate recognition by AcpS.

Two equivalents of acetyl-CoA and a catalytic amount of AcpS were added to a buffered 300 μM solution of the free [^{15}N] A1 peptide to convert A1 from the apo form to the holo form (acetyl-Ppant-[^{15}N]A1). ^{15}N-HSQC spectra of A1 peptide during this conversion process, and ^{15}N-edited 2D-Noesy spectrum of holo A1 peptide were recorded. We were able to observe the formation of the product and can describe the intermediate-stage of this conversion reaction as a binding of apo-A1 and the release as holo-A1 by NMR spectroscopy. The ^{15}N-HSQC spectra of apo A1 peptide and holo A1 peptide were superimposed and shown in Figure 2b.

The combined chemical shift change of the S_3 amide is about $\Delta ppm = 0.83$ ppm and 0.71 ppm in the proton dimension only, when acetyl-Ppant cofactor is covalently attached as a phosphoester. This reflects the dramatic change in the chemical environment for S_3. S_3's C-terminal following residues L_4, M_6, L_7 also showed measurable chemical shift differences between apo and holo. According to the recorded ^{15}N-edited 2D-Noesy spectrum of holo A1 peptide (data not shown), no stable secondary structure is formed.

Peptide kinetics of truncated A1 peptides

According to the ^{15}N-HSQC spectra of [^{15}N] A1 in the titration experiment, 6 residues (S_3, L_4, D_5, M_6, W_9, L_{11}) showed distinct chemical shift changes, reflecting strong and specific interactions with the PPTase AcpS. The amino acid residues S_3, L_4, D_5 and M_6 are responsible for the AcpS recognition core and form the phosphopantetheinylation site. Although W_9 and L_{11} are not directly adjacent to the phosphopantetheinylation core site, they are specifically recognized by AcpS and become the barriers to further reduce the size of the A1 peptide. To evaluate these predictions, we made a series of truncated peptides (A-1, A-2, A-3, A-4, A-6), and tested the specific recognition and enzymatic activity of AcpS. The kinetics of AcpS-catalyzed phosphopantetheinylation was determined for all truncated forms of the A1 peptides (Figure 4a). A-1 showed similar k_{cat}/K_m value compared with A1, consistent with the NMR results that M_{12} is not recognized. A-2 and A-3 have lower k_{cat}/K_m values than A1, due to the loss of L_{11}, which is one of the residues recognized by AcpS. We further removed the unrecognized G_1 to yield the octapeptide A-4, which remains an efficient and specific substrate of AcpS (Figure 4b). With the truncation A-6, no formation of the holo-peptide (Ppant-A-6) as detectable product could be observed, consistent with loss of the two essential residues W_9 and L_{11}. Thus, we reduced the size of AcpS peptide substrate to 8 residues (D_2-W_9), as the limit.

The enterobactin EntB aryl carrier protein is post-translationally modified by the iron-regulated PPTase EntD but is not a substrate for AcpS, nor is the derived peptide E0[7]. No Ppant introduction was detected after incubating E0 (Figure 4d) with AcpS and CoA. With a change of 4 residues (E0->E2), E2 showed comparable k_{cat}/K_m to A1 for AcpS-catalyzed phosphopantetheinylation (Figure 4c and 4d). This further proves that our NMR studies revealed the most important specific interactions between the AcpS and A1 peptide. This information can guide engineering of noncognate carrier proteins to be recognized by AcpS for the reprogramming of polyketide synthases (PKSs) and nonribosomal peptide synthetases (NRPSs) for the production of novel engineered natural products as potential antibiotics, fungicides and cytotoxins[8,9].

In vitro protein labeling with embedded A-4 peptide tag

The signaling domain of mammalian sonic hedgehog (ShhN) protein, which involves lipid post-translational modification at both its N-terminus and C-terminus during maturation[10-12] was next tested to demonstrate that the shortened, octapeptide tag is suitable for specific protein labeling. The A-4 peptide was inserted at an internal loop region between residues $T_{56}L_{57}$-$G_{58}A_{59}$ or at the C-terminus of ShhN protein, resulting in ShhN-(A-4_{57-58}), and ShhN-A-4, respectively (Figure 5a). ShhN-(A-4_{57-58}), and ShhN-A-4 were incubated with Alexa Fluor 633-CoA (red), and Alexa Fluor 350-CoA (violet), in the presence of 8.5 μM AcpS. After 10 minutes of incubation, the reactions were quenched and the proteins were separated on SDS-PAGE. Labeled ShhN proteins were excited with UV 302 nm. Alexa Fluor 633 and Alexa Fluor 350 were efficiently linked to ShhN-(A-4_{57-58}) and ShhN-A-4. No nonspecific introduction of flurophores was observed for ShhN protein without A-4 peptide sequence (Figure 5b). No labeling was observed in the absence of AcpS (data not shown). Proteins on the same gel were also visualized after coommasie staining to verify that similar amount of proteins were loaded on each lane (Figure 5c). Alexa Fluor 546-CoA (orange),

Bodipy-CoA (green), Texas Red-CoA (red) and Alexa 488-CoA (green) were also efficiently utilized by AcpS for specific labeling of the ShhN-A4 and ShhN-(A-4$_{57-58}$) proteins (data not shown). Properties of the labeled ShhN in cellular assays will be described elsewhere.

