Identification of the amino terminal subunit of the glycoprotein of Borna disease virus

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Abstract The only surface membrane glycoprotein of Borna disease virus (BDV) is synthesized as a polypeptide with a molecular mass of 57 kDa and *N*-glycosylated to a precursor glycoprotein (GP) of about 94 kDa. It is processed by the cellular protease furin into the C-terminal membrane-anchored subunit GP-C, also known as gp43, and a presumptive N-terminal subunit GP-N, that is highly glycosylated and has a molecular mass of about 51 kDa. However, up to now the latter remained undetected in BDV-infected material. We describe a novel approach to identify glycan masked linear antigenic epitopes. In the present study, GP-N was identified in BDV-infected cells by a combination of lectin precipitation, enzymatic deglycosylation on blot and immunochemistry using an N-terminal specific antiserum. The GP-N has an apparent molecular mass of 45–50 kDa in its glycosylated form and 27 kDa in its deglycosylated form. *N*-glycan analysis revealed that the precursor GP contains only mannose-rich *N*-glycans, whereas GP-N and GP-C contain mannose-rich and complex-type *N*-glycans.

Key words: Borna disease virus; Glycoprotein; Amino terminal subunit; Carbohydrate masking; Blot deglycosylation

1. Introduction

Borna disease virus (BDV) is the ethological agent of Borna disease which is observed in a wide range of vertebrates, in particular horses and sheep. Borna disease is a progressive disease of the central nervous system caused by a T-cell-mediated immunoreaction. It is also suspected to play a role in human psychiatric disorders. The fine structure and morphogenesis of BDV analyzed by electron microscopy revealed spherical enveloped virus particles, approximately 90–130 nm in diameter. The virions are assembled at the cell surface from where only small numbers of virions are released by budding [1]. The BDV nucleocapsid contains a non-segmented, single-stranded RNA of negative sense polarity, which is replicated and transcribed in the nucleus of BDV-infected cells. Because of these unusual properties, the few characterized BDV isolates form the family *Bornaviridae* within the order *Mononegavirales*. The BDV 8.9 kb RNA genome comprises six open reading frames (ORFs): ORF I codes for the nucleoprotein p40 (NP), ORF II for the phosphoprotein p24 (P), ORF III for the matrix protein p16 (M), ORF IV for the glycoprotein p57, ORF V for p180/190, the phosphorylated polymerase (L) [2]. A further ORF overlaps with the P gene and encodes the protein p10 (X) [3].

The BDV glycoprotein precursor (GP) molecule encoded by ORF IV is a type I membrane protein, consisting of 503 amino acids with a molecular mass of 57 kDa in the deglycosylated form (p57) and 84 or 94 kDa in the *N*-glycosylated form previously designated as gp84, gp94 or G (Fig. 1A) [4–6]. Precursor GP is cleaved at a basic tetrapeptide motif, C-terminally of arginine 249, by furin or by other subtilisin-like endoproteases. Proteolytic processing of GP is essential for fusion capacity of BDV and thus for BDV infectivity [7,8]. Cleavage of GP results in the previously detected C-terminal, membrane-anchored subunit GP-C (gp43) and a not yet identified N-terminal subunit GP-N. GP-N seems to be responsible for receptor binding as recently shown by a pseudotype approach of vesicular stomatitis virus (VSV) containing a BDV GP/VSV-G chimeric glycoprotein [9].

In this report, we identified the subunit GP-N present in BDV-infected cells and in the virus-enriched preparations by a combined method including: (i) the enrichment of different BDV glycoprotein forms by lectin precipitation, (ii) endoglycosidase treatment, and (iii) immunochemical detection using an antiserum raised against peptides corresponding to amino acids 81–107 of the N-terminal part of GP. Furthermore, the *N*-glycans of GP, GP-N and GP-C were characterized by endoglycosidases and lectins.

