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# Fatty acid acylation at the single cysteine residue in the cytoplasmic domain of the glycoprotein of vesicular-stomatitis virus

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The cysteine residue in the cytoplasmic domain at position 489 of the sequence of the glycoprotein (G protein) isolated from vesicular-stomatitis virions is completely blocked for carboxymethylation. After release of covalently bound fatty acids by hydroxylamine at pH 6.8, this cysteine residue could be specifically labelled by iodo[$^{14}$C]acetic acid. Reaction products were analysed after specific cleavage of labelled G protein at asparagine–glycine bonds by hydroxylamine at pH 9.3, which generated a *C*-terminal peptide of $M_r$ 15300 containing only the single cysteine residue. Bromelain digestion of [$^3$H]palmitic acid-labelled membrane fractions of vesicular-stomatitis-virus-infected baby-hamster kidney cells removed almost completely the $^3$H radioactivity from the cytoplasmic domain of the G protein, whereas the ectodomain was completely protected by the microsomal membrane. This result indicates that the acylation site of the G protein is exposed on the cytoplasmic side of intracellular membranes. Taken together, both biochemical techniques strongly suggest that the single cysteine-489 residue, which is located six amino acid residues distal to the putative transmembrane domain, is the acylation site. The thioester bond between palmitic acid and the G protein is quite resistant to hydroxylamine treatment (0.32 M at pH 6.8 for 1 h at 37 °C) compared with the reactivity of the thioester linkage in palmitoyl-CoA, which is cleaved at relatively low concentrations of hydroxylamine (0.05 M).

## INTRODUCTION

The covalent modification of proteins with long-chain fatty acids was first detected in membrane glycoproteins of Sindbis virus and vesicular-stomatitis virus (Schmidt *et al.*, 1979; Schmidt & Schlesinger, 1979) and has been subsequently found with numerous cellular and viral proteins (for review see Schmidt, 1983). Two groups of acylated proteins have been described, which contain chemically different fatty acid–protein linkages; hydroxylamine-sensitive ester bonds are formed by palmitic acid, whereas myristic acid is bound in a hydroxylamine-insensitive amide linkage to the *N*-terminal residue of several proteins (Magee & Courtneidge, 1985; McIlhinney *et al.*, 1985; Olson *et al.*, 1985). The chemical identification of acyl linkages proved to be very difficult owing to the extreme hydrophobicity of acyl-peptides. Only ester linkages to threonine in bovine brain lipophilin (Stoffel *et al.*, 1983) and thioester linkages to cysteine residues in a histocompatibility antigen (Kaufman *et al.*, 1984) and p21$^{ras}$ (Chen *et al.*, 1985) have been directly identified by chemical analysis so far.

For the glycoprotein (G protein) of VSV enrichment of serine residues in [$^3$H]palmitic acid-labelled peptide preparations was reported (Schmidt & Schlesinger, 1979). Because of the lability of protein-bound palmitic acid to alkaline methanolysis and the production of a membrane-protected peptide labelled by [$^3$H]palmitic acid after proteolytic digestion of intact viral particles, palmitic acid was thought to be esterified to serine residues of G protein on the outer surface of the viral envelope (Schmidt & Schlesinger, 1979; Petri & Wagner, 1980; Capone *et al.*, 1982; Rose & Gallione, 1981; Schlesinger *et al.*, 1981).

This localization of the acyl-acceptor site and its chemical nature were inconsistent with results obtained by Rose *et al.* (1984), who produced a mutant G protein that, when expressed in COS cells, was not modified by fatty acids any longer because cysteine-489 had been changed to serine by site-directed mutagenesis. This loss of acylation in the mutant G protein could be explained by (i) eliminating the primary acyl-acceptor site and thus blocking a subsequently occurring acyl shift from cysteine to serine, as has been observed in fatty acid synthase (Cardon & Hammes, 1983), or (ii) by a conformational change of the mutagenized peptide sequence rendering the acyl-acceptor site inaccessible for the acyltransferase. Similarly, Magee *et al.* (1984) suggested from their hydroxylaminolysis studies of G protein that thiol groups may be important in fatty acylation. They observed the formation of disulphide-linked dimers through the cytoplasmic cysteine residue of the G protein, although the kinetics of deacylation by hydroxylamine pointed to an ester linkage between fatty acids and protein sequence. Because of the limitations of the techniques used, we decided to analyse in a more direct approach: (i) the topological orientation of the acylation site and (ii) the involvement of the putative cysteine-489 as palmitic acid-acceptor site.

