Amino acid residue penultimate to amino-terminal Gly residue strongly affects two cotranslational protein modifications, N-myristoylation and N-acetylation

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Abstract

In order to examine the amino-terminal sequence requirements for cotranslational protein N-myristoylation, series of site-directed mutagenesis of N-terminal region were performed using tumor necrosis factor (TNF) as a non-myristoylated model protein. Subsequently, the susceptibility of these mutants to protein N-myristoylation was evaluated by metabolic labeling in an in vitro translation system or in transfected cells. It was found that the amino acid residue at position 3 in an N-myristoylation consensus motif, Met-Gly-X-X-Ser-X-X-X, strongly affected the susceptibility of the protein to two different cotranslational protein modifications, N-myristoylation and N-acetylation; 10 amino acids (Ala, Ser, Cys, Thr, Val, Asn, Leu, Ile, Gln, His) with radius of gyration smaller than 1.80 Å directed N-myristoylation, two negatively charged residues (Asp, Glu) directed N-acetylation, and two amino acids (Gly, Met) directed heterogeneous modification with both N-myristoylation and N-acetylation. The amino acid requirements at this position for the two modifications were dramatically changed when Ser at position 6 in the consensus motif was replaced with Ala. Thus, the amino acid residue penultimate to N-terminal Gly residue strongly affected two cotranslational protein modifications, N-myristoylation and N-acetylation, and the amino acid requirements at this position for these two modifications were significantly affected by downstream residues.
INTRODUCTION

A number of eukaryotic cellular proteins are found to be covalently modified with the 14-carbon saturated fatty acid, myristic acid (1-6). Many of the myristoylated proteins play key roles in regulating cellular structure and function. Protein N-myristoylation is the result of co-translational addition of myristic acid to a Gly residue at the extreme N-terminus after removal of the initiating Met. The requirement for Gly at the N-terminus is absolute and no other amino acid can take its place. A stable amide bond links myristic acid irreversibly to proteins. The N-myristoyltransferase (NMT)\(^1\) which catalyzes the transfer of myristic acid from myristoyl-CoA to the N-terminal Gly has been purified and cloned from several organisms (7-10). The precise substrate specificity of this enzyme has been characterized using purified enzyme and synthetic peptide substrates (2, 11, 12). In general, Ser or Thr is preferred at position 6 and the N-terminal consensus motifs such as Met-Gly-X-X-Ser/Thr-X-X (13) or Met-Gly-X-X-Ser/Thr-X-X-X (5) that direct protein N-myristoylation have been defined. However, Ser or Thr at position 6 is neither sufficient nor critical for the recognition of the protein substrate by the NMT. For instance, the peptide Gly-Gln-Ala-Ala-Ala-Lys-Lys derived from the N-terminus of cAMP-dependent protein kinase catalytic subunit was found to be a good substrate for the yeast NMT, and the peptide Gly-Gln-Ala-Ala-Ala-Arg-Arg was used as a reference substrate for the yeast NMT in earlier reports on the substrate specificity of this enzyme (7, 14). Some amino acid preferences were also reported at other positions such as 3, 7 and 8 (2, 15); however, the precise amino acid requirements at these positions were not fully characterized.

Protein N-myristoylation in intact cells is not a single enzymatic reaction catalyzed by NMT. This modification appears to be a highly regulated reaction involving the coordinated participation of the protein synthesis machinery (ribosomes) and several different enzymes/proteins such as N-methionyl-aminopeptidase, fatty acid synthase, long chain acyl-CoA synthetase, acyl-CoA-binding proteins, etc. Therefore, the substrate specificity of NMT analyzed by using purified NMT and synthetic peptide substrates may
not fully reflect the substrate specificity of NMT in intact cells. In addition, other cotranslational protein modification such as protein N-acetylation might also affect the reaction. It has been estimated that as many as 70% of soluble proteins (cytoplasmic or nucleoplasmic) in eukaryotes bear this modification (16). In fact, several proteins having N-terminal Gly residue, such as ovalbumin (17), cytochrome c (18) and actin (19), were found to be N-acetylated. However, the difference in the N-terminal sequence requirement for protein N-myristoylation and protein N-acetylation has not been characterized so far.

Since in vitro translation system using rabbit reticulocyte lysate contains the components involved in cotranslational protein N-myristoylation and N-acetylation (17, 19, 20), the use of this system to study cotranslational protein modification seems to be appropriate. In fact, we previously demonstrated that tumor necrosis factor (TNF), a non-myristoylated model protein, could be efficiently myristoylated in the in vitro translation system when an N-myristoylation motif of Rasheed leukemia virus-gag protein or Gi1α protein was linked to the mature domain of TNF (21, 22).

In this study, in order to examine the N-terminal sequence requirements for the cotranslational protein N-myristoylation and to reveal the difference in the N-terminal sequence requirements for protein N-myristoylation and N-acetylation, several series of site-directed mutagenesis of the N-terminal region of protein were performed using TNF as a non-myristoylated model protein. Subsequently, the susceptibility of these mutants to the cotranslational N-myristoylation and N-acetylation reactions was evaluated by an in vitro transcription/translation system using the rabbit reticulocyte lysate.

**EXPERIMENTAL PROCEDURES**

*Materials* —Restriction endonucleases, DNA-modifying enzymes, RNase inhibitor, and Taq DNA polymerase were purchased from Takara Shuzo, Japan. The mCAP RNA capping kit and proteinase K were from Stratagene. RNase was purchased from Boehringer-Mannheim, Germany. Rabbit reticulocyte lysate was from Promega. [3H]-leucine, [3H]-myristic acid, [3H]-acetyl-CoA, [35S]-methionine and Amplify were from
Amersham, UK. The Dye Terminator Cycle Sequencing kit was from Applied Biosystems. Anti-human TNF polyclonal antibody was purchased from R&D systems. Protein G Sepharose was from Pharmacia Biotech. Plasmid pET-22b-OVA which contains the full-length chicken ovalbumin cDNA was provided by Dr. Akio Kato, Yamaguchi university, Japan. Other reagents purchased from Wako Pure Chemical, Daiichi Pure Chemicals, and Seikagaku Kogyo (Japan) were of analytical or DNA grade.

