Cytoplasmic tail length influences fatty acid selection for acylation of viral glycoproteins

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We report remarkable differences in the fatty acid content of thioester-type acylated glycoproteins of enveloped viruses from mammalian cells. The E2 glycoprotein of Semliki Forest virus contains mainly palmitic acid like most other palmitoylated proteins analysed so far. However, the other glycoprotein (E1) of the same virus, as well as the HEF (haemagglutinin esterase fusion) glycoprotein of influenza C virus, are unique in this respect because they are acylated primarily with stearic acid. Comparative radiolabelling of uninfected cells with different fatty acids suggests that stearate may also be the prevailing fatty acid in some cellular acylproteins. To look for further differences between palmitoylated and stearoylated glycoproteins we characterized stearoylation in more detail. We identified the acylation site of HEF as a cysteine residue located at the boundary between the transmembrane region and the cytoplasmic tail. The

attachment of stearate to HEF and E1 occurs post-translationally in a pre-Golgi compartment. Thus, stearoylated and palmitoylated proteins cannot be discriminated on the basis of the fatty acid linkage site or the intracellular compartment, where acylation occurs. However, stearoylated acylproteins contain a very short, positively charged cytoplasmic tail, whereas in palmitoylated proteins this molecular region is longer. Replacing the short cytoplasmic tail of stearoylated HEF with the long influenza A virus haemagglutinin (HA) tail in an HEF–HA chimera, and subsequent vaccinia T7 expression in CV-1 cells, yielded proteins with largely palmitic acid bound. The reverse chimera, HA–HEF with a short cytoplasmic tail was not fatty acylated at all during expression, indicating that conformational or topological constraints control fatty acid transfer.

INTRODUCTION

The covalent attachment of fatty acids in a thioester-type linkage to membrane proteins (palmitoylation) is a widespread modification of cellular as well as viral polypeptides (reviewed in [1–4]). The biological function of this protein modification is not well defined. The carbon chain may cause targeting and membrane binding of proteins, which have no stretch of hydrophobic amino acids long enough to span the lipid bilayer [5–7]. However, in integral membrane proteins the fatty acids are not required for stable membrane anchoring and intracellular transport of the respective acylprotein (e.g. [8]). Recently, it has been suggested that the fatty acids are involved in protein–protein interactions between peripheral and integral membrane proteins. These interactions are required for assembly and release of virus particles [9] and for signal-transduction from membrane receptors to G-proteins in eukaryotic cells [10,11].

This latter hypothesis is supported by the analysis of fatty acid-binding site(s) in integral membrane proteins. The carbon chain is usually bound to cysteine residues located at the boundary between the transmembrane region and the cytoplasmic tail [8,12–17]. The attachment of fatty acids in a thioester-type linkage is a post-translational event, whereby the Co-A fatty acid esters serve as lipid donors [18]. For glycoproteins in transit from the endoplasmic reticulum (E. R.) to the plasma membrane palmitoylation occurs at a pre-Golgi site, i.e. after oligomerization but before carbohydrates are trimmed in the Golgi apparatus [19–21]. However, with some proteins palmitoylation is a late event, probably occurring at the plasma membrane after deacylation of the initially bound fatty acid [22–24].

Another important issue concerning the biochemistry of palmitoylation is the question of which fatty acid species are linked to a given acylprotein. This is of considerable interest, because carbon chains which differ by only two C-atoms (e.g. palmitic acid and stearic acid) show a significant difference in their hydrophobicity. Thus, acylation of proteins with fatty acids of different chain length has probably also an influence on the degree of hydrophobicity of their acylated domain. Furthermore, it has been shown, that [³H]palmitic acid, which is used to label acylated proteins, can be converted into other fatty acid species of different chain length or saturation before it is attached to the acylprotein [25]. Thus, if radioactivity derived from [³H]palmitic acid labelling is eventually detected in a given protein, it does not necessarily mean that palmitic acid (C_{16:0}) is exclusively or even predominantly present as the protein-bound acyl chain species. Instead, varying amounts of other fatty acid species including myristic (C_{14:0}), stearic (C_{18:0}) and oleic acids (C_{18:1}) are also detected [26–31].

We described recently that the haemagglutinin esterase fusion glycoprotein (HEF) of influenza C virus is unique in this respect, because it contains mainly stearic acid as protein-bound fatty acid [16]. Subsequently, thioester-type acylation of some proteins with unsaturated fatty acids like palmitoleic (C_{16:1}), arachidonic (C_{20:2}) and eicosapentanoic acids (C_{20:5}) has been reported for yeast and mammalian cells [32–34]. This shows that acylation of cysteine residues is more heterogeneous with respect to the utilized carbon chain than originally anticipated. However, it is not known whether these ‘unconventional’ fatty acids are linked to the same cysteine residue(s) as palmitate. Furthermore, the signals which determine the attachment of fatty acids other than

Abbreviations used: HEF, influenza C virus haemagglutinin esterase fusion glycoprotein (C/JHB/1/66 strain); HA, influenza A virus haemagglutinin (subtype H7); PAT, S-protein acyltransferase(s); HEF–HA and HA–HEF, chimeric proteins; E. R., endoplasmic reticulum; DMEM, Dulbecco's minimal essential medium; SFV, Semliki Forest virus; SV 40, simian virus 40; MDBK, Madin Darby bovine kidney; MDCK, Madin Darby canine kidney; BHK, baby hamster kidney.

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palmitate, and the timing of acylation with unusual fatty acids during the biosynthesis and maturation of these proteins are completely unknown at present.