Abbreviations used: VSV, vesicular-stomatitis virus; BHK cells, baby-hamster kidney cells.

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## MATERIALS AND METHODS

### Materials

[$^{35}$S]Cysteine (specific radioactivity 1000 Ci/mmol), iodo[2-$^{14}$C]acetic acid (specific radioactivity 53 mCi/mmol) and [$^{14}$C]methylated protein markers (specific radioactivity 10–50 $\mu$Ci/mg) were purchased from Amersham Buchler. [9,10-$^{3}$H]Palmitic acid (specific radioactivity 30 Ci/mmol) was obtained from New England Nuclear.

### Cell and virus growth

BHK cells were grown as monolayers in Joklik minimal essential medium (Flow Laboratories) supplemented with 5% (v/v) calf serum (Seromed). The San Juan strain of VSV (serotype Indiana, obtained from J. K. Rose, San Diego, CA, U.S.A.) was grown in BHK cells and purified by the use of sodium/potassium tartrate gradients as previously described (Graeve *et al.*, 1986; Mack *et al.*, 1987).

For the preparation of [$^{35}$S]cysteine- or [$^{3}$H]palmitic acid-labelled VSV, monolayers of BHK cells were inoculated with a multiplicity of infection of 10 plaque-forming units/cell. After 3 h of infection cells were washed with prewarmed phosphate-buffered saline (Dulbecco & Vogt, 1954) and labelled either with 10 $\mu$Ci of [$^{35}$S]-cysteine/ml in a medium containing one-tenth the normal concentration of cysteine supplemented with 2% (v/v) calf serum or with 20 $\mu$Ci of [$^{3}$H]palmitic acid/ml in a medium supplemented with 2% (v/v) calf serum. After an additional 17 h at 37 °C the medium was collected and the cell debris was removed by centrifugation at 18000 *g* for 10 min at 4 °C. Labelled viral particles were sedimented from the supernatant through a 1.5 ml cushion of 50% (v/v) glycerol at 100000 *g* for 1 h at 4 °C, resuspended in 20 mM-Tris/HCl buffer, pH 7.5, and stored at −20 °C.

### Preparation and bromelain digestion of [$^{3}$H]palmitic acid-labelled membrane fractions of VSV-infected BHK cells

Confluent monolayers of BHK cells were inoculated with VSV at a multiplicity of infection of 40 plaque-forming units/cell. At 4.5 h after infection monolayers were washed with prewarmed phosphate-buffered saline and labelled for 30 min at 37 °C in 3 ml of serum-free medium with 400 $\mu$Ci of [$^{3}$H]palmitic acid. Labelling was terminated by washing the cells twice with ice-cold phosphate-buffered saline. Cells were scraped off, collected by low-speed centrifugation and resuspended in hypo-osmotic buffer (15 mM-KCl/1.5 mM-$MgCl_2$/10 mM-Tris/HCl buffer, pH 7.5). After swelling on ice for 15 min the cells were homogenized by 20–30 strokes with a tight-fitting Dounce homogenizer. Nuclei were removed by centrifugation at 800 *g* for 5 min. Membranes were sedimented from the supernatant by centrifugation at 18000 *g* for 15 min at 4 °C, resuspended in iso-osmotic buffer (0.2 M-sucrose / 50 mM-KCl / 2 mM-magnesium acetate/1 mM-dithiothreitol/20 mM-Hepes/KOH buffer, pH 7.5) at a concentration of 300–400 $\mu$g of protein/ml, and stored in small portions at −70 °C.

Portions of these membrane preparations were incubated with bromelain (50 $\mu$g/60 $\mu$l) for 30 min at 37 °C. The reaction was stopped by the addition of 1 vol. of ice-cold 20% (w/v) trichloroacetic acid. Proteins were collected by centrifugation, washed with cold acetone and solubilized in electrophoresis sample buffer.

