Antigenic relationship and further characterization of two major Borna disease virus-specific proteins

N. Thiedemann, 1 P. Presek, 2 R. Rott 1 and L. Stitz 1*

1 Institut für Virologie and 2 Rudolf-Buchheim-Institut für Pharmakologie, Justus-Liebig-Universität Giessen, Frankfurter Strasse 107, 6300 Giessen, Germany

After immunization of mice with isolated Borna disease virus (BDV)-specific proteins having Mr's of 38/39K and 24K, monoclonal antibodies (MAbs) were obtained which were specific for one of the antigens in Western blot analysis. However, in immunoprecipitation assays it was found that some MAbs of each specificity reacted exclusively with their respective antigen from BDV-infected cells, whereas other MAbs coprecipitated the heterologous protein. The relationship between the 38/39K and 24K proteins was demonstrated by two-dimensional peptide mapping, which revealed four identical peptides. Additionally, it was found that neither the 38/39K nor the 24K protein is glycosylated, but that the 24K protein is phosphorylated at serine residues. Experiments employing various cell separation protocols revealed that the 38/39K and the 24K proteins are evenly distributed within infected cells; this was confirmed by immunofluorescence techniques using 38/39K- or 24K-specific MAbs. Iodination experiments clearly demonstrated that only the 38/39K protein is expressed on the surface of virus-infected cells.

Introduction

Borna disease virus (BDV) induces a meningoencephalomyelitis causing severe neurological symptoms in horses and sheep, and in various species of experimental animals (Zwick, 1939).

Several properties of BDV have been established during recent years, including its sensitivity to u.v. irradiation, lipid solvents and proteolytic enzymes, suggesting the presence of an enveloped virus (Heinig, 1969; Duchala et al., 1989; our unpublished observations). Recently, cDNA clones have been isolated from rats with Borna disease (BD) and characterized, and it has been shown that BDV is an ssRNA virus (DelaTorre et al., 1990; Lipkin et al., 1990; VandeWoude et al., 1990).

The pathogenesis of BD in Lewis rats has been clearly shown to be immune-mediated (Narayan et al., 1983a; Herzog et al., 1985; Rott et al., 1988; Stitz et al., 1989), virus-specific CD4+ T cells and macrophages (Richt et al., 1989, 1990; Deschl et al., 1990), but also CD8+ cells (Stitz et al., 1991a; Stitz, et al., 1992), apparently playing important roles. Strict neurotropism of the causative virus has been demonstrated in vivo (Narayan et al., 1983b; Morales et al., 1988; Carbone et al., 1989), which is paralleled by the in vitro finding that cells of neural origin can be easily infected (Herzog & Rott, 1980). However, after cocultivation several cell types of non-neural origin produce infectious virus and express virus-specific proteins (Herzog & Rott, 1980; Duchala et al., 1989). After natural infection of horses or after inoculation of BDV into susceptible experimental animals a vigorous antibody response can be observed. Interestingly, sera from human patients with psychiatric disorders have been shown to react with BDV-specific antigens (Rott et al., 1985, 1991; VandeWoude et al., 1990). This suggests that BDV or a related virus might also be a human pathogen.

BDV-specific antibodies from naturally diseased or experimental animals react with proteins of 60K, 38/39K, 24K and 14.5K (Schädler et al., 1985; Haas et al., 1986; Ludwig et al., 1988; Rott et al., 1988; Stitz et al., 1991b). In the present study we report the specificity and reactivity of monoclonal antibodies (MAbs) obtained after immunization of mice with isolated 38/39K and 24K proteins. We characterized these major viral proteins further, established their antigenic relationship and determined their localization in various cellular compartments.