In this study we have addressed some of these open questions using the HEF protein of influenza C virus [35], which is acylated mainly with stearic not palmitic acid as are most other viral glycoproteins. We have determined the fatty acid linkage site for HEF as a cysteine residue located at the boundary between the transmembrane region and the cytoplasmic tail. We have identified another viral glycoprotein, E1 of togavirus, which contains predominantly stearic acid. Both stearylabeled acylproteins (E1 and HEF) contain a very short, positively charged cytoplasmic tail, whereas in palmitoylated proteins [E2 and influenza A viral haemagglutinin (HA)] this molecular domain is longer. When we replaced the short cytoplasmic tail of HEF with the long cytoplasmic C-terminus of HA in a chimera HEF-HA, stearyl- acylation decreased and palmitoylation increased significantly. The reverse chimera HA-HEF was not acylated at all despite functional acylation sites and a membrane location of the expression product. Comparative labelling of various uninfected mammalian cells with [^3H]palmitic acid or [^3H]stearic acid of the same specific radioactivity indicates that stearate may be the prevailing fatty acid in specific sets of cellular acylproteins. We therefore suggest that in acylated transmembrane proteins the length of the cytoplasmic tails could influence the substrate selection for fatty acid transfer.

MATERIALS AND METHODS

Cells and virus

Mardin Darby bovine kidney (MDBK), Madin-Darby canine kidney (MDCK), baby hamster kidney (BHK) cells and a kidney cell line derived from African green monkeys (CV-1) were cultured in Dulbecco's minimal essential medium (DMEM) supplemented with 5% (v/v) fetal calf serum. For the preparation of viral acylproteins confluent monolayers of susceptible cell types were infected at a multiplicity of infection of 10 to 50 with one of the following viruses: influenza C virus (strain JHB/1/66), influenza A virus (A/chick/Germany/N/49, H10N2, virus N), Semliki Forest virus (SFV; strain Osterrieth) or recombinant simian virus 40 (SV 40)/HEF virus.

Labelling and/or purification of acylproteins

Virus-infected cells

SFV-infected BHK cells, influenza virus-infected MDCK cells or SV 40/HEF virus-infected CV-1 cells were labelled with ^3H -fatty acids ([9,10- ^3H]myristic acid or [9,10- ^3H]palmitic acid, 30–50 Ci/mmol, DuPont, Bad Homburg, Germany; 1 mCi/ml of medium) at 3 (BHK), 7 (MDCK) or 48 h (CV-1) after infection for the periods of time indicated in the Figure legends. The cells were then lysed in SDS-containing RIPA-buffer, clarified from insoluble material by centrifugation at 20000 *g* for 15 min and the supernatant was subjected to immunoprecipitation using polyclonal rabbit sera against influenza virus or SFV particles as detailed previously [36]. Immunoprecipitates were separated by SDS/PAGE using 10% polyacrylamide gels under reducing (influenza virus) or non-reducing (SFV) conditions. To minimize a possible cleavage of thioester-linked fatty acids by reducing agents [13,29], heating of samples prior to SDS/PAGE was avoided. The gels were then analysed by fluorography using the salicylate method [37].

To analyse the timing of E1 and E2 acylation, protein synthesis in SFV-infected BHK cells was blocked by the addition of cycloheximide (final concentration of 10 $\mu\text{g}/\text{ml}$) for 10, 20, 30, 40

or 70 min and cells were labelled with [^3H]palmitic acid during the last 10 min of this treatment. Viral proteins were then immunoprecipitated and subjected to SDS/PAGE and fluorography. The intensity of E1 and p62/E2 bands was quantified with a white light scanner (Hauteck) using the software 'PDI Quantity 1'.

To compare acylation of cellular proteins with different fatty acids, uninfected MDCK cells were labelled for 4 h with 500 $\mu\text{Ci}/\text{ml}$ each of [^3H]myristic, [^3H]palmitic or [^3H]stearic acids ([9-10- ^3H]stearic acid, 60 Ci/mmol; ARC, St. Louis, MO, U.S.A.). Specific radioactivity was adjusted to 20 Ci/mmol for each of the tritiated fatty acids prior to labelling. Cells were then lysed in gel-loading buffer containing SDS (2%) and mercaptoethanol (5%). The samples were boiled for 1 min and insoluble material was removed by centrifugation (20000 *g*, 15 min). Equal aliquots of each sample were subjected to SDS/PAGE in 10% gels and fluorography.

Virus particles

BHK cells were infected with SFV as described above and either labelled with [^3H]palmitic acid (20 $\mu\text{Ci}/\text{ml}$) 3 h after infection or left untreated. After 15 h the cell culture fluid was clarified from cell debris by low-speed centrifugation (2500 *g* for 15 min). Virus particles were pelleted from the resulting supernatant at 50000 *g* for 2 h. Acylproteins were purified by preparative SDS/PAGE.

Fatty acid analysis

TLC

The respective acylprotein bands were cut out off the gel and gel pieces were soaked in water for 2×15 min. Fatty acids were liberated by treatment of the dried gel slices with 6 M HCl for at least 16 h at 110 $^{\circ}\text{C}$ in tightly sealed ampoules. Fatty acids were then extracted three times with hexane, pooled and concentrated in a stream of nitrogen. To analyse the lipid-linked fatty acids of the viral envelope, [^3H]palmitic acid-labelled SFV particles were extracted with chloroform/methanol (2:1, v/v). Fatty acids were cleaved with 0.3 M NaOH in methanol and, after acidification, extracted with hexane [25]. Separation into individual fatty acid species was done on RP-18 TLC plates (Merck, Mannheim, Germany) with acetonitrile/acetic acid (1:1, v/v) as the solvent system. Fatty acid species were detected with a radiochromatogram scanner (LB 2821, Berthold, Wildbad, Germany) or visualized by fluorography after spraying the plates with EN 3 HANCE (DuPont). For identification of individual fatty acid species, radiolabelled reference substances ([^3H]myristic acid, [^3H]palmitic acid and [^3H]stearic acid) were run on the same plate.

