Type I interferon antagonistic properties of influenza B virus polymerase proteins

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Abstract
The innate immune system, in particular the type I interferon (IFN) response, is a powerful defence against virus infections. In turn, many if not all viruses have evolved various means to circumvent, resist, or counteract this host response to ensure efficient replication and propagation. Influenza viruses are no exception to this rule, and several viral proteins have been described to possess IFN-antagonistic functions. Although the viral nonstructural protein 1 appears to be a major antagonist in influenza A and B viruses (IAV and IBV), we have previously shown that a specific motif in the IAV polymerase proteins exerts an IFN-suppressive function very early in infection. The question remained whether a similar function would also exist in IBV polymerases. Here, we show that indeed a specific amino acid position (A523) of the PB1 protein in the IBV polymerase complex confers IFN-antagonistic properties. Mutation of this position leads to enhanced activation of the IFN-mediated signalling pathway after infection and subsequent reduction of virus titres. This indicates that inhibition of innate immune responses is a conserved activity shared by polymerase proteins of IAV and IBV.

KEYWORDS
diseases, immunology, infection, mechanism of action, virulence, viruses (phages)

1 | INTRODUCTION

Influenza A and B viruses (IAV and IBV) cocirculate in the human population and are still major health burdens especially for young children and the elderly. Although it is believed that IAV causes more severe infections, data show that clinical courses of disease in patients are very similar (Irving et al., 2012).

The type I interferon (IFN) system is an immediate host defence mechanism against viral and bacterial infections, counteracting invading pathogens and triggering the antiviral response. During infection with negative-strand RNA viruses, the cytosolic retinoic acid-inducible gene I (RIG-I) and the RIG-I-like receptor melanoma differentiation-associated gene 5 (MDA-5) sense pathogen-associated molecular patterns like 5'-triphosphate termini and RNA secondary base-paired structures, which are structural features of the viral ribonucleoprotein complexes (Loo et al., 2008; Rehwinkel et al., 2010; Weber et al., 2013). Upon recognition of viral RNA (vRNA) by RIG-I, the complex binds to the tripartite motif-containing protein TRIM25 and is recruited to the mitochondria by the scaffold protein mitochondrial antiviral-signalling protein (MAVS). This leads to the primary induction of IFNβ via TANK-binding kinase 1 and IFN regulatory factor (IRF)-3 activation. As a secondary response, released type I IFN binds to its cognate receptor (IFN-α/βR), which triggers the Janus kinase (JAK)/signal transducer and activator of transcription protein (STAT) signalling pathway, leading to the activation of a specific
transcription factor complex (Takaoka & Yanai, 2006; Randall & Goodbourn, 2008; Bonjardim, Ferreira, & Kroon, 2009; Kowalinski et al., 2011; Mäkelä et al., 2015). IRF9, STAT1, and STAT2 form the heterotrimeric transcription factor named IFN-stimulated gene factor (ISG)-3, which translocates to the nucleus, resulting in induction of numerous ISGs, like myxovirus resistance protein 1 (MxA), 2′-5′-oligoadenylate synthetase 1 (OAS), or ISG15 (Ehrhardt et al., 2010; Garcia-Sastre, 2011). ISGs, like the GTPase MxA or protein kinase R (PKR), amplify the host defence and lead to the establishment of an antiviral state (Pindel & Sadler, 2011; Xiao et al., 2013).

In turn, influenza viruses have evolved several temporally and spatially separated strategies to suppress these antiviral responses and to ensure efficient replication and spread. The nonstructural protein 1 (NS1) of IAV and IBV has been known as IFN antagonist for a long time (Wang & Krug, 1996; Garcia-Sastre et al., 1998; Yuan & Krug, 2001; Dauber, Heins, & Wolff, 2004; Dauber, Scheider, & Wolff, 2006). There is evidence that NS1 is incorporated in IAV and IBV virions. Nevertheless, the functionality of these NS1 molecules as IFN antagonist is still unknown, and the low number per virion might not be enough for sufficiently inhibiting the early IFN response (Hutchinson et al., 2014). Thus, the question arose on how IAV may suppress IFN responses immediately after virus entry. Along this line, we have previously identified two proteins of the viral polymerase complex, PB1 and PA, as part of the type I IFN-inhibitory strategy evolved by IAV (Liedmann et al., 2014a). A specific motif within the sequences of PB1 and PA subunits was identified to impact host type I IFN response (Liedmann et al., 2014a,b). Thus, the IFN-antagonistic function of PB1 and PA is pre-packaged in the virion and is already present during initial entry and viral ribonucleoprotein complex sensing by RIG-I, leading to suppression of the innate antiviral immune response (Liedmann et al., 2014b).

