Glycoprotein G is a virulence factor in infectious laryngotracheitis virus


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Infectious laryngotracheitis virus (ILT; Gallid herpesvirus 1) is an alphaherpesvirus that causes acute respiratory disease in chickens. The role of glycoprotein G (gG) in vitro has been investigated in a number of alphaherpesviruses, but the relevance of gG in vivo in the pathogenicity of ILTV or in other alphaherpesviruses is unknown. In this study, gG-deficient mutants of ILTV were generated and inoculated into specific-pathogen-free chickens to assess the role of gG in pathogenicity. In chickens, gG-deficient ILTV reached a similar titre to wild-type (wt) ILTV but was significantly attenuated with respect to induction of clinical signs, effect on weight gain and bird mortality. In addition, an increased tracheal mucosal thickness, reflecting increased inflammatory cell infiltration at the site of infection, was detected in birds inoculated with gG-deficient ILTV compared with birds inoculated with wt ILTV. The reinsertion of gG into gG-deficient ILTV restored the in vivo phenotype of the mutant to that of wt ILTV. Quantitative PCR analysis of the expression of the genes adjacent to gG demonstrated that they were not affected by the deletion of gG and investigations in vitro confirmed that the phenotype of gG-deficient ILTV was consistent with unaltered expression of these adjacent genes. This is the first reported study to demonstrate definitively that gG is a virulence factor in ILTV and that deletion of gG from this alphaherpesvirus genome causes marked attenuation of the virus in its natural host.

INTRODUCTION

Infectious laryngotracheitis virus (ILT; Gallid herpesvirus 1) is an alphaherpesvirus that causes acute respiratory disease in poultry. ILTV encodes glycoprotein G (gG), a glycoprotein that is conserved in most members of the subfamily Alphaherpesvirinae (Kongsuwan et al., 1993; Wild et al., 1996). The role of gG in vitro has been investigated in a number of different alphaherpesviruses. Studies on alphaherpesviruses with disruptions to, or deletions of, gG have shown that gG is not essential for virus growth in vitro in pseudorabies virus (PRV), herpes simplex virus 1 (HSV-1), bovine herpesvirus 1 (BHV-1), quine herpesvirus 1 (EHV-1) and equine herpesvirus 4 (EHV-4) (Huang et al., 2005; Kim et al., 1999; Longnecker & Roizman, 1987; Mettenleiter & Rauh, 1990; Nakamichi et al., 2001, 2002; Thomsen et al., 1987; Tran et al., 2000). In HSV-1, deletion studies have implicated gG in the facilitation of virus entry through the apical surfaces of polarized epithelial cells (Tran et al., 2000). In BHV-1, gG has been implicated in cell-to-cell spread and prevention of cell apoptosis (Nakamichi et al., 2001, 2002). The phenotypes of these deletion mutants, however, cannot be attributed definitively to gG, as studies in PRV have revealed that insertions into the gG gene reduced expression of the upstream protein kinase (PK) gene, which inhibits cell-to-cell spread (Demmin et al., 2001). Expression of the PK gene returned to normal following the restoration of the gG gene and hence the role of the PK gene cannot be separated from that of the gG gene (Demmin et al., 2001).

More recently, studies in vitro have shown that gG functions as a soluble broad-spectrum chemokine-binding protein (vCKBP) in some alphaherpesviruses, including BHV-1, bovine herpesvirus 5 (BHV-5), EHV-1, equine herpesvirus 3 (EHV-3) and feline herpesvirus 1 (FHV-1), but not in other alphaherpesviruses such as HSV-1 (Bryant et al., 2003; Costes et al., 2005). A number of poxviruses (Alcamí & Koszinowski, 2000; Alcamí et al., 1998) and also the murine gammaherpesvirus 68 (MHV-68) (Parry et al., 2000; van Berkel et al., 2000) also contain vCKBPs, which are important for the pathogenicity and in vivo phenotype of these viruses (Bridgegam et al., 2001; Martinez-Pomares et al., 1995; Reading et al., 2003). The relevance of gG in vivo in viral pathogenicity has not been extensively investigated. In one study, a mutant of BHV-1 with disruption to the gG gene was significantly attenuated in specific-pathogen-free (SPF) calves (Kaasheok et al., 1998). The phenotype of this mutant could not be attributed definitively to the lack of gG alone, however, since no rescue mutant was investigated and the expression of genes adjacent to the interrupted gG was not evaluated. Studies of gG deletion mutants of other alphaherpesviruses in vivo, such as HSV-1 and PRV, have
shown only a slight attenuation of virulence (Balan et al., 1994; Kimman et al., 1992a, b; Tran et al., 2000). In these studies, the role of gG in attenuation was again obscured by the unknown effect upon the expression of adjacent genes.

