THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 267, No. 6, Issue of February 25, pp. 3922-3931, 1992
Printed in U.S.A.

Myristoylation of Proteins in the Yeast Secretory Pathway*

(Received for publication, April 26, 1991)

Sanford M. Simon‡ and Alan Aderem§¶

From the ‡Howard Hughes Medical Institute, Laboratory of Cell Biology, and the §Laboratory of Signal Transduction, The Rockefeller University, New York, New York 10021-6399

Protein myristoylation was investigated in the yeast secretory pathway. Conditional secretory mutations were used to accumulate intemediaries in the pathway between the endoplasmic reticulum and Golgi (sec 18, 20), within the Golgi (*sec* 7), and between the Golgi and plasma membrane (*sec* 1, 3, 4, 5, 6, 8, 9). The accumulation of vesicles was paralled by the enrichment of a defined subset of proteins modified either via ester or amide linkages to myristic acid: Myristoylated proteins of 21, 32, 49, 56, 75, and 136 kDa were enriched between the endoplasmic reticulum and Golgi; proteins of 21, 32, 45, 56, 75, 136 kDa were enriched by blocks within the Golgi; and proteins of 18, 21, 32, 36, 49, 68, and 136 kDa were trapped in a myristoylated form by blocks between the Golgi and plasma membrane. This enrichment of myristoylated proteins was reversed upon returning the cells to the permissive temperature for secretion. The fatty acid was linked to the 21-kDa protein via a hydroxylamine-resistant amide linkage (*N*-myristoylation) and to the proteins of 24, 32, 49, 56, 68, 136 kDa via hydroxylamine-labile ester linkage (*E*-myristoylation). In addition, myristoylated proteins of 21, 56, and 136 kDa were glycosylated via amino linkages to asparagine. This suggests they are exposed to the lumen of the secretory pathway. Three proteins (24, 32, and 56) were *E*-myristoylated in the presence of protein synthesis inhibitors, indicating this modification can occur posttranslationally. After using cycloheximide to clear protein passengers from the secretory pathway the 21-, 32-, and 56-kDa proteins continued to accumulate in a myristoylated form when vesicular transport was blocked between the Golgi and plasma membrane. These data suggest that myristoylation occurs on a component of the secretory machinery rather than on a passenger protein.

The intracellular transport of vesicles plays an essential role in subcellular compartmentalization (1). Some of the biochemical machinery of vesicular transport has recently been identified using *in vitro* reconstitutions (2) and genetics (3, 4). However, little mechanistic detail is known. Results from two independent systems (viral membrane fusion and *in vitro* reconstitutions of vesicular transport) suggest that fatty acid acylation of proteins plays a role in vesicular transport and membrane-to-membrane fusion. Specifically, the influenza hemagglutinin protein loses its ability to induce membrane-to-membrane fusion when it is deacylated chemically with hydroxylamine or through genetic mutations (5–7). Further, Rothman and colleagues (8–10) have shown that acyl-CoA is required for the *in vitro* reconstitution of vesicular transport through the stacks of the Golgi apparatus (8–10). It has not been known, though, if the fatty acid acylation of protein is required *in situ* either for vesicular transport or membrane-membrane fusion. We have addressed this question by examining the fatty acid acylation of proteins during the formation of transport vesicles in yeast. *Saccharomyces* was selected because mutants can be used to conditionally block different loci in the secretory pathway (11), resulting in the accumulation and, thus, selective enrichment of specific populations of vesicles and their proteins.

Three forms of fatty acid modification of proteins have been previously described: a carboxyl-terminal phosphodiester bond to a phosphatidyl inositol-containing glycan (12); an amide bond of myristic acid to an amino-terminal glycine (*N*-myristoylation) (13); and an ester linkage (14) of palmitic acid to cysteine or, occasionally, to serine or threonine. While some forms of palmitoylation have been shown to be reversible, *N*-myristoylation is believed to be irreversible and obligatorily cotranslational (15). [Throughout this paper the term myristoylation is used for all covalent linkages of myristic acid to protein. This includes both myristic acid amide linked to amino-terminal glycines (*N*-myristoylation) and myristic acid associated with protein via hydroxylamine sensitive linkages (*E*-myristoylation)].

In yeast the palmitoylation of some proteins has been described (14, 16) and the *N*-myristoyl transferase has been characterized, purified (13), cloned, sequenced, and demonstrated to be essential (17). However, the myristoylation of yeast proteins has not been fully described. The recent identification of numerous yeast homologues for mammalian proteins acylated with fatty acids, such as the small G-proteins, provides further impetus for a description of myristoylation in yeast. To establish benchmarks by which fatty acid acylation of the secretory machinery could be studied, this paper characterizes some of the basic phenomenology of myristoylation in yeast. (i) Myristic acid can be incorporated into proteins via either a hydroxylamine-resistant amide bond or a hydroxylamine-labile ester bond. (ii) *E*-Myristoylation of some proteins occurs in the presence of protein synthesis inhibitors, indicating this form of acylation can occur posttranslationally. (iii) A number of *E*-myristoylated proteins are also glycosylated via amino linkages to asparagine. (iv) Blocking secretory traffic with a conditional, reversible secretion mutant in yeast results in the selective enrichment of a subset of fatty acylated proteins.

Secretory mutants were used to accumulate intermediaries between the endoplasmic reticulum and Golgi apparatus (group I mutants), within the Golgi (group II), and between the Golgi and plasma membrane (group III). It was found that different mutations that block secretion at the same locus

* This work was supported in part by Grant AIRO12503 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Established investigator of the American Heart Association.

exhibited similar, though not identical, profiles of proteins bearing covalently bound myristic acid. Specific subsets of myristic acid-labeled proteins were enriched when secretion was blocked in group I, group II, and group III secretory mutants. Even after clearing the secretory pathway with protein synthesis inhibitors during subsequent accumulation of vesicles many of these proteins were enriched in a myristoylated form. These results suggest that each round of vesicular budding, targeting, and fusion is accompanied by a round of fatty acid acylation and deacylation of the secretory machinery.

MATERIALS AND METHODS

Chemicals—Cerulenin was from Calbiochem; endoglycosidase-H, Trasylol, phenylmethylsulfonyl fluoride, and L-1-tosylamido-2-phenylethylchloromethyl ketone were from Boehringer-Mannheim; [^{3}H] myristic acid, [^{3}H]palmitic acid, and En^{3}Hance were from Du Pont-New England Nuclear; yeast nitrogen base and Bacto-Peptone were from Difco. Trizma base and cycloheximide were from Sigma.

Yeast Strains—The wild-type *Saccharomyces cerevisae* strain X-2180 1A and the secretory mutants were obtained from the Yeast Genetic Stock Center, Berkeley, CA.

Growth Media—Yeast were grown in YPD$^{2\%}$ (1% yeast extract w/v, 2% Bacto-Peptone w/v, 2% dextrose w/v) or YPD$^{0.1\%}$ (same, except with 0.1% w/v dextrose).

Labeling with [^{3}H]Myristic Acid—Yeast were inoculated into YPD$^{2.0\%}$ at a 1:1000 dilution from a stationary stock and grown for 15 h to a density of 2×10^7 cells/ml at 25 °C in a rotary air shaker. Cells were harvested, and resuspended at a density of 2×10^8 cells/ml. Cerulenin (10 mg/ml in dimethyl sulfoxide) was added to a final concentration of 2.5 μg/ml. After 10 min [^{3}H]myristic acid (Du Pont-New England Nuclear) was added from a stock of 10 mCi/ml EtOH to a final concentration of 30 μCi/ml. Cells were incubated in the [^{3}H]myristic acid at either 25 or 37 °C in a rotary water bath for 1 h.

