Dengue virus (DENV) is a mosquito-transmitted pathogen, which together with other arthropod-borne viruses like yellow fever virus, West Nile virus and tick-borne encephalitis virus (TBEV) are members of the genus Flavivirus. Flaviviruses are small, enveloped, icosahedral viruses with a single copy of a positive-strand RNA genome. The viral envelope anchors two transmembrane proteins: the small (8 kDa) membrane protein M and the major (51–60 kDa) envelope glycoprotein E (Lindenbach & Rice, 2001). The E glycoproteins are organized in 90 homodimers, which lie flat on the viral surface (Kuhn et al., 2002; Kuhn & Rossmann, 2005).

Assembly of virus particles is initiated in the endoplasmic reticulum by formation of an immature virion. In immature particles, the E protein forms a heterodimeric association with prM, the precursor protein of M. Each particle contains 60 trimers of E–prM heterodimers and thus differs structurally from a mature virion (Kuhn et al., 2002, 2003; Elshuber & Mandl, 2005; Heinz et al., 1994b, 1994c, 1994d; Stadler et al., 1997). Maturation of flavivirus particles occurs during transport through the exocytic pathway. The viral envelope proteins are believed to undergo conformational changes triggered by the low pH in the lumen of the trans-Golgi network (TGN). Shortly before or during the final release of the virions, prM is cleaved by the host cell endoprotease furin into virion-associated M and a soluble peptide (Mackenzie & Westaway, 2001; Wengler & Wengler, 1989). The biological significance of the maturation process has been investigated in considerable detail, particularly for TBEV. Characterization of the infectious properties of prM-containing TBEV virions revealed that these particles cannot undergo the structural rearrangements required for membrane fusion (Guirakhoo et al., 1991; Heinz et al., 1994a; Stadler et al., 1997). Consequently, immature particles are considered to be non-infectious (Elshuber et al., 2003; Elshuber & Mandl, 2005; Heinz et al., 1994b; Stadler et al., 1997). These results led to the widely accepted hypothesis that prM protects the E protein from undergoing premature conformational changes during transit through the acidic TGN and that cleavage of prM to M is required to render the virus particle infectious (Guirakhoo et al., 1992; Heinz et al., 1994a).

Interestingly, the DENV cleavage of prM to M is not very efficient. Several studies have shown that both mammalian cells (BHK-21 or Vero) and insect cells (C6/36) infected with DENV type 2 (16681, NGC and PR159 S1) release high numbers of particles containing unprocessed prM (Anderson et al., 1997; He et al., 1995; Henchal et al., 1985; Murray et al., 1993; Putnak et al., 1996; Randolph et al., 1990; van der Schaar et al., 2007; Wang et al., 1999). These recurring in vitro observations have their authentication in vivo, since anti-prM antibodies are commonly found in sera of DENV-infected patients (Bray & Lai, 1991; Cardosa et al., 2002; Se-Thoe et al., 1999). Furthermore, it has been shown that the specific infectivity of prM-containing DENV-2 particles produced on chloroquine-treated BHK-21 cells is only reduced by six- to eightfold compared with that of a wild-type virus preparation, which suggests that immature DENV particles may retain considerable levels of infectivity (Randolph et al., 1990). Remarkably, the authors also observed that viral infectivity could be
restored to wild-type levels upon addition of prM antibodies to the titration reaction. The high proportion of immature particles released from DENV-infected cells and the only limited reduction of the specific infectivity of immature virus particles suggest that DENV may have evolved such that cleavage of prM to M is not required for infectivity per se. In an attempt to resolve this question, we reinvestigated the infectious properties of immature DENV particles. Unlike the approach used in the above study (Randolph et al., 1990), we prepared immature DENV particles in LoVo cells, which lack functional furin.

First, we assessed the growth of DENV-2 in LoVo cells (human adenocarcinoma cells), and compared it to virus production in C6/36 mosquito cells, a cell line commonly used for DENV production. LoVo cells have been used since Takahashi and co-workers demonstrated that these cells lack the endoprotease furin, which suggested that these cells should secrete fully immature DENV particles (Takahashi et al., 1993). LoVo cells were cultured in Ham’s medium (Invitrogen) supplemented with 20% fetal bovine serum (FBS) at 37 °C and 5% CO2. Aedes albopictus C6/36 cells (CRL-1660; ATCC) were maintained in minimal essential medium (Life Technologies) supplemented with 10% FBS, 25 mM HEPES, 7.5% sodium bicarbonate, penicillin (100 U ml−1), streptomycin (100 µg ml−1), 200 mM glutamine and 100 µM non-essential amino acids at 28 °C and 5% CO2. Cells were infected with DENV-2 strain 16681 at an m.o.i. of 10, to ensure initial infection of all cells, for 1.5 h at 37 °C. Following infection, viral inoculum was removed and the cells were washed twice with PBS. Subsequently, fresh medium was added and incubation was continued. The number of genome-containing particles (GCP) was determined in samples collected at 24 h time intervals, as described previously (van der Schaar et al., 2007). Briefly, viral RNA was extracted from the samples by use of QIAamp Viral RNA mini kit (Qiagen). Next, cDNA was synthesized from the RNA by RT-PCR, copies of which were quantified using quantitative-PCR.

