Yeast One-Hybrid Gγ Recruitment System for Identification of Protein Lipidation Motifs

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Abstract

Fatty acids and isoprenoids can be covalently attached to a variety of proteins. These lipid modifications regulate protein structure, localization and function. Here, we describe a yeast one-hybrid approach based on the Gγ recruitment system that is useful for identifying sequence motifs those influence lipid modification to recruit proteins to the plasma membrane. Our approach facilitates the isolation of yeast cells expressing lipid-modified proteins via a simple and easy growth selection assay utilizing G-protein signaling that induces diploid formation. In the current study, we selected the N-terminal sequence of Gα subunits as a model case to investigate dual lipid modification, i.e., myristoylation and palmitoylation, a modification that is widely conserved from yeast to higher eukaryotes. Our results suggest that both lipid modifications are required for restoration of G-protein signaling. Although we could not differentiate between myristoylation and palmitoylation, N-terminal position 7 and 8 play some critical role. Moreover, we tested the preference for specific amino-acid residues at position 7 and 8 using library-based screening. This new approach will be useful to explore protein-lipid associations and to determine the corresponding sequence motifs.

Introduction

A wide range of proteins are modified by covalent attachment of fatty acids and/or isoprenoids, modifications that play major roles in regulating protein structure and function [1]. Moreover, aberrant expression of lipidated proteins or their biosynthetic enzymes is associated with many diseases, ranging from cancer to neurological disorders [2]. Because recruitment of lipidated proteins to the plasma membrane influences complex signaling pathways that regulate specific physiological functions, there is a great interest in gaining a better understanding of protein trafficking via lipid modification.

Dual lipidation of proteins, which consist of myristoylation and palmitoylation at N-terminal residues, a posttranslational modification that has been conserved from yeast to humans, leads to transport of the modified proteins toward plasma membrane. For some lipid modification, the consensus motifs have been identified. For example, after removal of the N-terminal methionine residue by methionine aminopeptidase, myristate is attached to the N-terminal glycine of protein substrates with the consensus motif Met1-Gly2-Xaa3-Xaa4-Xaa5-Ser/Thr6-Xaa7-Xaa8 (where Xaa indicates any amino acid residue) [3] in a reaction catalyzed by myristoyl-CoA/protein N-myristoyltransferase (NMT) [4]. Further refinement of this consensus might be possible, however, as it has been shown that not all amino acid residues are allowable at the positions indicated by Xaa [4–5]. Cysteine residues within proteins can be acylated with the 16-carbon fatty acid palmitate. Although protein acyltransferases (PATs) have a common DHHC Cys-rich domain [6], the consensus motif for palmitoylation of proteins is still unclear, due at least in part to the diversity of substrates that can be recognized by multiple PATs, including 7 DHHC proteins in yeast and 23 in humans [7].

We previously developed the Gγ recruitment system (GRS), which uses yeast G-protein signaling (pheromone signaling) to detect protein–protein interactions [8–10]. This system is based on the observation that transduction of the signal requires localization of the γ subunit of G-proteins (Gγ) to the inner leaflet of the plasma membrane [11]. Deletion of lipidation sites in yeast Gγ (Gγcyto) completely disrupts G-protein signaling [8]; however, protein–protein interactions between a Gγcyto-fused target and a membrane-bound binding partner can restore G-protein signaling.

Here we suggest a new approach to the investigation of protein-lipid associations, yeast one-hybrid GRS (Fig. 1). If a Gγcyto-fused hybrid protein is localized to the plasma membrane, G-protein signaling is recovered, inducing a mating response. Hybrid proteins positive in the assay can then be detected by diploid growth selection [9–10]. Thus, using this system, it is possible to ask if a domain fused to Gγcyto associates with lipid molecules that localize the protein to the plasma membrane.

In the current study, we attached short signal sequences, from 6 to 10 amino acid residues (AA), derived from G-protein Gα subunits to N-terminus of Gγcyto to append dual lipid modifications, i.e., myristoylation and palmitoylation, resulting in trafficking to the plasma membrane. Gα subunits play a key role in signal transduction that is mediated by lipid modification. It has been reported that lipidation-defective Gα1 mutatns (G2A and C3A) have lost their intrinsic ability to localize to membranes [12–13]. Among human Gα proteins, most subunits in the Gα subfamily...
receive both myristoylation and palmitoylation at N-terminal residues, similar to yeast Gpa1. Indeed, human G"ε" contains the same sequence at N-terminal 6 AA (Met 1-Gly2-Cys3-Thr4-Val5-Met 6) as yeast Gpa1 [14–15]. We have evaluated the membrane-targeting ability of several N-terminal short sequences thought to receive myristoylation and palmitoylation using yeast one-hybrid GRS.