2. Materials and methods

2.1. Propagation of BDV-infected cells

MDCK and Vero cells (African green monkey kidney cells) persistently infected with the BDV [10,11] and uninfected MDCK and Vero cells as controls were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. The cells were kept at 37°C with 5% CO_2.

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Abbreviations: BDV, Borna disease virus; ConA, concanavalin A; Endo H, endoglycosidase H; GP, glycoprotein precursor (gp84/gp94); GP-C, carboxy terminal glycoprotein subunit (gp43); GP-N, amino terminal glycoprotein subunit (gp51); GNA, *Galanthus nivalis* agglutinin; PNGase F, peptide-*N*-glycosidase F; Rb-αGP-C, rabbit antiserum against carboxy terminal BDV GP; Rb-αGP-N, rabbit antiserum against amino terminal BDV GP; Vero cells, African green monkey kidney cells; WGA, wheat germ agglutinin

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2.2. Peptide antisera

The monospecific antiserum Rb-αGP-N was obtained by immunization of rabbits with a chemical synthesized GP-specific polypeptide comprising the amino acids 81–107 of BDV GP (M. Kraus, IMT, Marburg, Germany). The monospecific rabbit antiserum Rb-αGP-C, previously designated as Rb-αGP2, was described previously [7].

2.3. Electrophoresis and immunoblot analysis

BDV-infected material was dissolved with an equal volume of 2× electrophoresis sample buffer containing 100 mM Tris–HCl (pH 6.8), 4% sodium dodecyl sulfate (SDS), 10% 2-mercaptoethanol, 20% glycerol and 0.05% bromophenol blue, heated at 96°C for 5 min. Proteins were separated on 12% polyacrylamide gels by SDS–polyacrylamide gel electrophoresis (PAGE), and electrophoretically transferred onto nitrocellulose membranes (Schleicher and Schuell). The membranes were blocked at 4°C overnight with a solution of 3% bovine serum albumin (BSA) in phosphate-buffered saline with 0.1% Tween 20 (PBS-T). The blots were incubated for 1 h with peptide antisera, Rb-αGP-C 1:400 diluted and Rb-αGP-N 1:300 diluted in PBS-T, followed by an incubation of a 1:2000 diluted anti-rabbit immunoglobulin G from swine complexed with horseradish peroxidase (Dako). BDV-specific glycoprotein bands were visualized using the SuperSignal chemoluminescence substrate as described by the supplier (Pierce).

2.4. Glycoprotein binding to lectin agarose

Detached cells from one 75 cm^2 flask were collected by centrifugation, washed once with PBS and dissolved in 600 μl GDK1 buffer consisting of 50 mM Tris–HCl (pH 6.8), 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$ and 2% SDS. Solubilized cells were treated for 1 min with ultrasonic (Branson Sonifier, 40 W) and 100 μl aliquots were 1:10 diluted in GDK1 buffer.

The glycoproteins were precipitated by addition of 50 μl concanavalin A (ConA) agarose conjugate (Calbiochem-Novabiochem), *Galanthus nivalis* agglutinin (GNA) agarose conjugate or wheat germ agglutinin (WGA) agarose conjugate (Alexis) to the diluted cell lysates at 4°C for 16 h. The glycoproteins bound to the lectin agarose beads were washed three times with GDK1 buffer.

2.5. Deglycosylation by endoglycosidases

2.5.1. Deglycosylation before SDS–PAGE. Glycoproteins of cell lysates precipitated by lectin agarose were denatured in 16 μl electrophoresis sample buffer by heating for 10 min at 96°C, cooled on ice and centrifuged 10 min at 13 000 rpm (Biofuge, Heraeus). The supernatants were 10-fold diluted in an appropriate reaction buffer for deglycosylation enzymes, 50 mM sodium citrate (pH 5.5) or 50 mM sodium phosphate (pH 7.5) in 1% NP-40, and incubated for 2 h at 37°C with endoglycosidase H (Endo H) or peptide-*N*-glycosidase F (PNGase F) (New England Biolabs), respectively. Deglycosylated protein samples were subjected to SDS–PAGE and immunoblot analysis.

