Differential Fatty Acid Selection during Biosynthetic S-Acylation of a Transmembrane Protein (HEF) and Other Proteins in Insect Cells (Sf9) and in Mammalian Cells (CV1)*

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The transmembrane glycoprotein HEF and its acylation deficient mutant M1 were expressed in Sf9 insect cells infected with recombinant baculovirus and in CV1 mammalian cells using the vaccinia T7 system. In insect cells (Sf9), both wild type HEF and HEF(M1) are synthesized in their precursor form HEF0 which appears as a double band in SDS gels. Digestion with glycopeptidase F and endoglycosidase H reveals that the larger 84-kDa form is modified by the attachment of unprocessed carbohydrates of the high mannose type whereas the smaller 76-kDa form is non-glycosylated. As revealed by in vitro labeling experiments with palmitic acid another modification of HEF is the attachment of a long chain fatty acid to cysteine residue Cys-652 which is located at the internal border of the cytoplasmic membrane. After labeling with [3H]palmitic acid in both systems only HEF(WT) is acylated, whereas HEF(M1) is not. High performance liquid chromatography analysis of the fatty acids bound to HEF(WT) expressed in Sf9 insect cells reveals nearly 80% of palmitic acid. In contrast to this finding, the acylation pattern of HEF expressed in CV1 cells shows nearly the same amounts of stearic and palmitic acid (40%).

In this report, we therefore compare the biosynthesis of HEF after expression of the cloned gene in insect (Sf9) and mammalian (CV1) cells using baculovirus or vaccinia virus, respectively. In particular, we show here that HEF expressed in insect cells selects for palmitic acid during acylation, whereas mammalian cells prefer stearic acid to acylate HEF. Since we identify the same cysteine residue (position 652) of HEF as acylation site in both cell types, different cellular factors must be involved in HEF acylation in mammalian and insect cells, which contribute to the selection of fatty acid species in this process.

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MATERIALS AND METHODS
Site-directed Mutagenesis

To construct a HEF mutant (M1) where the putative acylation site cysteine 652 is replaced by a serine residue cDNA coding for HEF wild type (WT) (9) was cloned into the replicative form DNA of m13 bacteriophage using standard techniques (12). Site-directed mutagenesis was performed using a commercial kit (oligonucleotide-directed in vitro mutagenesis system; Amersham, Braunschweig, Germany) based on the phosphorothioate method (13).

HEF transmembrane glycoprotein is coded for by influenza C virus, which is an enveloped virus. HEF mediates the receptor-binding (hemagglutinin) and receptor-destroying activities (esterase) as well as the fusion activity of influenza C virus (1). It appears on the surface of influenza virus-infected cells and is a typical type I transmembrane protein. HEF is synthesized on membrane-bound ribosomes as an inactive precursor polypeptide (HEF0) and is biosynthetically assembled to trimers (2, 3). In addition HEF is subject to processing and terminal glycosylation of N-linked carbohydrates (4). Another posttranslational modification occurring only in certain cell types is the proteolytic cleavage of the precursor HEF0 by a cellular protease, which is required for the viral fusion activity (5, 6). The cleavage products HEF1 and HEF2 are held together by disulfide bonds (7). The mobility of HEF0 analyzed with SDS-PAGE under reducing conditions suggests a molecular mass of about 85 kDa, which is in accordance with the size range expected from the published sequence data (8, 9). Recently Veit et al. (10) detected a further posttranslational modification of HEF, the attachment of fatty acids via a thioester-type linkage. In contrast to the majority of acylated proteins, which are mainly modified with palmitic acid, the predominant fatty acid bound to HEF in influenza C-infected MDBK cells was found to be stearic acid (10). The reason for this preference of stearic acid could be related to specific structural features of HEF and/or depend on the cell type under study. While substantial evidence points to a crucial role of the length of the C-tail in acyl selection (11), nothing is known about the potential contribution of cellular factors in this process.

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Fig. 1. Expression of HEF protein in insect cells. SF9 insect cells were infected with AcMNPV-HEFwt (lane 1) or AcMNPV-HEFm1 (lane 2). Two days postinfection, cells were labeled with \(^{35}\)S)methionine. Cell extracts were immunoprecipitated with a polyclonal influenza C virus antiserum and subjected to SDS-PAGE under reducing conditions followed by fluorography. The electrophoretic mobility of \(M\), markers is indicated on the left.

