Protein fatty acyltransferase is located in the rough endoplasmic reticulum

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The fatty acid acylation of polypeptides was studied in vivo and in vitro by incorporation of radiolabeled palmitic acid into Semliki Forest viral polypeptides. Utilizing a cell-free system for acylation protein fatty acyltransferase was characterized as an integral membrane protein. No acylation activity was detected in the cytosol. During subcellular fractionation of a variety of mammalian or avian cells the enzyme was localized to the rough endoplasmic reticulum. Therefore this posttranslational hydrophobic modification starts earlier in the biosynthesis of acylated polypeptides than previously believed.

Acyl protein *Protein fatty acyltransferase* *Rough endoplasmic reticulum*

1. INTRODUCTION

Acylation with long chain fatty acids has gained much interest recently among the numerous covalent modifications of polypeptides. This is due partly to the extension of this modification from viral membrane glycoproteins where it was originally detected [1] to various cellular polypeptides with known physiological functions [2–6] and to proteins involved in the tumorigenic transformation of cells [7,8]. Although at the present time no general function can be assigned to protein-bound fatty acids, in specific cases their involvement in membrane fusion [9], anchorage in cellular lipid membranes [10], intracellular transport [11] and in protease protection [12,13] has been reported. We have been interested in the biosynthetic aspects of this new hydrophobic modification and report here on an in vitro assay for the transfer of fatty acyl chains onto polypeptide substrates. In applying this cell-free acylation system we show that protein fatty acyltransferases operate in the rough endoplasmic reticulum.

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2. MATERIALS AND METHODS

2.1. *Cell-free acylation*

Microsomal membranes were prepared as described [14] and incubated for 60 min at 37°C in a total of 250 μl TN buffer (20 mM Tris-HCl, 0.15 M NaCl, pH 7.4) with about 150 μg microsomal and 100–200 μg of exogenous acceptor protein. Either 0.2 μCi [^{14}C]palmitoyl-coenzyme A (fig.1, panel A) or 100 μCi [^{3}H]palmitic acid in the presence of 2 mM ATP were used as lipid substrates and the incubations terminated by adding 2 ml chloroform/methanol (2:1, v/v). Acceptor polypeptides were prepared from purified SFV by a 2 h treatment of about 2 mg virus with 2% NP 40 in phosphate-buffered saline (PBS) at room temperature and a subsequent 4 h incubation with 1 M hydroxylamine, pH 6.6, for release of fatty acids from the viral E1 and E2 proteins [15,16].

2.2. *Subcellular fractionation of BHK cells*

About 5×10^9 BHK cells were homogenized and fractionated according to Green et al. [17]. Briefly, a postmitochondrial supernatant was made to 55% sucrose in 50 mM Tris-HCl, 2 mM $MgCl_2$, pH 7.0,

