The BM2 protein of influenza B virus is synthesized in the late phase of infection and incorporated into virions as a subviral component

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The influenza B virus genome RNA segment 7 encodes the M1 and BM2 proteins. The BM2 protein is synthesized by a coupled translational termination–reinitiation mechanism at the overlapping stop–start pentanucleotide in a bicistronic mRNA transcribed from RNA segment 7. However, features and functions of this protein remain unclear. In this study the BM2 protein was characterized by using an antiserum raised to the BM2 protein of influenza virus strain B/Yamagata/1/73. In cells infected with B/Yamagata virus the α BM2 antibody specifically detected the BM2 protein with a molecular mass of 12 kDa and also a polypeptide with a molecular mass of 17 kDa. When infected cells were labelled with $^{32}\text{P}_i$ and immunoprecipitated with the α BM2 antibody, the ^{32}P -labelled 17 kDa polypeptide was specifically precipitated. In the presence of casein kinase inhibitor CKI-7 the synthesis of the 17 kDa and BM2 proteins was completely suppressed, although other viral proteins, except for the polymerase protein, were synthesized normally. These results suggest that the 17 kDa species is a phosphorylated form of the BM2 protein. These species were substantially synthesized in the late phase of infection and localized in the cytoplasm throughout infection. Moreover, they were transported to the plasma membrane and thereafter were incorporated into virions. These results therefore suggest that the BM2 and the 17 kDa proteins are necessary for the life-cycle of influenza B virus.

Introduction

Influenza A, B and C viruses are a group of RNA viruses containing negative-stranded, segmented RNA genomes. Both influenza A and B viruses have eight RNA segments that encode one or two virus proteins and are structurally and biochemically similar to each other. A major biochemical difference between influenza A and B viruses with respect to the coding strategy and features of the proteins is found with RNA segment 6. Influenza A virus RNA segment 6 encodes neuraminidase (NA) without a second overlapping reading frame (Lamb, 1989), whereas RNA segment 6 of influenza B virus encodes NA in addition to a virion-associated integral membrane protein NB, using a bicistronic mRNA containing

overlapping open reading frames (ORFs) (Betakova *et al.*, 1996; Brassard *et al.*, 1996; Shaw *et al.*, 1983; Williams & Lamb, 1989). NB protein has ion channel activity and is thought to play a role in the virus replication cycle equivalent to that of the integral membrane protein M2 of influenza A virus (Sunstrom *et al.*, 1996).

Another major difference between influenza A and B viruses is found on RNA segment 7. Influenza A virus RNA segment 7 encodes both the membrane (matrix) protein M1, which is translated from a collinear transcript mRNA (Lamb, 1989), and the M2 protein, which is translated from a spliced mRNA (Lamb *et al.*, 1981). The M2 protein, like the NB protein, shows ion channel activity, which is responsible for dissociation of the M1 protein from viral ribonucleoprotein (vRNP) during the uncoating process and for preserving the native conformation of the haemagglutinin (HA) during virion assembly (Holsinger *et al.*, 1994; Pinto *et al.*, 1992; Steinhauer *et al.*, 1991). Similarly, RNA segment 7 of influenza B virus encodes two proteins, M1 and BM2, by a bicistronic mRNA (Briedis *et al.*, 1982; Hiebert *et al.*, 1986). However, the

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translational strategy of the BM2 protein is quite different from the bicistronic mRNA transcribed from influenza B virus RNA segment 6. In the mRNA transcribed from RNA segment 7, there is a pentanucleotide, residues 769–773, in which the AUG initiation codon for the BM2 protein overlaps with the termination codon for the M1 protein (Horvath *et al.*, 1990). The BM2 protein is synthesized by a coupled translational stop–start mechanism at the pentanucleotide which is dependent upon the initiation and termination of the upstream M1 protein (Horvath *et al.*, 1990). Since the amino acid alignment in the BM2 ORF has been highly conserved for at least 39 years, from influenza B/Lee/40 virus to B/Singapore/222/79 virus (Hiebert *et al.*, 1986), the BM2 protein is thought to have an important role(s) in the life-cycle of influenza B virus. Although the BM2 protein (molecular mass of 12 kDa) has been identified in influenza B virus-infected cells (Horvath *et al.*, 1990), its function(s) remains unknown.