**Baculovirus Expression System and SF9 Cell Culture**
cDNA coding for HEF(WT) and mutated HEF(M1) were cloned into the Smal site of baculovirus expression vector pVL1393. The plasmids and linearized AcMNPV DNA (Invitrogen, Leek, The Netherlands) were transfected into Spodoptera frugiperda insect cells (SF9) by Insectin (Invitrogen) following the manufacturer's procedure. Recombinant viruses AcMNPV-HEFwt and AcMNPV-HEFm1 containing HEF(WT) or HEF(M1), respectively, were plaque-purified and amplified (14). Positive viral clones were monitored for HEF expression by \(^{35}\)S)methionine labeling of infected cells and immunoprecipitation with polyclonal influenza C virus antiserum, followed by SDS-PAGE and fluorography. SF9 cells were grown in TC-100 medium (Life Technologies, Inc., Berlin, Germany) supplemented with 5% (v/v) fetal calf serum. For metabolic labeling SF9 insect cells (1 \(\times\) 10^6) in 35-mm diameter culture dishes were infected with recombinant baculoviruses at a multiplicity of infection of 10 virus particles/cell. Metabolic labeling was performed 2 days after infection.

**Vaccinia T7 Expression System and CV1 Cell Culture**
cDNA coding for HEF(WT) and mutated HEF(M1) were cloned into the Smal site of pTM1 plasmid (15) behind the promotor of baculovirus T7 polymerase. CV1 mammalian cells were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc., Berlin, Germany) containing 5% (v/v) fetal calf serum. Grown to 80% confluence in 35-mm diameter culture dishes, CV1 cells were infected with T7-3 vaccinia virus (16) at a multiplicity of infection of 10 plaque-forming units per cell. At 2 h postinfection the virus inoculum was replaced by a transfection mix containing 5 \(\mu\)g of pTM1-HEF(WT) or pTM1-HEF(M1) DNA and 10 \(\mu\)l of Lipofectin (Life Technologies, Inc.) in 1 ml of Dulbecco’s modified Eagle’s medium. Metabolic labeling was performed 4 h after transfection.

**Metabolic Labeling**
Cells were labeled for 4 h with \(^{35}\)S)methionine (50 \(\mu\)Ci/ml in medium without methionine, 1200 \(\mu\)Ci/mmol, Expre-35S-S) protein labeling mix, DuPont, Bad Homburg, Germany) or with \(^{3}H\)palmitic acid (1 \(m\)Ci/ml medium; 33 Ci/mmol, DuPont) for the time periods as indicated in the figure legends. Processing of the cells after labeling period was as follows.

**SF9 Insect Cells**—Cells were scraped from the culture dishes in culture medium, transferred to Eppendorf tubes, pelleted (3 min, 3000 \(\times\) g), washed with ice-cold phosphate-buffered saline (140 mM NaCl, 3 mM KCl, 2 mM KH\(_2\)PO\(_4\), 6 mM Na\(_2\)HPO\(_4\), pH 7.4) and lysed in 600 \(\mu\)l of RIPA buffer (1% Triton X-100, 1% deoxycholate, 0.1% SDS, 0.1% NaCl, 20 mM Tris, 10 mM EDTA, 10 mM iodoacetamide). Cells were freeze-thawed three times, and insoluble material was removed by centrifugation (5 min, 20000 \(\times\) g).

**CV1 Mammalian Cells**—CV1 cells were washed with ice-cold phosphate-buffered saline and subsequently lysed in 600 \(\mu\)l of RIPA buffer. Cell lysate was transferred to Eppendorf tubes, and insoluble material was removed as described above.

**Immunoprecipitation**
Polyclonal serum against influenza C virus was added at a dilution of 1:100 to the cell lysates, and samples were agitated overnight at 4 \(^\circ\)C. After an additional incubation with 30 \(\mu\)l of Protein-A-Sepharose Cl-4B (1:1 in RIPA buffer; Sigma) for 2 h, antigen-antibody complexes were washed three times with RIPA buffer. Antigen was solubilized by incubation in gel-loading buffer containing 2% SDS and 5% mercaptoethanol for 2 min at 95 \(^\circ\)C and subjected to SDS-PAGE in 10% polyacrylamide gels (17), followed by fluorography for 2 days (\(^{35}\)S) or 7 days (\(^{3}H\)) using salicylate (18).

