Thogoto virus ML protein is a potent inhibitor of the interferon regulatory factor-7 transcription factor

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INTRODUCTION

Thogoto virus (THOV) is a tick-transmitted orthomyxovirus with a genome consisting of six single-stranded RNA segments that encode seven structural proteins (Hagai et al., 2004; Nuttall et al., 1995; vanRegenmortel et al., 2000). THOV is highly sensitive to the action of type I interferons (IFN), mostly mediated by the IFN-induced antiviral Mx proteins (Haller et al., 1995; Kochs & Haller, 1999; Pavlovic et al., 1995). In order to avoid the antiviral effects of IFN, THOV developed a strategy to suppress the induction of the IFN system. We have previously shown that the ML protein, an extended version of the viral matrix (M) protein, is a viral IFN antagonist (Hagai et al., 2003, 2004). Previous work with a recombinant THOV lacking ML expression showed that ML is essential for virus growth and pathogenesis in an IFN-competent host (Pichlmair et al., 2004). Further studies demonstrated that in the presence of ML the activation and/or action of the interferon regulatory factor-3 (IRF3) is severely affected (Jennings et al., 2005). This effect depends on direct interaction of ML with the transcription factor IIB (TFIIB) (Vogt et al., 2008), suggesting that complex formation of ML with TFIIB specifically disturbs the function of certain transcription coactivators such as IRF3.

Members of the IRF family of transcription factors are involved in all aspects of IFN induction and action (Haller et al., 2006; Mamane et al., 1999; Randall & Goodbourn, 2008; Taniguchi & Takaoka, 2002). IRF3 is constitutively expressed in most cells and is essential for the induction of IFN-β as an early response to virus infection (Lin et al., 1998; Yoneyama et al., 1998) or to Toll-like receptor (TLR) agonists like dsRNA or lipopolysaccharide that bind to TLR3 and TLR4, respectively (Pitha, 2004). A positive feedback loop then leads to the induction of IRF7, which upon activation by virus infection leads to the amplification of the IFN signal mainly by transcriptional activation of the IFN-α genes in addition to IFN-β (Marie et al., 1998; Sato et al., 1998).

Two independent signal transduction pathways lead to the activation of IRF3 and IRF7 (Hiscott et al., 2006; Levy & Marie, 2004). TRIF is an essential component of the TLR-associated signalling complex that leads to IRF activation (Jiang et al., 2004). In contrast, RIG-I and Mda5 are intracellular receptors for viral dsRNA (Andrejeva et al., 2004; Yoneyama et al., 2004) that need the adaptor molecule IPS-1 for signalling (Kawai et al., 2005). The TRIF- and IPS-1-dependent pathways both lead to the activation of the unconventional IκB kinases (IKK), TANK-binding kinase-1 (TBK-1) and IKKε that are responsible for IRF3/7 phosphorylation (Fitzgerald et al., 2003; Sharma et al., 2003). Phosphorylation then triggers the following activation steps: dimerization, nuclear accumulation and assembly in a promoter-bound transactivating complex on IFN-responsive promoter regions (Lin et al., 2000; Marie et al., 2000). A special feature has been described for plasmacytoid dendritic cells (pDCs), the main producers...
of IFN-α in vivo (Colonna et al., 2002). It was found that, in contrast to other cells, pDCs show a constitutive expression of IRF7 (Honda et al., 2005), leading to a direct and strong expression of IFN-α upon virus infection (Barchet et al., 2002) or binding of TLR7 or TLR9 agonists like ssRNA and CpG DNA (Diebold et al., 2004; Hemmi et al., 2000).

In the present study, we investigated the effect of ML on different pathways involved in IFN induction by measuring reporter gene expression under the control of IRF3 and IRF7. We found that ML suppresses IRF7 in a similar manner as it suppresses IRF3. Further experiments revealed that ML associates with IRF7 and prevents IRF7 dimerization and interaction with TRAF6. Thus, ML prevents activation of IRF-dependent antiviral host defence in infected fibroblasts (IRF3) as well as in pDCs (IRF7).