Cell Extracts—After 1 h of incubation in [^{3}H]myristic acid 4 ml of cells were added to 10 ml of ice-cold 10 mM NaN_3, and harvested in a Sorvall SA-6000 at 1500 cpm. The pellet was resuspended in 1 ml of ice-cold 10 mM sodium azide, harvested with a 30-s spin in a microcentrifuge (15k rpm) and resuspended in 200 μl of lysis buffer (10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 150 mM NaCl, 10% glycerol, 1% Triton, 1% sodium deoxycholate, 1% SDS)[1] with additional protease inhibitors (1% Trasylol, 1 mM phenylmethylsulfonyl fluoride, 0.1 mg/ml L-1-tosylamido-2-phenylethylchloromethyl ketone). Approximately 200 μl of glass beads (Thomas Scientific no. 5663 R50), rinsed with lysis buffer, were added and the cells were vortexed vigorously in the cold room for 4 min. The tubes were spun for 10 min in a tabletop microcentrifuge at maximum speed. The supernatant was removed and the protein concentration determined with a Bio-Rad dye binding assay.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis—All gels were made with gradients of 6–12% acrylamide at pH 9.2. Samples were resuspended in sample loading buffer, boiled for 3 min, and 50 μg of protein were loaded per lane. The gels were stained, destained, prepared for fluorography by soaking in EN^{3}HANCE (Du Pont-New England Nuclear) for 1 h, soaked in 3% glycerol for 30 min, dried, and exposed to preflashed Kodak X-Omat film at −80 °C for 10–15 days.

Scanning of Gels—All fluorographs were scanned with an LKB 2222-020 Ultrascan XL laser densitometer. The data was imported to an IBM-AT for analysis and plotting.

Characterization of [^{3}H]Myristate—Samples were run on preparative SDS-PAGE with prestained molecular weight markers at both ends of the gel. Prior to staining the gels were cut in 3-mm-wide horizontal strips. A 2-cm vertical column with the molecular weight standards and part of the sample was left intact and stained, destained, and prepared for fluorography. A 5-mm piece from each horizontal strip was homogenized and suspended in Aquasol, and the tritium bands were identified in a liquid scintillation counter. This identification was later confirmed by comparing the liquid scintillation counts of the horizontal strips with the fluorograms of the vertical column from the gel. The protein in the horizontal bands was extracted and analyzed with reversed phase HPLC for fatty acids as previously described (18). The strips were homogenized in 1 ml of 0.1% SDS; 0.05 M NH_4HCO_3; 5% (v/v) 2-mercaptoethanol. The samples were heated at 100 °C for 5 min and then incubated overnight in 0.2 mg/ml trypsin at 37 °C in a rotary water bath. The trypsin digested solution was filtered through a 0.22-μm millex filter. The filtered solution was acidified by the addition of 50 μl of 1 M formic acid. 1 ml of petroleum ether was added, and the solution was vortexed and then allowed to stand. The petroleum ether was removed. Few counts partitioned into the organic phase. The aqueous phase was lyophilized overnight and then resuspended in 83% (v/v) methanol and 17% (v/v) HCl (final concentration of HCl = 2 M). Each sample received 10 μl each of 20 mg/ml stocks of myristic acid and palmitic acid, and it was then heated in a Pierce low vacuum tube at 110 °C for 60 h at reduced pressure. After the samples cooled to room temperature the reaction solution was extracted with three 1-ml washes of petroleum ether. All of the ^{3}H counts partitioned in the organic phase. Samples were evaporated and then resuspended in 250 μl of methanol. Separation and identification of the petroleum ether-extractable radioactivity were achieved by reversed-phase HPLC using a 4.6-mm × 250-cm Beckman Ultrosphere ODS column eluted with 80% (v/v) acetonitrile, 0.1% trifluoroacetic acid, 0.02% triethylamine as the mobile phase at a flow rate of 1 ml/min. 1-ml fractions were collected and their radioactivities were measured in Hydrofluor (National Diagnostics, Somerville, NJ). The absorbance elution profile of the methyl ester and free fatty acid internal standards were used to identify radioactive methanolysis products.

Hydroxylamine Treatment—The myristic acid-protein bonds were tested for sensitivity to hydroxylamine either by separating the proteins on a gel and then soaking the gel in hydroxylamine or by first treating the cell extracts with hydroxylamine and then separating the proteins on a gel.

To pretreat the samples with hydroxylamine, 100 μg of cell extract protein was added to an equal volume of either 2 M Tris, pH 10, or freshly made 2 M hydroxylamine, pH 10. After 1 h at room temperature 100% trichloroacetic acid was added to a final concentration of 10%. The precipitate was washed with 70% EtOH (4 °C). The precipitated protein was resuspended in Neville gel-loading buffer to a concentration of 1 mg/ml. The proteins were run on SDS-PAGE as described above.

To treat the samples with hydroxylamine after gel electrophoresis, cell extracts were run in duplicate on SDS-PAGE as described above. The gel was stained, destained, washed for 10 min in H_2O, and then cut in half to separate the duplicated lanes. One half of the gel was soaked for 12 h in 1 M hydroxylamine, pH 10, with the other half in 1 M Tris, pH 10. The bathing solutions were replaced three times.

Endoglycosidase-H (Endo-H) Digestion—18 μl of cell extract was boiled with 2 μl of 10% SDS/0.5 M Tris HCl/0.5 M dithiothreitol for 2 min. After cooling 35 μl of 0.3 M sodium citrate, pH 5.5, 3 μl of protease inhibitors (20% Trasylol, 20 mM phenylmethylsulfonyl fluoride, 4 mM L-1-toxylamido-2-phenylethylchloromethyl ketone) and 3 μl of either 1 unit/ml Endo-H or H_2O were added. The samples were then incubated at 37 °C for 18 h after which they were resolved by SDS-PAGE and fluorography.

Secretion Assays—Cells were grown to a density of 2×10^7 cells/ml in YPD$^{2.0\%}$, harvested, and resuspended at a density of 2×10^8 cells/ml in YPD$^{0.1\%}$ to induce invertase production. At select times 200 μl samples of cells were added to 1.2 ml of ice-cold 10 mM NaN_3. The cells were harvested and assayed for secreted invertase as described by Goldstein and Lampen (19). The harvested cells were resuspended in 500 μl of ice-cold 0.1 M NaOAc pH 5.3, 10 mM NaN_3, 0.1 M sucrose and incubated at 37 °C for 10 min. The tubes were chilled and 0.5 ml of 0.5 M potassium phosphate buffer, pH 7.0, was added, and the tubes were then vortexed and boiled. After the tubes were cooled, the cells were pelleted in a microcentrifuge and 20 μl of the supernatent was added to 0.5 ml of glucose oxidase/peroxidase/potassium phosphate buffer, pH 7.0/*O*-dianisidine/glycerol and incubated at 30 °C for 30 min. The OD was read at 540 nM.

RESULTS

[^{3}H]Myristic Labeling in Wild-type and sec 18 Yeast—Wild-type yeast x-2180 were incubated with [^{3}H]myristic acid and cerulenin (which inhibits fatty acid biosynthesis, see below), and the incorporation of the fatty acid into proteins was examined by SDS-PAGE and fluorography (Fig. 1). [^{3}H] Myristic acid was incorporated into proteins of apparent

[1] The abbreviations used are: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; Endo-H, endonuclease-H.

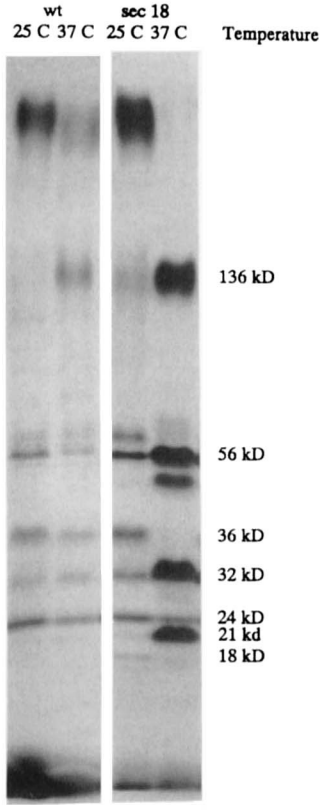

FIG. 1. **Labeling of yeast proteins with [^{3}H]myristic acid at 25 and 37 °C.** Wild-type yeast x-2180 or *sec* 18 were grown overnight in YPD to a density of 2×10^7 cells/ml. They were harvested and resuspended to a density of 1×10^8 cells/ml in YPD$^{0.1\%}$. [^{3}H]Myristic acid (Du Pont-New England Nuclear) was added from a stock of 10 mCi/ml EtOH to a final concentration of 30 μCi/ml, and the cells were incubated at either 25 or 37 °C. After 60 min of labeling, 3.75 ml of cells were added to 10 ml of ice-cold 10 mM NaN_3, harvested, washed with ice-cold 10 mM NaN_3, and resuspended in 200 μl of ice-cold lysis buffer in 1.5-ml tubes. Extracts of the cellular proteins were obtained by adding 200 μl of glass-beads and vortexing the cells vigorously for 4 min in the cold room. The tubes were spun in a microcentrifuge at 4 °C for 10 min to pellet unbroken cells. The supernatant was transferred to another tube and a Bio-Rad Coomassie Blue assay was used to determine protein concentration. 50 μg of protein was run in each lane of a 6–12% SDS-PAGE gel. The gels were stained, soaked in EN^{3}HANCE for fluorography, dried, and exposed to Kodak X-Omat film for 14 days.

molecular mass of 24, 32, 36, 56, 68, 75, 136, and 240 kDa. Labeling of proteins from wild-type yeast was similar at 25 and 37 °C, with the exception that, at 37 °C, the 136-kDa band increased and the 240-kDa band decreased.