In this study we developed an antiserum against the hexahistidine–BM2 fusion protein expressed in *Escherichia coli*. Using the antiserum we show the features of the BM2 protein in cells infected with influenza B/Yamagata/1/73 virus and in the virion. In infected cells the BM2 protein was found to be phosphorylated and the product was detected as a 17 kDa polypeptide. These molecules were localized in the cytoplasm and then incorporated into virions, suggesting that they are necessary for the growth of influenza B virus.

Methods

■ **Culture cells and virus.** MDCK cells were cultured in Eagle's minimal essential medium supplemented with 10% foetal calf serum (FCS). COS-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS. Influenza viruses B/Yamagata/1/73 and A/WSN/33 were propagated in MDCK cells in Opti-MEM I (Gibco BRL) containing 4 µg/ml *N*-acetyl trypsin.

■ **Plasmid construction and antiserum preparation.** The full-length cDNA copy of genome RNA segment 7 of influenza B/Yamagata virus was amplified by PCR using primers 5' GCGCGCGGATCCCT-TGAACCATTTTCAGATTCTTTCAA and 5' GCGCGCAAGCTTTTA-ATGCAATTCTTCTACCTCCAAACT. For construction of the BM2 expression vector, the PCR products containing the BM2 ORF, nucleotide residues 771–1100, were inserted into the *Eco*RI and *Nhe*I sites in the expression vector pCAGGS/MCS (Niwa *et al.*, 1991). To express the BM2 protein in mammalian cells, the resultant vector, pCAGGS/BM2, was transfected into COS-1 cells after incubation with LipofectAmine Plus reagent (Gibco BRL). For production of the hexahistidine-tagged BM2 fusion protein in *E. coli*, the PCR products ranging from residues 774 to 1100 and unique restriction enzyme sites were inserted into the *Bam*HI and *Hind*III sites in pTrcHis A (Invitrogen). The hexahistidine-tagged BM2 protein was expressed in and purified from *E. coli* strain JM109 according to protocols provided by the manufacturer.

To produce antiserum specific for the BM2 protein, a rabbit was immunized with 1.0 mg of the affinity-purified hexahistidine-tagged BM2 protein in complete Freund's adjuvant, followed by three booster

injections of 500 µg each in incomplete Freund's adjuvant at 2-week intervals. Similarly, the antiserum specific for nucleoprotein (NP) was made using the B/Yamagata virus NP purified from the vRNP complex (Parvin *et al.*, 1989) by SDS–PAGE.

■ **Virus infection and treatment with a protein kinase inhibitor.** MDCK cells grown in 8 cm² dishes were infected with virus at an m.o.i. of 10 p.f.u. per cell and incubated at 34 °C. At various times post-infection (p.i.), the cells were lysed in 50 µl of radioimmuno-precipitation assay (RIPA) buffer (200 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS and 50 mM Tris–HCl pH 8.0) and maintained for 30 min on ice. After clarification by centrifugation, the supernatant was mixed with an equal volume of SDS sample buffer as described previously (Peluso *et al.*, 1977), boiled for 2 min at 95 °C and subjected to SDS–PAGE as described below.

To inhibit phosphorylation of viral proteins, virus-infected cells were treated at various times p.i. with Opti-MEM I containing 200 µM CKI-7 [*N*-(2-aminoethyl)-5-chloro-isoquinoline-8-sulfonamide] (Seikagaku) to inhibit casein kinase I and II (Chijiwa *et al.*, 1989; Krantz *et al.*, 1997).

■ **Metabolic labelling of mammalian cells.** ³²P_i-labelling of virus-infected MDCK cells was carried out by the methods described previously (Whittaker *et al.*, 1995). Briefly, at 7 h p.i. virus-infected cells were labelled for 3 h with phosphate-free DMEM containing 0.5 mCi/ml ³²P_i (Amersham Life Science). At the end of the labelling period, cells were washed twice with PBS and lysed in RIPA buffer. For production of ³²P_i-labelled virions, virus-infected cells were labelled for 2 days with phosphate-free DMEM containing 0.1 mCi/ml ³²P_i. The labelled virus in the culture medium was purified through a 30–50% sucrose gradient as described previously (Odagiri *et al.*, 1982).

For production of ³⁵S-labelled virions, virus-infected cells were labelled with DMEM deficient in methionine and cysteine containing 20 mM HEPES pH 7.1 with 70 µCi/ml ³⁵S-methionine/cysteine (Amersham Life Science) and one-quarter volume of Opti-MEM I. After incubation for 2 days, the labelled virions were purified as described above.

