Protein *N*-myristoylation and prenylation in an insect cell-free protein synthesis system and their identification by mass spectrometry

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Objective

The functional analysis of proteins in postgenomic studies has been attracting considerable attention, and interest in analyzing posttranslational modifications (PTMs) of proteins is increasing. Cell-free protein synthesis systems are assumed to be powerful tools for these objectives. Protein *N*-myristoylation and prenylation are the important lipid modifications of proteins, and they play crucial roles in regulating reversible protein-membrane and protein-protein interactions. Metabolic labeling is an effective strategy for analysis of these modifications. A rabbit reticulcyte lysate cell-free system has been widely utilized for the strategy, because it contains all the components involved in these protein modifications. However , metabolic labeling cannot be used to identify the exact location of the modification.

In this study, to establish a novel and effective strategy to analyze these protein modifications, human truncated gelsolin (tGelsolin) and rhoC, which are a natural *N*-myristoylated and geranylgeranylated protein respectively, were synthesized using an insect cell-free protein synthesis system. Synthesized proteins were analyzed by MALDI-TOF MS and MALDI-quadrupole ion trap (QIT)-TOF MS.

Materials and methods

Transdirect *insect cell*, which is based on the *Spodoptera frugiperda* 21 (Sf21) extract, is a commercial product of Shimadzu (Kyoto, Japan). Epitope-tagged human tGelsolin and rhoC were synthesized using the insect cell-free protein synthesis system with or without the addition of a specific substrate for *N*-myristoylation or prenylation, such as myristoyl-CoA, farnesyl pyrophosphate, and geranylgeranyl pyrophosphate. The affinity purified proteins were reduced and alkylated, and then digested with trypsin or lysyl endopeptidase (Lys-C). The mass spectra and MS/MS spectra were acquired in reflectron positive ion mode with an AXIMA-CFR-plus MALDI-TOF MS instrument and an AXIMA-QIT MALDI-QIT-TOF hybrid mass spectrometer (Shimadzu/Kratos, Manchester, UK).

Results

In the case of the tGelsolin, when myristoyl-CoA was added to the translation reaction mixture, a peak corresponding to the *N*-myristoylated tryptic peptide was clearly observed, whereas this peak was hardly detected when myristoyl-CoA was not added. The peptide peak was identified as the *N*-myristoylated peptide fragment by tandem MS analysis using MALDI-QIT-TOF MS. We also constructed the Gly-2 to Ala (G2A) mutant, in which the *N*-myristoylation motif was disrupted, and analyzed by the same strategy. *N*-myristoylation did not occur on the G2A mutant, whereas this mutant was found to be *N*-acetylated after removal of the initiating Met.

In the case of the rhoC, it was found that it could serve as a substrate for both prenyltransferases in the presence of either farnesyl or geranylgeranyl pyrophosphate, whereas geranylgeranylation was only observed when both prenyl pyrophosphates were added to the translation reaction mixture. These results indicated that the rhoC protein was a better substrate for geranylgeranyltransferase I than for farnesyltransferase.

Conclusions

These results indicate that the insect cell-free protein synthesis system, as is the case with the rabbit reticulocyte lysate system, possesses *N*-myristoyltransferases and both prenyltransferases. In conclusion, a combination of the cell-free protein synthesis system with MS is an effective strategy to analyze protein *N*-myristoylation and prenylation.

References

1) Suzuki,T., Ito, M., Ezure, T., Shikata, M., *et. al.*,
N-Terminal protein modifications in an insect cell-free protein synthesis system and their identification by mass spectrometry.
Proteomics, 6, 4486-4495 (2006)

2) Suzuki, T., Ito, M., Ezure, T., Shikata, M., *et. al.*,
Protein prenylation in an insect cell-free protein synthesis system and identification of products by mass spectrometry.
Proteomics, 7, 1942-1950 (2007).

