Myristoylation of the arterivirus E protein: the fatty acid modification is not essential for membrane association but contributes significantly to virus infectivity

Bastian Thaa,1 Aleksander Kabatek,1 Jessika C. Zevenhoven-Dobbe,2 Eric J. Snijder,2 Andreas Herrmann3 and Michael Veit1

1Department of Immunology and Molecular Biology Veterinary Faculty, Free University, Berlin, Germany
2Molecular Virology Laboratory, Department of Medical Microbiology, Leiden University Medical Center, The Netherlands
3Department of Biology, Molecular Biophysics, Humboldt University, Berlin, Germany

The envelope of equine arteritis virus (EAV) contains two glycoprotein complexes (GP2b/GP3/GP4 and GP5/M) and the small, non-glycosylated E protein. As E is essential for the production of infectious progeny but dispensable for assembly and release of virus-like particles, it probably mediates virus entry into cells, putatively in concert with the GP2b/GP3/GP4 complex. The E protein contains a central hydrophobic domain and a conserved potential site for N-terminal myristoylation, a hydrophobic modification usually pivotal for membrane targeting of the modified protein. Here, it was shown by radiolabelling that E is myristoylated at glycine-2, both in transfected cells as a fusion protein with yellow fluorescent protein (YFP) and in virus particles. Biochemical fractionation revealed that E–YFP with an inactivated acylation site was still completely membrane-bound, indicating that the putative transmembrane domain of E mediates membrane targeting. Confocal microscopy showed that both myristoylated and non-myristoylated E–YFP were localized to the endoplasmic reticulum and Golgi complex, the membranes from which EAV buds. The presence of a myristoylation inhibitor during replication of EAV, whilst completely blocking E acylation, reduced virus titres by 1.5 log10. Similarly, a mutant EAV with non-myristoylatable E grew to a titre five- to sevenfold lower than that of the wild-type virus and exhibited a reduced plaque size. Western blotting of cell-culture supernatants showed that N and M, the major structural proteins of EAV, are released in similar amounts by cells transfected with wild-type and mutant genomes. Thus, E myristoylation is not required for budding of particles and probably has a function during virus entry.

INTRODUCTION

Equine arteritis virus (EAV) is the prototype member of the family Arteriviridae, which is grouped together with the coronaviruses and roniviruses in the order Nidovirales. In infected horses, EAV replicates primarily in endothelial cells and macrophages. EAV infection can be asymptomatic or can lead to a variety of symptoms, including fever, depression, oedema, conjunctivitis, nasal discharge, abortion and, infrequently, death in young foals (Balasuriya & MacLachlan, 2004; Del Piero, 2000). The positive-stranded RNA genome of EAV encodes two large replicate polyproteins and seven structural proteins: the nucleocapsid protein (N) and six membrane proteins, glycoproteins GP2b, GP3, GP4 and GP5 and the unglycosylated proteins E and M (Snijder & Meulenberg, 1998). The membrane proteins form disulfide-linked heterodimeric (GP5/M) or heterotrimeric (GP2b/GP3/GP4) complexes, but E is present as a monomer (Snijder et al., 1999, 2003; Wieringa et al., 2003a, b). N, M and GP5 are major virion components, whilst E together with GP2b, GP3 and GP4 are minor structural proteins. All seven structural proteins are essential for virus infectivity, but many details of their functions during virus assembly, maturation and entry remain to be elucidated. Reverse genetics experiments have revealed that cells transfected with mutants lacking expression of E, GP2b, GP3 or GP4 release virus-like particles (VLPs) consisting of RNA and the GP5, M and N proteins (Wieringa et al., 2004; Zevenhoven-Dobbe et al., 2004). This finding suggests that the latter proteins form the structural core of the arterivirus particle, whereas the four minor structural proteins probably play a role in
receptor binding and/or virus entry. Inactivation of EAV E expression prevents incorporation of the GP2b/GP3/GP4 complex into VLPs, whereas in the absence of the GP2b/GP3/GP4 complex, the amount of E present in VLPs is greatly (60–80%) reduced (Wieringa et al., 2004). Thus, the E protein and the GP2b/GP3/GP4 complex appear to interact structurally or at least functionally during virus assembly.