2.5.2. Deglycosylation after SDS–PAGE. Proteins of lysed cells or enriched lectin precipitations were separated by SDS–PAGE, transferred onto nitrocellulose membranes, equilibrated with 50 mM sodium acetate buffer (pH 5.5) for 20 min, and then incubated with 0.05 U/ml Endo H, diluted in sodium acetate buffer (pH 5.5) for 16 h at 37°C [12]. Then, the nitrocellulose membranes were washed with 50 mM sodium acetate buffer (pH 5.5) followed by a PBS washing and then blocked at 4°C for at least 16 h with a solution of 3% BSA in PBS-T and subjected to immunoblot analysis.

3. Results and discussion

3.1. Identification of the subunit GP-N

BDV GP is proteolytically processed similar to other viral envelope glycoproteins [4,7,9]. However, only the membrane-anchored subunit GP-C was described in BDV-infected cells and purified BDV virions. The other cleavage product(s), i.e. one or several N-terminal subunit(s) remained unidentified. In order to characterize N-terminal cleavage product(s), we used the monospecific antiserum Rb-αGP-N. When proteins of lysed BDV-infected cells were separated by SDS–PAGE and electrophoretically blotted on nitrocellulose membrane, Rb-αGP-N specifically reacts only with one band with the approximate molecular mass of 94 kDa indicating uncleaved GP (Fig. 1B, lower blot, lanes 1 and 2). No other BDV-specific band could be detected from BDV-infected and uninfected control cells. When a parallel blot was incubated with the Rb-αGP-C serum, uncleaved GP, and in addition the subunit GP-C were detected (Fig. 1B, upper blot, lanes 1 and 2). The lack of any detectable N-terminal cleavage fragment by the Rb-αGP-N raised the question whether GP-N is instable and rapidly degraded or whether the putative subunit GP-N remains undetectable because the linear antigenic epitope for the Rb-αGP-N antiserum was shielded by *N*-glycans after cleavage of the precursor GP. In order to analyze the latter possibility, the BDV glycoprotein was first enriched by precipitation with various lectin agarose beads. Secondly, after separation of the glycoproteins by SDS–PAGE and electroblotting onto nitrocellulose membranes, the blots were extensively treated with Endo H to remove mannose-rich carbohydrates and, thus, accomplish access to the linear antigenic epitope for the Rb-αGP-N serum. After these procedures, the BDV glycoprotein was detected by immunochemistry using Rb-αGP-N serum. Several lectins, among them ConA, GNA and WGA, precipitated the BDV glycoprotein from lysates of BDV-infected cells. WGA recognizes *N*-acetyl-β-(1,4)-D-glucosamine, ConA α-D-glucose and α-D-mannose, and GNA high mannose containing *N*-glycans. The subunit GP-N and the precursor GP of the BDV-infected cells immunochemically reacted with Rb-αGP-N serum on the blots after deglycosylation on blot indicating that the linear epitope of the BDV GP-N is indeed covered by *N*-glycans (Fig. 1B, lanes 4, 6, 8, lower blot). GP-C and GP were detected by Rb-αGP-C serum BDV-infected under the same conditions (Fig. 1B, lanes 4, 6, 8, upper blot). GP appears as a band with an apparent molecular mass in the range of 84–94 kDa on both blots. GP-N appears as a broad band with a molecular mass between 45 and 55 kDa (Fig. 1B, lanes 4, 6, 8, upper blot) and GP-C as a 43 kDa band (Fig. 1B, lanes 4, 6, 8, lower blot). Furthermore, GP-N was detected in virus-enriched preparations (data not shown). The experimentally determined molecular mass of GP-N is in accordance with the calculated molecular mass of about 51 kDa composed of the non-glycosylated GP-N with a molecular mass of 27 kDa and an additional mass of 27 kDa for potential *N*-glycans. GP-N contains nine predicted *N*-glycans, each with an average molecular mass of 3 kDa.