■ **Immunoprecipitation, SDS–PAGE and Western blotting.** For immunoprecipitation, the samples, lysed in RIPA buffer, were incubated with antiserum αBM2#3, αNP, αM1 (mouse monoclonal antibody against B/Nagasaki/1/87) (Nakagawa *et al.*, 1999) or pre-immune normal rabbit serum (final dilution of each antibody was 1:20) at 4 °C overnight. The immune complexes were incubated with protein A–Sepharose for 30 min at room temperature. The samples were washed twice with washing buffer I (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.1% Triton X-100) and twice with washing buffer II (50 mM Tris–HCl pH 8.0, 150 mM NaCl) before resuspension in SDS–PAGE sample buffer. Proteins were analysed by SDS–PAGE using a 15% polyacrylamide gel as described previously (Odagiri & Tobita, 1990). Rainbow coloured protein molecular mass standards (Amersham Life Science) were used as markers.

For Western blot analysis, the proteins were resolved by SDS–PAGE and electroblotted onto PVDF membrane (Amersham Life Science). After incubation with an antibody, the membrane was treated with horseradish peroxidase-conjugated anti-rabbit Ig and enhanced chemiluminescence (Amersham Life Science) and exposed to RX-U film (Fuji Photo).

■ **Fractionation of purified virions.** Purified B/Yamagata/1/73 virus was fractionated by the method described previously (Odagiri & Tashiro, 1997; Parvin *et al.*, 1989) with modifications. Briefly, viruses disrupted by incubation in 1.5% Triton N-101, 10 mg/ml lysolecithin,

0.1 M Tris-HCl pH 8.0, 0.1 M KCl, 5 mM MgCl₂, 5% glycerol and 1.5 mM DTT were subjected to a step-wise gradient consisting of 1.5 M CsCl and 30% glycerol, 2 M CsCl and 35% glycerol, 2.5 M CsCl and 40% glycerol, and 3 M CsCl and 45% glycerol containing 100 mM NaCl and 50 mM Tris-HCl pH 7.6 and centrifuged for 20 h at 45 000 r.p.m. in an SW55Ti rotor at 4 °C. Samples (300 µl) of the CsCl gradient were collected from the top to the bottom and pelleted by centrifugation for 120 min at 45 000 r.p.m. The pellets were resuspended in 25 µl PBS and an equal volume of each fraction was subjected to SDS-PAGE and detected by silver staining and Western blotting.

■ **Indirect immunofluorescence.** MDCK cells on glass coverslips were infected with B/Yamagata virus and incubated for various periods at 34 °C. Cell monolayers were washed three times with PBS, fixed in 4% paraformaldehyde for 10 min at room temperature and permeabilized with 0.2% Triton X-100 in PBS for 2 min. After washing with PBS, α BM2#3 antibody, which had been adsorbed with lysates of uninfected MDCK cells, was added to a final dilution of 1:1000. After incubation for 60 min, the cells were washed with PBS and incubated for 30 min with FITC-conjugated goat anti-rabbit IgG (H+L) (Funakoshi) at a final dilution of 1:150. After washing with PBS, the coverslips were mounted onto glass slides and examined by fluorescence microscopy.

Results

Detection of influenza B virus BM2 protein in infected cells by an anti-BM2 antibody

Previously, it has been demonstrated that the electrophoretic mobility of the BM2 protein is variable among influenza B virus strains (Horvath *et al.*, 1990). To show whether or not the BM2 protein of influenza B/Yamagata/1/73 strain could represent the prototype BM2, we initially determined the complete nucleotide sequence of a cloned full-length DNA copy of genome RNA segment 7. The result has been deposited in the GenBank database (accession no. AF077348) (Fig. 1A). RNA segment 7 of B/Yamagata virus consisted of 1188 nucleotides and the identity to the nucleotide alignment of B/Lee virus was 91.7%. The overlapping translational stop-start pentanucleotide, TAATG, was found at nucleotide residues 769–773, as reported with B/Lee virus (Briedis *et al.*, 1982). When the amino acids deduced from the +2 ORF, nucleotide residues 513–1100, were compared between these viruses, 31 out of the 195 residues were different (84.1% identity). In the BM2 ORF, nucleotide residues 771–1100, only 14 out of the 109 amino acids were different (87.2% identity), indicating that the amino acids in the BM2 protein of B/Lee virus were highly conserved by B/Yamagata virus. It is, therefore, likely that the BM2 protein of B/Yamagata virus is also translated from codon 771ATG in the pentanucleotide in the same manner as B/Lee virus.