Methods

Virus and cells. The Giessen strain He/80 of BDV was used throughout this study (Herzog & Rott, 1980). Lysates of persistently BDV-infected and uninfected Madin–Darby canine kidney (MDCK) cells also served as sources of antigen preparations for Western blot analysis. Alternatively, BDV-infected astrocytes were used in immunofluorescence assays (IFAs) (Richt et al., 1990).
Mabs. BALB/c mice were immunized intraperitoneally (i.p.) with either the 38/39K or 24K protein electroeluted from gels after immunoprecipitation of BDV-infected MDCK cell lysates. Two weeks later, mice received a second i.p. injection and were boosted again intravenously 4 days before fusion of spleen cells with the mouse myeloma X63-Ag8.653 (Kearney et al., 1979). Additionally, spleen cells from BDV-infected Lewis rats were immunized with the same antigen preparation and fused with the rat myeloma Y3-Ag 1.2.3 (Galfrè et al., 1979). All hybridoma supernatants were screened by Western blot analysis and Mabs were selected according to their specificity for the 38/39K or 24K protein.

Western blot analysis. Western blot analyses were performed with lysates from persistently infected cells or brain extracts from BDV-infected rats. Proteins were transferred from gels onto a protein binding membrane (Immobilon-P-Membrane; Millipore) as described (Porter & Porter, 1984).

Radioimmunoprecipitation (RIP). This was done as described by Kessler (1975). Briefly, confluent persistently infected or mock-infected MDCK cells were labelled overnight with [35S]methionine or [35S]cysteine (250 μCi/10 ml), washed three times with PBS and lysed with extraction buffer (10 mM-sodium phosphate buffer pH 7.4, 100 mM-NaCl, 1 mM-EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1% w/v aprotinin). Lysates were centrifuged for 5 min at 13000 g at 4 °C and the pellet was discarded. Aliquots of 100 μl were incubated for 1 h at 4 °C with either 2 μl of polyclonal antibodies obtained from BDV-infected rats or 100 μl of supernatant containing Mabs. Then 20 μl of a 10% suspension of inactivated Staphylococcus aureus strain Cowan 1 was added for a further 45 min. After centrifugation, the pellet was washed three times with extraction buffer and once with 10 mM-Tris-HCl pH 7.4. The pellet was resuspended in 20 μl of Laemmli buffer and boiled for 5 min. Finally, the suspension was centrifuged and run on an SDS-polyacrylamide gel. Gels were treated with Amplify (Amersham) for 30 min before drying.

Partial peptide mapping. After RIP and PAGE the 38/39K and the 24K bands were cut out of gels and one-dimensional protein digestion was carried out as described (Cleveland et al., 1977). Endoprotease Glu-C from S. aureus (V8 protease) was used at various concentrations (5 ng to 5 μg).

Two-dimensional peptide analysis. The immunoprecipitated [35S]labelled 38/39K and 24K proteins from BDV-infected MDCK cell lysates were digested with TPCK-treated trypsin for 24 h at room temperature as described (Reuter et al., 1990). After 12 h of co-incubation, additional trypsin was added.

Detection of carbohydrates. Peptides were examined for the presence of carbohydrate side-chains by using a glycan detection kit (Boehringer Mannheim), which reveals the presence of OH residues of carbohydrates by oxidizing them to aldehyde residues. These residues were linked covalently to the steroid hapten digoxigenin, which can be identified by using an immuno-enzyme assay. The test was performed according to the manufacturer’s protocol. Additionally, gels were reacted with Schiff’s reagent or biotinylated lectins (wheatgerm agglutinin or concanavalin A) and used for Western blot analysis.

Radioactive labelling of carbohydrates was performed using [2-3H]mannose, [3-3H]mannose, [3H]glucosamine, [14C]mannose or [14C]glucosamine (100 μCi/2 ml; 5 cm Petri dishes). BDV-infected MDCK cells were labelled with radioactive carbohydrates for 4 to 15 h. Thereafter, RIP was carried out as described above.

Determination of phosphorylation. BDV-infected MDCK cells and mock-infected control cells were grown in phosphate-free medium for 2 h prior to labelling overnight with [32P]orthophosphate (1 μCi/ml, carrier-free; Amersham) in phosphate-free medium according to the method described by Presek & Reuter (1987). The lysates obtained were immunoprecipitated as described above. Phosphoamino acids of radioimmunoprecipitated and isolated [32P]-labelled 24K proteins were identified by two-dimensional separation as described by Hunter & Sefton (1980).

