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A Method to Generate and Analyze Modified Myristoylated Proteins

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Abstract

Covalent lipid modification of proteins is essential to their cellular localizations and functions. Engineered lipid motif coupled with bio-orthogonal chemistry has been utilized to identify myristoylated or palmitoylated proteins in cells. However, whether modified proteins have similar properties as endogenous ones has not been well investigated mainly due to lack of methods to generate and analyze purified proteins. We have developed a method that utilizes metabolic interference and mass spectrometry to produce and analyze modified, myristoylated small GTPase ADP-ribosylation factor 1 (Arf1). The capacities of these recombinant proteins to bind liposomes and load and hydrolyze GTP are measured and compared with the non-modified myristoylated Arf1. The ketone-modified myristoylated Arf1 can be further labeled by fluorophore-coupled hydrazine and subsequently visualized through fluorescence imaging. This methodology provides an effective model system to characterize lipid-modified proteins with additional functions before applying them to cellular systems.

Keywords

Small GTPase; ADP-ribosylation factor; Myristoylation; Membrane proteins; Protein modifications

Introduction

Approximately 2% of the human genome encodes proteins with lipid modifications, with myristoylation, palmitoylation, and prenylation as the most frequently studied^[1]. Myristoylation adds a C14, and palmitoylation adds a C16 fatty acid chain while prenylation transfers either a farnesyl or a geranyl-geranyl moiety to the target protein. Some lipid modifications such as myristoylation are irreversible^[2], while others such as palmitoylation are reversible. Both types of lipidation are important for regulation of proteins' localizations

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and dynamic functions. For example, prenylation of the small GTPase Ras is essential to its enzymatic activity and transformative capability^[3]. Likewise, the palmitoylation of Wnt proteins is necessary for their secretion and interaction with receptors^[4]. Consequently, targeting lipid modification has been an increasingly important strategy to understand protein functions and produce novel therapeutic interventions.

Using lipid analogs to metabolically engineer lipid-modified proteins is one major application in this regard. Initially, either [³H] or [¹²⁵I] labeled myristate or palmitate is used to identify myristoylated or palmitoylated proteins^[5]. However, low sensitivity of autoradiographic detection of [³H], toxicity associated with [¹²⁵I] and challenges in synthesizing radiolabelled lipids limit the applications of this method. The introduction of “click” chemistry^[6] and the advancement of mass spectrometry prompted the development of azide- and alkyne-modified myristic acids and palmitic acids for identification of myristoylated^[7] or palmitoylated^[8] proteins, respectively. In this approach, the modified lipids were metabolically transferred to a fraction of proteins in cells, and the modified proteins are then tagged with biotin or a fluorophore through copper-catalyzed [3+2] Huisgen cycloaddition for proteomics studies, or the Staudinger ligation for live cell imaging because of the toxicity of copper^[9]. This strategy has also been combined with proximity ligation on antibodies to image subcellular localizations of palmitoylated proteins with high resolution^[10]. However, these elegant studies did not address whether the modifications on the lipids change the properties of the corresponding lipidated proteins^[11]. Previously, some oxygen-substituted myristic acids had been metabolically incorporated into human immunodeficiency virus 1 (HIV1) gag polyprotein precursor but markedly diminished its capacity to associate with cell membrane and to inhibit virus production, highlighting the need to analyze functions of modified lipidated proteins in pure forms^[12]. The biochemical characterization of lipidated proteins is generally limited in the literature, primarily due to lack of methods to generate and analyze purified proteins with modified lipids under non-denatured conditions.

