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Biochemical and Functional Analysis of the Borna Disease Virus G Protein

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The Borna disease virus (BDV) antigenome is comprised of five major open reading frames (ORFs). Products have been reported only for ORFs I, II, and III, encoding N (p40), P (p24/p23), and M (gp18), respectively. ORF IV predicts a 57-kDa protein with several potential glycosylation sites. Analysis of radiolabeled extracts from BDV-infected C6 cells and BHK-21 cells transfected with a Semliki Forest virus vector that contains ORF IV demonstrated the presence of a 94-kDa protein (G protein) which was sensitive to tunicamycin, endoglycosidase F/N-glycosidase, and endoglycosidase H but not to *O*-glycosidase. Sera from BDV-infected rats detected the G protein and had neutralization activity that was reduced following immunoadsorption with the G protein. Preincubation of cells with the G protein interfered with BDV infectivity. This effect was enhanced by treatment of the G protein with the exoglycosidase α-mannosidase and reduced after subsequent treatment with *N*-acetyl-β-D-glucosaminidase. In concert these findings indicate that ORF IV encodes a 94-kDa N-linked glycoprotein with extensive high mannose- and/or hybrid-type oligosaccharide modifications. The presence of neutralization epitopes on the G protein and its capacity to interfere with infectivity suggest that the G protein is important for viral entry.

Borna disease virus (BDV) is a neurotropic virus that causes movement and behavioral disturbances in a wide range of warm-blooded hosts (18, 25, 30, 31, 41). Although sporadic epidemics of Borna disease were first reported in horses and sheep in southern Germany, studies over the last decade indicate that both the natural host range and geographic distribution of BDV may be larger than previously appreciated (6, 19, 25, 35). A role for BDV in pathogenesis of human disease is not established; however, accumulated data from several laboratories suggest that it may be associated with neuropsychiatric disturbances, including schizophrenia and affective disorders (4, 5, 17, 26, 37). The expanding geographical prevalence and host range of BDV has heightened the importance of understanding the role of virus-specific proteins in the BDV life cycle.

BDV is an enveloped, nonsegmented, negative-strand RNA virus of the order *Mononegavirales* (2, 8, 40). The viral antigenome is comprised of approximately 8.9 kb and contains five major open reading frames (ORFs) (3, 9, 10, 28). BDV establishes a noncytolytic, persistent infection with limited gene expression. To date, products are reported only for the most abundant proteins encoded by the first three ORFs (15, 24, 27, 34). ORF I encodes the nucleoprotein (N) also known as p40 (21, 24). ORF II encodes the phosphoprotein (P) also known as p23 (34). ORF III encodes a glycosylated matrix-like protein (M) also known as gp18 (15, 27). ORF V is the most 3′ ORF on the viral antigenome and predicts a protein that contains motifs characteristic of viral RNA-dependent RNA polymerases (3, 9). ORF IV predicts a protein of 57 kDa with N-glycosylation sites, O-glycosylation sites, and hydrophobic domains at the amino and carboxyl termini reminiscent of the endoplasmic reticulum signal peptide sequence and transmembrane domains found in rhabdovirus G proteins (11).

Preliminary infectivity studies with purified BDV particles suggested that carbohydrate residues play a role in viral entry into the host cell (32). While previous studies have shown that the BDV M protein is glycosylated and contains neutralizing epitopes (15), a second glycoprotein was proposed to be encoded by ORF IV (G-ORF) (3, 9). We initiated this study to identify and characterize the gene product of the G-ORF and to address the role of the G protein carbohydrates in infectivity. To facilitate the biochemical characterization of the BDV G protein, the G-ORF was expressed in eukaryotic cells by using a Semliki Forest virus (SFV) vector system.

MATERIALS AND METHODS

BDSe. Twenty adult Lewis rats (Charles River) were infected with BDV strain He80-1 (29) by intranasal inoculation. Animals were bled by terminal exsanguination 1 and 4 months after viral inoculation (12). In all of the immunoprecipitation experiments the BD-rat sera (BDSe) were pooled prior to use.