The aim of the study reported here was to assess the role of gG in the pathogenicity of ILTV during infection of the natural host. This was achieved by generating gG-deficient mutants of ILTV and also an ILTV rescue mutant, into which the gG gene had been reinserted. These mutants were characterized in chickens to assess viral attenuation and in cell culture to assess the effect of the deletion of the gG gene upon the expression of adjacent genes.

METHODS

Virus strains and propagation. ILTV mutants were generated from the virulent Australian CSW-1 ILTV strain (wt ILTV), which was previously known as NSW virulent ILTV (Fahey et al., 1983). Virus was propagated in primary chick embryo kidney (CEK) cells, harvested from 17- to 18-day-old embryonated eggs by trypsin dis-aggregation. The chicken liver tumour cell line LMH (Kawaguchi et al., 1987) was used for transfection and viral plaque assays. Cell monolayers were cultured as described previously (Devlin et al., 2006). Virus quantification by median embryo infective dose (EID₅₀) assay was performed by viral inoculation of the choiologia-toxic membrane of 10-day-old embryonated chicken eggs.

Construction of ILTV mutants. A gG-deficient mutant of ILTV that expressed enhanced green fluorescent protein (eGFP; Clontech) in place of gG was constructed by homologous recombination. The eGFP sequence was then removed to generate ΔgG ILTV. A rescue mutant, ΔgG(R) ILTV, was constructed by reinserting the gG gene into the genome of ΔgG(eGFP) ILTV.

Construction of ΔgG(eGFP) ILTV. This mutant was created by recombination between plasmid pGu-eGFP-Gd and wt ILTV genomic DNA (Fig. 1). Plasmid pGu-GFP-Gd contained the eGFP gene sequence flanked by DNA sequences that extend 1004 bp upstream (Gu) and 989 bp downstream (Gd) of the gG gene of ILTV. Gu and Gd were amplified by PCR from wt ILTV DNA using the primer pairs h/lh and h/rr, respectively (Table 1). The PCR to amplify the eGFP sequence utilized the primers hgf and hgr (Table 1). The technique of gene splicing by overlap extension PCR (SOE PCR) (Horton et al., 1989) was used to assemble Gu–eGFP–Gd. This reaction utilized the primers lf and rr (Table 1) and gel-extracted products from the above Gu and Gd PCR as template. The resultant construct (Gu–eGFP–Gd) was inserted into the plasmid pGEM-T (Promega) to form pGu-eGFP-Gd (Fig. 1).

DNA was extracted from ILTV-infected CEK cells as described previously (Fuchs & Mettenleiter, 1996). LMH cells were infected with 1 µg ILTV DNA, 1 µg linearized pGu-eGFP-Gd and 1 µg pRc-ICP4 as described previously (Devlin et al., 2006). Plasmid pRc-ICP4 increases the infectivity of viral DNA (Fuchs et al., 2000). Viral plaques expressing eGFP were identified using fluorescent microscopy and picked with a micropipette as described previously (Devlin et al., 2006). The virus was plaque-purified three times under an overlay medium containing 1 % (w/v) methylcellulose. The recombination region of the ΔgG(eGFP) ILTV was amplified by PCR using primers srvf and srrv (Table 1) and sequenced using Big Dye Terminator version 3.1 chemistry (ABI PRISM).

Construction of ΔgG ILTV. The eGFP sequence was removed from ΔgG(eGFP) ILTV by recombination between plasmid pGu-Gd and extracted ΔgG(eGFP) ILTV genomic DNA (Fig. 1). Gu and Gd were amplified by PCR as described above, except that primer hrgGneg was used instead of hrf and primer hfgGneg was used instead of hlr (Table 1). These PCR products were used as template in an SOE PCR to assemble Gu–Gd as described above. Gu–Gd was ligated into pGEM-T to form pGu-Gd. One microgram of ΔgG(eGFP) ILTV DNA, 2 µg linearized pGu-Gd and 2 µg pRc-ICP4 were co-transfected into LMH cells and recombinant virus, which did not express eGFP, was identified by fluorescence microscopy. The virus was purified and the recombinant region was sequenced as described for ΔgG(eGFP) ILTV.

