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Palmitylation of Viral Membrane Glycoproteins Takes Place after Exit from the Endoplasmic Reticulum*

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Palmitylation of vesicular stomatitis virus G and Sindbis virus E1 glycoproteins has been studied in relation to the transport from the endoplasmic reticulum (ER) to the Golgi complex. Incubation of infected cells at 15 °C prevents the transport of newly synthesized membrane proteins from the ER to the Golgi (Saraste, J., and Kuismanen, E. (1984) Cell 38, 535–549). In these conditions, also palmitylation of G protein and of E1 glycoprotein is blocked. When the transport is restored by increasing the temperature, palmitylation occurs quickly and is followed by the complete trimming of peripheral mannose residues due to mannosidase I (a putative cis-Golgi function). Immunofluorescence analysis showed that the G glycoprotein accumulated at 15 °C in structures distinct from both ER and Golgi. These studies suggest that transport from the ER to the cis-Golgi involves intermediate compartments.

A number of viral and cell surface proteins contain palmitic acid residues, covalently linked by thioester bonds to cysteines (1). The palmitylation of surface proteins is a posttranslational event: it occurs about 20 min after protein synthesis and before glycoproteins acquire complex type carbohydrates (1). The exact intracellular location of palmitylation has not yet been defined. Cell fractionation studies of pulse-labeled cells have suggested that palmitylation occurs in the cis-Golgi cisternae or in the endoplasmic reticulum (ER)[1] (2–4). *N*-Linked carbohydrate structure analysis of a palmitylated chimeric membrane protein indicated an ER location of fatty acylation (5). In yeast cells, an ER location has been deduced from studies using secretory mutants (6). To extend these studies we have used incubations at low temperature (15 °C) in VSV ts045- and SV-infected cells. It has been shown previously that incubation at 15 °C prevents the entry of newly synthesized membrane and secretory proteins into the Golgi complex (7–9). The results we present in this paper show that palmitylation takes place between the site where membrane proteins accumulate at 15 °C and the site where mannosidase I activity is fully carried out. Moreover they reinforce the view that membrane proteins accumulated at 15 °C are in an intermediate location between ER and cis-Golgi.

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[1] The abbreviations used are: ER, endoplasmic reticulum; BHK, baby hamster kidney; CEF, chicken embryo fibroblasts; Endo D, endoglycosidase D; SV, Sindbis virus; VSV, vesicular stomatitis virus; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; MEM, minimal essential medium; FCS, fetal calf serum; CHO, Chinese hamster ovary; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

EXPERIMENTAL PROCEDURES

Materials

All culture reagents were supplied by BRL-Gibco, Kalsrhue, Federal Republic of Germany and all culture plasticware by Falcon Labware, Becton, Dickinson and Co., Heidelberg, FRG. Cycloheximide and SDS were obtained from BDH Chemicals Ltd., Poole, United Kingdom. Protein A-Sepharose CL-4B was from Pharmacia, Uppsala, Sweden. Endoglycosidase D was supplied from Boehringer, Mannheim, FRG. [^{35}S]Methionine (specific activity >1000 Ci/mmol) and [^{3}H]palmitic acid (specific activity 30 Ci/mmol) were obtained from Amersham, Braunschweig, FRG. Entensify was supplied by Du-Pont-New England Nuclear. Moviol was purchased from Thomas Scientific, Philadelphia, PA. Solid chemicals and liquid reagents were obtained from Sigma Chemical Gmbh, Munich, FRG and E. Merck, Darmstadt, FRG, respectively.

Cells and Viruses

VSV ts045 and SV HR strains were propagated on BHK cells and CEF, respectively (10, 11). Vero cells were grown in MEM/5% FCS/nonessential amino acids; CHO 15B cells in MEM/10% FCS; BHK cells in G-MEM/10% triptose phosphate broth/5% FCS; CEF cells in MEM/10% triptose phosphate broth/4% newborn calf serum/1% chicken serum. All cells were maintained in a 95% air, 5% CO_2 incubator.

Virus Infection

VSV ts045—Vero or CHO 15B cells growing in 35-mm or 6-cm Petri dishes or on glass coverslips were infected at a multiplicity of 50–100 plaque-forming units/cell for 1 h at 31 °C in phosphate-buffered saline containing Ca^{2+}, Mg^{2+}, and 1% FCS. After the incubation the medium was replaced with an appropriate volume of MEM containing one-sixth of normal $NaCHO_3$, 10 mM Hepes, pH 7.2, 1% FCS (infection medium). Infection was allowed to proceed in water bath at 39 °C as shown in Fig. 1.

SV—CEF cells growing in 6-cm Petri dishes were infected at a multiplicity of 50 plaque-forming units/cell as above, except that all manipulations were performed at 37 °C.