This paper was selected as an "In This Issue" of the PROTEOMICS journal. The key points of the work have been available for download in the June edition of the PROTEOMICS podcast(http://www.podcast.proteomics-journal.com).

N-myristoylation

Protein *N*-myristoylation

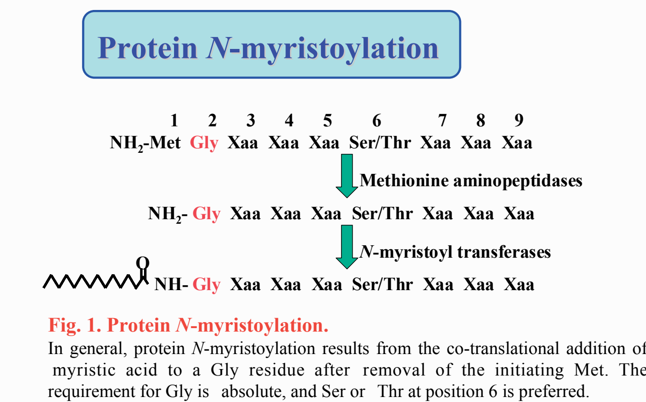

Fig. 1. Protein *N*-myristoylation.
In general, protein *N*-myristoylation results from the co-translational addition of myristic acid to a Gly residue after removal of the initiating Met. The requirement for Gly is absolute, and Ser or Thr at position 6 is preferred.

Expression and purification of tGelsolin

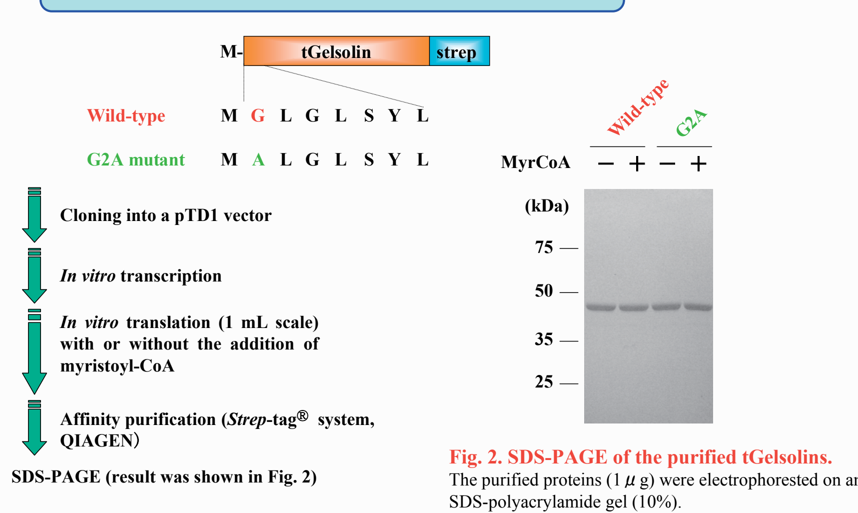

Fig. 2. SDS-PAGE of the purified tGelsolins.
The purified proteins (1 μg) were electrophoresed on an SDS-polyacrylamide gel (10%).

PMF analyses of the tryptic digests of tGelsolin proteins

	N-terminal structures	Theoretical	Observed
Wild-type	MGLGLS··VER	1846.9	ND
	GLGLS··VER	1715.9	1716.0
	(myristoyl)-GLGLS··VER	1926.1	1926.2
G2A mutant	MALGLS··VER	1860.9	ND
	ALGLS··VER	1729.9	1729.8
	(myristoyl)-ALGLS··VER	1940.1	ND

Fig. 3. Probable N-terminal structures of tryptic digests.