Construction of ΔgG(R) ILTV. The sequence encoding gG was reinserted into the genome of ΔgG(eGFP) ILTV by recombination between plasmid pGu-gG-Gd and extracted ΔgG(eGFP) ILTV DNA (Fig. 1). Gu–gG–Gd was amplified by PCR using primers lf and rr (Table 1). Extracted wt ILTV DNA was used as template. Gu–gG–Gd was ligated into pGEM-T to form pGu-gG-Gd. One microgram of ΔgG(eGFP) ILTV DNA, 2 µg linearized pGu-gG-gd and 2 µg pRc-ICP4 were co-transfected into LMH cells. Recombinant virus, which did not express eGFP, was identified, purified and the recombinant region was sequenced as described for ΔgG ILTV.
Pathogenicity of gG-deficient ILTV in chickens

**Pathogenicity of ΔgG(eGFP) ILTV.** An investigation of the pathogenicity of ΔgG(eGFP) ILTV was conducted in a pilot experiment, prior to the generation of the other mutants. Three-week-old SPF chickens were weighed and assigned randomly to one of three groups. Each group was held in a separate isolator and provided irradated feed and water ad libitum. The first group of five chickens was inoculated intratracheally with 3000 plaque-forming units (p.f.u.) of wt ILTV suspended in 300 μl Dulbecco’s minimal essential medium (DMEM). The second group of 10 chickens was similarly inoculated with 3000 p.f.u. ΔgG(eGFP) ILTV. Another group of 15 chickens was inoculated intratracheally with 300 μl sterile DMEM. The number of birds per group varied, as some birds in the last two groups were required for additional studies, subsequent to this investigation. This experimental design allowed the smallest number of experimental animals to be used overall.

Clinical signs of respiratory disease were scored at 4 days after infection. Mild dyspnoea, consisting of an increased respiratory effort without open beak breathing, was scored as 1. Moderate dyspnoea, with occasional open beak breathing, was scored as 2. Severe dyspnoea, with constant open beak breathing, was scored as 3. Very severe dyspnoea, with occasional gasping, was scored as 4. Extreme dyspnoea, with constant gasping, was scored as 5. Birds with no clinical signs of respiratory disease were scored as 0. Scores for clinical signs were compared between groups of birds using a Mann–Whitney test. At 4 days after infection, five birds in each group were killed by exposure to the anaesthetic agent halothane and then weighed and the larynx and trachea of each bird were removed aseptically. A scraping was taken from each trachea for virus isolation and titration by plaque assay. Percentage weight gains and viral titres were compared between groups using Student’s t-test.

**Pathogenicity of ΔgG ILTV.** Following the pilot study into the pathogenicity of ΔgG(eGFP) ILTV, mutants ΔgG ILTV and ΔgG(R) ILTV were generated as outlined above. To investigate the pathogenicity of these mutants, 3-week-old SPF chickens were weighed and assigned to one of four groups, with each group consisting of 16 birds. The birds were housed and maintained as described above. Each of the birds in the first three groups was inoculated intratracheally with 10^4 EID_{50} of either wt ILTV, ΔgG ILTV or ΔgG(R) ILTV. Each dose of virus was suspended in 300 μl DMEM. Each bird in the final group was inoculated intratracheally with 300 μl sterile DMEM.

Four days after inoculation, all the birds were weighed and the percentage weight gain for each bird was calculated. These weight gains were compared between groups using Student’s t-test. Clinical signs of disease in 10 randomly selected birds in each group were also scored at 4 days after inoculation. Dyspnoea was scored on a scale of 0–4: birds with no signs of respiratory disease were scored as 0, those showing a mild increase in respiratory effort but without open beak breathing were scored as 1, those showing open beak breathing were scored as 2, those showing gasping were scored as 3 and those showing severe gasping with neck extension were scored as 4. Signs of conjunctivitis were scored on a scale of 0–2: birds with no signs of conjunctivitis were scored as 0, those showing partial closure of the eye were scored as 1 and those showing complete closure of the eye were scored as 2. The