Yeast secretory mutant *sec* 18 secretes normally at 25 °C but at 37 °C accumulates proteins in the endoplasmic reticulum (11). At the permissive temperature for secretion the pattern of myristoylation of proteins was indistinguishable from wild-type cells. However, at the restrictive temperature there were distinct differences in the pattern of protein acylation: proteins of apparent molecular mass 21, 32, 49, 56, and 136 kDa were more intensely labeled while those of 36, 68, and 240 kDa were not labeled at all (Fig. 1, *right*).

Effect of Cerulenin on [^{3}H]Myristic Acid Labeling—Previous incubations of yeast cells with ^{3}H-labeled fatty acids has resulted in the labeling of a limited number of proteins (14, 16). Cerulenin blocks fatty acid biosynthesis (20) and could potentially increase the specific activity of the [^{3}H]myristoyl CoA pool. We found that adding cerulenin increased the efficiency of incorporation of [^{3}H]palmitic acid or [^{3}H]myristic acid more than 10-fold without affecting which proteins were being labeled (data not shown). Thus, with cerulenin, we could study labeling after a 15-min incubation which is shorter than the cell cycle (90 min) and short enough to allow labeling in the presence of protein synthesis inhibitors (see below).

Effect of Cerulenin on Secretion—Because cerulenin was being used to increase the radiolabeling of proteins accumulated in secretory mutants it was important to ascertain if it affects the secretory pathway. Secretion was assayed by monitoring the extracellular activity of the secretory enzyme invertase which was induced by resuspending yeast in low glucose (0.1%). The presence of cerulenin (Fig. 2, *empty circles*) had no detectable effect on the kinetics or magnitude of invertase induction or secretion (*filled circles*, mock-treated cells).

To test for potential effects of cerulenin on the reversibility of the secretory mutants, secretion was studied in *sec* 18. The cells were induced to secrete invertase, but were kept at the restrictive temperature for secretion. After 1 h of accumulating invertase, cycloheximide (200 μg/ml) was added to block further protein synthesis (this level of cycloheximide blocks 99% of [^{35}S]methionine incorporation into trichloroacetic acid-precipitable counts, data not shown). The cells were returned to the permissive temperature for secretion in the presence or absence of cerulenin. Once again, there was no difference in the kinetics or the magnitude of the secretion of the accumulated invertase secretion between the control (Fig. 2, *filled triangles*) or cerulenin treated (*empty triangles*) cells. Therefore, the concentration of cerulenin (2.5 μg/ml) used to increase the sensitivity of [^{3}H]myristic acid labeling neither affected the functioning of the secretory pathway (Fig. 2, *circles*) nor the reversibility of the secretory mutant (*triangles*).

Cerulenin blocks growth in yeast in minimal media (21). However, the cerulenin-induced block of yeast growth is reversed by the addition of either 100 μg/ml myristic or palmitic acid (22). The results presented here demonstrate that cerulenin has no observable short term effects on the secretory system. Either cerulenin can block secretion only after long

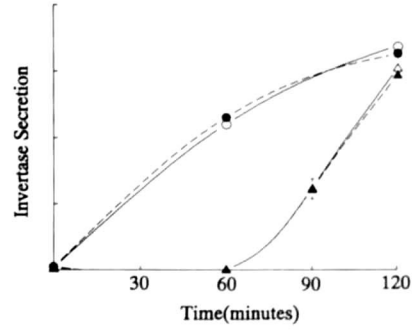

FIG. 2. **Cerulenin does not affect secretion of invertase.** *circles, sec* 18 were grown overnight in YPD to a density of 2×10^7 cells/ml. They were harvested and resuspended to a density of 1×10^8 cells/ml in YPD$^{0.1\%}$, to induce invertase production and incubated in a rotary bath at 25 °C. At 15, 30, 60, 90, and 120 min after resuspension 3.75 ml were harvested, washed, and external invertase was assayed by measuring the conversion of sucrose to glucose. The invertase activity was plotted as a function of time after resuspension in YPD$^{0.1\%}$ either in the presence (empty symbols) or absence (filled symbols) of 2.5 μg/ml cerulenin. *triangles*, after the cells were resuspended in YPD$^{0.1\%}$ to induce invertase production they were kept at 37 °C, the nonpermissive temperature for secretion, in the presence or absence of 2.5 μg/ml cerulenin. After 1 h they were returned to 25 °C, the permissive temperature. Samples were taken 0, 30, and 60 min after shifting the cells to 25 °C and assayed for invertase activity as described above. The *error bars*, shown when larger than the data symbol, represent the standard deviation of six samples.

incubations, or exogenous fatty acids in the media's yeast extract was sufficient to offset its effects.

Identification of the ^3H Label—Since cells can metabolize myristic acid it was important to determine if the tritium label was still associated with myristic acid. The ^3H-labeled bands in the gel were cut out, subjected to acid methanolysis, and the fatty acids separated by reversed-phase HPLC (18). Throughout the procedure, none of the counts partitioned into the aqueous phase, indicating that little of the ^3H label had been incorporated into amino acids. The elution profile of the ^3H label from the 21- and 32-kDa bands are plotted in Fig. 3. Approximately 85% of the ^3H label associated with the 21- and 32-kDa proteins eluted with the myristate or methyl myristate standards. The remaining 15% of the radiolabel from both protein bands comigrated with the palmitate or methyl palmitate standards. Similar results were obtained for the 24-, 36-, 49-, 56-, and 68-kDa bands (data not shown).

Characterization of Myristate-Protein Bonds—Fatty acid acylation of proteins occurs via hydroxylamine-labile ester bonds or hydroxylamine-resistant amino-terminal amide bonds (23). The fatty acid-protein bond on the 21-kDa protein was >95% resistant to hydroxylamine treatment at 25 °C, while at 37 °C it was 50% resistant. Both the shape of the ^3H scan and the fluorogram indicate that this increased hydroxylamine sensitivity at 37 °C is due to the induction of a second protein of slightly slower mobility (greater apparent molecular weight). In many of these 6–12% gradient gels it was difficult to distinguish the bands we refer to as 21 and 24 kDa at 37 °C. When the [^3H]myristoylated form of the 21-kDa band is enriched at the restrictive temperature it sometimes obscures the 24-kDa band. When the samples harvested at 25 and 37 °C are run side-by-side the two bands can be distinguished as seen in Fig. 1 and 3. The 32-, 36-, 49-, 56-, 68-, 136-, and 240-kDa proteins were linked to fatty acid via hydroxylamine labile bonds (Fig. 4, *A–C*). Indistinguishable results were obtained when samples were run in parallel lanes on a gel,