Construction of SFV-G and electroporation of BHK-21 cells. Total RNA isolated from BDV (strain He80-1)-infected C6 cells (C6BDV) was used as template for reverse transcription-PCR amplification of the G-ORF (nucleotides 2229 to 3744) by using primers 5′-CGCAATCAATGCAGC and 5′-TTCCTGC CACCGGCCG. The amplified product was purified from a 1% agarose gel by using the USBioclean DNA extraction system (U.S. Biochemicals) and cloned into the SFV vector pSFV-1 (Life Technologies) prepared with 3′ T overhangs at the *Sma*I restriction site (20). The resulting cDNA clone, pSFV-G or pSFV-LacZ (control provided by manufacturer), was used as template for the in vitro transcription of capped SFV genomic RNA encoding the G-ORF or the β-galactosidase-ORF, respectively. Ten micrograms of each purified RNA (SFV-G or SFV-LacZ) was electroporated into 10^5 BHK-21 cells (SFV-G and SFV-LacZ cells). The transfected cells were plated onto 35-mm-diameter dishes for metabolic labeling or onto glass chamber slides (Nunc) for immunofluorescence assays (IFA). The efficiency of transfection for SFV-G cells was assessed by IFA.

Metabolic labeling of cells. Approximately 2×10^4 SFV-G and SFV-LacZ cells (16 h after electroporation) or BDV-infected and noninfected C6 cells were incubated for 2 h in 1 ml of methionine-minus modified Eagle's medium (Life Technologies). Thereafter, 0.2 mCi of ^{35}[S]Met-Cys-protein labeling mix (New England Nuclear) was added to the cells for 5 h to radiolabel newly synthesized proteins. Following metabolic labeling, cells were washed twice with ice-cold phosphate-buffered saline and lysed in 0.4 ml of lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 0.1% NaN_3, 1 mM phenylmethylsulfonyl fluoride, 25 μg of aprotinin per ml, 1 mg of bovine serum albumin) per ml. The lysates were centrifuged at 15,000 × *g* for 30 min at 4°C, and the supernatants were analyzed for the presence of BDV-specific proteins by immunoprecipitation (IP) with pooled BDSe. For N-linked glycosylation inhibition experiments, the transfected

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cells were pretreated for 2 h with 10 μg of tunicamycin (Boehringer Mannheim) per ml in methionine-minus modified Eagle's medium prior to a 5-h incubation with ^{35}[S]Met-Cys-protein labeling mix.

IP of radiolabeled proteins. Cell lysates were subjected to IP by the method of Yamashita et al. (39) with slight modifications. Briefly, cell lysates were split into two 200-μl aliquots and incubated with 5 μl of normal rat sera (NLSe) for 1 h at 4°C followed by an additional 1 h of incubation at 4°C with 16 mg of protein G-Sepharose (Sigma). The lysates were clarified by centrifugation and incubated with 2 μl of pooled BDSe or NLSe for 1 h at 4°C. Eight milligrams of protein G-Sepharose beads were added for overnight incubation at 4°C. Immunoprecipitated proteins were subjected to SDS-polyacrylamide gel electrophoresis (PAGE; 10% polyacrylamide) and autoradiography (Kodak XTR).

Carbohydrate analysis by glycosidase digestion. Radiolabeled proteins immunoprecipitated with pooled BDSe were eluted from the Sepharose beads by incubation with 60 μl of 50 mM Tris-HCl (pH 6.8)–0.4% SDS–0.1 M 2-mercaptoethanol at 95°C for 10 min and digested with endoglycosidase H (endoH; Boehringer Mannheim), endoglycosidase F and *N*-glycosidase F (endoF/PNGaseF; gift from J. Elder), endoH followed by neuraminidase (Boehringer Mannheim) then *O*-glycosidase (Boehringer Mannheim), or neuraminidase followed by *O*-glycosidase. Methods for carbohydrate digestion followed the protocols of the manufacturer (endoH, neuraminidase, and *O*-glycosidase) or Alexander and Elder (endoF/PNGaseF) (1), using endoH, 2.0 mU; endoF/PNGaseF, 25 mU; *O*-glycosidase, 0.8 mU; and neuraminidase, 1.0 mU in 40-μl reactions. Controls for these reactions included incubation of proteins with digestion buffers alone (16). Radiolabeled G protein was prepared by in vitro transcription/translation of G-ORF in rabbit reticulocytes in the absence of microsomal membranes, using the manufacturer's protocols (Life Technologies) to provide a nonglycosylated G protein standard. Proteins were subjected to SDS-PAGE (10% polyacrylamide) and analyzed by autoradiography.