### Table 1. Primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Orientation</th>
<th>Sequence (5′–3′)</th>
<th>Target sequence</th>
</tr>
</thead>
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<tr>
<td>hgf</td>
<td>Forward</td>
<td>tcgaaagtcaagagacatacgATGTTGAGCAAGGGGCA</td>
<td>7225–7244, eGFP</td>
</tr>
<tr>
<td>hgr</td>
<td>Reverse</td>
<td>caaaaaacctctctgtggttcAGTTATTCTTAGATCCGGTGAT</td>
<td>8143–8124, eGFP</td>
</tr>
<tr>
<td>lf</td>
<td>Forward</td>
<td>GCTGGGCTTTGTTGACAGTA</td>
<td>6241–6261</td>
</tr>
<tr>
<td>hlr</td>
<td>Reverse</td>
<td>TCGCCCTTGTTCACCATCATGtagtcttcaagtcg</td>
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</tr>
<tr>
<td>hrf</td>
<td>Forward</td>
<td>ATCCACCGGATCTAGATAACTGAccacccgagagtlttttg</td>
<td>eGFP, 8124–8143</td>
</tr>
<tr>
<td>rr</td>
<td>Reverse</td>
<td>CGTCAATGCTGGGATGTG</td>
<td>9113–9097</td>
</tr>
<tr>
<td>rrs</td>
<td>Reverse</td>
<td>TTAGCAGACACACGAAAAC</td>
<td>8250–8231</td>
</tr>
<tr>
<td>hfgNeg</td>
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<td>7231–7247, 8124–8142</td>
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<td>hrGneg</td>
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</tr>
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<tr>
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<td>Reverse</td>
<td>CGATGGGAGTITTTTTCCTTC</td>
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<td>5759–5741</td>
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<td>7535–7514</td>
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<td>ICP4 of ILTV</td>
</tr>
<tr>
<td>ICP4r</td>
<td>Reverse</td>
<td>AGGAGGAAGAGAAGAGAAGA</td>
<td>ICP4 of ILTV</td>
</tr>
<tr>
<td>fCyclophilin</td>
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<td>GAGGGAGACAAAGCCAAGT</td>
<td>Cyclophilin</td>
</tr>
<tr>
<td>rCyclophilin</td>
<td>Reverse</td>
<td>GTTTACGTTTAACAGGCCG</td>
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</tr>
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<td>UL15f</td>
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<td>UL15 of ILTV</td>
</tr>
<tr>
<td>UL15r</td>
<td>Reverse</td>
<td>GAAAAATGTTAGTGGCGGCAAT</td>
<td>UL15 of ILTV</td>
</tr>
</tbody>
</table>
general demeanour of the birds was scored on a scale of 0–2: birds showing a normal demeanour were scored as 0, those showing a depressed demeanour were scored as 1 and those showing a severely depressed demeanour were scored as 2. These scores were summed to give an overall clinical signs score. These scores were compared between groups using a Mann–Whitney test.

At 4 days after infection, eight birds in each group were killed as described above. Their tracheas were removed aseptically and a transverse section of proximal trachea was collected and transferred to a microcentrifuge tube containing 500 μl of 4 M guanidine isothiocyanate, 15 mM PIPES/NaOH (pH 7·6) and 5 μl β-mercaptoethanol. Viral DNA from these sections was extracted using Qiaex II suspension (Qiagen) for use in detection and quantification of ILTV by quantitative PCR using SYBR Green master mix (Invitrogen). This PCR utilized the forward primer UL15f and the reverse primer UL15r (Table 1), which amplify a 115 bp region from the ILTV UL15 gene. The template was 2 μl extracted product or sterile water for the contamination control reactions or 5 μl 10-fold dilutions of a sample containing 4·0 × 10^6 copies of the ILTV UL15 sequence in pGEM-T to generate a standard curve.

Another transverse section of proximal trachea was collected and preserved in Bouin’s fixative prior to histopathological examination. Sections were stained with haematoxylin and eosin (H&E) and examined using light microscopy. The severity of histopathological lesions was scored using a previously described system (Guy et al., 1990). These lesion scores were compared between groups using a Mann–Whitney test. The thickness of the tracheal mucosa, excluding the epithelium, was calculated as a measure of inflammatory cell infiltration. This was achieved by averaging the thickness of the mucosa at four points transected by vertical and horizontal lines (Nunoya et al., 1987). Tracheal mucosal thickness was compared between groups using Student’s t-test.

Bird mortalities were monitored for 21 days following inoculation. Birds that were found dead or that required euthanasia due to severe dyspnoea were recorded as mortalities. Bird mortalities were compared between the groups using Fisher’s exact test. At 21 days after inoculation, all remaining birds were killed as described above.

