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Timing of palmitoylation of influenza virus hemagglutinin

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The timing of the attachment of fatty acids to the hemagglutinin (HA) of influenza A virus was studied. Treatment of virus infected cells with brefeldin A (BFA), a drug which blocks intracellular transport along the exocytic pathway at a pre-Golgi site, does not prevent palmitoylation of HA. The relationship of HA-palmitoylation to the oligomerisation and to the proteolytical cleavage of the protein revealed that the uncleaved trimer of HA is the substrate for the acylating enzyme in virus infected cells. The results are discussed with regard to the intracellular site of palmitoylation.

Influenza virus; Hemagglutinin; Intracellular transport; Palmitoylation; Oligomerisation; Brefeldin A

1. INTRODUCTION

The hemagglutinin of influenza virus is a typical type I membrane glycoprotein with a large N-terminal ectodomain, one membrane spanning region and a short cytoplasmic tail. Besides its important role for virus infectivity [1], HA is a model system for glycoproteins in general, because its structure, biosynthesis and posttranslational modifications have been studied in great detail [2]. The polypeptide chain is synthesized on membrane-bound ribosomes and translocated into the lumen of the endoplasmic reticulum (ER), where signal-peptide cleavage, N-linked glycosylation, folding and disulfide-bond formation occur. Post-translationally but still in the ER, three HA molecules assemble to a trimer [3–5]. Subsequent to its transport through the recently described intermediate compartment or *cis*-Golgi network (CGN) [6], trimming of HA-linked carbohydrates occurs in medial-Golgi vesicles and terminal glycosylation in the *trans*-Golgi network (TGN). Also in the TGN HA is cleaved into the membrane-spanning HA_2 and the large N-terminal HA_1 subunits by the cellular endoprotease furin [7]. HA is then transported to the plasma membrane and incorporated into virus particles during the budding process. Half-times of 7–10 min for oligomerisation [3,4], of 15–20 min for carbohydrate processing and proteolytical cleavage, and of 60 min between translation and exposure at the cell surface have been reported [8].

Another posttranslational modification of HA is the attachment of fatty acids, mainly palmitic acid, to three conserved cysteine residues in the cytoplasmic tail of the molecule [9–11]. The timing of HA palmitoylation relative to the other above-mentioned protein modifications is not known. Results in this respect have been reported only for other viral acylproteins. Using pulse-chase experiments on toga- or rhabdovirus infected cells, it was shown, that palmitoylation occurs posttranslationally before the carbohydrate-chains of the respective glycoprotein are trimmed and before proteolytical cleavage of the precursor proteins [12]. From subcellular fractionation studies it has been suggested that palmitoylation occurs either in the *cis*-Golgi cisternae or in the endoplasmic reticulum [13–15]. Using low temperature (15°C) to block transport of proteins along the exocytotic pathway at a pre-Golgi site and temperature-sensitive transport mutants of viral acylproteins, palmitoylation was located to a post ER/pre-Golgi compartment [16,17]. However, a number of cellular acylproteins are palmitoylated also at the plasma membrane, and their fatty acids are apparently subject to cycles of de- and reacylation [18–20].

Here we have studied the timing of palmitoylation of influenza A virus HA by two novel approaches. Intracellular transport of HA was blocked at a pre Golgi-site using the fungal metabolite brefeldin A [21]. The temporal relation of palmitoylation and oligomerisation of HA was studied using short-pulse labeling and sucrose-gradient centrifugation. This is of considerable interest, because oligomerisation is also believed to occur at intracellular sites considered above for palmitoylation [5].