Identification of neutralization activity in BDSe. Affinity adsorption of BDSe to lysates of SFV-G cells or SFV-LacZ cells was performed to remove antibodies directed against the G protein prior to determination of the neutralization titer. The affinity adsorption and neutralization experiments were performed as previously described (7, 12, 23). Briefly, heat-inactivated sera from three BDV-infected rats (4 months postinfection) were diluted 1:10 in Tris-buffered saline and incubated with membrane-bound lysates of SFV-G or SFV-LacZ (control) cells. The capacity of the sera to neutralize virus infectivity was assessed following adsorption to lysates of SFV-G or SFV-LacZ cells. Virus neutralization assays were performed by incubating serial dilutions of the adsorbed BDSe ($G^{(-)}$ BDSe and $LacZ^{(-)}$BDSe), nonadsorbed BDSe, and NLSe with 50 focus-forming units of BDV for 1 h at 37°C prior to applying the mixture to rabbit fetal glial cells. The number of infected cells was determined 4 days following inoculation. Neutralization titers, defined as the dilution required for 50% reduction in BDV infectivity, are reported as averages, with error bars representing the standard error of the mean.

Partial purification of G protein. Approximately 1.5×10^6 SFV-G or SFV-LacZ cells were grown for 16 h after electroporation and harvested by scraping the cells in ice-cold phosphate-buffered saline. The cells were collected by centrifugation at $500 \times g$ for 5 min at 4°C. The cell pellet was homogenized in 1.5 ml of lysis buffer (10 mM Tris-HCl [pH 7.5], 0.5% SDS, and 1% Nonidet P-40) by sonication on ice for 100 pulses at a duty cycle of 0.5, 50% power (Braun-Sonic U). Following centrifugation of the cell homogenate at $15{,}000 \times g$ for 10 min at 4°C, the supernatant was removed and frozen. After thawing the sample, a precipitant was observed and collected by centrifugation at $18{,}000 \times g$ for 30 min at 4°C. The supernatant, which contained soluble detergent, was removed and the pellet was resuspended in 0.5 ml of Tris-buffered saline (pH 8.0). The total protein concentration of the partially purified fraction from SFV-G cells or the corresponding fraction from SFV-LacZ cells was determined by using the Bio-Rad protein microassay (Bio-Rad), and the relative amount of protein impurities was determined by SDS-PAGE (10%) and silver stain analysis.

BDV infectivity interference assays. The partially purified protein fractions from SFV-G and SFV-LacZ cells were assessed for the ability to block BDV infectivity. The protein fractions were serially diluted in ice-cold Dulbecco's modified Eagle's medium (DMEM) and applied to 10^4 rabbit fetal glial cells on ice for 15 min prior to the addition of 50 focus-forming units of BDV stock in DMEM for a final volume of 50 μl. After incubation for 1.5 h at 37°C in 5% CO_2, the medium was replaced with fresh DMEM and cells were incubated for 4 days and then assayed for infectious virus as previously described (23). The assay was performed with virus in the absence of exogenous protein to determine maximal infectivity (defined as 100%). In addition, the assay was performed in the absence of virus to serve as a negative control.

To investigate the effects of specific carbohydrates on infectivity interference, partially purified G protein was treated with either α-mannosidase (0.5 mU/μl; Boehringer Mannheim), *N*-acetyl-β-D-glucosaminidase from *Diplococcus pneumoniae* (GlcNAcase; 1.0 mU/μl; Boehringer Mannheim), or α-mannosidase followed by GlcNAcase and assayed for the potential to inhibit BDV infectivity. Twenty-five microliters of purified protein fractions from SFV-G or SFV-LacZ cells was treated with the exoglycosidases in citrate buffer (25 mM sodium citrate, 25 mM citric acid, pH 5.5) or with buffer alone (mock) in a final volume of 50 μl at 30°C for 18 h. For the sequential exoglycosidase treatment, the protein fractions were digested at 30°C with α-mannosidase (0.5 mU/μl) for 9 h and then with GlcNAcase (1.0 mU/μl) for an additional 9 h at 30°C. Twenty-five microliters of mock- and exoglycosidase-treated protein fractions was serially diluted and analyzed for the ability to interfere with BDV infectivity as described above.

