The NB protein is an integral component of the membrane of influenza B virus

Tatiana Betakova,† Milan V. Nermut and Alan J. Hay

1 National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK
2 National Institute for Biological Standards and Control, South Mimms, Herts EN6 3QG, UK

The results of biochemical and immunoelectron microscopic studies provide evidence that the NB protein is an integral component of the influenza B virion. Its glycosylation and orientation in the membrane were shown to be equivalent to that of NB in the plasma membrane of virus-infected cells. Sensitivity to proteinase K showed that the N terminus is exterior to the virion and gold immunolabelling of freeze-fractured replicas showed that the C terminus is located in the interior of the virion. The similarities between NB of influenza B and M2 of influenza A viruses in structural features, their presence in the virion and possession of an ion channel activity suggest that, by analogy with the M2 protein, NB may also have a role in virus entry.

Introduction

The NB glycoprotein of influenza B viruses is an integral membrane protein encoded by a bicistronic mRNA of the NA gene which contains overlapping reading frames for the neuraminidase and the 100 amino acid NB protein (Shaw et al., 1983). A hydrophobic domain comprising amino acids 19–40 anchors the protein in the membrane with the 18 amino acid N-terminal domain exposed externally and the C-terminal 60 amino acid segment located within the cytoplasm (Williams & Lamb, 1986). Polylactosaminoglycan side-chains are attached to asparagine residues 3 and 7 (Williams & Lamb, 1988). The role(s) of the NB protein in virus replication are not known. On the basis of structural similarities with the M2 protein of influenza A viruses, it was suggested that NB may play an equivalent role in virus entry to that of the M2 ion channel of influenza A viruses (Sugrue & Hay, 1991; Pinto et al., 1992). Recent electrophysiological studies of purified NB incorporated into lipid bilayers (Sunstrom et al., 1996) or NB expressed in mouse erythroleukaemia (MEL) cells (I. Chizhmakov and T. Betakova, unpublished results) have shown that the protein has an ion channel activity. Although it is not clear whether this does relate to a role in virus entry equivalent to that perceived for the M2 protein (Martin & Helenius, 1991) it raises the question of whether or not the protein is a component of influenza B virions.

In this paper we describe the results of biochemical and immunoelectron microscopic experiments which provide evidence that NB is an integral component of the virus membrane and support the notion that it may well have a role in the process of virus infection. During preparation of this manuscript a paper by Brassard et al. (1996) reported a similar conclusion.

Methods

• Virus and cells. Influenza virus B/Johannesburg/26/94 was grown in 10-day-old fertile hen’s eggs. Madin–Darby canine kidney (MDCK) cells were cultured in Eagle’s minimum essential medium (MEM) containing 10% calf serum.

• Antisera. Anti-NB serum was raised in rabbits against a 16 amino acid C-terminal peptide of NB (PSFLPGNLSESTPN) coupled with glutaraldehyde to keyhole limpet haemocyanin (KLH). Antiserum against whole virus was raised in rabbits.

• Purification of virus. Virus in allantoic fluid or tissue culture fluid was pelleted and purified successively on a discontinuous sucrose (30/60%) gradient, a linear sucrose (15–60%) gradient and a self-generating gradient of Iodixanol (Optiprep; Nycomed). Virus in 25% (w/v) Iodixanol was centrifuged at 350,000 g for 4 h in a Beckman TLA 100.3 rotor. To remove non-virus NB protein, virus was further purified...
on a column of protein A-Sepharose 4 Fast Flow (Pharmacia) with bound (4 °C overnight) anti-C-terminal NB antiserum before the Iodixanol gradient.

[^35S]Cysteine-labelled virus was obtained from virus-infected MDCK cells labelled with[^35S]cysteine (10 μCi/ml; Amersham) in MEM minus cysteine without calf serum. Virus was pelleted and purified as above. Purified virus was treated with proteinase K (100 μg/ml in PBS) for 20 min at 37 °C and once again purified on an Iodixanol gradient.