[^{35}S]Methionine Labeling

3 h post-infection the infection medium of CHO 15B cells was replaced with medium lacking methionine and preequilibrated at 39 °C. 3.5 h post-infection the cells were pulsed for 10 min with 100 μCi/ml [^{35}S]methionine, contained in infection medium lacking methionine. Cells were chased with infection medium preequilibrated at the different temperatures and containing a 10-fold excess of cold methionine and 10 μg/ml cycloheximide. The same procedure was adopted in the case of SV-infected CEF, except that the labeling was performed for 5 min 6 h post-infection.

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[^{3}H]Palmitic Acid Labeling

VSV ts045-infected Vero cells (3.5 h post-infection) and SV-infected CEF (6 h post-infection) were labeled with 200 μCi/ml [^{3}H] palmitic acid. Labeling was in infection medium preequilibrated at the different temperatures and containing cycloheximide but lacking FCS. Unless specified, VSV ts045-infected cells were labeled for 30 min at 39 or 31 °C and 60 min at 15 °C.

Immunoprecipitation and Endo D Treatment of G Protein

After the labeling, G protein was immunoprecipitated exactly as described (12); in the case of Endo D treatment, the washed Protein A-Sepharose beads were treated as specified in (13) except that $NaPO_4$ buffer (0.2 M), pH 6.4, and Endo D (1 unit/ml) were used.

Preparation of Cell Extracts from SV-infected CEF

Methanolic KOH Treatment—Infected CEF were lysed with 20 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1% SDS; total extracts were passed few times through a syringe needle and aliquots were analyzed on SDS-PAGE. In the case of methanolic KOH treatment, duplicate aliquots were incubated with 9 volumes of acetone for 1 h at −20 °C and the precipitated proteins collected by centrifugation. 100 μl of 0.2 M KOH in methanol or 0.2 M Tris/HCl, pH 7.4, was added to the dried pellets which were incubated for 30 min at room temperature. After an additional cycle of acetone precipitation, the pellets were treated for SDS-PAGE analysis.

SDS-PAGE—Proteins were resolved on linear 9% polyacrylamide gels after reduction and alkylation as described before (14). After the run, the gels were fluorographed by treatment with Entensify or 2,5-diphenyloxazole/dimethyl sulfoxide (15).

Indirect Immunofluorescence—Vero cells were grown on glass coverslips, infected with VSV ts045, and manipulated as shown in Fig. 1. Cells were fixed with 3% formaldehyde and labeled with either a monoclonal antibody directed against G protein, P5D4 (12), or an antibody directed against endogenous ER protein (16). The second antibodies (goat anti-mouse and goat anti-rabbit), kindly provided by Dr. T. Kreis (European Molecular Biology Laboratory), were affinity purified and conjugated to rhodamine or fluorescein. The fixed and labeled coverslips were mounted in Moviol and viewed by epifluorescence on a Zeiss axiophot photomicroscope. For confocal microscopy cells were fixed using a modification of the method of Berod *et al.* (17) as modified by Bacallao and Stelzer (18). The fixed and stained cells were mounted in 50% glycerol in phosphate-buffered saline for viewing in the EMBL confocal laser scanning microscope (19).

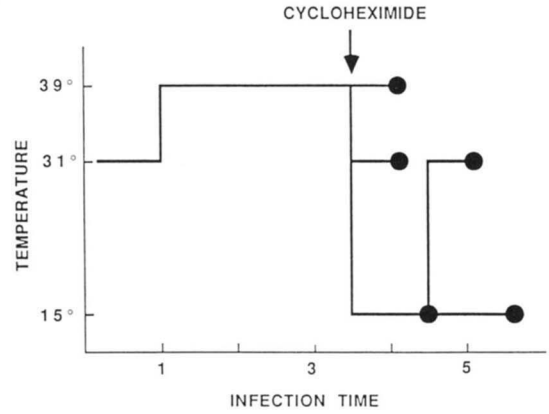

FIG. 1. **Diagram of temperature handling of VSV ts045-infected CHO 15B or Vero cells.** *Solid circles* represent time points after cycloheximide addition at which infected cells were subjected to biochemical or morphological analysis. Unless specified, all infections were carried out in this way. For details and labeling protocol see "Experimental Procedures."