Cell surface protein labeling with A-4 peptide tag

The A-4 peptide tag was also used as a fusion to the extracellular C-terminus of human transferrin receptor 1 (TfRI). NIH/3T3 cells were transfected with pCIG-TfR1-A-4 plasmid, which also contains a nucleus-GFP transfection marker. Transfected cells were incubated with 5 μM AcpS and 2 μM Texas-Red for 30 minutes at 37 °C and images are shown on Figure 6. Cells were stained with DAPI (4′,6-diamidino-2-phenylindole) for nuclei. In Figure 6, panel **a** shows the nuclei of all the cells in the field, panel **b** shows the nucleus-GFP marker for transfected cells, panel **c** shows the labeled TfR1-A-4 receptors, and panel **d** shows the merged colors for a-c. Labeling was specific to transfected cells, and no labeling was seen for neighboring untransfected cells without nucleus-GFP marker. A strong fluorescent labeling signal was detected for cell 1 (lower left), as it received a relatively large amount of plasmid indicated by the relative strong GFP signals; and as expected, a weaker labeling signal was detected for cell 2 (upper right), which received less plasmid. NIH/3T3 cells were also transfected with control pCIG plasmids, and no labeling was detected for either transfected or untransfected cells under the identical microscopic setting (Supporting Information Figure S1). To exam the functionality of TfR1-A-4, NIH/3T3 cells were transfected with pcDNA3.1-TfR1-A-4. TfR1-A-4 on live cell surfaces was labeled with Texas Red for 30 minutes, incubated with 10 μg/ml Alexa Fluor 488-transferrin for 10 minutes, fixed and imaged. In Figure 7, normal transferrin internalization was seen in cells labeled with Texas Red (cell 1), and no internalization was seen in unlabeled cells (cell 2). The expected colocalization of transferrin and TfR1-A-4 was also observed (Figure 7 **panel d**). These results suggest that the tagged TfR1-A-4 is functional for binding to and internalize transferrin, and the C-terminal A-4 tag does not disturb these functions of TfR1 receptors.

3. Discussion

The ^{15}N labeled 12-mer peptide used for described NMR titration experiments was made in *E. coli* inexpensively and the readily overproduced AcpS[13] on titration with ^{15}N-enriched peptide substrate led to unambiguous chemical shift changes of the backbone amides for six of the 12 residues. These findings enabled two distinct ways to proceed. First, it led to synthesis of an octamer peptide containing the conserved sequence from D_2 to W_9 and validation that this was a minimal substrate for phosphopantetheinylation. Second, it suggested a route, successfully executed, to convert a nonribosomal peptide synthetase (NRPS) carrier protein that was not a substrate for AcpS, the EntB aryl carrier protein of enterobactin synthetase, into a substrate. This may be generalizable for engineering other NRPS carrier proteins expressed in *E.coli* to be modifiable from their inactive apo forms to their active holo forms.

The kinetic characterization of the minimized peptides for phosphopantetheinylation, guided by the NMR-based structural approach for AcpS recognition, has led to a ten fold reduction in size from the 80-100 residues of the native carrier protein to the octapeptide fragment. This is the size of a FLAG tag or a His$_{6-10}$ tag widely used to purify proteins by affinity methods. It is 25 fold smaller than the GFP class of fluorescent protein tags. The NMR analysis of the substrate peptide indicated the absence of secondary structure both free in solution and when bound in the AcpS active site, suggesting that the eight-residue peptide would not impose a disruptive secondary structural element when embedded in proteins and consequently minimize the likelihood of functional disruption. Indeed, we have previously shown the larger 11-mer YbbR peptide could be inserted at the N-terminus, the C-terminus or at internal sequences of EGFP proteins without disrupting function and still be post-translationally

accessible for Sfp phosphopantetheinylation[14]. A variety of fluorescent CoA derivatives are available and serve as substrates for the promiscuous phoshopantetheinyl transferases to create different fluorophores in a site-specific manner at S_3 of the embedded octapeptide moiety, as exemplified here with sonic hedgehog protein and transferrin receptor. The k_{cat}/K_m for A-4 peptide modification by AcpS was determined to be 0.021 min^{-1}, and the *in vitro* or *in vivo* labeling efficiency may vary depending on the accessibility of AcpS and the position of peptide tag in a protein.

Mammalian cells with A-4 tagged transferrin receptor labeled with Texas Red by AcpS were healthy and functional for binding to and internalization of transferrin ligands. The concentrations of fluorescent-CoA and AcpS used were no greater than 2 μM and 5 μM respectively for efficient labeling and no detectable toxicity was observed. Previously, Johnsson and coworkers reported that yeast cells labeled with different fluorophores by AcpS could functionally grow, form buds and respond to mating hormones[15]. The time frame of AcpS labeling was 30 minutes for A-4 tag on cell surface, and it is comparable with other enzyme-catalyzed cell surface receptor labeling methods.

