Expression of Recombinant Small Hydrophobic Protein for Serospecific Detection of Avian Pneumovirus Subgroup C

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The small hydrophobic (SH) gene of the avian pneumovirus (APV) Colorado isolate (CO), which belongs to subgroup C (APV/C), was expressed with a baculovirus vector. The recombinant SH protein was evaluated as a potential subgroup-specific diagnostic reagent in order to differentiate infections resulting from APV/C from those induced by APV/A, APV/B, and human metapneumovirus (hMPV). When the recombinant baculovirus was used to infect insect cells, a 31- to 38-kDa glycosylated form of the SH protein was produced and subsequently tested for reactivity with antibodies specific for APV/A, APV/B, APV/C, and hMPV. Western blot analysis showed that the expressed recombinant SH protein could only be recognized by APV/C-specific antibodies. This result was consistent with sequence analysis of the APV/C SH protein, which had very low (24%) amino acid identity with the corresponding protein of hMPV and no discernible identity with the SH protein of APV/A or APV/B. A recombinant SH protein-based enzyme-linked immunosorbent assay (ELISA) was developed, and it further confirmed the lack of reactivity of this protein with antisera raised to APV/A, APV/B, and hMPV and supported its designation as a subgroup-specific antigen. This finding indicated that the recombinant SH protein was a suitable antigen for ELISA-based detection of subgroup-specific antibodies in turkeys and could be used for serologically based differential diagnosis of APV and hMPV infections.

Avian pneumovirus (APV) causes turkey rhinotracheitis, an acute upper respiratory tract infection of turkeys, and is associated with swollen syndrome in chickens, which is usually accompanied by secondary bacterial infections that increase mortality. It was first reported in the late 1970s in South Africa, and viruses were subsequently isolated in Europe, Israel, and Asia (4, 7, 16). APV is a member of the Paramyxoviridae family, subfamily Pneumovirinae, and has been proposed as the type species for the newly defined genus Metapneumovirus (22), which was classified into two subgroups, designated APV/A and APV/B (17). In 1997, the first U.S. APV isolate (APV/C) was obtained from commercial turkeys in Colorado after an outbreak of turkey rhinotracheitis and proposed as the prototype of a new subgroup, designated APV/C (22). Several reports showed that the APV/C isolate was genetically and antigenically different from virus isolates belonging to European subgroups APV/A and APV/B (27, 31). In general, APV infection can be diagnosed by serology, reverse transcription (RT)-PCR, and virus isolation assays (10, 29). Although virus isolation can be performed with tracheal organ cultures, chicken embryo fibroblasts, or Vero cells (10), it is time-consuming and often unsuccessful. APV RNA can be detected by RT-PCR for only a short period (2 to 10 days postinfection) in tracheal and cloacal swabs (7, 29). Antibodies to APV are detectable for many weeks by enzyme-linked immunosorbent assay (ELISA), which is more rapid and economical than virus isolation or RT-PCR as an indicator of infection (5, 11). How-ever, discrepancies in the results of an ELISA have been reported when the coating antigen consisted of crude cell lysates produced by infection with one virus type (9). This problem was highlighted during the first 10 months of the recent APV outbreak in the United States when it was not possible to detect virus activity by serological methods, owing to the lack of cross-reactivity of antibodies specific for the newly emerged APV/C isolate with antigen derived from European APV isolates (12).

APV is a negative-sense, nonsegmented single-stranded RNA virus that contains eight genes, namely, nucleocapsid (N), phosphoprotein (P), matrix (M), fusion (F), second matrix (M2), small hydrophobic (SH), attachment protein (G), and RNA-dependent RNA polymerase (L) in the order 3′-N-P-M2-SH-G-L-5′ (Fig. 1). Antigenic diversity of APV/A and APV/B has been reported (3), and these variations are mainly in the three envelope glycoproteins, SH, G, and F. The APV/C SH gene is 525 nucleotides in length and encodes a polypeptide of 175 amino acids including four potential glycosylation sites. The recombinant APV/C SH protein was produced in baculovirus-infected insect cells in order to evaluate it as a potential subtype-specific diagnostic reagent and to have a better understanding of its antigenic and genetic relationship to the SH protein of APV/A, APV/B, and human metapneumovirus (hMPV). The results reported in this paper demonstrate the potential utility of the recombinant SH protein as a serological assay reagent for differentiating APV/C infections from those induced by APV/A, APV/B, and hMPV.

