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COTRANSLATIONAL ATTACHMENT OF FATTY ACIDS TO NASCENT PEPTIDES IN GASTRIC MUCUS GLYCOPROTEIN

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SUMMARY - Using gastric mucous cells which are involved exclusively in the synthesis of secretory O-glycosidic glycoprotein (mucin), the relationship between protein core synthesis and its acylation with fatty acids was investigated. Labeling of the cells with [^{3}H]palmitic acid and [^{35}S]methionine followed by isolation of peptidyl-tRNA and release of nascent peptides, indicated that these peptides contain covalently bound fatty acids. The high performance thin layer chromatography, SDS-gel electrophoresis, and radioactivity scanning revealed that the preparation contained three fractions labeled with palmitate (Mr 15,000-3,600) and two (Mr 1,500 and less) without this label. Based on these data and the nascent peptides amino acid analysis, we conclude that the protein core of the O-glycosidic glycoprotein is acylated with fatty acids during translation, when the peptide chain is longer than 21 amino acid residues.

INTRODUCTION - In contrast to present understanding of the synthesis and processing of Asn-glycoproteins, less is known about the intracellular sites and mechanisms of the O-glycosidic mucus glycoprotein assembly and modifications (1). The exact temporal relationship between formation of the core protein of O-glycosidic glycoprotein, its acylation with fatty acids, and addition of the carbohydrate residues has not been established and the critical experiments to explore the aspects of fatty acylation, and initial O-glycosylation as to their co-or posttranslational occurrence have not been carried out. Studies on glycophorin, which has 1 N-linked oligosaccharide and 15 O-linked glycosyl units suggest that this membrane glycoprotein could be N- and O-glycosylated in the rough endoplasmic reticulum, and that both processes may by co-translational events (2). The data on the kinetics of acylation of membrane and soluble proteins indicate that the acylation with myristic acid might be an extremely early modification that takes place cotranslationally, while the

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acylation with other fatty acids is posttranslational (3). In secretory glycoproteins (gastric mucin), we have found that palmitate is added prior to or concomitantly with O-glycosylation of mucin apoprotein (4). Here, we provide evidence that palmitate is added to nascent peptides of mucus glycoprotein at the time of translation when the chains contain more than 21 amino acid residues.

MATERIALS AND METHODS - The columnar epithelium (mucous cells), removed from surface of gastric mucosa from twenty rat stomachs, was gently dispersed in Dulbecco's modified Eagles' minimum essential medium (D-MEM), centrifuged and rinsed several times with D-MEM. The suspension of the rinsed viable cells was preincubated under 95% O_2-5% CO_2 atmosphere (30 min at 37°C), and labeled with 9,10-[^{3}H]palmitic acid (30Ci/mmol) and [^{35}S]-methionine (800Ci/mmol). In pulse-chase experiments, the mucous cells were labeled for 4 min and chased with 100μg/ml methionine and palmitic acid for 4-12 min. After labeling, the cells were treated with 2mg/ml cycloheximide, suspended in 10 volumes of hypotonic buffer A (10mM TRIS·HCl, pH 7.4, 10mM KCl, 1.5mM $MgCl_2$, 1mM phenylmethylsulfonyl fluoride, 0.2TIU/ml aprotinin, 20μg/ml pepstatin, and 0.2mg/ml ribonuclease inhibitor from human placenta) for 10 min and collected by centrifugation (1,000xg, 5 min). The pellet of swollen cells was suspended in 1 volume of the same buffer, the cells were disrupted with 10 strokes of an all-glass Dounce homogenizer and the homogenate was immediately adjusted to 0.2M sucrose. The postnuclear supernate was prepared by centrifugation (600xg, 5 min) and layered over three step sucrose gradient (0.5, 1.0, 2.0M), prepared in 50mM TRIS·HCl, pH 7.4, 25mM KCl, 5mM $MgCl_2$ and 5mM 2-mercaptoethanol, and centrifuged at 4°C for 5h at 46,000xg. The microsomes pelleted on 2.0M sucrose cushion were treated with 1% NP-40 in buffer A (2h at 4°C), layered on buffered 2.0M sucrose and centrifuged at 4°C for 16h at 195,000xg. The pellet (polyribosomes) was resuspended in 10% buffered sucrose (50mM TRIS·HCl, pH 7.4, 80mM KCl, 5mM $MgCl_2$ and 5mM mercaptoethanol) and layered on the top of linear 20-30% (m/w) sucrose gradient, prepared in 50mM TRIS·HCl, pH 7.4, 80mM KCl, and 10mM EDTA, and centrifuged at 4°C for 16h at 25,000rpm. After centrifugation, the tubes were placed in a Beckman fraction recovery system and 0.5ml fraction were collected from the bottom of the tube. The peptidyl-tRNA, recovered from fractions 6-12, was chromatographed on a DEAE-Sephadex column (0.9 x 20cm) equilibrated with 50mM TRIS·HCl, pH 7.4, 0.15M NaCl (5). After initial development with the above buffer, the column was eluted with 0.2 - 1.0M NaCl gradient in the same buffer.