2. MATERIALS AND METHODS

2.1. *Virus infection, metabolic labeling*

Confluent chicken embryo fibroblasts (CEF) were infected with influenza A virus (A/FPV/Rostock/34 [H7,N1], fowl plague virus) at a multiplicity of infection of 50. Four hours after infection the cells were labeled with [^{35}S]methionine (100 μCi/ml Dulbecco medium without methionine, 1000 Ci/mmol, Amersham) or [^{3}H]palmitic acid (1 mCi/ml Dulbecco medium, 54 Ci/mmol, Amersham) for the times

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indicated in the text. For pulse-chase experiments with [^{35}S]methionine the chase was initiated by the addition of unlabeled methionine to a final concentration of 10 mM. At the end of the chase the cell monolayers were washed on ice with phosphate-buffered saline and subsequently lysed with RIPA-buffer [10] or Triton-lysis buffer (1% Triton X-100, 20 mM MES, 30 mM Tris, 100 mM NaCl, 40 mM EDTA, 10 mM iodoacetamide, 1 mM PMSF, 5% Trasylol). HA was immunoprecipitated with a polyclonal rabbit serum against fowl plague virus or the monoclonal antibody HC-2 [31] and subjected to SDS-PAGE and fluorography as described previously [10].

2.2. *Cycloheximide and brefeldin A treatment*

Influenza virus infected monolayers were treated with cycloheximide (Sigma, Deisenhofen, Germany; 50 μg/ml final concentration) or BFA (Epicentre technologies, Madison, WI, USA; 6 μg/ml) at the times indicated in the text before metabolic labeling. Both inhibitors were also present throughout the labeling and chase periods. Stock solutions of BFA (10 mg/ml methanol) and cycloheximide (50 mg/ml ethanol) were stored at −20°C.

2.3. *Sucrose-gradient centrifugation*

Virus-infected cells were lysed with Triton-lysis buffer. Cleared lysates (100,000 × *g* for 30 min) were layered onto a linear sucrose gradient (5% to 20% sucrose in 100 mM NaCl, 20 mM MES, 30 mM Tris, 10 mM $CaCl_2$, 1% Triton X-100) and monomers and trimers of HA separated by centrifugation at 40,000 rpm for 16 h at 4°C in a SW 41 rotor (Beckman). The material was fractionated from the bottom of the tube to 1ml per fraction. Each fraction was diluted 1:4 with RIPA- buffer and HA was immunoprecipitated.

3. RESULTS

To relate palmitoylation to the proteolytical cleavage of HA, influenza A virus infected chicken embryo fibroblasts (CEF) were pulse-chase labeled with [^{3}H]palmitic acid. HA was immunoprecipitated from cell extracts and subjected to SDS-PAGE and fluorography. As seen from the results shown in Fig. 1A, freshly acylated, pulse-labeled HA is uncleaved (lane 1) and Endo-H digestion revealed that it has not undergone trimming of its carbohydrate side chains (not shown). After 15 min of chase approximately 60% of [^{3}H]palmitic acid labeled HA is cleaved (Fig. 1A, lane 2) and proteolytic processing of HA is complete after about one hour of chase (lane 3). No loss of [^{3}H]palmitic acid label was detectable during the chase periods, which indicates that the fatty acids are stably attached to the viral glycoprotein during its transport to the plasma membrane and thus no substrate for deacylation.

To examine the dependence of palmitoylation on ongoing protein synthesis, polypeptide translation in influenza virus infected cells was blocked by the addition of cycloheximide. Cells were then labeled for 10 min at 0, 5, 10 or 15 min after adding cycloheximide. Fig. 1B (lanes 1 and 2) shows that [^{3}H]palmitic acid labeling of HA decreases continually until 10 min after cycloheximide addition and is virtually absent 15 (lane 4) and 60 (not shown) min after the block in protein synthesis. This indicates, that palmitoylation of HA is a post-translational, but relatively early event in the maturation of the protein.