In this work, we describe an approach to generate myristoylated proteins with various modified myristic acids under non-denaturing conditions. In this method, bacterial cells are transformed with plasmids that encode the protein of interest and yeast N-myristoyltransferase 1 (NMT1) (Fig. 1A). The cells are cultured in the presence of modified myristic acids, which are converted to the corresponding myristoyl-CoA by acetyl-coenzyme A synthetase, and subsequently incorporated into protein of interest by NMT1^[7c, 7d, 13]. The myristoylated protein is purified from nonmyristoylated protein using hydrophobic interaction chromatography, and subsequently analyzed by mass spectrometry, membrane association and other functional assays. The substrate specificity of NMT1 has previously been thoroughly investigated by using a synthetic octapeptide substrate and 81 fatty acid analogs^[14]. When recombinantly co-expressed with a natural substrate protein, NMT1 has been shown to transfer a myristic acid derivative bearing a terminal azide or alkyne to the substrate protein for directly labeling in cell lysates without purification and biochemical characterization of the modified proteins^[15].

ADP-ribosylation factor 1 (Arf1) is one member of the Ras-superfamily of small GTPases that plays critical roles in cargo trafficking through the Golgi apparatus^[16]. Although

myristoylation of Arf1 at its N-terminal helix is not absolutely necessary to load GTP in the presence of high concentration of phospholipids *in vitro*^[17], it contributes significantly to its capacity to dynamically interact with other proteins and membranes *in vivo*^[18].

Myristoylation is usually not considered reversible. However, elimination of N-myristoylation of Arf1 was observed after *Shigella* infection through proteolytic cleavage of the amide bond between glycine-2 and asparagine-3 by virulence factor IpaJ^[19], suggesting functional consequences of Arf1 myristoylation in human health and disease. Consequently, we choose to use Arf1 as the model system to demonstrate the feasibility of the new method to incorporate various modified myristic acids into proteins.

Results and Discussion

Generation of purified, myristoylated Arf1 with modified myristic acids

Myristoylated Arf1 has been expressed and purified from bacterial cells transformed with plasmids encoding Arf1 and NMT1 in the presence of myristic acid^[20]. We hypothesize that a metabolic interference strategy, where modified myristic acid is used would harness the bacteria's synthetic machinery to generate myristoylated Arf1 with modified lipid chain. Taken the previously published article on NMT1 substrate specificity into account^[14, 21], we have designed and synthesized five derivatives of myristic acid (Fig. 1B). These modifications are diverse, and also add potential new functions to the corresponding myristoylated proteins. For example, keto-modified myristic acid introduces a bioorthogonal functional group^[22] that enables the corresponding myristoylated proteins to be labeled with fluorescent dyes for potential *in vivo* imaging. Likewise, probes with diazirine groups generate reactive carbenes upon light illumination, thereby have been employed to crosslink ligands with their interacting partners^[23]. The perfluorinated groups C₆F₁₃ and C₈F₁₇ are incorporated because replacing multiple C-H bonds with C-F bonds represents a major structural perturbation, and has been shown as a separation strategy to enrich the tagged substrates from a complex mixture^[24]. Finally, selenium linkers have been shown to undergo oxidative cleavage^[25], and consequently, may serve as a cleavable site on the selenium incorporated lipid motif of the modified myristoylated proteins.

The modified myristic acids were synthesized according to routes shown in Scheme 1. The synthesis of the keto-modified myristic acid **1b** started with octane-1,8-diol **2** (Scheme 1A). Monosilylation followed by Swern oxidation generated aldehyde **3**, which subsequently reacted with the Grignard reagent hexylmagnesium bromide to form **4**. Removal of the silyl protection followed by oxidation with pyridinium dichromate (PDC) provided **1b**. The C₆F₁₃- and C₈F₁₇-modified myristic acids **1c** and **1d** were synthesized from addition of the corresponding perfluoro radicals to oct-7-enoic acid/hex-5-enoic acid **6** followed by reduction in the presence of zinc. To synthesize the selenium-modified **1e**, 1-bromodecane **7** was coupled with selenium and 3-bromopropanoic acid **8** under reducing conditions. The intermediate **4** for synthesis of **1b** was also used as the starting material for the diaz-modified myristic acid **1f**. Oxidation by pyridinium chlorochromate (PCC) yielded **9**, which was converted to the diazirdine **10** in a one-pot, 3-step sequence. The primary alcohol in **10** was then oxidized by PDC to form **1f**.