3.2. Specification of N-glycans in BDV-infected cells

To specify the *N*-glycans of GP, GP-N and GP-C, the glycoprotein of BDV-infected cells was first enriched from lysed cells by precipitation either with ConA agarose beads (Fig. 2A) or with GNA agarose beads (Fig. 2B). Subsequently, the BDV glycoprotein was treated either with Endo H or PNGase F as indicated and the deglycosylated proteins were separated by SDS–PAGE. Using this approach, the removal of mannose-rich (Endo H sensitive) and complex-type (PNGase F sensitive) *N*-glycans from BDV GP, GP-N and GP-C was achieved. Treatment of GP with either Endo H or PNGase F results in the totally deglycosylated form with the expected molecular mass of 57 kDa (Fig. 2A, lanes 4 and 6; Fig. 2B, lanes 4 and 6). These results are in agreement with the concept that all or nearly all *N*-glycosylation sites of GP are occupied with mannose-rich glycans. The digestion of GP-

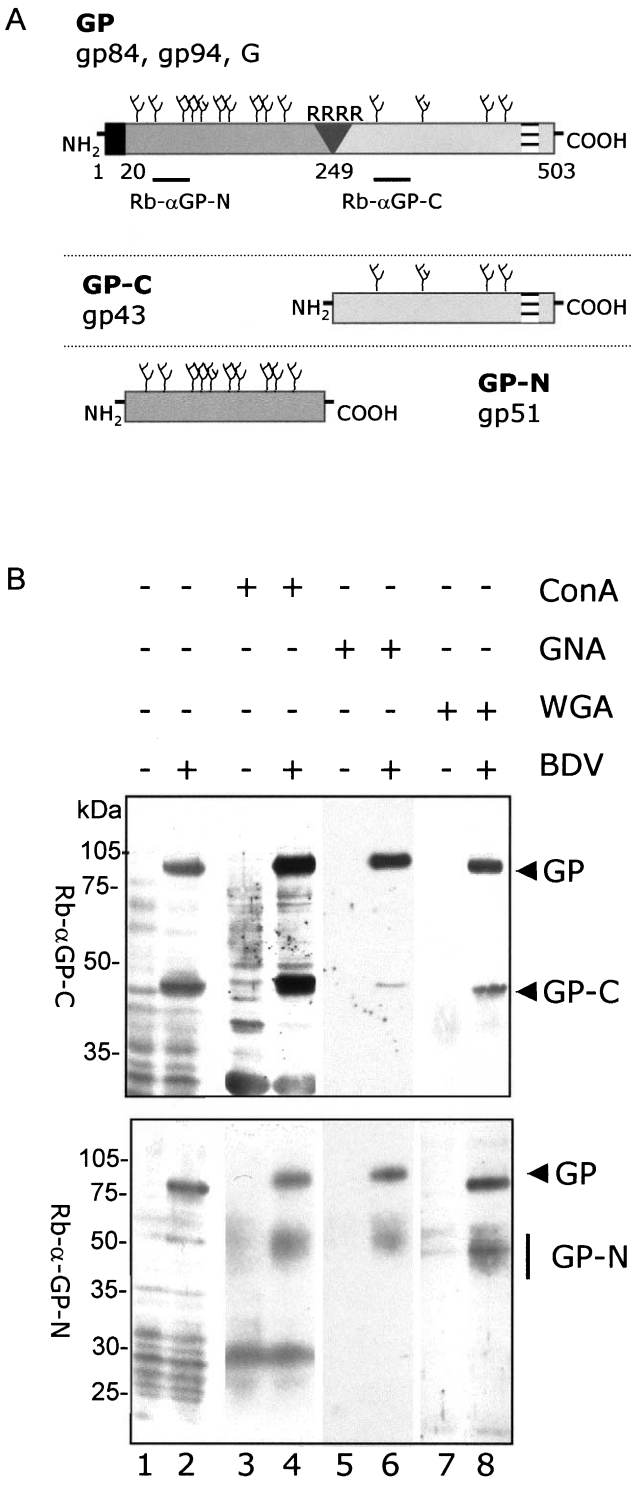

Fig. 1. A: Schematic overview of the BDV glycoprotein. The GP (gp84, gp94, G) and the subunits GP-C (gp43) and GP-N (gp51) are shown. Signal peptide (black box), furin cleavage site (filled triangle), transmembrane domain (strips), antisera recognition sites for Rb-αGP-N and Rb-αGP-C (black beams) and potential attachment sites for *N*-glycans (tree-like symbols) are indicated. Numbers show the amino acid positions. B: Identification of BDV GP-N. Immunoblot analysis of material of BDV-infected Vero cells (lanes 1 and 2) and MDCK cells (lanes 3–8) and respective control cells using the N-terminal specific (Rb-αGP-N) and C-terminal specific (Rb-αGP-C) anti-peptide sera. Glycoproteins of dissolved cells were precipitated by ConA, GNA, or WGA. Uncleaved BDV GP, and subunits GP-N and GP-C are indicated. Prior to immunodetection the glycoproteins were enzymatically deglycosylated by Endo H.

C with PNGase F resulted in the non-glycosylated form GP-C** with a molecular mass of about 30 kDa (Fig. 2A, lane 6). Interestingly, Endo H only partially removed the *N*-glycans from GP-C and led to a semi-deglycosylated GP-C* with a molecular mass of about 35 kDa (Fig. 2A, lane 4). Two of four potential *N*-glycosylation sites of GP-C may be occupied