**Plasmid construction** — Plasmid pBluescript II SK(+) lacking Apa I and HinD III sites was constructed as previously described (23), and designated pB. Plasmid pBpro-TNF, which contains the full-length human pro-TNF cDNA, and plasmid pBΔpro-TNF, containing a cDNA coding for the mature domain of TNF, were constructed as described (21, 23). Plasmid pBV2G-TNF was constructed by utilizing PCR. For this procedure, pBΔpro-TNF served as a template and two oligonucleotides (V2G, B1) as primers (Table I).

**Table I**

After digestion with Bam HI and Pst I, the amplified product was subcloned into pB at BamHI and Pst I sites. cDNAs coding for other TNF-mutants (designated R3X-TNF) in which Arg at position 3 in V2G-TNF was replaced with each of the 19 other amino acids, were constructed by a method similar to that of V2G-TNF. The mutagenic primers used in these procedures are listed in Table I. Plasmids pBGag-TNF and pBGα-α-TNF were constructed as described previously (22). Plasmid pBOVA which contains the full-length chicken ovalbumin cDNA was constructed by using PCR. In this case, pET-22b-OVA served as a template and two oligonucleotides (OVA-N, OVA-C) as primers (Table I). After digestion with Bam HI and Eco RI, the amplified product was subcloned into pB at Bam HI and Eco RI sites. cDNA coding for OVA60-TNF in which N-terminal 60 residues of ovalbumin was linked to the N-terminus of the mature domain of TNF was constructed by using PCR. For this procedure, pBOVA served as a template and two oligonucleotides (OVA-N, OVA-60) as primers (Table I). After digestion with Bam HI and Xho I, the amplified product was subcloned into pBGag-TNF at Bam HI and Xho I sites. cDNAs
coding for R3X, S6A-TNF in which Ser at position 6 in R3X-TNF mutants was replaced with Ala were constructed by using PCR. In this case, each of the pBR3X-TNF constructs served as a template and two oligonucleotides (T3, S6A) as primers (Table I). After digestion with Sac I and Ava I, the amplified product was subcloned into pΔpro-TNF at Sac I and Ava I sites. cDNAs coding for Gag-Q3K-TNF and Gi1α-C3K-TNF in which the amino acid at position 3 in Gag-TNF or Gi1α-TNF was replaced with Lys were constructed by using PCR. In this case, pBGag-TNF or pBGi1α-TNF served as a template and two oligonucleotides (Gag-Q3K, B1 and Gi1-C3K, B1, respectively) as primers (Table I). After digestion with Bam HI and Pst I, the amplified products were subcloned into pB at Bam HI and Pst I sites. cDNAs coding for Arf6-TNF and Hippocalcin-TNF in which N-terminal 10 residues of Δpro-TNF was replaced with those of Arf6 or Hippocalcin were constructed by using PCR. For this procedure, pBΔpro-TNF served as a template and two oligonucleotides (Arf6, B1 and HC, B1, respectively) as primers (Table I). After digestion with Bam HI and Pst I, the amplified products were subcloned into pB at Bam HI and Pst I sites. The DNA sequences of these recombinant cDNAs were confirmed by the dideoxynucleotide chain termination method (24).

In vitro transcription and translation —Methods essentially identical to those described previously were employed (23). T3 polymerase was used to obtain transcripts of these cDNAs subcloned into pB vector. These were purified by phenol-chloroform extraction and ethanol precipitation prior to use. Subsequently, the translation reaction was carried out using the rabbit reticulocyte lysate (Promega) in the presence of [3H]-leucine, [35S]-methionine, [3H]-myristic acid or [3H]-acetyl-CoA under conditions recommended by the manufacturer. The mixture (composed of 20.0 µl of rabbit reticulocyte lysate; 1.0 µl of 1 mM leucine- or methionine-free amino acid mixture, or 1 mM complete amino acid mixture; 4.0 µl of [3H]-leucine (5µCi), [35S]-methionine (1µCi), [3H]-myristic acid (25µCi), or [3H]-acetyl-CoA (2µCi); and 4.0 µl of mRNA) was incubated at 30°C for 90 min.
Transfection of COS-1 cells and determination of N-myristoylated proteins — The simian virus 40-transformed African Green monkey kidney cell line, COS-1, was maintained in Dulbecco modified Eagle’s medium (DMEM, GIBCO BRL) supplemented with 10% fetal calf serum (FCS, GIBCO BRL). Cells (2 x 10^5) were plated onto 35-mm-diameter dishes 1 day before transfection. pcDNA3 construct (2 µg; Invitrogen, San Diego, Calif.) containing mutant TNF cDNA was used to transfect each plate of COS-1 cells along with 4 µl of LipofectAmine (2 mg/ml; GIBCO BRL) in 1 ml of serum-free medium. After incubation for 5 h at 37°C, the cells were refed with serum-containing medium and incubated again at 37°C for 24 h. The cells were then washed twice with 1 ml of serum-free DMEM and incubated for 5 h in 1 ml of DMEM with 2% FCS containing [³H]-myristic acid (100 µCi/ml). Subsequently, the cells were washed three times with Dulbecco’s phosphate-buffered saline (DPBS) and collected with cell scrapers, followed by lysis with 200 µl of RIPA buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, proteinase inhibitors] on ice for 20 min. The cell lysates were centrifuged at 15,000 rpm at 4°C for 15 min in a microcentrifuge (HITACHI-CF15D2) and supernatants were collected. After immunoprecipitation with anti-TNF antibody, the samples were analyzed by SDS-PAGE and fluorography.