Fig. 1(a) shows that comparable numbers of GCP were secreted from LoVo and C6/36 cells at each time point examined. This indicates that the release of viral particles, based on quantification of RNA copies, is not impaired in LoVo cells and that these cells are at least as permissive to DENV infection as mosquito cells. In both cell lines, maximal virus particle release was observed at 48 h post-infection (p.i.).

Subsequently, we analysed the protein composition of the virus particles produced in LoVo and C6/36 cells by SDS-PAGE. To allow accurate visualization and quantification of the protein bands, [35S]methionine-labelled virus was prepared. Briefly, 2 h after infection of both cell lines at an m.o.i. of 10, 400 µCi (14.8 MBq) of [35S]methionine (Amersham Biosciences) was added to 20 ml medium and incubation was continued overnight. At 23 h p.i., the medium was supplemented with an additional 200 µCi (7.4 MBq) of the radioactive label. At 72 h p.i., the supernatant containing the viral particles was cleared from cell debris by low-speed centrifugation and the virions were pelleted by ultracentrifugation at 4 °C in a Beckman type SW41 rotor for 2.5 h at 35 000 g. Subsequently, virus pellets were resuspended in HNE buffer (5 mM HEPES, 150 mM NaCl, 0.1 mM EDTA, pH 7.4) and further purified on a discontinuous (20 and 55% w/v) Optiprep gradient (Axis-Shield) ultracentrifugation in a Beckman type SW41 rotor, 4 °C at 210 000 g for 2 h. Viruses were harvested from the gradient interface and subjected to SDS-PAGE analysis. The results showed that virus particles secreted from LoVo cells do not contain the M protein, since only E, prM and C protein bands were detected (Fig. 1b, lane 2). In contrast, virus preparations harvested from C6/36 mosquito cells did show the presence of the M protein (lane 1). Next, we quantified the prM and M contents of these virus preparations by phosphorimaging analysis using ImageQuant TL software (Amersham Biosciences). The percentage of prM and M in virions was determined by relating the intensity of prM and M
bands to that of E, on the basis of the relative numbers of methionine residues in the distinct viral proteins and with the assumption of uniform labelling. The number of methionine residues in the E, prM and M protein of DENV-2 16681 are 19, 11 and 5, respectively. Three independent SDS-PAGE analyses revealed that DENV particles released from LoVo cells contain on average 94 ± 9% prM, this number representing the observed amount of prM relative to the theoretical prM content of a completely immature virus preparation calculated on the basis of the intensity of E. Conversely, DENV-infected mosquito cells yielded primarily mature virions with an average M content of 89 ± 13.4%. In agreement with other studies, we also detected a substantial amount of prM in C6/36-produced virus (Fig. 1b, lane 1), quantification of which revealed an average content of 28.7 ± 9.8%. In both DENV preparations, the identity of the prM protein was ensured by Western blot analysis using the anti-prM antibody 2H2 (Fig. 1c). Collectively, these results clearly demonstrate that DENV-infected LoVo cells secreted fully immature prM-containing viruses.

Next, we determined the infectious properties of the distinct virus preparations using an infectious centre assay (ICA) (van der Schaar et al., 2007). Briefly, serial dilutions of virus samples harvested during the growth curve analysis (Fig. 1) were used to infect BHK-15 cells. Cells were fixed at 24–27 h p.i. and stained intracellularly with monoclonal antibody (mAb) 3H5 (Chemicon International) against the DENV E glycoprotein. Fig. 2(a) shows that the infectious titre of immature DENV-2 was severely reduced compared with that of virions produced in the insect cells at any given time point. Indeed, very few DENV-infected cells were observed (Fig. 2b). Viruses were also titrated on Vero and C6/36 cells with very similar results (data not shown). Subsequently, we calculated the ratio of GCP to infectious units (IU) for each of the virus samples (Table 1). The results show that the specific infectivity of immature DENV was at least 10 000-fold lower than that of virus generated in C6/36 cells.