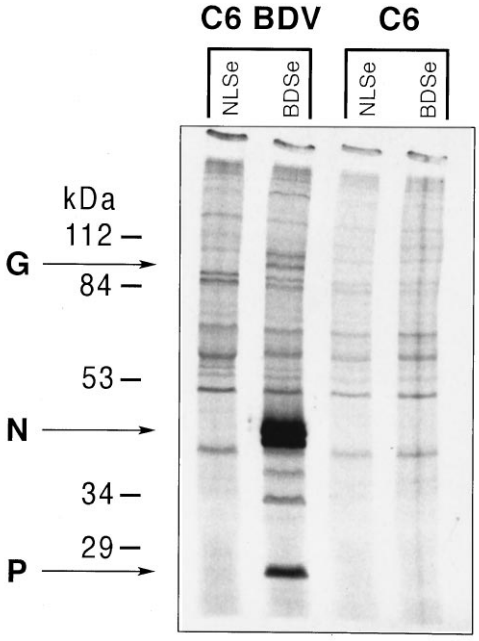

FIG. 1. Identification of BDV G protein in infected C6 cells. Radiolabeled proteins from BDV-infected (C6BDV) and noninfected (C6) cultured cell lysates were immunoprecipitated by using NLSe or pooled BDSe. Several BDV-specific proteins, including G, N, and P, were immunoprecipitated from C6BDV by BDSe but not by NLSe.

RESULTS

Identification of a BDV-specific 94-kDa protein in BDV-infected C6 cells. Lysates of metabolically labeled C6BDV and noninfected C6 cells were used for IP experiments with pooled BDSe or NLSe. After SDS-PAGE and autoradiography, at least five BDV-specific proteins were detected in lysates from C6BDV cells by BDSe. These included proteins of 94, 40, 36, 33, and 23 kDa (Fig. 1). None of these proteins was detected in lysates of C6BDV cells by using NLSe or in lysates of noninfected C6 cells by using BDSe. The 40- and 23-kDa proteins represent the BDV N and P proteins, respectively. Although the remaining BDV-specific proteins were of unknown origin, the apparent molecular weight of the 94-kDa protein was consistent with a posttranslationally modified product of the G-ORF. To facilitate biochemical characterization, the G-ORF was expressed in BHK-21 cells by using an SFV expression system.

Expression of a 94-kDa protein from the BDV G-ORF by using an SFV expression vector. Capped RNAs encoding either the entire G or β-galactosidase (control) ORF were transcribed using pSFV-G or pSFV-LacZ as template, respectively. After RNA transfection into BHK-21 cells, the expressed protein was detected by IFA. These results revealed that approximately 80% of transfected cells could be labeled with sera from BDSe and none was immunoreactive with NLSe (data not shown). Lysates of metabolically labeled SFV-G and SFV-LacZ cells were used for IP experiments with pooled BDSe. After SDS-PAGE and autoradiography, a 94-kDa protein was detected in lysates of SFV-G cells but not in lysates of SFV-LacZ cells (Fig. 2). The 94-kDa SFV-G cell-specific protein comigrated with the 94-kDa BDV-specific protein identified in C6BDV cells (Fig. 2).

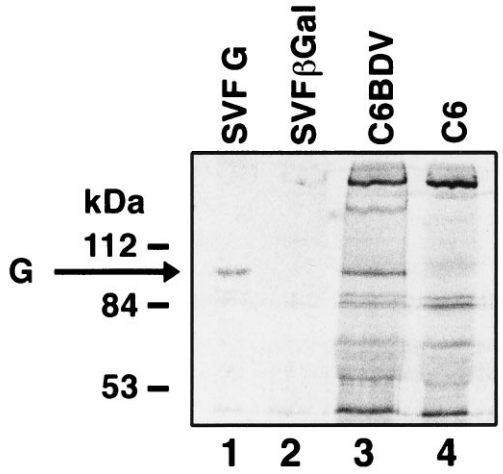

FIG. 2. G protein expressed in SFV-G cells comigrates with a 94-kDa BDV-specific protein. Pooled BDSe precipitated a radiolabeled 94-kDa protein from SFV-G (lane 1) but not SFV-LacZ (lane 2) cells. The 94-kDa protein comigrated with a 94-kDa protein precipitated from infected (lane 3) but not from noninfected (lane 4) C6 cells.

Characterization of the BDV G protein. (i) Tunicamycin analysis. To identify N-linked carbohydrate modifications of the expressed protein, metabolically labeled SFV-G and SFV-LacZ cells were treated with 10 μg of tunicamycin per ml to inhibit N-linked glycosylation. Cell lysates from tunicamycin-treated and untreated cells were used in IP experiments with BDSe or NLSe prior to SDS-PAGE and autoradiography. BDSe immunoprecipitated proteins of 94 and 64 kDa from SFV-G cell lysates. The 94-kDa protein was detected in untreated cells (Fig. 3, lane 3) but not in tunicamycin-treated SFV-G cells. Conversely, the 64-kDa protein was detected in treated cells (Fig. 3, lane 1) but not in untreated SFV-G cells. Neither protein was detected in SFV-LacZ cells (Fig. 3, lanes 5 to 8).