Western blotting — TNF samples immunoprecipitated from in vitro translation products or total cell lysates of each group of transfected cells were resolved by 12.5% SDS-PAGE and then transferred to an Immobilon-P transfer membrane (Millipore). After blocking with nonfat milk, the membrane was probed with a specific goat anti-hTNF antibody as described previously (25). Immunoreactive proteins were specifically detected by incubation with horseradish peroxidase-conjugated anti-goat IgG antibody (Santa Cruz). The membrane was developed with ECL Western blotting reagent (Amersham Corp.) and exposed to an X-ray film (Kodak). Quantitative analysis of immunoreactive proteins on the membrane was carried out using the storage phosphor imaging screen and GS-250 Molecular Imager (Bio-Rad).
Immunoprecipitation — Samples containing TNF mutants were immunoprecipitated with a specific goat anti-hTNF polyclonal antibody (R&D systems) as described (23).

SDS-PAGE and fluorography — Samples were denatured by boiling for 3 min in SDS-sample buffer followed by analysis by SDS-PAGE on a 12.5% gel. Thereafter, the gel was fixed and soaked in Amplify™ (Amersham) for 30 min. The gel was dried under vacuum and exposed to an X-ray film (Kodak) for an appropriate period. Quantitative analysis of the labeled proteins was carried out by scanning the fluorogram using an imaging densitometer (Bio-Rad GS-700).

Analysis of bound fatty acids — Fatty acid-labeled TNF mutants immunoprecipitated from in vitro translation products were resolved by 12.5 % SDS-PAGE and then transferred to an Immobilon-P transfer membrane. The region of membrane containing the labeled TNF mutant, identified by western blotting with anti-hTNF antibody, was excised and hydrolyzed in 6 N HCl at 110°C for 16 hr. The released fatty acids were extracted in hexane and run on a thin-layer chromatography plate (RP18, Merck) with acetonitrile/acetic acid (9:1) as solvent system. Radioactivity on the thin-layer plate was made visible by spraying with En³Hance (NEN).

RESULTS

Amino acid at position 3 in N-myristoylation consensus motif strongly affects protein N-myristoylation and N-acetylation — In order to examine the amino-terminal sequence requirements for cotranslational protein N-myristoylation, and to reveal the difference in the N-terminal sequence requirement for protein N-myristoylation and N-acetylation, the N-terminal nine residues of the mature domain of TNF including initiating Met were changed to the N-myristoylation consensus motif, and the susceptibility to cotranslational protein N-myristoylation and N-acetylation was evaluated by an in vitro translation system. Since Δpro-TNF, a mature domain of TNF in which the initiating Met was introduced at the N-terminus, has Met and Ser residues at positions 1 and 6, respectively, Val at position 2 was replaced with Gly to obtain V2G-TNF in which the N-
terminal 9 residues were adapted to the N-myristoylation consensus motif, Met-Gly-X-X-
X-Ser-X-X-X (Fig.1).

Fig. 1.

As shown in Fig.2 (lanes 1,2), translation of mRNAs coding for Δpro-TNF and V2G-TNF in the presence of [3H]-leucine gave rise to two translation products; one is the major product with an expected molecular mass (17 kDa) and the other is a fainter band with an ~2 kDa larger molecular mass. However, no incorporation of [3H]-myristic acid and [3H]-acetyl CoA was detected in these translation products as shown in Fig.2 (lanes 5,6,9,10).

From early experiments performed by Towler et al. using purified yeast N-
myristoyltransferase (NMT) and the synthetic peptide substrates, it has been reported that an amino acid residue at position 3 affects the susceptibility to protein N-myristoylation (2). We therefore prepared two additional constructs, R3A- and R3D-TNF, in which Arg at position 3 in V2G-TNF was replaced with Ala and Asp, respectively. [3H]-leucine labeling revealed an efficient expression of R3A- and R3D-TNF as observed with V2G-TNF (Fig.2 lanes 3,4). In R3A-TNF, significant incorporation of [3H]-myristic acid, but not [3H]-acetyl CoA, was observed (lanes 7,11). Conversely, significant incorporation of [3H]-
acetyl CoA, but not [3H]-myristic acid, was observed with R3D-TNF (lanes 8,12).

Fig. 2.

In order to determine whether the incorporation of [3H]-myristic acid into R3A-
TNF was comparable to that into proteins having a natural N-myristoylation motif, incorporation of [3H]-leucine and [3H]-myristic acid into R3A-TNF was compared with those into Gag-TNF and G11α-TNF (22) in which the N-terminal 10 residues of the Gag protein or G11α were linked to the N-terminus of the mature domain of TNF. As shown in Fig.3 [A] (lanes 1-3,7-9), incorporation of [3H]-leucine and [3H]-myristic acid into these three TNF-mutants were found to be comparable, indicating that R3A-TNF is efficiently myristoylated, similar to proteins having a natural N-myristoylation motif. Next, we compared the incorporation of [3H]-acetyl CoA into R3D-TNF with that into ovalbumin, a
naturally acetylated protein (17). In this case, since specific antibody against ovalbumin was not available for the immunoprecipitation of the in vitro-translated products, OVA60-TNF in which N-terminal 60 residues of ovalbumin was linked to the N-terminus of TNF was used. As expected, only a low level of $[^{3}H]$-leucine- and $[^{3}H]$-acetyl CoA incorporation was observed with ovalbumin, as shown in Fig.3 [A] (lanes 6, 18). In contrast, efficient $[^{3}H]$-leucine- and $[^{3}H]$-acetyl CoA incorporation was detected with OVA60-TNF (lanes 5, 17). As shown in Fig. 3 [A] (lanes 4,5,16,17), incorporation of $[^{3}H]$-leucine and $[^{3}H]$-acetyl CoA into R3D TNF was comparable to those of OVA60-TNF, indicating that R3D-TNF is as efficiently acetylated as the naturally acetylated protein. Analysis of the $[^{3}H]$-labeled fatty acid attached to the R3A-TNF by thin layer chromatography (TLC) confirmed the presence of $[^{3}H]$-myristic acid (Fig. 3 [B] lane 3). In contrast, $[^{3}H]$-labeled fatty acid attached to the R3D-TNF was not detected on the TLC plate (lane 4). However, acetic acid liberated from the acetylated protein is volatile and will be evaporated by the extraction and concentration procedure. Therefore, this result is consistent with the fatty acid attached to the R3D-TNF being acetic acid. Taken together, it is suggested that the amino acid residue at position 3 in the Met-Gly-X-X-X-Ser-X-X-X motif strongly affects the susceptibility of the protein to two different cotranslational protein modifications, N-myristoylation and N-acetylation.