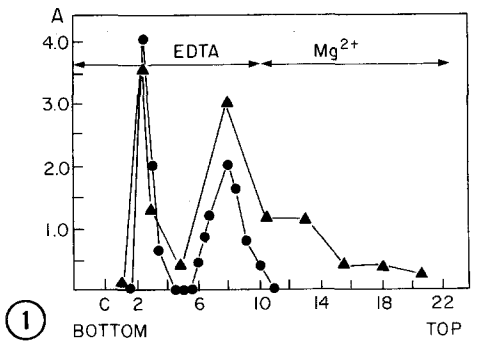
The [^{3}H]palmitic acid and [^{35}S]methionine labeled peptidyl-tRNA, recovered from DEAE-Sephadex with buffered 0.4-0.5M NaCl was dialyzed, treated with RNase or 1.8M TRIS·HCl, pH 8.9 (6) and subjected to two dimensional high performance thin layer chromatography in solvent systems: chloroform/acetone/methanol/acetic acid/water (6:8:2:2:1, by vol.), 1-butanol/acetic acid/water (3:1:2, v/v/v), 1-propanol/25% ammonia/water (7:1:2, v/v/v) and 1-propanol/water (7:3, v/v). Following radioactivity scanning (Berthold LB285 Linear Analyzer System), the nascent peptides were recovered by extracting silica gel with 1-propanol/water (1:1, v/v).

The recovered peptides were subjected to acid hydrolysis (6N HCl at 118°C for 20h) and extraction with hexane. The hexane phase containing labeled palmitic acid, was chromatographed along the palmitate standard and its radioactivity counted (4). The aqueous hydrolyzates were dried over KOH, the residues were dissolved in 20μl of 0.2M $NaHCO_3$, pH 8.75, and 40μl of dabsyl chloride 5nmol/μl acetone) was added (7). After 10 min of incubation with shaking at 70°C, a 6μl of derivatized mixture was injected directly to HPLC for amino acid analysis. The analysis was conducted on a Perkin-Elmer Series 4 Liquid Chromatograph using a Pecosphere-3C C18 (4.6 x 83mm) column and series of gradients consisting of solvent A - 12mM phosphate buffer, pH 6.5, and

solvent B-acetonitrile, both with 4% dimethylformamide. Detection of dabsyl amino acid derivatives was at 436nm using Perkin-Elmer LC-95 UV/VIS spectrophotometer. SDS-gel electrophoresis was conducted in 10-12% polyacrylamide slab gel containing 6M urea and 0.1% SDS (8), and developed with silver stain (9).

RESULTS - The gastric mucous cells incubated for 4 min with [^{3}H]palmitic acid and [^{35}S]methionine incorporated both radioactive precursors into its polysome fraction. Upon polysomes dissociation in the presence of EDTA, the ^{3}H and ^{35}S labels remained with peptidyl-tRNA fraction (Fig. 1). On DEAE-Sephadex, the peptidyl-tRNA preparation and [^{3}H]palmitic acid and [^{35}S]methionine were bound to the column while a small fraction of tRNA-free peptides eluted in the unbound fraction (Fig. 2). The peptidyl-tRNA that eluted with 0.4-0.5M buffered NaCl, was susceptible to alkaline hydrolysis and RNase treatment. After hydrolysis of the complex, the peptides were no longer retained on the ion exchange column but [^{3}H]palmitic acid and [^{35}S]methionine persistently appeared with the nascent peptide fraction.

On SDS-PAGE, the fraction released from tRNA separated into several bands ranging in size from 18,000 to 1,500 (Fig. 3), and gave on the two-dimensional thin layer chromatography 5 major and several minor components (Fig. 4).