MATERIALS AND METHODS

Construction of recombinant plasmid. APV/C (lot number 193ADV9902; Animal and Plant Health Inspection Service, National Veterinary Service Laboratories, Ames, Iowa) was propagated in QT-35 cells (25), and virion-associated RNA was extracted from infected cells with the RNasy Mini Kit (QIAGEN, Toronto, Ontario, Canada) in accordance with the manufacturer’s instructions.
The SH protein gene was amplified by RT-PCR with primers APV-SHf (5'-GT AATGGAAGGCTGAAATGCCTG-3') and APV-SHr (5'-H11032/AATGGAGCCCCTGAAAGTCTCTG-3'-CCAAAAAAAC), which were based on the published sequence of APV/C SH and G genes (GenBank accession number AF513020). The RT-PCR amplicon was cloned into a pCR2.1 vector (Invitrogen, Burlington, Ontario, Canada), and then the full-length SH and G genes were subcloned into the baculovirus transfer vector pBlueBac4.5 (Invitrogen). Figure 1 shows the construction of the plasmid DNA used in this study and indicates the inserted foreign gene. Nucleotide sequence analysis was conducted with an ABI 377 sequencer with a fluorescent dye terminator kit (Applied Biosystems, Streetsbridge, Ontario, Canada). Hyperimmune sera specific for APV/A, A. californica multiple nucleopolyhedrosis virus.

FIG. 1. Construction of recombinant plasmid pBlueBac4.5-APV/CO-SH. A 1,308-bp segment containing the combined SH and G genes of APV/C was amplified and cloned into baculovirus transfer vector pBluBac4.5 under the control of the polyhedrin promoter. The TAG stop codon for the SH gene is indicated by a bar with an asterisk at nucleotide positions 526 to 528. The primer set used for amplification is indicated by arrows.

ELISA antigen preparation. Subconfluent SF21 cell monolayers were infected with the recombinant baculovirus Ac-Bac-APV/CO-SH at a multiplicity of infection between 3 and 5. Infected cells were harvested at 3 days postinfection, when a maximal cytopathic effect was observed. The infected cells were lysed in insect cell lysis buffer (containing 1% Triton X-100, 130 mM NaCl, 10 mM Tris [pH 7.5], 10 mM NaF, 10 mM Na₂PO₄, and 10 mM Na₂PP₃), and the lysates were clarified by centrifugation at 2,700 × g for 10 min. The clarified cell lysate was used as the coating antigen for an ELISA. Various dilutions of the antigen preparation were tested for reactivity with anti-APV/C serum as described below in order to determine the optimum concentration of the antigen to be used in the SH-based ELISA. A recombinant protein (VP7) from bluetongue virus (BTV) was prepared in a manner similar to that described for the SH protein and used as a control antigen to monitor nonspecific assay reactivity.

ELISA. Each well of a 96-well plate (Nunc Immunoplate; Nunc, Mississauga, Ontario, Canada) was incubated overnight at 4°C with 200 ng of recombinant Ac-Bac-APV-SH- or Ac-Bac-BTV-VP7-infected cell lysate. The plate was then washed to remove nonadherent material, and nonspecific sites were blocked with buffer containing 0.2% Tween 20. Sera were diluted from 1:100 to 1:1,600 in the blocking buffer, and 100 μl of each dilution was applied to the wells. After incubation for 1 h at 37°C, the plate was extensively washed, horseradish peroxidase-labeled anti-turkey immunoglobulin G (Mandel, Guelph, Ontario, Canada) was added to each well, and the plate was incubated for 1 h. After a final washing step, the reactions were visualized by addition of a substrate solution (FAST o-phenylenediamine dihydrochloride; Sigma, St. Louis, Mo., Color development proceeded for 30 min, after which time it was stopped by the addition of 3 M HCl. A₄₅₀ was measured with a multichannel spectrophotometer (SPECTRA Max Plus; Molecular Devices, Sunnyvale, Calif.).