**Fig. 3.**

As shown in Fig.2 (lanes 3,4,7,12), protein N-myristoylation and N-acetylation were specifically observed in the lower $[^{3}H]$-leucine-labeled band, with no modification of the upper band observed. To clarify the basis for this, differential labeling of these two protein bands with $[^{3}H]$-leucine, $[^{35}S]$-methionine, $[^{3}H]$-myristic acid and $[^{3}H]$-acetyl CoA were performed using Δpro-, R3A- and R3D-TNF mRNA. In these three TNF variants, the initiating Met is the only Met residue in the entire molecule; in contrast, these TNFs contain several residues of Leu in their amino acid sequences. As shown in Fig.4, $[^{35}S]$-methionine was specifically incorporated into the upper band, whereas $[^{3}H]$-leucine was incorporated into both bands. Incorporation of $[^{3}H]$-myristic acid into R3A-TNF and $[^{3}H]$-
acetyl CoA into R3D-TNF was observed exclusively in the lower band. Since protein N-myristoylation and protein N-acetylation occurs on Gly-2 after removal of the initiating Met and there is no Met residue in the mature domain of TNF, these results clearly indicated that the upper band corresponds to the protein species retaining the initiating Met residue and the lower band to the one lacking this residue.

**Fig. 4.**

*Effect of amino acid residue at position 3 in N-myristoylation consensus motif on the efficiency of cotranslational N-myristoylation and N-acetylation reaction* — To investigate the amino acid requirement at position 3 in Met-Gly-X-X-X-Ser-X-X-X motif for protein N-myristoylation and N-acetylation, twenty mutants, each with a different amino acid at position 3, were generated and their susceptibility to the two cotranslational modifications was evaluated by the same method as above. The results of 20 amino acids are arranged according to their radius of gyration. All of these mutants were efficiently expressed as determined by the incorporation of $[3^\text{H}]$-leucine as shown in the upper panels of Fig. 5 [A]. The ratio of the amount of the two $[3^\text{H}]$-leucine-labeled protein bands was almost the same in these 20 mutants, indicating that there is no significant difference in the efficiency of the removal of the initiating Met residue in these mutants.

**Fig. 5.**

The labeling with $[3^\text{H}]$-myristic acid revealed a strong correlation between the radius of gyration of the amino acid at position 3 and the efficiency of protein N-myristoylation as shown in the lower panels of Fig. 5 [A]. The relationship between the relative N-myristoylation efficiency and the radius of gyration of amino acid at position 3 is summarized in Fig. 5 [B]. The presence of Gly, Ala, Ser, Cys, Thr, Val, Asn, Leu, Ile, Gln and His residues, each having a radius of gyration smaller than 1.80Å, at position 3 led to efficient $[3^\text{H}]$-myristic acid labeling. In contrast, the presence of amino acids with a radius of gyration larger than 1.80Å (Phe, Lys, Tyr, Trp, and Arg) at this position completely inhibited the $[3^\text{H}]$-myristic acid incorporation. The presence of Met residue which has an intermediate radius of gyration (1.80Å) led to a diminished efficiency of N-
myristoylation. In addition to the restriction by the radius of gyration of the amino acid, it was also revealed that the presence of negatively charged residues (Asp, Glu) and Pro residue at this position completely inhibited the myristoylation reaction. Labeling of these TNF mutants with $[^3H]$-acyetyl CoA revealed that non-myristoylated mutants with Asp or Glu at position 3 and weakly-myristoylated mutant having Met at this position, were efficiently acetylated as shown in the lower panels of Fig. 6 [A]. In addition, a low level of $[^3H]$-acyetyl CoA incorporation was observed with an effectively myristoylated mutant having Gly at position 3. These results indicate that the amino acid at position 3 in Met-Gly-X-X-X-Ser-X-X-X motif affected differently the two cotranslational protein modifications, N-myristoylation and N-acetylation.

**Fig. 6.**

*Effect of lack of Ser residue at position 6 in N-myristoylation consensus motif on protein N-myristoylation and N-acetylation* — It is generally accepted that Ser or Thr is preferred at position 6 for protein N-myristoylation. However, Ser or Thr at this position is neither sufficient nor critical for protein N-myristoylation. Therefore, we next examined the effect of lack of Ser residue at position 6 in N-myristoylation consensus motif on cotranslational protein N-myristoylation and N-acetylation. In this experiment, R3X,S6A-mutants in which the Ser residue at position 6 in each of R3X-mutants was changed to Ala were generated and their susceptibility to N-myristoylation and N-acetylation reaction was determined by metabolic labeling. As shown in the middle panels of Fig. 6 [B], dramatic changes in the amino acid requirement at position 3 were observed for protein N-myristoylation. The number of amino acid residues which can direct protein N-myristoylation was strikingly reduced; only two amino acids, Asn and Gln, could direct efficient modification. In contrast, the number of amino acid residues that direct protein N-acetylation was increased to include Ser and Thr. These results show that the amino acid residue at position 6 strongly affects the amino acid requirement at position 3 for both protein N-myristoylation and protein N-acetylation. The susceptibility of R3X- and R3X,S6A-TNFs to protein N-myristoylation and N-acetylation is summarized in Fig. 7.
Fig. 7.

Amino acid requirements for protein N-myristoylation found in the in vitro translation system are applicable to the protein N-myristoylation in intact cells — In order to determine whether the amino acid requirements for protein N-myristoylation found in the in vitro translation system are applicable to N-myristoylation reaction in intact cells, R3X-mutants were expressed in COS-1 cells and their susceptibility to N-myristoylation was evaluated by metabolic labeling with [3H]-myristic acid. The same experiment with [3H]-acetyl CoA could not be performed because of the limited supply of [3H]-acetyl CoA. As shown in the upper panels of Fig. 8, Western blotting analysis of the expressed proteins immunoprecipitated from the total cell lysates revealed that a remarkable difference in the level of protein expression was present within these mutants. Two mutants having Cys or Val residue at position 3 were not expressed in COS-1 cells. Interestingly, only low levels of protein expression were equally observed with the mutants in which the radius of gyration of amino acids at position 3 is larger than 1.80Å, namely, Phe, Lys, Tyr, Trp, and Arg. In contrast to the results obtained by the in vitro translation system, only a single protein band with an expected molecular mass was detected in all these mutants in the Western blotting analysis, indicating that the initiating Met residue in these mutants was completely removed in intact cells.