The E protein, encoded by ORF2a in the case of EAV, has been identified as a component of arterivirus particles. It is a small protein (8 kDa) composed of three domains: an N-terminal domain containing a potential myristoylation signal, a hydrophobic domain, which probably represents a transmembrane region, and a hydrophilic, polybasic C-terminal domain (Snijder et al., 1999). The topology of E within the viral membrane is not known. The hydrophobic domain might span the membrane once, resulting in exposure of either the N or C terminus to the extraviral milieu. Alternatively, the protein might adopt a hairpin topology with both the N and C terminus exposed on the same side of the membrane. Recently, the E protein of the closely related porcine reproductive and respiratory syndrome virus was shown to form non-covalently linked oligomers that exert an ion channel-like activity, possibly allowing uncoating of the viral genome in the acidic pH of the endosome (Lee & Yoo, 2005, 2006). As cell entry of EAV also requires clathrin-dependent virus uptake and exposure to a low-pH compartment (Nitschke et al., 2008), a similar function for the E protein of EAV is conceivable.

Myristoylation involves the attachment of myristate via an amide linkage to the amino group of a glycine residue at the N terminus of eukaryotic and viral proteins. N-terminal myristoylation is an irreversible process that occurs co-translationally after the initiator methionine has been removed. The enzyme N-myristoyltransferase (NMT) is highly specific for the 14-carbon saturated fatty acid and for the N-terminal glycine residue, but not all proteins with such a glycine are acylated, as myristoylation also depends on the presence of specific amino acids downstream (see the consensus motif in Fig. 1a). Myristoylated proteins are found in diverse intracellular locations such as the cytosol, the nucleus and the cytoplasmic side of intracellular membranes or the plasma membrane (Farazi et al., 2001; Gordon et al., 1991; Maurer-Stroh & Eisenhaber, 2004).

A function often attributed to myristoylation is targeting of the modified protein to membranes. However, as the C14:0 carbon chain itself is not sufficiently hydrophobic to confer permanent anchoring to membranes (Peitzsch & McLaughlin, 1993), stable binding to lipids is mediated by other means, such as subsequent palmitoylation of nearby cysteine residues, the presence of clusters of basic amino acids to allow interaction with negatively charged lipids or even by specific phospholipid-binding domains. As palmitoylation in principle is reversible, and negative charges can be masked by binding of a ligand or neutralized by phosphorylation of neighbouring amino acids, membrane binding of the myristoylated protein can be regulated. In some proteins (or protein conformations), the myristoyl

Fig. 1. The E protein is myristoylated at the N-terminal glycine. (a) Alignment of the consensus motif for myristoylation at the N-terminal glycine residue (Gordon et al., 1991) and its presence in the EAV E protein, as well as in the homologues of the other arteriviruses: porcine reproductive and respiratory syndrome virus (PRRSV), lactate dehydrogenase-elevating virus (LDV) and simian hemorrhagic fever virus (SHFV). (b) Schematic of the E–YFP fusion protein. The amino acid sequence of the N-terminal domain of E with the glycine residue in bold and the border of the putative transmembrane domain (TMD) is shown above. The methionine located in the TMD, which might serve as the start of protein translation in the truncated version of E–YFP, termed E–YFP, is underlined. E and YFP are connected by the 7 aa linker RDPPVAT as a result of cloning (hatched). (c) CHO cells were transfected with E–YFP-wt or E–YFP-G2A, the mutant with a G–A replacement, or were left untransfected (Ø), and radiolabelled with [35S]methionine ([35S]) or [3H]myristic acid ([3H]) for 4 h as indicated, lysed and subjected to immunoprecipitation with an anti-GFP antibody, followed by SDS-PAGE and fluorography. Molecular mass markers (in kDa) are shown on the left. (d) A gel containing immunoprecipitated E–YFP-wt prepared as in (c) was treated with hydroxylamine (NH₂OH) to cleave thioester-linked fatty acids and was subjected to fluorography.
chain is sequestered inside the molecule, but is exposed after an appropriate signal has been received, such as binding of calcium or GTP, a mechanism that provides another level of regulation (Resh, 2006).

Myristoylation of cellular proteins is often crucial for signal transduction and other vital functions of the cell and, as a consequence, deletion of NMT is lethal in yeast (Farazi et al., 2001; Resh, 2006). Myristoylation of structural proteins of non-enveloped viruses affects protein–protein interactions involved in assembly and cell entry of virus particles (Chow et al., 1987; Krausslich et al., 1990; Krauzewicz et al., 1990; Marc et al., 1990). In enveloped viruses, myristoylation of peripheral membrane proteins, such as Gag of retroviruses and Z of Lassa virus, is required for targeting of the modified protein to (microdomains of) membranes or even to specific lipids, such as phosphatidylinositol-4,5-diphosphate (Hearps & Jans, 2007; Perez et al., 2004; Saad et al., 2006). An essential function of myristoylation in virus entry has been documented for the large glycoprotein of the enveloped hepatitis B virus, where myristate is directly involved in interactions with the cellular receptor (Bruss et al., 1996; De Falco et al., 2001; Gripon et al., 1995). Thus, the fatty acid is essential for the function of most myristoylated viral proteins studied so far, affecting either assembly or cell entry of enveloped and non-enveloped viruses.