Materials and Methods

Strains and Media

Detailed information about Saccharomyces cerevisiae strains BY4741 [16] and MCF4741 [17], as well as about other strains used in this study, including genotypes, are shown in Table 1. Yeast cells were grown in YPD medium containing 1% yeast extract, 2% peptone and 2% glucose, or in SD medium without histidine (SD–His) containing 0.67% yeast nitrogen base without amino acids (Becton Dickinson and Company, Franklin Lakes, NJ, USA), 2% glucose, 20 mg/l uracil, 30 mg/l leucine and 20 mg/l methionine. In either case, 2% agar was added to make solid media (plates).

Construction of Plasmids

The sequences of oligonucleotides used in this study are shown in Table 2. The plasmids shown in Table 1 were made as follows. Using BY4741 genomic DNA as a template, each DNA fragment encoding Gγ, Gγcyto, MP6-Gγcyto, MP6L2-Gγcyto, MP10Y-Gγcyto, MP10Hiii-Gγcyto, MP10Hii-Gγcyto, MP10Hi-Gγcyto, or MP6L2Y-Gγcyto was amplified with oligonucleotide pair o3 and o4, o3 and o5, o6 and o5, o7 and o6, o8 and o7, o9 and o8, o10 and o9, o11 and o8, o12 and o7, and inserted in place of the EGFP at the NotI-BamHI sites of pHY-PGA, yielding the plasmids pHY-Gγ, pHY-Gγcyto, pHY-6Gγcyto, pHY-10YGγcyto, pHY-10HiGγcyto, pHY-10HiiGγcyto, pHY-10HiGγcyto, and pHY-6L2YGγcyto, respectively.

Using plasmid pHY-10YGγcyto as a template, each DNA fragment encoding G2A-Gγcyto, C3A-Gγcyto, or G2A-C3A-Gγcyto was amplified with oligonucleotide pair o13 and o18, o15 and o18, or o16 and o14, and inserted in place of the EGFP at the NotI-BamHI sites of pHY-PGA [17], yielding the plasmids pHY-G2A-Gγcyto, pHY-C3A-Gγcyto, and pHY-GCA-Gγcyto, respectively.

For expression analyses, using BY4741 genomic DNA as a template, each DNA fragment encoding Gγ, Gγcyto, MP6-Gγcyto, MP6L2-Gγcyto, MP10Y-Gγcyto, MP10Hi-Gγcyto, MP10Hi-Gγcyto, MP8YLGγcyto, MP6LY-Gγcyto, G2A-Gγcyto, C3A-Gγcyto, or G2A-C3A-Gγcyto was amplified with oligonucleotide pair o3 and o4, o3 and o5, o6 and o5, o7 and o6, o8 and o7, o9 and o8, o10 and o9, o11 and o8, or o12 and o7, and inserted into the NotI-SalI sites of pHY-PGA, yielding the plasmids pHY-Gγ, pHY-Gγcyto, pHY-6Gγcyto, pHY-10YGγcyto, pHY-10HiGγcyto, pHY-10HiGγcyto, and pHY-6L2YGγcyto, respectively.

Using plasmid pHY-10YGγcyto as a template, each DNA fragment encoding G2A-Gγcyto, C3A-Gγcyto, or G2A-C3A-Gγcyto was amplified with oligonucleotide pair o13 and o18, o15 and o18, or o16 and o14, and inserted into the NotI-SalI sites of pHY-PGA, yielding the plasmids pHY-G2A-Gγcyto, pHY-C3A-Gγcyto, and pHY-GCA-Gγcyto, respectively.

Construction of Yeast Strains and Yeast Transformation

The plasmid pUYG-Cre [17], which expresses Cre recombinase under the control of the promoter of the GAL1 gene, was introduced into MCF4741 using the lithium acetate method [18]. To exclude the kanMX4 gene between the two loxP sites, a transformant harboring pUYG-Cre was cultivated in Sgal media (containing 6.7% yeast nitrogen base without amino acids; Becton Dickinson and Company, Franklin Lakes, NJ, USA, and 2% galactose) without uracil but containing 20 mg/l histidine, 30 mg/l leucine and 20 mg/l methionine. Then, the cells were maintained on YPD media containing 5-fluoroorotic acid (5-FOA) to select ura3" strains that have lost the plasmid pUYG-Cre, yielding MCF-B1.