### Deacylation by hydroxylamine and iodo[$^{14}$C]acetic acid labelling of the G protein of VSV

G protein was selectively extracted from purified VSV by using 50 mM-octyl $\beta$-D-glucopyranoside (Calbiochem) in 10 mM-sodium phosphate buffer, pH 8.0 (Miller *et al.*, 1980). Extracts had a concentration of 300–500 $\mu$g of G protein/ml and were stored at −20 °C.

After the addition of Nonidet P-40 (Sigma Chemical Co.) to a concentration of 1%, 500 $\mu$l portions of solubilized G protein were deacylated at 37 °C for 2 h in 1 M-hydroxylamine (Fluka), pH 6.8. These conditions resulted in a complete release of $^{3}$H-labelled protein-bound palmitic acid from the G protein. G protein treated with 1 M-Tris/HCl buffer, pH 6.8, served as a control. G protein was precipitated with 10 vol. of methanol cooled to −20 °C and collected by centrifugation at 10000 *g* for 15 min. Pellets were solubilized twice in reaction buffer (6 M-guanidinium hydrochloride/0.1 M-Tris/HCl buffer, pH 8.0) and precipitated with methanol. Finally, the G protein was solubilized in 100 $\mu$l of reaction buffer and reduced with 0.25 mM-dithiothreitol (final concentration) for 1 h at 21 °C. The reduced protein was carboxymethylated by addition of 1 $\mu$Ci of iodo[$^{14}$C]acetic acid for 1 h at 21 °C in the dark. Unlabelled iodoacetic acid was added to a final concentration of 5 mM and the G protein was recovered by precipitation with methanol cooled to −20 °C.

In order to block unmodified thiol groups, isolated VSV G protein was precipitated with methanol, solubilized in 200 $\mu$l of 6 M-guanidinium chloride/0.1 M-Tris/HCl buffer, pH 8.0, reduced with 1 mM-dithiothreitol for 1 h at 21 °C, and subsequently carboxymethylated in the dark for 1 h at 21 °C with 2 mM non-radioactive iodoacetic acid. Modified G protein was recovered by precipitation with methanol, and the pellet was washed with methanol. Half of the material was deacylated in 1 ml of 1 M-hydroxylamine/6 M-guanidinium chloride, pH 6.8, for 2 h at 37 °C. As a control the remaining half was incubated in 6 M-guanidinium chloride/0.1 M-Tris/HCl buffer, pH 8.0, for 2 h at 37 °C. G protein was recovered by precipitation with methanol, and remaining disulphide bonds were reduced and carboxymethylated with 0.5 $\mu$Ci of iodo[$^{14}$C]acetic acid. Finally, the carboxymethylated G protein was solubilized in gel sample buffer and separated from contaminants by SDS/polyacrylamide-gel electrophoresis.

### Polyacrylamide-gel electrophoresis and immunoblotting

Radioactively labelled VSV proteins were electrophoretically separated on 10% polyacrylamide gels by use of the discontinuous system of Laemmli (1970). G protein bands were excised from polyacrylamide gels, washed four times in cold 5% (v/v) methanol and chemically cleaved at asparagine–glycine bonds by hydroxylamine, pH 9.3, as previously described (Saris *et al.*, 1983). Protein fragments were analysed by electrophoresis on SDS/15% polyacrylamide gels. Electrophoretic separation was carried out under reducing conditions at 15 mA (constant current) for 11 h. [$^{14}$C]Methylated proteins were used as $M_r$ standards. Gels were processed as previously described (Mack *et al.*, 1987).

Polypeptides separated by SDS/polyacrylamide-gel

electrophoresis were electrophoretically transferred on to nitrocellulose paper (BA 85, Schleicher und Schüll) with 20% (v/v) methanol/25 mM-Tris/192 mM-glycine as transfer buffer (Towbin *et al.*, 1979). The VSV G protein was detected with a rabbit antiserum raised against purified G protein (Garreis-Wabnitz & Kruppa, 1984). Bound antibodies were developed with anti-(rabbit IgG) antibody coupled to peroxidase (Dakopatts) and detected with *o*-dianisidine dihydrochloride (Sigma Chemical Co.).