**Analyses of virus-infected cells.** MDCK cells grown in 12-well plates (10⁶ cells per well) were infected with undiluted virus-containing allantoic fluid (0.5 ml, m.o.i. 10^-100), washed and incubated in MEM. To inhibit glycosidases, a mixture of N-methyldeoxynojirimycin (NMdNM; 5 mM), 1,4-dideoxy-1,4-imino-D-mannitol hydrochloride (DIM; 0.25 mM) and deoxygalactonojirimycin (dGJ; 1 mM) (Oxford Glyco system, UK) was added to medium immediately after infection. Endoglycosidase-treatment: cells were washed twice with PBS and incubated for 3 h at 37 °C with 250 μl incubation buffer (20 mM-sodium phosphate pH 7.5, 50 mM-EDTA) containing 1.5 units endoglycosidase F/peptide-N-glycosidase F (Oxford Glyco Systems, UK), washed with PBS and extracted with lysis buffer.

Cells were treated with 100 μg/ml proteinase K in PBS for 1 h at 37 °C, spun down, washed with PBS and extracted with lysis buffer.

**Protein analyses.** Virus, or cells washed with PBS, were lysed in 1% NP40, 150 mM-NaCl, 2 mM-MgCl₂, 1 mM-EDTA, 20 mM-Tris pH 7.5 containing 0.2% soya bean trypsin inhibitor, 10 μg/ml aprotinin and 1 mM-PMSF.

Samples of lysates were made 1% in SDS and 0.1 M in dithiothreitol, heated at 100 °C for 3 min and analysed by electrophoresis on 12% or 17.5% polyacrylamide gels containing 4 M-urea. Immunoblotting was done as described by Grambas et al. (1992) using anti-NB serum (diluted 1:1000), protein A-horseradish peroxidase conjugate and enhanced chemiluminescence (ECL) reagent (Amersham). Gels were stained using a Silver Stain Plus kit (Bio-Rad).

**Electron microscopy – gold immunolabelling.** Purified virus was adsorbed to glow discharged carbon films on 400 mesh grids, washed in PBS and fixed in 1% glutaraldehyde (10 min), extensively washed in distilled water and negatively stained with 4% silicotungstate pH 6.9. Some grids were washed briefly in distilled water after adsorption and negatively stained with 2% uranyl sulphate. The same procedure was applied to virus particles treated with proteinase K.

Gold immunolabelling of virus preparations with anti-NB antibody was as described by Nermut & Nicol (1987) except that the conditioning buffer was replaced by 0.1% bovine serum albumin C (Aurion) and 0.1% fish gelatin in 20 mM-Tris-buffered saline pH 8.2 (TBG). Rabbit antiserum recognizing the C-terminal end of NB or whole virus was used. Preimmune rabbit serum was employed as a control. All antisera were diluted 1:10 in TBG.

For gold immunolabelling of ultrathin sections of virus-infected MDCK cells, cells were fixed in 3% paraformaldehyde 12, 18 or 21 h post-infection (p.i.) and processed using a progressive lowering of temperature protocol (Carlemalm et al., 1982). Lowicryl HM20 embedded specimens were polymerized at -45 °C. Thin sections were labelled with polyclonal antibody to NB as described above.

Gold immunolabelling of freeze-fractured replicas of purified virus suspensions was done essentially as described by Fujimoto (1995). Virus particles were adsorbed to a positively charged piece of mica and fractured as described by Nermut & Williams (1977). Shadowed replicas were floated onto 0.5% NP40 followed by glycin buffer containing 1 M-NaCl and 10 mM-EDTA pH 10 (GNE) for 15 min or onto 5% mercaptoethanol (ME) for 17 min. This treatment removed most of the internal proteins, as observed by negative staining, and improved the access of antibody to the C terminus of NB, as illustrated in the diagrams in Fig. 5. This was followed by repeated washing in Tris-buffered saline pH 7.4, incubation in TBG and gold immunolabelling with antibody to NB or antibody to whole virus. All specimens were observed in a Philips CM12 electron microscope operating at 80 kV.
Results and Discussion