Deletion of STE18 gene by substitution with LEU2 was carried out as follows. The upstream region of STE18 was termed STE5', and the downstream region of STE5' was termed STE3'. The LEU2 gene was amplified with oligonucleotide pair o19 and o20, using pLY-3GC [17] as a template. Using genomic DNA from strain BY4741 as the template, STE5' was amplified with oligonucleotide pair o21 and o22, and STE3' was amplified with oligonucleotide pair o23 and o24. DNA fragments containing STE3'-LEU2-STE3' were combined using oligonucleotide pair o21 and o24 from the amplified fragments STE5', LEU2 and STE3', and then used to transform MG-F1 using the lithium acetate method. Transformants were selected on SD plates without uracil (SD – Leu) but containing 20 mg/l histidine, 20 mg/l uracil and 20 mg/l methionine. The resulting strain was named MCF-B1L. Each plasmid shown in Table 1 was introduced into MCF-B1L using the lithium acetate method and the transformants were used for the following analyses.

Diploid Growth Assay

Evaluation of mating ability was performed as follows. Each engineered yeast strain was cultivated in 1 ml of YPD medium with the mating partner BY4742 at 30°C for 1.5 h setting the
Table 1. Yeast strains and plasmids used in this study.

| Name               | Description                                                                 | Reference source            |
|--------------------|------------------------------------------------------------------------------|-----------------------------|
| **Yeast strains**  |                                                                              |                             |
| BY4741             | MATa his3Δ1 ura3Δ0 leu2Δ0 met15Δ0                                          | Brachmann et al. [16]       |
| MCF4741            | BY4741 fig1Δ: FIG1-EGFP-loxP-kanMX4-loxP                                      | Fukuda et al. [17]          |
| MCF-B1             | BY4741 fig1Δ: FIG1-EGFP-loxP                                                  | Present study               |
| MCF-B1L            | MCF-B1 ste18Δ: LEU2                                                           | Present study               |
| BY4742             | MATa his3Δ1 ura3Δ0 leu2Δ0 lys2Δ0                                            | Brachmann et al. [16]       |
| BY4743             | MATa his3Δ1/His3Δ1 leu2Δ0/Leu2Δ0 lys2Δ0 met15Δ0/MET15Δ0 ura3Δ0/ura3Δ0         | Brachmann et al. [16]       |
| **Plasmids**       |                                                                              |                             |
| pHY-2GA            | 2 µ ori, HIS3 marker, and Pst2T-EGFP                                        | Fukuda et al. [17]          |
| pLY-3GC            | 2 µ ori, LEU2 marker, and Pst2T-EGFP                                        | Fukuda et al. [17]          |
| pHY-PGA            | 2 µ ori, HIS3 marker, and PPGK1-EGFP                                        | Present study               |
| pHY-Gy             | 2 µ ori, HIS3 marker, and PPGK1-Gy                                          | Present study               |
| pHY-Gyc            | 2 µ ori, HIS3 marker, and PPGK1-GyC                                         | Present study               |
| pHY-66Gyc          | 2 µ ori, HIS3 marker, and PPGK1-MP6-Gyc                                       | Present study               |
| pHY-6LGyc          | 2 µ ori, HIS3 marker, and PPGK1-MP6L-Gyc                                      | Present study               |
| pHY-10GYc          | 2 µ ori, HIS3 marker, and PPGK1-MP10Gy                                       | Present study               |
| pHY-10iiGYc        | 2 µ ori, HIS3 marker, and PPGK1-MP10ii-Gyc                                    | Present study               |
| pHY-10iiGyc        | 2 µ ori, HIS3 marker, and PPGK1-MP10ii-GyC                                    | Present study               |
| pHY-8YLGyc         | 2 µ ori, HIS3 marker, and PPGK1-MP8YL-GyC                                     | Present study               |
| pHY-6L2YGyc        | 2 µ ori, HIS3 marker, and PPGK1-MP6L2-GyC                                     | Present study               |
| pHY-G2A-Gyc        | 2 µ ori, HIS3 marker, and PPGK1-G2A-GyC                                      | Present study               |
| pHY-C3A-Gyc        | 2 µ ori, HIS3 marker, and PPGK1-C3A-GyC                                       | Present study               |
| pHY-GCA-Gyc        | 2 µ ori, HIS3 marker, and PPGK1-GCA-GyC                                      | Present study               |
| pHY-Gy             | 2 µ ori, HIS3 marker, and PPGK1-Gy                                          | Present study               |
| pHY-Gyc            | 2 µ ori, HIS3 marker, and PPGK1-GyC                                          | Present study               |
| pHY-66Gyc          | 2 µ ori, HIS3 marker, and PPGK1-MP6-GyC                                       | Present study               |
| pHY-6L2GYc         | 2 µ ori, HIS3 marker, and PPGK1-MP6L-GyC                                      | Present study               |
| pHY-10iGYc         | 2 µ ori, HIS3 marker, and PPGK1-MP10i-GyC                                     | Present study               |
| pHY-8YLGyc         | 2 µ ori, HIS3 marker, and PPGK1-MP8YL-GyC                                     | Present study               |
| pHY-6L2YGyc        | 2 µ ori, HIS3 marker, and PPGK1-MP6L2-GyC                                     | Present study               |
| pHY-G2A-Gyc        | 2 µ ori, HIS3 marker, and PPGK1-G2A-GyC                                       | Present study               |
| pHY-C3A-Gyc        | 2 µ ori, HIS3 marker, and PPGK1-C3A-GyC                                       | Present study               |
| pHY-GCA-Gyc        | 2 µ ori, HIS3 marker, and PPGK1-GCA-GyC                                      | Present study               |
| pUYG-Cre           | 2 µ ori, URA3 marker, and PPGK1-Cre                                          | Fukuda et al. [17]          |