## RESULTS

### Extent of acylation of G protein isolated from VSV particles

In order to analyse the degree of acylation in the *C*-terminal domain of the G protein we used the chemical cleavage at asparagine–glycine bonds by hydroxylamine at alkaline pH (Bornstein & Balian, 1977). The G protein contains three potential cleavage sites for hydroxylamine, at asparagine residues 166, 336 and 387 (Rose & Gallione, 1981). Since asparagine-336 is *N*-glycosylated, this site is expected to be resistant against cleavage by hydroxylamine (Bornstein & Balian, 1977). After limited cleavage of [$^{35}$S]cysteine-labelled G protein by hydroxylamine, pH 9.3, five labelled fragments (I–V) are detected in addition to uncleaved G protein (Fig. 1, lane 1). The three peptides with the highest mobility in SDS/polyacrylamide-gel electrophoresis (III–V) represent fragments after complete cleavage of G protein by hydroxylamine, whereas the other two fragments (I and II) represent intermediates in which only one of the two possible sites was cleaved. Those three fragments can be aligned with the amino acid sequence of the G protein (Rose & Gallione, 1981). The smallest fragment (V) corresponds to the cytoplasmic and transmembrane domains and contains 75 amino acid residues of the ectodomain of the G protein ($M_r$ 15300). Fragment II is the *N*-terminal portion of the G protein ($M_r$ 19700), and fragment III is the sequence joining these two other fragments, which contains both glycosylation sites of the G protein ($M_r$ 34700). The intensity of the [$^{35}$S]cysteine-labelled fragments is proportional to five and seven cysteine residues in the *N*-terminal and the glycosylated fragments respectively (Fig. 1, lane 1). The *C*-terminal fragment V contains only the single cysteine residue at position 489. For this reason cleavage of the G protein by hydroxylamine, pH 9.3, is ideally suited for analysis of the extent of acylation of G protein and the involvement of the single cysteine-489 in the cytoplasmic domain.

G protein when directly carboxymethylated with iodo[$^{14}$C]acetic acid did not contain any radioactivity in the corresponding *C*-terminal fragment after hydroxylamine cleavage (Fig. 1, lane 5). This result indicated that the cysteine residue in the cytoplasmic tail of the G protein isolated fom VSV particles is completely blocked for carboxymethylation.

### Carboxymethylation of deacylated G protein at the single cytoplasmic cysteine residue

Isolated G protein was deacylated by 1 M-hydroxylamine, pH 6.8, which resulted in a complete release of protein-bound fatty acids (see below, and Magee *et al.*, 1984). In contrast with the chemical cleavage of proteins by 2 M-hydroxylamine, pH 9.3, treatment with 1 M-