Fig. 8.

Incorporation of [3H]-myristic acid into these COS-1 cells is shown in the lower panels in Fig. 8. As for the mutants in which the expression of the protein was observed, exactly the same patterns of [3H]-myristic acid incorporation as with the in vitro translation system were observed. These results indicated that the amino acid requirements at position 3 in the N-myristoylation consensus motif found in the in vitro translation system are consistent with those observed for the N-myristoylation reaction in intact cells.

Lysine residue is permitted at position 3 in naturally occurring N-myristoylation motif — In order to determine whether the amino acid requirements at position 3 for protein N-myristoylation found in the present study are consistent with the amino acids at this
position in naturally occurring N-myristoylation motifs, the number of each amino acid residue located at position 3 in 88 N-myristoylated proteins listed in two recent reviews (5, 6) were counted and summarized in Fig. 9. Ninety percent (79 out of 88) of these proteins had amino acid residues at position 3 which were consistent with the amino acid requirements at position 3 for protein N-myristoylation found in this study. Seven out of nine proteins in which the amino acid at position 3 is inconsistent with our present results have Lys residue at this position. Thus, Lys residue seems to be permitted at position 3 in naturally occurring myristoylation motif.

Fig. 9.

One possible explanation for this discrepancy might be our use of a non-myristoylated model protein to study the amino acid requirement instead of using a naturally myristoylated protein. To test this possibility, the amino acid at position 3 in Gag-TNF and G_{i1}\alpha-TNF, which have a natural myristoylation motif at the N-terminus, was changed to Lys and the susceptibility to N-myristoylation was evaluated. As shown in Fig. 10 (lanes 1-4), myristoylation of Gag-TNF and G_{i1}\alpha-TNF was remarkably reduced by replacing the amino acid at position 3 with Lys; only a very low level of [3H]-myristic acid incorporation was observed with Gag-Q3K- and G_{i1}\alpha-C3K-TNF. These results clearly indicated that the discrepancy in the amino acid requirement at position 3 was not due to the use of a non-myristoylated model protein. Interestingly, however, when the N-terminal 10 residues of the mature domain of TNF were changed to those of Arf6 or Hippocalcin, both having Lys 3 in their myristoylation motif, remarkable myristoylation was detected as with Gag-TNF or G_{i1}\alpha-TNF as shown in Fig. 10 (lanes 7, 8). These results suggested that the N-myristoylation motif having Lys 3, such as that of Arf6 or Hippocalcin, might have some specific structural determinant that permits the Lys residue at position 3, while still directing protein N-myristoylation.

Fig. 10.
DISCUSSION

Since all protein synthesis begins at the N-terminus, this region provides an initial and important site of cotranslational protein processing. Removal of the initiator methionine and modification of the α-amino group are examples of commonly encountered N-terminal modifications. Three of these modifications, excision of the initiator methionine, N-acetylation, and N-myristoylation potentially affect many of the eukaryotic cytoplasmic proteins and variously correlate with their stability, physiological function and degradation. These three modifications are catalyzed by methionine aminopeptidases, N-acetyltransferases (NATs) and N-myristoyltransferases (NMTs), respectively.

Methionine aminopeptidases involved in the cotranslational removal of the initiator Met are defined by a highly conserved substrate specificity, which is dictated by the residue adjacent to the Met residue (26, 27). The seven amino acids that have the smallest radii of gyration are substrates for methionine aminopeptidases, while those with the 13 largest side chains are not. Residues downstream of the specificity-determining residue have little impact on the reaction.

NATs that catalyze cotranslational protein N-acetylation also have a restricted number of substrates (28-31). Studies both in yeast and in higher eukaryotic cells showed that the principal substrates of NATs are proteins that have N-terminal Gly, Ala, Ser, or Thr residues (GAST substrates), or Met residue (M substrates) (32-34). Unlike the methionine aminopeptidases, this specificity is clearly affected by downstream residues, making the rules that guide acetylation less clear. In the case of M substrates, N-acetylation only occurs if the adjacent residue is an Asp, Glu or Asn residue. However, the amino acid requirements for the adjacent residue of the GAST substrates have not been defined as yet.

Protein N-myristoylation is a cotranslational protein modification catalyzed by two enzymes, methionine aminopeptidase and NMT. Proteins destined to become myristoylated begin with the sequence : Met-Gly. The initiating methionine is removed.
cotranslationally by methionine aminopeptidase and then myristic acid is linked to Gly-2 via an amide bond by NMT. NMT catalyzes the transfer of myristic acid from myristoyl-CoA to the N-terminus of protein substrates. However, not all proteins with an N-terminal glycine are N-myristoylated and the ability to be recognized by NMT depends on the downstream amino acid sequence.

Some amino acid preferences have been observed at distinct positions downstream of the N-terminal glycine (2, 13, 15). In general, Ser or Thr is preferred at position 6 and N-terminal consensus motifs such as Met-Gly-X-X-Ser/Thr-X-X (13) or Met-Gly-X-X-Ser/Thr-X-X-X (5) have been defined. In addition to the preference for Ser/Thr residue at position 6, positively charged residues (lysine or arginine) are known to be preferred at positions 7 and/or 8 (2, 15). These amino acid preferences were confirmed by recent studies on the NMT structure as determined by X-ray crystallography (35). In this study, the structure of the yeast *Saccharomyces cerevisiae* NMT1p was solved as a ternary complex and revealed how myristoyl CoA and peptide substrates bind to the enzymes. The determined structure allows identification of specific residues within NMT that serve to restrict substrate specificity to 14-carbon fatty acids and account for the preference of Gly-2, Ser-6, and basic amino acids at positions 7 and 8 of the peptide substrate.