initial OD₆₀₀ of each haploid cell at 0.1. After cultivation, yeast cells were harvested, washed, and resuspended in distilled water. Setting the OD₆₀₀ at 1, 0.1 and 0.01, 10 µl of each cell suspension were spotted on an SD plate without methionine and lysine but containing 20 mg/l histidine, 30 mg/l leucine, and 20 mg/l uracil (SD – Met, Lys plate) for growth selection of diploid cells. After incubation at 30°C for 2 days, we recorded image data of diploid colonies generated on solid medium. For quantitative evaluation, cell suspensions were spread on SD – Met, Lys plates using the appropriate dilution factor for each strain. After incubation at 30°C for 2 days, the colony number was determined for each strain and multiplied by its dilution factor to estimate the number of diploid cells generated in an equivalent volume of 1 ml of cell suspension, with the OD₆₀₀ set at 1.0.

**Fluorescent Reporter Assay**

The EGFP gene was fused to the C-terminus of G'yC and used as a fluorescent reporter to indicate expression of G'yC-fused hybrid proteins in yeast cells. The cells were incubated in SD – His medium at 30°C for 18 h, harvested and washed with distilled water. The cells were resuspended in 100 µl of distilled water to an optical density of 5.0 at 600 nm (OD₆₀₀ = 5.0). GFP fluorescence intensities were measured on an Infinite 200, fluorescence microplate reader (Tecnat Japan Co., Ltd., Kawasaki, Japan). The excitation wavelength was set at 485 nm with the bandwidth of 20 nm and emission wavelength was set at 535 nm with the bandwidth of 25 nm for green fluorescence detection; the gain was set at 50.
Table 2. Sequence of oligonucleotides used to construct plasmids and yeast strains.

| Number | Sequence |
|--------|----------|
| 1      | 5’-tttcccccgccggaagatgctccgatgctgggtgcc-3’ |
| 2      | 5’-aaaaaccggccccgctttttatagtttgtga-3’ |
| 3      | 5’-ttttgccgccgcagcatacgatgcagtttcaacaac-3’ |
| 4      | 5’-ccccgcatcactgactagctgaacatagtgaacacgagagaggtac-3’ |
| 5      | 5’-ccccgccgatgctgggtacagtgaacacgagagaggtac-3’ |
| 6      | 5’-aaaaaccggccccgcatggggtgtacagtgaacacgagagaggtac-3’ |
| 7      | 5’-aaaaaccggccccgcatggggtgtacagtgaacacgagagaggtac-3’ |
| 8      | 5’-aaaaaccggccccgcatggggtgtacagtgaacacgagagaggtac-3’ |
| 9      | 5’-aaaaaccggccccgcatggggtgtacagtgaacacgagagaggtac-3’ |
| 10     | 5’-aaaaaccggccccgcatggggtgtacagtgaacacgagagaggtac-3’ |
| 11     | 5’-aaaaaccggccccgcatggggtgtacagtgaacacgagagaggtac-3’ |
| 12     | 5’-aaaaaccggccccgcatggggtgtacagtgaacacgagagaggtac-3’ |
| 13     | 5’-ataaaaaaccggccccgcgttctttgtcatggcatgatgac-3’ |
| 14     | 5’-ccccagtggattgtgattgtgatcagttcaaaac-3’ |
| 15     | 5’-ataaaaaaccggccccgcgttctttgtcatggcatgatgac-3’ |
| 16     | 5’-ataaaaaaccggccccgcgttctttgtcatggcatgatgac-3’ |
| 17     | 5’-ttttgtcatcactgactacgatgctgggtacagtgaacacgagagaggtac-3’ |
| 18     | 5’-ttttttgtcatcactgactacgatgctgggtacagtgaacacgagagaggtac-3’ |
| 19     | 5’-ttttttgtcatcactgactacgatgctgggtacagtgaacacgagagaggtac-3’ |
| 20     | 5’-ttttttttgtcatcactgactacgatgctgggtacagtgaacacgagagaggtac-3’ |
| 21     | 5’-ttttttttgtcatcactgactacgatgctgggtacagtgaacacgagagaggtac-3’ |
| 22     | 5’-ttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt...
lipidations, restored signaling, as evidenced by the production of diploid colonies.