Based on the observations, we synthesized a hexahistidine-tagged BM2 protein to generate anti-BM2 antibody as described in Methods and shown in Fig. 1(A). The antiserum, designated α BM2#3, was shown by Western blot and dot blot analyses to be specific for both the hexahistidine-BM2 fusion protein and a synthetic peptide corresponding to amino acid residues 38–57 of the BM2 ORF (data not shown).

To examine whether the α BM2#3 antibody specifically detected the BM2 protein of B/Yamagata virus, virus-infected cells were harvested at 9 h and 10 h p.i. and examined by Western blot analysis after SDS-PAGE. The α BM2#3 antibody detected two polypeptides with molecular masses of about 12 kDa and 17 kDa (Fig. 1B, lanes Y-9h and Y-10h). This antibody, however, did not react with any other viral proteins, indicating that it was specific for only those two polypeptides. Interestingly, the amount of 17 kDa species increased with the incubation period. In contrast, no specific reactants were detected in cells infected with A/WSN virus and in uninfected cells (Fig. 1B, lanes W and M). The results indicated that these two polypeptides were encoded by B/Yamagata virus, and were not host products induced by virus infection. Since the BM2 ORF of B/Yamagata virus was shown to encode a 12.6 kDa maximum polypeptide by computer analysis, the 12 kDa polypeptide was considered to correspond to the BM2 protein of B/Yamagata virus. To demonstrate this, we constructed an expression plasmid, pCAGGS/BM2, containing the BM2 ORF of B/Yamagata virus as described in Methods and examined whether this vector expressed the 12 kDa polypeptide in transfected COS-1 cells. As expected, it expressed the 12 kDa polypeptide by 20 h post-transfection, although an unexpected band was also detected (Fig. 1B, lane BM2–20h). In contrast, no specific band was detected in cells transfected with vector without the BM2 ORF (Fig. 1B, lane Vect). Therefore, we concluded that the 12 kDa polypeptide in infected cells was the BM2 protein of B/Yamagata virus. It should be noted that when the pCAGGS/BM2-transfected cells were incubated for 30 h, a band corresponding to the 17 kDa species in addition to BM2 was also detected (Fig. 1B, lane BM2–30h).

BM2 protein is synthesized in the late phase of infection

We next examined the kinetics of viral protein synthesis during the infection cycle. When MDCK cells were infected with B/Yamagata virus and pulse-labelled for 30 min at 10 h p.i. with a mixture of [³⁵S]methionine and [³⁵S]cysteine, the viral proteins, polymerase (Ps), HA, NP, nonstructural protein (NS1) and M1, were readily detected (data not shown). However, the BM2 and 17 kDa species were not detected by metabolic labelling, even when the cells were labelled for 1 h (data not shown). Therefore, the viral proteins synthesized every hour were analysed by high-sensitive Western blotting using a mixture of the α BM2#3 antibody and the α B/Yamagata antibody, which was raised to the whole virion of B/Yamagata virus. Before analysis we ensured that the kinetics of major viral protein synthesis, which were detected by Western blotting, corresponded well to that detected by the metabolic labelling and that the α B/Yamagata antibody did not interfere with the reaction between the α BM2#3 antibody and the BM2 and 17 kDa protein (data not shown).

Fig. 1. Synthesis of a hexahistidine-tagged BM2 protein for production of an antiserum to the BM2 protein and detection of the BM2 protein in virus-infected cells. (A) The B/Yamagata/1/73 segment 7 cDNA encodes the M1 protein consisting of 248 amino acids (aa) (shaded box). The +2 frame contains an untranslated ORF and BM2 ORF encoding 109 aa (hatched box). To produce an inducible hexahistidine-tagged BM2 protein in *E. coli*, the BM2 ORF corresponding to aa residues 2–109 was constructed in pTrcHis. The fusion protein in *E. coli* was purified and used for immunization of a rabbit. (B) MDCK cells infected with B/Yamagata/1/73 virus were incubated for 9 h (lane Y-9h) and 10 h (lane Y-10h) and the BM2 protein in the cell lysates was detected by Western blotting using the anti-BM2 antibody. Lanes W and M indicate the A/WSN virus-infected cells and uninfected cells, respectively. COS-1 cells were transfected with the pCAGGS/BM2 vector, which contained the BM2 ORF of B/Yamagata virus, and incubated for 20 h and 30 h (lanes BM2-20h and BM2-30h). The BM2 protein expressed from the vector was detected in the same manner. Lane 'Vect' indicates the cells transfected with vector without the BM2 ORF. Molecular masses of protein bands were calculated from standards.