**Fatty Acid Preparation**
HEF-bound Fatty Acids—\(^{3}H\)Palmitic acid-labeled HEF was purified by immunoprecipitation and SDS-PAGE. After localizing HEF bands by fluorography, the respective regions were excised from the dried gel and scintillator was removed by washing with water. Subsequently, gel pieces were dried in a desiccator and fatty acids were cleaved by treatment with 6 \(\times\) HCl for 16 h at 110 \(^\circ\)C in tightly closed glass tubes. Fatty acids were extracted from the hydrolysate three times with hexane and the upper phases combined.

**Fatty Acids Derived from Whole Cellular Protein**—After metabolic labeling, cells were extracted three times with 1 ml of chloroform/methanol (v/v 2:1) and twice with 1 ml of methanol. Total delipidated cellular protein was pelleted (15 min, 20,000 \(\times\) g) and hydrolyzed in 6 \(\times\) HCl for 16 h at 110 \(^\circ\)C. Fatty acids were extracted from the hydrolysate three times with hexane and the upper phases combined.

**Fatty Acid Separation**
Combined hexane extracts from the above described procedures were dried under nitrogen and redissolved in 50 \(\mu\)l of methanol containing 100 \(\mu\)g of unlabeled myristic, palmitic, and stearic acid. Samples were separated by HPLC using a Nova-Pak C\(_{18}\) column (Waters, Echshorn, Germany) and 90% acetonitrile as a solvent at a flow rate of 0.5 or 0.8 ml/min (see figure legends). Unlabeled fatty acids were monitored by an
RESULTS AND DISCUSSION

A full-length HEF-gene wild type (WT) and a HEF mutant, designated HEF(M1), coding for a serine instead of the putative acylation site cysteine (Cys-652) were cloned into the baculovirus expression plasmid pVI1393 behind the strong promotor of the polyhedrin gene. As described under "Materials and Methods," SF9 insect cells were infected with high titer, recombinant baculovirus stock and expression of HEF was monitored by radiolabeling, immunoprecipitation and PAGE analysis of immunoprecipitates. Fig. 1 shows that two populations of HEF can be resolved under reducing conditions with an estimated size of about 76 and 84 kDa, corresponding to the size of HEF calculated from sequence data (8, 9). Therefore it can be concluded that, in insect cells, biosynthesis of recombinant HEF proceeds without proteolytic cleavage to HEF\(_1\) and HEF\(_2\), which have molecular masses of 65 and 30 kDa, respectively (7). Utilizing glycopeptidase F and endopeptidase H, we reveal in Fig. 2A that the 76-kDa population of recombinant HEF in insect cells must represent a non-glycosylated form, because its molecular mass does not shift after digestion with these endoglycosidases. All the carbohydrates present in the 84-kDa form of the same protein must be in the high mannose form, since no material remains in the 84-kDa region of the gel after endopeptidase H treatment (Fig. 2A, two lanes on right). This is in accordance with former results on the "glycosylation machinery" of insect cells, which apparently lack the enzymes for terminal processing of N-linked carbohydrates (19–21). The same results as shown in Fig. 2A are obtained when HEF(M1) is expressed in this system instead of wild type HEF (data not shown).