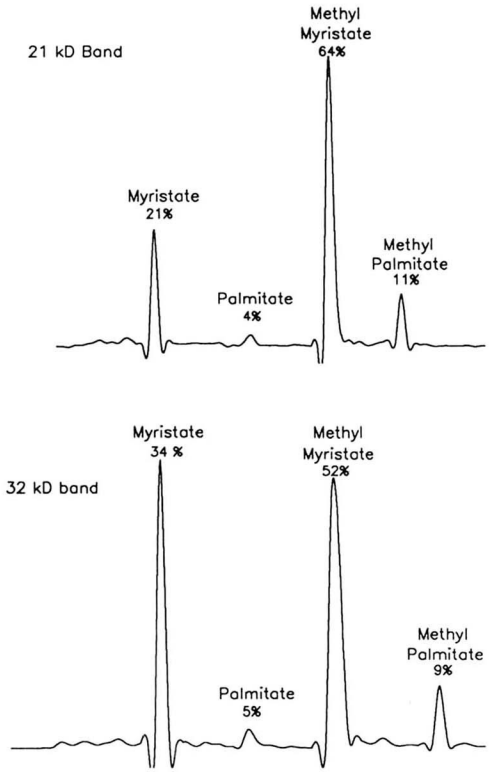

FIG. 3. **^3H label is myristic acid.** Cells were labeled with [^3H] myristic acid as in Fig. 1. 500 μg of cell extracts was run on preparative SDS-PAGE. The ^3H bands were cut out and the protein extracted from the gel. After digesting the protein with trypsin and acid methanolysis to release the label, none of the counts partitioned into the aqueous phase. (This suggests that none of the ^3H label was reincorporated into amino acids.) The nonpolar phase was separated on reversed-phase HPLC (18). The elutant was collected in 80 fractions which were counted for tritium. Superimposed on the tritium profiles are the locations of the absorbance peaks for the palmitic acid, methyl-palmitate, myristic acid, and methyl-myristate standards which were run with the samples. The *upper tracing* shows the ^3H profile for the hydroxylamine-resistant 21-kDa band, while the *lower tracing* is the hydroxylamine-sensitive 32-kDa band. With both samples 85% of the tritium label migrated with methyl myristate or myristic acid.

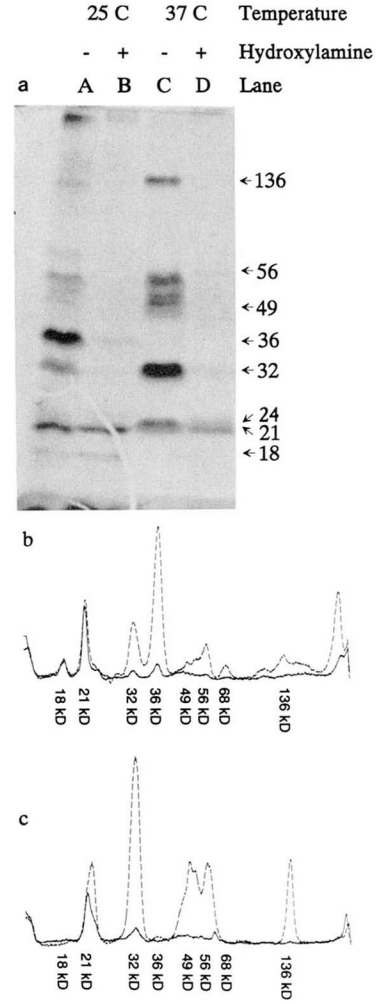

FIG. 4. **Sensitivity of the [^3H]myristic acid-protein bonds to hydroxylamine.** *sec* 18 were labeled as in Fig. 1 with [^3H]myristic acid at 25 °C, the permissive temperature for secretion, and 37 °C, the restrictive temperature for secretion. Cell extracts were incubated either with 1 M Tris or 1 M hydroxylamine, pH 10, for 1 h at room temperature. The samples were precipitated with 10% trichloroacetic acid, resuspended in gel loading buffer, and the proteins separated on SDS-PAGE and prepared for fluorography as in Fig. 1. *a*, fluorogram of [^3H]myristoylated proteins from *sec* 18 cell extracts grown at 25 °C (*lanes A* and *B*) or 37 °C (*lanes C* and *D*). Prior to running the proteins on the gel the samples in *lanes A* and *C* were soaked in 1 M Tris and the samples in *lanes B* and *D* were incubated in 1 M hydroxylamine, pH 10. Scans of the fluorogram of gels of *sec* 18. *b*, scans of proteins labeled at the permissive temperature from *lanes A* and *B*. The scans of the Tris-HCl-treated lanes are plotted as *dashed lines*, and the hydroxylamine treated lanes are plotted as *solid lines*. *c*, scans of proteins at the restrictive temperature from *lanes C* and *D*. The scans of the Tris-HCl-treated lanes are plotted as *dashed lines* and the hydroxylamine-treated lanes are plotted as *solid lines*.

and then half of the gel was incubated in hydroxylamine for 10 h.

Some of the Acylated Proteins are N-Glycosylated—If a protein is *N*-linked glycosylated then it must at least partially face the lumen of the secretory pathway (24, 25). To further characterize the [^{3}H]myristoylated bands the cell extracts from *sec* 18 were digested with endoglycosidase H, which specifically cleaves off sugar groups *N*-linked to asparagines (Fig. 5). Endo-H digestion resulted in the complete shift in molecular mass of the 21-, 56-, and 136-kDa proteins indicating that they must, at least partially, face the lumen of the secretory pathway.

Myristoylation of Yeast Secretory Mutants—The temperature-sensitive mutations used in this work can be divided into three groups based on where they block within the secretory pathway. Group I mutants block between the endoplasmic reticulum and the Golgi (*sec* 18, 20). Group II block within the Golgi (*sec* 7), and group III block secretion between the Golgi and the plasma membrane (*sec* 1, 3, 4, 5, 6, 8, 9). The data is depicted in Fig. 6, *a* and *b*, and summarized in Table I. Briefly, blocking the transport at any point in the secretory pathway resulted in an increased myristoylation of a 21-kDa band. The fatty acid linkage to this band at the permissive temperature is hydroxylamine-resistant, suggesting that it is bound via an amide linkage (*N*-myristoylation). The increased labeling obtained at the nonpermissive temperature is hydroxylamine-sensitive, suggesting that it is bound via an ester bond (*E*-myristoylation). The profile of myristic acid labeling depended on the location of the secretory block. For example, blocking secretion in group I mutants (*sec* 18, 20) resulted in the additional enrichment of myristoylated proteins of apparent molecular mass of 32, 49, 56, and 136 kDa (Fig. 6, *a* and *b*, and Table I). The myristic acid protein bond was hydroxylamine labile in all four cases, suggesting an ester linkage (Table I). The 36- and 68-kDa bands seen in the wild-type and in the mutants at the permissive temperature were no longer [^{3}H]myristate labeled (Fig. 6, *a* and *b*, and Table I). When transport was blocked within the Golgi using a group II mutant *sec* 7 there was an increased [^{3}H]myristate labeling of 21-, 32-, 56-, and 136-kDa bands as seen with group I mutants. However, there was no change in the labeling of a 36-kDa band (which decreased in group I). There was also a decreased labeling of a 24-kDa band (which did not change in group I) and an increased labeling of a 45-kDa band (which was not labeled in group I) (Fig. 6, *a* and *b*, and Table I). Similar data were obtained using *sec* 14 which also blocks within the Golgi (data not shown).

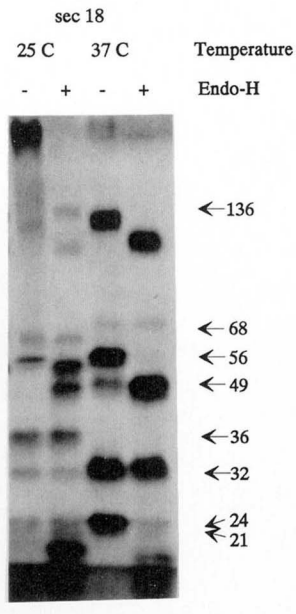

FIG. 5. **Some of the myristoylated proteins are *N*-linked glycosylated.** Effects of Endo-H digestion on total cell extracts from *sec* 18. Cell extracts were made, as described in Fig. 1, from *sec* 18 cells labeled with [^{3}H]myristic acid at 25 and 37 °C. The extracts were incubated 18 h at 37 °C in citrate buffer either with 1 μl of ddH_2O (−) or 1 μl of Endo-H, 1 unit/ml (+) and then subjected to SDS-PAGE and fluorography.