NB synthesized in virus-infected cells

Following infection of MDCK cells with B/Johannesburg/26/94, virus production, as measured by HA titre, increased from 4 h p.i. and reached a maximum at about 12 h p.i. (Fig. 1a). The NB protein was initially detected in immunoblots at about 4 h p.i. mainly as a 18 kDa band (as shown in Fig. 1b) which corresponds to the high mannose form, previously described by Williams & Lamb (1986). As infection progressed different glycosylated forms, presumably with heterogeneous polylactosaminoglycan side-chains (Williams & Lamb, 1988), with apparent molecular masses of 22 kDa, 23–28 kDa and 32–55 kDa became more prominent. Late in infection, after 10 h, a band of about 12 kDa appeared and increased in proportion at later times such that at 24 h it was the predominant form of NB. That this corresponded to unglycosylated NB was shown by treatment of virus-infected cells with endoglycosidase F/peptide-N-glycosidase F, which resulted in a single band corresponding to the 12 kDa form (Fig. 2, lane 2). Treatment of virus-infected cells with proteinase K, which removes most of the extracellular domain of 18 amino acids, gave rise to a single band of about 10-5 kDa (Fig. 2, lane 1). Together, the results of these enzymatic treatments indicate that the majority of the NB protein is expressed on the surface of infected cells. Cycloheximide chase experiments which examined changes in the pattern of NB over 12 h following inhibition of protein synthesis by cycloheximide (50 μg/ml), added at, e.g., 9 h p.i., showed that the non-glycosylated 12 kDa form of the protein was stable over this time, contrary to previous reports of the relative instability of NB (Shaw & Chopin, 1984; Williams & Lamb, 1986). The same experiments showed that removal of the carbohydrate side-chains had a half-life of 2–3 h. Deglycosylation thus appears to be due to extracellular glycosidases and could be prevented by including the glycosidase inhibitors N-methyldeoxynojirimycin (5 mM), 1,4 dideoxy-1,4-imino-D-mannitol hydrochloride (0.25 mM) and deoxygalactonojirimycin (1 mM) in the culture medium throughout infection, although inhibition of trimming of the high mannose precursors resulted in two glycosylated forms of 20 kDa and 23 kDa (data not shown).

NB is associated with purified virus

After purification of virus on sucrose and Iodixanol gradients a low level of non-virion associated NB was observed in virus preparations by immunoelectron microscopy. Such contamination, which may originate from disrupted virus or fragments of plasma membrane of virus-infected cells, was
removed by passage of virus through a column containing antibody against the C terminus of NB.

Western blot analysis of virus samples purified in this way and electrophoresed on 17.5% SDS–polyacrylamide gels containing 4 M-urea indicated the presence of the various forms of NB identified in virus-infected cells. The unglycosylated (12 kDa) and glycosylated (32–55 kDa) forms were present in similar amounts in the sample of egg-grown virus shown in Fig. 3(a), although the relative proportions of the different glycosylated forms varied between different preparations. Similar results were obtained for virus purified from virus-infected MDCK cells.

Treatment of virus with proteinase K converted NB to a 10.5 kDa truncated form detectable with the C-terminal antibody (Fig. 3b, lane 2), as observed after similar treatment of virus-infected cells. If virus was disrupted with 1% NP40 prior to proteinase K treatment no NB was detected, indicating that the C terminus was protected against proteolytic digestion, presumably due to its location within the virion. The heterogeneous glycosylated forms of NB were not detectable by staining or by analysis of [35S]cysteine-labelled proteins (Fig. 3c, lane 2, and Fig. 3d, lane 1, respectively). However, when trimmed with proteinase K a band corresponding to about 10.5 kDa was observed (Fig. 3c, lane 1, and 3d, lane 2, respectively). The [35S]-labelled band (Fig. 3d, lane 2) corresponded to the NB band detected by subsequent immunoblotting (Fig. 3d, lane 3). The low level of radioactivity and the relatively high background precluded accurate estimates of the content of NB. However, taking account of their respective cysteine contents NB appears to be present in molar amounts equivalent to those of the individual polymerase (P) components.

These results indicate that NB is an integral component of the virus membrane oriented with the N-terminal domain external to the virion.