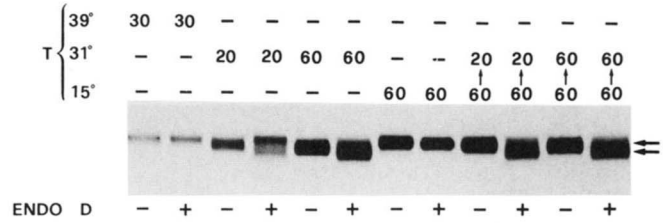

FIG. 2. **Incubation at 15 °C prevents reversibly G protein acquisition of Endo D sensitivity.** Pulse labeling with [^{35}S]methionine of VSV-infected CHO 15B cells, immunoprecipitation, and SDS-PAGE analysis as detailed under "Experimental Procedures." Numbers on the *top* indicate min of chase time after cycloheximide addition at the different temperatures. *Vertical arrows* indicate subsequent shift up from 15 to 31 °C. The immunoprecipitated G protein was divided into 2 aliquots and treated or mock-treated with Endo D as indicated on the bottom. *Horizontal arrows* indicate the position of resistant and sensitive G protein forms.

RESULTS

The VSV ts045 mutant encodes a G protein that exhibits temperature-sensitive cell surface transport (20). At 39 °C G protein accumulates in the ER in an aggregated form (21); if the temperature is then lowered to 31 °C the aggregated G protein trimerizes correctly and leaves the ER (21, 22). Therefore, if infection is performed at 39 °C and the temperature is then lowered in the presence of cycloheximide it is possible to follow the export of G from the ER both morphologically and biochemically (22–24). To perform our analysis we used the protocol detailed in Fig. 1.

Incubation at 15 °C Prevents Post-ER Oligosaccharide Remodeling of G Protein—Endo D cleaves *N*-linked oligosaccharide side chains only when they are trimmed to the $Man_5GlcNAc_2$ form. The mannosidase activity that completes this trimming is due to 1,2-mannosidase I, and it has been suggested that this enzyme is in the cis-Golgi (24). In wild type cells, the $Man_5GlcNAc_2$ chains are modified as soon as they are produced by the action of GlcNAc acetyltransferase I, whereas in the mutant CHO 15B cell line this enzyme activity is missing (25). Therefore, oligosaccharide remodeling will not proceed any further in these cells, and the acquisition of sensitivity to Endo D digestion can be used to monitor the entry of G protein into the Golgi complex (26). As shown in Fig. 2, ts045 G glycoprotein was fully resistant to Endo D when the cells were kept at 39 °C or shifted to 15 °C but became sensitive upon shift to the permissive temperature. If the cells were first kept for 1 h at 15 °C and then shifted to 31 °C, Endo D sensitivity was detected. These results confirmed that incubation at 15 °C blocks G protein before mannose trimming and that this block is reversible (24). Similar results have been obtained with influenza virus hemagglutinin (27). A detailed analysis of the timing of Endo D sensitivity acquisition is shown in Fig. 3. In our conditions, G protein became sensitive with a half-time of about 45 min and 18 min when previously accumulated at 39 or 15 °C, respectively. Control experiments have been performed on infected Vero cells using the acquisition of resistance to endoglycosidase H as a probe to monitor the transport of G protein to medial/trans-Golgi (28). As expected, at 15 °C the formation of complex-type oligosaccharide side chains was totally prevented.

Site of Palmitylation of VSV ts045 G Glycoprotein—Infected Vero cells were processed for [^{3}H]palmitic acid labeling as detailed in Fig. 4. *Lanes 1–3* show that palmitylation of G protein was clearly detected only upon shift to the permissive temperature. Some labeling was variably observed at 39 °C, probably due to leakiness in the mutant phenotype. If the cells were first shifted for 1 h to 15 °C and then labeled at 15 or 31 °C, palmitylation was detected only at 31 °C (Fig. 4, *lanes 4* and *5*), in a slightly sharper and slower migrating band (Fig. 4, compare *lanes 2* and *5*). To completely rule out the unlikely possibility that either G protein itself or the acylating enzyme were responsible for the lack of palmitylation at 15 °C, we carried out an infection at the permissive