We next attached the N-terminal 10 AA from the N-terminus of Gpa1 (yeast G\textsubscript{a} subunit) to G\textsubscript{cyto}, (MP10Y-G\textsubscript{cyto}) in order to create a potential lipidation site receiving myristoylation and palmitoylation. As shown in Figure 2, MP10Y-G\textsubscript{cyto} successfully restored signaling, resulting in diploid cells, although the number of diploid cells generated from MP10Y-G\textsubscript{cyto} was lower than from wild-type G\textsubscript{y}. These results suggest that it is possible to evaluate the membrane-targeting ability of additional motifs fused to G\textsubscript{cyto}, as described in Figure 1. We additionally investigated the contribution of myristoylation and palmitoylation to the membrane-targeting ability of MP10Y-G\textsubscript{cyto}. Neither lipidation-defective mutants G2A- (lacking myristoylation-acceptor site), C3A- (lacking palmitoylation-acceptor site) and G2A-C3A-G\textsubscript{cyto} (lacking both sites) restored the signal transduction at all, suggesting that both lipid modifications are required.

**Contribution of the N-terminal Sequence from Position 7 to 10 to Membrane Targeting**

Yeast Gpa1 and human G\textsubscript{c2}, receiving both myristoylation and palmitoylation, has the common N-terminal 6 AA (MP6 sequence; Met\textsubscript{1}-Gly\textsubscript{2}-Cys\textsubscript{3}-Thr\textsubscript{4}-Val\textsubscript{5}-Ser\textsubscript{6}). To confirm its function for N-terminal palmitoylation, has the common N-terminal 6 AA (MP6 sequence; Met\textsubscript{1}-Gly\textsubscript{2}-Cys\textsubscript{3}-Thr\textsubscript{4}-Val\textsubscript{5}-Ser\textsubscript{6}). To confirm its function for N-terminal palmitoylation, we used the yeast one-hybrid assay to test the mating ability of yeast cells expressing G\textsubscript{cyto}, intact G\textsubscript{y}, MP10-G\textsubscript{cyto}, G2A-G\textsubscript{cyto}, C3A-G\textsubscript{cyto} or G2A-C3A-G\textsubscript{cyto}. To confirm a direct relationship between membrane-localization of G\textsubscript{cyto} hybrids and recovery of signal transduction, we used the yeast one-hybrid assay to test the mating ability of yeast cells expressing G\textsubscript{cyto}, intact G\textsubscript{y}, MP10-G\textsubscript{cyto}, G2A-G\textsubscript{cyto}, C3A-G\textsubscript{cyto} or G2A-C3A-G\textsubscript{cyto}.

**Figure 2. Assessment of the function of N-terminal motifs derived from yeast G\textsubscript{a}** The diploid growth assay was used to test the mating ability of yeast cells expressing G\textsubscript{cyto}, intact G\textsubscript{y}, MP10-G\textsubscript{cyto}, G2A-G\textsubscript{cyto}, C3A-G\textsubscript{cyto} or G2A-C3A-G\textsubscript{cyto}. doi:10.1371/journal.pone.0070100.g002

To further investigate the contribution of the sequence from position 7 to position 10 to N-terminal dual lipid modifications, two kinds of G\textsubscript{cyto} hybrids were prepared (Fig. 3B). MP6L2Y-G\textsubscript{cyto} contains an irrelevant linker sequence (Gly-Gly) at position 7 and 8 of the N-terminal 10 AA sequence derived from Gpa1 and MP9YL-G\textsubscript{cyto} contains an irrelevant linker sequence (Gly-Ser) at position 9 and 10. In the diploid growth assay, MP9YL-G\textsubscript{cyto} restored diploid cell formation but MP6L2Y-G\textsubscript{cyto} resulted in only a few diploid colonies (Fig. 3B). This suggests that in addition to the MP6 sequence, the N-terminal positions 7 and 8 also contribute largely to dual lipid modifications, and that positions 9 and 10 have subtle but definite effects on these modifications.

**Quantitative Assessment of the Membrane-targeting Ability of G\textsubscript{cyto} Hybrids**

To compare expression levels among G\textsubscript{cyto} hybrids shown in Figure 4A, a GFP reporter was fused to the C-terminus of G\textsubscript{y}, or wild-type G\textsubscript{y}. Figure 4B shows the fluorescence intensity of each yeast cell suspension. All G\textsubscript{cyto} hybrids were successfully synthesized in yeast. Moreover, based on GFP fluorescence, we found that the levels of expression of the hybrid proteins were higher than that observed for wild-type G\textsubscript{y}. We next used the number of colonies generated in the diploid growth assay as a quantitative measure of the membrane-targeting ability of each G\textsubscript{cyto} hybrid (Fig. 4C). With the exception of wild-type G\textsubscript{y}, MP10Y-G\textsubscript{cyto} produced the most diploid colonies, suggesting that the N-terminal 10 AA derived from Gpa1 lead to efficient attachment of both myristate and palmitate.