RESULTS

Construction of recombinant APV/C SH protein and sequence analysis. Because of the availability of only one combined APV/C SH and G gene sequence containing 1,308 nu-
cleotides (GenBank accession number AF513020), a primer set specific for the 5′/H11032 SH gene and the 3′/H11032 G gene was designed in order to produce a full-length SH gene by RT-PCR amplification. As shown in Fig. 1, the amplified segment containing the combined SH and G genes of APV/C was inserted into baculovirus transfer vector pBlueBac4.5. Recombinant baculovirus was then successfully obtained following the co-transfection of SF21 cells with the respective recombinant plasmid transfer construct and wild-type AcNPV DNA. Sequencing results showed that a stop codon (TGA) of the SH gene was located at nucleotide positions 526 to 528 of the combined SH and G genes, which was consistent with the report of Toquin et al. (32). As a result, the open reading frame of the SH gene of APV/C was 525 bases long, encoding a predicted protein of 175 amino acids (Fig. 1). It exhibited 80 and 100% amino acid identity with the recently published sequences of two APV/C SH proteins (32, 33). The deduced SH protein had a predicted molecular mass of 19.5 kDa and exhibited a high serine and threonine content (22.3%), which included four N-glycosylation sites (Fig. 2).

FIG. 2. Alignment of the predicted amino acid sequence of the SH protein of the APV/C Colorado isolate (accession number CAD30203) with those of APV/A strain CVL 14/1 (accession number AAB22546), APV/B strain UK/11/94 (accession number CAD42709), and hMPV strain CAN98-75 (accession number AAQ67689). The amino acid sequences of the proteins were published in the National Center for Biotechnology Information GenBank database. Amino acid residues that are strictly conserved in the consensus sequence are shaded in red (four identical sequences), bright green (three identical sequences), or olive or blue (two identical sequences each). A dot indicates the absence of an amino acid relative to the majority of sequences. The putative glycosylation sites in the APV/C sequence are boxed. Sequences were aligned with CLUSTAL W (30).

Antigenic characterization of recombinant APV/C SH protein. In order to further characterize the SH protein, it was examined by Western blotting. As shown in Fig. 3A, a broad protein band migrating at about 31 to 38 kDa was specifically recognized by anti-APV/C serum, thereby confirming its antigenicity. By contrast, Western blot reactivity was not observed with polyclonal antibodies specific for APV/A (Fig. 3C), APV/B (Fig. 3B), or hMPV (Fig. 3D) or with sera obtained from naive, uninfected turkeys (data not shown).

Evaluation of SH-specific ELISA. The baculovirus-expressed SH protein was evaluated for suitability as a coating antigen for specific detection of APV/C antibodies in a direct ELISA. To accomplish this, sera prepared against APV/A (CVL 14/1), APV/B (Hungary 657/4), and APV/C (Colorado) were titrated in duplicate over a twofold series dilution range and the results were expressed as the mean A₄₅₀. Clarified infected cell lysates containing recombinant BTV-VP7 were also used as a coating antigen, and as shown in Fig. 4, the mean (± the standard deviation) background level of serum reactivity (A₄₅₀ of 0.074 ± 0.023) was determined. Antiserum was considered positive if the A₄₅₀ was ≥0.1. The APV/C SH protein was shown to specifically react with only APV/C-specific antibodies, resulting in A₄₅₀ values of ≥0.3.

To further confirm the group-specific immune reactivity of the recombinant APV/C SH protein, 34 field serum samples obtained from turkeys and chickens in Canada and received at the diagnostic laboratory were tested. Of these, 4 were positive and 30 were negative for the presence of APV/A-specific antibodies by an ELISA described by Heckert et al. (14). None of the field serum samples demonstrated ELISA reactivity with the recombinant APV/C SH protein.