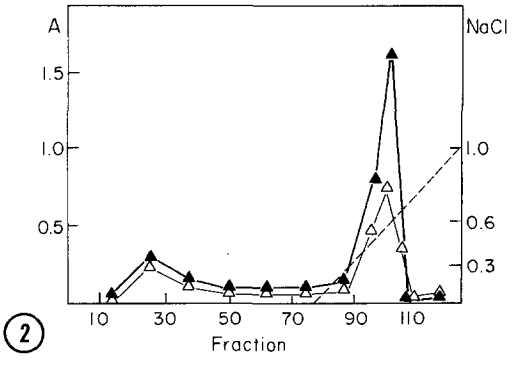
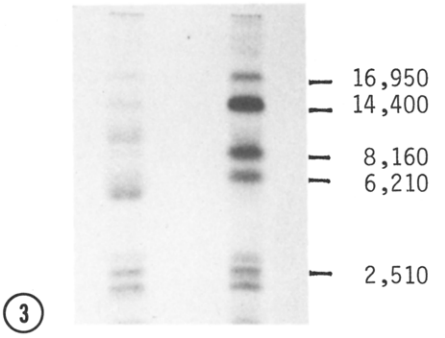
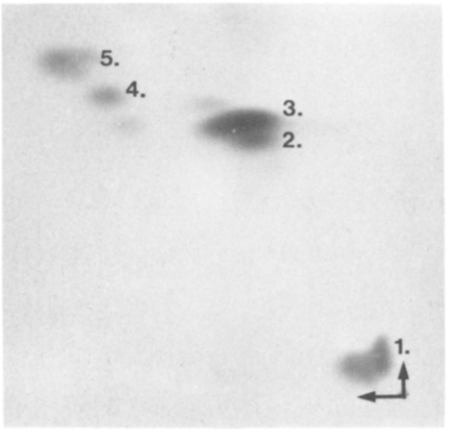


Fig. 1. Purification of peptidyl-tRNA on 20-30% (w/v) linear sucrose gradient. Polyribosomes, prepared as described in Materials and Methods were applied in buffered (pH 7.4) 10% sucrose on the top of linear sucrose gradient containing 10mM EDTA and centrifuged for 16h at 25,000rpm. Using Beckman fraction recovery system 0.5ml fractions were collected, their absorbance at 260 and 280nm monitored, and the peptidyl-tRNA present in fractions 6-12 was recovered. C - sucrose cushion.

Fig. 2. DEAE-Sephadex column chromatography of the peptidyl-tRNA. Fractions 6-12 from sucrose gradient (Fig. 1) were dialyzed against 50mM TRIS·HCl, 0.15M NaCl, pH 7.4, and chromatographed on a DEAE-Sephadex column. The peptidyl-tRNA bound to the ion exchange column was recovered by elution with 0.4 - 0.5M NaCl in 50mM TRIS·HCl buffer, pH 7.4.



(3)



(4)

Fig. 3. SDS-polyacrylamide gel electrophoresis of nascent peptides released from the peptidyl-tRNA. The peptides released from tRNA by alkaline hydrolysis were analyzed by electrophoresis on 12% SDS-polyacrylamide gel containing 6M urea. First lane (left) mucus glycoprotein nascent peptides released from tRNA, second lane, low molecular weight standards.

Fig. 4. Two dimensional high performance thin layer chromatography of the mucus glycoprotein nascent peptides. The plate was developed with chloroform/acetone/methanol/acetic acid/water (6:8:2:2:1, by vol.) and 1-butanol/acetic acid/water (3:1:1, v/v/v) and in the second dimension with 1-propanol/ammonia/water (7:2:1, v/v/v) and 1-propanol/water (7:3, v/v). After drying, the peptides were visualized with Nin-Print (Pierce).

After 4 min labeling, the [^{3}H]palmitic acid was detected in peptides 2 and 3, whereas peptides 1,4, 5 were not labeled (Fig. 5).

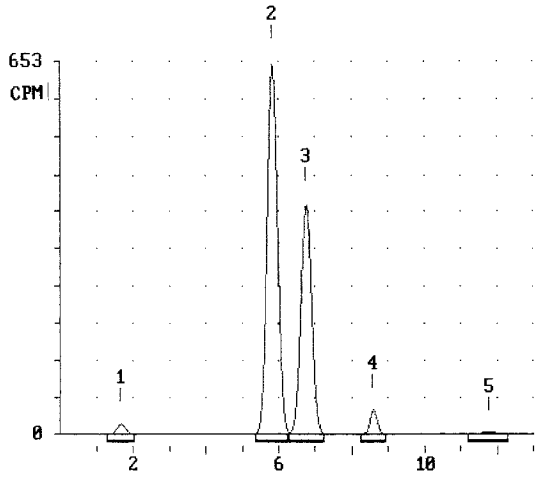


Fig. 5. Thin layer chromatogram of [^{3}H]palmitic acid labeled mucus glycoprotein nascent peptides. The silica gel plate was developed in chloroform/acetone/methanol/acetic acid/water (6:8:2:2:1, by vol.) and 1-butanol/acetic acid/water (3:1:1, v/v/v), and then subjected to radioscanning using Berthold LB285 Linear Analyzer System. The positions of nascent peptides are indicated by numbers as in Fig. 4.