We previously reported the orthogonality of AcpS and Sfp for dual-color labeling with peptide tags[6]. There has been a continuous interest for developing small tag-based orthogonal labeling methods with tools like lipoic acid ligase[16] and O^6-alkylguanine-DNA alkyltransferase labeling[17]. Thus, A-4 tag may be combined with other tags to track multiple components on cell surfaces. One limitation to date for *in vivo* analysis is the poor penetration of CoA derivatives across cell membranes, and the current reagents are most appropriate for analysis of cell surface events involving the tagged proteins. Development of fluorescent pantetheine analogs which could be uptaken and converted to CoA analogs by *E. coli* for cytosolic protein labeling was reported[18], and cell membrane-penetrable CoA analogs to mammalian cells would be of interest for future research.

4. Materials and methods

Molecular cloning

DNA primers ZZP175 (5′-GATATACATATGTCCCCTATACTA-3′) and ZZP170 (5′-GCGCTCGAGTTACATCAAAGACCACTCCAACATATCAAGAGAATCTCCGCCCTG AAAATACAGGTTTTCACCAGAACCACTAGTTGAACC-3′) were used for PCR amplification of GST gene from a pET41a plasmid. The PCR fragment of GST-A1 gene was digested by NdeI and XholI, and cloned into a pET22b vector between NdeI and XholI site to construct pET22b-GST-A1 plasmid.

DNA primer ZZP181 (5′- GCGCGCCATATGATTATCGGGCCCGGCAGGGGGTTT-3′) was paired with ZZP277 (5′-CGCGCTCGAGGCCGCCGGATTTGGCCGC-3′) and ZZP276 (5′-CGCGCTCGAGCCACTCCAACATATCAAGAGAATCGCCGCCGGATTTGGCCGC-3′) for PCR amplification of coding sequences of mice sonic hedgehog signaling domain gene from the pBS-Shh plasmid derived from a full length mouse Shh clone[19]. The corresponding PCR fragments of ShhN and ShhN-A-4 were digested with NdeI and XholI, and cloned into pET22b vectors to construct pET22b-ShhN and pET22b-ShhN-A-4 constructs. Primer ZZP 296 (5′-ATTCCCAACGTAGCCGAGAAGACCCTAGACTCTCTTGATATGTTGGAGTGGGGG GCCAGCGGCAGATATGAAGGGAAG-3′) was used for full plasmid amplification of pET22b-ShhN plasmid to construct pET22b-ShhN-(A-4)$_{57-58}$ plasmid.

DNA Primers ZZP280 (5′- AACTAGAGAACCCACTGCTTAC-3′) was paired with ZZP281 (5′-

GGGCCCTCTAGATTACCACTCCAACATATCAAGAGAATCAAACTCATTGTCAAT
GTCCC-3′) for PCR amplification of TfR1-A-4 gene from pcDNA3.1-TfR1-A1 plasmid[6]. The
PCR fragment of TfR1-A-4 gene was digested by EcoRI and XbaI, and cloned into a pcDNA3.1
vector between EcoRI and XbaI sites to construct pcDNA3.1-TfR1-A-4 plasmid. ZZP280 was
paired with ZZP298 (5′-CACACACCCGGGTTACCACTCCAACATATCAAG-3′) for PCR
amplification of TfR1 gene from pcDNA3.1-TfR1-A-4 plasmid. The PCR fragment of TfR1-
A-4 gene was digested by EcoRI and XmaI, and cloned into a pCIG vector between EcoRI and
XmaI sites to construct pCIG-TfR1-A-4 plasmid.

Sequencing was performed at the Dana Farber Cancer Institute Molecular Biology Core
Facilities.

Expression and purification of apo ^{15}N labeled A1 peptide

E. coli BL21 DE3 (star) strains (Invitrogen) were transformed with pET22b-GST-A1
constructs, grown in Luria Broth at 37 °C to saturation, and inoculated to 6 L M9 minimal
medium (pH 7.5) containing 6.78 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 0.5 g/L NaCl, 0.2 mM
$MgSO4$, 0.1 mM $CaCl_2$, 0.1 g/L glycerol, 2 g/L glucose, 1 g/L [^{15}N] NH_4Cl, 0.1 mg/ml
ampicillin, and vitamin mixture supplement. The vitamin mixture supplement contains 80 μg/
L choline chloride, 100 μg/L folic acid, 100 μg/L pantothenic acid, 100 μg/L nicotinamid, 200
μg/L myo-inositol, 100 μg/L pyridoxal hydrochloride, 100 μg/L thiamin hydrochloride, 10
μg/L riboflavin, 20 μg/L adenosine and 20 μg/L biotin. The *E .coli* culture was grown in the
described M9 minimal media to OD_{600} of 0.6 and then induced with 400 μM IPTG. [^{15}N] GST-
A1 protein was overexpressed at 15 °C overnight. *E. coli* cells were harvested by centrifugation,
suspended in PBS, lysed and clarified by centrifugation. The supernatant were incubated with
6×1 mL of Glutathoine Sepharose™ 4B resin (GE Healthcare) in 6 columns. The protein loaded
resin was washed with PBS, and 0.5 mg TEV protease was added into each column and
incubated with the resin overnight. The ^{15}N-enriched A1 peptide was eluted with PBS
supplemented with 1.5 M NaCl and 1 mM DTT to avoid nonspecific hydrophobic adsorption
of the A1 peptide by the resin. The eluted peptide solution was purified by HPLC and dissolved
in a buffered solution of 50 mM MES (pH 6.0), 10 mM $MgCl_2$ and 5 % glycerol. The identity
of the ^{15}N labeled peptide was confirmed with MALDI-TOF mass ([M+H]=1467).