In the present study, we demonstrate a similar type I IFN-antagonistic property of the IBV polymerase complex and provide evidence of a comparable pattern within the PB1 polymerase.

### RESULTS AND DISCUSSION

To study a putative IFN-antagonistic function of the IBV polymerase complex, A549 cells were transfected with constructs encoding the IBV proteins PB1, PB2, PA and the nucleoprotein (NP), which constitutes a functional polymerase complex. To induce type I IFN expression, commercially available 5′-triphosphate RNA was used as a stimulus in comparison with a negative-control-5′-ppp-dsRNA (InvivoGen). 5′-ppp-dsRNA is the specific ligand for RIG-I to trigger activation of IRF3, resulting in the release of IFN-β (Hornung et al., 2006; Mäkelä et al., 2015). The interaction of newly produced and secreted type I IFN with the IFN-α/β receptor complex leads to the activation of the intracellular associated Janus tyrosine kinases JAK1 and Tyk2. These kinases subsequently phosphorylate STAT1/2 (Takaoka & Yanai, 2006). Thus, IRF3 phosphorylation and STAT1 phosphorylation serve as markers for primary induction of type I IFN response.

**FIGURE 1** Expression of the influenza B polymerase complex proteins inhibit IRF3 and STAT1 activation. (a) The IBV polymerase proteins (PB1, PB2, PA) and NP were overexpressed in A549 cells (PPPNP). pHW2000 transfected cells served as negative control. 24 h post transfection (p.t.) cells were stimulated with 1 μg μl⁻¹ commercially available 5′-ppp-dsRNA or negative-control-5′-ppp-dsRNA (neg. control) for 6 h, respectively. Unstimulated cells (−) served as additional negative control. IRF3 and STAT1 activation were analysed by validating the phosphorylation states respectively. The expression of viral proteins was controlled using an anti-NP antibody. ERK2 served as loading control. (b, c) Western blot quantification of the IRF3 and STAT1 activation of (a). The Western blots were quantified with LiCor Image Studio™ (V5.2.5). Data represent mean ± SD of five independent experiments. pHW2000 was arbitrarily set to 1.0. A paired t test using the vector transfection as control was used to calculate statistical significance (**p ≤ 0.01; ***p ≤ 0.001)
IFNβ and secondary stimulation of type I IFN-mediated signalling. In Western blot analysis, a reduction of 5'-ppp-dsRNA-induced IRF3 and STAT1 phosphorylation was observed in the presence of the IBV polymerase complex (Figure 1). These results indicate a lower IFN release, which suggests IFN-antagonistic properties of the IBV polymerase complex. Thus, we hypothesised a similar mode of action as already described for the polymerase complex proteins of IAV, which was shown to interact with RIG-I at the mitochondria and thereby inhibits the vRNA sensing pathway (Liedmann et al., 2014a,b). In IAV, a highly IFN-antagonistic amino acid combination within the PB1 and PA subunits, referred to as ESIE motif, was identified. The IFN-antagonistic activity was lost when this motif was changed to GGRK (Liedmann et al., 2014a).

