Characterization of Lipid Modifications on Regulator of G Protein Signaling 4 (RGS4) from Sf9 Cells by Mass Spectrometry

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Introduction
RGS4 is a member of the family of GTPase activating proteins (GAPs). GAPs can interact with a G protein to activate its intrinsic GTPase, switching off the G protein signaling pathway. There are three potential palmitoylation sites in RGS4, at Cys2, Cys12, and Cys95, and the palmitoylation state of RGS4 plays an essential role in regulating its membrane association, orientation, and GAP activity. Palmitoylation on RGS4 was previously detected by metabolic labeling with [^{3}H] palmitoyl-CoA. Although the metabolic labeling approach is sensitive, it cannot differentiate palmitoylation from other types of endogenous *S*-acylation, and this limitation can lead to incorrect assignment of palmitoylation. Here we report a mass spectrometry (MS) study to characterize lipid modifications on RGS4 in its native form.

Methods
His-tagged RGS4 was overexpressed by infection of Sf9 insect cells with Baculovirus. Cells were lysed and His-tagged RGS4 was enriched with Ni-NTA beads. A portion of the purified proteins was separated by SDS-PAGE, in-gel digested by trypsin, and analyzed by MALDI-TOF MS for protein ID. Another portion of the proteins was concentrated and buffer-exchanged with 50 mM Tris (pH 7.4), 0.1% RapiGest using Amicon Ultra Filters (MWCO 10 kDa), followed by in-solution trypsin digestion. The lipid-modified peptides were enriched by stepwise elution of the digest from the POROS R1 50 resin. The eluent containing lipid-modified peptides was analyzed by online LC-MS/MS on an LTQ-Orbitrap instrument equipped with a C4 column.

Preliminary Results/Abstract
SDS-PAGE of the purified proteins showed a major band at ~25 kDa which was identified as RGS4 by peptide mass fingerprinting. For analysis of lipid-modified peptides from RGS4, in-solution trypsin digestion was performed in 50 mM Tris buffer (pH 7.4), 0.1% RapiGest, but without reductive alkylation to minimize S-acyl losses. The RGS4 tryptic peptides were loaded onto POROS R1 50 resin with 5% ACN/0.1% TFA, and sequentially eluted with 20% ACN/0.1% TFA, 40% ACN/0.1% TFA, and 60% ACN/0.1% TFA. Nearly all the hydrophobic peptides were recovered in the 40% ACN/0.1% TFA eluent. To characterize these hydrophobic peptides, C4-LC-MS/MS analysis was performed with CID as the fragmentation mode on an Orbitrap mass spectrometer, and this analysis unveiled two types of cysteine modifications resulting in a mass shift of either 220.182 Da or 238.192 Da. Hydroxylamine treatment of these peptides led to the loss of the 238.192-Da modification, while leaving the 220.182-Da modification intact. Thus, the 220.182-Da modification can be tentatively assigned as hydroxyfarnesylation based on its accurate mass and resistance to hydroxylamine treatment. Meanwhile, the 238.192-Da modification, although vulnerable to hydroxylamine treatment, cannot be assigned as palmitoylation (238.2297 Da), because the mass error lies outside the acceptable range for Orbitrap mass measurement. The results presented in this study contradict previous reports that RGS4 is palmitoylated in Sf9 cells. Further characterization of released lipids from RGS4 by GC-MS is currently underway, and this should validate the identification of these lipid modifications and determine the structural details of the hydroxyfarnesyl groups.
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Novel Aspect
New types of lipid modifications and new modification sites on RGS4 from Sf9 cells were discovered.