Expression and purification of AcpS

E. coli RL101 strain[13] was inoculated into 6 L of Luria Broth, and grown at 37 °C to OD_{600}
of 0.6, induced with 100 μM IPTG, and overexpressed at 30 °C for 4 hours. Cells were
harvested, suspended in buffer A with 50 mM MES (pH 6.0), 10 mM $MgCl_2$, 1 mM DTT and
lysed. Cell lysate were clarified by centrifugation. The AcpS protein was purified at 4 °C from
the supernatant by cation exchange chromatography with a MonoS column (Amersham) with
buffer A and buffer B (50 mM MES at pH 6.0, 10 mM $MgCl_2$, 1 mM DTT and 1 M NaCl).
The eluted fractions containing the AcpS protein were concentrated and passed through a
Superdex 200 size exclusion chromatography column to change the buffer to peptide
compatible conditions. The eluate was concentrated to 10.6 mg/ml (759 μM).

^{15}N-edited 2D NOESY and the ^{15}N-HSQC spectra of the ^{15}N-A1 peptide

All spectra of the ^{15}N A1 peptide were recorded at 298 K on a Bruker 500 MHz NMR
spectrometer equipped with a 5 mm triple resonance z-gradient probe at the laboratories of
Gerhard Wagner (HMS). The peptide was dissolved in the buffer mentioned above, 10 %
D_2O and 2 mM DSS, as an internal standard, were added to a total sample volume of 500 μl.
All ^{15}N-HSQC spectra were recorded with a resolution of 1024 data points in the proton and
256 data points in the nitrogen dimension. A zero filling to 2048 data points was applied
previous the fourier transformation using a square sine bell window function. For all titration
steps the temperature and the pH were kept identical and the dilution by adding the buffered

AcpS solution to the peptide was corrected by concentrating the sample to a total volume of 500 μl for each step. Both ^{15}N-edited 2D-Noesy spectra for the apo and the holo form of the A1 peptide were recorded with a mixing time of 180 ms and all further variables were kept from the ^{15}N-HSQC spectra.

Phosphopantetheinylation assays for A1, A-1, A-2, A-3, A-4, A-6, E0 and E2 peptides

Peptides were purchased from AnaSpec, CHI Scientific or the HMS BCMP Biopolymers Laboratory and purified by HPLC. The kinetics of PPTase-catalyzed phosphopantetheinylation reaction with CoASH was measured following the procedures previously reported[14] at modified conditions: for AcpS, 10~1000 μM A1, A-1, A-2, A-3, A-4, E0 and E2 peptides were incubated with 0.15~5 μM AcpS for 10 minutes at 37 °C; for Sfp, peptides were incubated with 2~5 μM Sfp for 10 minutes at 37 °C.

In vitro labeling of ShhN proteins

ShhN, ShhN-(A-4)$_{57-58}$, ShhN-(A-4) proteins were expressed in *E. coli* BL21 (star) DE3 strains at 15°C overnight with standard protocols. Cell were harvested, resuspended in solutions containing 25 mM Tris, pH 8.0 and 300 mM NaCl, and clarified by ultracentrifugation. ShhN, ShhN-(A-4)$_{57-58}$, ShhN-(A-4) proteins were purified at room temperature from supernatants with standard Ni-NTA purification protocols, with a salt concentration of 300 mM NaCl in all solutions. Elutes were dialyzed with solutions containing 25 mM Tris (pH 8.0), 300 mM NaCl, 10% glycerol and 0.5 mM DTT. The identities of ShhN and variants were verified with MALDI-TOF mass at the Dana Farber Molecular Core facility.

CoASH (Sigma) were reacted with Alexa Fluor 350 C5 maleimide (Invitrogen, A30505) and Alexa Fluor 633 C5 maleimide (Invitrogen, A20342) to synthesize Alexa Fluor 350-CoA and Alexa Fluor 633-CoA, following manufacture's standard protocol or our previously reported standard protocol for flurophore-CoA synthesis[20].

40 μM ShhN proteins were reacted with 50-150 μM flurophore-CoAs in the solutions containing 21 μM Tris, pH 7.5, 300 mM NaCl, 8.5 μM AcpS and 8.5 mM MgCl$_2$ at 37 °C for 10 minutes. Labeled proteins were separated by SDS-PAGE and visualized over a transilluminator (VWR). ShhN proteins were excited with UV at 302 nm and images were taken with a Nikon CCD camera.

Labeling of cell surface TfR1 receptors

NIH/3T3 cells (American Tissue Culture Collection, ATCC) were maintained in DMEM (ATCC) supplemented with 10% calf serum and glutamine. One day prior transfection, cells were plated on gelatin coated coverslips. Cells were transfected with plasmids (pCIG-TfR1-A-4 or pCIG) in Lipofectamine 2000 (Invitrogen) following the manufacturer's protocols. After culturing for another 2 days, labeling reactions were performed: live cells were placed in DMEM containing 50 mM HEPES, pH 7.5, 5 mM MgCl$_2$, 5 μM AcpS and 2 μM Texas Red-CoA[6] at 37 °C for 30 minutes. Labeled cells were washed with PBS for 5 times, fixed, stained with a nucleus-DAPI dye and mounted for microscopic analysis.