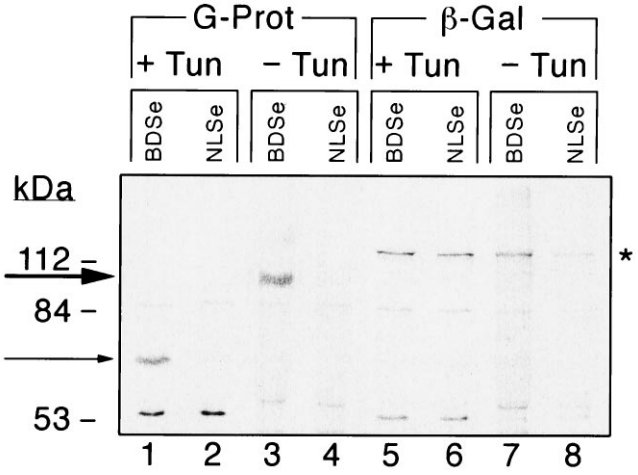

FIG. 3. Tunicamycin treatment of SFV-G cells alters the apparent molecular weight of the G protein. Cells transfected with SFV-G or SFV-LacZ were radiolabeled in the presence (+ Tun) or absence (− Tun) of tunicamycin. Lysates were subjected to IP with either NLSe or pooled BDSe and then analyzed by SDS-PAGE and autoradiography. Lanes: 1 to 4, IP of lysates from SFV-G cells: 5 to 8, IP of lysates from SFV-LacZ cells. Large arrow indicates G protein in lane 3 (approximately 94 kDa). Small arrow indicates G protein in lane 1 (approximately 64 kDa). Asterisk indicates β-galactosidase protein precipitated nonspecifically (lanes 5 to 8).

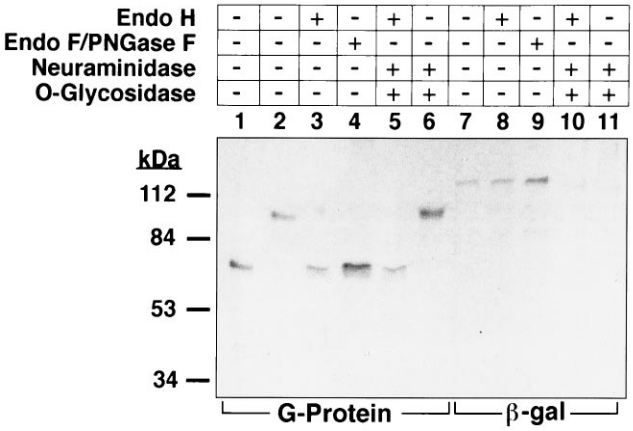

FIG. 4. Glycosidase sensitivity profile of G protein expressed in SFV-G cells. Lanes: 1, radiolabeled G protein prepared by in vitro translation in rabbit reticulocyte lysates in the absence of microsomal membranes (nonglycosylated G protein standard); 2, G protein incubated with digestion buffer alone (glycosylated G protein standard); 3, G protein incubated with endoH; 4, G protein incubated with endoF/PGNaseF; 5, G protein incubated with endoH, neuraminidase, and *O*-glycosidase; 6, G protein incubated with neuraminidase and *O*-glycosidase; 7, β-galactosidase protein incubated with digestion buffer alone; 8, β-galactosidase protein incubated with endoH; 9, β-galactosidase protein incubated with endoF/PGNaseF; 10, β-galactosidase protein incubated with endoH, neuraminidase, and *O*-glycosidase; 11, β-galactosidase protein incubated with neuraminidase and *O*-glycosidase.

(ii) Glycosidase analysis. Radiolabeled protein immunoprecipitated by using pooled BDSe and protein-G-Sepharose beads was eluted and treated with endoH, endoF/PNGaseF, endoH/neuraminidase/*O*-glycosidase, or neuraminidase/*O*-glycosidase prior to SDS-PAGE and autoradiography. Treatment with endoH (Fig. 4, lane 3) or endoF/PNGaseF (Fig. 4, lane 4) resulted in an apparent shift in molecular weight from 94 to approximately 64 kDa, consistent with the apparent molecular weight of nonglycosylated G protein (Fig. 4, lane 1). Treatment with neuraminidase/*O*-glycosidase did not allow resolution of a shift (compare lanes 2 and 6). Therefore, to enhance the resolution of SDS-PAGE for identifying small shifts in molecular weight, protein was incubated with endoH prior to *O*-glycosidase treatment. Comparison of protein digested with endoH alone (Fig. 4, lane 3) and protein digested with endoH/neuraminidase/*O*-glycosidase (Fig. 4, lane 5) revealed no discernible shift in molecular weight. Furthermore, when samples treated with endoH alone or endoH/neuraminidase/*O*-glycosidase were combined prior to SDS-PAGE analysis, the treated protein migrated as a single 64-kDa band (data not shown), suggesting insensitivity to neuraminidase/*O*-glycosidase digestion.