E. coli. competent strain BL21[DE3] cells were transformed with both plasmids encoding human Arf1 and yeast NMT1, respectively, were used to incorporate the modified myristic acids into Arf1. Co-expression of NMT1 is necessary because bacterial cells do not have the myristoyl-CoA transferase activity required for the myristoylation. Modified myristic acids (100 μ M) were supplemented to the bacterial culture before induction of expression of both Arf1 and NMT1 by isopropyl-D-1-thiogalactopyranoside (IPTG). The myristoylated Arf1 was first purified using anion exchange and size exclusion chromatography, and then separated from non-myristoylated Arf1 using phenyl sepharose hydrophobic interaction chromatography^[26]. The non-myristoylated Arf1 does not adhere to the column at high salt concentration, whereas the myristoylated Arf1 binds and can be eluted by lowering salt concentration. The purity of the protein was determined by SDS-PAGE (Fig. S1). For all myristoylated Arf1 expressed with either wild type or modified myristic acids, the yields were in the range of 0.18–1.0 mg of protein per liter of culture, and the myristoylation efficiency is 10–25% as quantified by peak integration of FPLC chromatogram.

MS analysis of modified myristoylated Arf1

To confirm the incorporation of modified myristic acids into Arf1, the purified, intact proteins were analyzed by performing nanoLC-MS/MS experiments on a high-sensitive and high-resolution LTQ Velos Orbitrap mass spectrometry. One representative spectrum of wild type myr-Arf1 is shown in Fig. 2A. Accordingly, the molecular weight of the protein was measured as the m/z of 20775 daltons, which is consistent with the calculated value of 20775.8 based on its sequence and myristoylation. Similarly, the molecular weight of keto-myr-Arf1 was found to be 20789 (Fig. 2B) and is consistent with the calculated value (20789.8), suggesting that the modified myristic acid was incorporated into Arf1. This notion is further confirmed by mass spectra of other modified, myristoylated Arf1 proteins (Fig. S1).

To further demonstrate that the myristoylation takes place at the N-terminal glycine-2 residue, tandem LC-MS/MS measurements were carried out. Non-myristoylated Arf1 was first digested by trypsin and the N-terminal peptide GNIFANLFK was identified and subjected to MS/MS sequencing (Fig. 2C). Both b ions and y ions were clearly identified. Next, keto-modified myristoylated Arf1 was analyzed in a similar manner. In the MS/MS spectrum, all the y ions were identical as those in non-myristoylated Arf1. The b ions, however, showed a shift of 224 Daltons which is the mass of keto-modified myristoyl group. Taken together, these data clearly demonstrate that modified myristic acids were incorporated into Arf1 at the glycine-2 residue.

Lipid membrane association and binding and hydrolysis of GTP

Myristoylation of Arf1 is critical for its localizations and various functions such as coat and adaptor protein recruitment^[16, 18b, 18c]. Consequently, it is essential to evaluate the impacts of modifications on myristoylation. We chose to first investigate Arf1's capacity to bind to lipid membranes by using lipid association assay that has been used to study lipid association of GTPase activating protein of ADP-ribosylation factor 1 (ArfGAP1)^[27]. In this assay, non-myristoylated, wild type myristoylated or modified myristoylated Arf1 was loaded with GTP at low Mg^{2+} concentration in the presence of small unilamellar vesicle

(SUV) containing fluorescent lipid nitrobenzoxadihexadecanoyl-phosphatidylethanolamine (NBD-PE), and mixed with sucrose solution to reach 35% (v/v). Two layers of cushions with lower concentration of sucrose were then overlaid on top. A lipid-aqueous interface was created by ultracentrifuge of the mixture, with liposomes floated to the top of the sucrose cushion (Fig. S2) as judged by the presence of NBD-PE. The bottom and middle layers containing different concentrations of sucrose solution were sequentially removed before the top layer was collected. Arf1 in each layer was resolved on SDS-PAGE and quantified by SYPRO-RUBY staining and fluorescence detection (inset, Fig. 3A). As shown in Fig. 3A, the non-myristoylated Arf1 does not effectively associate with the lipids under our assay conditions, which could be either due to insufficient GTP loading or incapable of associating with membrane in the absence of myristoylation group. On the other hand, both wild type and modified myristoylated Arf1 proteins primarily partitioned in the top layer with little differences, suggesting that the modification in myristoyl group did not significantly diminish the membrane binding capability of the resulting Arf1 proteins. A small amount of protein remained in the lower layers, likely due to a small fraction of non-myristoylated protein or disturbance of layers when sampling.