Subcellular fractionation of BDV-infected MDCK cells. To determine the distribution of BDV-specific antigens within cells, we used the detergent Triton X-114, which allows the separation of soluble proteins from membrane-associated proteins (Bordier, 1981). A confluent monolayer of BDV-infected MDCK cells in 5 cm Petri dishes was washed twice with ice-cold PBS and lysed with 200 μl 0.5% (v/v) Triton X-114 in PBS containing the protease inhibitor 1% (v/v) aprotinin. The cells were scraped off and centrifuged at 13000 g for 3 min at 4 °C.

The supernatant (free of nuclei) was incubated for 3 min at 30 °C, followed by centrifugation at 600 g for 3 min to precipitate the membrane proteins. The procedure was performed twice, with adjustment of all resulting solutions to a Triton X-114 concentration of 0.5%. After centrifugation, the pellet was considered to contain the proteins associated with the cellular membrane (membrane proteins). The supernatant was precipitated using a final concentration of 10% TCA for 1 h on ice, washed with ethanol, ethanol:ether (1:1) and ether, and finally dried (soluble proteins). The samples were resuspended in Laemmli buffer and separated by SDS-PAGE, followed by Western blot analysis.

In a second separation method, BDV-infected rat brains or MDCK cells were lysed and homogenized using a tightly fitting Dounce homogenizer. The homogenate was centrifuged at 400 g for 30 min and the supernatant was collected, which contained the nuclei, was washed and treated with DNase (pellet fraction). The supernatant was centrifuged for 40 min at 25000 g to separate the cytosol fraction (supernatant) from the membrane fraction (pellet). Analysis of the various fractions was performed by Western blotting. Accordingly, Vero cells were lysed in hypertonic buffer, homogenized with a Dounce homogenizer and centrifuged for 10 min at 1000 g (Duchala et al., 1989). The sonicated nuclear pellet and enucleated cells with the soluble proteins were tested by Western blot analysis.

Iodination of virus-specific proteins on the surface of BDV-infected MDCK cells. Iodination was performed using Bolton and Hunter reagents (Bolton & Hunter, 1973). Briefly, after washing with medium, confluent monolayers of BDV-infected and mock-infected MDCK cells were incubated for 15 min with 200 μl of Bolton and Hunter reagents in a total volume of 500 μl. The reaction was stopped by washing the cells with PBS containing 0.2 M-glycine. The viability of the cells was determined by trypsin blue exclusion and revealed that more than 99% of cells were intact. Cells were lysed with extraction buffer and immunoprecipitation was performed as described above.

IFA. To demonstrate the distribution of viral antigens, BDV-infected cells were grown in chamber slides in medium containing 0.5% foetal calf serum for 6 days and then cultured for 12 h in normal medium. Cells were fixed overnight in acetone at 20 °C. MAb (100 μl; dilution 1:100 to 1:12800) in PBS was added for 1 h, followed by incubation with species-specific biotinylated antibodies (100 μl/chamber; 1:1000) and streptavidin-fluorescein isothiocyanate (FITC) (100 μl/chamber; 1:200) for 30 min.

Results

Reactivity of virus-specific proteins with Mabs

Mabs against the 38/39K and 24K proteins of BDV were produced from mice immunized with either 38/39K or 24K protein preparations. The antigens from brains of
infected rats and persistently infected MDCK cells were obtained by electroelution of immunoprecipitated proteins from gels. Antisera from immunized mice reacted in Western blot analysis exclusively with the protein used for immunization, i.e. either the 38/39K or the 24K protein (data not shown). Immunoblot analysis revealed MAbs induced by immunization with the 38/39K protein, which again reacted exclusively with the corresponding protein (Fig. 1a, lane 3 and data not shown (MAb 38/17C2)), but not with the 24K protein, whereas MAbs obtained with the 24K antigen recognized the 24K band (Fig. 1a, lanes 2 and 4), but not the 38/39K protein.

