Formation of virus-like particles from cloned cDNAs of Thogoto virus

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Thogoto virus (THOV) is the type species of tick-transmitted orthomyxoviruses. Here, we describe the generation of virus-like particles (VLP) of THOV from cloned cDNAs. To synthesize the six structural proteins of THOV in mammalian cells, we used T7-controlled expression plasmids and a recombinant vaccinia virus producing T7 RNA polymerase. A minireplicon encoding a reporter gene flanked by THOV promoter sequences was expressed by the cellular RNA polymerase I. The recombinant proteins were functional in encapsidation, amplification and transcription of the minireplicon RNA. Furthermore, the artificial nucleocapsids were packaged into THO-VLPs that transferred the minireplicon to indicator cells. This system should be helpful in generating recombinant THOV entirely from cloned cDNAs.

Several systems have been established to generate virus-like particles (VLPs) of Influenza A virus (FLUAV; Mena et al., 1996; Neumann et al., 2000). In these systems, a minireplicon consisting of a reporter gene flanked by viral promoter sequences was cotransfected with plasmids encoding the structural proteins of FLUAV. The minireplicon was amplified by the reconstituted viral polymerase and packaged into infectious VLPs. Recently, successful generation of recombinant FLUAV entirely from cloned cDNAs was achieved by replacing the minireplicon with eight plasmids containing full-length sequences of the genomic segments (Neumann et al., 1999; Fodor et al., 1999). This system was a breakthrough in the field of orthomyxovirus research and allows manipulation of all genomic segments of FLUAV.

It is conceivable that such a rescue system could also be established for tick-borne orthomyxoviruses. This system may be a useful tool for several reasons. Firstly, THOV can serve as a simple model for study of the orthomyxovirus life-cycle since it encodes only the basic set of proteins required for multiplication and propagation of an orthomyxovirus genome, but lacks additional proteins such as the M2 ion channel, the NS1 of FLUAV. Secondly, unlike FLUAV, THOV does not cause shutoff of host-cell protein synthesis (Siebler et al., 1996). Finally, THOV has been established as an ideal tool to investigate the function of the antivirally active, interferon-induced Mx proteins (Haller et al., 1995; Frese et al., 1995; Kochs & Haller, 1999; Weber et al., 2000).

We recently demonstrated the reconstitution of the functional RNA polymerase of THOV by expression of the three subunits of the viral polymerase (PB1, PB2 and PA) from cDNA plasmids in eukaryotic cells. Together with recombinant NP, the polymerase complex was able to transcribe a minigenome RNA containing THOV promoter sequences (Weber et al., 1998, 2000). In the present study, we employed an RNA polymerase I-based expression system for intracellular generation of minireplicons and extended the system by additionally expressing GP and M to generate infectious THO-VLPs.

To detect newly formed VLPs at a single cell level, we generated a negative-sense minireplicon construct encoding the green fluorescent protein (GFP), pPolI-THOV/GFP(−). A
Fig. 1. Generation of THO-VLPs entirely from cloned cDNAs. (A) Vero cells (10\(^6\) cells) were transfected with T7-driven expression plasmids encoding the six structural proteins of THOV (500 ng pG7-NP, 100 ng pBS-PB2, 100 ng pBS-PB1, 100 ng pG7-PA, 100 ng pBS-GP, 50 ng pBS-M) together with an RNA polymerase I-driven minireplicon construct encoding GFP [100 ng of pPolI-THOV/GFP(−)] and infected with MVA-T7 at an m.o.i. of 10. In a control experiment (C), the plasmid encoding GP was omitted from the plasmid mixture. (B, D) Supernatants of the cells transfected with (B) or without (D) GP-expression plasmids were harvested and passed onto THOV-infected indicator cells. Cells were fixed 48 h post-transfection and 24 h after incubation with the supernatants, respectively, and GFP expression was detected by fluorescence microscopy (magnification × 400).

