Characterization of the assembly and processing of infectious laryngotracheitis virus glycoprotein B

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Introduction

Infectious laryngotracheitis virus (ILTV) is an alpha-herpesvirus that causes severe upper respiratory infections in chickens. Although ten putative ILTV glycoprotein genes have been identified by sequence analysis, no ILTV glycoprotein has been extensively characterized. In order to delineate the synthesis and processing pathway of ILTV glycoprotein B (gB), rabbit polyclonal antibodies were raised against a Cro–gB–β-galactosidase fusion protein. Through immunoprecipitation analysis of ILTV-infected chicken embryo liver cells it was determined that ILTV gB is initially synthesized as a 110 kDa monomeric precursor protein which rapidly assembles into homodimers composed of 100 kDa subunits. The dimer form of ILTV gB is rapidly cleaved to form two disulphide-linked species of 58 kDa. The apparent reduction in mass (from 110 to 100 kDa) of the mature form of gB during processing in the Golgi apparatus appears to be a common feature of avian herpesvirus gB proteins.

Methods

Virus. The USDA challenge strain of ILTV (National Veterinary Services Laboratory, Ames, Ia., USA) was propagated on monolayers of primary chicken embryonic liver (CEL) cells as previously described (Keeler et al., 1993).

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Infectious laryngotracheitis virus (ILTV) is an alpha-herpesvirus which causes severe upper respiratory infections of chickens, resulting in substantial losses to the poultry industry (Bagust & Johnson, 1995; Cover, 1996). The ILTV genome has been mapped and numerous ILTV-encoded polypeptides have been identified on polyacrylamide gels (Johnson et al., 1991; Prideaux et al., 1992; Wild et al., 1996).

The envelope glycoproteins of herpesviruses mediate virus entry, cell fusion and virus egress (Pereira, 1994; Spear, 1993). In addition, they are important immunogens and potential targets of cell-mediated and humoral immune responses. Viral glycoproteins produced by cells infected with ILTV have been recognized and studied (York et al., 1987). With the sequencing of the unique short region of the ILTV genome, up to ten ILTV-encoded glycoprotein genes, including glycoprotein B (gB), have now been identified (Fuchs & Mettenleiter, 1996; Griffin, 1991; Johnson et al., 1995a, b; Kingsley et al., 1994; Kongsuwan et al., 1991, 1993, 1995; Poulsen et al., 1991; Wild et al., 1996; C. L. Keeler, unpublished results).

A gB homologue has been identified in every herpesvirus studied to date, and gB is the most highly conserved herpesvirus structural glycoprotein (Pereira, 1994; Spear, 1985). Glycoprotein B is required for herpesvirus infectivity and is involved in virus penetration. In addition, gB elicits neutralizing antibodies (Keller et al., 1984), cell-mediated immune responses (Zarling et al., 1986), and has been shown to be a candidate antigen for recombinant, subunit vaccines (Cantin et al., 1987; Nazerian et al., 1992). Like other glycoproteins that mediate virus entry, gB homologues assemble into oligomers and most are proteolytically cleaved (Pereira, 1994).

Information on the antigenic and structural properties of the ILTV gB homologue is limited. Using a polyclonal rabbit antiserum to ILTV gB, we have characterized the product of the ILTV gB gene and have examined the post-translational modifications and the process pathway kinetics of the ILTV gB protein. The gB homologue of ILTV is processed in a manner similar to the gB homologues of Marek’s disease virus (MDV) and varicella-zoster virus (VZV). In this report, we find that the ILTV gB protein is initially synthesized in the endoplasmic reticulum (ER), rapidly converted to an oligomeric form (homodimer), and then transported to the Golgi apparatus where it is further modified and proteolytically processed. This information will aid in the ongoing efforts to characterize the involvement of gB in immunity against ILTV infection.
Expression cloning and antiserum production. A 1287 bp HindIII–Bcl fragment (codons 172–601) of the ILTV gB gene, representing approximately 70% of the extracellular domain of the protein (Poulsen et al., 1991), was cloned into the HindIII and BamH I sites of the expression vector pC412 (Robbins et al., 1986) using standard cloning techniques (Sambrook et al., 1989). Escherichia coli NF1829 cells (Robbins et al., 1986) containing the resulting plasmid, pDP12, overexpress a 164 kDa Cro–gB–β-galactosidase fusion protein. Methods for the partial purification of insoluble protein aggregates and the production of rabbit antiserum have been previously described (Kingsley et al., 1994). The anti-ILTV antiserum used in this study has also been previously described (Kingsley et al., 1994).