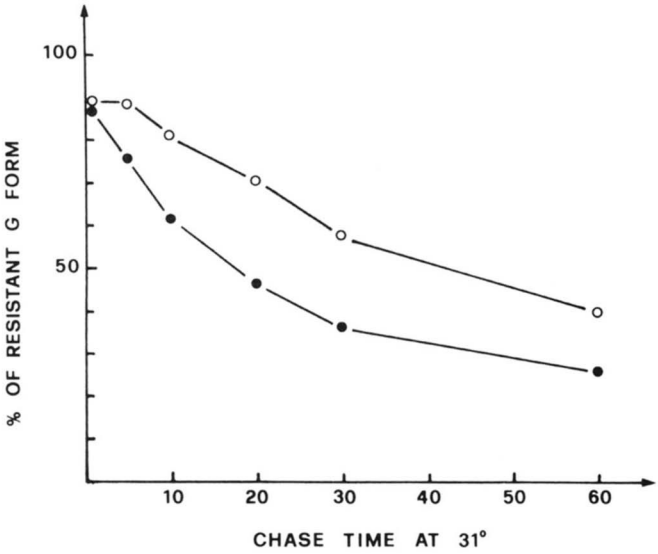

FIG. 3. **Half-time of G protein acquisition of Endo D sensitivity.** Parallel cultures of infected CHO 15B cells were pulse-labeled for 10 min with [^{35}S]methionine. One set of cultures was shifted directly to 31 °C for the indicated times (in minutes, *open circles*); a second set was first incubated for 1 h at 15 °C and then shifted to 31 °C for the same time points (*solid circles*). The amount of resistant and sensitive G protein forms was determined by liquid scintillation counting of the gel slices containing the relative bands.

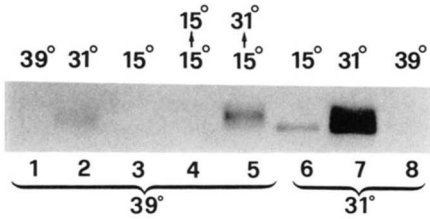

FIG. 4. **Incubation at 15 °C prevents reversibly the palmitylation of G protein.** *Lanes 1–5,* parallel cultures of infected Vero cells were labeled with [^{3}H]palmitic acid. *Numbers on the top* indicate labeling temperatures (see text for details). *Lanes 6–8,* parallel cultures of infected Vero cells were kept at 31 °C throughout the infection. After cycloheximide addition, the cells were labeled with [^{3}H] palmitic acid at the temperatures indicated on the *top*.

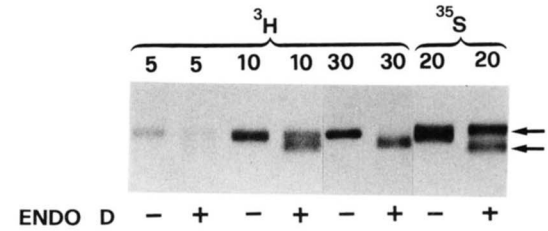

FIG. 5. **Palmitylation precedes G protein acquisition of Endo D sensitivity.** Parallel cultures of CHO 15B cells were infected as in Fig. 1 and pulse-labeled at 31 °C with [^{3}H]palmitic acid for the time indicated on the *top* (in minutes). Immunoprecipitated G protein was divided into 2 aliquots and treated or mock-treated with Endo D as indicated on the bottom. As a marker, a [^{35}S]methionine sample derived from a 10-min pulse, 20 min chase was treated and analyzed in parallel. *Horizontal arrows* indicate the position of Endo D-resistant and -sensitive G protein forms.

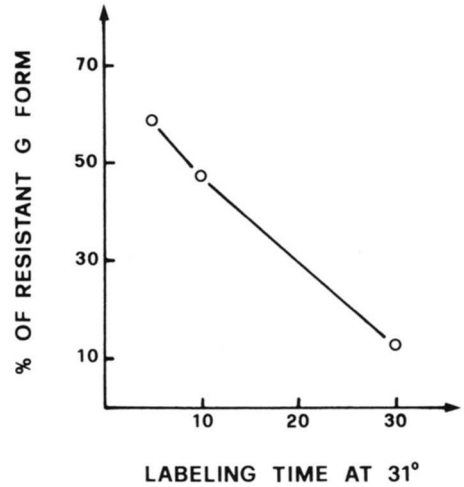

FIG. 6. **Quantitation of the fluorogram shown in Fig. 5.** The amount of Endo D-resistant and -sensitive G protein forms of the [^{3}H]palmitic acid-labeled samples was determined as in Fig. 3.

temperature. At this temperature G glycoprotein spread along the entire intracellular pathway to the surface. The infected cells were then labeled with [^{3}H]palmitic acid at the relevant temperatures in the presence of cycloheximide (Fig. 4, *lanes 6–8*). Labeling of G protein at 15 °C was clearly detected, with practically no labeling at 39 °C and a higher level at 31 °C. The most likely explanation of the labeling patterns that we obtained with the two different protocols of infection is that G protein, synthesized at 39 °C and chased at 15 °C, could not reach the site where palmitylation takes place. Therefore, we examined whether mannosidase I trimming and palmitylation were temporally coincident or separate events. To achieve this we again infected CHO 15B cells as outlined in Fig. 1. First, we confirmed with this cell line the finding that palmitylation of G protein is subsequent to the 15 °C block (data not shown). Then, we performed Endo D digestion of G protein pulse-labeled with [^{3}H]palmitic acid for different times. As shown in Figs. 5 and 6, the majority of G protein palmitylated in a 5-min pulse was Endo D-resistant, whereas about 50% was detectable after a 10-min pulse. Little resistant form was observed after a 30-min pulse. This behavior suggests that palmitylation takes place before mannose trimming is completed and that the two events are separated by a few min at 31 °C.