HPLC

Fatty acids from acylated proteins and host cell lipids were prepared as described for fatty acid analysis by TLC and for lipid-linked fatty acids of the viral envelope, respectively. The dried hexane extracts were redissolved in 50 μl of methanol containing 100 μg of non-labelled myristic, palmitic and stearic acids as internal standards. HPLC was performed using a Nova-Pak C $_{18}$ column (Waters, Eschborn, Germany) and 90% acetonitrile as eluent at a flow rate of 1 ml/min. Fractions were collected every 30 s and analysed by scintillation counting. Fatty acid species were identified by the retention time of the unlabelled standard fatty acids detectable with an absorbance detector set at 214 nm.

GC analysis

The respective proteins were excised from the preparative gel (0.5 cm thick) and purified by electrophoretic elution. After dialysis for 5 days against 50 mM NH_4HCO_3 buffer, the proteins were lyophilized. After two extractions with chloroform/methanol (2:1, v/v), covalently bound fatty acids were released from the protein by a 20 min treatment with 0.3 M NaOH in 90 % (v/v) methanol at 100 °C and, after acidification, extracted three times with hexane. The combined extracts were dried in a stream of nitrogen and the fatty acids were methylated with 14 % borotrifluoride in methanol for 75 min at 100 °C. Methyl esters of fatty acids were again extracted three times with hexane and were concentrated. GC of the methylated fatty acids with heptadecanoic acid methyl ester as internal standard was performed with a Sulpeco GP 10 SP-2330 column heated at 200 °C in a Perkin Elmer F 20 gas-liquid chromatograph. The injection temperature was 210 °C. Fatty acids were detected by flame ionization [25].

Recombinant DNA methods

HEF mutants

Recombinant DNA methods were performed as described elsewhere [36,38]. Briefly, site-specific mutagenesis of the cloned HEF gene was done on M13mp18/HEF single-stranded DNA using a commercial kit (Amersham, Braunschweig, Germany) based on the phosphorothioate method. A synthetic oligonucleotide was used which converted the triplet coding for cysteine (TGC) to one encoding serine (AGC). Mutant and wild-type HEF were cloned into the SV 40-based plasmid pA11SVL3 and expressed in CV-1 cells with a SV 40 system exactly as described before [36]. Expression of both proteins was verified by radiolabelling of recombinant SV 40-infected cells with [^{35}S]methionine (EXPRE ^{35}S , 1200 Ci/mmol, DuPont; 50 $\mu\text{Ci}/\text{ml}$ of medium without methionine) followed by immunoprecipitation, SDS/PAGE and fluorography.

HEF-HA chimera

A new *Nde*I restriction site was inserted into the HEF gene by silent mutagenesis of position 1971. After subcloning the mutated HEF gene and wild-type HA into pTM1 plasmid [39], both genes (without the DNA sequences coding for the cytoplasmic tails) were cut out of the vector again by double digestion with *Xba*I and *Nde*I. Subsequently the HEF fragment was ligated to the pTM1 plasmid containing the DNA sequences coding for the C-tail of HA and the HA fragment to pTM1 plasmid containing the DNA sequences coding for the C-tail of HEF. For transient expression CV-1 cells were infected with vTF7-3 vaccinia virus [40] at a multiplicity of infection of 10. Two hours after infection the virus inoculum was replaced by a transfection mix containing 3 μg of pTM1 plasmid DNA with the appropriate insert and 10 μl of lipofectin (BRL) in 1 ml of DMEM. Then 3 h after transfection cells were labelled for 3 h with [^3H]palmitic acid or, as an expression control, with [^{35}S]methionine.

Indirect immunofluorescence

At 48 h after infection with SV 40/HEF virus, CV-1 cells grown on coverslips were fixed with paraformaldehyde (3 % in PBS) for 15 min. Unreacted paraformaldehyde was quenched with 0.1 M glycine. Cells were then permeabilized with Triton X-100 (0.1 % in PBS). The first antibody [anti-(influenza C virus) rabbit serum, diluted 1:500 in PBS containing 2 % BSA] and the second antibody (fluorescein-conjugated porcine immunoglobulins dir-

ected against rabbit immunoglobulins, diluted 1:40; Dakopatts, Denmark) were adsorbed successively to the cells for 1 h. Unbound antibodies were washed off at every step, and coverslips were mounted in 90 % (v/v) glycerol. Cells were examined under a microscope (Zeiss Axiophot) with UV optics and the results were recorded photographically at $\times 630$ magnification.

RESULTS

Fatty acid analysis of HEF immunoprecipitated from influenza C virus-infected cells

Our previous report, that HEF contains predominantly stearic acid as protein-bound acyl chain, was solely based on fatty acid analysis of HEF present in mature virus particles, which had been grown in cells labelled with [^3H]myristic acid or [^3H]palmitic acid for long periods of time [16]. Such experiments did not exclude the possibility that during its transport along the exocytotic pathway HEF was initially acylated with palmitic acid, then deacylated and during assembly of virus particles again re-acylated, but this time with stearic acid. Therefore we analysed also the fatty acid content of HEF present in virus-infected cells. Influenza C virus-infected MDCK cells were labelled for 30 min with either [^3H]myristic acid or [^3H]palmitic acid. Cell-associated HEF was purified by immunoprecipitation and SDS/PAGE and its fatty acid content was analysed by TLC. The acyl chains of HA were also determined using the same experimental conditions. Scanning of the TLC plates revealed, that cell-associated HA contains mainly palmitic acid (Figures

Figure 1 Fatty acid analyses of cell-associated HEF and HA

Influenza C virus- (A and B) or influenza A virus- (C and D) infected MDCK cells were labelled for 30 min with [^3H]myristic acid (A and C) or [^3H]palmitic acid (B and D). The viral acylproteins HEF (A, B) and HA (C, D) were then purified by immunoprecipitation and SDS/PAGE. Protein-bound fatty acids were liberated by acid treatment, extracted and analysed by TLC. A radiochromatogram scan is shown, where relative radioactivity (y axis) is plotted against migration on the TLC plate (x axis). Abbreviations: m, myristic acid; p, palmitic acid; s, stearic acid. The one or two minor peaks on the left-hand side of the plate represent material which remained at the origin or was trapped between the concentration and separation zone of the TLC plate.