**In vitro characterization of gG-deficient ILTV**

**Growth kinetics.** Wild-type ILTV, ΔgG ILTV, ΔgG (eGFP) ILTV and ΔgG(R) ILTV were inoculated onto confluent LMH cells in 6-well trays, in duplicate, at an m.o.i. of approximately 0·002. After an adsorption period of 1 h, the supernatant was removed and replaced with 2 ml fresh medium. Incubation was continued and, at an adsorption period of 1 h, the supernatant was removed and replaced with 2 ml methylcellulose overlay medium. At 12 h after infection, the cells were washed with PBS and total RNA was extracted using an RNeasy RNA Isolation Kit (Qiagen). The concentration and purity of the RNA was determined by spectrophotometry (Eppendorf Biophotometer). Two micrograms of the extracted RNA was treated with 2 μl DNase (Invitrogen) and then 1 μg of this RNA was used to generate cDNA using Superscript II RNase reverse transcriptase (SSII RT; Invitrogen) and 100 μg random hexamers (Generewks). Control reactions without SSII RT were also performed for each sample. Amplification of cDNA was performed using quantitative PCR (Mx3000; Stratagene). The reaction mixture (20 μl) contained 5 μl diluted cDNA, 0·3 μM primers (gJf/Gr, gGf/GGr or U47f/U47r; Table 1) and 10 μl SYBR Green master mix. Two replicates were assayed for each sample. For each replicate, cycle threshold (C_t) values for the gl, UL47 and gG genes were determined by automated threshold analysis using Stratagene Mx3000 version 1·0 software. The C_t values for ICP4, which was known to be present at 12 h p.i., were also determined for each sample in duplicate. These reactions utilized the ICP4f and ICP4r primers (Table 1). The cellular housekeeping gene for cyclophilin was used as an internal control for the amount of RNA in each tube (CyCyclophilin and rCyclophilin primers; Table 1). Control reactions that did not contain cDNA were also performed for each sample and each primer pair. Values of 50–C_t for the gl, UL47 and gG genes were calculated for each sample and the relative abundance of mRNA from the gl and UL47 genes was calculated by normalizing these values against the 50–C_t value for ICP4 mRNA for the same sample. The relative abundances of mRNA were compared between groups using Student’s t-test.

**RESULTS**

**Construction of ILTV mutants**

Two gG-deficient mutants of ILTV and also a revertant virus in which the gG gene had been reinserted were constructed by targeted homologous recombination. Sequence analysis of the recombination regions showed that successful recombination had occurred in all of the mutants and that no coding mutations were introduced into the flanking regions of any of the mutants or in the gG coding region of ΔgG(R) ILTV.

**Pathogenicity of gG-deficient ILTV in chickens**

To determine whether gG deletion would affect the pathogenicity of ILTV, a pilot experiment was conducted in which groups of birds were inoculated with wt or ΔgG(eGFP) ILTV or remained as mock-infected controls. At 4 days after inoculation, the median clinical sign score for birds inoculated with ΔgG(eGFP) ILTV was 1·5 (range 0–3). The median clinical sign score for the wt ILTV-inoculated birds was 4 (range 1–5). These scores were significantly different (P=0·012). None of the birds in the negative control group developed clinical signs of respiratory disease. The mean percentage weight gain (±SD) of birds inoculated with ΔgG(eGFP) ILTV was 52·4±14·3%. This was significantly greater than the weight gain in birds inoculated with the wt ILTV (16·0±14·3%) (P=0·006). Birds in the negative control group showed a significantly greater weight gain than birds in both the virus-inoculated groups (69·8±11·6%). The mean ILTV titre in the tracheas of...
wt ILTV-inoculated birds was 6.92 ± 0.73 log₁₀ p.f.u. per scraping. This titre was not significantly different from that of ΔgG(eGFP) ILTV-inoculated birds (5.59 ± 1.12 log₁₀ p.f.u. per scraping) (P = 0.068). No ILTV was detected in the birds in the negative control group.

Since these first experiments showed that the pathogenicity of ΔgG(eGFP) ILTV appeared significantly reduced compared with wt ILTV, further studies were initiated. The viruses ΔgG ILTV and ΔgG(R) ILTV were constructed and used to infect birds to compare the pathogenicity of these viruses to wt ILTV. The clinical scores, percentage weight gain and ILTV titres at 4 days p.i. for wt-, ΔgG- and ΔgG(R)-infected birds, and also uninfected birds, are summarized in Table 2. Birds inoculated with ΔgG ILTV had significantly less severe clinical signs than those inoculated with wt ILTV (P = 0.010) or ΔgG(R) ILTV (P = 0.011). No significant difference was found between the clinical scores of birds inoculated with ΔgG ILTV and uninfected birds (P = 0.271). No significant difference was found between the clinical scores of birds inoculated with ΔgG(R) ILTV and wt ILTV (P = 0.712). Birds inoculated with ΔgG ILTV had weight gains significantly greater than the wt ILTV-inoculated group (P = 0.029) and the ΔgG(R) ILTV-inoculated group (P = 0.043). No significant difference was detected between the weight gains of birds inoculated with ΔgG ILTV and uninfected birds (P = 0.218). No significant difference was detected between the weight gains of birds inoculated with wt ILTV and birds inoculated with ΔgG(R) ILTV (P = 0.483). There was no significant difference in the tracheal titres of ILTV in the groups inoculated with ΔgG ILTV, wt ILTV and ΔgG(R) ILTV. Virus was not detected in the tracheas of any of the birds in the negative control group.