To confirm a direct relationship between membrane-localization of G\textsubscript{cyto} hybrids and recovery of signal transduction, we observed yeast cells expressing G\textsubscript{cyto} hybrid-GFP using a confocal microscopy (Fig. 4D). While G2A-G\textsubscript{cyto}, G2A-C3A-G\textsubscript{cyto}, MP6L-G\textsubscript{cyto}, MP10HiIII-G\textsubscript{cyto} and MP10HiII-G\textsubscript{cyto} were localized in cytoplasm as well as G\textsubscript{y}, G\textsubscript{cyto} that completely lacks membrane-targeting ability, other G\textsubscript{cyto} hybrids were detected at plasma membrane with different degrees of intensity. The intensity of green fluorescence from the juxtamembrane region, except for C3A-G\textsubscript{cyto} relatively reflected the efficiency of MP6-G\textsubscript{cyto}, MP10Y-G\textsubscript{cyto}, MP9YL-G\textsubscript{cyto} and MP6L2Y-G\textsubscript{cyto} at which signal transduction was restored.

1: MP10Y-G\textsubscript{cyto}  
\[\text{MGC TVS TQTI MTSV...NSV}\]  
\[\text{Gpa1}\]  
\[\text{G_y}\]  
2: G2A-G\textsubscript{cyto}  
\[\text{MAC TVS TQTI MTSV...NSV}\]  
\[\text{G_y}\]  
3: C3A-G\textsubscript{cyto}  
\[\text{MGA TVS TQTI MTSV...NSV}\]  
\[\text{G_y}\]  
4: G2A-C3A-G\textsubscript{cyto}  
\[\text{MAA TVS TQTI MTSV...NSV}\]  
\[\text{G_y}\]
Exploration of Amino Acid Residue Preference via Screening of a Library Randomized at N-terminal Positions 7 and 8

A randomized DNA library encoding Met1-Gly2-Cys3-Thr4-Val5-Ser6-Xaa7-Xaa8-Thr9-Ile10-Gcyto was prepared by PCR amplification using primers with NNK codons (N represents an equal mixture of A, G, C, and T; K represents an equal mixture of G and T) covering all 20 amino acids, followed by introduction into the MCF-B1L strain (initial yeast library). After dilution and isolation of initial yeast library colonies on solid media, the population of amino acid residues present at positions 7 and 8 was determined by DNA sequencing of plasmids from 100 randomly picked colonies (‘initial’ in Fig. 5).

We next randomly picked 100 colonies grown in the diploid growth assay and determined the frequency of each amino acid residue at positions 7 and 8 (‘final’ in Fig. 5). The ratio of enrichment for each amino acid residue was defined as the value for final population divided by that for initial population (Table 3). Uncharged hydrophilic residues, i.e., Ser, Thr, Asn and Gln, were preferred at positions 7 and 8. No preference was observed for charged residues, i.e., Asp, Glu, Arg, Lys and His, with the exception of a preference for His at position 8. We never observed Asp at position 7, or Arg or Lys at position 8 (ratio of enrichment of 0 in all three cases), suggesting that those residues are incompatible with lipid modification. Cys is known to be a potent acceptor for palmitoylation. In this assay, Cys was enriched at position 7 but not at position 8. Aromatic residues Phe and Tyr, but not Trp (the largest amino acid), were preferred at positions 7 and 8. Among hydrophobic residues, Ala, Val, Ile and Met were preferred at position 7, and Val, Leu and Met were enriched at position 8 (‘final’ in Fig. 5). The ratio of enrichment for each amino acid residue was defined as the value for final population divided by that for initial population (Table 3). Uncharged hydrophilic residues, i.e., Ser, Thr, Asn and Gln, were preferred at positions 7 and 8. No preference was observed for charged residues, i.e., Asp, Glu, Arg, Lys and His, with the exception of a preference for His at position 8. We never observed Asp at position 7, or Arg or Lys at position 8 (ratio of enrichment of 0 in all three cases), suggesting that those residues are incompatible with lipid modification. Cys is known to be a potent acceptor for palmitoylation. In this assay, Cys was enriched at position 7 but not at position 8. Aromatic residues Phe and Tyr, but not Trp (the largest amino acid), were preferred at positions 7 and 8. Among hydrophobic residues, Ala, Val, Ile and Met were preferred at position 7, and Val, Leu and Met were enriched at position 8 (‘final’ in Fig. 5).