**RESULTS**

Type I IFN induction by THOV is blocked by ML

THOV has been shown to induce a strong innate host response in infected mice (Haller et al., 1995; Pichlmair et al., 2004). In order to study type I IFN induction by THOV, we infected mouse embryo fibroblasts (MEF) and pDCs. To evaluate the effect of the IFN antagonist ML in infected cells, we used recombinant THOV encoding either the ML protein, THOVML+ or THOV with a single nucleotide insertion in segment 6 that disrupts the ML open reading frame, THOVML− (Hagmaier et al., 2003). MEFs were isolated from reporter mice carrying the IFN-β gene replaced by the firefly luciferase (FF-Luc) cDNA, thus allowing quantitative detection of IFN-β promoter activation. The expression of luciferase in the lysates of these cells correlates with the induction of endogenous IFN-β (Lienenklaus et al., 2009). Infection of these cells with different doses of recombinant THOV showed a strong activation of the IFN-β promoter by THOVML− (Fig. 1a). This upregulation of the IFN-β promoter activity was reduced by about 10-fold when cells were infected with THOVML+. To study the effect of THOV on dendritic cells, bone marrow cells were differentiated into pDCs in the presence of Flt3-L. We used bone marrow from IFN-β-deficient mice as a source of pDCs because it was shown previously that knockout of IFN-β reduces background induction of the IFN system in primary cell cultures (Koerner et al., 2007). As expected, untreated primary cells from mice with defective IFN-β alleles showed nearly undetectable levels of secreted IFN-α (Fig. 1b, ctrl). pDCs infected with THOVML− for 24 h secreted IFN-α as quantified by ELISA of the cell culture supernatant. However, infection with THOVML+ reduced production of IFN-α to about 60 % (Fig. 1b). These data indicate that THOV expressing ML is potent in reducing induction of the innate immune response in primary fibroblast cells and also functions to some extent in pDCs.
ML inhibits activation of type I IFN promoters

The induction of type I IFN depends on cellular signalling pathways leading to IRF3 and IRF7 activation (Hiscott et al., 2006; Levy & Marie, 2004; Pitha, 2004). We therefore characterized the molecular mechanism of ML-mediated inhibition of IFN expression by studying its effect on upstream components of the IRF activation cascade. cDNA constructs expressing cellular signalling molecules that are involved in IRF activation were transfected together with an ML expression construct into 293T cells, and the effect on the IFN-β promoter was determined by reporter assays. We tested TRIF as a component of the TLR3/4-associated signalling complex, the intracellular receptors for viral RNA, RIG-I and Mda5, and their downstream adaptor molecule IPS-1. These pathways lead to the activation of TBK-1 that is responsible for IRF phosphorylation. Individual overexpression of these key molecules led to a strong activation of the IFN-β promoter (Fig. 2a). However, coexpression of ML suppressed reporter gene expression in all cases. These results indicate that ML affects a step in the IRF-dependent IFN-β induction cascade located downstream of TBK-1.

The IKK-related kinase TBK-1 activates IRF3 as well as IRF7 that is mainly responsible for the amplification of IFN-α synthesis (Sharma et al., 2003). To analyse the effect of ML on this pathway, we compared constitutively active mutants IRF3(5D) and IRF7(2D), using reporter constructs for their cognate promoters (IFN-β promoter for IRF3 and IFN-α promoter for IRF7). In both molecules critical C-terminal phosphorylation sites, which are involved in activation of the IRFs, were converted into phosphomimetic aspartic acid residues (Lin et al., 1998, 2000). Expression of the constitutively activated IRFs led to high stimulation of the IFN promoters, as expected. However, coexpression of ML suppressed the activity of IRF3(5D) and IRF7(2D) to a similar extent (Fig. 2b and c), suggesting a direct effect of ML on IRF3 and IRF7-dependent activation of the IFN promoters.

Activation of both IRF3 and IRF7 by TBK-1 leads to their dimerization and nuclear translocation (Sharma et al., 2003). For IRF3, we have previously shown that ML prevents its dimerization (Jennings et al., 2005). To test an influence of ML on IRF7 oligomerization, cDNA constructs were used that encode wild-type IRF7 with an N-terminal Flag- or haemagglutinin (HA)-tag. Overexpression of these constructs led to oligomerization of IRF7, as shown by coimmunoprecipitation of the HA-tagged IRF7 together with the Flag-tagged IRF7 (Fig. 2d). Coexpression of ML reduced the ability of HA-tagged IRF7 to associate with Flag-tagged IRF7 (Fig. 2d), suggesting an inhibition of IRF7 dimerization. We also investigated nuclear translocation of IRF7 by infecting cells that were cotransfected with expression constructs for GFP–IRF7 and ML. Newcastle disease virus (NDV) was used to induce IRF7 activation and nuclear translocation (Fig. 2e, left cell). In uninfected cells, GFP–IRF7 remained in the cytoplasm (Fig. 2e, right cell). Interestingly, coexpression of ML in NDV-infected cells (Fig. 2e, left cell) did not affect nuclear accumulation of GFP–IRF7. Thus, consistent with the effects on IRF3 activation (Jennings et al., 2005), ML specifically interferes with IRF7 dimerization, but not with its nuclear accumulation.

