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Fatty acylation of yeast glycoproteins proceeds independently of *N*-linked glycosylation

P.S. Appukuttan and Henry C. Wu

Department of Microbiology, Uniformed Services University of the Health Sciences, Bethesda, MD 20814-4799, USA

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The relationship between protein glycosylation and fatty acylation of glycoproteins was studied in the wild-type and asparagine-linked glycosylation-deficient mutants (*alg1* and *alg2*) of *Saccharomyces cerevisiae*. At the non-permissive temperature (37°C), both mutant cells exhibited increased incorporation of [^{3}H]palmitate into five polypeptides based on SDS-PAGE. In contrast, the wild-type yeast cells contained [^{3}H]palmitate-labeled polypeptides of higher molecular weights, which were converted to the bands seen in the mutant cells upon treatment of the cell extract with endoglycosidase H prior to SDS-PAGE. In addition, labeling of the wild-type yeast cells with [^{3}H]palmitate in the presence of tunicamycin revealed the incorporation of [^{3}H]palmitate into the same five bands as found in the *alg1* and *alg2* mutants at the non-permissive temperature without tunicamycin. These results indicate that fatty acylation of glycoproteins proceeds independently of protein *N*-glycosylation in yeast cells.

Fatty acylated protein; Glycoprotein

1. INTRODUCTION

Glycosylation and fatty acylation represent co-translational or post-translational modifications of soluble or membrane proteins. Fatty acids are attached to proteins in eukaryotes by an amide linkage (myristate) to the amino-terminal glycine, by an *O*- or thio-ester linkage (palmitate) to serine/threonine or cysteine residues, respectively, or as part of a glycophospholipid attached to the carboxy-terminal amino acid of the membrane proteins [1-3]. The functions of fatty acylation of proteins are largely unknown. A role for the acylation, deacylation and reacylation in protein transport has been suggested [4], and recent studies have revealed that the *N*-myristoyl transferase activity is essential for vegetative growth of yeast cells [5].

Previous studies of the effect of inhibition of *N*-glycosylation on palmitoylation of glycoproteins have yielded different results depending on the nature of the proteins under study. Tunicamycin, an inhibitor of *N*-linked glycosylation [6], also inhibits the palmitoylation of viral glycoprotein and its transport to the cell surface [7]. On the other hand, tunicamycin does not affect the palmitoylation of the transferrin receptor or its transport to the cell surface [8]. We have previously reported that the perturbation of the *N*-linked oligosaccharide structure results in an altered incorporation of [^{3}H]palmitate into specific proteins in Chinese hamster ovary cells [9]. In the present study, we have examined the effect of a mutational defect in *N*-glycosylation on palmitoylation of glycoproteins in yeast cells using defined mutants deficient in the glycosylation pathway.

Correspondence address: H.C. Wu, Department of Microbiology, Uniformed Services University of the Health Sciences, Bethesda, MD 20814-4799, USA

2. MATERIALS AND METHODS

Wild-type yeast (*Saccharomyces cerevisiae*) strain DBY 640 and two asparagine-linked glycosylation-deficient mutants (*alg1* and *alg2*) were used in the present study. *alg1* mutant cells are expected to contain glycoproteins substituted with GlcNAc2-Asn, while *alg2* mutant cells should contain Man1-2GlcNAc2-Asn-substituted glycoproteins [10]. YPD medium (1% yeast extract, 2% bactopeptone, and 2% glucose)

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was used for the growth of yeast cells. [^{3}H]Palmitic acid (20 Ci/mmol), [^{35}S]methionine (800 Ci/mmol) and 'ENHANCE' for fluorography were purchased from New England Nuclear. Molecular weight standards were purchased from Sigma Chemical Co. Endoglycosidase H was obtained from Boehringer-Mannheim Biochemicals.

2.1. *Labeling experiment*

Yeast cells at A_{600nm} of 2.5 were grown for 4 h to mid-exponential phase at 26°C, and half of the culture was shifted to 37°C. 30 min later, 4 ml samples of each culture was labeled with either [^{3}H]palmitate (20 μCi/ml) or [^{35}S]methionine (100 μCi/ml). For labeling in the presence of tunicamycin, the drug was added to a final concentration of 5 μg/ml, 15 min prior to the addition of radioactive precursors. Labeling was continued for 4 h, and the cells were collected by centrifugation, and washed three times with ice-cold 10 mM NaN_3. Cells were resuspended in 100 mM Tris-HCl buffer (pH 6.8) containing 10 mM phenylmethylsulfonyl fluoride, 2% SDS and 10% glycerol, and disrupted with the aid of glass beads (40 μm) first by vortex mixing and then in a Branson bath sonicator. Finally, the solubilized sample was incubated at 95°C for 3 min.