To identify a potential similar motif in the IBV polymerases, we did sequence comparison using the NIH NCBI (http://www.ncbi.nlm.nih.gov) influenza database and aligned >9,000 influenza B/PB1 as

**FIGURE 2** Motif comparison in the influenza A and B polymerase complexes. (a) Structural analysis of IAV (left) and IBV (right) polymerase complexes revealed a comparable spatial orientation of the motif-forming amino acids. The 3D structures of the polymerase complexes were calculated by using pymol (2.2.0), and the crystallographic structure analysis of Pflug et al. (2014) and Reich et al. (2014). PB1 and PA are labelled in blue and green, respectively, whereas PB2 is black. The motif-forming amino acids in the IAV polymerase complex have been matched by sequence alignment and are depicted in yellow spheres. The bound vRNA with the 5'- and 3'-ends is visible (orange). (b) Sequence alignment of influenza A/Puerto Rico (H1N1), A/Panama (H3N2), A/little yellow-shouldered bat (H17N10), and influenza B/Lee/40 viruses. Influenza A/Puerto Rico/8/34 (PR8) virus PB1 and PA sequences were aligned with A/Panama/1/68 (H3N2), A/little yellow-shouldered bat/Guatemala/060/2010 (H17N10), and 9360 PB1 sequences and 9379 PA sequences from 1940 to 2019. The comparison of the sequences revealed a highly conserved amino acid motif for influenza B viruses referred to as EARE motif (A523: 100%; E399: 99.97%; PA: 99.85%) instead of the influenza A/PR8 ESIE motif. The NIH NCBI (http://www.ncbi.nlm.nih.gov) influenza database was used. (c) The ESIE and GGRK mutants have a lower ability to inhibit STAT1 activation. A549 cells were infected with IBV/Lee/40 wild type (wt) or the ESIE and GGRK mutants (MOI 5). Cells were lysed after 8 h and subjected to SDS-PAGE. STAT1 phosphorylation and viral protein expression was analysed by Western blotting using anti-pSTAT1 (Y701), anti-STAT1, and anti-M1 antibodies. STAT1 and ERK2 served as loading controls. Data represent mean ± SD of three independent experiments. One-way ANOVA followed by Dunnett’s multiple comparisons test using the wt virus as control was used to calculate statistical significance (*p ≤ 0.05, **p < 0.01). The IBV wt-infected sample was arbitrarily set as one and served as control. (d) The mutations of the EARE motif within the polymerase complex of IBV have no effect on the polymerase activity. HEK 293T cells were transfected with the IBV wt and mutated polymerase proteins as indicated NP and the reporter gene construct pPolI/SapI-B/NS-luc. Within the negative control (mock), the pHW2000 vector was used instead of PB1. Twenty-four hours p.t., the polymerase activities were analysed with the reporter gene assay. Data represent mean ± SD of four independent experiments. One-way ANOVA followed by Dunnett’s multiple comparisons test was used to calculate statistical significance. The IBV wt-transfected sample was arbitrarily set as one and served as control. MOI, multiplicity of infection.
well as influenza B/PA sequences in comparison with influenza A/Puerto Rico/8/34 (H1N1), A/Panama/1/68 (H3N2), and the distinctly related A/little yellow-shouldered bat/Guatemala/060/2010 (H17N10) virus (Figure 2b). Interestingly, the sequential and structural comparison of the influenza A and B polymerase complexes revealed a similar amino acid combination that is highly conserved, referred to as EARE motif (PB1: E399/A523/R562; PA: E350). To compare the physical configuration of the ESIE motif of IAV and the respective EARE motif of IBV, structure analysis was performed using published structural data (Pflug et al., 2014; Reich et al., 2014). Interestingly, a comparable spatial orientation of the respective amino acids of the IAV and IBV polymerase complexes was observed (Figure 2a).

To elucidate whether the IBV EARE motif is also functional in suppressing IFN responses, the ESIE motif and the GGRK motif of IAV were introduced in the IBV PB1 and PA polymerase proteins of the B/Lee/40 virus via site-directed mutagenesis. Virus mutants were compared with wild-type (wt) virus with respect to their impact on STAT1 phosphorylation. Western blot analysis revealed a stronger STAT1 phosphorylation upon infection of A549 cells with IBV mutants encoding the ESIE and GGRK motifs in comparison with IBV wt (Figure 2c), indicating a disturbed IFN-inhibitory potential of the mutants. Of note, viral M1 protein expression was changed upon mutation of the IBV polymerase complex proteins PB1 and PA. Infection with the IBV ESIE mutant exhibited a higher M1 protein expression and a slight but not significant enhancement of the polymerase activity revealed by mini replicon driven luciferase assay (Figure 2d). Interestingly, infection with the IBV GGRK mutant resulted in a very low expression of M1 but a fivefold higher STAT1 phosphorylation than did the infection with IBV wt (Figure 2c). The polymerase activity was not significantly changed (Figure 2d). To exclude effects on the IFN-mediated signalling due to the production of non-infectious particles, we measured the ratio of infectious/non-infectious particles in a haemagglutinin assay. The agglutination of chicken erythrocytes of wt compared with GGRK and ESIE mutants showed no alterations in the different assays (data not shown).