1C and 1D), as has been reported before for HA present in virus particles [16,25]. In contrast, HEF is acylated predominantly with stearic acid, regardless of whether [^3H]myristic acid or [^3H]palmitic acid was used for labelling (Figures 1A and 1B). These results show that the difference in the fatty acid content of virus-bound HA and HEF is also detectable in the cell-associated forms of these acylproteins. Furthermore, it is also evident that 30 min of labelling is sufficient for the cells to elongate the carbon chains of both [^3H]myristic and [^3H]palmitic acid into [^3H]stearic acid.

Determination of the acylation site in the HEF glycoprotein

The different fatty acid content of HA and HEF may be simply explained by the possibility that these proteins are acylated by different acyltransferases with pronounced substrate specificities. Possibly these proteins are acylated at different intramolecular sites, e.g. serine versus cysteine or cytoplasmic versus luminal region. We therefore determined the fatty acid linkage site of HEF. It is well established that integral membrane proteins with palmitate as prevailing fatty acid (including HA) are acylated at cysteine residues located at the boundary between the transmembrane region and the cytoplasmic tail [4,13]. The stearylated HEF glycoprotein of influenza C virus also contains one cysteine residue in this region. This cysteine was changed by site-specific mutagenesis of the cloned gene to a serine residue. Subsequently the mutant (M1) and wild-type (WT) HEF were expressed with an SV 40 system in mammalian CV-1 cells. Labelling with [^{35}S]methionine, immunoprecipitation, SDS/PAGE and fluorography revealed, that wild-type and mutant HEF are expressed to similar extents as proteins with an apparent molecular mass of 80 kDa (Figure 2, ^{35}S -MET). To test for acylation of wild-type and mutant HEF, recombinant SV 40-infected cells were labelled for 4 h with [^3H]palmitic acid (^3H -PAL). As apparent in Figure 2, [^3H]palmitic acid-derived labelling was clearly detectable in the wild-type HEF, but not in its cysteine-mutant (M1). This reveals, that both stearic acid and the smaller amount of palmitic acid, which were determined as HEF-bound fatty acids (see Figure 1), are probably attached to the same intramolecular site, Cys-652 (Figure 2, bottom). This is consistent with our previous observation, that the fatty acid bond in HEF is sensitive to neutral hydroxylamine, which had already indicated a thioester-type linkage [16]. With no differences in the acylated amino acid species or its intramolecular topology between HEF and 'conventional' palmitoylated proteins (such as HA), some other, hitherto undefined, molecular feature encoded in this region must determine which fatty acid is preferentially added to a protein.

Acylproteins with a very short, positively charged cytoplasmic tail contain predominantly stearic acid

One characteristic structural feature of the HEF glycoprotein is its very short, positively charged cytoplasmic tail (Arg⁺ Thr Lys⁺). A similar cytoplasmic tail (Arg⁺ Arg⁺) is present in the E1 acylprotein of the togaviruses SFV and the related Sindbis virus, whereas the other acylated glycoprotein (E2) of the same viruses contains a longer cytoplasmic domain. The fatty acid content of E1 plus E2 has been shown before to consist mainly of palmitic acid [25]. However, both acylproteins had been combined for this analysis and the 3–5-fold larger amount of fatty acids linked to E2 could have obscured a possible differential acylation of both proteins. We therefore purified E1 and E2 from [^3H]palmitic acid-labelled SFV particles and determined their fatty acid content separately. Lipid-linked fatty acids from the viral envelope were also analysed. Fluorography of the TLC plate

Figure 2 Identification of the acylation site in the HEF glycoprotein

Wild-type (WT) HEF and the HEF mutant, where the only cysteine in the transmembrane region was replaced by serine (M1), were expressed with an SV 40 system in CV-1 cells and labelled for 4 h with [^{35}S]methionine (^{35}S -MET) or [^3H]palmitic acid (^3H -PAL). HEF was then immunoprecipitated from cell extracts and subjected to SDS/PAGE and fluorography. Abbreviations: UN, uninfected CV-1 cells; SV, cells infected with non-recombinant SV 40; MW, ^{35}S -labelled molecular-mass markers (phosphorylase B 92.5 kDa, BSA 69 kDa, ovalbumin 46 kDa and carbonic anhydrase 30 kDa). The lower panel shows the amino acids at the boundary between the transmembrane region and the cytoplasmic tail of WT and M1 HEF. Wild-type HEF, but not the fatty acid-free mutant, is present in this gel as a doublet band. However, two bands of HEF were not always resolved by SDS/PAGE (results not shown), albeit identical conditions for electrophoresis were used each time. The occasional occurrence of doublet bands of HEF has been described, but their origin and significance is unclear (see [35]).

revealed that [^3H]palmitic acid is the predominant fatty acid in viral lipids, as well as in the E2 glycoprotein. In contrast, stearic acid is the main fatty acid in the E1 protein (Figure 3). Practically identical fatty acid patterns were obtained for [^3H]palmitic acid-labelled E1 and E2 of the closely related Sindbis virus (results not shown). These results are consistent with our previous observation that labelling of SFV proteins with [^{14}C]stearic acid revealed a larger amount of label appearing in E1, although E2 is acylated at severalfold higher stoichiometry [25].