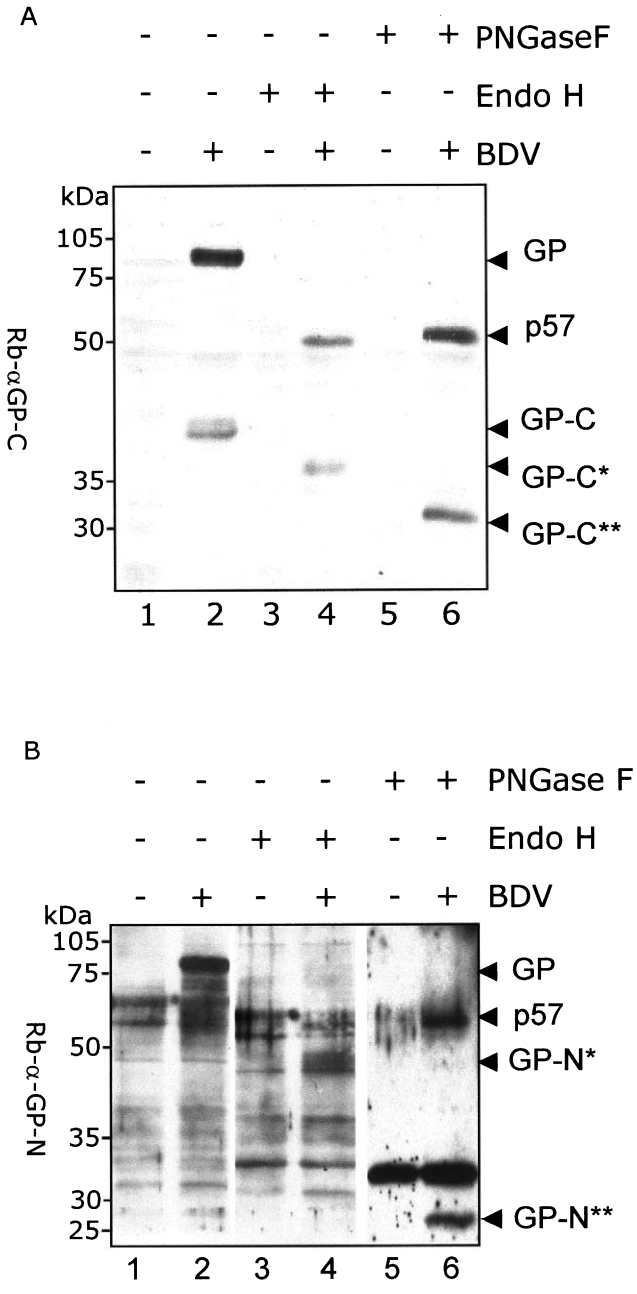

Fig. 2. Various deglycosylated forms of the BDV glycoproteins. A: Immunoblot analyses were performed with material of BDV-infected MDCK cells or of non-infected MDCK cells as controls which was first precipitated by ConA agarose beads and secondly treated with either Endo H or PNGase F. BDV GP, uncleaved and non-glycosylated BDV GP (p57), the membrane-anchored glycoprotein subunit with mature *N*-glycans GP-C, the C-terminal subunit with Endo H resistant *N*-glycans GP-C*, and the totally deglycosylated subunit GP-C** are shown. B: Immunoblot analyses of GNA precipitated and endoglycosidase treated material of BDV-infected MDCK cells and control cells were done according to A. The N-terminal subunit with Endo H resistant *N*-glycans GP-N*, and the totally deglycosylated GP-N** are shown.

by *N*-glycans of the complex type and two other sites may be occupied by mannose-rich glycans.

Deglycosylation of GP-N by PNGase F and immunoblot analysis with the N-terminal peptide antiserum thereafter resulted in the loss of all *N*-glycans and the remaining polypeptide GP-N** of 27 kDa (Fig. 2B, lane 6). When GP-N was treated with Endo H, only several partially deglycosylated bands were obtained on the blots (Fig. 2B, lane 4). The results for the GP-N were independent whether lectins with specificities for mannose-rich or complex-type *N*-glycans were used.

The data indicate that the precursor GP contains immature *N*-glycans, which are all Endo H sensitive, the GP-C possesses two complex and two mannose-rich *N*-glycans, whereas GP-N contains nine *N*-glycans of both glycotypes.