The tracheal mucosal thicknesses of uninfected birds and birds infected with wt, ΔgG and ΔgG(R) ILTV at 4 days p.i. are also summarized in Table 2. The tracheal mucosal thickness in ΔgG ILTV-inoculated birds was significantly greater than the tracheal mucosal thickness of wt ILTV- and ΔgG(R) ILTV-inoculated birds (P = 0.001 and 0.036, respectively). There was no significant difference in the thickness of the tracheal mucosa in birds inoculated with wt ILTV and ΔgG(R) ILTV (P = 0.160). The lesion scores for the ΔgG ILTV-inoculated group (median 3–5, range 2–4), the wt ILTV-inoculated group (median 4, range 3–6) and the ΔgG(R) ILTV-inoculated group (median 4, range 3–4) were not significantly different. The lesion scores for the uninfected birds (median 1, range 0–1) were significantly lower than those of all other groups. Fig. 2 shows photomicrographs of the tracheal mucosa in uninfected birds and birds infected with wt or ΔgG ILTV.

Mortality rates in groups of birds inoculated with different strains of ILTV and also in the group of uninfected birds are shown in Fig. 3. The mortality rate in the group of birds inoculated with ΔgG ILTV was not significantly different from that in the group of uninfected birds (P = 0.467), whilst mortality rates in the groups of birds inoculated with ΔgG(R) ILTV or with wt ILTV were significantly different from that in the group of uninfected birds (P = 0.007 and 0.026, respectively).

In vitro characterization of gG-deficient ILTV

There was no apparent difference in the in vitro growth kinetics of any of the ILTV strains (Fig. 4). There was no significant difference in the diameters of the plaques induced by ΔgG ILTV, ΔgG(R) ILTV or wt ILTV. The plaques induced by ΔgG(eGFP) ILTV were consistently larger than those induced by wt ILTV and larger than those induced by ΔgG ILTV at 24 h p.i. (Table 3). There was no significant difference in the amount of transcript produced from the gJ or UL47 genes in wt ILTV, ΔgG ILTV and ΔgG(R) ILTV (Table 4). There was no apparent difference in the amount of transcript produced from the gG gene in wt ILTV (50 – C values of 32–84 and 32–20) and ΔgG(R) ILTV (50 – C values of 31–58 and 31–90). No transcript from the gG gene was detected in ΔgG ILTV.

DISCUSSION

In these studies, the role of gG in ILTV was investigated by characterizing gG-deficient mutants of ILTV in vivo and in vitro. The in vivo studies revealed that gG-deficient ILTV was significantly attenuated compared with wt ILTV. Birds inoculated with the initial gG-deficient mutant, ΔgG(eGFP) ILTV, had less severe clinical signs and better weight gain than birds inoculated with wt ILTV. A more extensive investigation into the pathogenicity of ΔgG ILTV also

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**Table 2. Measures of ILTV pathogenicity at 4 days p.i. for uninfected birds and birds infected with wild-type ILTV, ΔgG ILTV or ΔgG(R) ILTV**

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<tr>
<th>Measure</th>
<th>wt ILTV</th>
<th>ΔgG ILTV</th>
<th>ΔgG(R) ILTV</th>
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<tr>
<td>Median clinical score (range)</td>
<td>1 (1–4)*</td>
<td>0 (0–2)†</td>
<td>2 (0–2)*</td>
<td>0 (0–0)†</td>
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<tr>
<td>Mean weight gain ± SD (%)</td>
<td>19.5 ± 18.4*</td>
<td>32.9 ± 12.9†</td>
<td>22.8 ± 13.2*</td>
<td>38.3 ± 10.3†</td>
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<td>Mean tracheal mucosal thickness ± SD (μm)</td>
<td>116 ± 42*</td>
<td>220 ± 61†</td>
<td>153 ± 49*</td>
<td>52 ± 21†</td>
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<td>Rate of ILTV detection</td>
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<td>8/8</td>
<td>7/8</td>
<td>0/8</td>
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<tr>
<td>Mean log₁₀ ILTV genome equivalents per section ± SD</td>
<td>4.80 ± 0.70*</td>
<td>4.95 ± 0.57*</td>
<td>4.50 ± 1.74*</td>
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*†‡Values with the same superscript symbols in the same row were not significantly different.
demonstrated attenuation with respect to clinical signs and weight gain and also mortality rate. A difference in tracheal mucosal thickness was also detected. The reinsertion of the gG gene into gG-deficient ILTV restored the in vivo phenotype of the mutant back to that of wt ILTV. In these experiments, the birds showed large weight gains over a short time period. This was useful for providing an objective parameter to measure the attenuation of gG-deficient ILTV. Similarly, tracheal mucosal thickness provided an objective parameter to measure the degree of inflammatory cell infiltration.