Transport is blocked between the Golgi and the plasma membrane in group III mutants. The proteins labeled with [^{3}H]myristic acid in these mutants at the permissive and restrictive temperature are shown for *sec* 1, 3, 4, 5, 6, 8, and 9 (Fig. 6, *a* and *b*). There was increased labeling of a 21-kDa band at the restrictive temperature in all the mutants (as seen with group I and II mutants). The labeling of a 36-kDa band was also substantially increased. This band was not labeled at the restrictive temperature in group I and did not change in group II mutants. In group III mutants there was a consistent decrease in labeling of the 56-kDa band whose labeling increased both in group I and II mutants (except *sec* 1). A 68-kDa band was more strongly labeled when transport was blocked after the Golgi. This band was not labeled in yeast with secretion blocks prior to the Golgi and its labeling was slightly increased when secretion was blocked within the Golgi. The labeling of the 136-kDa band increased substantially in all secretion mutants. However, it was also increased slightly when wild-type yeast were shifted to 37 °C.

Differences in Acylation Patterns within Each Group of Secretory Mutants—All secretory mutants within a single group did not yield identical patterns of myristoylation. Blocks in group III (between the Golgi and plasma membrane) resulted in an increased acylation of a 32-kDa band in *sec* 1 and 6 and had no effect on myristoylation in *sec* 3, 4, 8 and 9 (Fig. 6). Likewise, *sec* 1 and *sec* 6 yielded an increased [^{3}H] myristic acid labeling of a 24-kDa band at the restrictive temperature. Yet secretory blocks in *sec* 3, 5, 8, and 9 (post-Golgi), as well as *sec* 7 (within the Golgi), and *sec* 20 (before the Golgi) all decreased the labeling of the 24-kDa band. Several other small differences can be seen in Fig. 6. For example, in *sec* 4 a protein of approximately 43 kDa is acylated at the restrictive temperature but is not labeled in any other mutant.

Ten *sec* mutants have been identified that all block traffic between the Golgi and the plasma membrane. Some differences between the mutations have already been reported. While all block at 37 °C, some do so within minutes of the temperature shift, others take hours to do so. After returning the temperature to 25 °C, some of the mutants secrete all the proteins that accumulated during the block while others secrete only newly synthesized proteins. The small differences in protein acylation observed within group III mutants do not necessarily correlate with the phenotypic differences such as reversibility but they do further characterize these mutants.

Chase of [^{3}H]Myristic Acid Labeling after Reversing Block of the Secretory Pathway—Blocking secretory traffic results in a significant change in the profile of [^{3}H]myristoylated proteins (Fig. 6). It is possible that these changes are the result of indirect effects of the secretory block. We therefore compared the time course with which *sec* 18 at the restrictive temperature reverses its profile of protein myristoylation to the wild-type profile with the time course of reversal of the secretory phenotype. *sec* 18 was labeled with [^{3}H]myristic acid at 37 °C for 50 min (Fig. 7). Protein synthesis was subsequently inhibited with 200 μg/ml cycloheximide, and the cells were washed with an excess of cold myristic acid. After 10

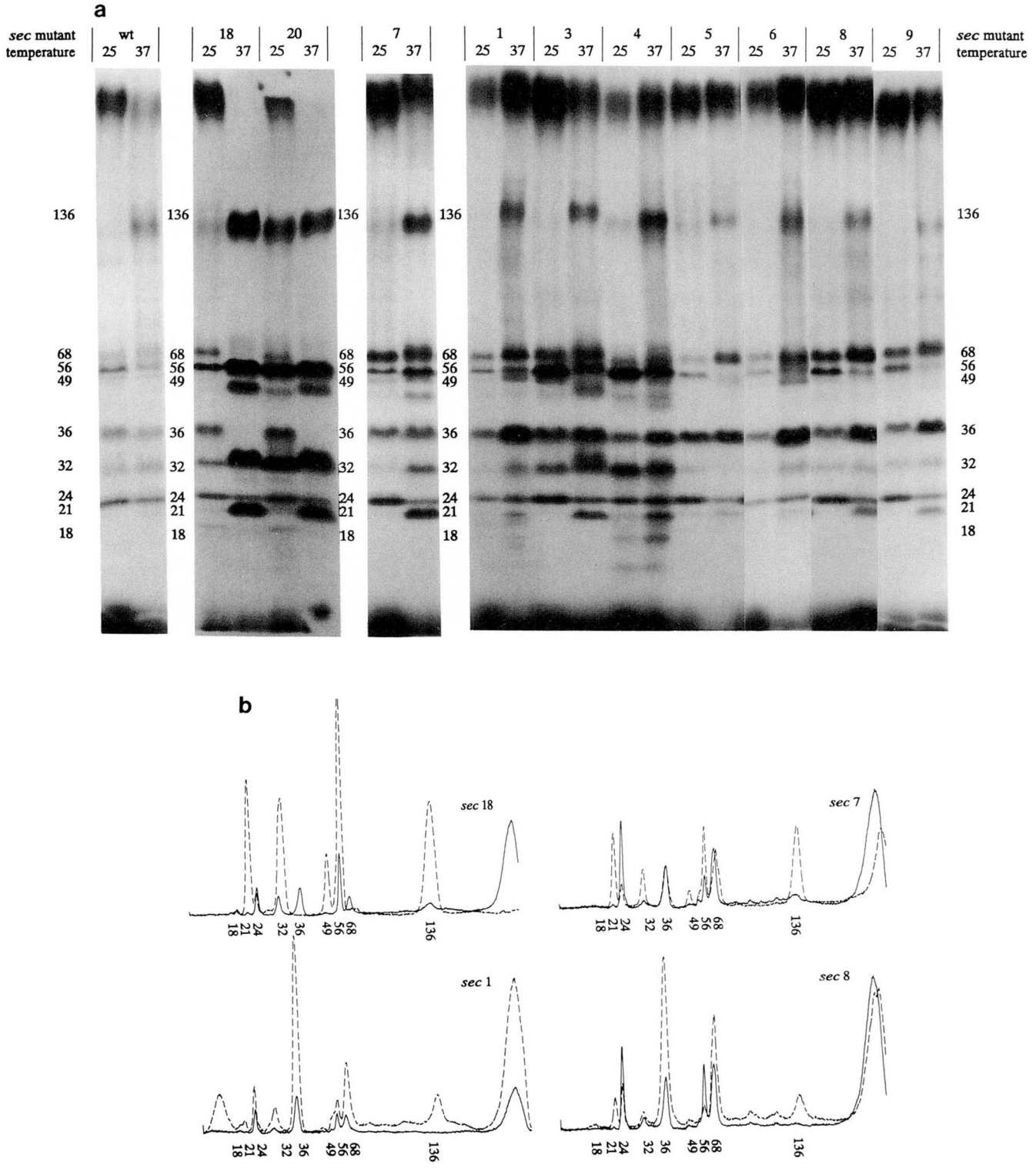

FIG. 6. *a*, [^{3}H]myristic acid labeling of proteins in yeast secretory mutants. The yeast secretory mutants were grown overnight at the permissive temperature, 25 °C, in YPD to a density of 2×10^7, then harvested and resuspended at a density of 1×10^8 in YPD$^{0.1\%}$. Four μl of 10 mg/ml cerulenin in dimethyl sulfoxide was added to each 15-ml culture (final concentration, 2.7 μg/ml). After 10 min [^{3}H]myristic acid (Du Pont-New England Nuclear) was added from a stock of 10 mCi/ml EtOH to a final concentration of 30 μCi/ml, and the cells were incubated in a rotary water bath at either 25 °C (the permissive temperature for secretion) or 37 °C (the restrictive temperature for secretion). After 60 min the cells were harvested and prepared for SDS-PAGE and fluorography as described in Fig. 1. *b*, scans of the fluorograms of [^{3}H]myristic acid labeling of the secretory mutants. Scans of the fluorograms in Fig. 6*a* for *sec* 1, 7, 8, and 18 are shown for the permissive temperature (*solid line*) and the restrictive temperature (*dashed line*).