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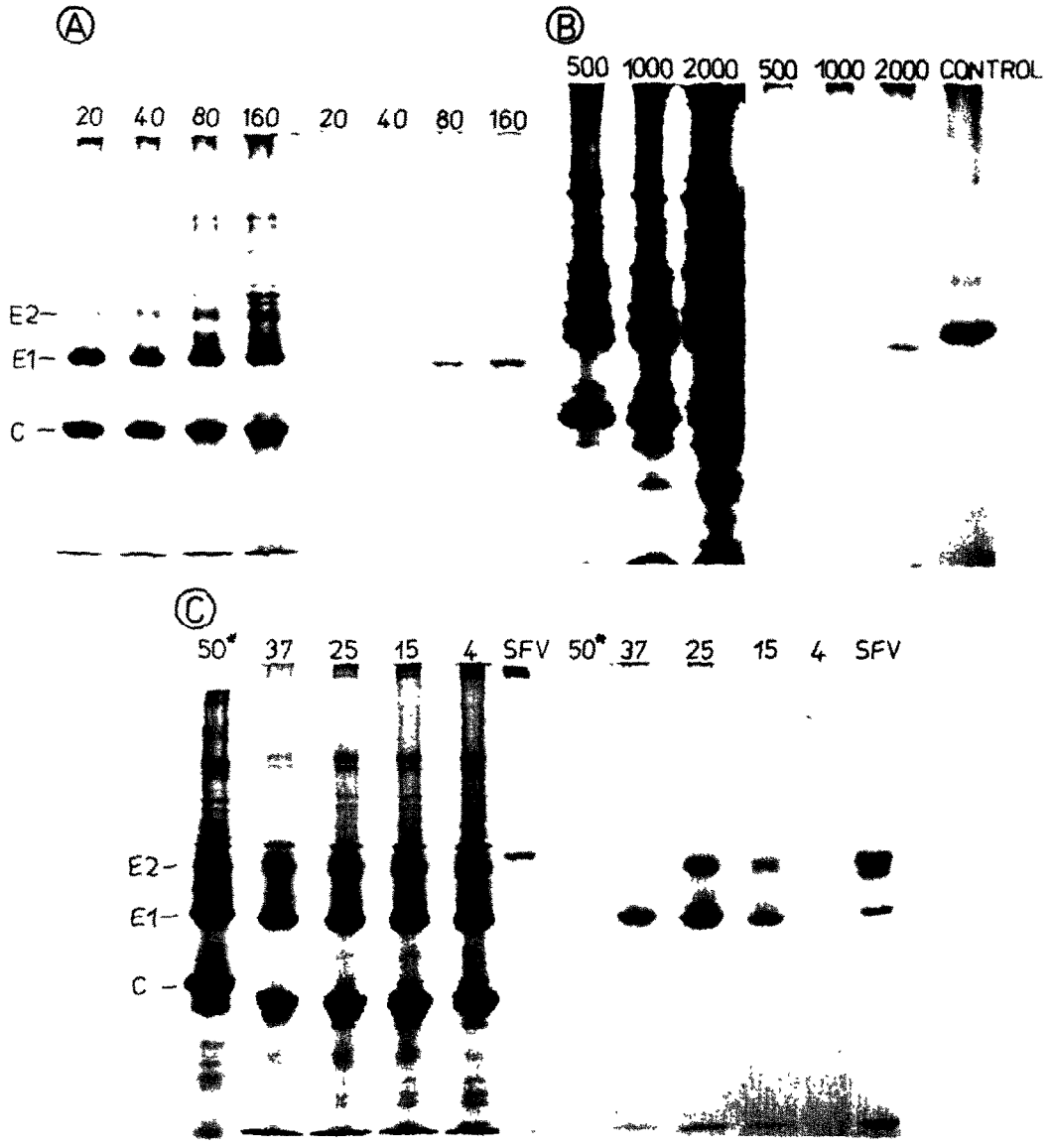

Fig.1. The fatty acid acylation of exogenous viral membrane glycoproteins in vitro. Microsomal preparations from BHK cells with increasing protein content (indicated as μg above the lanes) were used to transfer ^{14}C-labeled fatty acid from its activated form ([^{14}C]palmitoyl-coenzyme A) onto exogenous fatty acid-free viral polypeptide solubilized in NP 40 (panel A). Protein fatty acyltransferase is bound to intracellular membranes since cytoplasmic extracts (100000 × g supernatant), even with large amounts of protein present (given as μg above the lanes) fail to catalyze the acyltransfer reaction to a significant extent when compared to the control incubation with microsomal membranes (panel B). The transfer of acyl chains works best between 25 and 28°C (numbers above lanes refer to the temperatures of incubation). After a 50°C preincubation for 10 min of the microsomal membranes acylation is completely abolished (lane 50* in panel C). Coomassie staining patterns (left side) and fluorograms (right side) of the same gels are shown. Viral proteins in the gels are designated E1, E2 and C (capsid protein) according to the migration of polypeptides of [^{3}H]palmitic acid-labeled SFV reference virus (panel C).

and overlaid in Beckman SW 28 tubes with a step gradient of 46, 40 and 20% sucrose, respectively. After centrifugation for 2 h at 100000 × *g* the visible membrane bands at the interphases were diluted with Tris buffer to 10% sucrose and sedimented in a 1 h run at 100000 × *g* in an SW 28 Ti rotor. The membranes were suspended in a small volume of Tris buffer and aliquots split for measuring protein [18], the activity of 5'-nucleotidase, galactosyltransferase, glucose-6-phosphatase [17], and protein fatty acyltransferase. Small samples were subjected to thin sectioning for inspection by electron microscopy (kindly performed by Kurt Wahn, Giessen).