cDNA encoding the GFP reporter gene in negative-sense flanked by the noncoding end sequences of THOV segment 5 was inserted into the BsmBI site of pHH21 between the human RNA polymerase I promoter and terminator regions (kindly provided by Gerd Hobom, Justus Liebig-University, Giessen, Germany; Neumann et al., 1999). As a first step, this reporter plasmid was cotransfected with four T7-driven protein expression plasmids encoding the four components of the viral nucleocapsids (pG7-PA, pBS-PB1, pBS-PB2, pG7-NP) in Vero cells using LipofectAMINE (GibcoBRL). Six hours post-transfection, the cells were infected with MVA-T7, a recombinant vaccinia virus expressing the T7 RNA polymerase (kindly provided by Gerd Sutter, GSF-Neuherberg, Germany; Sutter et al., 1995), at an m.o.i. of 10 and incubated for 40 h. Subsequent examination of the transfected cells by fluorescence microscopy revealed high level expression of GFP (data not shown), indicating that the GFP minigenome was accepted by the recombinant polymerase complex and that transcriptionally active nucleocapsids were reconstituted.

We then added expression plasmids encoding GP and M of THOV to the transfection mixture. The ORFs encoding GP and M were amplified from THOV vRNA by RT–PCR and cloned into pBluescript under the control of the T7 RNA polymerase promoter to generate pBS-GP and pBS-M, respectively. In this extended system, cells were transfected with seven different plasmids. The highly attenuated recombinant vaccinia virus allowed long incubation times (over 50 h) to be used without any cytopathic effect. The expression of the two additional recombinant proteins had no obvious effects on the accumulation of GFP in the transfected cells (Fig. 1A). In order to demonstrate formation of THO-VLPs, supernatant from the transfected cells was transferred to a fresh Vero cell monolayer (indicator cells). Passaging the supernatants did not lead to detectable GFP signals in the indicator cells (data not shown).

Fig. 2. Viral proteins required for the formation of infectious VLPs. (A) Vero cells were transfected with different combinations of expression plasmids encoding the six structural proteins of THOV together with the CAT minireplicon expression plasmid, pPolI-THOV/CAT(−), and infected with MVA-T7. (B) At 55 h post-transfection, supernatants were collected and passaged onto indicator cells infected with THOV helper virus. Lysates of transfected or VLP-infected cells (10\(^6\) cells in 100 µl of buffer) were tested for CAT expression (1 to 10 µl of the lysates was incubated in the CAT assay at 37 °C for 1 h according to Gorman et al., 1982). CAT activity was quantified by phosphoimaging of the radiolabelled spots and is indicated as a percentage of the acetylated products compared to the chloramphenicol substrate used in the assays.
Virus-like particles of *Thogoto* virus

Fig. 3. Neutralization of VLP infectivity by a virus-specific antiserum. Vero cells were transfected with the six expression plasmids encoding the THOV proteins and the CAT reporter minireplicon, pPolI-THOV/CAT(−), and infected with MVA-T7. (A) CAT activity detected in the transfected cells. The supernatant was harvested and tested for the presence of infectious VLPs by inoculation of THOV-infected indicator cells (B; lane 1). Before inoculation of the indicator cells, VLP-containing aliquots of the cell supernatant were treated with RNase A (100 µg/ml at 37 °C for 10 min; lane 3) or with MAbs (1 h on ice) specific for the THOV glycoprotein (GP, lane 4), the THOV nucleoprotein (NP, lane 5), or with a polyclonal control serum directed against FLUAV (C, lane 6). Passage of the supernatant onto uninfected indicator cells was used as a control (lane 2). Transfected cells and indicator cells were harvested and the lysates were tested for CAT activity as described in Fig. 2.