Steady-state labelling and immunoprecipitation of ILTV glycoproteins. In steady-state labelling experiments, CEL cells were infected at an m.o.i. of 1 with the USDA challenge strain of ILTV and grown in Dulbecco’s MEM (DMEM) containing 2% foetal bovine serum (FBS) and 100 µCi/ml [3H]glucosamine or [3H]fucose (DuPont-NEN) for 13 h. The preparation of infected cell extracts and the immunoprecipitation procedure have been previously described (Robbins et al., 1984).

Pulse-chase and post-transcriptional analysis of ILTV gB proteins. The pulse-chase procedure was performed as described by Ryan et al. (1987) with the following modifications. CEL cells were infected at an m.o.i. of 1 with the USDA challenge virus. At 11–13 h post-infection, cells were cultured for 30 min in methionine-free DMEM. After methionine depletion, a radioactive pulse with 100 µCi/ml [35S]methionine (DuPont-NEN) was administered for 10 min. The radiolabelled methionine was removed, and the cells were incubated in the presence of a 200-fold excess (40 mM) of unlabelled methionine for various times. At the desired chase times, monolayers were harvested and immunoprecipitated with the rabbit ILTV gB antiserum.

Monensin (Sigma) was added at a final concentration of 10 µM at the time of infection. Tunicamycin (Sigma) was added at a final concentration of 10 µg/ml to ILTV-infected cultures 30 min prior to methionine depletion. In both cases, cells were labelled for 1 h at 11–13 h post-infection with 100 µCi/ml [35S]methionine. Infected cell lysates were prepared and immunoprecipitated with rabbit ILTV gB antiserum as described previously.

Endoglycosidase digestion of gB was performed with N-glycosidase F (PNGase-F) and endoglycosidase H (Endo-H) under conditions specified by the manufacturer (New England Biolabs). The substrate for the enzymes was ILTV gB that had been labelled for 1 h at 11–13 h post-infection with 100 µCi/ml [35S]methionine and immunoprecipitated with anti-gB antiserum.

Sucrose gradient sedimentation. Sedimentation of ILTV-infected CEL cell lysates in sucrose gradients was done essentially as previously described by Hampl et al. (1984) and modified by Whealy et al. (1990). Briefly, ILTV-infected cells were pulse-labelled with [35S]methionine and chased for 0, 45 or 90 min as described above. Infected cells were scraped from the plates and solubilized in non-denaturing lysis buffer [1% Triton X-100, 150 mM NaCl, 50 mM Tris (pH 7.5)] on ice for 10 min. Nuclei were removed by centrifugation at 12,000 g for 5 min at 4 °C. The supernatant was layered onto an 11 ml 8–20% linear sucrose gradient containing 0.1% Triton X-100, 150 mM NaCl, 50 mM Tris (pH 7.5), and was sedimented in an SW41 rotor at 190,000 g for 18 h at 4 °C. Fractions (750 µl) were collected from each gradient by positive displacement with an ISCO 185 density gradient fractionator and immunoprecipitated with anti-gB antiserum. The molecular masses of the monomeric and dimeric forms of ILTV gB were determined by running parallel gradients containing gel filtration standards (Sigma) as indicators of molecular mass.

Polyacrylamide gel analysis. All immunoprecipitates were loaded onto SDS–10% polyacrylamide slab gels. Electrophoresis was performed as described previously (Robbins et al., 1984). Samples examined under non-reducing conditions were resuspended in sample buffer without β-mercaptoethanol. Fluorography was conducted with sodium salicylate (Chamberlain, 1979) and was followed by autoradiography.