Next we asked whether acylation of HEF occurs in insect cells. This is indeed the case because wild type HEF can be strongly labeled with \(^{3}H\)palmitic acid (Fig. 2B) in a hydroxylamine-sensitive manner (results not shown). It is noteworthy that only the upper 84-kDa band shows fatty acid label (Fig. 2B) indicating either that the non-glycosylated population of HEF may not reach the intracellular site of palmitoylation or that it develops a conformation unsuitable for fatty acid transfer. If the mutation at the putative acylation site in position 652 (Cys-652 \(\rightarrow\) Ser) abolishes the acylation observed in HEF(WT), it can be surmised that this site is critical for fatty acid transfer, or serves for attachment of fatty acid. Fig. 2B (lane 2) reveals that this is indeed the case, since no radioactivity at all becomes visible in the respective region even during extended fluorography after PAGE of \(^{3}H\)palmitic acid-labeled HEF(M1). Thus, insect cells show the same requirement of Cys-652 for S-acylation of HEF as mammalian cells (11). Since almost all of the above described features of biosynthesis parallel those observed during expression of HEF in mammalian cells (22), it remained to be tested whether the unusual selectivity of HEF for stearic acid (15) would be retained during its biosynthesis in insect cells. In order to achieve this, we had to compare the \(^{3}H\)-fatty acid species present in HEF isolated from the protein after expression in the presence of \(^{3}H\)palmitate with the pattern of lipid-bound fatty acids from the same cells. Using fatty acid analysis by HPLC of the acyl chains bound to HEF(WT) expressed in insect cells, one main peak was revealed after scintillation counting of individual fractions (Fig. 3A). Comparison with the retention time of the unlabeled fatty acid standards showed that this peak represents \(^{3}H\)palmitic acid. Only very low amounts of stearic and myristic acid were detectable in HEF, although analysis of lipid-bound fatty acids reveals that considerable amounts of \(^{3}H\)stearic and \(^{3}H\)myristic acid are present after the 4-h labeling period with \(^{3}H\)palmitic acid utilized in this experiment (Fig. 3).

This selection for palmitic acid from the total acyl pool during HEF acylation in insect cells clearly differs from the reported stearoylation of HEF in MDBK cells infected with influenza C virus (15). This unusual acyl selection may be insect cell-specific, but can also be the result of the sole expression of stearic acid to HEF, HEF expressed in mammalian CV1 cells should also lose its specificity for stearic acid binding. We addressed this question by using the vaccinia T7 expression system (Fig. 4A). Fatty acid analysis after a 4-h labeling period with \(^{3}H\)palmitic acid revealed that nearly half of the recombinant HEF protein expressed in CV1 cells is acylated with stearic acid (Fig. 4B, left panel), although \(^{3}H\)palmitate conversion into stearic acid in CV1 cells was even lower than in insect cells (compare Figs. 3A and 4B, right panel). This indicates a strong preference of HEF for C18:0 chains from the different acyl substrates present in the mammalian cells. Therefore the nearly exclusive attachment of palmitate to HEF expressed in insect cells is a peculiarity of these cells and not due to the lack of other viral proteins.
This surprising result led us to ask whether acylation in mammalian cells and SF9 cells may differ in its substrate utilization. In order to ensure steady state conditions for acylation of general polypeptides in insect cells during [3H]palmitate labeling, we studied long term kinetics of acyl incorporation into total cellular lipid and protein. The results from these experiments presented below indicate that the selection for palmitic acid is not restricted to the HEF protein, but seems to be a general feature of SF9 insect cells. Analysis of all the protein-bound fatty acids in SF9 cells after increasing labeling periods up to 24 h with [3H]palmitic acid showed beside palmitate only traces of stearate covalently linked to total polypeptides (Fig. 5), which is in contrast to mammalian cells where up to 40% of stearic acid were detectable in proteins (23, 24), similar to our results with HEF (Fig. 4B, left panel). Fatty acid species in lipids did only marginally change from the pattern shown after 4 h (Fig. 3A, right panel) over the whole labeling. We therefore suggest that cellular factors are involved in the differential acylation of viral and cellular acylproteins in insect and mammalian cells. This may be the acyltransferases themselves, which could have evolved to higher complexity in mammalian cells to attach specific fatty acids to individual proteins. This would be also reminiscent of N-linked glycosylation in insect cells, which lacks the complexity described for mammalian cells (19–21).

Our results have implications for the current issue of chemical versus enzymatic acylation. Chemical acylation can be achieved in vitro by adding activated fatty acids (e.g. acyl-CoA) to purified donor proteins or peptides (25). Obviously, acylation without an enzyme should show no preference for a particular fatty acid, but reflect the availability of the individual carbon chains present in the cellular lipid pool. Such non-enzymatic acylation is very unlikely in vivo, since (i) significant differences occur apparently in the acylation pattern of HEF and other proteins in insect and mammalian cells, although the complete spectrum of acyl-CoA lipid substrates (e.g. stearoyl-CoA) is present in both cell types (Figs. 3 and 4) and (ii) the concentration of free acyl-CoA is very low in vivo (more that 95% of it is bound to a acyl-CoA-binding protein) (26, 27). The possible involvement of acyl-CoA-binding proteins in S-acylation and/or acyl chain selectivity is an interesting subject for future studies.

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