2.2. *Gel electrophoresis and fluorography*

SDS-polyacrylamide gel electrophoresis was performed in 13% gel according to Laemmli [11], but in the absence of 2-mercaptoethanol since thio-ester-linked fatty acids might be labile to the sulfhydryl reagent [12]. Molecular weight standards used in SDS-PAGE included myosin (200 000), β-galactosidase (166 000), phosphorylase *b* (97 400), bovine serum albumin (66 200), ovalbumin (42 700), ricin B chain (35 000), ricin A chain (30 000), soybean trypsin inhibitor (21 500) and myoglobin (16 900). Fluorography was used for gels containing [^{3}H]palmitate-labeled proteins, and the 'ENHANCE'-treated gels were exposed to Kodak XAR-5 film (8 inch × 11 inch). Gels containing [^{35}S]methionine-labeled samples were fixed and exposed for 24 h before developing.

2.3. *Endoglycosidase H treatment*

[^{3}H]Palmitate-labeled samples solubilized in SDS as described above were mixed with an equal volume of 0.3 M Na citrate buffer (pH 5.5), and endoglycosidase H was added to a final concentration of 4 μg (100 mU) per ml. Following a 20 h incubation at 37°C, proteins were precipitated with the addition of 4 vols of ice-cold acetone, and collected by centrifugation. The pellets were dried under a stream of N_2, and resuspended in the solubilizing buffer before electrophoresis.

3. RESULTS

N-linked glycosylation-deficient mutants *alg1* and *alg2* cells labeled with [^{3}H]palmitate at 37°C were found to contain several fatty acylated polypeptides not seen in the wild-type cells labeled under the same condition; proteins with apparent molecular weights of 158 000, 136 000, 41 000, 30 000 and 15 300, respectively, appeared to be prominently labeled with [^{3}H]palmitate, and to a lesser extent, two additional proteins with apparent molecular weights of 27 500 and 26 000 were noted (fig.1A). In contrast, the wild-type cells contained [^{3}H]palmitate-labeled polypeptides with apparent molecular weights of >200 000, 51 000, 31 000 and 27 000, respectively. The different profiles of [^{3}H]palmitate-labeled proteins between the wild-type strain and *alg1/alg2* mutants were not seen when [^{35}S]methionine-labeled proteins were analyzed with SDS-PAGE (fig.1B). Thus the different labeling patterns with [^{3}H]palmitate between the wild-type and the mutant cells do not reflect differences in the metabolic conversion of palmitate into amino acids, nor do they result from an increased synthesis of a subset of proteins in the mutant cells as compared to the wild-type cells. Intense labeling of high molecular weight proteins (>200 000) with [^{3}H]palmitate was not accompanied by a concomitant increase in the labeling with [^{35}S]methionine. Thus the polypeptides readily labeled with [^{3}H]palmitate represent fatty acylated proteins. Those [^{3}H]palmitate-labeled proteins unique to the *alg1* and *alg2* mutants may represent increased fatty acylation of proteins due to defective *N*-glycosylation, as shown previously in CHO cell mutants [9]. Alternatively, these proteins are both fatty acylated and glycosylated, and the apparent differences in the molecular weight distribution of fatty acylated proteins between the wild-type and the mutants result from the loss of *N*-linked oligosaccharides from the glycoproteins.

Two approaches were taken to ascertain the latter possibility. Treatment with endoglycosidase H was used to remove *N*-linked oligosaccharide chains from [^{3}H]palmitate-labeled glycoproteins. As shown in fig.2A, treatment of [^{3}H]palmitate-labeled proteins from the wild-type cells led to the appearance of four [^{3}H]palmitate-labeled polypeptides with apparent molecular weight of 169 000, 46 000, 38 000, and 16 600, respectively. These polypeptides might correspond to all but one of the [^{3}H]palmitate-labeled polypeptides seen in *alg1* and *alg2* mutants (fig.1A). The minor bands unaffected by endoglycosidase H treatment may be non-glycosylated proteins.