Low Temperature Prevents Palmitylation of Sindbis Virus Glycoprotein E1—SDS-PAGE analysis of pulse-chase labeling experiments revealed a complex maturation pattern of *Sindbis* virus glycoprotein E1 (29). Newly synthesized E1 (E1e, 53 kDa; Fig. 7, *lane 1*) was first converted to a faster migrating form (E1i, 50 kDa; Fig. 7, *lanes 2* and *3*) with a half-time of 15 min at 37 °C after a 5-min pulse of infected CEF (29). In similar conditions, it was found that SV glycoproteins exited from the ER with a half-time of about 10 min (30). Subsequently, E1i was converted to the mature E1 form (E1, 51.5 kDa; Fig. 7, *lane 4*). The conversion of E1i to mature E1 is due to terminal glycosylation (29), whereas the conversion of E1e to E1i is unrelated to glycosylation (29). The maturation of E1e to E1i is most likely due to palmitylation (Fig. 7). Very short pulses with [^{3}H]palmitic acid always revealed the E1i form, no labeling was ever detected in E1e (Fig. 7, *lanes 5* and *6*). Chemical removal of the acyl moiety (31) (Fig. 7, *lanes 11* and *12*) reverted the electrophoretic mobility of E1i to E1e (Fig. 7, compare *lanes 2* and *3* with *8* and *9*) and also shifted the mature E1 form upward (Fig. 7, *lanes 4* and *10*). There was a complete correlation between E1e and E1i maturation and palmitic acid labeling or chemical deacylation *in vitro* in all conditions tested (infection carried out in the presence of tunicamycin, deacylation by hydroxylamine, analysis of seven different host cell lines and four virus isolates, data not shown). Therefore we conclude that palmitylation results in

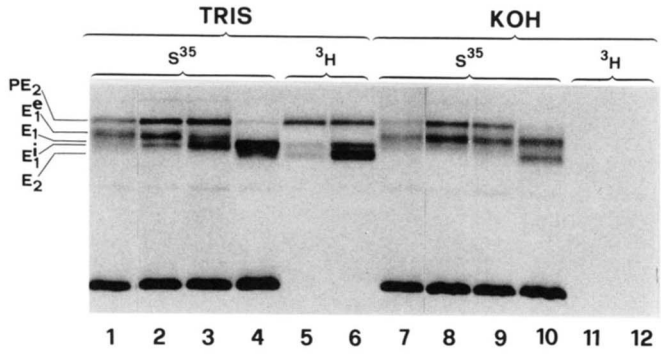

FIG. 7. **Maturation of SV glycoprotein E1e to E1i form is due to palmitylation.** Parallel cultures of CEF were infected with SV, pulse-labeled with [^{35}S]methionine for 5 min, and chased for 0 min (*lanes 1* and *7*), 10 min (*lanes 2* and *8*), 20 min (*lanes 3* and *9*), and 60 min (*lanes 4* and *10*). Aliquots of the cell lysates were treated or mock-treated with methanolic KOH before the SDS-PAGE analysis as indicated on the *top* (see "Experimental Procedures"). Separate cultures were labeled for 5 or 20 min with [^{3}H]palmitic acid (*lanes 5* and *11* or *6* and *12*, respectively) and treated or mock-treated with methanolic KOH as above. The migration of the SV glycoprotein forms is indicated on the *left*.

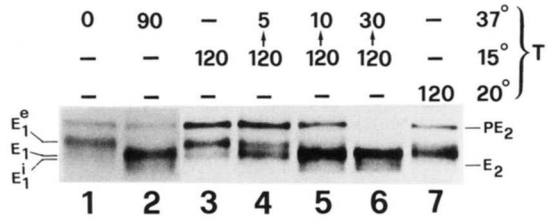

FIG. 8. **Incubation at 15 °C prevents maturation of SV glycoprotein E1e to E1i form.** Parallel cultures of CEF were infected with SV, pulse-labeled for 5 min with [^{35}S]methionine, and chased at different temperatures for different times (in minutes) as indicated on the *top*. The migration of the SV glycoprotein forms is indicated.