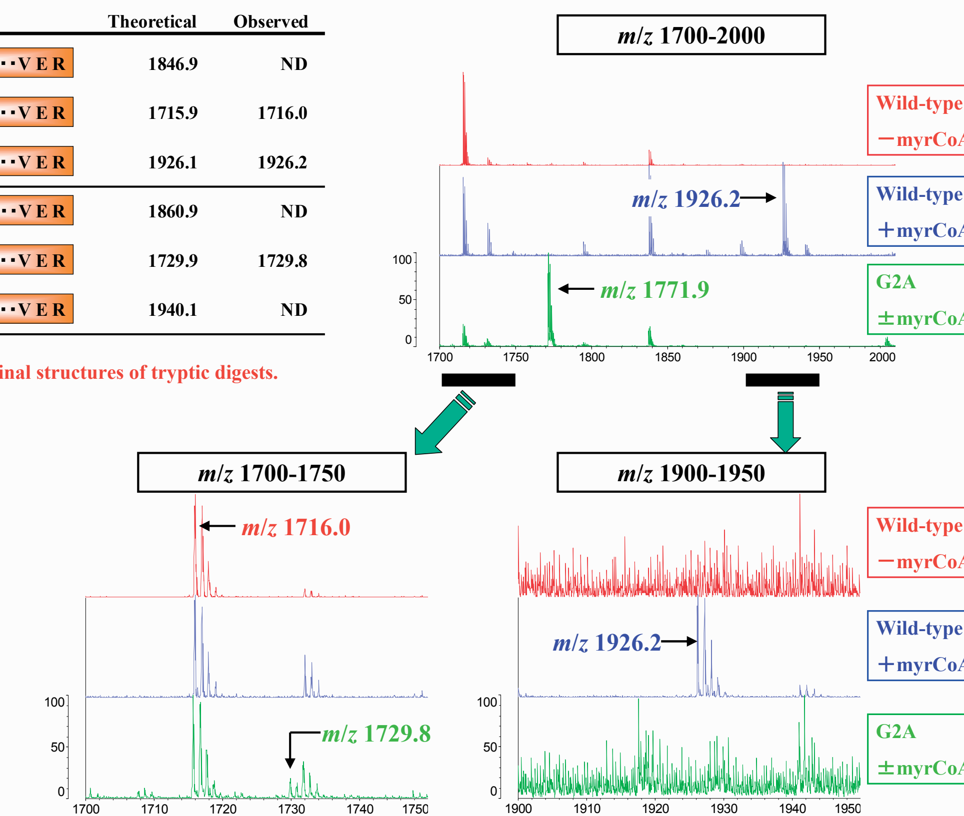

Fig. 4. MALDI-mass spectra of tryptic digests of the wild-type and G2A mutant.
The wild-type mRNA was translated without (upper) or with (middle) the addition of myristoyl-CoA. The G2A mutant was also translated under same conditions (lower). Arrows indicate probable N-terminal prptide peaks.

MS/MS analyses

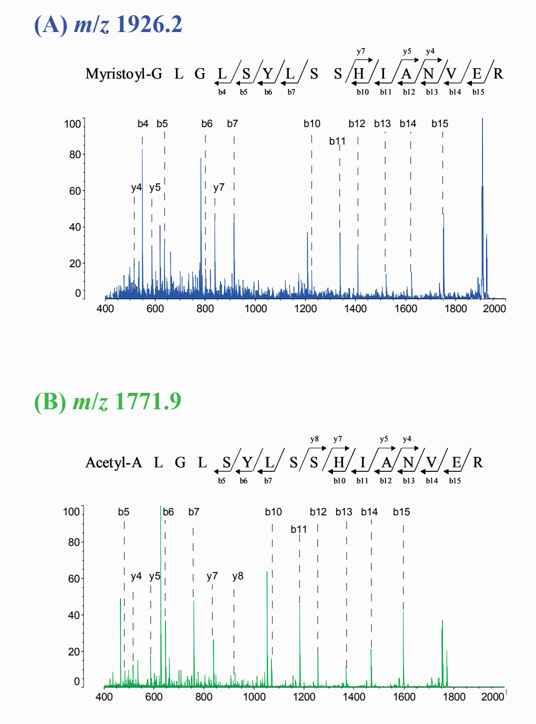

Fig. 5. MALDI-MS/MS spectra of the N-terminal peptides.
MS/MS analyses were performed for the peak at *m/z* 1926.2 observed in the wild-type (A) and at *m/z* 1771.9 for the G2A mutant (B).The observed fragment ions are indicated in the sequences shown.