One drawback of the method to determine only the ^3H -

Figure 3 Fatty acid analyses of E1 and E2 acylproteins of SFV particles

TLC of E1- and E2-linked fatty acids after labelling SFV particles with [^3H]palmitic acid and isolating the glycoproteins by preparative PAGE. Lip: analyses of fatty acids linked to the lipids of the viral envelope. Pal and Stear indicate the positions of authentic palmitic acid and stearic acid, respectively.

labelled fatty acids of a given protein after metabolic labelling, is the possibility that the result may not necessarily reflect the real fatty acid composition and stoichiometry in an acylprotein, but is superimposed by the metabolism of the ^3H -labelled fatty acids. Therefore the proteins were purified from virus particles on a larger scale by preparative PAGE and the unlabelled fatty acids linked to E1 and E2 were analysed, this time by GC. The results from duplicate analysis of 96 nmol of E1 and 42 nmol of E2 revealed that both proteins contain only traces of myristic acid ($< 3\%$) and approximately one-third of unsaturated fatty acids, which consist mainly ($> 90\%$) of oleic acid. Unsaturated fatty acids were not considered previously because they do not separate from saturated species by TLC, i.e. oleic acid runs to the same position as palmitic acid. However, the main fatty acid linked to E2 is palmitic acid (42%) versus 23% of stearic acid. In contrast to E2, and as expected from the experiments shown above (Figure 3), E1 contains predominantly stearic acid (49%) and only 13% of palmitic acid. Deviations between individual values in the two experiments were less than 10% (not shown in Figures, compare [41]). These results prove that E1 and E2 are indeed predominantly acylated with stearic and palmitic acids, respectively.

Timing of stearoylation of E1 and HEF

The differential acylation of E1 and E2 may not be caused by structures encoded in the protein itself, but may be due to the possibility that E1 and E2 are acylated at different intracellular sites where either palmitoyl-CoA or stearoyl-CoA are present as the prevailing fatty acid donor. To exclude this possibility we compared the timing of E1- and E2-acylation relative to the translation of their polypeptide chains. If this method reveals a severe difference this would argue for different intracellular sites for acylation of E1 and E2, because both proteins assemble to a hetero-oligomer in the E. R. and are therefore transported together with the same kinetics to the cell surface [42]. To analyse the timing of E1 and E2 acylation, SFV-infected cells were treated with cycloheximide for 10, 20, 30, 40 or 70 min and labelled with ^3H palmitic acid during the last 10 min of the translation block. As a control for the absolute amount of radioactivity incorporated in the acylproteins, untreated cells were also labelled for 10 min. Immunoprecipitation of viral proteins, SDS/PAGE, fluorography and quantification of bands revealed (Figure 4), that labelling of both E2 and its precursor p62 as well as E1 decreases continually with increasing time of cycloheximide treatment. This indicates that within the limitations of this method acylation of E1 and E2 occurs at roughly the same time after translation of their polypeptide chains, probably at the same intracellular site.

Next we examined the intracellular site where stearoylation of HEF occurs. We took advantage of our previous observation, that the cloned HEF gene expressed in mammalian cells in the absence of other influenza C virus proteins is not transported to the cell surface [35,38]. Despite its block in intracellular transport, the HEF glycoprotein is acylated (lane marked WT in Figure 2). Therefore, determination of the intracellular site where the transport of HEF is arrested would help to identify the site of its acylation. In order to determine the intracellular distribution of HEF in more detail we applied immunofluorescence studies on permeabilized, recombinant SV 40-infected cells. The results show (Figure 5A), that HEF is present in reticular structures, which are distributed throughout the cytoplasm of the cell and which are typical for the E. R. The bright staining of the perinuclear region, typical for glycoproteins in transit to the cell

Figure 4 Timing of E1 versus E2 acylation

SFV-infected BHK cells were treated with cycloheximide for 10, 20, 30, 40 or 70 min and labelled with ^3H palmitic acid during the last 10 min of this treatment. Untreated cells (0) were also labelled with ^3H palmitic acid for 10 min. Viral proteins were immunoprecipitated from cell extracts and subjected to SDS/PAGE. Upper panel: fluorography of the resulting gel. The fluorograms are different exposures of the same gel to make the lower amount of fatty acids linked to E1 visible. Exposure times were 1 week for p62/E2 and 3 weeks for E1. Using only short pulses of ^3H palmitic acid, radioactivity is incorporated mainly in the p62 precursor of E2, because p62 is the primary acceptor of the acyl chains. p62 is then proteolytically processed into E2 [18]. Lower panel: quantification of the bands shown in the upper panel by white light scanning. Relative radioactivity (untreated cells: 100%) as implied from the absorbance of each band is plotted against the time of cycloheximide treatment. ●, E1; ▲, p62/E2.

surface (shown in Figure 5, panel C, for the HA of influenza A virus) and usually representing the Golgi apparatus, was never observed for the HEF glycoprotein. This suggests that the intracellular transport of HEF along the exocytotic pathway is blocked at a pre-Golgi site. This assumption is also supported by the observation, that HEF expressed in CV-1 cells contains only endoglycosidase-H-sensitive, unprocessed carbohydrates (not shown in Figures). Since we had observed stearoylation of HEF under the same conditions, these results imply that acylation of HEF occurs before it reaches the Golgi apparatus.

Taken together, the attachment of stearic acid on to E1 and HEF occurs post-translationally, but at a site between the E. R. and the Golgi apparatus, which is similar to the site where the attachment of palmitate to other viral glycoproteins was located [19–21]. To examine a possible influence of acylation on the intracellular transport of HEF, similar immunofluorescence studies were done for the fatty acid-free mutant protein HEF-M1. As shown in Figure 5(B), the fluorescent staining of fatty acid-free HEF is clearly different from HA, but resembles the staining of acylated HEF. This indicates that acylation of E1 and E2 are both early modifications which do not occur at the plasma membrane. However, given the methodological limitations, subtle differences in the intracellular site of palmitoylation and stearoylation (e.g. late ER versus *cis*-Golgi network) cannot be completely excluded.

Figure 5 Intracellular distribution of HEF expressed with an SV 40 system in CV-1 cells

Recombinant SV 40/HEF- or SV 40/HA [36]-infected cells were analysed by intracellular immunofluorescence. All micrographs are shown at the same magnification with the nuclei visible in (A) and (B) indicating about 10 μm diameter. (A) WT HEF, (B) fatty acid-free HEF, (C) HA of influenza A virus, and (D) cells infected with non-recombinant SV 40.