G protein contains neutralization epitopes. Depletion of G protein antibodies by adsorption to a partially purified protein fraction from SFV-G cells decreased the mean neutralization titer of BDSe from 1:1,950 (prior to adsorption) to 1:480 (following adsorption) (Fig. 5). In contrast, adsorption to the corresponding protein fraction from SFV-LacZ cells (control) did not reduce the neutralization titer of BDSe.

Interference with BDV infectivity by G protein. To investigate the function of the BDV G protein, infectivity interference assays were performed by applying partially purified G protein fractions to rabbit fetal glial cells on ice prior to the addition of BDV. Lysates from SFV-LacZ cells were subjected to purification procedures identical to those of the SFV-G cell lysates and used as controls in infectivity interference assays. Results of SDS-PAGE and silver stain analysis of both protein

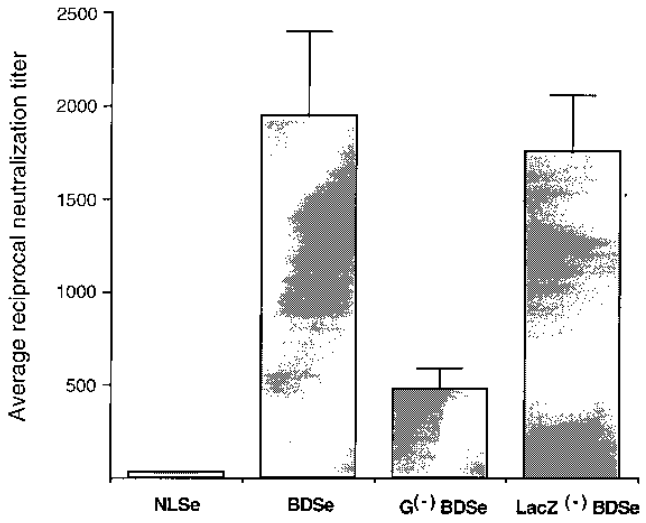

FIG. 5. Neutralizing anti-G protein antibodies in BDSe. Neutralization titers of NLSe and BDSe 4 months postinfection were determined prior to and following adsorption with lysates of SFV-G ($G^{(-)}$ BDSe) and SFV-LacZ ($LacZ^{(-)}$ BDSe) cells. Graph indicates average neutralization titer of three rat serum samples, with error bars representing standard error of the mean.

fractions revealed the same relative amounts of protein impurities. In addition, the G protein was estimated to represent approximately 15% of the total protein in the partially purified G protein fraction. The G protein fraction interfered with BDV infectivity, whereas the corresponding protein fraction of SFV-LacZ cells did not (Fig. 6).

The protein fractions from SFV-G cells and SFV-LacZ cells were treated with exoglycosidases to determine the effects of removal of specific carbohydrate moities on the ability of exogenous G to interfere with infectivity. Treatment of the G protein fraction from SFV-G cells with α-mannosidase, which removes terminal mannose residues, enhanced the interference activity of the G protein fraction relative to the mock (digestion buffer alone)-treated G protein fraction (Fig. 7A). Treatment of the corresponding fraction from SFV-LacZ cells with α-mannosidase had no effect on viral infectivity relative to mock-treated SFV-LacZ fractions (Fig. 7B). The enhancement of interference activity of the G protein fraction by α-mannosidase was reduced after treatment with GlcNAcase (Fig. 7A). Treatment of the G protein fraction with GlcNAcase alone, which removes only terminal GlcNAc residues, had no effect on interference activity (data not shown). In addition, D-mannose or monosaccharide GlcNAc did not interfere with infectivity at concentrations up to 0.1 M (data not shown). These results suggest that the GlcNAc residues important for G protein interference activity are at core positions and contribute to interference activity only when conjugated to protein.