To exam the function of TfR1-A-4, NIH/3T3 cells were similarly grown, transfected with pcDNA3.1-TfR1-A-4, and labeled with 2 μM Texas Red-CoA in the presence of 5 μM AcpS at 37 °C for 30 minutes. After labeling, live cells were washed for 5 times with PBS and incubated with 10 μg/ml Alexa Fluor 488-transferrin (Invitrogen) for 10 minutes to allow transferrin internalization. Alexa Fluor 488-transferrin treated cells were washed 5 times with PBS, fixed, stained with a nucleus-DAPI dye and mounted for microscopic analysis.

Confocal fluorescent images were acquired with Zeiss LSM510 META on an inverted microscope at the imaging centre of Molecular and Cellular Biology Department at Harvard University. Images were collected using a 63x (1.4 NA) or 40x (1.3 NA) oil-immersion objective lens and three laser lines (405, 488, 543 nm). All the images used for comparisons were collected under the identical microscopic setting.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENT

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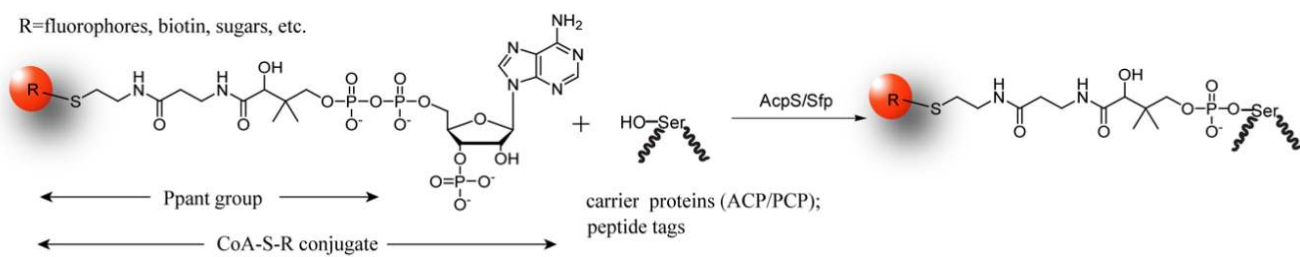(20). Yin J, Lin AJ, Golan DE, Walsh CT. Nat Protoc 2006;1:280–5. [PubMed: 17406245]

Figure 1.
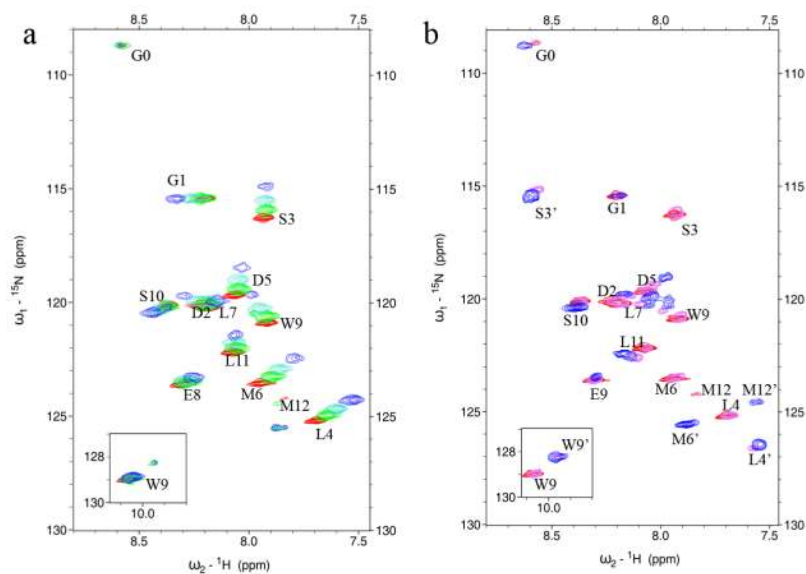The phosphopantetheinylation reaction catalyzed by PPTase.