DISCUSSION

This study demonstrated that the baculovirus-expressed APV/C SH protein may be used as an antigen in an ELISA or a Western blot assay for the exclusive detection of APV/C antibodies in turkeys. The SH protein was chosen as a likely candidate for a subgroup-specific serodiagnostic reagent because it demonstrated extensive amino acid sequence variation among the three APV antigenic subgroups and hMPV. Although the APV/C G protein has also been shown to be quite variable, demonstrating only 21 and 19% amino acid identity with the corresponding proteins of APV/A and APV/B, respec-
tively, and 20.6% amino acid identity with the hMPV G protein (32), cross-neutralization between these viruses (6, 27; unpublished observations) suggested that their G proteins may share immunodominant epitopes. Recently the *Escherichia coli*-produced recombinant matrix (M) protein of APV/C was used as a diagnostic reagent for detection of APV/C antibodies in turkey serum (12). However, it was not suitable for specific detection of APV/C antibodies because the M protein of APV/C shares 78 and 77% amino acid identity with APV/A and APV/B, respectively (24, 28). Interestingly, although the N protein of APV/C, like the M protein, shares amino acid identity with the corresponding proteins of APV/A and APV/B (8, 19), Gulati et al. (13) reported that the *E. coli*-produced recombinant N protein, when used in an indirect ELISA, was able to specifically detect APV/C antibodies in turkey sera whereas antisera to APV/A and APV/B failed to react in this assay at any dilution. This result was somewhat surprising since previous studies have shown that viral proteins with highly conserved sequences, such as the nucleocapsid, have not been suitable for antigenic variant detection of viruses such as vesicular stomatitis virus and measles virus (15, 18). In this regard, we examined the antigenicity of baculovirus-produced hMPV N protein by Western blot analysis (unpublished data). The preliminary results showed that the expressed recombinant N protein not only reacted strongly with anti-hMPV antibodies but also exhibited cross-reactivity with anti-APV/C antiserum. Consistent with this observation, the ELISA performed with recombinant hMPV N protein also showed a positive reaction with APV/C-specific antibodies (data not shown). These results provided additional evidence of a close antigenic relationship between APV/C and hMPV and confirmed the previously reported close genetic relationship between these viruses (1, 21, 33).

The baculovirus expression system has been used extensively to express large quantities of proteins that are antigenically similar to their native counterparts and can be used in standardized assays to provide consistent results in different laboratories. Insect cells, which also serve as the virus substrate, offer appropriate posttranslational modification of expressed proteins, demonstrate the ability for membrane protein secretion, and do not contain excessive lipopolysaccharide, which often contaminates proteins prepared from an *E. coli* expression system. The broad gel migration pattern of the baculovirus-expressed APV/C SH protein was consistent with the prediction of four potential N-linked glycosylation sites and likely reflects different usage of these sites during posttranslational modification in insect cells. Unlike some baculovirus-expressed proteins, typically viral nucleocapsids, the recombinant APV/C SH protein was found to be very stable and homogeneous in nature, even after several freeze-thaw cycles. These are impor-

**FIG. 3.** Western blot analysis of baculovirus-expressed APV/C SH protein and cross-reactivity with anti-APV/A and anti-APV/B antibodies. (A) SF21 cells infected with recombinant Ac-Bac-APV/CO-SH and control cells were analyzed by Western blot assay with anti-APV/C antibody. (B, C, and D) Reactivity with antisera specific for APV/B, APV/A, and hMPV, respectively. M, marker protein; SH, recombinant Ac-Bac-APV/CO-SH virus-infected cells; Cell, uninfected SF21 cell control. The locations of molecular size standards are shown on the left. The expressed recombinant is indicated by the arrow.

**FIG. 4.** Comparison of the cross-reactivity of antisera against APV/A, APV/B, and APV/C in a recombinant SH protein-based ELISA. The results shown were obtained with baculovirus-expressed SH protein as the coating antigen. Antisera against APV/A, APV/B, and APV/C were serially diluted in 2.5% milk–phosphate-buffered saline and allowed to react on preblocked plates as described in Materials and Methods. The results shown are representative of identical assays performed in duplicate, and the cutoff is indicated by the arrow on the left.
tant features of an assay reagent since they ensure data reliability and reproducibility. Equally important, the antigenicity and serospecificity of the APV/C SH protein make it potentially suitable for ELISA-based detection of APV/C infections in poultry. Efforts are currently under way to obtain positive avian sera from the United States in order to carry out validation studies to confirm this protein’s usefulness as a diagnostic reagent for standardized testing of poultry. This reagent could also be used to further elucidate the evolutionary relationship between APV/C and hMPV, since it has been suggested that the avian virus may have evolved from human precursor strains of hMPV that infected birds (1). If, in fact, coinfection or cocirculation of hMPV and APV is possible, then having a reagent capable of being used for differential diagnosis would be critical.

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