a significant increase in the electrophoretic mobility of SV E1 glycoprotein. An altered electrophoretic mobility has also been reported for the palmitylated form of p21 Ras (32). This behavior of Sindbis E1 provided us the possibility to monitor palmitylation during the intracellular transport, using [^{35}S] methionine and SDS-PAGE. As shown in Fig. 8, prolonged incubation at 15 °C prevented the E1e to E1i conversion (*lane 3*); when the temperature was subsequently shifted to 37 °C a quick conversion took place (*lanes 4–6*). In parallel, labeling experiments with [^{3}H]palmitic acid at 15 °C confirmed that low temperature per se does not arrest palmitylation (data not shown). Thus, these findings showed that prevention of palmitylation of newly synthesized proteins due to the block of transport at 15 °C could be reproduced in a third cell line with a different membrane protein. Moreover, E1e to E1i conversion took place more rapidly when assayed after a chase performed at 15 °C (compare Figs. 7 and 8). As a control, we performed a prolonged chase at 20 °C, a condition in which newly synthesized membrane proteins accumulate in the trans-Golgi network (33). As expected, at 20 °C the conversion of E1e to E1i was not prevented (Fig. 7, *lane 7*).

Immunofluorescence Analysis of the Intracellular Transport of VSV ts045 G Glycoprotein—As shown in Fig. 9, *panel a*, infected Vero cells kept at 39 °C showed a diffuse distribution of G glycoprotein. The staining that we obtained was very similar to those reported previously (12, 23), and almost coincident, in double labeling experiments, to the one shown by an anti-ER antibody (Fig. 9, *panels a* and *b*) (16). As expected, a perinuclear and surface labeling was observed after a shift up to the permissive temperature, indicating that G protein had entered the Golgi and had been transported to the surface (Fig. 9, *panel c*) (12). Conversely, when the infected cells were shifted to 15 °C, a totally different pattern was revealed (Fig. 9, *panel d*). The ER type of staining was very weak; instead, a punctate, discrete dotty staining was predominant. The dots appeared to be distributed all over the cell, with a variable concentration in a perinuclear area and no precise localization; by confocal beam scanning laser microscopy we could confirm that the staining was due to rounded, isolated dots dispersed in the cytoplasm (Fig. 10). These dots do not represent either Golgi or ER elements dispersed as a consequence of the low temperature because we observed normal staining of these organelles in double labeling experiments (data not shown). When the cells were shifted up to the permissive temperature for 10 min, dots were still detectable but staining in the perinuclear area became apparent (Fig. 9, *panel e*). After 30 min, there were no more dots and the staining was mostly in a perinuclear area or at the cell surface (Fig. 9, *panel f*). Previous reports have shown that VSV ts045 G glycoprotein enters the Golgi complex upon shifting from the nonpermissive to the permissive temperature (12, 23). Our images are very similar to those previously reported and our morphological and biochemical studies have been performed in the same conditions as described previously. Therefore, our immunofluorescence analysis showed that during the incubation at 15 °C VSV G protein moved from the ER to a new location characterized by a dotty appearance: shifting up the temperature, G protein progressively entered the Golgi complex to proceed further to the surface.

DISCUSSION

Our results indicate that palmitylation of membrane proteins in transit to the surface takes place between the sites defined by low temperature (15 °C) and 1,2-mannosidase I, respectively. This conclusion is based on the observation that low temperature prevents palmitylation of VSV ts045 G proteins and SV E1 glycoproteins in several host cell lines, whereas newly palmitylated G glycoprotein is not sensitive to Endo D digestion. Thus, our study defines more precisely the intracellular site of palmitylation. Cell fractionation analysis of Semliki Forest virus infected BHK cells, pulse-labeled with [^{3}H]palmitic acid, led Quinn *et al.* (2) to suggest that fatty acylation is localized in the cis- or medial Golgi. Similar results were obtained by Dunphy *et al.* (3) working with VSV-infected CHO 15B cells. On the other hand, Berger and Schmidt (4) proposed that the fatty acyltransferase is located in the ER on the basis of cell fractionation of Semliki Forest virus-infected BHK cells. A different approach was taken by Rizzolo *et al.* (5, 34). They constructed a chimeric membrane protein, composed of influenza hemagglutinin and rat growth hormone. In transient expression experiments in COS cells this protein failed to enter the Golgi complex and accumulated intracellularly in cisternae and vesicular elements, interpreted as post-ER/pre-Golgi structures (34). The chimeric protein was palmitylated and had oligosaccharide side chains of the structure $Gluc_1Man_8GlcNac_2$ and $Man_8GlcNAc_2$ (5). Both findings are in agreement with our observation that G protein acylation took place before mannosidase I activity, and that G protein, as well as SV E1 protein, were acylated in a post-ER location. However, the results obtained with the chimeric protein may be difficult to generalize because the protein was irreversibly blocked in its intracellular transport and only low levels of palmitylation could be detected. The intracellular site of palmitylation has been studied also in the yeast *Sac-*