Like other Ras-related GTP-binding proteins, Arf1 cycles between an inactive GDP-bound and an active GTP-bound form^[28]. GTPase-activating proteins for Arfs (ArfGAPs) promote Arf1 inactivation by stimulating GTP hydrolysis, whereas guanine nucleotide exchange factors of Arfs (ArfGEFs) catalyze the formation of active GTP-bound Arf1. Unlike other small GTP-binding proteins, such as Ras-family and Rho-family proteins, the intrinsic GTPase activity of Arf1 is almost undetectable in *in vitro* GTPase assays. We thus examined the free GTP-exchange rate and GAP-assisted GTP-hydrolysis of modified myristoylated Arf1 proteins using tryptophan fluorescence assay. This assay takes advantage of changes in intrinsic tryptophan fluorescence upon GTP/GDP-exchange on Arf1, which can be monitored in real time. The GTP-exchange was initiated by addition of EDTA and terminated with high concentration of MgCl₂. Subsequently, the hydrolysis of Arf1-bound GTP was catalyzed by full length ArfGAP1 and the fluorescence changes were monitored. As shown in Fig. 3B, the five modified myristoylated Arf1 proteins exhibited similar GTP-exchange rate as that of the wild type myristoylated Arf1. Moreover, the GAP assisted GTP-hydrolysis rates were also undistinguishable between the modified and wild type myristoylated Arf1 proteins. In contrast, the non-myristoylated Arf1 was not capable of loading GTP efficiently as compared to its myristoylated counterpart (Fig. 3B).

Labeling of keto-myr-Arf1 with a fluorescent dye

The modifications introduced in myristic acid derivatives enable additional functions in the corresponding myristoylated proteins. As a proof-of-principle, we incubated keto-myr-Arf1 with fluorescein-conjugated hydrazine, which reacts with keto-group under mild conditions^[29]. Wild type, myristoylated Arf1 was treated similarly as a control. The mixture was separated by gel electrophoresis and detected by both Coomassie staining (Fig. 4A) and fluorescence scanning (Fig. 4B). As shown in Fig. 4A, a new band was detected in the reaction of the dye with keto-myr-Arf1 but not that with wild type myr-Arf1. Moreover, only this new band was detected by fluorescent scanning. These data collectively demonstrated that keto-myr-Arf1 was selectively labeled by the fluorescein-conjugated hydrazine.

Conclusions

In this work, we have developed a general strategy to express, purify and characterize modified, myristoylated Arf1 proteins. The method features metabolic interference, non-denatured enrichment, and full length protein identification by mass spectrometry and could be extended to generate other lipid-modified proteins. Five different derivatives of myristic acid have been successfully incorporated into Arf1 suggesting that this method tolerates a broad range of modifications. The modified, myristoylated Arf1 proteins have similar affinity with lipid vesicles and capacity to bind and hydrolyze GTP as the parent Arf1. This is the first biochemical characterization of this kind to directly compare the modified with wild type myristoylated proteins and lays the foundation to use this strategy in cells. Although further characterization of the modified Arf1 for new functions is not the focus of this work, the selective labeling of keto-modified, myristoylated Arf1 with a fluorescent dye demonstrates the potential application of engineering the lipid modification to monitor and regulate Arf1's functions in cells. For example, the diazirine modified protein could be used to capture Arf1-interacting proteins in live cells while the selenium-modified Arf1 could be oxidatively cleaved to generate non-myristoylated correspondents.