DISCUSSION

This study was initiated to identify and characterize the product of BDV ORF IV. Since the levels of G protein expression are low in BDV-infected rat brain and cultured cells, the ORF was expressed using an SFV system to ensure high fidelity of posttranslational modifications. This expression system facilitated the identification and characterization of a previously undescribed protein, the BDV G protein. By using recombinant protein we detected anti-G protein antibodies in BDV-infected rats and determined that the G protein contained neutralization epitopes. These findings together with the observation that protein from SFV-G cells interfered with BDV infectivity suggest that the G protein participates in viral attachment and/or penetration.

Computer algorithm prediction analysis of the BDV G-ORF suggested that the protein is a type I membrane protein with a molecular weight of 57 kDa. Expression of this ORF in SFV-G cells yielded a 94-kDa protein that comigrated with a 94-kDa BDV-specific protein found in C6BDV cells. The increase in apparent molecular weight of the protein expressed in eukaryotic cells (94 kDa) relative to the molecular weight of the protein translated in rabbit reticulocyte lysates (64 kDa) is consistent with posttranslational modification predictions which included 13 potential N-glycosylation sites. Inhibition of carbohydrate conjugation by tunicamycin and sensitivity to endoF/PNGaseF indicated a primary role for N-linked carbohydrates. Sensitivity to endoH suggested that the N-linked carbohydrate is largely composed of high mannose- and/or hybrid-type oligosaccharides (33). The absence of a shift in apparent molecular weight after digestion with neuraminidase and *O*-glycosidase and the inability to bind the lectin *Helix pomatia* (data not shown) suggest that the protein does not contain O-linked carbohydrates (13, 14).

Glycoproteins from viruses of the order *Mononegavirales* play important roles in early events in the virus life cycle such as viral attachment and penetration (22, 36). These glycoproteins tend to be immunoreactive and are often targets for neutralizing antibodies (38). The BDV M protein is a glycoprotein which contains epitopes that bind neutralizing antibodies (12, 15). Because adsorption experiments using purified M protein did not completely abrogate neutralization activity in chronic BDSe, it was proposed that additional neutralization epitopes might be present on the BDV G protein (12). Consistent with this proposal, the levels of antibodies to G protein in infected rats increased dramatically from 1 to 4 months postinfection (data not shown), concurrent with the appearance of neutralizing antibodies (12). In addition, the neutralization titer in BDSe was reduced 75% after adsorption with

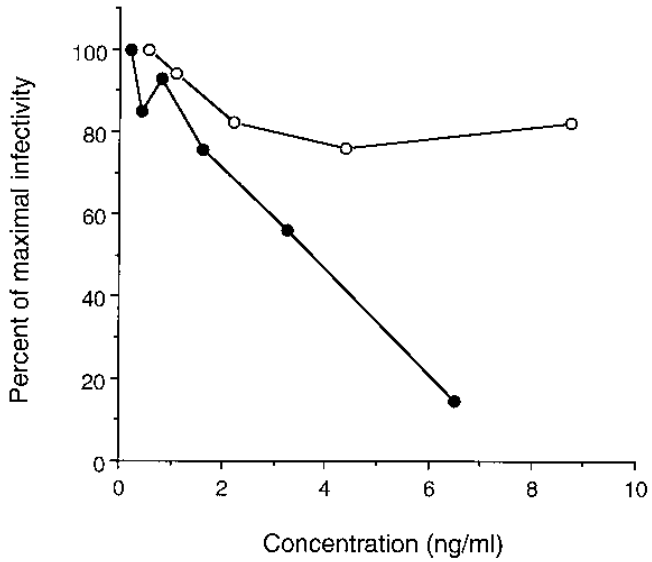

FIG. 6. Inhibition of BDV infectivity by G protein. Cells were incubated with serial dilutions of partially purified fractions of G protein from SFV-G cells (G) or corresponding fractions from SFV-LacZ cells (LacZ) prior to the addition of virus. Incubation with various concentrations of G (●) but not LacZ (○) inhibited infectivity. Data are expressed as a percentage of maximal infectivity determined in the absence of exogenous protein.