The effect of tunicamycin on the labeling pattern of [^{3}H]palmitate in the wild-type cells was studied. As shown in fig.2B, five [^{3}H]palmitate-labeled polypeptides were observed with apparent molecular weights of 151 000, 126 000, 43 000,

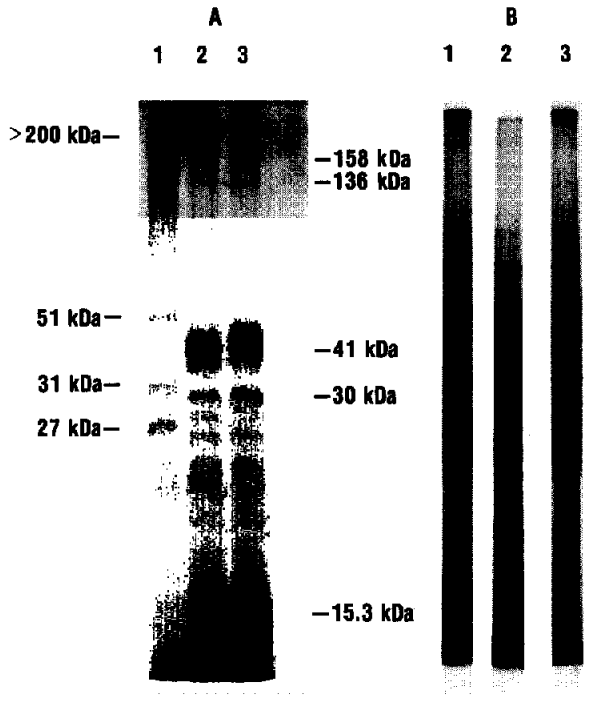

Fig.1. SDS-gel electrophoresis of fatty acylated proteins from the wild-type and mutant yeast cells. Crude extracts from cells labeled with [^{3}H]palmitate (panel A) or with [^{35}S]methionine (panel B) at 37°C were examined by SDS-gel electrophoresis. (A) [^{3}H]Palmitate-labeled cell extracts. Lane 1, wild-type yeast cells, strain DBY 640 (150 μg, 3 × 10^6 dpm); lane 2, *alg1* mutant cells (150 μg, 5 × 10^6 dpm); lane 3, *alg2* mutant cells (150 μg, 5 × 10^6 dpm). (B) [^{35}S]Methionine-labeled cell extracts from the wild-type DBY 640 (lane 1), *alg1* mutant (lane 2) and *alg2* mutant (lane 3). For [^{3}H]palmitate-labeled samples (panel A), fluorography with 'ENHANCE' was used, and the gels were exposed to Kodak XAR-5 film for 14 days at −70°C before developing. Gels containing [^{35}S]methionine-labeled samples were fixed and exposed to Kodak XAR-5 film for 24 h at −70°C before developing.