Experimental Section

All chemicals were purchased from Fisher Scientific or Sigma-Aldrich unless otherwise specified. Modified myristoylated Arf1 were expressed and purified similarly to wild type myristoylated Arf1. Full-length rat ArfGAP1 with a polyhistidine (His₆) tag was purified from SF9 cells using a modified method from described previously.

Protein Expression and Purification

Modified myristoylated Arf1 were expressed and purified based on published protocol for WT myristoylated Arf1^[26, 30]. A frozen stock of *E. coli* BL21 [DE3], transformed with both pET3 vector encoding human Arf1 and pBB131 plasmid encoding yeast NMT1, was a kind gift from Prof. Randazzo (National Cancer Institute, Bethesda, MD). A single colony was grown in LB media containing 100 µg/mL ampicillin and 25 µg/mL kanamycin for overnight, and 10 mL of such culture was used to inoculate 1 L of the same media. The culture was grown at 37 °C until O.D. reached 0.6, when ethanol-dissolved modified myristic acid was added to a final concentration of 100 µM. After 20 min, 1 mM IPTG was added to induce the protein expression. The incubation was continued at 25 °C for additional 12–16 h before harvesting by centrifugation.

The cell pellet was resuspended in lysis buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM MgCl₂, 1 mM DTT) supplemented with complete protease inhibitor cocktails (Roche), 1 mM PMSF, and 5 U/mL DNase I (Roche). The cell was lysed either by Emulsiflex homogenizer or probe sonication. Cell debris was removed by centrifugation at 100,000g for 1 h. The supernatant was loaded onto buffer A (20 mM TrisHCl, pH 8.0, 100 mM NaCl, 1 mM MgCl₂, 1 mM DTT, 10% glycerol (v/v)) equilibrated Hitrap Q column (GE healthcare) and eluted by the same buffer. The flow through containing Arf1 was further purified by a Superdex 75 column (GE healthcare) with buffer A. Fractions containing Arf1, determined by SDS-PAGE, were pooled and concentrated, and mixed with buffer B (20 mM TrisHCl,

pH 8.0, 4 M NaCl, 1 mM MgCl₂, 1 mM DTT) so that the final concentration of NaCl reached 3 M. The myristoylated Arf1 was then separated from non-myristoylated Arf1 by passing through a Hitrap Phenyl sepharose column (GE healthcare). The non-myristoylated Arf1 does not adhere to the column whereas the myristoylated Arf1 was eluted by a gradient of 3 M to 100 mM NaCl. An additional size exclusion chromatography is employed when necessary. The purity of the recombinant protein was examined by SDS-PAGE and the concentration was determined by the Bradford assay.

Full-length rat ArfGAP1 with a polyhistidine tag was purified from SF9 cells using a modified method from described previously^[31]. Frozen SF9 cells in buffer C (20 mM Tris-HCl, pH 7.4, 100 mM NaCl) was thawed and lyzed using homogenizer at 15,000 psi for 10 strokes, and NaCl was added to a final concentration of 250 mM followed by another 10 strokes. Cell debris was removed by centrifugation at 20,000g for 30 min. The supernatant was loaded onto Histrap columns (GE Healthcare), pre-equilibrated with buffer C containing 250 mM NaCl. Nonspecifically bound proteins were further washed off by 12% buffer D (20 mM Tris-HCl, pH 7.4, 200 mM imidazole). The ArfGAP1 was then eluted with a gradient of 7–100% buffer D. Fractions containing ArfGAP1 were further purified by a Superdex 75 column (GE Healthcare) using buffer C.