Figure 2.
NMR spectra of ^{15}N labeled A1 peptide. **a**. HSQC spectra of ^{15}N labeled A1 peptide sequentially titrated by AcpS. Free [^{15}N]-A1 was shown in red, and [^{15}N]-A1/AcpS 1:1.1 complex is shown in blue. Green and aqua represent the intermediate steps in the titration process. All the residues in the A1 peptide were assigned. G_0 is an additional residue at the N-terminus. **b**. Superposition of the ^{15}N-HSQC spectra of apo and holo ^{15}N labeled A1 peptides. The red spectrum represents the 100% apo [15]N A1 peptide. The blue spectrum represents 100% holo [15]N A1 peptide. The pink spectrum represents the mixture of apo and holo A1 as an intermediate stage of this conversion process.
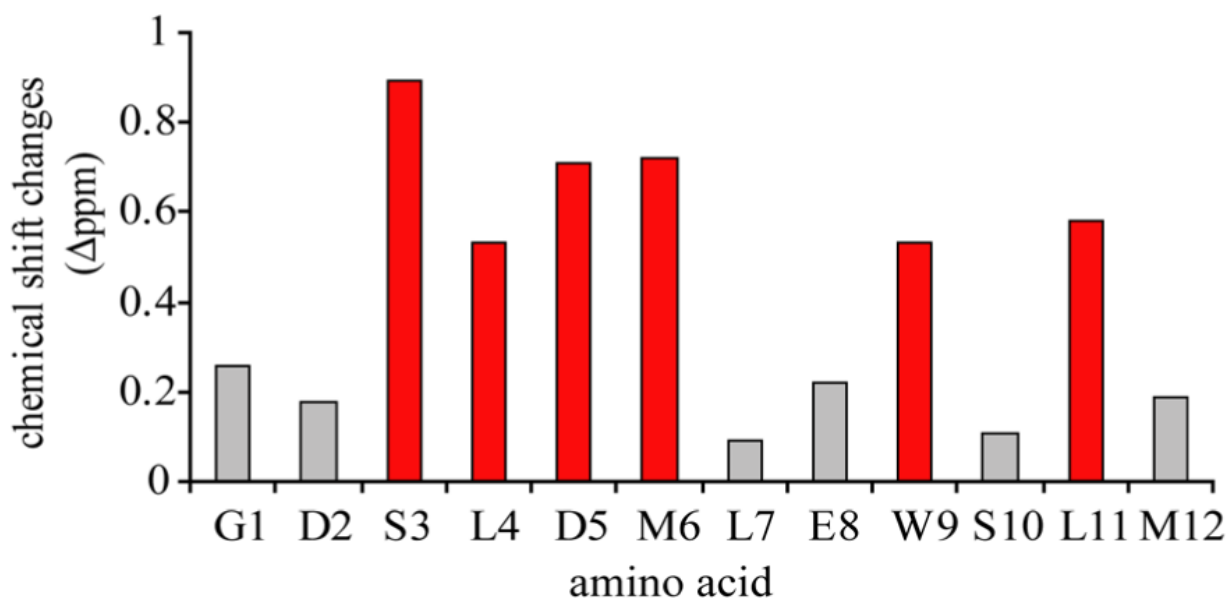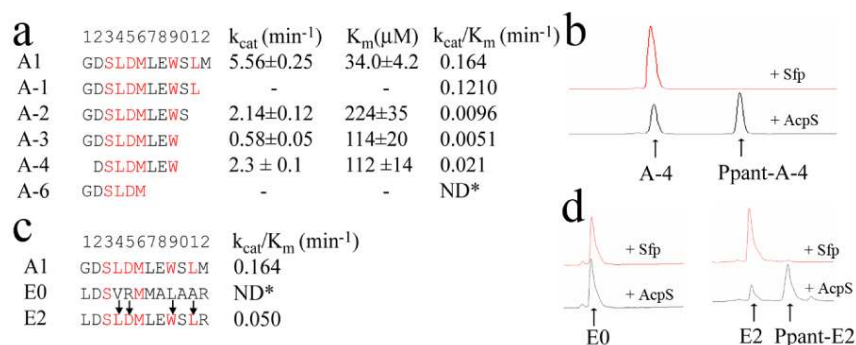
Figure 3.
Plot of ^{1}H/^{15}N combined chemical shift differences of the free A1 peptide compared to the 1:1.1 complex with AcpS. Selectively recognized residues by AcpS are colored in red. All residues with chemical shift changes below the cut off of 0.25 ppm are colored in gray.