**Immunoelectron microscopy**

MDCK cells were analysed at 12, 18 and 21 h p.i. The highest level of budding or released virus particles was observed in the 18 h sample, although the overall image was similar in all three samples. Gold particles were associated mainly with the plasma membrane, the microvilli and free virus particles (Fig. 4a–e). Labelling of free virus particles, although low, was significant when set against a very low background. Out of 300 particles counted, 77 (25%) were labelled with gold particles; the average number of gold particles per virus particle was 1.8 with a range of 1–4. This is a reasonable level of labelling taking into account the relatively low amounts of NB per virion indicated by gel analyses and the low efficiency of gold immunolabelling of sections.

To label the C terminus of NB without fixation and embedding, the monolayer freeze-fracture technique (Nermut & Williams, 1977) was used. In these experiments two types of fractured images of virus particles are produced (see diagram, Fig. 5). Concave fracture faces, where only the outer surface of the virion is exposed to the antibody, were more frequently observed. These structures were labelled well with antibody to whole virus (Fig. 6a) and were not labelled with anti-NB antibody (large arrow in Fig. 6d). In replicas of convexly fractured virus particles, about 20% in some experiments, the internal surface of the virion membrane was exposed: 15–20% of convex replicas were labelled with antibody to the C terminus of NB and a selection of such particles is shown in Fig. 6(b–d). These convex structures were not labelled with
antibody to the whole virus, indicating that most of the internal proteins were removed by treatment with NP40 followed by GNE or ME. The results of immunoelectron microscopy thus support the results of the biochemical analyses and indicate that NB is an integral component of the virus membrane oriented with its C terminus within the virus particle.

**Conclusion**

The NB protein seems, therefore, to be structurally and functionally analogous to the M2 protein of influenza A viruses. The two proteins have a similar size subunit (100 and 97 amino acids, respectively) and are located in the membrane with a short N-terminal external domain. Both are synthesized in virus-infected cells in amounts comparable to those of other virus membrane proteins but are incorporated into the virion membrane in relatively low amounts (Zebedee & Lamb, 1988). The M2 protein forms a proton channel (Shimbo *et al.*, 1996; Chizhmakov *et al.*, 1996) which can modulate the pH of the exocytic pathway (Sugrue *et al.*, 1990). In addition, it appears that during endocytosis of virus, M2 in the virion allows passage of protons into the virion interior to induce a low pH-dependent dissociation of the matrix (M1) protein from the ribonucleoprotein (RNP), which is necessary to allow entry of the RNP into the nucleus to initiate virus replication (Bukrinskaya *et al.*, 1982; Martin & Helenius, 1991). Although it has recently been shown that NB protein incorporated into lipid bilayers forms cation-permeable channels at physiological pH (Sunstrom *et al.*, 1996), its role in virus infection is unclear. The demonstration that the protein is a component of the virus membrane provides further support for the suggestion that its ion channel activity may play a role in virus entry.

We thank Dr D. J. Hockley (NIBSC) for assistance with low temperature embedding and electron microscopy. T. Betakova gratefully acknowledges the support of a Research Travelling Fellowship (042871/2/94) from the Wellcome Trust.

---

**Fig. 5.** Diagram illustrating convexly (b, left) and concavely (b, right) fractured virus particles and the effects of treatment with detergent and glycine buffer (GNE), as described in Methods. (c) Gold immunolabelling of the C terminus of NB with anti-NB antibody (left) and surface spikes with antibody to whole virus (right). VM, viral membrane; M1, M1 protein; NB, NB protein; NA, neuraminidase; NP, nucleoprotein complex; FE, freeze-etch; GNE, glycine buffer pH 10; CR, carbon replica.
References


Received 22 July 1996; Accepted 12 August 1996

Fig. 6. Shadowed replicas of freeze-fractured virus particles revealing concave fracture faces which were heavily labelled with antibody to the whole virus (o). Convex fracture faces were gold immunolabelled with antibody to the C terminus of NB after treatment of replicas with NP40 followed by GNE pH 10 (small arrows in b, c, d). Note that concavely fractured virus particles are not labelled with anti-NB antibody (large arrow in d). The bar represents 100 nm.