In RIP two different reaction patterns could be observed. MAbs specific for the 38/39K or 24K protein exclusively precipitated the respective protein (Fig. 1b, lane 2; Fig. 1c, lane 2). However, some MAbs coprecipitated the heterologous protein, i.e. MAb 38/15H7, specific for the 38/39K protein, also reacted with the 24K protein and MAb 24/36F1, prepared

Fig. 1. Patterns of reactivity of BDV-specific MAbs with 24K and 38/39K proteins in Western blot analysis (a) and RIP (b, c). Lanes 1 (a, b) show an irrelevant hybridoma supernatant; lanes 5 (a, b) and lane 1 (c) demonstrate the reaction of a polyclonal serum from a BDV-infected rat (control). (a, b) Lanes 2, 3 and 4, MAbs 24/28D12, 38/15H7 and 24/36F1, respectively; (c) lane 2, MAb 38/17C1.

Fig. 2. Two-dimensional peptide analysis of the 38/39K and 24K proteins. Tryptic fingerprint analysis was performed as described in Methods. (a) 38/39K protein; (b) 24K protein; (c) mixture of 38/39K and 24K. The horizontal electrophoresis was at pH 1.9 and followed by vertically ascending chromatography in the second dimension. Identical peptides are circled.
against the 24K protein, coprecipitated the 38/39K protein (Fig. 1b).

Relationship between major BDV-specific antigens

The results presented above indicate that the 38/39K and 24K proteins found in extracts from brains of BDV-infected rats or BDV-infected MDCK cells might be antigenically related. To examine this possibility further, tryptic fingerprint analyses were performed to establish the extent of the identical or overlapping peptides responsible for the antigenic cross-reaction. Therefore, the 38/39K and 24K proteins obtained by RIP and SDS-PAGE were digested with trypsin and the resulting peptides were run two-dimensionally on thin-layer cellulose plates. As shown in Fig. 2, these experiments revealed four identical peptides, after digestion of both the 38/39K (Fig. 2a) and the 24K (Fig. 2b) protein.

Glycosylation and phosphorylation of the major BDV-specific antigens

To characterize the BDV-specific 38/39K and 24K proteins further, we investigated whether they were glycosylated. In experiments employing radioactive carbohydrates, including [3H]glucosamine, [2-3H]mannose, [3-3H]mannose, [14C]glucosamine and [14C]mannose we did not find any evidence for the presence of carbohydrate moieties in virus-specific bands after immunoprecipitation (data not shown). Negative results were also obtained by using a glycan detection assay, demonstrating that neither the 38/39K nor the 24K protein contained carbohydrate side-chains (Fig. 3). Furthermore, neither Schiff's reagent nor biotinylated lectins such as wheatgerm agglutinin and
Characterization of BDV-specific proteins

1061

1 2 3

45K--

29K--

9K

18K--

14K--

6K--

(a)

1 2 3

45K--

29K--

9K

18K--

14K--

6K--

(b)

Fig. 5. Comparative partial peptide mapping of the $^{32}$P- and $^{35}$S-labelled 24K protein. The undigested $^{35}$S-labelled 24K protein is shown in lane 1, endoprotease Glu-C-digested 24K protein as a control in lane 2. Lane 3 shows the digested 24K protein labelled with $^{32}$P-orthophosphate. Three fragments with the same $M_r$ are marked by arrows.

concanavalin A showed any positive reaction with virus proteins in Western blot analyses (data not shown).

Additionally, we determined whether the virus-specific proteins were post-translationally modified in other ways. In experiments using $^{32}$P-orthophosphate-labelled BDV-infected cells we were able to establish that the 24K protein is phosphorylated, whereas the 38/39K protein is not (Fig. 4a). Phosphoamino acid analysis revealed that the 24K protein is phosphorylated exclusively at serine residues (Fig. 4b). One-dimensional peptide mapping of the phosphorylated 24K protein treated with endoprotease Glu-C resulted in at least three different phosphopeptides (Fig. 5).