Since insect cells are quite different from mammalian cells and might produce virus with a high specific infectivity for reasons other than the presence of functional furin, we also determined the specific infectivities of DENV generated in two mammalian cell lines, BHK-15 and Vero cells (p149). These cells were maintained at 37 °C, 5% CO2 in C6/36 medium without glutamine and non-essential amino acids and infected at an m.o.i. of 10 as described above. Samples were collected at different time points p.i. for analysis. As shown in Table 1, at all time points the GCP : IU ratio of virus produced in these cells was very similar to that of virus generated in C6/36 cells, thus confirming that immature DENV generated in LoVo cells is at least 10 000-fold less infectious due to the absence of functional furin.

To further substantiate that cleavage of prM to M by furin is essential for DENV infectivity, we investigated whether treatment of immature DENV with exogenous furin could...
restore viral infectivity. To this end, immature particles were incubated with furin (New England BioLabs) for 16 h at pH 6.0, after which the infectious properties of the particles were measured by ICA. Fig. 2(c) shows that the infectivity of the virus preparation was dramatically increased (approx. by a factor of 1000) upon in vitro treatment with furin. This observation indicates that immature DENV is actually non-infectious due to a complete lack of prM cleavage and hence demonstrates that processing of prM to M is a prerequisite for DENV infectivity.

Earlier studies have shown that immature DENV particles produced on cells treated with chloroquine had a reduced (factor six to eight), but still quite high, level of infectivity. However, this study clearly demonstrates that immature DENV particles are not infectious. This conclusion is in agreement with previous results obtained with TBEV (Elshuber et al., 2003; Elshuber & Mandl, 2005; Heinz et al., 1994b). Immature TBEV virions produced in LoVo cells, BHK-21 cells treated with furin inhibitor or particles in which the prM cleavage motif was mutated showed a significant drop (up to a factor of 10 000) in viral infectivity. A less profound effect (factor 20–50) on viral infectivity was seen when immature TBEV virions were generated in chicken embryo cells treated with acidotropic reagents (Heinz et al., 1994b). This may suggest that usage of acidotropic reagents is not sufficient to completely prevent viral maturation in the TGN and that the presence of M-containing particles in the viral preparations contributes to the residual infectious titre observed. The results presented in this manuscript show that, once cleavage of prM to M is completely blocked, DENV infectivity is essentially abolished.

While the very large majority of virus particles in the immature preparation were non-infectious, we did detect some infectious virions in the titration assay. A possible explanation for this finding may be that some prM-containing particles mature during cell entry, since furin, although predominantly localized in the TGN, also shuttles between early endosomes and the cell surface (Mollooy et al., 1999; Shapiro et al., 1997). This notion has been experimentally confirmed for Semliki Forest virus (SFV), an alphavirus (Zhang et al., 2003a). The authors observed that immature SFV particles can be processed during endocytic uptake in furin-containing cells, albeit with a low efficiency since the infectious titre increased only by a factor of 10. Envelope maturation during cell entry may therefore explain the low but detectable number of infectious particles in our immature DENV preparation.

Interestingly, antibodies against prM have been shown to enhance DENV infection (Henchal et al., 1985; Randolph et al., 1990; Huang et al., 2005, 2006). Enhancement of viral infection by prM antibodies was observed with both wild-type virus particles, presumably due to the substantial content of unprocessed prM in these preparations, and with DENV particles containing high levels of prM generated from cells in the presence of chloroquine. It is not yet clear how prM antibodies stimulate the infectious properties of these virions. As argued above, it is conceivable that DENV virus particles generated in cells in the presence of acidotropic agents exhibit a low residual level of maturation and corresponding infectivity, which might be enhanced in the presence of anti-prM antibodies. Also, one could speculate that prM antibodies enhance uptake of immature particles in cells, which might consequently lead to increased processing of prM to M and creation of potentially fusogenic virions within the target cell. This report shows for the first time that entirely immature and almost completely non-infectious DENV particles can be generated in furin-negative LoVo cells, and therefore may serve as a novel tool to elucidate the role of prM antibodies in DENV infectivity. Using fully immature DENV, we are currently investigating if prM antibodies only facilitate viral entry or whether other processes are involved that might render immature virions infectious.

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**Table 1.** Specific infectivities of DENV-2 16681 in time.

<table>
<thead>
<tr>
<th>DENV-2 production</th>
<th>GCP : IU ratio*</th>
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<tr>
<td></td>
<td>24 h p.i.</td>
</tr>
<tr>
<td>C6/36</td>
<td>139, 32</td>
</tr>
<tr>
<td>Vero</td>
<td>29, 22</td>
</tr>
<tr>
<td>BHK-15</td>
<td>4, 4</td>
</tr>
<tr>
<td>LoVo</td>
<td>$2 \times 10^{6}$, $7 \times 10^{5}$</td>
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*For all cell lines, two independent cultures were carried out. GCP : IU ratio for each of the cultures was determined at least twice.
References