Figure 3. Contribution of N-terminal positions 7 to 10 to the efficiency of dual lipidation. The diploid growth assay was used to test the mating ability of (A) yeast cells expressing MP6-Gcyto, MP6L-Gcyto, MP10Y-Gcyto, MP10Hi-Gcyto, or MP10HiIII-Gcyto, and (B) those expressing MP8YL-Gcyto or MP6L2Y-Gcyto.

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Yeast One-Hybrid Reveals Protein Lipidation Motifs
Figure 4. Expression levels and membrane-targeting ability of $G_{\gamma\text{cyto}}$-hybrids. (A) The N-terminal sequence of Gpa1 (yeast $G_{\alpha}$ subunit), which confers membrane-targeting ability to the protein. Gpa1 receives dual lipid modification comprised of myristoylation and palmitoylation at N-terminal Gly and Cys residues, respectively, leading to recruitment to the plasma membrane. The membrane-targeting ability of N-terminal sequences derived from several different $G_{\alpha}$ subunits were evaluated using yeast one-hybrid GRS. (B) A fluorescent reporter assay was used to
position 8. Moreover, Gly and Pro were rarely or never seen at either position 7 or position 8.

Verification of Amino Acid Residue Preferences Conferring Gc Membrane-targeting Ability

To verify the results of the preference test, we next looked at the membrane-targeting ability of the set of Gc-hybrids listed in Table 4 using the diploid growth assay (Fig. 6). DW-, GE- and WA-Gc-hybrids were used as representatives expected to confer low efficiency of lipid modification (Asp, Gly and Trp were enriched the least at position 7). FN-, QT- and TM-Gc-hybrids were used as representatives expected to confer efficient lipid modification (Phe, Gln and Thr were the most enriched residues at position 7). As shown in Figure 6, DW-, GE- and WA-Gc rarely generated diploid cells. In contrast, FN-, QT- and TM-Gc restored signal transduction with a higher efficiency than MP10Y-Gc, which contains the wild-type 10 AA from Gpa1. These results suggest that the amino acid residue biases identified using the yeast one-hybrid GRS (see Figure 5 and Table 3) are reliable predictions of protein activity in cells.

Discussion

The aim of this study was to establish a genetic system, yeast one-hybrid GRS, for analyzing membrane-targeting ability of proteins in a living cell (Fig. 1). Our system is based on the observation that G-protein signaling requires localization of Gc to the inner leaflet of the plasma membrane [11]. In the current study, we focused the sequence involved in N-terminal dual lipid modifications, i.e., myristoylation and palmitoylation. Protein lipidation is a critical modification that in most cases results in protein trafficking to the membrane. Because the attachment of a single lipid is often not sufficient for membrane targeting, an additional lipidation is typically required [19]. Modification via both myristoylation and palmitoylation at the N-terminus is an example of dual lipidation that can enhance the membrane-targeting ability of a protein.

We succeeded in construction of yeast one-hybrid GRS to investigate lipid-protein associations. Use of diploid growth caused by restoration of G-protein signaling has a potency to explore the sequences responsible for protein lipidations with high-throughput. In our system, both myristoylation and palmitoylation of Gc hybrids are required to restore the signal transduction (See Fig. 2). As shown in Figure 4, our system can relate eventual diploid cell formation to membrane-targeting ability of proteins with the exception of C3A-Gc. Although C3A-Gc receiving only

Table 3. Frequency of each amino acid residue at positions 7 and 8 before and after diploid growth screening.

| Residue | Position 7 Pi [%] | Pf [%] | Er | Position 8 Pi [%] | Pf [%] | Er |
|---------|------------------|-------|----|------------------|-------|----|
| G       | 13 1              | 0.08  |    | 11 1             | 0.09  |    |
| A       | 5 7               | 1.40  | 9 5 | 0.56             |       |    |
| V       | 4 11              | 2.75  | 5 22| 4.40             |       |    |
| L       | 7 4               | 0.57  | 7 14| 2.00             |       |    |
| I       | 2 6               | 3.00  | 3 3 | 1.00             |       |    |
| S       | 8 13              | 1.63  | 7 8 | 1.14             |       |    |
| T       | 1 5               | 5.00  | 1 7 | 7.00             |       |    |
| C       | 2 9               | 4.50  | 5 3 | 0.60             |       |    |
| M       | 2 6               | 3.00  | 1 7 | 7.00             |       |    |
| F       | 1 6               | 6.00  | 3 5 | 1.67             |       |    |
| W       | 8 2               | 0.25  | 8 2 | 0.25             |       |    |
| Y       | 4 8               | 2.00  | 1 3 | 3.00             |       |    |
| P       | 9 0               | 0.00  | 4 0 | 0.00             |       |    |
| E       | 5 5               | 1.00  | 4 2 | 0.50             |       |    |
| D       | 8 0               | 0.00  | 8 2 | 0.25             |       |    |
| Q       | 1 5               | 5.00  | 4 6 | 1.50             |       |    |
| N       | 2 4               | 2.00  | 3 7 | 2.33             |       |    |
| K       | 1 1               | 1.00  | 3 0 | 0.00             |       |    |
| R       | 4 5               | 1.25  | 8 0 | 0.00             |       |    |
| H       | 3 2               | 0.67  | 1 3 | 3.00             |       |    |
| *       | 10 0              | 0.00  | 4 0 | 0.00             |       |    |