TABLE I

Effect of secretory block on [^{3}H]myristoylated proteins

Each of the [^{3}H]myristic acid-labeled bands is characterized by (i) apparent molecular weight (kDa), (ii) whether the intensity of label increases or decreases during secretory block, (iii) whether the band is sensitive or resistant to digestion by Endo-H, (iv) whether the myristic acid-protein bond is hydroxylamine sensitive, (v) whether the [^{3}H]myristic acid labeling is blocked in presence of cycloheximide (S) or continues in its presence (R). The band 21a has a slightly faster mobility than the 21b band.

M_r	Group I				Group II	Group III			
	Label during block	Endo-H	Hydroxyl	Cyclohex	Label during block	Label during block	Endo-H	Hydroxyl	Cyclohex
14						Increase			
18	Decrease		R[a]	S	Decrease	Increase		R	
21a	Decrease	R	R	R		No change	R	R	S
21b	Increase	S	S	S	Increase	Increase	R	S	R
24	No change	S			Decrease	No change	R	S	S
32	Increase	R	S		Increase	Increase	S	S	R
33						Increase			
36	Decrease	R			No change	Increase	R	S	S
45					Increase				
49	Increase	R	S			Increase		S	
56	Increase	S	S		Increase	Decrease	R		R
60						Decrease			
68	Decrease	R			No change	Increase	R	S	S
75	Increase				Increase				
136	Increase	S	S		Increase	Increase	S	S	S

[a] R, resistant; S, sensitive.

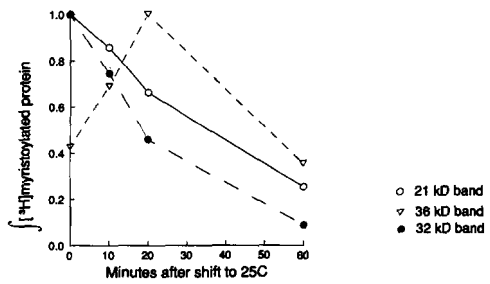

FIG. 7. **Protein acylation pattern reverses when cells are shifted from the restrictive to permissive temperature.** Cells were labeled with [^{3}H]myristic acid for 50 min at 37 °C as described in Fig. 1, and then 200 μg/ml of cycloheximide was added. After an additional 10 min (t = 60) half the cells were put at 25 °C and half were kept at 37 °C. At t = 60 (just before the temperature shift), t = 70 (10 minutes after), t = 20, and t = 60 aliquots of cells were taken. Cell extracts were made of each sample and the proteins were separated on SDS-PAGE and prepared for fluorography as described in Fig. 1. The [^{3}H]myristic acid labeling of the 21-kDa (*open circle*), 32-kDa (*closed circle*), and 36-kDa (*triangle*) proteins are shown.

min (t = 60 min) half the cells were put at 25 °C and samples were taken at 0, 10, 20, and 60 min after the temperature shift. Fluorograms of the gels of the cell extracts show that at 37 °C there was little change in the pattern of myristoylation (data not shown). However, at 25° C there was a rapid decrease of the 21-, 32-, (Fig. 7), and 56-kDa (data not shown) bands which specifically accumulate with secretory block. Simultaneously, bands appeared at 36 (Fig. 7) and 68 kDa (data not shown), both of which are seen in the wild-type phenotype. The band at 36-kDa only appeared transiently with a peak between 10 and 20 min. The time course of reversal of the profile of myristoylation to the wild-type phenotype correlates well with the time course of reversal of the *sec* phenotype in *sec* 18 (Fig. 2, *triangles*).

E-Myristoylation Can Occur Posttranslationally—To test if myristoylation in yeast could occur in the absence of protein synthesis, but while the secretory system was still carrying proteins, newly synthesized proteins were accumulated in the endoplasmic reticulum, or early post-endoplasmic reticulum, by keeping *sec* 18 cells at the restrictive temperature. After 40 min, 200 μg/ml of cycloheximide (which inhibits 99% of protein synthesis within 3 min) was added. After an additional 10 min, [^{3}H]myristic acid was added for a 10-min incubation. In the absence of cycloheximide four major bands were labeled with [^{3}H]myristic acid during the 10-min incubation: 21, 32, 56, and 136 kDa (Fig. 8*a*). The proteins at 32 and 56 kDa were labeled with [^{3}H]myristic acid to a similar extent in the presence of cycloheximide. Cycloheximide reduced the labeling of the protein at 21 kDa to 40% of the control and blocked the labeling of the 136-kDa protein (Fig. 8*a*). At 25 °C 100% of the 21-kDa protein is hydroxylamine insensitive. At 37 °C there is an increase of labeling which is hydroxylamine-sensitive, and thus presumably on a different site or different protein. The cycloheximide insensitive label that is observed at 37 °C is apparently on this same hydroxylamine-sensitive component.

The extent of labeling of the 32 and 56 kDa in the presence of cycloheximide implies either that the myristoylated proteins were hypoacylated, or that they can become hyperacylated, or that the protein-myristic acid linkage can turn over relatively rapidly. The decreased labeling of the 21- and 136-kDa proteins in the presence of cycloheximide could either indicate that their half-lives are shorter than the 10-min chase period or, alternatively, that they can only be labeled cotranslationally.

Effect of Cycloheximide on Vesicular Transport—We next attempted to distinguish whether the [^{3}H]myristic acid-labeled proteins were passengers in the secretory pathway or whether they constituted part of the secretory machinery. If the secretory pathway could be cleared of passenger proteins and if vesicular transport could be maintained, we could accumulate vesicles enriched in trafficking machinery. We first assayed how long it took to clear proteins from the secretory pathway during incubation with the protein synthesis inhibitor cycloheximide. Yeast were induced to produce and secrete invertase by incubation in a media of reduced (0.1%) dextrose (Fig. 8*b*). At various time points yeast were harvested and assayed for extracellular invertase activity (Fig. 8, *solid boxes*). When cycloheximide was added 30 min after