Prenylation

Protein prenylation

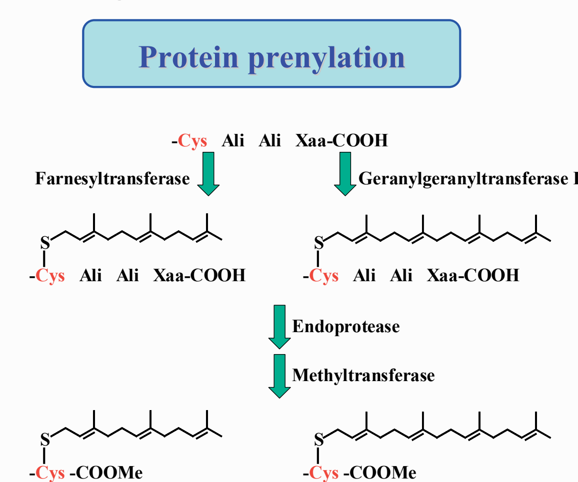

Fig. 6. Protein prenylation.
Farnesyltransferase (FTase) and geranylgeranyl-transferase (GGTase) I recognize a C-terminal Cys-Ali-Ali-Xaa motif (Ali is usually an aliphatic amino acid, and Xaa is one of a variety of amino acids) and covalently attach a farnesyl group from farnesyl pyrophosphate (FPP) or a geranylgeranyl group from geranylgeranyl pyrophosphate (GGPP) to the free cysteine residue. The C-terminal amino acid residue is the major determinant for the selection of which of the two types of prenyl group is to be attached to the cysteine residue.
The three amino acid peptide (Ali-Ali-Xaa) is cleaved by an endoprotease after prenylation. The newly exposed carboxyl group of the prenylated cysteine residue is then methylated by a methyl-transferase. These modifications occur on meicrosomal membranes.

Expression and purification of rhoC

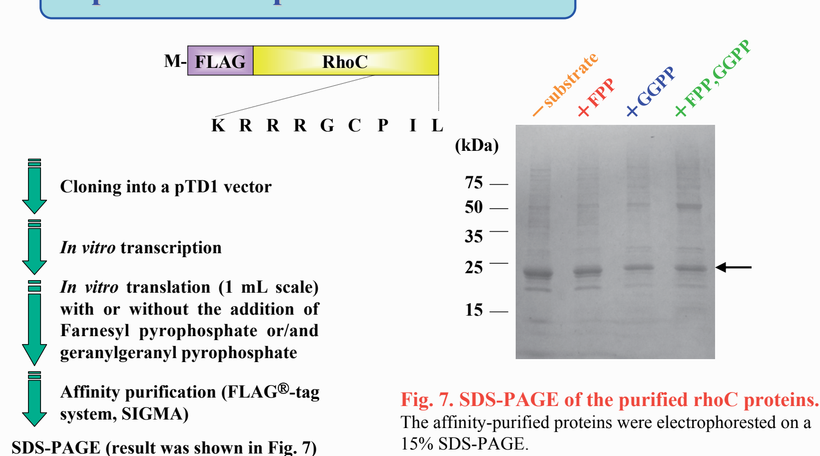

Fig. 7. SDS-PAGE of the purified rhoC proteins.
The affinity-purified proteins were electrophoresed on a 15% SDS-PAGE.