Effect of ML on the alternative IRF7 activation complex

IFN-α production in pDCs infected with THOV was clearly reduced by the viral ML protein (Fig. 1b). In pDCs, IRF7 is constitutively expressed as part of a TLR-associated multi-protein complex consisting of IRF7, MyD88 and TRAF6 that causes a TBK-1/Iκκα-independent induction of IFN-α (Kawai et al., 2004). Formation of this complex can be induced by ectopic expression of MyD88, TRAF6 and IRF7, leading to the activation of IRF7 and the IFN-α promoter (Honda et al., 2004; Kawai et al., 2004). To elucidate the effect of ML on this IRF7 activation pathway, a small amount (1 ng) of wild-type IRF7 expression plasmid was cotransfected with MyD88 or TRAF6, leading to the activation of the IFN-α promoter (Fig. 3a). This stimulatory effect was strongest upon cotransfection of all three components. However, addition of ML completely suppressed the activation of the IFN-α promoter (Fig. 3a).

We further investigated the effect of ML on the formation of the TLR-associated complex by coimmunoprecipitation. When Flag–IRF7 and Myc–TRAF6 were coexpressed, immunoprecipitation with the anti-Flag antibody showed an interaction of IRF7 with TRAF6 (Fig. 3b), consistent with published data (Honda et al., 2004; Kawai et al., 2004). Coexpression of HA–ML blocked this IRF7–TRAF6 interaction (Fig. 3b). When we analysed the Flag–IRF7 immunocomplex for the presence of HA–ML, we found ML associated with IRF7 (Fig. 3c). These results suggest that ML interacts with IRF7 and thus prevents its activation by TRAF6.

This assumption was supported by additional reporter assays using the IFN-α promoter construct. Transfection of increasing amounts of the IRF7 expression plasmid slightly reduced the inhibitory effect of coexpressed ML (Fig. 3d). However, IFN-α promoter inhibition was restored when ML levels were increased (Fig. 3e). This occurred without a pronounced effect of ML on expression of SV40 promoter driven Renilla luciferase (data not shown). In contrast to IRF7, overexpression of TRAF6 in this system somehow disturbed the formation of a proper activator complex, resulting in reduced activation of the IFN-α promoter (Fig. 3f). However, increasing the expression of TRAF6 did not change the suppressive effect of ML (Fig. 3f), indicating that TRAF6 is not a target of ML action. We recently found a direct interaction between ML and the general transcription factor TFIIIB (Vogt et al., 2008). Therefore, we examined whether TFIIIB influences the effect of ML on the IFN-α promoter activation by IRF7. Overexpression of TFIIIB significantly reversed the inhibitory effect of ML.
(Fig. 2), suggesting that ML by interacting with IRF7 and TFIIB disturbs the formation of a functional activator complex at the IFN-α promoter.

**DISCUSSION**

Our study reveals a remarkable feature of the orthomyxoviral IFN antagonist ML. ML blocks the function of IRF3 and IRF7 and is therefore able to interfere with several signal transduction pathways involved in innate immune response. In previous studies we showed that ML suppresses the action of IRF3, the critical factor involved in early type I IFN induction (Jennings *et al.*, 2005) and blocks induction of type I IFN in THOV-infected cells (Hagmaier *et al.*, 2003). However, an incomplete block of IFN synthesis might lead to an amplification of the IFN signal through IRF7. The IFN-induced IRF7 has been shown to be essential for the expression of the whole spectrum of type I IFN (Marie *et al.*, 1998; Sato *et al.*, 2000) and is also critical for IFN-α production by pDCs, specialized cells that produce large amounts of IFN-α (Colonna *et al.*, 2002; Honda *et al.*, 2005). Comparing virus activated IFN induction in primary embryo fibroblasts and pDCs as well as IFN promoter activation by transfected IRF3 and IRF7, we found a suppressive effect of ML. The minor effect of THOVML+ infection on the IFN-α synthesis in pDCs (Fig. 1b) might result from lower expression levels of viral proteins in this cell type, which could not be monitored due to the small amounts of
material from these cells. In addition, IFN expression by pDCs might be notably resistant to the action of viral IFN antagonists as previously described for influenza A virus and vesicular stomatitis virus (Diebold et al., 2004; Waibler et al., 2007). Further analysis demonstrated that ML associates with IRF7, prevents IRF7 interaction with TRAF6 and blocks IRF7 dimerization, indicating that THOVML can also alter the composition of the pDC-specific signalling pathway and prevent the assembly of a transcriptionally active IRF7 complex. Thus, targeting of the transcription factor IRF7 in addition to IRF3 by ML most probably contributes to the suppression of IFN production by THOV in cell culture and in vivo (Hagmaier et al., 2003; Pichlmair et al., 2004).