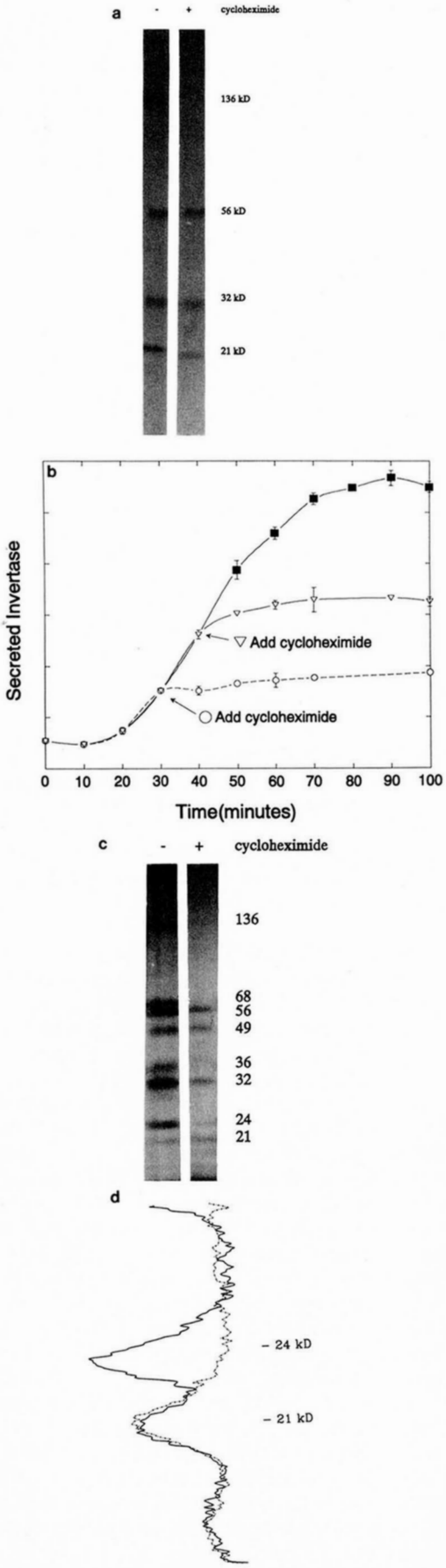

FIG. 8. *a*, effect of cycloheximide on [^{3}H]myristic acid labeling of proteins accumulated in secretory pathway. *sec* 18 was kept at 37 °C, the restrictive temperature for secretion, while newly synthesized proteins accumulated between the endoplasmic reticulum and the Golgi. After 40 min cycloheximide (200 μg/ml) was added to one half of the cells. After 10 additional min [^{3}H]myristic acid was added to both samples for a 10-min incubation. Cell extracts were made and run on SDS-PAGE and prepared for fluorography as described in Fig. 1. *b*, effect of cycloheximide on movement of proteins through secretory pathway. Yeast were grown overnight in YPD$^{5\%}$ to suppress all invertase production. After resuspension in YPD$^{0.1\%}$ (1×10^8 cells/ml) to induce invertase production, the cells were incubated in a rotary bath at 25 °C. After 30 (*empty circle*) or 40 (*empty triangle*) min cycloheximide (0.2 mg/ml) was added to cells. At various time points 0.2 ml of cells were added to 1.3 ml of ice-cold 10 mM sodium azide. These were harvested and assayed for invertase activity. The *error bars*, shown when *larger* than the *data symbol*, represent the standard deviation of four samples. *c*, effect of cycloheximide on [^{3}H]myristic acid labeling of *sec* 1. Cycloheximide (200 μg/ml) was added to *sec* 1 at a density of 2×10^7 cells/ml in YPD for 10 min at 25 °C. Next the cells were incubated at 37 °C for 15 min in the presence of [^{3}H]myristic acid and cycloheximide. The cells were then harvested and prepared for SDS-PAGE and fluorography as described in Fig. 1. *d*, effect of cycloheximide on [^{3}H]myristic acid labeling of *sec* 18.

the induction of invertase the appearance of invertase on the cell surface stopped in less than 5 min (Fig. 8*b*, *empty circles*). When cycloheximide was added during the maximal rate of invertase production (40 min after induction) it still took less than 5 min to clear the secretory pathway of all invertase (Fig. 8*b*, *empty triangles*). Similar results were found when yeast were assayed for phosphatase secretion (data not shown).

In the presence of cycloheximide the secretory pathway is cleared of passenger proteins within 5 min. However, under these conditions, transport of vesicles, devoid of their contents, continues. If a group III mutant is incubated at the restrictive temperature vesicles start accumulating in the bud until they fill the entire cell. This accumulation of vesicles is unaffected by 200 μg/ml cycloheximide.[2] Thus, by incubating cells in cycloheximide prior to placement at the restrictive temperature it should be possible to accumulate vesicles that are empty, or significantly depleted, of passenger proteins.

Effect of Cycloheximide on [^{3}H]Myristic Acid Labeling—The secretory pathway of a group III mutant (*sec* 1) was emptied of constituents by blocking protein synthesis with cycloheximide for 10 min. Next the cells were incubated at the restrictive temperature (to accumulate vesicles) for 15 min in the presence of [^{3}H]myristic acid and cycloheximide. The labeling was limited to 15 min because of concern about the cytotoxicity of prolonged incubation with cycloheximide. Cycloheximide blocked the enrichment of four acylated proteins (approximate molecular masses of 24, 36, 68, and 136 kDa) during vesicular accumulation (Fig. 8*c*). This does not distinguish whether these four proteins are passengers in the secretory pathway or whether they are part of the secretory machinery. However, enrichment of four myristoylated proteins (apparent molecular masses of 21, 32, 49, and 56 kDa) was either not affected, or only marginally reduced, by cycloheximide. These are not likely to be newly synthesized protein passengers in the secretory pathway and thus are candidates for components of the secretory machinery.

DISCUSSION

In recent years an increasing number of proteins have been recognized to have covalent bonds to fatty acids. Three different bonds between fatty acids and proteins have been described. One is an ester or thioester bond to palmitic acid which is synthesized posttranslationally (26, 27). A second is an amide linkage by myristic acid to an amino-terminal glycine which apparently occurs cotranslationally (26, 28). A

[2] S. M. Simon, manuscript in preparation.

third type of bond links the carboxyl-terminal of the protein to a phosphatidyl inositol containing glycan (12).

The results presented in this paper indicate that myristoylation of most yeast proteins resembles ester-linked acylation which is usually associated with palmitoylation: the myristate bonds are cleaved by hydroxylamine, suggesting they form an ester link (Fig. 4, *a* and *b*), and a number of the proteins can be myristoylated in the presence of protein synthesis inhibitors (Fig. 8, *a* and *c*). The similarity between palmitoylation and the myristoylation observed here is not the result of elongation of myristic acid (14 carbons) to palmitic acid since the ^{3}H label associated with each protein was shown to be myristic acid. However, it is possible that the "palmitoyl transferases" are promiscuous and, in the absence of palmitate, use myristic acid. This is supported by the observation that [^{3}H]palmitic acid can label some proteins whose mobility on SDS-PAGE is similar to those labeled with [^{3}H]myristic acid (data not shown). However, until these proteins are purified it cannot be determined which fatty acid is bound to the protein *in situ*.

Three ways have been suggested by which acylation can play a role in intracellular protein targeting. One, that acylation increases the hydrophobicity of proteins thereby anchoring them directly to membranes (29–31) or via a receptor for myristoylated proteins (32). Two, the addition of a glycophospholipid membrane anchor acts as an apical targeting signal for secretory proteins in polarized epithelial cells (39). Three, fatty acid acylation may be involved with vesicular trafficking or fusion since acetyl-CoA is needed in the *in vitro* reconstitution of vesicular traffic in the Golgi (8).

The findings presented here suggest that fatty acid acylation of proteins has a role in *in situ* vesicular budding, targeting, and fusion: (i) when transport through the secretory pathway is blocked so that vesicles accumulate, there is a buildup of proteins labeled with [^{3}H]fatty acid; (ii) reversal of the block reverses the accumulation; and (iii) acylation of proteins continues as long as there is vesicular movement and is independent of whether there are protein passengers in the vesicles.

Intermediary organelles were accumulated using yeast mutants that block at different steps in the secretory pathway. Enriching these intermediaries resulted in increased [^{3}H]fatty acylated labeling of selected proteins. Different mutants that block at the same step and therefore accumulate the same intermediaries of secretory machinery and passengers had very similar patterns of myristoylation. For example, all group III (post-Golgi) mutants accumulated 36-, 68-, and 136-kDa myristoylated protein bands. The 36- and 68-kDa bands were not labeled when transport was blocked in group I (prior to the Golgi) or in group II (within the Golgi). A complete summary of these results is shown in Fig. 6 and Table I.

Similar results have been previously reported for incubation in the presence of [^{3}H]palmitic acid (14). When transport is blocked pre-Golgi (*sec* 18) a highly acylated protein is observed at 32 kDa and when transport is blocked post-Golgi (*sec* 1) a highly acylated band is observed at 36 kDa. It was suggested that the palmitoylation was on a 32-kDa protein whose molecular mass increased to 36 kDa after glycosylation in the Golgi (14). The 36-kDa protein described in our study is Endo-H-resistant suggesting that it is not glycosylated. However, an important question is whether the labeling occurs on machinery of the secretory pathway or on protein passengers *en route* to other destinations. To distinguish between these possibilities group III mutants were incubated in cycloheximide to block protein synthesis then shifted to the restrictive temperature in the continued presence of cycloheximide to accumulate secretory vesicles. There is significant [^{3}H]myristic acid labeling of the 21-, 32-, 49-, and 56-kDa (Fig. 8*a*) proteins even though the secretory pathway was substantially depleted or empty of secretory proteins. The continued accumulation of the labeled form of the 21-, 32-, and 49-kDa bands in the presence of cycloheximide suggests they are not passengers in the secretory system, but components of the vesicular machinery. In contrast, when vesicles were accumulated in the absence of cycloheximide three additional bands at 24, 36, and 68 kDa increased their labeling with [^{3}H]myristic acid suggesting that they may be secretory proteins. Returning the cells to the permissive temperature reverses the secretion block and returns the [^{3}H]myristic acid labeling to the wild-type phenotype. These results suggest that some of the [^{3}H]myristic acid labeling occurs on the proteins involved in the functioning of the secretory pathway. One possible explanation is that some protein components are acylated with a fatty acid during vesicular budding and then deacylated during vesicular targeting and fusion. If the block is prior to the deacylation step intermediaries are accumulated which are enriched in fatty-acylated vesicular proteins.