The observation that enhanced IFN expression is only observed in case of IBV PB1 (A523) mutation suggests that this amino acid contributes to an IFN-antagonistic function of the protein. To analyse whether the alteration of the IFN response would also translate in an altered replication of the respective virus mutants, we performed multicycle replication assays. Indeed, we observed significantly reduced titres after 24 or 56 h, both with the single A523G mutant as well as with the A523G/E399G double mutant (Figure 3c), indicating that the IFN-enhancing activity translates into reduced progeny virus titres of the mutants.

In summary, we have identified the amino acid A523 in IBV PB1 as a contributing site to the IFN-antagonistic activity of IBV polymerases. As the A523 site is not exposed on the surface of the polymerase complex (Figure 2a), it is still enigmatic how a direct interaction with RIG-I-like proteins should occur. However, the site might have an indirect supportive function, for example, via other binding partners. Another explanation might be that specific temporal structural changes in the IBV polymerase complex may expose the site in the right timing when it is needed to interact with RIG-I.

For IAV, a motif of four amino acids in PB1 and PA was identified to be responsible for the IFN-suppressive activity (Liedmann et al. 2014b). The fact that A523 is the only site in the putative EARE motif of IBV PB1 displaying such a function indicates that although IAV and IBV polymerases are overlapping in their IFN suppressing activity, the structural constraints for such an action might differ between the two virus types.
**FIGURE 3** Effect of the A523G and E399G mutations on the interferon signalling and the viral life cycle. (a) The single and double mutations of PB1 (A523G and A523G/E399G) lead to higher activation of the JAK/STAT signalling pathway. A549 cells were infected with the IBV/Lee/40 wild type virus or the mutant viruses (A523G, E399G, A523G/E399G; MOI 5). 8 h p.i., cells were lysed and subjected to SDS-PAGE followed by Western blotting. STAT1 activation was analysed by using anti-pSTAT1 (Y701), anti-STAT1, and anti-NP antibodies. STAT1 and ERK2 served as loading controls. (b) Increased mRNA levels of MxA, PKR, RIG-I, and MDA5 after A523G mutant infection. A549 cells were infected with IBV/Lee/40 wild-type virus or the mutant viruses (A523G, E399G, A523G/E399G; MOI 5). 8 h p.i., mRNA expression of the ISGs MxA and PKR, as well as RIG-I like proteins RIG-I and MDA5, was measured by qRT-PCR. Viral gene expression was analysed by measuring the NP, M1, and NS1 mRNA expression and was normalised to the housekeeping gene GAPDH. Values represent expression of IBV wt-infected cells. Data represent mean ± SD of three independently repeated experiments. One-way ANOVA followed by Dunnett’s multiple comparisons test was used to calculate statistical significance (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001). The IBV wt sample was arbitrarily set as one. (c) Multicycle replication kinetics of the IBV/Lee/40 wild-type (wt) virus and mutated viruses. MDCK II cells were infected with the IBV/Lee/40 wild-type virus or the mutated viruses (A523G, E399G, A523G/E399G; MOI 0.1). Supernatants of infected cells were analysed after the indicated time points by standard plaque titration. Data represent mean ± SD of five independent experiments. One-way ANOVA followed by Dunnett’s multiple comparisons test was used to calculate statistical significance of each time point separately (*p ≤ 0.05). (d, e) The A523G mutant does not induce increased STAT1 phosphorylation in MAVS knockout cells. (d) A549 crispr control cells (Crispr Ctrl) or (e) A549 crispr-cas9 MAVS knockout cells (MAVS-KO) were infected with wt or mutated IBV (A523G, E399G, A523G/E399G; MOI 5). 8 h p.i., cells were lysed and subjected to SDS-PAGE followed by Western blotting. STAT1 activation was analysed using anti-pSTAT1 (pY701) and anti-STAT1 antibodies. MxA and RIG-I expression levels were analysed using specific antibodies. MAVS knockout was validated using a specific antibody against MAVS. The infection was controlled using an NP-specific antibody. ERK2 served as loading control. (a, d, e) The Western blots were quantified with LiCor Image Studio™ (V5.2.5). Data represent mean ± SD of three independent experiments. The wt was arbitrarily set to 1.0. One-way ANOVA followed by Dunnett’s multiple comparisons test was used to calculate statistical significance (**p ≤ 0.01, ***p ≤ 0.001).
3 | EXPERIMENTAL PROCEDURES