Results

Characterization of ILTV gB

In order to identify the molecular forms of ILTV gB which are synthesized and processed within infected avian cells, rabbit antibodies were raised against a partially purified Cro–ILTV gB–β-galactosidase fusion protein as described in Methods. Extracts prepared from [3H]glucosamine- or [3H]fucose-labelled ILTV-infected cells were reacted with this antiserum or with anti-ILTV virion antiserum as a control. The anti-gB antiserum specifically immunoprecipitated two [3H]glucosamine-labelled ILTV proteins of 110 kDa and 58 kDa (Fig. 1, lane 1). These results were reproduced using chicken kidney cells or chicken embryo fibroblasts infected with other strains of ILTV (data not shown).

Many glycoproteins are modified by the addition of fucose in the Golgi apparatus. Consequently, differential labelling of the two species of gB by fucose would indicate which form had passed through the Golgi during transport and processing. Only the 58 kDa form of ILTV gB is labelled with fucose (Fig. 1, lane 3). This result supports the hypothesis that the 110 kDa glycoprotein is a precursor form of gB localized to the ER and

Fig. 1. Immunoprecipitation of ILTV gB labelled with [3H]glucosamine or [3H]fucose. CEL cells were infected at an m.o.i. of 1 with the USDA challenge strain of ILTV and labelled with 100 µCi/ml [3H]glucosamine (lanes 1 and 2) or [3H]fucose (lanes 3 and 4) for 13 h. Infected cells were harvested, lysed, and samples were immunoprecipitated with ILTV gB antiserum (lanes 1 and 3) or ILTV virion antiserum (lanes 2 and 4). Immunoprecipitates were resolved on SDS–10% polyacrylamide gels and visualized by fluorography. The molecular masses of the gB species are indicated in kDa to the left of the gel while the positions and sizes of molecular mass standards are shown to the right of the gel.
Fig. 2. Endoglycosidase (A) and metabolic inhibitor (B) analysis of ILTV gB synthesis and processing. CEL cells were infected with the USDA challenge strain of ILTV at an m.o.i. of 1 and incubated at 37 °C. At 11–13 h post-infection, infected cells were labelled with [35S]methionine for 1 h. Cells were then harvested, lysed, and samples were immunoprecipitated with ILTV gB antiserum. (A) Samples which were untreated (lane 2), treated with Endo-H (lane 3) or treated with PNGase-F (lane 4) after immunoprecipitation. In lane 1 the infected cell lysate was immunoprecipitated with normal rabbit serum. (B) Samples which were immunoprecipitated from untreated infections (lane 1), infections performed in the presence of 10 µM monensin (lane 2) or infections in which tunicamycin was added at a concentration of 10 µg/ml 30 min prior to methionine depletion (lane 3). Samples were resolved on SDS–10% polyacrylamide gels and visualized by fluorography. The molecular masses of the gB species are indicated in kDa to the right of each panel, while the positions and sizes of molecular mass standards are shown to the left of each panel.

early Golgi compartments, while the 58 kDa glycoprotein has acquired modifications within the Golgi and is further processed.

The relationship between the two forms of ILTV gB was further characterized through the use of metabolic inhibitors of glycosylation and by endoglycosidase treatment of [35S]methionine-labelled and immunoprecipitated ILTV gB. Treatment with Endo-H (Fig. 2A, lane 3), an enzyme that removes only the high-mannose product of N-linked glycosylation that occurs as the protein crosses the membrane of the ER, shifted the 110 kDa form of gB to a faster migrating species with an apparent molecular mass of 95 kDa. The 58 kDa protein was resolved into two protein species, 56 kDa and 50 kDa in size, following Endo-H treatment. This suggests that the 58 kDa glycosylated form of ILTV gB is composed of two distinct protein species resulting from the cleavage of a
larger precursor. Upon treatment with PNGase-F, an endoglycosidase which cleaves all glycans with an N-link between asparagine and the carbohydrate moiety, an additional mobility shift to 50 kDa was observed for the 56 kDa protein species (Fig. 2A, lane 4). A further reduction in mass was not detected for the other two forms of gB observed after Endo-H treatment, indicating that these species contain only high-mannose form oligosaccharide side-chains.