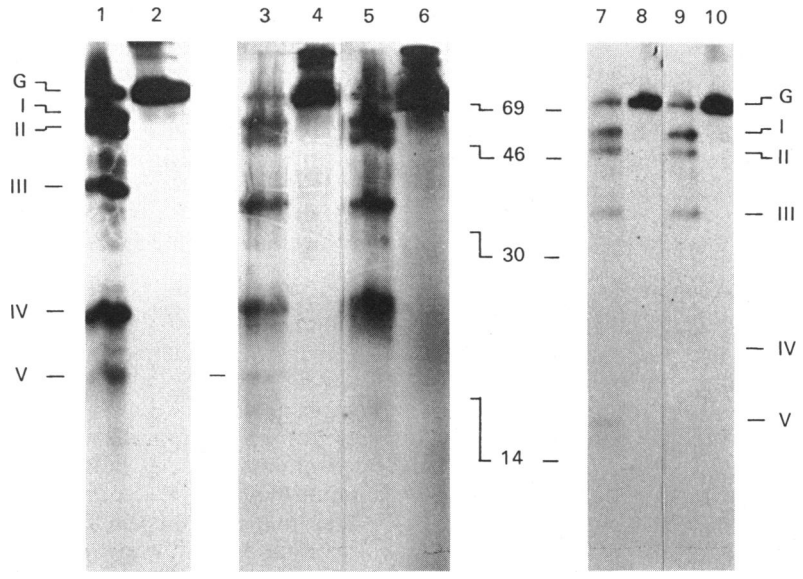


**Fig. 1. Hydroxylamine cleavage of [$^{35}$S]cysteine-labelled and iodo[$^{14}$C]acetic acid-modified G protein**

G protein metabolically labelled with [$^{35}$S]cysteine (lane 2) was isolated and cleaved by hydroxylamine for 4 h (lane 1). Deacylated (lane 3) and non-deacylated G protein (lane 5) were directly carboxymethylated with iodo[$^{14}$C]acetic acid and then cleaved by hydroxylamine for 6 h. In another experiment reduced G protein was first carboxymethylated with non-radioactive iodoacetic acid in order to block free thiol groups. One half of the material was subsequently deacylated with hydroxylamine at pH 6.8 (lane 7) and the other half, which served as a control, was treated with Tris/HCl buffer, pH 6.8 (lane 9). Finally, both G protein samples were carboxymethylated with iodo[$^{14}$C]acetic acid, and cleaved by hydroxylamine for 5 h. Deacylated and non-deacylated controls (lanes 4, 6, 8 and 10) were not cleaved by hydroxylamine. The controls and the fragments were analysed by electrophoresis on SDS/15% polyacrylamide gels, which were fluorographed (Chamberlain, 1979). The fragments of the G protein generated by hydroxylamine cleavage at pH 9.3 are marked I–V. G, VSV G protein.

hydroxylamine, pH 6.8, does not lead to a detectable alteration of proteins, as verified by SDS/polyacrylamide-gel electrophoresis, amino acid sequence analysis and binding assays with monoclonal antibodies (Magee *et al.*, 1984; Schmidt & Lambrecht, 1985). As a control G protein was treated wth 1 M-Tris/HCl buffer, pH 6.8, which does not lead to a release of covalently linked fatty acids (Magee *et al.*, 1984).

G protein samples were then reduced by dithiothreitol and carboxymethylated by iodo[$^{14}$C]acetic acid. $^{14}$C-labelled G protein was isolated by preparative SDS/polyacrylamide-gel electrophoresis and cleaved by hydroxylamine, pH 9.3. Only the *C*-terminal fragment of the deacylated sample was labelled by iodo[$^{14}$C]acetic acid, indicating that a reactive thiol group had been exposed after release of the fatty acids bound to the G protein (Fig. 1, lanes 3 and 4).

This result became more evident when G protein was first carboxymethylated with non-radioactive iodoacetic acid. Replicate samples were then deacylated by 1 M-hydroxylamine, pH 6.8, or were treated with Tris/HCl buffer as a control, and remaining free thiol groups were carboxymethylated by iodo[$^{14}$C]acetic acid. Hydroxylamine cleavage of this material shows, besides residual labelling of larger fragments, specific labelling of the *C*-terminal fragment V of the deacylated sample (Fig. 1, lane 7), whereas labelling of the *C*-terminal fragment of the Tris-treated control could not be detected (Fig. 1, lane 9).

The specificity of labelling of cysteine residues by iodo[$^{14}$C]acetic acid under the conditions employed was checked. To this end iodo[$^{14}$C]acetic acid-labelled G protein was hydrolysed and the amino acid composition was determined by using the Waters PICO-TAG system. Over 80% of the radioactivity was identified as carboxymethylcysteine. The remaining radioactivity was eluted from the column in two peaks, which were interpreted as degradation products of carboxymethylcysteine because they accumulated when the duration of hydrolysis was increased from 24 h to 48 h and to 72 h. Similar products were observed with G protein treated with 1 M-hydroxylamine, pH 6.8, or 1 M-Tris/HCl buffer, pH 6.8 (results not shown).

### Removal of the palmitic acid-acceptor site from membrane-integrated G protein by bromelain digestion

In order to demonstrate that the acceptor site of palmitic acid is in the cytoplasmic domain of the G protein, we made use of the observation that the cytoplasmic domain of the G protein is exposed in intracellular membrane preparations of VSV-infected cells and can be removed by proteinases, whereas the ectodomain is protected by the microsomal membrane (Morrison & McQuain, 1978).

Since cysteine-489 is located six amino acid residues distal to the putative transmembrane domain of the G protein (Rose & Gallione, 1981), we attempted to remove proteolytically the [$^3$H]palmitic acid label from the ectodomain of the G protein.

Membrane fractions of VSV-infected BHK cells that had been labelled with [$^3$H]palmitic acid for 30 min were prepared and digested with bromelain. The resulting polypeptides were separated by SDS/polyacrylamide-gel electrophoresis and blotted on to nitrocellulose. G-protein-related polypeptides were detected with a monospecific rabbit antiserum raised against purified G protein. The blot was then impregnated with diphenyloxazole (Southern, 1979) and fluorographed.