3.1 | Cell lines, virus strains, and infection conditions

The human lung epithelial cell line (A549), the human embryonic kidney cell line (HEK 293T) and the African green monkey kidney epithelial cell line (Vero) were cultivated in Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich). The Madin–Darby canine kidney cell line (MDCK II) was cultivated in minimum essential medium (MEM; Sigma-Aldrich), both supplemented with 10% fetal calf serum (Merck Millipore). All cell lines were grown at 37 °C, 5% CO₂, and humidified conditions.

Human IBV strain B/Lee/40 wt and mutated viruses were generated by use of the pHW2000-based eight plasmid reverse genetics system as described elsewhere (Hoffman et al., 2000). Site-directed mutagenesis was used to introduce the following mutations and confirmed by sequencing: B/Lee/40 PB1 E399G, A523S, A523G, E399G/A523G, B/Lee/40 PA E350K. The following primers were used:

B/Lee E399G fwd: tcaatgaaggagaacggca,
B/Lee E399 rev: tgcgtctctcttcctagg,
B/Lee PB1 A523S fwd: gcagagactgcaataggaatg,
B/Lee PB1 A523G fwd: gcagagactggaataggaatg,
B/Lee PB1 A523 rev: catctctatgcagctgtcgc,
B/Lee PB1a R562M fwd: aatgccacatgaggattcc,
B/Lee PB1b M562I fwd: aaatgccacataggagattcc,
B/Lee PB1 R562 rev: ggaatctcctatgtggcattt,
B/Lee PA E350K fwd: ctaaagaactttaaataggcaac,
B/Lee PA E350K rev: ttgctgtttcttcattgttgatg.

HEK 293T cells were transfected with the eight plasmids, each at 1 μg. The transfection reagent TransIT-LT1 (Mirus) was used for the transfection according to the manufacturer’s protocol. Cells were cultivated in DMEM supplemented with 0.6% bovine serum albumin (35%; MP Biomedicals), 1% CaCl₂/MgCl₂, and 1% 100 U ml⁻¹ of penicillin/streptomycin (Invitrogen) and 0.3% bovine serum albumin, 1% CaCl₂/MgCl₂, and 1% 100 U ml⁻¹ of leupeptin, 5 μg ml⁻¹ of aprotinin, 0.2 mM of Pefabloc, 1 mM of sodium vanadate, and 5 mM of benzamidine). Centrifugation (14,000 r.p.m., 4°C, 10 min) was used to clear the lysates. The protein concentrations were determined by Bradford assay. The samples were prepared with 5x Laemmli sample buffer (312 mM of Tris–HCl pH 6.8, 10% SDS, 50% glycerol, 25% β-mercaptoethanol, and 0.01% bromophenol blue) and heated for 5 min at 95 °C. Equal protein amounts were separated in an SDS–polyacrylamide gel electrophoresis (SDS-PAGE; 20 ma/gel) using electrophoresis buffer (25 mM of Tris, 250 mM of glycine, 0.1% SDS). Proteins were transferred to nitrocellulose membranes (GE Healthcare) within a wet blotting system (400 mA, 50 min) using transfer buffer (25 mM of Tris, 192 mM of glycine, 20% methanol). The induction of type I IFN response was defined by analysis of IRF3 phosphorylation with a phosho-specific rabbit anti-IRF3 (S396) polyclonal antibody (pAb; Millipore) and STAT1 phosphorylation with a phosho-specific mouse anti-STAT1 (pY701) monoclonal antibody (mAb; BD Biosciences). The total amount of IRF3 was determined with the rabbit anti-IRF3 mAb and of STAT1 with the rabbit anti-STAT1 pAb (Cell Signaling). Protein expression of RIG-I was evaluated using a rabbit anti-RIG-I pAb (abcam), of MxA using a mouse anti-MxA mAb (Acris) and a mouse anti-NP mAb (Serotec). Rabbit anti-ERK 2 pAb (clone C-14, Santa Cruz) was used as loading control. Antibodies were diluted in TBST buffer (50 mM of Tris–HCl pH 7.5, 150 mM of NaCl, 0.05% Tween-20) and incubated overnight at 4°C. Horseradish-labelled secondary antibodies anti-mouse and anti-rabbit (Cell Signaling) were diluted in TBST buffer and incubated for 1 h at room temperature. Standard enhanced chemiluminescence reaction was used to visualise protein bands.