Tunicamycin is a metabolic inhibitor which blocks the formation of dolichol phosphate–carbohydrate intermediates, therefore partially or completely blocking N-linked glycosylation (Poss et al., 1989; Spear, 1985). Monensin is an ionophore which interferes with transport of proteins from the ER to the Golgi. The effects of tunicamycin and monensin on ILTV gB processing are shown in Fig. 2(B). Growth of ILTV-infected cells in the presence of monensin resulted in the accumulation of the 110 kDa uncleaved, immature protein (Fig. 2B, lane 2). Growth of ILTV-infected cells in the presence of tunicamycin resulted in the production of a single 95 kDa protein (Fig. 2B, lane 3). The unmodified ILTV gB protein is predicted to be 852 amino acids in length and to have a molecular mass of 96.7 kDa.

Taken together these results confirm that the 110 kDa species represents an immature precursor form of gB, while the 58 kDa species represents the mature processed form of gB. In addition, these results suggest that the 58 kDa band is composed of two protein species, one of which contains only high-mannose glycans, while the other carries both high-mannose and complex oligosaccharide side-chains.

**Pulse-chase analysis of ILTV-encoded gB**

To further investigate the precursor–product relationship between the 110 kDa and 58 kDa forms of gB, and to determine whether any intermediate processed forms of the protein exist, the kinetics of gB synthesis was examined in a pulse-chase experiment as described in Methods. Duplicate immunoprecipitated samples were electrophoresed on 10% polyacrylamide gels under either reducing or non-reducing conditions. The results are shown in Fig. 3. The processing of ILTV gB is rapid and efficient. The mature, 58 kDa, form of gB appeared within a 60 min chase period, and the majority of the labelled protein had been converted to the mature form after 2 h. The 110 kDa gB precursor protein is converted to the processed 58 kDa form with a $t_{1/2}$ of 60–75 min.

Due to the large reduction in size of the mature form of gB, immunoprecipitated samples were also electrophoresed under non-reducing conditions (Fig. 3B). Under these conditions, the 110 kDa protein was chased to a 100 kDa species. The presence of the 100 kDa species under non-reducing conditions further confirms that the 58 kDa mature form of ILTV gB is composed of two proteolytically cleaved subunits which form a disulphide-linked heterodimer.

**Oligomer formation of glycoprotein B**

Most viral envelope glycoproteins that mediate virus entry are assembled into oligomers (Doms et al., 1993; White, 1990). Given that the herpesvirus gB proteins studied to date form oligomers (Niikura et al., 1992; Sarmiento & Spear, 1979; Whealy et al., 1990) we reasoned that ILTV gB would also form an oligomeric structure. In order to examine the kinetics of oligomer formation, a pulse-chase experiment was performed. At various times after a 10 min pulse with $[^35]S$methionine, chase samples were solubilized in 1% Triton X-100 and layered on an 8–20% sucrose gradient as described in Methods. Gel filtration standard markers were run in parallel gradients. The gradients were fractionated, and samples were...
immunoprecipitated with rabbit anti-gB antiserum. Until this point, no reducing agents were used during the procedure. Immunoprecipitated proteins were boiled under reducing conditions and examined by electrophoresis on 10% polyacrylamide gels as described in Methods.

The results of this experiment are shown in Fig. 4. Immediately following the 10 min pulse, only the 110 kDa immature form of gB was detected. The majority of the labelled protein sedimented as a broad peak approaching the predicted size of the homodimer (lanes 8–12) and not as a monomer (lanes 5 and 6). This indicated that dimerization occurred rapidly after synthesis within the ER. After a 45 min chase the majority of labelled protein sedimented as dimers (fractions 11–13) and the 58 kDa processed form of gB started to appear. By 90 min the majority of the labelled protein sedimented as mature (58 kDa) dimers. The fully processed 58 kDa form of gB was only observed in association with the dimeric form of gB, indicating that dimerization occurs prior to cleavage. The presence of higher order oligomers was not observed.