Rothman and colleagues (8) have demonstrated that acyl-CoA is needed to reconstitute the *in vitro* transport of vesicles from *cis* to *medial* Golgi. The work presented here is consistent with this observation and suggests that the fatty acid acylation of proteins also plays a role in the *in situ* intracellular transport of vesicles. There are at least two potential functions for acylation in the cycle of vesicular budding, targeting, and fusion which would require a round of fatty acid acylation and deacylation. Acylation may allow the transient attachment of trafficking machinery to the vesicle or may be necessary to prime the machinery to allow membrane-to-membrane fusion.

Membrane localization of a number of proteins is affected by fatty acid acylation (33). A number of small, 21–24 kDa, GTP-binding ras-like molecules which are involved in the secretory pathway, such as rab3A (34), YPT1 (35), and SEC4 (36), cycle between cytosolic and membrane-bound forms. Some have been shown to be acylated (16). Bourne (37) has suggested that binding of GTP to these small proteins may be involved in attaching these molecules to vesicles and that hydrolysis of GTP, upon vesicular targeting, would dissociate these molecules. In line with the observations of Rothman and colleagues (8), fatty acid acylation could transiently associate these proteins with a budding vesicle. Upon targeting, these proteins could be deacylated and returned to the cytosolic pool. One obvious advantage to a soluble targeting mechanism over an integral membrane protein is that it would preclude having to retrieve the address molecule from the plane of the target membrane once the vesicle fuses.

For some proteins myristoylation alone is necessary but not sufficient for membrane localization. Evidence exists for a specific membrane-receptor for myristoylated p60^{src} (32, 38). Vesicles may also have specific receptors for myristoylated proteins which are involved in targeting and transport. The specificity of targeting will be determined by the transient attachment of the proper myristoylated protein to the vesicle's receptor. This vesicular protein could be a generic addressing molecule receptor. After it became part of the target membrane it could be used for targeting vesicles to a subsequent step in the secretory pathway upon attachment of a new targeting molecule.

Alternatively, fatty acylation may be necessary to prime components of the fusion machinery. Acylation of some viral fusion proteins is necessary for their fusagenic ability. For

instance, hydroxylamine treatment of influenza viruses decreases hemagglutinin-mediated fusion (5, 6). Further, deletion of any of the three potential acylation sites severely inhibits, or abolishes, the ability of influenza virus hemagglutinin to induce membrane fusion (7). Thus, acylation of a fusion component of the vesicular trafficking machinery may be necessary to allow vesicular-target membrane interaction or fusion in a mechanism analogous to the influenza virus fusion. Either during or after fusion this complex would be deacylated of its fatty acid.

Acknowledgments—S. M. S. is grateful for the support of an Irma T. Hirschl-Monique Weill-Caulier Career Scientist Award and would like to thank Rachael D. Migler and Tohru Sato for technical assistance, Teresa Jones for comments on the manuscript, and Daniel Lew for many fruitful discussions that laid the foundations for this work.

REFERENCES

1. Palade, G. E. (1975) *Science* **189,** 347–358
2. Rothman, J. E., and Orci, L. (1990) *FASEB J.* **4,** 1460–1468
3. Novick, P., Ferro, S., and Schekman, R. (1981) *Cell* **25,** 461–469
4. Rothman, J. H., Yamashiro, C. T., Raymond, C. K., Kane, P. M., and Stevens, T. H. (1989) *J. Cell Biol.* **109,** 93–100
5. Lambrecht, B., and Schmidt, M. F. (1986) *FEBS. Lett.* **202,** 127–132
6. Schmidt, M. F., and Lambrecht, B. (1985) *J. Gen. Virol.* **66,** 2635–2647
7. Naeve, C. W., and Williams, D. (1990) *EMBO J.* **9,** 3857–3866
8. Glick, B. S., and Rothman, J. E. (1987) *Nature* **326,** 309–312
9. Pfanner, N., Orci, L., Glick, B. S., Amherdt, M., Arden, S. R., Malhotra, V., and Rothman, J. E. (1989) *Cell* **59,** 95–102
10. Pfanner, N., Glick, B. S., Arden, S. R., and Rothman, J. E. (1990) *J. Cell Biol.* **110,** 955–961
11. Novick, P., Field, C., and Schekman, R. (1980) *Cell* **21,** 205–215
12. Cross, G. A. M. (1987) *Cell* **48,** 179–181
13. Towler, D. A., Adams, S. P., Eubanks, S. R., Towery, D. S., Jackson Machelski, E., Glaser, L., and Gordon, J. I. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 2708–2712
14. Wen, D., and Schlesinger, M. J. (1984) *Mol. Cell Biol.* **4,** 688–694
15. Wilcox, C., Hu, J. S., and Olson, E. N. (1987) *Science* **238,** 1275–1278
16. Molenaar, C. M., Prange, R., and Gallwitz, D. (1988) *EMBO J.* **7,** 971–976
17. Duronio, R. J., Towler, D. A., Heuckeroth, R. O., and Gordon, J. I. (1989) *Science* **243,** 796–800
18. Aderem, A. A., Keum, M. M., Pure, E., and Cohn, Z. A. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 5817–5821
19. Goldstein, A., and Lampen, J. O. (1975) *Methods Enzymol.* **76,** 504–511
20. Omura, S. (1976) *Bacteriol. Rev.* **40,** 681–697
21. Ngsee, J. K., Hansen, W., Walter, P., and Smith, M. (1989) *Mol. Cell. Biol.* **9,** 3400–3410
22. Awaya, J., Ohno, T., Ohno, H., and Omura, S. (1975) *Biochim. Biophys. Acta* **409,** 267–273
23. Magee, A. I., Koyama, A. H., Malfer, C., Wen, D., and Schlesinger, M. J. (1984) *Biochim. Biophys. Acta* **798,** 156–166
24. Kukuruzinska, M. A., Bergh, M. L. E., and Jackson, B. J. (1987) *Annu. Rev. Biochem.* **56,** 915–944
25. Hirschberg, C. B., and Snider, M. D. (1980) *Annu. Rev. Biochem.* **56,** 63–87
26. Magee, A. I., and Courtneidge, S. A. (1985) *EMBO J.* **4,** 1137–1144
27. Olson, E. N., Towler, D. A., and Glaser, L. (1985) *J. Biol. Chem.* **260,** 3784–3790
28. Olson, E. N., and Spizz, G. (1986) *J. Biol. Chem.* **261,** 2458–2466
29. Cross, F. R., Garber, E. A. Pellman, D., and Hanafusa, H. (1984) *Mol. Cell. Biol.* **4,** 1834–1842
30. Pellman, D., Garber, E. A., Cross, F. R., and Hanafusa, H. (1985) *Nature* **314,** 374–377
31. Kamps, M. P., Buss, J. E., and Sefton, B. M. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 4625–4628
32. Resh, M. D. (1989) *Cell* **58,** 281–286
33. Towler, D. A., Gordon, J. I., Adams, S. P., and Glaser, L. (1988) *Annu. Rev. Biochem.* **57,** 69–99
34. Fischer von Mollard, G., Südhof, T. C., and Jahn, R. (1991) *Nature* **349,** 79–81
35. Zahraoui, A., Touchot, N., Chardin, P., and Tavitian, A. (1989) *J. Biol. Chem.* **264,** 12394–12401
36. Goud, B., Salminen, A., Walworth, N. C., and Novick, P. J. (1988) *Cell* **53,** 753–768
37. Bourne, H. R. (1988) *Cell* **53,** 669–671
38. Schultz, A. M., Henderson, L. E., Oroszlan, S., Garber, E. A., and Hanafusa, H. (1985) *Science* **227,** 427–429
39. Lisanti, M. P., Caras, I. W., Davitz, M A., Rodriguez-Boulan, E. (1989) *J. Cell Biol.* **109,** 2145–2156