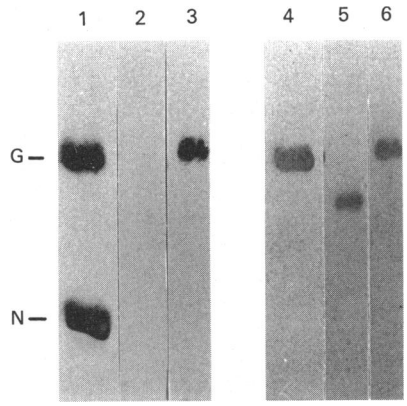


**Fig. 2. Digestion of membrane-integrated [$^3$H]palmitic acid-labelled G protein by bromelain**

Portions of [$^3$H]palmitic acid-labelled membrane fractions of VSV-infected BHK cells were incubated with bromelain for 30 min at 37 °C (lanes 2 and 5). As a control bromelain was omitted (lanes 3 and 6). Proteins were separated by electrophoresis on a SDS/10% polyacrylamide gel and blotted on to nitrocellulose. G-protein-related polypeptides were detected with a monospecific rabbit antiserum raised against purified G protein of VSV (lanes 4–6). The blot was then impregnated with 20% (w/v) diphenyloxazole in toluene, air-dried and exposed to Cronex 4 X-ray film at −70 °C (Southern, 1979). A fluorogram of the blot is shown (lanes 1–3). Lanes 1 and 4 contain [$^{35}$S]methionine-labelled VSV G protein ($M_r$ 69000) and N protein ($M_r$ 50000) as markers.

As shown in Fig. 2 (lanes 5 and 6), an amino acid sequence of an apparent $M_r$ 5100 was removed from the G protein, whereas the ectodomain of the G protein was completely protected by the microsomal membrane. The [$^3$H]palmitic acid label was completely removed from the G protein by digestion with bromelain (Fig. 2, lanes 2 and 3), indicating that the palmitic acid-binding site of the G protein is exposed on the cytoplasmic side of intracellular membranes.

### Deacylation of the G protein by hydroxylamine

Ester bonds of long-chain fatty acids linked to acylproteins are sensitive to cleavage by hydroxylamine at neutral or slightly alkaline pH (Magee *et al.*, 1984). The cleavage of acyl linkages by hydroxylamine under defined conditions compared with the cleavage of reference compounds such as palmitoyl-CoA, *O*-acetylserine and palmitoylserine has been used to distinguish between ester linkages to serine and thioester linkages to cysteine (Schmidt & Lambrecht, 1985).

In order to investigate the stability of the acyl linkage in G protein we have incubated [$^3$H]palmitic acid-labelled VSV with various concentrations of hydroxylamine at pH 6.8 for 1 h at 37 °C. As a control served [$^3$H]palmitic acid-labelled VSV that was incubated with 1 M-Tris/HCl buffer, pH 6.8. After separation of polypeptides by SDS/polyacrylamide-gel electrophoresis and fluorography the intensities of [$^3$H]palmitic acid-labelled G protein bands were quantified as described in the legend to Fig. 3. A significant release of [$^3$H]palmitic acid bound to the G protein could be detected, starting from 0.32 M-hydroxyl-