**METHODS**

**Cell culture and virus infections.** Cell culture, virus infection and plaque assays were carried out as described previously (Veit et al., 2008a). 2-Hydroxymeristate (2-hydroxytetradecanoic acid; Sigma-Aldrich) was prepared as a 100 mM stock solution in DMSO and added to the medium to achieve the indicated concentrations. The drug was added at 4 h post-infection (p.i.) and remained present throughout the infection experiment. Hydroxylamine treatment of the polyacrylamide gel was carried out as described previously (Veit et al., 2008).

**Transient expression of E.** The full-length E gene (ORF2a) was PCR-amplified from the EAV ORF2a expression vector pAV102a (Snijder et al., 1999) and cloned between the XhoI and BamHI sites of pEYFP-N1 (Clontech), downstream of the human cytomegalovirus (CMV) immediate-early promoter, to produce an E protein C-terminally tagged with yellow fluorescent protein (E-YFP). To inactivate the putative myristoylation signal of E, a G2A mutation was introduced by PCR mutagenesis. Confluent monolayers of Chinese hamster ovary (CHO) cells grown in 10 cm2 dishes were transfected with 4 µg plasmid DNA using Lipofectamine 2000 (Invitrogen).

**Metabolic labelling of proteins and virus.** Before protein labelling with [35S]methionine, transfected cells were starved for 2 h in medium lacking methionine and cysteine (Eagle’s minimal essential medium with Earle’s balanced salt solution; PAN Biotech). Cells were labelled with Trans35S-label containing [35S]methionine and [35S]cysteine (5 µCi ml−1) in methionine- and cysteine-deficient medium; MP Biomedicals) or with [3H]myristic acid (250 µCi ml−1) in complete medium, [9,10-2H]myristic acid; Moravak Biochemicals) for 4 h. After metabolic labelling, the medium was removed and the cells were lysed for 15 min on ice with RIPA buffer [20 mM Tris/HCl (pH 7.4), 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 150 mM NaCl, 10 mM EDTA, 10 mM iodoacetamide, 1 mM PMSF). For pulse-chase experiments, cells were labelled for 2 h with Trans35S-label and incubated in complete medium in the absence of radioactivity for 1, 4 or 21 h prior to cell lysis. Cell lysates were cleared (5000 g, 5 min, 4°C) and the supernatants were used for immunoprecipitation.

To prepare radiolabelled virus particles, baby hamster kidney (BHK-21) cells infected with EAV at an m.o.i. of 50 were labelled for 4 h after infection with Trans35S-label [3 µCi ml−1 in methionine- and cysteine-deficient medium containing 2% fetal calf serum (FCS)] or with [3H]myristic acid (10 µCi ml−1 in complete medium containing 2% FCS) for 20 h at 39.5°C. Cell culture supernatants were cleared (30000 g, 15 min, 4°C) and virus particles were pelleted (100000 g, 2 h, 4°C).

**Membrane fractionation and flotation assays.** Membrane isolations were performed as described previously (Thaa et al., 2009). Briefly, the cleared homogenates of transfected cells were subjected to centrifugation at 100000 g at 4°C for 1 h or were loaded at the bottom of a Nycodenz density gradient and centrifuged at 100000 g at 4°C for 16 h. Samples were then precipitated with trichloroacetic acid and analysed by Western blot.

**Immunoprecipitation, Western blotting and SDS-PAGE.** For immunoprecipitation, 8 µg anti-green fluorescent protein (GFP) antibody (cross-reacts with YFP, Molecular Probes) or 1:10-diluted rabbit antiserum raised against the E protein (Snijder et al., 1999) was added to the cleared cell lysate (in RIPA buffer) or to the virus preparation [in immunoprecipitation (IP) buffer; 20 mM Tris/HCl (pH 7.6), 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 0.1% sodium deoxycholate, 0.1% SDS] and these mixtures were incubated under agitation for 16 h at 4°C. Next, 40 µl of a 1:1 slurry of protein A-Sepharose (Sigma-Aldrich) in RIPA buffer was added, and samples were shaken at 4°C for an additional 2.5 h. The protein A-Sepharose with bound antigen–antibody complexes was pelleted (2000 g, 1 min), the supernatant was discarded and complexes were resuspended in 800 µl RIPA or IP buffer without SDS and sodium deoxycholate. Immune complexes were washed three times. For SDS-PAGE, 20 µl 3× reducing sample buffer was added to the protein A-Sepharose and the samples were boiled for 5 min at 100°C.