MS analysis of modified myristoylated Arf1

The purified, myristoylated Arf1 protein (1 mg/ml) was dialyzed against water for 8 h at room temperature, and further cleaned by spin column (PepClean C18, Pierce) to remove any residual, interfering contaminants following the manufacture's protocol. The protein was lyophilized and re-suspended into 0.1% formic acid in H₂O (buffer A) and loaded onto a C18 trap column (Integritrap, 100 μ m $\times$ 2.1 cm, New Objective) with a flow rate of 1 μ L/min for 20 min before eluting into the mass spectrometer. The elution buffer is a mixture of buffer A and buffer B (0.1% formic acid in CH₃CN) with the following gradient: 2% to 40% buffer B in 15 min and an additional 10 min of 40% buffer A following by a gradient of 40% to 80% buffer B in 10 min. The flow rate was 200 nL/min. The mass data were collected using Orbitrap (two microscans, 100,000 resolving power at m/z = 400) with an m/z range of 300–2,000 and a target value of 1,000,000 with a maximum injection time of 100 ms.

For MS/MS analysis, the myristoylated Arf1 (1 mg/ml) was subjected to trypsin digestion after dialysis against water. The digested samples were desalted using C18 ziptip (Millipore) according to the manufacture's protocol and the peptides were lyophilized and re-suspended into buffer A prior to LC separation. MS analyses were performed using LTQ Orbitrap Velos (Thermo Scientific) coupled with a nanoLC-Ultra system (Eksigent, Dublin, CA). Samples (5 μ L) were loaded onto an IntegraFrit column (C18, 75 μ m $\times$ 15 cm, 300 Å, 5 μ m) and eluted with a linear gradient that started with 100% A and ended with 40% B in 110 min followed by another gradient started with 40% B and ended with 80% B in 10 min. The flow rate was 200 nL/min. The data were acquired in a data-dependent mode using XCalibur software (version 2.1, Thermo Scientific) and processed through Proteome Discoverer platform (version 1.2, Thermo Scientific).

Lipid membrane association assay

Lipids in chloroform or powder were purchased from Avanti Polar Lipids except PE (Sigma) and NBD-PE (Molecular Probes). The ingredients were derived from Golgi-mix as described previously, with addition of diacylglycerol that has been previously shown to enhance the GAP activity of ArfGAP1. A dried lipid film was prepared by mixing 35% egg PC, 19% egg PE, 5% brain PS, 10% liver PI, 16% cholesterol, 15% 1-2-dioleoyl-sn-glycerol and 0.2% NBD-PE in chloroform and drying under an air stream for 30 min and vacuum for another 30 min. The dried lipids were re-suspended in HKM buffer (50 mM HEPES, pH 7.2, 120 mM KAc, 1 mM MgCl₂, and 1 mM DTT) and subsequently frozen and thawed five times. The liposome suspension was then extruded through a polycarbonate membrane (30 nm pore size) using a hand extruder (Avanti) and analyzed with dynamic light scattering using a DynaPro Plate Reader (Wyatt) for size distribution. The liposome was used within 24 h after preparation.

The modified myristoylated Arf1 (3 μ M) was incubated with the prepared liposome (1 mM) and GTP (0.3 mM) in the HKM buffer for 10 min, and GTP exchange was initiated by adding EDTA to a final concentration of 2 mM. The mixture was incubated at 37 °C for 40 min before MgCl₂ was added to 3 mM to stop the reaction. Subsequently, GTP-loaded Arf1 (final concentration 1 μ M) was mixed with NBD-PE liposome (final concentration 1 mM) in HKM buffer in a total volume of 150 μ L. A solution of 75% (w/v) sucrose solution (100 μ L) in HKM buffer was then added to adjust the sucrose concentration to 30%. Subsequently, 25% sucrose (200 μ L) in HKM buffer and HKM buffer (50 μ L) were sequentially overlaid on top of the bottom layer. The mixture was centrifuged at 240,000g in a TLS 55 rotor (Beckman) for 1 h at 4 °C. The fluorescent lipids in the centrifuge tube were visualized using a CCD camera before and after the ultracentrifugation. The bottom (250 μ L), middle (150 μ L) and top (100 μ L) layers were then removed sequentially using syringes. Myristoylated Arf1 in each layer was separated by SDS-PAGE, stained with SYPRO-RUBY (Molecular Probes), detected with a Typhoon 9400 Imager (GE Healthcare) and quantified using the ImageQuant software.