IRFs consist of an N-terminal DNA-binding domain that shows a homologous structure in the different IRFs and a less conserved C-terminal effector domain that is the target of regulatory modifications and contains the transactivation potential (Mamane et al., 1999). Coimmunoprecipitation of ML together with IRF7 suggests a direct association of ML with this factor. Previous studies with IRF3 mutants excluded the N-terminal DNA-binding domain as well as the C-terminal autoinhibitory domain.
that is present in IRF3 and IRF7 as possible targets of ML (Jennings et al., 2005). The heterogeneity of the remaining part of the IRF sequences argues against IRFs as a direct target of ML but for a factor involved in IRF-dependent transactivation. In addition, ectopic overexpression of IRF7 had only a limited effect on the ML inhibitory activity, indicating an additional factor as the prime target of ML.

Activated IRF3 and IRF7 are part of a complex that includes the coactivators CBP and p300, NFκB, and the chromatin-associated HMG proteins (Suhara et al., 2000; Thanos & Maniatis, 1995; Weaver et al., 1998; Yang et al., 2003). This multi-protein complex contacts components of the general transcription machinery (Abraham et al., 1993; Wang et al., 1996; Xia et al., 2004). We recently identified the transcription factor TFIIB as an ML-interacting protein and showed that this interaction blocks activation of IRF3 and NFκB-dependent promoters (Vogt et al., 2008). In the present study, we demonstrate that overexpression of TFIIB also outcompetes the inhibitory effect of ML on the IRF7-mediated activation of the IFN-α promoter. Therefore, we would speculate that ML prevents the association of IRFs with downstream elements of the general transcription machinery, like TFIIB. This might destabilize IRF7 dimerization and the formation of a promoter-specific activator complex. By contrast, early activation steps, resulting in nuclear translocation of IRFs are not affected by ML (Jennings et al., 2005; this study). Our data suggest a unique mechanism of ML action by perturbing the assembly and function of an IRF-dependent coactivator complex via interaction with both members of promoter-specific activator proteins like IRF7 and components of the general transcription initiation complex.

**METHODS**

**Cells and viruses.** MEFs were prepared from 13-day-old embryos of transgenic C57BL/6 knock-in mice carrying firefly luciferase (FF-Luc) cDNA instead of the IFN-β gene under the control of the endogenous IFN-β promoter (Lienenklaus et al., 2009). Bone marrow-derived pDCs were prepared from IFN-β deficient C57BL/6 mice (IFN-β−/−) (Erlandsson et al., 1998) by cultivating bone marrow cells for 8 days in medium supplemented with Flt3-L (100 ng ml−1; R&D systems) as described previously (Waibler et al., 2007). Such cultures consisted of 20–40 % B220+ CD11c+ pDCs.

Recombinant THOV expressing ML (THOVML+) or lacking ML (THOVML−) were described previously (Hagamaier et al., 2003) and were propagated on HK2-21 cells to titres of 1×107 p.f.u. ml−1.

**In vitro stimulation of the cells and quantification of IFN.** In vitro differentiated pDCs and MEFs were seeded at 1×106 cells per well into culture plates and were infected at an m.o.i. of 1 or 0.1 with THOV for 18 or 24 h. Cell-free supernatants were collected from pDCs and analysed using a mouse IFN-α-specific ELISA kit (PBL Biomedical Laboratories). Activation of the IFN-β promoter was detected in MEFs by analysis of FF-Luc activity in the cell lysates as described by the manufacturer (Luciferase Reporter Assay System; Promega).