2.3. *In vivo short pulse labeling*

At 4 h after infection with Semliki Forest virus (SFV) about 10^8 subconfluent BHK cells were labeled with a total of 1.5 mCi [^{3}H]palmitic acid (23.5 Ci/mmol from New England Nuclear) in medium 199 (Gibco). After rapid processing on an ice-cold bench the cell homogenate was fractionated as described above and membrane fractions analysed by polyacrylamide gel electrophoresis.

2.4. *SDS-polyacrylamide gel electrophoresis (SDS-PAGE)*

SDS gels of 10% polyacrylamide were used throughout utilizing the buffer system as described [14]. Coomassie brilliant blue was used for staining of polypeptides and a standard fluorography procedure was employed to detect radiolabeled polypeptides. Alternatively gels were sliced into 1 mm fractions and radioactivity measured by liquid scintillation counting.

3. RESULTS

From in vivo experiments on fatty acylation of polypeptides it is now widely accepted that this modification occurs as a posttranslational event (review [13]). However, the intracellular location of protein acylation has not been determined with certainty although data are available which point to a Golgi-near location [19–21]. For a direct proof of the enzyme's localization we have established a microsomal system which allows the cell-free acylation of SFV E1 polypeptide. The features of this in vitro system are summarized in fig.1 which shows the transfer of palmitic acid from [^{14}C]palmitoyl-coenzyme A (PalCoA) as a function of the concentration of microsomal protein present during the incubation (panel A). The results depicted in fig.1, panel B, show that all protein fatty acyltransferase detectable in BHK cells is membrane bound since no such enzymatic activity is detected in cytosolic protein. Likewise no acyltransferase activity could be washed off the membranes by treatment with high salt (0.5 M KCl) at pH 11 indicating that the enzyme is an integral protein. These findings also apply to other cell types, for instance, chick embryo fibroblasts and organ extracts from liver (rat, pig) or kidney (pig, bovine) (not shown). If the microsomes are preincubated at 50°C prior to the in vitro assay, no palmitoylation of acceptor polypeptides is observed suggesting that the protein fatty acyltransferase has been inactivated thermally during the preincubation (lane 50*, panel C). The optimal temperature for the acyl transfer itself was found to be 25–28°C as shown in the other lanes of panel C.

To analyze the intracellular location of protein fatty acyltransferase in a direct approach BHK cells were fractionated and the various membrane preparations tested for acyl transfer activity. The quality of the rough endoplasmic reticulum (rER), Golgi and plasma membrane fractions was verified by electron microscopic examination (fig.2, panels A,B) and by measuring the activity of the characteristic guide enzymes, respectively (table 1). Samples of these 3 fractions were incubated with viral acceptor polypeptides and the incubation mixtures subsequently analyzed by SDS-PAGE. It becomes apparent from the right panel of fig.2 that the rER shows a much higher level of [^{14}C]palmitoyl transfer onto the E1 polypeptide than do the other fractions, Golgi and plasma membrane. Accordingly, the rER affords clearly the highest specific activity of protein fatty acyltransferase (table 1).