GTP binding and hydrolysis

The fluorescence of myristoylated Arf1 was measured with a SPEX Fluorolog-3 fluorometer (Horiba Jobin Yvon) with excitation wavelength at 297.5 nm (bandwidth 3 nm) and emission wavelength at 340 nm (bandwidth 14 nm) at 37 °C. Cuvette containing modified Arf1 (0.4 μ M) and liposome (0.4 mM) in HKM buffer was incubated in the heating chamber for 5 min. Solutions of GTP and EDTA were then added to final concentrations of 40 μ M and 2 mM, respectively, to initiate the GTP-exchange reaction. The reaction was monitored for 10 min and stopped by adding MgCl₂ to a final concentration of 3 mM. Arfgap1 was then added to a final concentration of 27 nM to initiate the GTP hydrolysis.

Labeling of keto-myr-Arf1 with a fluorescent dye

Purified myr-Arf1 or keto-Arf1 was dialyzed against phosphate buffered saline (PBS) overnight at 4 °C. The samples were incubated with either fluorescein-hydrazide (1 mM in DMF) or DMF in PBS for 16 h. Un-reacted dye and resulting byproducts were removed by dialysis against PBS buffer. The protein samples were then separated by SDS-PAGE and

detected by both coomassie blue staining and fluorescent scanning with a Typhoon 9400 imager (GE Healthcare).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Generation of modified myristoylated proteins through metabolic interference. A. Schematic illustration of the method. NMT1 was co-expressed with Arf1 and responsible for transferring the modified myristoyl group to the N-terminus of Arf1. B. Chemical structures of modified myristic acids. The calculated and observed molecular weights of modified Arf1 are also shown.

Figure 2.

Analysis of modified myristoylated Arf1 by mass spectrometry. A. Mass spectrum of myristoylated Arf1. The mass to charge (m/z) and charges (z) are labeled for the ionized proteins. B. Mass spectrum of keto-modified myristoylated Arf1, keto-myristoylated Arf1. C. Tandem mass spectra of the N-terminal peptide of the trypsin digested non-myristoylated Arf1 (top) and keto-myristoylated Arf1 (bottom). B ions and y ions are labeled based on non-myristoylated Arf1.

Figure 3.

Biophysical and biochemical comparisons between myr-Arf1 and modified, myristoylated Arf1 proteins. A. Affinity of modified myristoylated Arf1 proteins with liposome by lipid membrane association assay. Amounts of Arf1 in each layer were quantified by SYPRO-RUBY staining. The inset shows a representative image of staining. The yields were calculated as the average of three independent experiments, with the control sample before ultracentrifugation as 100%. B. Intrinsic fluorescence assay of modified Arf1. Arf1 (0.4 μ M) and liposome (0.4 mM) was incubated at 37 $^{\circ}$ C for 5 min. The GTP exchange was initiated by adding GTP and EDTA to a final concentration of 40 μ M of and 2 mM, respectively. After 10 min, additional MgCl₂ was added to stabilize the Arf1-GTP complex. Subsequently, ArfGAP1 was added to a final concentration of 27 nM to initiate the GAP-

catalyzed GTP hydrolysis. The fluorescence intensity of myr-Arf1 before and after GTP loading was standardized to be 0% and 100% relative intensity, respectively. Non-myristoylated Arf1 was standardized to GTP-loaded myristoylated Arf1 due to lack of significant change in GTP-loading process.

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Figure 4.

Fluorescein labeling of keto-myr-Arf1. Purified myr-Arf1 or keto-myr-Arf1 was either incubated with 1 mM fluorescein-hydrazide (pre-dissolved in DMF) or DMF only in PBS for 16 h at 4 °C. Excess dye was removed by dialysis against PBS. The samples were analyzed by SDS-PAGE and detected by both coomassie blue staining (A) and fluorescent scanning (B).

Scheme 1.
Synthesis of modified myristic acids.