In the present study, in order to examine the amino-terminal sequence requirements for the cotranslational N-myristoylation of proteins, several series of mutants were prepared by site-directed mutagenesis of the N-terminal region of the protein, using tumor necrosis factor (TNF) as a non-myristoylated model protein. Subsequently, the susceptibility of these mutants to the cotranslational N-myristoylation reaction was evaluated by an in vitro transcription/translation system using the rabbit reticulocyte lysate. It was found that the radius of gyration of the amino acid residue at position 3 in an N-myristoylation consensus motif, Met-Gly-X-X-Ser-X-X-X, strongly affected the cotranslational protein N-myristoylation. Amino acids with a radius of gyration larger than 1.80 Å (Phe, Lys, Tyr, Trp and Arg) could not direct efficient protein N-myristoylation in the in vitro translation system. From the study on the X-ray crystallography of NMT, it
was proposed that during the catalytic process, the amine nucleophile of the N-terminal Gly of the protein substrate must move in the active site of NMT toward the thioester carbonyl of myristoyl-CoA, which is positioned in the oxyanion hole formed by the main chain amide NH groups of Phe170 and Leu171 (35). Therefore, the presence of amino acids having large radii of gyration at position 2 might cause a steric hindrance of the movement of the N-terminal Gly. This notion is supported by the finding that the presence of Pro at this position completely inhibits the reaction.

As mentioned earlier, similar limitation by the radius of gyration of the amino acid residue penultimate to the N-terminal residue was observed on the substrate specificity of methionine aminopeptidases. Since both NMTs and methionine aminopeptidases function cotranslationally while the nascent polypeptide chain is still attached to the ribosomes (20, 36, 37), it might be possible to speculate that these two enzymes may share similar restrictions with respect to their substrate specificities.

In addition to the restriction by the radius of gyration of the amino acid, the presence of either negatively charged residues (Asp, Glu) or Pro at this position completely inhibited protein N-myristoylation. Labeling of these TNF mutants with [3H]-acetyl CoA revealed that non-myristoylated mutants having Asp or Glu residue at position 3 and weakly-myristoylated mutant having Met at this position were efficiently acetylated. These results clearly indicated that the amino acid at position 3 in Met-Gly-X-X-X-Ser-X-X-X motif strongly affected two different cotranslational protein modifications, N-myristoylation and N-acetylation. Most of the cotranslationally modified TNF-mutants were modified exclusively with one of the two modifications, indicating that the amino acid requirements for protein N-myristoylation and protein N-acetylation are generally different. However, some partially N-myristoylated mutants such as R3G-TNF and R3M-TNF were found to be partially N-acetylated, suggesting that the N-terminal sequence requirements for the two cotranslational modifications partially overlap.

When Ser at position 6 in Met-Gly-X-X-X-Ser-X-X-X motif was replaced with Ala, amino acid requirements at position 3 for the two cotranslational modifications were
dramatically changed. As for protein N-myristoylation, only two amino acids, Asn and Gln, could direct this modification. This result clearly indicates that the amino acid requirements at position 3 for protein N-myristoylation are significantly affected by the downstream residue(s).

In contrast, in the case of protein N-acetylation, two additional amino acids, Ser and Thr, could direct efficient protein modification. Two different interpretations can be derived to explain this phenomenon. It may simply imply that the amino acid requirement at position 3 for protein N-acetylation is affected by amino acid residue at position 6, as is the case with protein N-myristoylation. Alternatively, the N-myristoylation reaction predominates over the N-acetylation reaction and inhibition of the N-myristoylation reaction by replacing Ser-6 with Ala resulted in the two N-terminal sequences (Gly-Ser-, and Gly-Thr-) to be N-acetylated. Further analysis is required to fully characterize these experimental observations.

It is also interesting to investigate whether the amino acid requirements at position 3 observed for R3X- and R3X,S6A-TNF for protein N-acetylation are applicable to the other GAST-substrates of N-acetyl transferase. We are currently investigating these issues.

Another issue to be clarified is whether the amino acid requirements for protein N-myristoylation and N-acetylation found in the in vitro translation system are fully applicable in intact cells. As shown in Fig. 8, metabolic labeling of COS-1 cells transfected with R3X-TNFs revealed that the amino acid requirements at position 3 in N-myristoylation consensus motif found in the in vitro translation system are consistent with those observed in N-myristoylation reaction in intact cells. Therefore, it is quite possible that the amino acid requirements for both protein N-myristoylation and N-acetylation found in the in vitro translation system are applicable to intact cells. In fact, when N-terminal sequences of 88 N-myristoylated proteins with Met-Gly-X-X-Ser/Thr-X-X-X motif listed in two recent reviews (5, 6) were examined, 90% (79 out of 88) of the amino acid residue at position 3 of these proteins were found to be consistent with the amino acid requirements at position 3 for protein N-myristoylation found in this study. Analysis of the
N-myristoylation motifs in which the amino acid at position 3 is inconsistent with our present results revealed that Lys residue could be permitted at position 3 in naturally occurring N-myristoylation motif. In this case, however, it seems likely that N-myristoylation motifs having Lys 3 have some specific structural determinant that permits the Lys residue at position 3, while still directing protein N-myristoylation. We are currently searching for the specific determinant in these N-myristoylation motifs. In addition, when N-myristoylated proteins with N-terminal Met-Gly-X-X-Ala-X-X-X-sequences listed in these reviews were examined, 4 out of 5 proteins were found to possess Asn or Gln residue at position 3. These results strongly suggest that the amino acid requirements found in this study well correlated with those for the myristoylation reaction in intact cells.

Thus, analysis of amino acid requirements for cotranslational protein modifications in the in vitro translation system is an effective strategy for characterization of the consensus amino acid sequences that direct cotranslational protein modifications.

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   *Biol.* **5**, 1091-1097

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FOOTNOTES

1 The abbreviations used are: NMT, N-myristoyltransferase; NAT, N-acetyltransferase; TNF, tumor necrosis factor; PCR, polymerase chain reaction; FCS, fetal calf serum; DPBS, Dulbecco’s phosphate-buffered saline; DMEM, Dulbecco modified Eagle’s medium; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; ECL, enhanced chemiluminescence.