Figure 4.
Peptides derived from A-1 peptide. **a**. Kinetics of truncated A1 peptides: A-1, A-2, A-3 and A-4 and A-6. Recognized residues are colored in red. **b**. A-4 phosphopantetheinylation. A-4 peptide was incubated with coenzyme A in the presence of 5 μM AcpS or Sfp for 10 minutes and products were monitored by HPLC. **c**. Engineering of a peptide from aryl carrier protein EntB in enterobactin synthetase. E0 is the native sequence from EntB. V_4, R_5, L_9, A_{11} in E0 were mutated to L, D, W, and L respectively. Recognized residues are colored in red. **d**. HPLC trace of E0 and E2 phosphopantetheinylation. E0 and E2 peptides were incubated with coenzyme A in the presence of 3 μM AcpS or Sfp for 10 minutes and products were monitored by HPLC
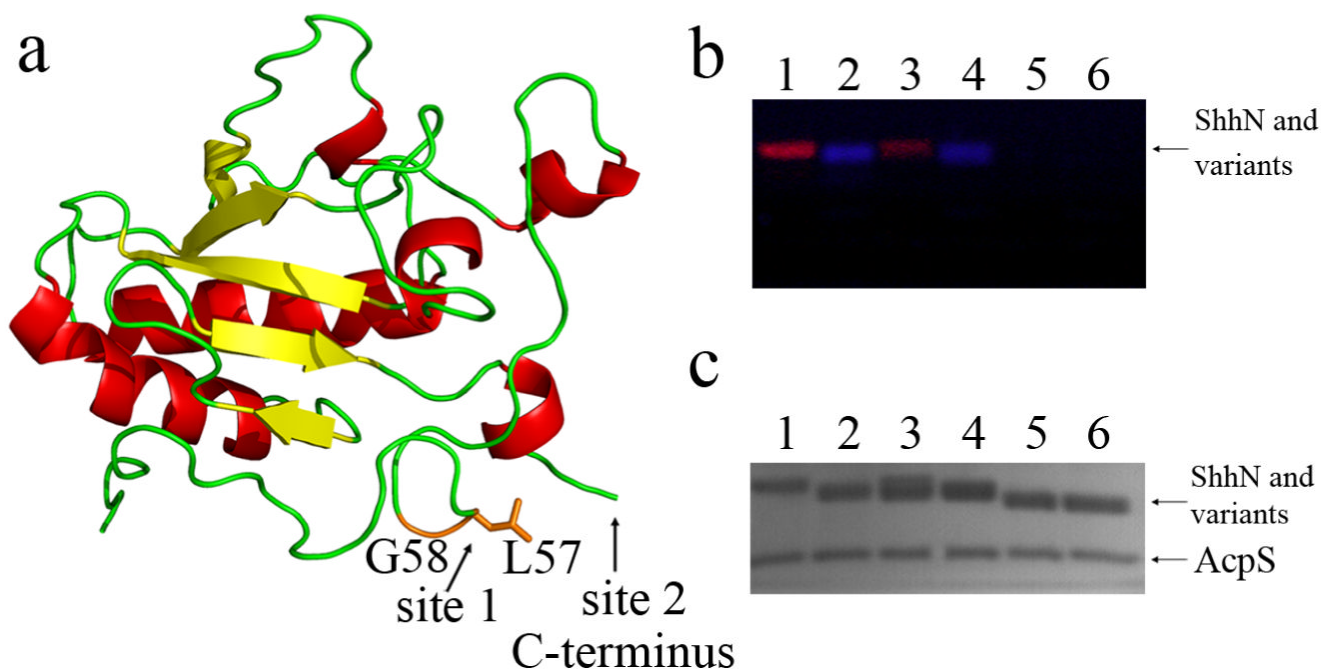
Figure 5.
Scheme of A-4 peptide insertion into the ShhN protein. A-4 tag were either inserted at site 1 (loop region between residues L_{57}-G_{58}) or site 2 (C-terminus) of ShhN protein. Structural figures were prepared with Pymol and Protein Data Bank file 1VHH. **b**. Fluorescent image of ShhN proteins labeled with diverse flurophores. ShhN-(A-4$_{57-58}$) protein reacted with Alexa Fluor 633-CoA and Alexa Fluor 350-CoA in the presence of AcpS are shown in