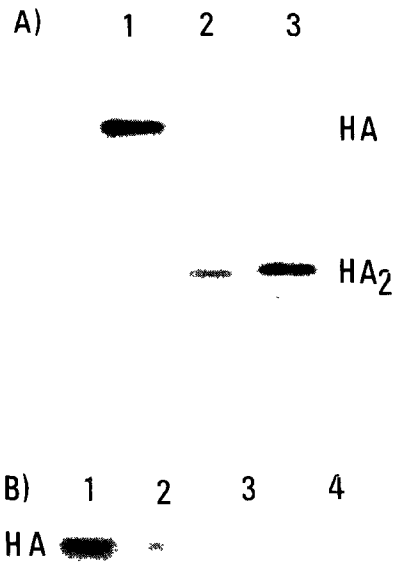

Fig. 1. Palmitoylation of HA occurs before its proteolytical cleavage. (A) CEF cells infected with influenza A virus were labeled for 5 min with [^{3}H]palmitic acid and chased for 0 min (lane 1), 15 min (lane 2) or 60 min (lane 3). HA was immunoprecipitated with influenza virus antiserum and subjected to SDS-PAGE and fluorography. (B) Influenza virus infected CEF cells were treated with cycloheximide (50 μg/ml) and labeled simultaneously (lane 1) or 5 min (lane 2), 10 min (lane 3) and 15 min (lane 4) after adding the drug each for 10 min with [^{3}H]palmitic acid. HA was immunoprecipitated and subjected to SDS-PAGE and fluorography.

To pinpoint the intracellular site of HA-palmitoylation more closely, intracellular transport of HA was blocked at a pre-Golgi site using the fungal metabolite brefeldin A (BFA). Because cell-type and species-specific differences in the action of the drug have been reported [21], we firstly determined whether BFA inhibits transport in our system using the cleavage of HA as a marker, since this event depends on transport. Influenza A virus infected chicken embryo fibroblasts were treated with BFA one hour before pulse-chase labeling with [^{35}S]methionine. Immunoprecipitation of influenza virus specific proteins revealed, that pulse-labeled HA is uncleaved in BFA-treated and untreated cells (Fig. 2A, lanes 1 and 2). Due to the proteolytical processing, uncleaved HA is no longer detectable in untreated cells after a chase of one hour (Fig. 2A, lane 3), but prevails in large amounts in BFA-treated cells (lane 4). This control experiment shows that BFA indeed inhibits exocytotic transport in our cell system (CEF).

Labeling with [^{3}H]palmitic acid in the presence of the drug revealed that BFA has no effect on palmitoylation of HA (Fig. 2B). Because BFA is known to cause disintegration of Golgi-vesicles thereby leaving the CGN and the ER intact [22], these results support a pre-Golgi-site for palmitoylation of HA.

Next we defined the temporal relation between palmitoylation and oligomerisation of HA, a modification

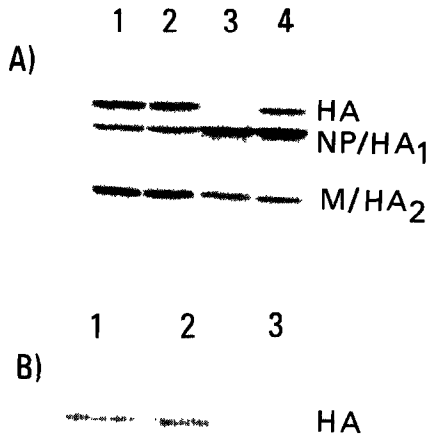

Fig. 2. BFA treatment does not abolish palmitoylation of HA. (A) Influenza virus infected CEF were left untreated (lanes 1 and 3) or were treated with brefeldin A (lanes 2 and 4). Cells were labeled 1 h later with [^{35}S]methionine for 10 min and chased for 0 min (lanes 1 and 2) or 60 min (3 and 4). Influenza virus specific proteins were immunoprecipitated from cell extracts and subjected to SDS-PAGE and fluorography. HA cleavage products HA_1 and HA_2 are not separated from the other viral proteins NP and M, respectively. (B) Infected CEF were labeled with [^{3}H]palmitic acid for 10 min in the presence (lane 1) or absence (lane 2) of BFA. The drug was added at 6 μg/ml final concentration 1 h before labeling. Lane 3 = uninfected control cells.