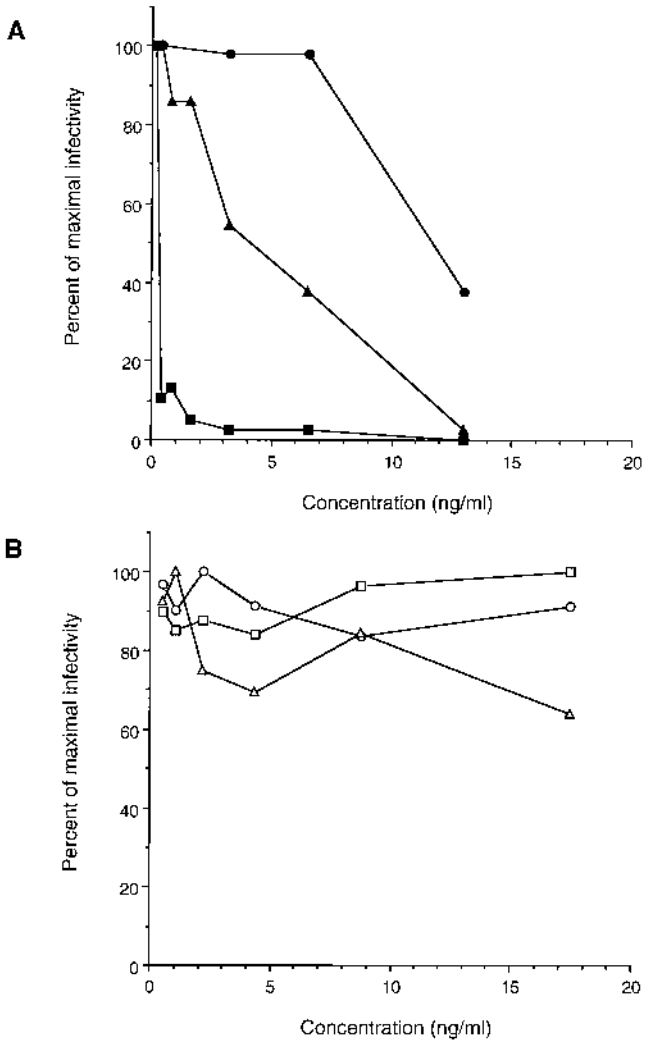

FIG. 7. Mannose and GlcNAcase residues of the BDV G protein play a role in its ability to interfere with infectivity. Partially purified fractions of G protein from SFV-G cells (G) or corresponding fractions from SFV-LacZ cells (LacZ) were treated with either α-mannosidase alone or α-mannosidase followed by GlcNAcase prior to being assayed for the potential to inhibit BDV infectivity. Effects on infectivity with various concentrations of G or LacZ are indicated as a percentage of maximal infectivity determined in the absence of exogenous protein. (A) Treatment of G with α-mannosidase alone (■) enhanced the ability of G to interfere with infectivity relative to mock treatment of G with buffer alone (●). Treatment of G with α-mannosidase followed by GlcNAcase (▲) reduced the enhancement of infectivity inhibition observed following treatment of G with α-mannosidase alone (■). (B) Treatment of LacZ with α-mannosidase alone (□), α-mannosidase followed by GlcNAcase (△), or mock treatment with digestion buffer alone (○) had a minimal effect on BDV infectivity.

partially purified G protein, suggesting that the G protein contains epitopes which bind neutralizing antibodies (Fig. 5).

The observation that incubation of cultured cells with partially purified G protein prior to the addition of virus reduces infectivity supports the hypothesis that the G protein is important for viral attachment and/or penetration. Stoyloff et al. performed both endoglycosidase and exoglycosidase studies of purified virus (32). Their results indicated that combined digestion with the exoglycosidases α-mannosidase and GlcNAcase reduced viral infectivity, whereas digestion with either exoglycosidase alone had no effect (32). Although it was suggested that both mannose and GlcNAc residues act together to facilitate viral entry into the cell (32), an alternative explanation is that removal of terminal mannose by α-mannosidase was required for removal of proximal GlcNAc by GlcNAcase. Our findings support the latter hypothesis. Data presented here indicate that the BDV G protein contains both mannose and GlcNAc residues. To determine the role of mannose and GlcNAc in G protein function, carbohydrate residues were sequentially removed by exoglycosidase treatment and the treated protein was assayed for the ability to interfere with viral infectivity. Intriguingly, removal of terminal mannose residues by treatment with α-mannosidase enhanced inhibition of infectivity, presumably by exposing functionally important residues. Sequential treatment with α-mannosidase and GlcNAcase reduced the enhancement effect of α-mannosidase alone. Treatment of the G protein with GlcNAcase alone had no effect on the ability of G protein to interfere with infectivity, suggesting that the GlcNAc residues important for viral infectivity are not located in terminal positions. In concert, these observations are consistent with core G protein-conjugated GlcNAc oligosaccharides serving a key role in BDV infectivity.

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