We analysed which of the THOV structural proteins is required for packaging of a minireplicon RNA into infectious VLPs. To quantify reporter gene expression in the transfected and the indicator cells, we used the negative-sense CAT minigenome. Plasmid pPolI-THOV/CAT(−) contains the CAT ORF in negative-sense orientation flanked by the noncoding regions of THOV segment 5, as previously described (Weber *et al.*, 1998, 2000). The effect of the expression of M and GP on minireplicon expression in the transfected cells was assayed. Production of M or GP had only a minor effect on CAT reporter gene expression, whereas there was an absolute requirement for the four components of the viral nucleocapsids, PB1, PB2, PA and NP (Fig. 2A). For the formation of infectious VLPs able to transfer the minireplicon to indicator cells, all six structural proteins of THOV were necessary and sufficient (Fig. 2B, lane 1). Omission of the M or GP expression plasmids abolished the formation of infectious VLPs (Fig. 2B, lanes 3, 4 and 5). The plasmids encoding M and GP were then titrated. Highest CAT activity was detected in the indicator cells when 50 ng of M and 100 ng of GP expression plasmid were used in the transfection mixture (data not shown). These amounts were used in the experiments described below. It should be noted that we cannot exclude the possibility that low amounts of minigenome-containing particles were formed in the absence of GP or M as described for *Rabies virus* (Mebatsion *et al.*, 1996) or *Human parainfluenza virus* type 1 (Coronel *et al.*, 1999), but such putative particles are not infectious and therefore cannot be detected in our system.

We further analysed whether the VLPs indeed had characteristics of THOV particles. Supernatants containing THO-VLPs were treated with RNaseA (100 µg/ml at 37 °C for 10 min) or a monoclonal antibody directed against THOV NP (MAb2; kindly provided by Patricia A. Nuttall, Institute of Virology and Environmental Microbiology, Oxford, UK; Portela *et al.*, 1992). These treatments did not affect the infectivity of the VLPs, suggesting that neither uncomplexed minireplicon RNA nor unenveloped vRNPs had been transmitted to the indicator cells (Fig. 3B, lanes 3 and 5). In contrast, the infectivity of the VLPs was neutralized by a monoclonal antibody directed against THOV GP (MAb10; Portela *et al.*, 1992; Fig. 3B, lane 4). This antibody is also highly effective against wild-type THOV, reducing infectivity in a conven-
Structural similarity to orthomyxoviruses. The system described here might serve as a basis for the rescue of a THOV passaging or might be encoded by one of the six THOV genes. This THOV system can be used to further analyse the function of the six viral proteins and of THOV proteins with FLUA-VLP formation (e.g. the M2 ion channel or the baculovirus glycoprotein gp64). THOV has only six structural proteins, all of which are needed for VLP formation, indicating that functions required for FLUA-VLP formation (e.g. the M2 ion channel or the NS2/NEP nuclear export protein) might be dispensable for THOV passaging or might be encoded by one of the six THOV genes. This THOV system can be used to further analyse the function of the six viral proteins and of cis-acting sequence elements regulating virus replication, transcription, as well as particle morphogenesis. Furthermore, the system described here might serve as a basis for the rescue of a recombinant THOV entirely from cloned cDNAs.

In summary, this plasmid-based reverse genetics system for THOV allows the reconstitution of functional vRNPs that can be packaged into infectious VLPs. All six structural proteins of THOV were required for this rescue. In previous experiments with FLUAV, the nine viral structural proteins were required for the generation of infectious FLUA-VLPs (Mena et al., 1996; Gomez-Puertas et al., 1999; Neumann et al., 2000). THOV has only six structural proteins, all of which are needed for VLP formation, indicating that functions required for FLUA-VLP formation (e.g. the M2 ion channel or the NS2/NEP nuclear export protein) might be dispensable for THOV passaging or might be encoded by one of the six THOV genes. This THOV system can be used to further analyse the function of the six viral proteins and of cis-acting sequence elements regulating virus replication, transcription, as well as particle morphogenesis. Furthermore, the system described here might serve as a basis for the rescue of a recombinant THOV entirely from cloned cDNAs.

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References


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