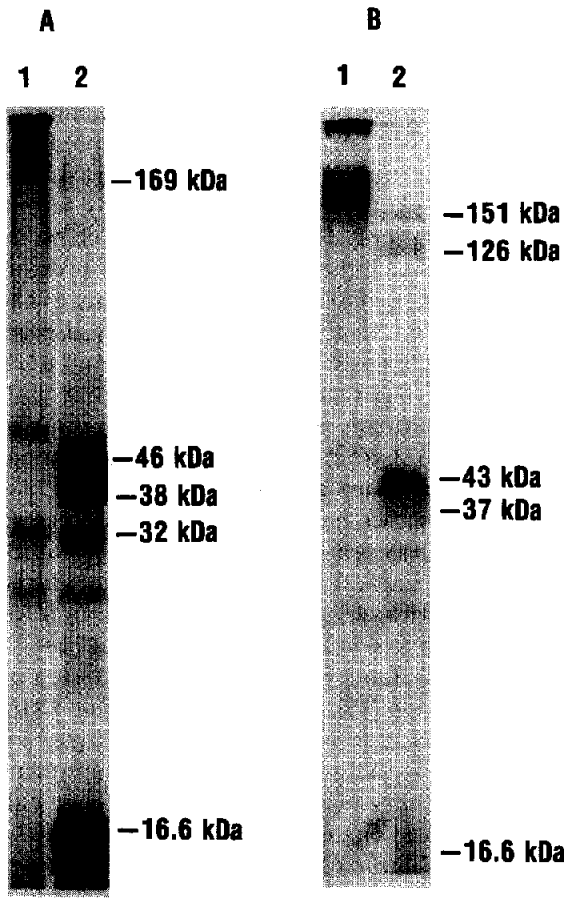

Fig.2. Effects of tunicamycin or endoglycosidase H treatment on the apparent mobilities of fatty acylated proteins from wild-type yeast cell extract. Crude cellular extracts from wild-type DBY 640 yeast cells labeled at 37°C with [^{3}H]palmitate were examined by SDS-gel electrophoresis. (A) Lanes: 1, untreated sample containing 1.2 × 10^6 dpm; 2, endoglycosidase H-treated sample containing 1.2 × 10^6 dpm. (B) Wild-type yeast cells labeled with [^{3}H]palmitate in the absence (lane 1) or presence (lane 2) of tunicamycin (5 μg/ml). 180 μg labeled proteins were applied to each lane.

37 000 and 16 600, respectively. These five fatty acylated polypeptides corresponded very well in apparent molecular weights with those seen in *alg1* and *alg2* mutants labeled at the non-permissive temperature in the absence of tunicamycin. Significantly, the relative intensities of [^{3}H]palmitate-labeled polypeptides in tunicamycin-treated wild-type cells and those following endoglycosidase H treatment of [^{3}H]palmitate-labeled wild-type cells are similar to those seen in *alg1* and *alg2* mutant cells. These results taken together strongly suggest that these palmitate-labeled polypeptides are also *N*-glycosylated, and the difference in [^{3}H]palmitate-labeling profiles between the wild-type and mutant cells is due to a reduction in apparent molecular weights of these fatty acylated glycoproteins as a consequence of a shortening of the *N*-linked oligosaccharide chains. The observations that the high molecular weight (>200 000) palmitate-labeled polypeptides in the wild-type cells disappeared following endoglycosidase H treatment or when the labeling was done in the presence of tunicamycin support this conclusion.

4. DISCUSSION

Glycosylation and fatty acylation are covalent modifications of proteins which are biochemically distinct processes. A temporal and/or topological coincidence, if not interdependence, may exist between these two processes as suggested by several recent observations. Thus results of pulse-chase experiments indicate that palmitoylation of proteins takes place in the Golgi region where many of the maturation steps in glycosylation pathway also occur [13]. Acyl transferase activity was found to be enriched in Golgi-rich microsomal fractions [14]. Many glycoproteins and fatty acylated proteins also appear to share a common subcellular destination, e.g. the plasma membrane.

Previous studies have suggested an unexpected relationship between fatty acylation of glycoproteins and its intracellular trafficking through the secretory pathway. Increased incorporation of [^{3}H]palmitate into yeast glycoproteins was observed in yeast mutants defective in an early step of protein secretion [15]. We have reported that CHO mutants deficient in *N*-glycosylation contained increased palmitoylation of membrane glycoproteins [9]. These results suggest that glycoproteins with altered *N*-linked oligosaccharide structures may be over-acylated due to either conformational changes in the proteins or an increased exposure to the acylating enzymes as a result of defective intracellular transport.

In the present study, we have employed yeast cells to investigate the relationship between a defective *N*-glycosylation and fatty acylation of proteins. Our results show that all the major palmitoylated proteins in *alg1* and *alg2* mutants can be generated in the wild-type cells if the labeling is performed in the presence of tunicamycin. Likewise, they can be produced by endoglycosidase H treatment of wild-type cells labeled with palmitate in the absence of tunicamycin. Abolition of *N*-glycosylation does not result in an increase in fatty acylation of proteins otherwise unmodified in the wild-type cells under conditions of unperturbed glycosylation. Thus it would appear that in yeast cells, glycosylation is not a prerequisite for proteins to enter the compartment where fatty acylation takes place, nor does the presence or absence of glycosylation affect the extent of fatty acylation. This conclusion is consistent with the observation of Slomiani et al. that acylation of rat gastric mucus glycoproteins occurs at the protease-susceptible non-glycosylated region of the polypeptide, away from the protease-resistant glycosylation sites [14].

The change in apparent molecular weights of almost all of the palmitate-labeled proteins following endoglycosidase H treatment or with labeling carried out in the presence of tunicamycin indicates that palmitoylation in yeast cells is limited largely to glycoproteins. This selective palmitoylation of glycoproteins may be merely a reflection of a common subcellular localization of two distinct and independent modification reactions. Yeast cells may differ in this respect from other eukaryotic cells in which many palmitoylated proteins are not glycoproteins [3].

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