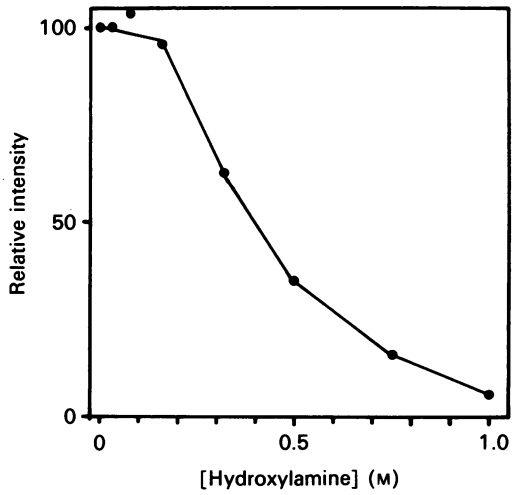


**Fig. 3. Deacylation of [$^3$H]palmitic acid-labelled G protein by increasing concentrations of hydroxylamine**

About 5 $\mu$g of [$^3$H]palmitic acid-labelled VSV in 20 $\mu$l of 20 mM-Tris/HCl buffer, pH 7.5, was mixed with an equal volume of an appropriate hydroxylamine solution, pH 6.8, leading to final concentrations of 1 M-, 0.75 M-, 0.5 M-, 0.32 M-, 0.16 M-, 0.075 M- and 0.025 M-hydroxylamine. Control samples were mixed with an equal volume of 2 M-Tris/HCl buffer, pH 6.8. After incubation for 1 h at 37 °C viral proteins were precipitated with 10 vol. of methanol that had been precooled to −20 °C. Precipitations were left for 10 min on ice. Proteins were recovered by centrifugation, solubilized in gel sample buffer and separated by SDS/polyacrylamide-gel electrophoresis. A fluorogram of the gel was quantified with an Ortec densitometer as described previously (Kruppa, 1983). The peak areas were determined and relative intensities calculated by taking the intensity of Tris/HCl-treated controls as 100%.

amine, pH 6.8, increasing to an almost complete release of labelled palmitic acid at 1 M-hydroxylamine, pH 6.8 (Fig. 3).

## DISCUSSION

Acylation of proteins has been connected with important cellular functions such as membrane anchorage and growth regulation (Klockmann & Deppert, 1983; Schultz *et al.*, 1985), morphogenesis and virus maturation (Schlesinger & Malfer, 1982; Bolanowski *et al.*, 1984; Rein *et al.*, 1986), receptor assembly (Olson *et al.*, 1984) proteinase protection (Slomiany *et al.*, 1983) and membrane fusion (Lambrecht & Schmidt, 1986).

Some of the proposed functions of acylated proteins would imply that the acyl-acceptor site should be in close proximity to the lipid bilayer. Originally, in agreement with this view an ester linkage between serine residues and fatty acids at the surface of the viral envelope was assumed for the G protein of VSV (Schmidt & Schlesinger, 1979). This location and the chemical nature of the acyl-acceptor site are inconsistent with our results. In the present study we were able to demonstrate that the single cysteine residue in the cytoplasmic domain of the G protein is completely blocked for carboxymethylation by iodo[$^{14}$C]acetic acid under reducing conditions (Fig. 1, lane 5). This cysteine residue can only be modified by iodo[$^{14}$C]acetic acid by exposure of this thiol group after removing the blocking long-chain fatty acid through hydroxylaminolysis (Fig. 1, lanes 3 and 7).

From the kinetics of the release of palmitic acid from the G protein by hydroxylamine Magee *et al.* (1984) concluded that the fatty acid was bound to serine residues. An involvement of the cytoplasmic cysteine residue in the acylation reaction was not excluded in their opinion, since they observed a dimerization of G protein. Fatty acid transfer to serine in the cytoplasmic tail of the G protein is impossible because this domain does not contain any serine residues (Rose & Gallione, 1981). Our data do also not allow for an acyl shift from cysteine-489 to serine residues at the other side of the lipid bilayer because bromelain digestion removed the [$^3$H]palmitic acid label of membrane-integrated G protein when conditions of proteolysis were chosen in such a way that the ectodomain of the G protein was completely protected by the microsomal membrane (Fig. 2).

The negative result obtained by Rose *et al.* (1984), who observed a loss of acylation after changing cysteine-489 to serine by oligonucleotide-directed mutagenesis, did not prove that fatty acid is linked to cysteine-489 and remained therefore inconclusive. Our data clearly demonstrate that cysteine-489 is not only essential for the initial transfer of palmitic acid but that the fatty acid remained also bound to this amino acid in mature virally integrated G protein. The amount of non-acylated G protein in VSV particles seems to be very low. This high degree of acylation had been previously encountered during studies of [$^3$H]palmitic acid transfer on to G protein of viral particles *in vitro* (Mack *et al.*, 1987).

One can speculate that the modification of the thiol group at the single cysteine residue in the cytoplasmic domain serves the purpose of inhibiting the formation of G protein dimers. An artificial dimerization of G protein could be detrimental for the virus, and might inhibit the normally occurring trimerization of G protein molecules that is thought to be essential for the vectorial transport from the endoplasmic reticulum to the plasma membrane (Doms *et al.*, 1987).