Replacement of the short C-tail in HEF with the long one of HA in chimeric HEF–HA increases palmitoylation at the expense of stearylation

In order to test our above hypothesis that the length of the C-tail in transmembrane proteins may influence the selection of fatty acid species for acylation, we constructed the two obvious chimeric molecules. HEF–HA and HA–HEF were prepared as described in the Materials and methods section and then expressed by the vaccinia T7 system in CV-1 cells in the presence of [^3H]palmitic acid (see Figure 7). Protein bands of the HEF–HA chimera were excised from polyacrylamide gels and processed for fatty acid analysis by HPLC. Likewise, total lipids of CV-1 cells were analysed to determine the general acyl-chain pattern present in CV-1 cells after [^3H]palmitate labelling for increasing periods of time. The results from a comparative kinetic analysis of conversion of the input of [^3H]palmitic acid show that CV-1 cells are less efficient than BHK cells in this respect [25]. The former cells yield approximately 10 and 15 % stearic acid and 5 and 7 % myristic acid from total fatty acids present in cellular lipid after labelling with [^3H]palmitic acid for 2 and 4 h, respectively. In contrast BHK cells yield up to 20 % of stearic acid after the same labelling period with [^3H]palmitic acid (4 h) (results not shown in Figures). Although quite a low proportion of [^3H]stearic acid is

found in total cellular lipids (Figure 6A), HEF protein expressed in the same cells clearly selected for stearic acid from this pool (Figure 6D) while HA protein contained predominantly [^3H]palmitic acid with only moderate binding of [^3H]stearate (Figure 6B), both as expected (see Discussion). HEF–HA, which has the long C-tail of HA, shows clearly increased palmitoylation (Figure 6C) when compared with HEF (Figure 6D). This supports the above hypothesis that the length of the C-tail contributes to acyl selection during acylation. Our attempts to substantiate this finding further with the reverse chimera HA–HEF yielded an unexpected finding, which precluded the projected fatty acid analysis. This construct was successfully expressed, but no labelled fatty acid could be incorporated at all (Figure 7). The reason for this is unclear at present, although preliminary results suggest its transport is blocked inside the E. R. Therefore, either the conformation of this particular chimera (HA–HEF) is not suitable for acyl transfer, or it fails to reach the acylation site during its biosynthesis.

Stearic acid labelling of cellular proteins

Because most viral glycoproteins fully depend on cellular enzymes for their synthesis and post-translational processing, we wondered whether there were also cellular proteins which contain

Figure 6 Fatty acid analysis of HEF, HA and HEF-HA expressed in CV-1 cells

HA, HEF-HA and HEF (panels **B**, **C** and **D**) were expressed in CV-1 cells with the vaccinia T7 system and labelled with [3 H]palmitic acid. Fatty acids from proteins and cellular lipids (from WT vaccinia-infected CV-1 cells, panel **A**) were analysed by HPLC. Radioactivity measured in the HPLC-fractions is plotted against the retention time and the percentage share of each fatty acid species indicated for each particular experiment shown in the different panels. In two separate repeat experiments the deviations in percentage distribution were below 10% of each of the values shown. The retention times for authentic standard fatty acids were 2 min for myristic acid, 6.5 min for palmitic acid and 11 min for stearic acid, respectively.

mainly stearic acid. For detection of preferential acylation of cellular proteins with stearic acid, uninfected MDBK cells were labelled for 4 h with either [3 H]myristic acid (Myr), [3 H]palmitic acid (Pal) or [3 H]stearic acid (Stear), all with the same specific radioactivity, and cellular extracts were subjected to SDS/PAGE and fluorography. The results in Figure 8 show a very distinct protein pattern for the myristoylated species as expected on the basis of previous reports by others [43,44]. However, despite some overlap with a few proteins, [3 H]palmitate- and [3 H]stearate-labelled polypeptides appear at different positions in the gel (Figure 8). As with the MDBK cells shown here, palmitoylated cellular polypeptides can also be differentiated from stearoylated ones in BHK-, CV-1- and CHO-cells (results not shown in Figures). Since it is very likely that cells contain many different transmembrane proteins, it is conceivable that this apparent fatty acid selection may correlate with different C-tail structures of these proteins.

Figure 7 Vaccinia T7 expression of HEF-HA and HA-HEF chimeras

HEF-HA and HA-HEF chimeras were expressed in CV-1 cells using the vaccinia T7 system. Cells were labelled for 3 h with [35 S]methionine (**A**) or with [3 H]palmitic acid (**B**); chimeric proteins were immunoprecipitated with polyclonal rabbit sera directed against influenza C virus (HEF-HA chimera) or influenza A virus (HA-HEF chimera) and analysed with SDS/PAGE followed by fluorography.

Figure 8 Comparative labelling of uninfected cells with different fatty acids

Uninfected MDBK cells were labelled for 4 h with [3 H]myristic acid (Myr), [3 H]palmitic acid (Pal) or [3 H]stearic acid (Stear) and total cellular proteins were subjected to SDS/PAGE and fluorography. Cellular lipids co-migrate with the dye-front during SDS/PAGE. Since the lane which shows the proteins after labelling with [3 H]palmitic acid (designated Pal_(A)) appeared overexposed in the 3-week original fluorography of the complete gel, an 8-day exposure for this lane is shown separately (designated Pal_(B)). Positions of the molecular-mass marker proteins are indicated on the left.