3.2 | Lysis of cells and Western blotting

For the analysis of viral protein expression and cellular protein phosphorylation, cells were washed with PBS and lysed with cold RIPA lysis buffer (25 mM of Tris–HCl pH 8.0, 137 mM of NaCl, 10% glycerol, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP40, 2 mM of EDTA pH 8.0, 5 μg ml⁻¹ of leupeptin, 5 μg ml⁻¹ of aprotinin, 0.2 mM of Pefabloc, 1 mM of sodium vanadate, and 5 mM of benzamidine). The quantity of infectious particles in the supernatants was determined via standard plaque titration at the indicated time points post infection as described elsewhere (Dudek et al., 2011).

3.3 | RNA isolation, cDNA synthesis, and quantitative real-time PCR

Total RNA isolation was performed by use of the RNeasy Mini Kit (Qiagen) according to the manufacturers protocol. Total RNA concentration was measured with the Nanodrop ND-1000 spectrometer (Peglab); 1 μg of RNA, the Revert AID H Minus Reverse Transcriptase (Thermo Fisher Scientific), and oligo (dT) primers (Eurofins MWG Operon) were used for synthesis of cDNA. qRT-PCR was performed with the Mx Pro 3005P cycler system (Stratagene) and the qRT-PCR reaction mix (Brilliant III SYBR Green QPCR Master Mix) from Agilent Technologies.

The following primers were used:

- B/Lee A523G fwd: gcagagactggaataggaatg
- B/Lee A523S fwd: gcagagactgcaataggaatg
- B/Lee PA E350K fwd: ctaaagaactttaaataggcaac
- B/Lee PA E350K rev: ttgctgtttcttcattgttgatg

For the analysis of viral protein expression and cellular protein phosphorylation, cells were washed with PBS and lysed with cold RIPA lysis buffer (25 mM of Tris–HCl pH 8.0, 137 mM of NaCl, 10% glycerol, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP40, 2 mM of EDTA pH 8.0, 5 μg ml⁻¹ of leupeptin, 5 μg ml⁻¹ of aprotinin, 0.2 mM of Pefabloc, 1 mM of sodium vanadate, and 5 mM of benzamidine). The quantity of infectious particles in the supernatants was determined via standard plaque titration at the indicated time points post infection as described elsewhere (Dudek et al., 2011).
The determination of relative RNA levels was conducted after 40 amplification cycles using the $2^{-\Delta\Delta Ct}$ method (Livak et al., 2001).

3.4 | Reporter gene assay

For the reporter gene assays, MDCK II cells were transfected with 0.5 µg of the reporter gene constructs pPol/Sapl/B-NS-luc, pTATA-luc-4x-IRF3, or pGL-IFN-luc and the polymerase subunits PB2, PB1, PA (pHW2000-B/Lee: 0.25 µg each) and NP (pHW2000-B/Lee: 0.5 µg) using TransIT-LT1 transfection reagent (Mirus Bio) according to the manufacturer’s protocol. As negative control, the same set of plasmids was used without the PB1 construct. 24 h post transfection, luminescence was measured after cell harvesting as described earlier (Hrinicis et al., 2012).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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