Localization of BDV-specific proteins in infected cells

Using MAbs against the 38/39K or 24K protein which did not coprecipitate the heterologous protein, we investigated the subcellular localization of BDV-specific proteins. By this mean we wished to answer two pertinent questions: first, whether both proteins were found in distinct cellular compartments and, second, which of the virus proteins is exposed on the surface of BDV-infected cells. The latter point is important for considering the function of immunogenic BDV-specific antigens in the pathogenesis of the disease.

Various fractionation methods were employed. Persistently BDV-infected MDCK cells were treated with

Fig. 6. Distribution of BDV-specific antigens in infected MDCK cells. (a) Distribution using Triton X-114 treatment and BDV-infected MDCK cell lysates; (b) pattern obtained using Dounce-homogenized and centrifuged cell extracts from BDV-infected Vero cells (Duchala et al., 1989). In (a) BDV-specific antigens are present in the nucleus (lane 2; arrows indicate the localization of the 24K protein in the preparation from the nuclear fraction), in the cytoplasm as soluble proteins (lane 3) and in the fraction containing membrane and membrane-associated proteins (lane 4). As a control, the reaction of a polyclonal serum from a BDV-infected rat with virus antigens is shown (lane 1). (b) BDV-specific proteins in the whole cell extract (lane 1), the soluble fraction (lane 2) and nucleus (lane 3) of infected Vero cells.
Fig. 7. Detection of virus-specific proteins on the surface of BDV-infected MDCK cells. Cell surface proteins were labelled with Bolton and Hunter reagent. Lane 1 shows the reaction of 35S-labelled BDV-specific proteins immunoprecipitated with a serum from a BDV-infected rat as a control. Lanes 2 and 3 show 125I-labelled BDV-specific proteins immunoprecipitated with serum from a BDV-infected (lane 2) or an uninfected (lane 3) rat. Lanes 4 and 5 show the reaction of uninfected MDCK cells with the same sera.

Discussion

Several attempts have been made to define the nature of BDV and to characterize BDV-related antigens found either in infected tissue culture cells or in the tissue of infected animals. These investigations revealed presumably virus-specific components of 60K, 38K to 40K, 22K to 24K and 14-5K (reviewed in Ludwig et al., 1988; Rott et al., 1988; Stitz & Rott, 1992). Of these, only the 38/39K protein has been purified and characterized (Haas et al., 1986). Data have been published on the molecular characterization of BDV that provide evidence for it being an ssRNA virus (DelaTorre et al., 1990; Lipkin et al., 1990; VandeWoude et al., 1990). In addition, in vitro transcription and translation of a BDV-specific cDNA clone has resulted in the synthesis of BDV-specific proteins of 24K and 14-5K (VandeWoude et al., 1990).

To enable characterization, BDV-specific MAbs were produced against either the 38/39K or 24K protein by immunizing mice with material that had been electrophoresed from gels. Since both protein bands are easily distinguishable on gels owing to their Mr, monospecificity of these antibodies could be assumed. This was proven by the reaction of polyclonal antibodies present in the sera of immunized mice. Western blot analysis showed that the sera exclusively recognized the protein used for induction of the antibody response. The same was found when the specificity of hybridoma supernatants or affinity chromatography-purified MAbs was determined by Western blot analysis.

In immunoprecipitation assays, some MAbs exclusively recognize the antigen against which they were raised. However, other MAbs react with the 38/39K protein and coprecipitate the 24K protein; similarly, some MAbs directed against the 24K protein coprecipitate the 38/39K component. These results can be interpreted as follows. The proteins share epitopes that are recognized by certain conformation-dependent MAbs (e.g. MAbs 24/36F1 and 38/15H7), resulting in the reaction with the homologous and heterologous proteins. Coprecipitation of the proteins in RIP and the lack of reactivity with the heterologous protein in Western blot analysis might suggest that the proteins renature after

reaction pattern in both the nucleus and the cytoplasm (data not shown). All these experiments show that both the 38/39K and the 24K proteins can be detected in the nucleus and cytoplasm, and in membrane fractions.