FIGURE LEGENDS

Fig. 1. Schematic representation of V2G-TNF generation.
cDNA coding for Δpro-TNF which contains the mature domain of TNF was first generated from pro-TNF cDNA by deleting the nucleotide sequence encoding the propeptide region of pro-TNF. Subsequently, V2G-TNF cDNA was generated from Δpro-TNF cDNA by site-directed mutagenesis.

Fig. 2. In vitro translation of mRNAs coding for Δpro-, V2G-, R3A-, and R3D-TNF.
The mRNAs encoding Δpro-, V2G-, R3A-, and R3D-TNF were translated in vitro in the presence of [3H]leucine, [3H]myristic acid, or [3H]acetyl CoA using rabbit reticulocyte lysate. Following immunoprecipitation with anti-TNF antibody, the labeled translation products were analyzed by SDS-PAGE and fluorography.

Fig. 3. Characterization of protein N-myristoylation and N-acetylation of TNF mutants and ovalbumin.
The mRNAs encoding Gag-, Giα-, R3A-, R3D-, OVA60-TNF, and ovalbumin were translated in vitro in the presence of [3H]leucine, [3H]myristic acid, or [3H]acetyl CoA using rabbit reticulocyte lysate. The labeled translation products were analyzed directly.
(I.P. -) or following immunoprecipitation with anti-TNF antibody (I.P. +) by SDS-PAGE and fluorography (A). [3H]fatty acids attached to the R3A- and R3D-TNF were analyzed by thin layer chromatography. [3H]fatty acid-labeled protein band of R3A- or R3D-TNF on the transfer membrane, identified by western blotting, was excised and the fatty acids were liberated by acid treatment and extracted with hexane. The extracts from R3A- and R3D-TNF ([B] lane 3 and 4, respectively), together with control [3H]palmitic acid (lane1), [3H]myristic acid (lane 2), and [3H]acetyl CoA (lane 5), were separated by thin layer chromatography and detected by fluorography. The migration positions of control [3H]myristic acid (Myr) and [3H]palmitic acid (Pal) are indicated. The arrows indicate the origin (B).

**Fig.4.** Differential labeling of the two distinct protein bands obtained by in vitro translation of mRNAs coding for Δpro-, R3A-, and R3D-TNF.

The mRNAs encoding Δpro-, R3A-, and R3D-TNF were translated in vitro in the presence of [3H]leucine, [35S]methionine, [3H]myristic acid, or [3H]acetyl CoA using rabbit reticulocyte lysate. Following immunoprecipitation with anti-TNF antibody, the labeled translation products were analyzed by SDS-PAGE and fluorography.

**Fig.5.** Effect of the amino acid residue at position 3 in N-myristoylation consensus motif on the efficiency of cotranslational N-myristoylation reaction.

The mRNAs encoding R3X-TNFs were translated in vitro in the presence of [3H]leucine or [3H]myristic acid using rabbit reticulocyte lysate. Following immunoprecipitation with anti-TNF antibody, the labeled translation products were analyzed by SDS-PAGE and fluorography. Results of the 20 amino acids were arranged according to their radius of gyration. Three independent experiments showed similar labeling patterns (A).

Efficiency of protein N-myristoylation ([3H]myristic acid incorporation / [3H]leucine incorporation) of R3X-TNF was compared by quantitative analysis of the fluorogram of [3H]myristic acid- and [3H]leucine-labeled proteins shown in the upper and lower panels of
(A). Relative N-myristoylation efficiency of each R3X-TNF was expressed as % of the myristoylation efficiency of R3A-TNF. Results of the 20 amino acids were arranged according to their radius of gyration. Data are expressed as mean ± S.D. of three independent experiments (B).

**Fig.6.** Effect of the amino acid residue at position 3 on the protein N-myristoylation and N-acetylation of R3X-TNFs and R3X,S6A-TNFs.

The mRNAs encoding R3X-TNFs and R3X,S6A-TNFs were translated in vitro in the presence of [3H]leucine, [3H]myristic acid, or [3H]acetyl CoA using rabbit reticulocyte lysate. Following immunoprecipitation with anti-TNF antibody, the labeled translation products were analyzed by SDS-PAGE and fluorography. Results of the 20 amino acids were arranged according to their radius of gyration. (A) and (B) are results with R3X-TNFs and R3X,S6A-TNFs, respectively.

**Fig.7.** Summary of cotranslational N-myristoylation and N-acetylation of R3X-TNFs and R3X,S6A-TNFs.

Susceptibility of R3X- and R3X,S6A-TNFs to protein N-myristoylation and N-acetylation as determined by the metabolic labeling experiments in Fig. 6 is summarized. ++; effectively modified, +; weakly modified, ±; slightly modified.

**Fig.8.** N-myristoylation of R3X-TNFs expressed in COS-1 cells.

The cDNAs encoding R3X-TNFs were transfected into COS-1 cells, and their expression and N-myristoylation were evaluated by Western blotting analysis and [3H]myristic acid-labeling, respectively. Results of the 20 amino acids were arranged according to their radius of gyration.

**Fig.9.** Amino acid residues at position 3 in naturally occurring N-myristoylation motifs.
The number of each amino acid residue located at position 3 in 88 N-myristoylated proteins listed in two recent reviews by Boutin, J.A. (5) and Resh, M.D. (6) were counted and arranged according to their radius of gyration.

: amino acid residue consistent with the amino acid requirements at position 3 found in this study.

: amino acid residue inconsistent with the amino acid requirements at position 3 found in this study.

**Fig.10.** Effect of Lys residue at position 3 in N-myristoylation consensus motif on protein N-myristoylation.

The mRNAs encoding Gag-, Gag-Q3K-, G\(_i\)\(\alpha\)-, G\(_i\)\(\alpha\)-C3K-, R3A-, R3K-, Arf6-, and Hippocalcin-TNF were translated in vitro in the presence of \([^3]H\)leucine or \([^3]H\)myristic acid using rabbit reticulocyte lysate. Following immunoprecipitation with anti-TNF antibody, the labeled translation products were analyzed by SDS-PAGE and fluorography.
**Table I.** Nucleotide sequences of oligonucleotides used for the construction of mutant TNF cDNAs

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<td>R3P</td>
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Fig. 2. Utsumi, T. et al.