From our data we cannot exclude that fatty acids are linked to other amino acids in addition to cysteine-489; however, those would have also to be located in the cytoplasmic domain. The enrichment of serine in acyl-peptide preparations that was previously reported (Schmidt & Schlesinger, 1979) may be explained by co-purification of serine-containing hydrophobic peptides with [$^3$H]palmitic acid-labelled acyl peptides. On the other hand, detection of cysteine is often quite difficult because it is frequently almost completely destroyed during acid hydrolysis for amino acid analysis (Scoffone & Fontana, 1975).

The release of covalently bound palmitic acid by hydroxylamine under defined conditions from model compounds, e.g. *O*-acetylserine, palmitoylserine and palmitoyl-CoA, has been used to discriminate acyl linkages to specific amino acids like serine and cysteine (Schmidt & Lambrecht, 1985). Schmidt & Lambrecht (1985) reported a significant release of fatty acids from $E_1$ and $E_2$ glycoproteins of Semliki Forest virus and the haemagglutinin of influenza virus after treatment with 0.32 M-hydroxylamine, pH 6.6, for 1 h at 37 °C. Since *O*-acetylserine was also significantly cleaved starting from 0.32 M-hydroxylamine but cleavage of the thioester palmitoyl-CoA occurred already at 0.05 M-hydroxyl-

**Table 1. Amino acid sequences surrounding covalent attachment sites of fatty acids to acyl-proteins**

| Protein | Amino acid sequence | Reference |
|---|---|---|
| VSV G protein | Ile His Leu Cys* Ile Lys Leu | Rose & Gallione (1981) |
| HLA-B7 heavy chain | Ala Val Met Cys* Arg Arg Lys | Ploegh *et al.* (1981) |
| HLA-D invariant chain | Glu Ser Lys Cys* Ser Arg Gly | Claesson *et al.* (1983) |
| Transferrin receptor | Pro Lys Arg Cys* Ser Gly Ser | McClelland *et al.* (1984) |
| $p21^{ras}$ | Ser Cys Lys Cys* Val Leu Ser | Capon *et al.* (1983) |
| Lipophilin | Ser Ile Gly Thr* Leu Cys Ala | Stoffel *et al.* (1983) |

* Amino acid residue in which palmitic acid is covalently attached.

amine, they assigned the acyl linkages of those viral glycoproteins to serine esters.

We also found a significant release of [$^3$H]palmitic acid from the G protein starting from 0.32 M-hydroxylamine, pH 6.8, under similar conditions, showing that thioester linkages of fatty acids to cysteine residues may be much more stable than previously considered from the analysis of palmitoyl-CoA, in which the palmitic acid is bound in a thioester linkage to CoA (Fig. 3). At present, both thioester and ester bonds of long-chain fatty acids to proteins have been described (Table 1). It seems questionable that cleavage of acyl linkages by hydroxylamine can be used at all to differentiate between ester and thioester linkages of long-chain fatty acids. Nevertheless, it would be useful to develop a simple procedure for distinguishing ester and thioester linkages of fatty acids to proteins by a simple chemical reaction. To reach this goal reference compounds have to be used that resemble the type of acyl linkage in acyl-proteins to a greater extent, i.e. palmitic acid linked to the respective amino acids or to defined peptides.

The site for fatty acid attachment has been determined for only a few palmitoylated proteins. In addition to the G protein, these are the HLA-B7 heavy chain (Kaufman *et al.*, 1984), the HLA-D invariant chain (Koch & Hämmerling, 1986), the human transferrin receptor (Jing & Trowbridge, 1987), $p21^{ras}$ (Chen *et al.*, 1985), in which thioester linkages to cysteine residues have been identified, and bovine brain lipophilin (Stoffel *et al.*, 1983), in which palmitic acid is linked to a threonine residue. In proteins acylated via thioester bonds to cysteine, the fatty acids are topologically located on the cytoplasmic side of intracellular membranes, whereas threonine residues that covalently bind palmitic acid are thought to reside on the extracellular side of the plasma membrane. In Table 1 amino acid sequences surrounding the fatty acid binding sites are compiled for comparison. No common sequences can be identified that could act as a signal for the protein fatty acyltransferase except that positively charged amino acids occur in the immediate vicinity of the modified amino acid residue. Another common feature of membrane proteins acylated at cysteine residues is the localization of their modified cysteine residues close by the transmembrane domains at the cytoplasmic side.

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