PMF analyses of Lys-C digests of the rhoC proteins

C-terminal structures	Theoretical
RRRGC(SCH_2CONH_2)PIL	1027.6
RRRGC(S-farnesyl)PIL	1174.8
RRRGC(S-geranylgeranyl)PIL	1242.8

Fig. 8. Probable C-terminal structures of Lys-C digests.

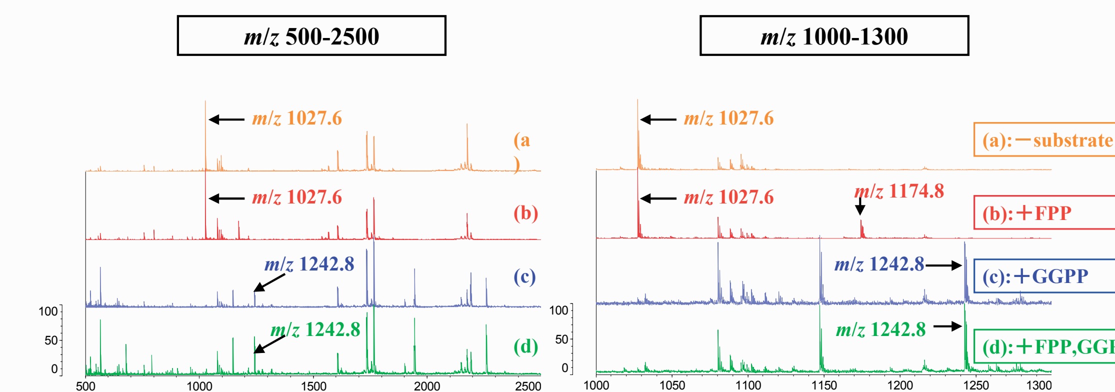

Fig. 9. MALDI-mass spectra of Lys-C digests of the rhoC proteins.
The rhoC mRNA was translated without the addition of prenyl pyrophosphate (a) or with the addition of FPP (b), GGPP (c), or FPP and GGPP (d). Arrows indicate peaks corresponding to the C-terminal Lys-C digests.

MS/MS analyses

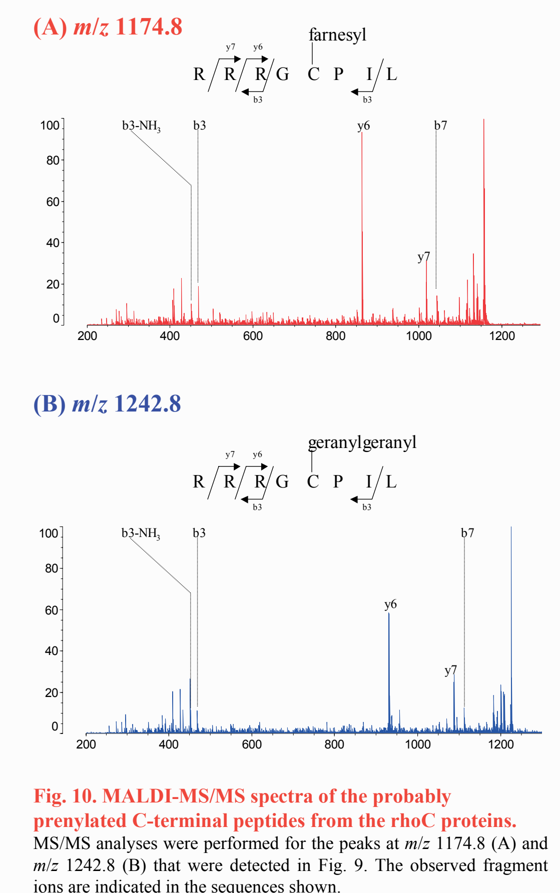

Fig. 10. MALDI-MS/MS spectra of the probably prenylated C-terminal peptides from the rhoC proteins.
MS/MS analyses were performed for the peaks at *m/z* 1174.8 (A) and *m/z* 1242.8 (B) that were detected in Fig. 9. The observed fragment ions are indicated in the sequences shown.