Discussion

This report defines the assembly and processing pathway of the gB glycoprotein of ILTV, the first such characterization of a glycoprotein from this avian alphaherpesvirus. The ILTV gB molecule is initially synthesized within the ER as a 110 kDa monomeric precursor protein which rapidly assembles into homodimers. This immature dimeric form of gB is then transported to the Golgi where it acquires further modifications, including the addition of fucose and the conversion of at least some of the high-mannose form oligosaccharide side-chains to complex carbohydrates. These modified gB dimers undergo a rapid and efficient proteolytic cleavage event to form two disulphide-linked species, each with an apparent molecular mass of 58 kDa. The mature forms of gB from herpes simplex virus type 1 (HSV-1), MDV and pseudorabies virus (PRV) also contain both high-mannose and complex glycans (Wensky et al., 1982; Whealy et al., 1990; Yoshida et al., 1994). The presence of high-mannose oligosaccharides on mature proteins may result from the glycans being sequestered within the immature dimer during the initial folding of the nascent protein, rendering them inaccessible to modification by Golgi enzymes.

Many herpesvirus gB homologues are cleaved by a type I cellular endoprotease which recognizes the motif R X K/R R (Breshnahan et al., 1993; Gainer, 1993). A potential cleavage sequence (R E R R) is located near the centre of the ILTV gB protein. Cleavage of gB at this site would produce two subunits, each with a predicted molecular mass (unmodified) of about 47 kDa. Endo-H treatment of immunoprecipitated gB and electrophoresis under non-reducing conditions both confirm that the 58 kDa form represents two protein subunits of equal mass, which result from cleavage of a 110 kDa precursor. Similar processing pathways have been determined for the gB homologues of PRV, human (HCMV) and murine cytomegalovirus (MCMV), VZV and MDV (Loh, 1991; Montalvo & Grose, 1987; Niikura et al., 1992; Whealy et al., 1990).

A comparison of gB processing models revealed characteristics unique to the avian herpesviruses. While the mature form of gB in mammalian herpesviruses exhibits an increase in mass following Golgi maturation, processing of glycans associated with the avian gB homologues (MDV and ILTV) results in an apparent reduction of mass from 110 kDa to 100 kDa. Comparing the processing of MDV gB and ILTV gB to the processing of mammalian herpesvirus gB proteins may point to differences in glycan processing in mammalian and avian cells. A comparison of the monosaccharide composition of the immature and mature forms of both mammalian and avian gB molecules may also reveal differences in their carbohydrate modifications.

Analysis of gB oligomerization kinetics has determined that dimeric molecules form shortly after synthesis for both the HSV-1 and PRV homologues (Claesson-Welsh & Spear, 1986; Highlander et al., 1991; Pereira, 1994; Whealy et al., 1990). We observed similar dimerization kinetics for ILTV gB. HSV-1 gB has been shown to oligomerize through the interactions of a 28 amino acid domain (Laquerre et al., 1996). The ILTV gB amino acid sequence contains a region with 75% similarity to this sequence. Included in this region of homology is a conserved cysteine residue, which contributes to HSV-1 gB oligomer formation and stability, and a core charged sequence (HRRY) which forms a β-sheet structure. It is interesting to note that a short sequence of 14 amino acids (SSSPESQFSANSTE), unique to ILTV gB, is located 18 amino acids upstream of the region corresponding to the potential dimerization domain. Future studies involving the analysis of deletion mutations within this region are required to determine the importance, if any, of this short serine-rich sequence on the formation of dimers.

A previous study has reported different molecular masses for the components of the ILTV gB complex. York et al. (1987) reported that a class of monoclonal antibodies raised against partially purified ILTV virions reacted with a complex of glycoproteins with molecular masses of 205, 115 and 90 kDa. This complex was later reported to represent the ILTV gB protein family, although their relationship was not examined (Kongsuwon et al., 1993). The 115 kDa and 90 kDa proteins may correspond to the 110 kDa and 100 kDa species we observed, and the 205 kDa protein may represent the dimeric form of gB. It is possible we did not detect a 205 kDa dimeric form of gB and that York et al. did not observe the 58 kDa species due to the more stringent reducing conditions of our preparations. Alternatively, our normal rabbit serum may have recognized a 200 kDa cellular protein (Fig. 2A, lane 1), which may have obscured the 205 kDa protein species in our analysis.

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