N-myristoylation motif

\[ \text{Met} - \text{Gly} - X - X - X - \text{Ser} - (X)_3 \]

\begin{align*}
\textbf{Apro-TNF} & \quad \text{Met} \quad \text{Val} \quad \text{Arg} \quad \text{Ser} \quad \text{Ser} \quad \text{Ser} \\
\textbf{V2G-TNF} & \quad \text{Met} \quad \text{Gly} \quad \text{Arg} \quad \text{Ser} \quad \text{Ser} \quad \text{Ser} \\
\textbf{R3A-TNF} & \quad \text{Met} \quad \text{Gly} \quad \text{Asp} \quad \text{Ser} \quad \text{Ser} \quad \text{Ser} \\
\textbf{R3D-TNF} & \quad \text{Met} \quad \text{Gly} \quad \text{Asp} \quad \text{Ser} \quad \text{Ser} \quad \text{Ser} 
\end{align*}

\begin{tabular}{c|c|c|c|c|c|c|c}
\hline
 & [\text{H}]-\text{leucine} & [\text{H}]-\text{myristic acid} & [\text{H}]-\text{acetylCoA} \\
\hline
\text{Apro} & \text{V2G} & \text{R3A} & \text{R3D} & \text{Apro} & \text{V2G} & \text{R3A} & \text{R3D} & \text{Apro} & \text{V2G} & \text{R3A} & \text{R3D} \\
\hline
\text{kDa} & \text{17} & \text{17} & \text{17} & \text{17} & \text{17} & \text{17} & \text{17} & \text{17} & \text{17} & \text{17} & \text{17} & \text{17} \\
\hline
\end{tabular}
Fig. 3. Utsumi, T. et al.

[A]  

$^{[3]}$H-leucine  $^{[3]}$H-myristic acid  $^{[3]}$H-acetyl CoA

|                | Gag  | Gg  | R3A  | R3D  | OVAl60- | Ovalbumin | Gag  | Gg  | R3A  | R3D  | OVAl60- | Ovalbumin | Gag  | Gg  | R3A  | R3D  | OVAl60- | Ovalbumin | Gag  | Gg  | R3A  | R3D  | OVAl60- | Ovalbumin |
|----------------|------|-----|------|------|---------|-----------|------|-----|------|------|---------|-----------|------|-----|------|------|---------|-----------|------|-----|------|------|---------|-----------|------|-----|------|------|---------|-----------|
| [1]            |      |     |      |      |         |           |      |     |      |      |         |           |      |     |      |      |         |           |      |     |      |      |         |           |
| [2]            |      |     |      |      |         |           |      |     |      |      |         |           |      |     |      |      |         |           |      |     |      |      |         |           |
| [3]            |      |     |      |      |         |           |      |     |      |      |         |           |      |     |      |      |         |           |      |     |      |      |         |           |
| [4]            |      |     |      |      |         |           |      |     |      |      |         |           |      |     |      |      |         |           |      |     |      |      |         |           |
| [5]            |      |     |      |      |         |           |      |     |      |      |         |           |      |     |      |      |         |           |      |     |      |      |         |           |
| [6]            |      |     |      |      |         |           |      |     |      |      |         |           |      |     |      |      |         |           |      |     |      |      |         |           |
| [7]            |      |     |      |      |         |           |      |     |      |      |         |           |      |     |      |      |         |           |      |     |      |      |         |           |
| [8]            |      |     |      |      |         |           |      |     |      |      |         |           |      |     |      |      |         |           |      |     |      |      |         |           |
| [9]            |      |     |      |      |         |           |      |     |      |      |         |           |      |     |      |      |         |           |      |     |      |      |         |           |
| [10]           |      |     |      |      |         |           |      |     |      |      |         |           |      |     |      |      |         |           |      |     |      |      |         |           |
| [11]           |      |     |      |      |         |           |      |     |      |      |         |           |      |     |      |      |         |           |      |     |      |      |         |           |
| [12]           |      |     |      |      |         |           |      |     |      |      |         |           |      |     |      |      |         |           |      |     |      |      |         |           |
| [13]           |      |     |      |      |         |           |      |     |      |      |         |           |      |     |      |      |         |           |      |     |      |      |         |           |
| [14]           |      |     |      |      |         |           |      |     |      |      |         |           |      |     |      |      |         |           |      |     |      |      |         |           |
| [15]           |      |     |      |      |         |           |      |     |      |      |         |           |      |     |      |      |         |           |      |     |      |      |         |           |
| [16]           |      |     |      |      |         |           |      |     |      |      |         |           |      |     |      |      |         |           |      |     |      |      |         |           |
| [17]           |      |     |      |      |         |           |      |     |      |      |         |           |      |     |      |      |         |           |      |     |      |      |         |           |
| [18]           |      |     |      |      |         |           |      |     |      |      |         |           |      |     |      |      |         |           |      |     |      |      |         |           |

kDa  | 43  | 24  | 17  |

L.P.  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +

[B]  

Myr  Pal

1  2  3  4  5
Δpro-TNF
NH₂-Met Val Arg Ser Ser Ser

R3A-TNF
NH₂-Met Gly Ala Ser Ser Ser

R3D-TNF
NH₂-Met Gly Asp Ser Ser Ser

Fig. 4. Utsumi, T. et al.
Fig. 5. Utsumi, T. et al.
### Table

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### Figures

**Fig. 7.** Utsumi, T. et al.
Amino acid residue at position 3

Number of protein
Amino acid residue penultimate to amino-terminal Gly residue strongly affects two cotranslational protein modifications, N-myristoylation and N-acetylation
Toshihiko Utsumi, Masahiro Sato, Kengo Nakano, Daisuke Takemura, Hiroyuki Iwata and Rumi Ishisaka

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