Pi indicates initial population, Pf indicates final population, Er indicates Enrichment ratio, and symbol * indicates amber stop codon.

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myristoylation partially localized at the plasma membrane, it did not restore the signal transduction at all (Fig. 2). It has been reported that G-proteins, G-protein-coupled receptors and other regulators are accumulated in lipid rafts in order to trigger intracellular signal transduction [20–22]. Because the combination of myristoylation and palmitoylation functions as a defined lipid raft-targeting signal in protein trafficking [23], C3A-G<sub>cyto</sub> lacking palmitoylation might have failed to localize in lipid rafts. Therefore, the number of generated diploid cells seems to reflect the amount of dually-lipidated G<sub>cyto</sub> hybrids in a cell.

N-terminal residues position 7 and 8 play some critical role, but it is unclear if these residues are required for either the myristoylation, palmitoylation, or both as shown in Figure 5. Because it has been reported that Ala<sub>7</sub>-Glu<sub>8</sub> within MP10Hi-G<sub>cyto</sub> and MP10Hi1-G<sub>cyto</sub>, were not suitable as substrates of yeast NMT [4–5], these positions are probably involved in myristoylation directly. Also these positions are at least indirectly involved in palmitoylation because myristoylation is a prerequisite for palmitoylation [11].

Using yeast one-hybrid GRS, we investigated sequences at position 7 and 8 required for dual lipid modifications which can give membrane-targeting ability to proteins (Fig. 5 and 6). We classified the unsuitable or unacceptable residues for dual lipid modifications into three broad categories. Charged residues are common in N-terminal positions 8 to 10 of human G<sub>x</sub> subunits in the G<sub>x</sub>16 subfamily [14]. As described above, differences in substrate specificity of NMT between yeast and humans suggest that Glu at position 8 is unacceptable, at least for myristoylation. The second category comprised residues with the highest (Gly) or lowest (Pro) degree of rotational freedom [24], which can have extreme effects on main-chain structure. Finally, Trp, the largest amino acid residue, can be classified as the sole member of a last category of amino acids with a high steric barrier.

In conclusion, we have established a new approach, yeast one-hybrid GRS, to facilitate investigation of protein-lipid associations. Using N-terminal sequences derived from G<sub>x</sub> subunits as a model case, we showed that it is possible to test membrane-targeting ability of N-terminal motifs for their ability to receive dual lipid modification, i.e., myristoylation and palmitoylation. Our method approved to be reliable and versatile, and as such, use of the method should help promote further advancement in functional analyses of proteins within a living cell.

Author Contributions

Conceived and designed the experiments: NF. Performed the experiments: NF. Analyzed the data: NF MD SH. Wrote the paper: NF SH.

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Table 4. N-terminal sequences of G<sub>cyto</sub> hybrids used to verify amino acid residue preferences.

| Name | Description |
|------|-------------|
| DW-G<sub>cyto</sub> | Met–Gly–Cys–Thr–Val–Ser–Asp–Trp–Thr–Ile–Glu–G<sub>cyto</sub> |
| GE-G<sub>cyto</sub> | Met–Gly–Cys–Thr–Val–Ser–Gly–Glu–Thr–Ile–G<sub>cyto</sub> |
| WA-G<sub>cyto</sub> | Met–Gly–Cys–Thr–Val–Ser–Trp–Ala–Thr–Ile–G<sub>cyto</sub> |
| TM-G<sub>cyto</sub> | Met–Gly–Cys–Thr–Val–Ser–Thr–Met–Thr–Ile–G<sub>cyto</sub> |
| QT-G<sub>cyto</sub> | Met–Gly–Cys–Thr–Val–Ser–Gln–Thr–Ile–G<sub>cyto</sub> |
| FN-G<sub>cyto</sub> | Met–Gly–Cys–Thr–Val–Ser–Phe–Asn–Thr–Ile–G<sub>cyto</sub> |

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