The in vitro studies revealed that the transcription of UL47 and gl, the genes adjacent to gG, was not affected by the deletion of the gG gene. Also, there was no apparent difference between the transcription of gG in wt and ΔgG(R) ILTV. The in vitro phenotypes of the mutants were consistent, with an unaltered expression of UL47 and gl. There was no deficiency in cell-to-cell spread, as would be expected if the UL47 gene (Kopp et al., 2002) or, to a lesser extent, the gl gene had been downregulated (Fuchs et al., 2005). The small increase in the plaque diameter of ΔgG(eGFP) ILTV, compared with wt ILTV, was not thought to represent an enhanced ability for cell-to-cell spread, but instead may be attributable to the cytotoxic effects of high concentrations of eGFP, which can resemble viral CPE. The gG-deficient mutants did not appear to show a significant reduction in maximum virus titres, as would be expected if the gl gene had been significantly downregulated (Fuchs et al., 2005). Expression of the US3 (PK) gene, which was reduced by insertions in the gG gene in PRV (Demmin et al., 2001), was not investigated in this study as the UL47 gene, rather than US3 gene, is immediately upstream of the gG gene in ILTV (Wild et al., 1996). However, downregulation of US3 would also be expected to inhibit cell-to-cell spread (Demmin et al., 2001).

The attenuation of gG-deficient ILTV was not due to a reduced capacity for in vivo replication, as gG-deficient ILTV and wt ILTV reached similar titres in the trachea,
Table 3. Plaque sizes of ΔgG(eGFP) ILTV, ΔgG ILTV, ΔgG(R) ILTV and wt ILTV on LMH cells

<table>
<thead>
<tr>
<th>Time (h p.i.)</th>
<th>ΔgG(eGFP) ILTV</th>
<th>ΔgG ILTV</th>
<th>ΔgG(R) ILTV</th>
<th>wt ILTV</th>
</tr>
</thead>
<tbody>
<tr>
<td>48</td>
<td>838 ± 168 *</td>
<td>650 ± 184 †</td>
<td>713 ± 137 †</td>
<td>588 ± 177 †</td>
</tr>
<tr>
<td>72</td>
<td>850 ± 175 †</td>
<td>738 ± 168 †</td>
<td>713 ± 168 †</td>
<td>636 ± 118 *</td>
</tr>
<tr>
<td>96</td>
<td>1151 ± 165 †</td>
<td>1063 ± 137 †</td>
<td>1025 ± 156 †</td>
<td>912 ± 159 *</td>
</tr>
</tbody>
</table>

*†Values with the same superscript symbols in the same row were not significantly different.

which is the major site for ILTV replication (Bagust & Guy, 2003). Tracheal titres and other parameters were assessed only at 4 days p.i., the time point associated with maximal viral titres (Bagust et al., 1986). However, it can be reasonably expected that any significant attenuation in the replication of gG-deficient ILTV, and hence any associated differences in the time-course of disease, would be manifested in a reduced viral titre of gG-deficient ILTV compared with wt ILTV at that time point. Twice daily observations of the ΔgG ILTV inoculated birds until 21 days p.i. supported the view that there was no difference in the time-course of clinical disease resulting from infection with ΔgG ILTV or wt ILTV.