In view of the hypothetical location of protein fatty acyltransferases to the Golgi apparatus in the literature [19–21] the above result necessitates confirmation by a different approach. Since subcellular fractionation especially of BHK cells is a well established and reliable procedure [17] there was no need to diverge from this technique. However, since there may be limitations to the in-

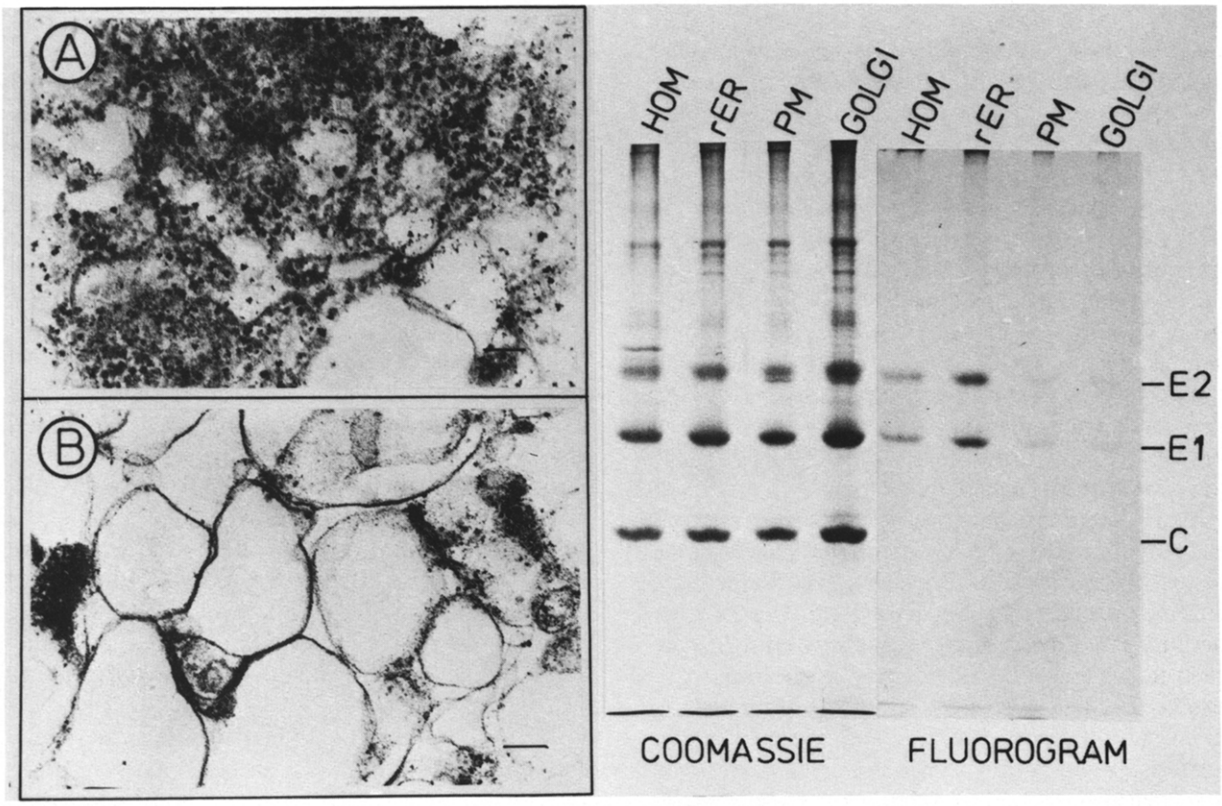

Fig.2. Cell fractions from BHK cells as depicted for the rER (panel A) and Golgi (panel B) in the electron micrographs (120000-fold magnification) on the left were tested for their content of protein fatty acyltransferase with the in vitro acylation system. Aliquots with 200 μg of protein were incubated for 10 min as described in section 2. The fluorogram of a polyacrylamide gel of the various incubation mixtures shows the highest levels of [^{14}C]palmitic acid in the acceptor proteins when incubated with protein from the endoplasmic reticulum (right). Exposure time was for 3 weeks.