**Western blots** were performed on Hybond LFP (PVDF) membranes (GE Healthcare) using either the above-mentioned anti-GFP antibody at a dilution of 1:1000 and anti-rabbit secondary antibody (Sigma-Aldrich) at a dilution of 1:5000, or an anti-M protein rabbit antiserum (de Vries et al., 1992) and anti-N protein monoclonal antibody 3E2 (MacLachlan et al., 1998), corresponding biotinylated secondary antibodies and a Cy3-conjugated anti-biotin antibody (Dako). Protein bands were visualized using a Typhoon imager (GE Healthcare).

**Immunofluorescence and confocal microscopy.** Twenty-four hours after transfection with plasmids expressing wild-type E (E-YFP-wt) or mutant E (E–YFP-G2A), cells grown on coverslips were fixed using 3% (w/v) paraformaldehyde in PBS for 10 min, followed by permeabilization with 0.5% (v/v) Triton X-100 in PBS for 3 min. Epitopes were blocked by applying blocking solution [3% (w/v) BSA in PBS] for 60 min. The cells were then incubated with primary antibody diluted in blocking solution for 60 min [endoplasmic reticulum (ER) marker, rabbit anti-calreticulin (1:200; Calbiochem); Golgi marker, mouse anti-membrin (1:500; Abcam)]. Coverslips were washed three times with PBS between each step. The cells were then incubated with an Alexa Fluor 568-conjugated anti-mouse or anti-rabbit secondary antibody (1:1000; Molecular Probes) for 60 min and mounted on a microscope slide.
Microscopy was carried out using a Leica TCS SP2 confocal laser-scanning microscope. The fluorescence of YFP was excited at 514 nm with an argon laser and recorded between 528 and 550 nm (pseudocoloured green); the excitation of Alexa Fluor 568 fluorescence was at 543 nm with a helium–neon laser, the emission was recorded between 580 and 664 nm (pseudocoloured red). Images were processed using Adobe Photoshop.

Reverse genetics. To generate an EAV mutant carrying the G2A mutation in the E protein, a G→C mutation was introduced at nt 9755 of the genome in the EAV full-length cDNA clone pEAV211 (van den Born et al., 2005; van Dinten et al., 1997). The mutation was first engineered in a BamHI–EcoRI fragment (nt 9150–11488), which was sequenced (GATC Biotech) and transferred to pEAV211. Full-length clones were linearized with XhoI and in vitro-transcribed using T7 RNA polymerase, and 5 μg RNA was then introduced into 4 × 10⁶ BHK-21 cells using Amaza Nucleofector technology (Nucleofector kit T; program T-20) and incubated at 39.5 °C for 16 or 24 h. Plaque assays were performed for 3 days using Dulbecco’s minimal essential medium containing 1.2% Avicel (RC-581, FMC biopolymer; Matrosovich et al., 2006), 3% FCS and 50 mM HEPES.

RESULTS AND DISCUSSION

Bioinformatic analysis suggests arterivirus E protein myristoylation

Recently, a large-scale, cross-genome analysis predicted the myristoylation of numerous proteins that contain a transmembrane region instead of one of the other above-mentioned additional signals for stable membrane binding, such as subsequent palmitoylation of nearby cysteine residues, the presence of clusters of basic amino acids or even specific phospholipid-binding domains (Maurer-Stroh et al., 2004), but none of the proteins identified in such a manner is known to be acylated. The arterivirus E protein also belongs to this group, as it has a (predicted) transmembrane region but no basic amino acids or cysteines as potential sites for palmitoylation in its vicinity (Snijder et al., 1999). However, the algorithm used in the study by Maurer-Stroh et al. (2004) (http://mendel.imp.ac.at/myristate/SUPLpredictor.htm) does not predict the E protein of EAV to be myristoylated, whereas another program (http://expasy.org/tools/myristoylator/) does, albeit with low confidence. EAV E protein contains a glycine directly after the N-terminal methionine and fulfils the requirements of the myristoylation consensus motif (Fig. 1a). Moreover, the same is true for the E proteins of all other arteriviruses (Snijder et al., 1999), as illustrated in Fig. 1(a). We were therefore interested in analysing whether EAV E is myristoylated and to investigate what role this modification plays in the arterivirus life cycle.