DISCUSSION

Although the ester-type acylation of proteins was the first one of the hydrophobic modifications of proteins described in the literature [26,45], our knowledge about its biochemistry, enzymology and biological significance is still rudimentary. Palmitoylation has now been reported for about 100 proteins of viral as well as cellular origin, including polypeptides of medical significance like various membrane receptors, α -subunits of heterotrimeric G-proteins and tyrosine kinases [2,4,11]. However, many investigators show only labelling of the respective proteins with [3 H]palmitic acid and characterize its linkage, but do not analyse the actual protein-bound fatty acids. In the cases where

the fatty acid content of acylproteins has been analysed, palmitate was the prevailing fatty acid, although significant differences in the relative amounts of individual fatty acid species have been reported [26–31]. Adding to this variation, we showed recently that the glycoprotein HEF purified from influenza C virus particles is acylated mainly with stearic acid, regardless of whether [^3H]myristic acid or [^3H]palmitic acid was used for labelling [16]. In this study we investigated this unusual attachment of stearic acid in more detail. We identified two other glycoproteins, E1 of SFV and of Sindbis virus, which contain mainly stearic acid, whereas the second glycoprotein of both these viruses (E2) is acylated primarily with palmitic acid (shown for SFV in Figure 3).

How can a cell achieve such differential acylation of proteins? There are at least two possibilities to explain the different fatty acid content of acylproteins. First, acyl selectivity may simply be determined by the relative amounts of individual fatty acid-CoA species present at the particular intracellular site where acylation occurs. These fatty acid-CoA pools may differ in each cell type or even at specific intracellular locations. Alternatively, it is possible that a given acylprotein itself selects its fatty acid from a mixed acyl-CoA pool by an unknown mechanism, thereby involving a low-specificity S-protein acyltransferase (PAT). At least for the differential acylation of E1 and E2 of SFV and Sindbis virus the second possibility is evident, because both proteins are synthesized in the same cell during the same time and are most likely acylated at the same intracellular site (see below).

This acyl specificity is not restricted to viral acylproteins. Comparative labelling of different types of uninfected mammalian cells with palmitate and stearate suggests that preferential acylation with stearic acid also occurs for some cellular proteins (Figure 8). This is consistent with previous observations, that stearic acid accounts for up to 40% of total ester-bound fatty acids in whole cellular proteins after labelling with [^3H]palmitic acid [46,47]. However, cellular proteins with stearic acid as the predominant protein-bound carbon chain remain to be identified.

In the case of N-terminal myristoylated and ester-type palmitoylated proteins it is now well established that they are acylated by completely different enzymes, which modify proteins at distinct molecular sites and in a different type of linkage [48,49]. However, different fatty acid linkage sites are not obvious in palmitoylated and stearylized proteins, although both fatty acid types are sensitive to hydroxylamine treatment (results not shown in Figures). We identified the linkage site for stearate in HEF by mutational analysis and show here that replacing a single cysteine residue with serine by site-specific mutagenesis completely abolishes acylation of HEF (Figure 2). This cysteine residue is located at the boundary between the transmembrane region and the cytoplasmic tail, which is the same molecular region of palmitoylated proteins where fatty acids are usually attached to cysteine residues (reviewed in [4]). The possibility that the recombinant DNA approach used here does not identify the acylation site, but destroys only part of a specialized signal required for the attachment of stearic acid elsewhere, seems unlikely, because direct biochemical analysis of the linkage site in glycoprotein E1 of SFV and of vesicular stomatitis virus G-protein revealed a cysteine residue in this molecular region [13,50], although it was not known at the time that E1 contains mainly stearic acid (Figure 3).

Differential acylation with palmitate and stearate could also be explained, if the attachment of fatty acids occurs at different intracellular sites perhaps by different enzymes. However, acylation of both SFV E1 and E2 occurs approximately at the same time after synthesis of their polypeptide chains (Figure 4). Because

E1 and E2 assemble to an hetero-oligomer in the E. R. and are transported with the same kinetics to the cell surface [42], this result shows that acylation of both proteins is an early modification. Using immunofluorescence methods to study the intracellular distribution of HEF glycoprotein, we show that stearylization occurs in a pre-Golgi compartment (Figure 5). This is consistent with an earlier report, that transfer of stearate to E1 occurs before its carbohydrates are processed by the Golgi-located enzyme mannosidase I [20]. It is unlikely that stearylized proteins are initially acylated with palmitate in this compartment, then deacylated and re-acylated with stearate at a different intracellular site, because short-pulse labelled, intracellular HEF has the same preference for stearic acid as long-term labelled HEF purified from virus particles (Figure 1; [16]).

Thus, palmitoylated and stearylized proteins cannot be discriminated on the basis of their fatty acid linkage site and the intracellular compartment where acylation occurs. It is therefore possible that there is only one type of PAT, which links both fatty acid species to cysteine residues. This enzyme's specificity for particular lipid donors may be regulated by the protein which is destined to be acylated. Alternatively, there may be two or even more related enzymes with similar intracellular distribution and membrane topology, but different, fixed specificities for activated fatty acids. In this case distinct affinities of these enzymes for a given acylprotein may determine which fatty acids are preferentially attached. However, in both cases the acyltransferase(s) must recognize structural features in an integral membrane protein besides the acylation site. This assumption is consistent with our preliminary data, that introducing a cysteine residue in the palmitoylation area of a normally not palmitoylated integral membrane protein is not sufficient to cause acylation [51]. However, to answer these open questions requires purification of PAT and characterization of its lipid and acceptor specificities beyond the depth of previous reports [49,52] and of a recent paper by Berthiaume and Resh [53]. The need to purify this difficult enzyme has become even more important since results from *in vitro* assays are emerging which are interpreted to indicate non-enzymic mechanisms of acylation ([54]; C. Bano and A. I. Magee, personal communication), but which could not explain differential acylation (this report) and the lack of acylation of our HA-HEF chimera (Figure 7, see below) and of mutant proteins with new acylation sites [51].