**Plasmids and reporter assays.** Expression constructs of ML and HA-tagged ML under the control of the chicken β-actin promoter (pCAGGS) have been described previously (Hagamaier et al., 2004; Jennings et al., 2005). Expression plasmid pCAGGS-TFIIH has been described previously (Vogt et al., 2008).

Constitutively active IRF3(5D) (Lin et al., 1998) and IRF7(2D) (Lin et al., 2000), as well as GFP–IRF7 fusion construct were cloned into pCAGGS. Expression constructs for the N-terminal fragment of RIG-I (Yoneyama et al., 2004), Mdα5 (Andrejeva et al., 2004), TBK-1 (Sharma et al., 2003), IPS-1 (Kawai et al., 2005) and TRIF (Jiang et al., 2004) were kindly provided by Washington Cardenas (MSSM, NY, USA), by Richard E. Randall (University of St. Andrews, UK), by John Hiscott (McGill University, Montreal, Canada), by Shizuo Akira (Osaka University, Japan), and by Xiaoxia Li (Cleveland Clinical Foundation, OH, USA), respectively. Expression plasmids encoding Flag-tagged IRF7, Myc-tagged MyD88 and Myc-tagged TRAF6 were kindly provided by Shizuo Akira (Kawai et al., 2004). The reporter plasmid pRL_SV40 carrying Renilla luciferase (REN-Luc) under the control of the constitutive SV40 promoter was purchased from Promega.

For reporter assays, plasmids carrying the FF-Luc gene under the control of the IFN-β promoter (p125Luc) (kindly provided by Takashi Fujita, Tokyo Metropolitan Institute of Medical Science, Japan) (Yoneyama et al., 1998), the IFN-24 promoter (kindly provided by Shizuo Akira) (Kawai et al., 2004), and a reporter plasmid encoding REN-Luc under the control of the constitutive SV40 promoter, pRL-SV40 (Promega), were used.

Reporter assays were performed in Metafectene (Biontex) transfected 293 or 293T cells. Cell monolayers were transfected with 0.5 μg FF-Luc-encoding reporter construct together with 50 ng pRL-SV40, 0.5 μg IRF-activator and 0.25–0.5 μg ML expression plasmid. To measure activation of the promoters, cells were harvested and lysed in 200 μl passive lysis buffer (Promega) at 24 h post-transfection. An aliquot of 10 μl was used to measure FF-Luc and REN-Luc activities as described by the manufacturer (Dual-Luciferase Reporter Assay System; Promega).

**Immunofluorescence analysis.** GFP–IRF7 nuclear translocation was assayed as described previously (Jennings et al., 2005). Vero cells were transfected with the expression constructs encoding GFP–IRF7 and ML (1 μg each). At 16 h post-transfection, cells were infected with 3 p.f.u. per cell NDV (strain H53) (Bazzigher et al., 1992) or mock infected for 8 h. Then cells were fixed with 3 % paraformaldehyde and stained with a polyclonal rabbit serum directed against the ML protein (Kochs et al., 2000) and a mouse polyclonal serum specific for NDV combined with fluorophore-conjugated secondary donkey antibodies (Dianova). The samples were analysed using a Leica confocal laser scanning microscope.

**Coimmunoprecipitation.** Interaction between distinct protein partners was tested in the lysates of transiently transfected 293T cells. Cells (106 cells) were transfected with 2 μg each expression plasmid. After 24 h, the cells were lysed in buffer containing 50 mM Tris, 150 mM NaCl, 1.0 % NP-40, 1 mM EDTA, pH 7.5 and subjected to immunoprecipitation using protein A–Sepharose beads (Amersham-Pharmacia) preadsorbed with the monoclonal antibody directed against the Flag-tag (1 μg) (Sigma-Aldrich). After incubation overnight at 4 °C, the protein A–Sepharose beads were washed three times in lysis buffer. The precipitated proteins were separated by SDS-PAGE and detected by Western blot analysis using rabbit antibodies directed against the Flag-tag (Sigma), HA-tag (Santa Cruz) or the Myc-tag (Santa Cruz). Primary antibodies were visualized using horseradish peroxidase-labelled secondary antibodies, the ECL-detection reagent (GE Healthcare) and a ChemiDoc XRS system (Bio-Rad).
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