The translated sequence of BDV ORF IV represents a protein of 57 kDa [13], which was later described as an *N*-glycosylated envelope protein of BDV (gp83, gp94, GP) [4,6,7]. Analogous to other viral glycoproteins of the *Mononegavirales* order, the glycoprotein cleavage by soluble furin was shown, which is important for virus propagation [7]. Until now, only the membrane-anchored C-terminal subunit and not the distal N-terminal subunit of the BDV glycoprotein was described for BDV-infected material. The first experimental indication for an N-terminal subunit of the BDV glycoprotein was obtained from in vitro transcription and translation of unglycosylated 57 kDa polypeptide of the BDV ORF IV [7]. Furin cleaved the unglycosylated precursor BDV GP in two fragments of distinct sizes, 29 and 27 kDa, respectively. This finding argued for the usage of the furin cleavage site at amino acid position 249, although two other furin cleavage motifs are present within BDV GP. The non-glycosylated in vitro generated fragments represent the corresponding subunits of GP-C and GP-N, obtained from BDV-infected cells after deglycosylation as shown in this report. The difficulties for GP-N identification were based on the lack of suitable antibodies for detection of the N-terminal part of the BDV glycoprotein and an appropriate detection method. The immunodetection is complicated due to the low antigenicity of the BDV GP-N and the high content of *N*-glycans which shield antigenic sites. About 50% of the total molecular mass of GP-N is attributed to its *N*-glycans. For other highly glycosylated molecules it might be of interest that glycan removal by Endo H prior to SDS–PAGE did not result in detection after immunoblot analyses. But the detection was successful after removal of the glycans on blot. Obviously, the same glycan structures appear to be only accessible for deglycosylation after denaturation of an immobilization on nitrocellulose. High glycan content might protects the BDV glycoprotein surface against the host immune attack and supports the establishment of a persistent infection.

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