In which part of an acylated molecule may the signal for preferential acylation with stearic acid be located? Figure 9 shows an alignment of published amino acid sequences near the acylation sites of integral membrane proteins. This alignment is based on the widely believed assumption, that the first charged amino acid marks the boundary between the transmembrane region and the cytoplasmic domain. Assuming that this is indeed the case, it is striking, that stearylized glycoproteins contain a very short, positively charged cytoplasmic tail (HEF: Arg⁺ Thr Lys⁺; E1: Arg⁺ Arg⁺) whereas in palmitoylated glycoproteins this cytoplasmic domain is longer, at least 11 amino acids in the case of HA. This suggests that the length and/or charge of the cytoplasmic tail determines which fatty acid is added to a given protein. But even HA and HEF-HA select stearate over total cellular fatty acids. Compared with most cellular glycoproteins (e.g. membrane receptors, compare Figure 9) HA also contains a relatively short cytoplasmic tail. Thus, it is tempting to speculate that the amount of stearate decreases if the length of a cytoplasmic domain increases. Interestingly, the short cytoplasmic tail of E1 is conserved through all togaviruses, but its function is still mysterious. Deletion of both basic amino acids in infectious cDNA clones of SFV has no effect on the replication of the virus, at least in cell culture [55]. However, the influence of this

PALMITOYLATED PROTEINS:

FLU A HA:	MGLVFICV	KNGNMRTICV-COOH
		↓
VSV G:	IIGLFLVL	RVGIHLCKLKKRQIYTDIEMNRLGK-COOH
SFV E2:	SCYMLVAA	RSKCLTPYALTPGAAPVWTLGILCCAPRAHA-COOH
	■ ■	↓ ↓
Sindbis E2:	TVAVLCAC	KARRECLTPYALAPNAVIPTSLALLCCVRSANA-COOH
	↓	↓
CD4:	GIFFCV	RCRHHRRQAERMSQIKRLSEKKTQC.....-COOH
		↓ ↓
Rhodopsin:	VYIMMN	KQFRNCMVTTLCCGKNPLGDDEASTT.....-COOH
	↓ ↓	
Transferrin-rec:	YCISGSC	RKPKTVNAKTNNANEEDVALKM.....NH ₂
	↓ ↓	
Marburg virus GP:	LSCIC	RIFTKYIG-COOH
	↓	
F-MuLV env:	LFGPCILN	RLVQFVKDRISVVQAL-COOH
	↓	↓ ↓
HEF/HA chimera	GIAICV	KNGNMRTICV-COOH

STEAROYLATED PROTEINS:

Flu C HEF:	VISGLAIC	RTK-COOH
	↓	
SFV E1:	VVVTCIGL	RR-COOH
	■	
Sindbis E1:	CSMMLTST	RR-COOH

transmembrane cytoplasmic

Figure 9 Primary structure of the cytoplasmic tail of palmitoylated and stearoylated acylproteins

Amino acid sequences in the cytoplasmic tail and the inner leaflet of the transmembrane region of palmitoylated and stearoylated acylproteins. The putative boundary between the transmembrane region and the cytoplasmic tail is indicated by a gap in the sequence. Arrows indicate cysteine residues which have been identified as acylation sites. ■ indicates cysteine residues as putative acylation sites. + indicates positively charged amino acids in the cytoplasmic tail of stearoylated acylproteins. ... indicates additional amino acids before the stop-codon. Abbreviations of proteins and references for the identification of acylation sites and/or protein-bound fatty acids: FLU A HA, haemagglutinin of influenza A virus [25,36,65–67]; VSV G, G-protein of vesicular stomatitis virus [8,26]; SFV and Sindbis E2, E2 protein of the togaviruses SFV and Sindbis virus ([9] and this work); CD 4, surface antigen of T-helper cells [17]; rhodopsin, [14]; transferrin-receptor [12,22]; Marburg virus GP [57]; F-MuLV env [56]; HEF–HA chimera [16,25,36]; see also Figure 7); Flu C HEF, glycoprotein of influenza C virus ([16] and this work); SFV and Sindbis E1, E1 protein of the togaviruses SFV and Sindbis ([13] and this work).

mutation on acylation and on the type of the attached fatty acid, was not investigated. The construction and expression of chimeric molecules, where the cytoplasmic tails of palmitoylated and stearoylated proteins were exchanged, supports the hypothesis that a long cytoplasmic tail determines the preferential attachment of palmitic acid (Figures 6 and 7), while short C-tails are preferentially stearoylated (Figures 1, 3 and 9). The possibility, that not the length and/or charge of the cytoplasmic tail, but the location of the acylation site in the transmembrane region determines preferential attachment of stearic acid, cannot

be excluded completely from our experiments. However, this assumption is very unlikely, because two viral glycoproteins (Marburg virus GP and F-MuLV env) and the cellular CD-4 receptor with an acylated cysteine residue at the same location as HEF and E1, but a longer cytoplasmic tail, are acylated mainly with palmitic acid [17,56,57].

The biological function of thioester-linked fatty acids and of differential acylation of integral membrane proteins is not completely understood, but some evidence is accumulating that acyl-chains are involved in protein–protein interactions at the inner leaflet of membrane bilayers [9–11,58–62]. These interactions may include physical contact between individual amino acids as well as between fatty acids and amino acids or even between individual fatty acids linked to different molecules. Obviously, hydrophobic interactions would be stronger if stearic acid is present instead of palmitate as the protein-bound fatty acid. It is therefore tempting to speculate that proteins with a very short cytoplasmic tail require stearic acid in order to increase the interactive capacity of this region. There is a precedence in the literature that the attachment of different fatty acids has indeed an influence on the strength of protein–protein interactions. Transducin, a heterotrimeric G-protein of the retinal rod is heterogeneously acylated at an N-terminal glycine of its α -subunit with lauric acid ($C_{12:0}$), myristic acid ($C_{14:0}$) and two unsaturated derivatives of myristic acid ($C_{14:1}$, $C_{14:2}$). Functional experiments with $C_{12:0}$ - and $C_{14:0}$ -modified peptides suggest that the myristoylated subpopulation of transducin interacts more strongly with its $\beta\gamma$ -subunit than one which is modified by $C_{12:0}$ [63,64]. Clearly, the hypotheses discussed above need to be further substantiated by future experimental data. However, it emerges from this work and other recent studies [32–34] that in hydrophobic modification of proteins the use of carbon chains is more heterogeneous than originally anticipated.

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