The results from the investigation of tracheal mucosal thickness may provide a possible explanation for the attenuation of gG-deficient ILTV. In chickens, measuring the thickness of the tracheal mucosa is an established system for evaluating the degree of inflammatory cell infiltration in response to Mycoplasma gallisepticum infection (Nunoya et al., 1987). The increase in tracheal mucosal thickness in the ΔgG ILTV-inoculated birds, compared with the birds inoculated with wt or ΔgG(R) ILTV, was consistent with a significantly increased inflammatory infiltrate in the tracheal mucosa of ΔgG ILTV-infected birds compared with those birds infected with ILTV containing gG. This finding suggests that gG plays a role in influencing the inflammatory response at the site of ILTV infection. As chemokines are involved in the recruitment of inflammatory cells to the sites of virus infection, this finding is consistent with the proposed function of gG as a vCKBP. The scoring system described by Guy et al. (1990) incorporates the degree of inflammatory cell infiltration with other parameters such as syncytium formation and degree of epithelial loss. The lack of any apparent differences in these other parameters may explain why this system could not detect any significant difference in the severity of the lesions induced by the different ILTV strains.

A reduction in virulence due to the deletion of a vCKBP gene contrasts with findings from poxvirus studies. In poxviruses, investigations in vivo have shown that deletion of genes encoding vCKPBs increase leukocyte migration into infected tissue but either do not affect virulence or result in increased virulence (Graham et al., 1997; Lalani et al., 1999; Martinez-Pomares et al., 1995; Reading et al., 2003). However, the ultimate effect of vCKPBs on viral pathogenicity would not necessarily be expected to be the same for poxviruses and alphaherpesviruses. In particular, the relative degrees to which infiltrating inflammatory cells either contribute to pathology or prevent viral-induced pathology need to be considered. This may be influenced by the site of infection, the efficacy of the local immune response and the role of the infiltrating inflammatory cells in aiding virus spread (Reading et al., 2003). In ILTV, as in many alphaherpesviruses, the local cell-mediated immune response appears to be particularly relevant in preventing disease (Fahey et al., 1983; Fahey & York, 1990). Also, a large infiltration of inflammatory cells into the trachea is not likely to be as physiologically compromising for the host as a large infiltration of inflammatory cells into the lungs, as seen in infections with a vCKBP-deficient poxvirus (Reading et al., 2003). In addition, it has been speculated that other proteins expressed by some poxviruses may interfere with the antiviral activity of the infiltrating leukocytes (Reading et al., 2003). This would not necessarily be the case for ILTV.

Table 4. Relative abundance of mRNA for gJ and UL47 at 12 h p.i. of LMH cells with ΔgG ILTV, ΔgG(R) ILTV or wt ILTV

<table>
<thead>
<tr>
<th>Gene</th>
<th>wt ILTV</th>
<th>ΔgG ILTV</th>
<th>ΔgG(R) ILTV</th>
</tr>
</thead>
<tbody>
<tr>
<td>gJ</td>
<td>1-72 ± 0-17 *</td>
<td>2-23 ± 0-68</td>
<td>2-05 ± 0-72 *</td>
</tr>
<tr>
<td>UL47</td>
<td>1-66 ± 0-19 *</td>
<td>2-05 ± 0-64</td>
<td>2-00 ± 0-75 *</td>
</tr>
</tbody>
</table>

*Values with the same superscript symbols in the same row were not significantly different.
attenuated ILTV vaccine strains is limited by their ability to establish latency and also by their relatively high level of pathogenicity (Bagust & Guy, 2003). The attenuation seen in gG-deficient ILTV, combined with a high level of immunogenicity and a disruption to establishment of latency, would be highly desirable in a vaccine strain. However, because of the very different mechanisms of the establishment of latency by MHV-68, a gammaherpesvirus that establishes latency in B lymphocytes, macrophages and dendritic cells (Flano et al., 2000; Sunil-Chandra et al., 1992; Weck et al., 1999), and ILTV, an alphaherpesvirus that establishes latency in nervous tissue (Williams et al., 1992), a deficit in latency in gG-deficient ILTV could not necessarily be expected.

Avian immunology is a rapidly expanding field. The most significant advance in the knowledge of avian chemokines has come from the chicken genome sequence. Analysis of the chicken genome sequence has identified 24 probable chemokine-encoding genes (Kaiser et al., 2005; Wang et al., 2005). However, their functions remain largely uncharacterized and they appear to have a low degree of homology with mammalian chemokines (Wang et al., 2005). Future developments in the availability of avian chemokine reagents will enable the interaction between ILTV gG and avian chemokines to be investigated, as has been done for EHV-1, EHV-3, BHV-1, BHV-5 and other mammalian alphaherpesviruses (Bryant et al., 2003). At present, however, further work to determine the role of gG in ILTV is likely to involve further experiments in vivo. Fortunately, as demonstrated by this study, the characterization of gG-deficient ILTV in the natural host species is a convenient and powerful system to study the role of this glycoprotein.

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