To determine which virus-specific proteins are exposed on the surface of BDV-infected cells, iodination was employed. As shown in Fig. 7, only the 38/39K protein was readily detected on the surface of BDV-infected cells.
elution from the gel. However, the 38/39K and 24K proteins also possess epitopes not present on the heterologous protein (M Abs 38/17C1 and 24/28D12). This interpretation assumes the presence of overlapping common region(s) and regions that are not present on the heterologous protein. This view is supported by the work of VandeWoude et al. (1990), who suggested shared common sequences in mRNAs, which could represent a ‘nested set’ of partially overlapping mRNAs or mRNAs with common 5’ leader or 3’-specific sequences. Taken together these results show that the 24K protein cannot be a direct product of proteolytic processing of the 38/39K protein, in which case the 24K protein would be a protease-resistant part of the 38/39K protein, carrying a relevant epitope.

Tryptic fingerprint analysis demonstrated the presence of four identical peptides in both the 38/39K and 24K proteins, and provided unequivocal evidence that these two major virus proteins are related.

The fact that these major BDV-specific proteins are related does not simplify the picture of the genomic organization of BDV. From their data VandeWoude et al. (1990) have concluded that a 10.5 kb RNA found by hybridization in BDV-infected rat brain could represent the virus genome. But BDV RNA consists also of 3.6 kb, 2.1 kb and 0.85 kb species which may be products of splicing of the 10.5 kb RNA because their hybridization requires identical sequences. Therefore, it also seems reasonable to view the 38/39K and 24K proteins as products of alternative splicing, which would produce identical N-terminal sequences which could be recognized by the same antibody. However, the possibility that the proteins are derived from the same polyprotein cannot be excluded, nor can other mechanisms such as RNA editing (Cattaneo et al., 1989; Vidal et al., 1990a, b) or frame shifting (Brierley et al., 1987, 1989; Garcia-Barreno et al., 1990), which have been reported for other virus systems.

We have demonstrated that the 24K protein is phosphorylated at serine residues. There is evidence that phosphorylated virus proteins may be involved in uncoating, maturation or assembly, and it has been demonstrated that binding of virus proteins to nucleic acid is affected by phosphorylation (reviewed by Leader & Katan, 1988). Additionally, a functional significance of phosphorylation has been shown for the NS protein of vesicular stomatitis virus, which seems to be involved in RNA synthesis (Banerjee, 1987). Whether the phosphorylated BDV-specific 24K protein might be involved in the regulation of BDV replication or persistence awaits further investigation.

In addition, we have demonstrated that neither protein carries carbohydrate side-chains. The finding that neither protein is glycosylated is unusual because enveloped viruses carry glycoproteins, and we have provided evidence that the 38/39K protein is exposed on BDV-infected cells. Both the 38/39K and the 24K protein were localized to cellular compartments such as the nucleus, cytosol and membrane of BDV-infected cells.

The fact that the 38/39K protein is exposed on the surface of BDV-infected cells is in good agreement with previous work on the pathogenesis of BD. It is possible to induce specific T cells after immunization of Lewis rats with the 38/39K protein and successive restimulation in vitro. This T cell line is phenotypically CD4+ and capable of inducing BD after passive transfer into BDV-infected immuno-suppressed rats (Richt et al., 1989, 1990). This result is supported by the finding that major histocompatibility complex class II-expressing BDV-infected cells or cells primed with purified 38/39K protein serve as antigen-presenting as well as target cells for BDV-specific T cells (Richt & Stitz, 1992). Since we have now demonstrated that the 38/39K protein is expressed on the surface of BDV-infected cells, this protein must be regarded as being of crucial importance for the triggering and/or targeting of 38/39K protein-specific T cells in the brains of infected animals.

We thank Dr H. Becht for his help and Dr B. Boscheck for critically reading the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft (Forschergruppe ‘Pathogenitätmechanismen von Viren’) and is in partial fulfilment of the requirements for the degree Dr rer. nat. for N. Thiedemann, Fachbereich Biologie, University of Giessen, Germany.

References


(Received 1 October 1991; Accepted 7 January 1992)