Evidence for myristoylation from E expression studies

For transient expression studies, we engineered a plasmid expressing an EAV E–YFP fusion protein from a CMV promoter (Fig. 1b). To test whether E–YFP is myristoylated, transfected CHO cells were labelled with [35S]methionine or [3H]myristic acid. E–YFP was immunoprecipitated from cellular extracts with antibodies against GFP and subjected to SDS-PAGE and fluorography. Labelling with [35S]methionine showed a 36 kDa band, slightly larger than the predicted size for E (8 kDa) plus YFP (27 kDa). The fluorogram also revealed clear labelling of E–YFP with [3H]myristate (Fig. 1c). To determine the type of fatty acid linkage present in E–YFP, we treated the gel with hydroxylamine, which is known to cleave labile thioester-linked fatty acids from proteins, whereas stable amide-linked fatty acids are not affected (Veit et al., 2008b). The labelling of E–YFP with [3H]myristic acid was not diminished by hydroxylamine treatment of the gel (Fig. 1d); thus, the fatty acid was concluded to be attached through an amide linkage. To demonstrate the requirement for glycine-2 for myristoylation of E–YFP, this residue was changed to alanine. The resulting mutant, E–YFP-G2A, only marginally incorporated [3H]myristate, although it was expressed at a similar level to E–YFP-wt (Fig. 1c). The faint labelling was probably due to metabolism of the [3H]myristic acid, resulting in [3H]labelled amino acids that were subsequently incorporated into proteins. E–YFP was not labelled when [3H]palmitic acid was used (data not shown).

Besides the full-length E protein with the expected molecular mass, a truncated version, termed Ei–YFP, was also observed in [35S]methionine-labelled cells (Fig. 2a). As a 2.5 kDa shorter version of E was also observed in EAV-infected cells (Snijder et al., 1999), this product is probably not an artefact of the expression system. It was speculated that truncation of E might be due to proteolytic degradation of the N-terminal domain or caused by translation initiation at the second AUG codon, which is in frame with the first AUG (Snijder et al., 1999). Our [3H]myristate labelling results are compatible with both assumptions, as Ei–YFP was not labelled, indicating that it does not contain the N-terminal amino acids of the full-length protein. As noted previously (Snijder et al., 1999), variable amounts of Ei–YFP were found in different experiments; typically, Ei–YFP levels were higher in Western blots than in immunoprecipitation experiments (compare, for example, Fig. 2a with Fig. 1c), possibly due to differences in folding between E–YFP and Ei–YFP and therefore weaker antibody binding to the latter in immunoprecipitation.

Myristoylation is not required for membrane association of E

A possible function of myristoylation of E would be to target the protein to membranes. We therefore compared membrane association of myristoylated E–YFP-wt with that of the non-myristoylated mutant E–YFP-G2A. Cells expressing these proteins were homogenized and cell lysates were separated by ultracentrifugation into a particulate (100 000 g pellet) and soluble (100 000 g supernatant) fraction. Western blotting clearly showed that both E–YFP-wt and E–YFP-G2A were exclusively

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complication, they might pellet at 100,000 g and, if large protein aggregates resulted from this absence of myristate might cause misfolding of E–YFP–present in the membrane fraction (Fig. 2b). However, the absence of myristate might cause misfolding of E–YFP–G2A and, if large protein aggregates resulted from this absence of myristate might cause misfolding of E–YFP–G2A.

Myristoylation is not required for membrane binding and stability of E. (a) Western blot (anti-GFP antibody) of lysates from CHO cells transfected (+) or not (−) with E–YFP-wt. Molecular mass markers (kDa) are shown on the left. (b) Membrane fractionation: cleared supernatants of homogenized CHO cells expressing E–YFP-wt or E–YFP-G2A were centrifuged for 1 h at 100,000 g. The resulting supernatant (Cyt) and pellet (Mem) were subjected to SDS-PAGE and Western blotting (anti-GFP). Cell: aliquot of the cell homogenate prior to fractionation (analysed on a different gel in parallel). (c) Membrane floatation assay: cleared supernatants of homogenized CHO cells expressing E–YFP-wt or E–YFP-G2A were centrifuged in a Nycodenz gradient. Fractions from the top (F1, membranes) to the bottom (F4) were analysed by SDS-PAGE and Western blotting (anti-GFP). (d) Pulse–chase experiment: CHO cells expressing E–YFP-wt or E–YFP-G2A were labelled with [35S]methionine for 2 h, followed by incubation with non-radioactive medium for the indicated time periods, lysis, immunoprecipitation with an anti-GFP antibody, SDS-PAGE and fluorography.