Table 1. Amino acid composition of the mucus glycoprotein nascent peptides

Amino acid	Peptide			
	1	2	3	4
	residues/mol			
Aspartic acid	2.8 (3)	1.1 (1)	--	--
Glutamic acid	3.8 (4)	2.1 (2)	0.9 (1)	0.9 (1)
Serine	35.4 (35)	8.8 (9)	4.1 (4)	1.9 (2)
Threonine	15.4 (15)	1.7 (2)	0.8 (1)	0.4 (0)
Glycine	37.6 (38)	11.7 (12)	7.3 (7)	2.8 (3)
Alanine	9.2 (9)	4.6 (5)	4.8 (5)	1.8 (2)
Proline	5.8 (6)	3.1 (3)	1.9 (2)	1.7 (2)
Valine	14.1 (14)	7.1 (7)	3.3 (3)	2.6 (3)
Methionine	--	0.8 (1)	1.0 (1)	0.8 (1)
Isoleucine	3.3 (3)	3.1 (3)	2.1 (2)	1.4 (1)
Leucine	4.0 (4)	2.7 (3)	2.3 (2)	0.9 (1)
Phenylalanine	4.7 (5)	3.0 (3)	1.9 (2)	1.3 (1)
Lysine	0.8 (1)	1.0 (1)	0.9 (1)	0.6 (1)
Histidine	0.8 (1)	0.9 (1)	--	--
Tyrosine	--	--	--	--

In pulse-chase experiments after 4 min labeling, the palmitic acid incorporated into peptides 2 and 3 and during chase also appeared in peptide 1. Within 4 min, the [^{35}S]methionine incorporated into peptides 2-5, but was not detected in peptide 1 even after 12 min chase. The amino acid analysis of the major peptide fractions (1,2,3 and 4) showed that palmitic acid label was present on peptides containing 43-142 amino acid residues, but was not detected on peptides containing 21 or less amino acids (Fig. 4 and Table I).

DISCUSSION - The acylation with fatty acids has been reported for O-glycosidic secretory glycoprotein in healthy and cystic fibrosis individuals (10,11), however, studies on the specificity of this modification and on the biosynthetic events leading to the attachment of fatty acid to protein core have only begun (12). The actual time or stage at which fatty acids are attached to protein core still remains poorly defined (4). Since palmitate was detected on the

partially glycosylated mucus glycoprotein subunits it was deduced that acylation is either an early modification or is concomitant with glycosylation. The results of this study demonstrate that fatty acylation is an early cotranslational event, since the incomplete peptides released from peptidyl-tRNA complex already contain covalently bound fatty acids. The attachment of fatty acid residue to the growing peptide chain takes place at the time when chain is 21-43 amino acid long. The peptides shorter than 21 amino acid residues are either of insufficient length or the amino acid sequence required for fatty acid attachment has not been translated. The high degree of incorporation of palmitate to peptides larger than 21 amino acid residues, suggest the existence of fatty acyltransferase enzyme in the vicinity of ribosomal subunit or on the cytoplasmic surface of endoplasmic reticulum. The facts that acylation site is at least 20 residues away from the amino terminal and that palmitate residues are retained on fully assembled molecules remarkably agrees with signal sequence hypothesis (13-16), which postulate that about 15-20 amino acid, so-called "leading sequence", are cleaved either on cytoplasmic or luminal site of endoplasmic reticulum (13). It seems that peptide 1, which is missing N-terminal methionine but not its palmitate, represent such processed component.

Although the precise function of fatty acylation of protein remains to be determined, it appears that the role of these fatty residues might be to limit proteolysis and translocate the nascent peptides through the endoplasmic membrane. The driving force for the nascent peptide to cross the endoplasmic reticulum membrane is unknown, but is quite reasonable to believe that fatty acyl residue may serve as an anchor in binding of the peptide emerging from the ribosome and influence its protein-lipid interaction with the lipid core of membrane. If indeed the fatty acyl residues are involved in regulation of the leading sequence cleavage and the interaction and transfer of the peptide across endoplasmic reticulum, then perhaps the acylating enzyme and "docking protein" (14) are synonymous names for protein which coordinates the synthesis of the nascent presecretory peptide with the recognition and insertion into the endoplasmic membrane (17). Because of the diversity of acyl protein func-

tions which could be attributed to fatty acyl residue (3,18), it would be interesting to determine whether these acyl residues play role in recognition of the apoprotein substrate by specific glycosyltransferases and whether fatty acyl moiety is a marker for the intracellular sorting of the synthesized protein and glycoconjugates.

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