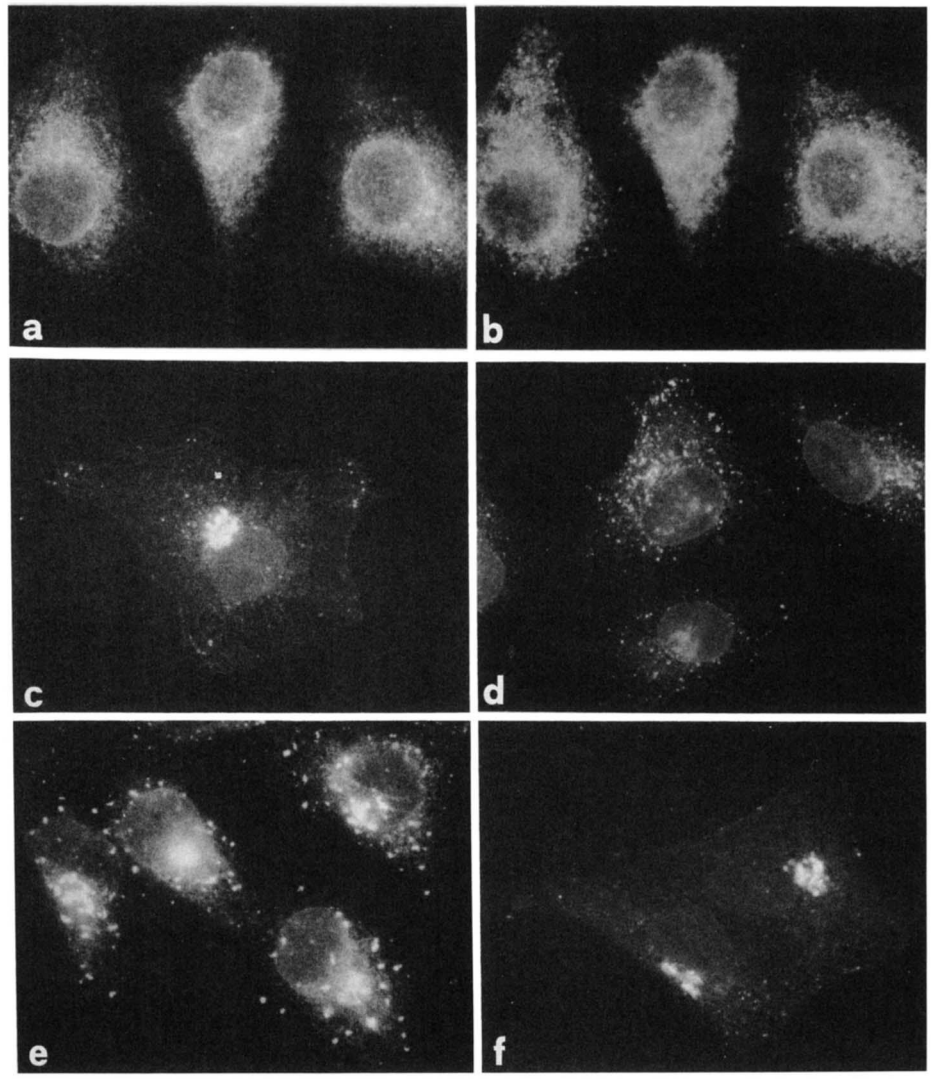

FIG. 9. **Immunofluorescence analysis of the intracellular transport of G protein.** Parallel cultures of infected Vero cells were manipulated as in Fig. 1 and processed for indirect immunofluorescence analysis (see "Experimental Procedures"). *Panel a*, cells kept for 30 min at 39 °C; *panel b*, same cells stained in parallel with anti-ER antibodies; *panel c*, cells incubated for 30 min at 31 °C; *panel d*, cells incubated for 60 min at 15 °C; *panel e*, cells incubated for 10 min at 31 °C after a 60-min incubation at 15 °C; *panel f*, cells incubated for 30 min at 31 °C after a 60-min incubation at 15 °C.

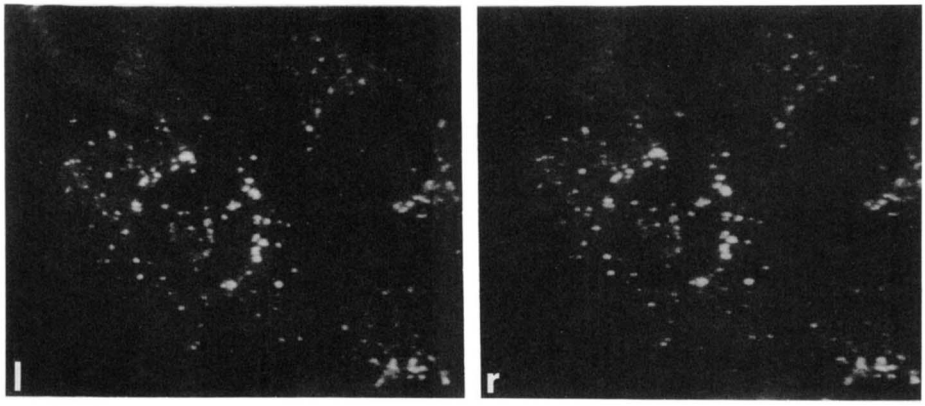

FIG. 10. **Confocal beam scanning laser microscopy of cells treated as in *panel d* of Fig. 9.** The resulting computerized stereo images of the analysis are presented.