which is believed to occur in the ER just before the newly translated protein leaves this compartment [3–5,23]. To follow trimerization of HA experimentally, influenza A virus infected chicken embryo fibroblasts (CEF) were pulse-chase labeled with [^{35}S]methionine. Cells were lysed in non-ionic detergent and monomers and trimers were separated by sucrose-gradient centrifugation and fractionation of the gradient. Immunoprecipitation, SDS-PAGE and fluorography revealed that after short-pulse labeling with [^{35}S]methionine HA bands appear in fractions 4–6 of the gradient, which represents the monomeric form of the molecule (Fig. 3A). As shown in Fig. 3B, HA is closer to the bottom of the gradient after a 15 min chase period (fractions 1–3), which is the position of the trimer and cleavage of the protein to HA_1 and HA_2 has begun. This is in agreement with previous studies on the kinetics of HA-oligomerisation [3,4]. However, after short-pulse labeling with [^{3}H]palmitic acid HA bands to fractions 1–3 and is thus completely trimerized (Fig. 3C). As shown in the same figure this material is still uncleaved, which indicates that palmitoylation occurs after oligomerisation of HA, but before its cleavage to HA_1 and HA_2. Results from cross-linking experiments with dimethylsuberimidat (DMS) also reveal trimerization of HA before palmitoylation (not shown) which supports our interpretation, that the uncleaved trimer of HA serves as the substrate for the palmitoylating enzyme in influenza virus-infected cells.

4. DISCUSSION

We describe here the timing of fatty acid attachment to the influenza virus hemagglutinin in relation to some other well-established modifications of this protein. Short-pulse [^{3}H]palmitic acid labeled and therefore freshly acylated HA is completely trimerized (Fig. 3C), but has not undergone proteolytical cleavage (Figs. 1 and 3) or trimming of its N-linked carbohydrates. This would suggest that trimerization and palmitoylation are not only sequential events but that they occur in different intracellular compartments. Our results further exclude a possible involvement of HA-bound fatty acids in the initiation of oligomerization.

This interpretation is in agreement with similar conclusions from published results using a different approach. In these studies acylation of Semliki-Forest virus (SFV) E-proteins and vesicular stomatitis virus (VSV) G-protein was blocked at 15°C [17], whereas oligomerisation was not affected [5,23].

Continuous acylation of HA is a posttranslational event which depends on ongoing protein-synthesis since a loss of [^{3}H]palmitic acid label of the hemagglutinin was not observed during at least 1 h of chase (Fig. 1).