Table 1

Characterization of cell fractions

Enzyme activities	Specific activities of enzymes in cell fractions from BHK cells			
	Homogenate	rER fraction	PM fraction	Golgi fraction
Glucose-6-phosphatase (μmol/mg per h)	0.101	0.728	0.511	0.367
Galactosyltransferase (cpm/mg per h)	1175	2997	3625	5670
5'-Nucleotidase (μmol/mg per h)	0.082	0.090	0.210	0.071
Protein fatty acyltransferase (μmol/mg per h)	11512	14226	5091	8172

Cell fractionation was done in triplicate and mean values are given. Deviation of specific activities in the individual experiments was less than 10%. Activity of protein fatty acyltransferase was measured as the incorporation of [^{14}C]palmitic acid from [^{14}C]palmitoyl-coenzyme A into E1 polypeptide mediated by the protein present in the various fractions

However, since there may be limitations to the interpretation of the in vitro data presented above we switched to a more physiological situation by performing experiments in which we labeled SFV-infected BHK cells (at 4 h after infection) for an extremely short period of time (20 s) with [^{3}H]palmitic acid. We then asked in which cell compartment we would trace the freshly acylated viral membrane proteins p62 (a precursor of the SFV proteins E2 and E3) and E1. As demonstrated in fig.3 we could indeed identify these acyl protein species within the rER fraction although quite large amounts of acylated proteins were also found associated with Golgi membranes. This latter fraction of p62 and E1 represents polypeptides which have been translocated into the Golgi apparatus within the period of labeling and processing for cell fractionation. The fatty acid labeled p62 found in the plasma membrane represents a slight cross contamination with ER or Golgi membranes because this precursor polypeptide is known to be cleaved to E2 and E3 before or at the time as it reaches the cell surface [17,22,23]. If pulse periods longer than 2 min were used for the initial in vivo labeling all fatty acid labeled polypeptides were detected in the Golgi or even in the plasma membrane fraction ([20,21]; Berger and Schmidt, unpublished).

We know from previous experience that fatty acylation of viral membrane proteins is clearly a posttranslational event which occurs about 20 min after the translation of the respective polypeptide at a time only about 5 min before the trimming of its oligosaccharides has been completed [19,24]. These facts together with the data reported here indicate that fatty acylation of polypeptides occurs in such regions of the endoplasmic reticulum which are predestined to become translocated to the Golgi apparatus shortly after the hydrophobic modification of the partially mature membrane glycoprotein. These membranes active in protein acylation could well represent transport vesicles with similar physical properties to rER membranes. If so fatty acylation of membrane proteins could be functionally involved in intracellular membrane traffic as has been suggested previously [11].

Whether the enzyme also acylates the recently discovered myristilated polypeptides [2,3,5,25] and transforming proteins [7,8,10,26,27] is not yet known. However, since the acyl transfer activity reported here and by others seems to lack the strict specificity for myristic (tetradecanoic) acid [15,28,29] postulated for the aminoterminally acylated polypeptides, this possibility seems unlikely at first glance.

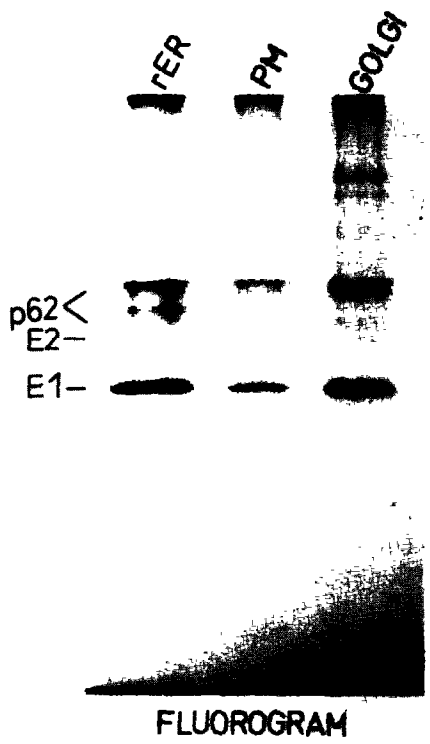

Fig.3. BHK cells infected with SFV were pulse-labeled with [^{3}H]palmitic acid for 20 s and the cells processed on a 4°C bench by double washing with phosphate-buffered saline, scraping with a rubber policeman and 10 strokes with a tight-fitting Dounce homogenizer. The homogenate was fractionated by centrifugation and fatty acid labeled polypeptides in the fractions analysed by SDS-PAGE. After exposure for 6 weeks the fluorogram shows fatty acid labeled p62 and E1-protein mainly in the rough ER and the Golgi fraction.

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