charomyces cerevisiae (6), in one of the sec mutants, isolated by Schekman and coworkers (35). The Sec 18 mutant accumulates ER membrane and vesicles at the nonpermissive temperature (36). Although ER to Golgi transport was blocked, several glycoproteins were palmitylated (6), suggesting an ER location for palmitylation. In conclusion, all the previously published evidence indicates that palmitylation occurs late in ER, or in an intermediate location, or early in the Golgi. Assuming that mannosidase I is a cis-Golgi marker, our study suggests a post-ER/pre-Golgi location for fatty acylation.

Our results also clearly show that the viral membrane proteins accumulate at 15 °C in an intermediate location between the ER and the sites where palmitylation and mannosidase I action take place. This conclusion is based on morphological as well as on kinetic evidence. The immunofluorescence pattern of G protein, blocked at 15 °C, was very different from both the ER and the Golgi patterns; and G protein reached the mannose trimming site faster when coming from the 15 °C block than when coming from the ER (39 °C). The SV E1 glycoprotein was palmitylated more rapidly after accumulation at 15 °C than after the short pulse at

37 °C. These observations are in agreement with the results of Saraste and Kuismanen (7) and with the findings that folding and trimerization of newly synthesized membrane proteins take place in the ER and these are not prevented by low temperature (21, 27).

The presence of an intermediate compartment (or more than one) between ER and Golgi has been suggested by several authors on the basis of different observations. Saraste and Kuismanen (7) observed structures which they called pre-Golgi vacuoles after immunoperoxidase staining of cells, infected with a ts mutant of Semliki Forest virus and incubated at 15 °C. These vacuoles had an average diameter of about 300–500 nm and were seen in close contact to the cis-Golgi cisternae. Tooze *et al.* (37) have reported a biochemical and morphological characterization of coronavirus E1 glycoprotein transport from ER to the Golgi. They showed that these are tubulo-vesicular structures located between the ER and Golgi. This is the intracellular site of coronaviruses budding and the site where *N*-acetylgalactosamination of coronavirus E1 glycoprotein takes place (the first step in *O*-linked carbohydrate side chain formation). Recently, Schweizer *et al.* (38) reported the characterization of a membrane protein which is located in a tubular structure between the ER and the Golgi. What could be the function of this (or these) intermediate compartment(s)? Presently, the most attractive hypothesis derives from the work of Munro and Pelham (39, 40). According to their hypothesis, soluble resident proteins are kept in the ER by continuous retrieval, mediated by a recycling receptor protein. This receptor would bind to the Lys-Asp-Glu-Leu sequence, located at the carboxyl terminus of ER proteins, and retrieve the ER proteins from the intermediate "salvage" compartment, which would be the station in the exocytic pathway responsible for cycling proteins back to the ER. Recently, Pelham (41) showed that when the Lys-Asp-Glu-Leu sequence was attached to the carboxyl terminus of a lysosomal enzyme, cathepsin D, the chimeric protein was retained in the ER and underwent the first phosphorylation reaction, the addition of GlcNAc phosphate to peripheral mannose. At 15 °C this phosphorylation step was blocked (41). This finding would suggest that the site, defined by 15 °C, and the hypothetical site defined by the salvage reaction do not coincide. As working hypothesis, we suggest that low temperature (15 °C) leads to accumulation of proteins in vesicular structures related to transitional elements (9, 42), and that acetylgalactosamination, palmitylation, and lysosomal enzyme phosphorylation take place subsequently but before entry of the proteins into the cis-Golgi. More work is clearly necessary to pinpoint the locations of all these post-translational modifications. One problem is that it is difficult to compare and generalize results, obtained by so many approaches in different experimental systems. Another problem is that the cis-Golgi is still poorly defined. The only specific marker is a recently described 58-kDa protein (43). We plan to extend the dissection of the events happening during the transit from ER to Golgi by using immunocytochemistry at the ultrastructural level; we believe this approach, in conjunction with studies based on perforated cells (13, 26), will allow a more detailed understanding of this complex intracellular transport segment.

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