Finally, we analysed whether myristoylation might affect the long-term stability of E in transfected cells. In pulse–chase experiments with [35S]methionine, the intensity of labelling of both proteins did not decrease with increasing chase times (Fig. 2d). Thus, both E–YFP-wt and E–YFP-G2A were largely stable for (at least) 21 h, a period that is longer than the replication cycle of EAV in BHK cells (Veit et al., 2008a).

The truncated E$_i$–YFP protein showed a completely different cell fractionation pattern compared with full-length E–YFP. E$_i$–YFP was present exclusively in the cytosolic fraction of the cell homogenate and did not float with membranes in the gradient (Fig. 2b, c). Thus, it lacked all membrane-binding activity, suggesting that at least a part of the transmembrane region was either removed or not synthesized in the first place. As proteolytic degradation of E–YFP in pulse–chase experiments was not detectable (Fig. 2d), translation initiation at the second AUG, which would skip the first 6 aa of the predicted hydrophobic domain, is a probable explanation for the origin of E$_i$–YFP. The biological function of this truncated and soluble version of E, if any, remains to be investigated.

**Lack of myristoylation does not affect the intracellular localization of E**

So far, we have shown that myristoylation is not required for membrane association of E–YFP, but it might well be that the fatty acid retains the protein in the ER and/or Golgi complex, the intracellular membranes from which EAV budding occurs (Snijder & Meulenberg, 1998). To investigate whether E–YFP-wt and E–YFP-G2A localized to different cellular membranes, both proteins were transiently expressed in CHO cells, which were immunolabelled after 24 h using antibodies recognizing marker proteins of the ER and Golgi, calreticulin and membrin, respectively. E–YFP-wt showed a bright perinuclear and reticular staining, which overlapped some parts of the ER and the entire Golgi complex (Fig. 3, upper panels). Similar results have been reported for the native E protein produced in EAV-infected cells (Snijder et al., 1999), suggesting that fusion to YFP does not greatly compromise targeting of E. Also E–YFP-G2A localized partially to the ER and the Golgi complex, demonstrating that lack of myristoylation does not lead to detectable mistargeting of the E protein (Fig. 3, lower panels).

**Myristoylated E is present in the EAV particle**

As myristoylation is apparently not required for accumulation of E in intracellular membranes and because E is dispensable for assembly of the core arterivirus particle (Wieringa et al., 2004), we investigated whether myristoylation of E might be essential for the entry of EAV into
target cells. To this end, we attempted to chemically block E myristoylation during EAV replication and to analyse the released virus particles for their infectivity. From the many known inhibitors of myristoylation, we chose 2-hydroxy-myristate, which, after activation to its coenzyme A derivative inside cells, effectively blocks myristoylation but not palmitoylation. The drug binds to NMT, but, unlike other inhibitors, the fatty acid is not transferred to the protein destined to be myristoylated, resulting in the synthesis of proteins that are devoid of any hydrophobic modification (Paige et al., 1990). To determine the concentration of 2-hydroxymyristate required to block myristoylation, cells expressing E–YFP-wt were labelled with $[^3H]$myristate in the presence of increasing concentrations of the inhibitor. Considerable inhibition of myristoylation of E–YFP-wt was detectable at 0.1 mM 2-hydroxymyristate and complete inhibition at a concentration of 2 mM (Fig. 4a), which is in line with published reports analysing the inhibition of myristoylation of various other proteins (Paige et al., 1990). We then labelled EAV-infected cells with $[^3H]$myristate in the presence or absence of 1 mM 2-hydroxymyristate and pelleted virus particles from the cell culture supernatant. Protein analysis of virus particles revealed three major bands, representing GP5, M and N, the main proteins of EAV (Fig. 4b). Between GP5 and M, various bands were visible representing the (heterogeneously glycosylated) minor proteins GP2b, GP3 and GP4 (Veit et al., 2008a; Wieringa et al., 2002). The E protein could not be identified unambiguously, probably because the mature protein contains only a single methionine. However, SDS-PAGE and fluorography of EAV particles labelled with $[^3H]$myristate showed a band running well below the N protein, which most likely represents E. This band did not appear when EAV was grown in the presence of 1 mM 2-hydroxymyristate, indicating that its myristoylation was completely blocked. Coomassie Brilliant Blue R250 staining of the gel used to create this fluorogram revealed no difference in the band pattern between virus grown in the presence or absence of 2-hydroxymyristate (not shown), suggesting that similar numbers of virus particles (or at least viral proteins) were released.