lanes 1 and 2; ShhN-A-4 reacted with the same two fluorescent CoA conjugates in the presence AcpS are shown in lanes 3 and 4; ShhN reacted with the same two fluorescent CoA conjugates in the presence of AcpS are shown in lanes 5 and 6. **c**. Coomassie staining of ShhN proteins on the same gel in panel b.
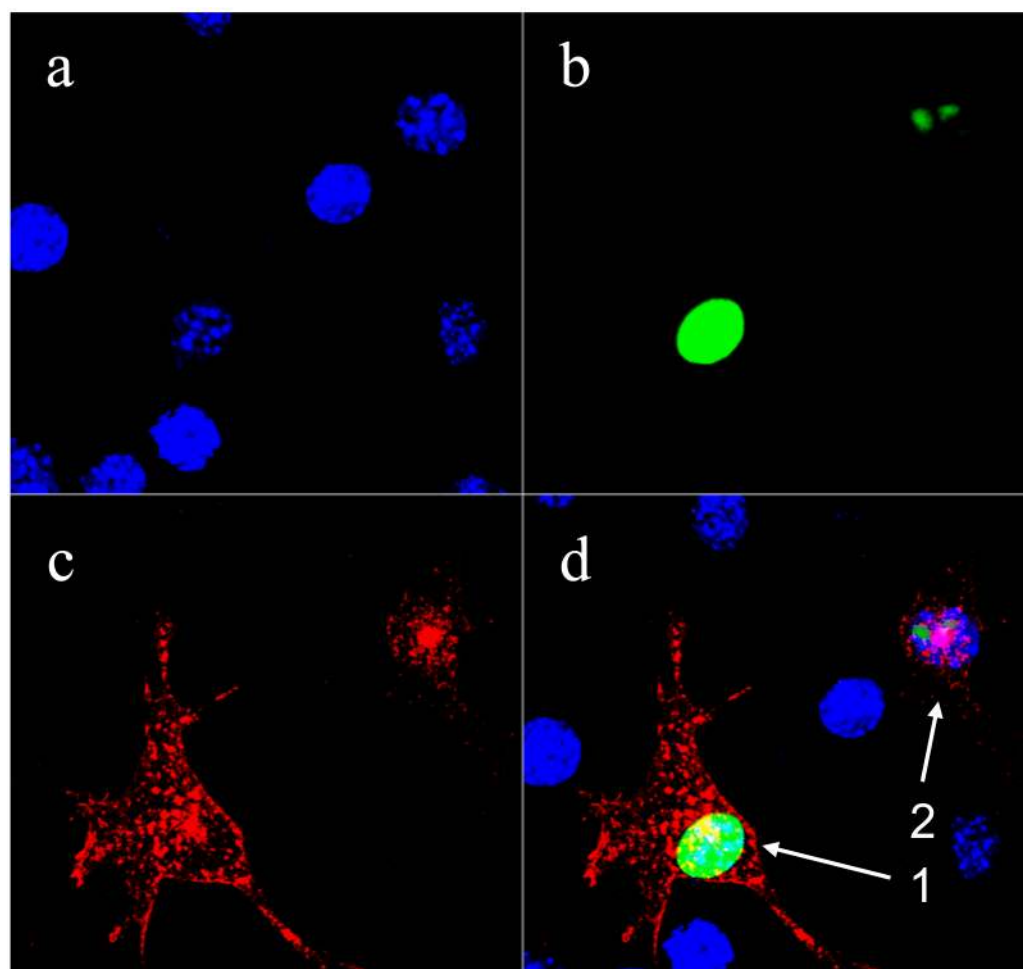
Figure 6.
Labeling of expressed transferrin receptors on live NIH/3T3 cell surfaces. TfR1-A-4 was labeled with 5 μM AcpS and 2 μM Texas Red-CoA for 30 minutes. DAPI, green and red channels are shown in panels **a**, **b**, **c** respectively, and panel **d** shows merged colors. Cells transfected with pCIG-TfR1-A-4 (see arrows) were labeled with Texas Red efficiently, and no labeling was seen for neighboring untransfected cells.
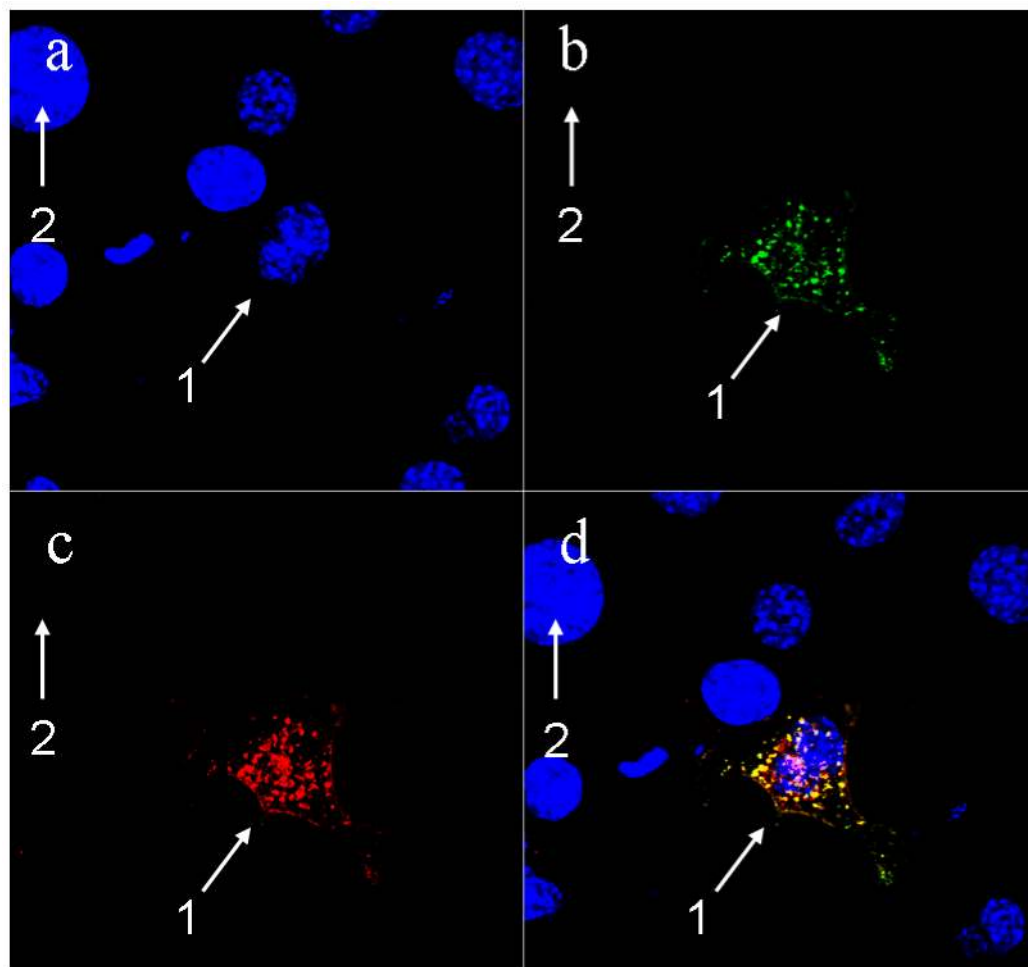
Figure 7.
Transferrin internalization and colocalization. NIH/3T3 cells transfected with pcDNA3.1-A-4 were labeled with 5 μM AcpS and 2 μM Texas Red-CoA for 30 minutes, and then incubated with Alexa Fluor 488-transferrin. Panel **a** (DAPI channel) shows the nuclei of all the cells; panel **b** (green channel) shows Alexa Fluor 488-transferrin; panel **c** (red channel) shows labeled TfR1-A-4; panel **d** shows combined colors of **a-c**.