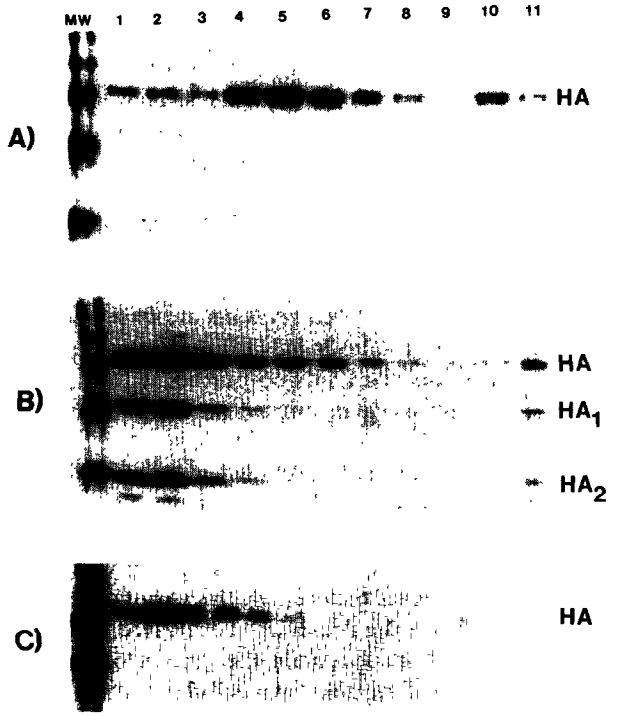

Fig. 3. Palmitoylation of HA occurs after its trimerization. CEF infected with influenza virus were labeled with [^{35}S]methionine (panels A and B) or [^{3}H]palmitic acid (C) for 3 min. Cells were lysed with non-denaturing detergent immediately (panels A and C) or after a chase period of 15 min (B). Cleared cell extracts were centrifuged in a 5–20% sucrose gradient containing 1% Triton X-100 at 40,000 rpm for 16 h. The gradient was fractionated and HA immunoprecipitated from every fraction with the monoclonal antibody HC-2 and subjected to SDS-PAGE and fluorography. Fraction 1 represents the bottom, fraction 11 the top of the gradient.

This indicates, that the fatty acids are stably attached to the viral glycoprotein and are not subject to cycles of deacylation and reacylation. This is in agreement with our earlier studies on the palmitoylation of Sindbis and VSV glycoproteins [12]. However, 'turn-over' of ester-linked fatty acids has been described for several cellular acylproteins, notably the transferrin-receptor, the ras-protein and ankyrin [18–20]. The reason for this difference between viral and cellular acylproteins, which are both acylated at cysteine residues [24] is not known. The viral proteins may escape action of the acylesterase, because they leave the host cell into budding virus particles. The alternative possibility that cellular acylproteins may have specific structural features, which are recognized by the acylesterase and which are not present in viral acylproteins seems unlikely, because cleavage of thioester-linked fatty acids has been described for the E2 protein of Semliki-Forest virus in a cell-free system [25].

Blocking intracellular transport at a pre-Golgi site with brefeldin A (BFA) does not abolish palmitoylation of HA (Fig. 2). This is in accordance with studies on two cellular acylproteins (p62 and p63), where BFA-treatment even causes an enhancement of palmitoylation [26,27].

Based on the evidence that (i) trimerization of HA is completed in the ER [3–5,23], (ii) HA leaves this compartment rapidly after its oligomerisation [3–5,23], and (iii) BFA treatment allows proteins to reach the CGN [21,22], our results show that palmitoylation of HA localizes to the CGN. This supports similar conclusions by others who related the timing of palmitoylation and oligosaccharide processing [16,17]. Furthermore, one particular palmitoylated protein (p63) could be located exclusively to the CGN [27]. The differing data on the location of freshly acylated proteins are based soley on cell fractionation methods [13–15] and may be due to the possibility that the CGN co-purifies with the ER in one experimental system, but to the Golgi-cisternae in an other.

However, it cannot be excluded at present that the protein-acyltransferase (PAT) is present at multiple intracellular sites besides the CGN. Several observations point to this possibility. Vesicle-mediated in vitro transport of glycoproteins between all the various Golgi cisternae depends on the presence of activated fatty acids [28,29]. The authors speculate that cycles of acylation and deacylation of an unknown cellular protein are required for budding, targeting and fusion of transport vesicles. This assumption would imply that acylating enzymes are located at all these different intracellular sites. Recently palmitoylating activity was detected in extracts from ghosts prepared from human red blood cells (Schmidt, Burns and McIlhinney, unpublished results). Furthermore, palmitoylation of the mature form of several cellular membrane proteins has been reported [18,24], suggesting that PAT is also present at the plasma membrane. Palmitoylating enzymes located distally to the CGN are not active on viral glycoproteins, probably because all potential acylation sites are filled during the first encounter with an acylating enzyme and because the fatty acids are stably attached to the viral glycoprotein. Definite proof for any of these hypotheses requires biochemical purification of PAT [30] and the examination of its intracellular location with immunological methods. This would also help clarify whether we are dealing with one PAT or with a number of acylating enzymes with similar properties.

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