![Fig. 3. Myristoylation is not required for localization of E to intracellular membranes. CHO cells transfected with E–YFP-wt or E–YFP-G2A (pseudocoloured green) were stained 24 h later with ER-specific (calreticulin) or Golgi-specific (membrin) antibodies, and Alexa Fluor 568-coupled secondary antibodies (pseudocoloured red). Co-localization of proteins was analysed by confocal microscopy.](image)

![Fig. 4. Inhibition of E myristoylation. (a) CHO cells expressing E–YFP-wt were labelled with $[^35S]$methionine ($[^35S]$) or $[^3H]$myristic acid ($[^3H]$-Myr) for 4 h prior to lysis; 2-hydroxymyristate (Myr-OH) was present in the medium throughout labelling at the indicated concentrations. E–YFP was then subjected to immunoprecipitation (anti-GFP), SDS-PAGE and fluorography. (b) BHK-21 cells infected with EAV at an m.o.i. of 50 were radiolabelled with $[^35S]$methionine or $[^3H]$myristic acid at 4 h p.i. To inhibit myristoylation, cells were treated with 2-hydroxymyristate at a concentration of 1 mM throughout the labelling with $[^3H]$myristic acid. Virus particles were pelleted from the culture supernatant at 20 h p.i. and subjected to SDS-PAGE and fluorography. Right panel: an aliquot of $[^3H]$myristic acid-labelled virus preparation subjected to 2-hydroxymyristate treatment (+) or not (−), immunoprecipitation using an E protein antiserum prior to SDS-PAGE and fluorography.](image)
In order to confirm the identity of the observed 8 kDa band, we immunoprecipitated E from this virus preparation using an anti-E antiserum (Snijder et al., 1999) and detected \(^{3}H\)myristate-labelled E in virus grown in the absence of 2-hydroxymyristate (Fig. 4b, right panel). This result showed that E is myristoylated not only as a fusion protein with YFP in transfected cells but also in the context of virus infection, and that the mature virion contains myristoylated E.

E protein myristoylation contributes to but is not essential for EAV infectivity

We next performed plaque assays with EAV harvested from cells infected in the absence or presence of increasing concentrations (0.01, 0.1, 1 and 2 mM) of 2-hydroxymyristate. This allowed us to determine the amount of released infectious progeny more precisely and to analyse whether EAV containing non-myristoylated E protein was infectious, i.e. able to enter target cells. In the absence of inhibitors, EAV grew to a titre of \(4 \times 10^7\) p.f.u. ml\(^{-1}\), as observed previously (Veit et al., 2008a). The presence of 2-hydroxymyristate, which was added 4 h p.i. and remained present throughout the infection, moderately reduced the titre of virus released after 20 h. At the highest concentration of 2-hydroxymyristate (2 mM), the reduction in virus titre was 40-fold (1 \(\times\) 10\(^6\) p.f.u. ml\(^{-1}\), Fig. 5a). To exclude the possibility that 2-hydroxymyristate is unstable and no longer functional by the time the majority of E molecules are synthesized, we also added the inhibitor at 12 h p.i. to virus-infected cells and harvested the virus 8 h later. In this case, a virus titre of 1.5 \(\times\) 10\(^6\) p.f.u. ml\(^{-1}\) was obtained in untreated cells, which was reduced by addition of the drug to titres of 1 \(\times\) 10\(^5\) (2 mM 2-hydroxymyristate), 1.5 \(\times\) 10\(^5\) (1 mM), 2 \(\times\) 10\(^5\) (100 \(\mu\)M) and 2.5 \(\times\) 10\(^5\) (10 \(\mu\)M) (data not shown). Thus, E myristoylation is apparently not essential for productive EAV infection in cell culture. In contrast, it has been shown that even 100 \(\mu\)M 2-hydroxymyristate can reduce the growth of a variety of viruses, such as herpes- and arenaviruses, by up to three orders of magnitude (Cordo et al., 1999; Harper et al., 1993; Perez et al., 2004; Strecker et al., 2006).

To exclude the possibility that the impact of 2-hydroxymyristate on virus replication is due to non-specific cytopathic effects, we used reverse genetics to engineer a recombinant EAV carrying the G2A mutation. BHK-21 cells were transfected with \textit{in vitro}-synthesized full-length wild-type or mutant EAV RNA. The transfection efficiency

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\centering
\includegraphics[width=0.8\textwidth]{fig5}
\caption{Lack of E myristoylation reduces EAV infectivity. (a) BHK-21 cells were infected with EAV and treated with different concentrations of 2-hydroxymyristate as indicated, added at 4 h p.i. After incubation for 20 h in the presence of the inhibitor, the virus-containing medium was removed and a plaque assay was performed in duplicate to determine the virus concentration. The experiment was performed three times to calculate mean titres ± SD. (b) Immunofluorescence to assess subcellular localization of wild-type E protein and G2A mutant at 16 h p.t. with recombinant viral RNA. (c) Plaque assay of progeny from the initial transfection (P0, 16 h p.t.) and the subsequent triplicate infection experiment (P1, 16 h p.i.), and representative images (P0, wild type at 10-fold higher dilution; left plate). (d) Western blot analysis of the particle content of cell culture supernatants from the initial transfection (P0) and the subsequent triplicate passaging experiment (P1). Samples (10 \(\mu\)l) of all supernatants were analysed directly using a rabbit antiserum recognizing the M protein and a mouse monoclonal antibody recognizing the N protein. Molecular mass markers (kDa) are shown on the left.}
\end{figure}
was verified by immunofluorescence microscopy and was found to be about 80% for both RNAs. Labelling of the E protein did not reveal any obvious differences in subcellular localization, with both wild-type and mutant E proteins displaying the previously documented staining of ER and Golgi membranes (Fig. 5b). Cell culture medium was harvested at 16 and 24 h post-transfection (p.t.) and the production of infectious progeny was analysed by plaque assay. Infectious progeny was released for both the wild-type and the G2A mutant, but plaques were clearly smaller for the mutant (Fig. 5c) and its titre was reduced about fivefold at 16 h (Fig. 5c) and sevenfold at 24 h (not shown). Subsequently, the 16 h harvests were used to infect fresh BHK cells in triplicate at an m.o.i. of 5. Again, medium was harvested at 16 h p.i. and progeny titres were determined by plaque assay, resulting in an approximately sixfold reduction in titre for the mutant (Fig. 5c). The finding that the EAV mutant expressing a non-myristoylated E protein could be rescued corroborates the conclusion that the fatty acid modification is not necessary for virus replication, but it is evident from both the inhibitor study and the reverse genetics approach that E myristoylation offers a five- to tenfold selective advantage before definitive conclusions about the function of its myristoylation can be drawn.

To analyse whether the reduced titre of the mutant was accompanied by a detectable reduction in particle production, cell culture supernatants from both the transfection experiment (16 h p.t.) and the triplicate transfection experiment (16 h p.i.) were clarified and analysed directly by Western blotting using antibodies recognizing the viral M and N proteins (Fig. 5d). For all samples, the signal for these two major structural proteins of EAV was approximately equal, suggesting that the G2A mutant produced the normal number of progeny particles, but that their infectivity was affected by the lack of E protein myristoylation. Formally, it should be noted that the G →A amino acid substitution at position 2 may also contribute to the observed phenotypic difference.

Our combined data are consistent with an important role for myristoylation of glycine-2 of the arterivirus E protein in virion infectivity. The hydrophobic modification is not essential for targeting and anchoring of the protein to intracellular membranes, indicating that both functions are probably performed by the central hydrophobic domain of the molecule. The release of infectious virus in the presence of the myristoylation inhibitor 2-hydroxymyristate and the infectivity of the G2A mutant with non-myristoylatable E were both reduced to a similar extent, in line with the reduced size of the plaques observed for the mutant. The normal subcellular localization of the mutant protein and the observation that the total release of particles was not affected in the mutant both suggest that E myristoylation is not required for budding of EAV particles and that it is more likely to have a function during virus entry, e.g. during binding of EAV to a receptor on the cell surface. This is also reflected by the fact that the myristoylation signal is strictly conserved in all members of the family *Arteriviridae* (Snijder et al., 1999; see also Fig. 1a). Myristoylation could be directly involved in the recognition of the cellular receptor for the virus, as described for the L protein of hepatitis B virus (Barrera et al., 2005). Alternatively, it is possible that myristoylation of E exerts its function indirectly, for example by modulating the conformation of the GP2b/GP3/GP4 complex, with which it interacts physically or at least functionally (Wieringa et al., 2004). Furthermore, assuming that EAV E also functions as an ion channel, as has been suggested for the E protein of porcine reproductive and respiratory syndrome virus (Lee & Yoo, 2006), the fatty acid modification might affect ion transport. Clearly, the structure and function of the arterivirus E protein must be defined in more detail before definitive conclusions about the function of its myristoylation can be drawn.

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