Fig. 2. Time-course of viral protein synthesis of the B/Yamagata/1/73 strain. Infected and uninfected (mock) cells were subjected to SDS-PAGE and analysed by Western blotting using a mixture of the anti-BM2 and the anti-B/Yamagata antibodies. The numbers at the top of lanes indicate time (h) after infection.

As shown in Fig. 2, the BM2 protein was first detected 4 h p.i. and its amount gradually increased with the incubation period. Synthesis of this protein was shown to occur at least 1 h and 2 h later than that of M1 and NP proteins, respectively. The kinetics of BM2 protein synthesis appeared similar to that of HA protein, which is synthesized in the late phase of infection. On the other hand, the synthesis of the 17 kDa species occurred 6 to 7 h p.i. (Fig. 2, lanes 6 and 7).

The 17 kDa polypeptide is a phosphorylated form of the BM2 protein

Since the synthesis of the 17 kDa species was found to occur at least 2 h later than that of the BM2 protein (Fig. 2), it was postulated that this molecule could be a posttranslationally modified product of the BM2 protein. We therefore examined the amino acid sequence of the B/Yamagata virus BM2 protein by the computer program Motif Libraries in the GenomeNet (ICR, Kyoto University) and found three motifs for casein kinase II-mediated phosphorylation at Thr-69, Ser-91 and Thr-101, and one motif for protein kinase C-mediated phosphorylation at Thr-69, as shown in Table 1. To demonstrate whether the BM2 protein is actually phosphorylated in infected cells to produce the 17 kDa molecule, virus-infected cells were labelled for 3 h at 7 h p.i. with $^{32}\text{P}_i$ and the cell lysate was immunoprecipitated with the $\alpha\text{BM2}\#3$ antibody (Fig. 3 A, lanes Y-IP and M-IP). In infected cells a broad ^{32}P -labelled band was detected at the position corresponding to the electrophoretic mobility of the 17 kDa polypeptide, whereas in uninfected cells no specific band of either 17 kDa or 12 kDa was detected. From these results, it was concluded that the 17 kDa species was a phosphoprotein.

In order to further confirm that the 17 kDa species was the phosphorylated product of the BM2 protein, virus-infected cells were treated with CKI-7, which is a casein kinase inhibitor (Chijiwa *et al.*, 1989; Krantz *et al.*, 1997), from 2 h and 4 h p.i. to 10 h p.i. and the viral proteins synthesized in the presence

Table 1. Putative phosphorylation sites in the BM2 protein of B/Yamagata/1/73 virus

BM2 Residue	Recognition site*	Recognition motif	Protein kinase
Thr-69	69 TIKE 72	S/TXXEX	Casein kinase II
Thr-69	69 TIK 71	S/TXT/R	Protein kinase C
Ser-91	91 SAEE 94	S/TXXEX	Casein kinase II
Thr-101	101 TVLE 104	S/TXXEX	Casein kinase II

* Phosphorylation residue in bold.

† From Hardie (1993).

Fig. 3. Detection of ^{32}P -labelled polypeptides by immunoprecipitation with the anti-BM2 antibody and synthesis of the BM2 protein in the presence of protein kinase inhibitor. (A) At 7 h p.i., virus-infected (lane Y-IP) and uninfected (lane M-IP) cells were labelled for 3 h with ^{32}P , and cell lysates were immunoprecipitated with the anti-BM2 antibody. The ^{32}P -labelled band was detected by SDS-PAGE followed by autoradiography exposed for 4 days. Simultaneously, the BM2 and 17 kDa proteins were detected by Western blotting as markers (lane Y). (B) Virus-infected cells were treated with protein kinase inhibitor CKI-7 at 2 h p.i. (lane Y+CKI-7-2h) and 4 h p.i. (lane Y+CKI-7-4h) and incubated for 10 h. Viral proteins synthesized in the presence or absence (lane Y) of inhibitor were detected by Western blotting using the anti-B/Yamagata antibody (i) and the anti-BM2 antibody (ii).

of the inhibitor were analysed by Western blotting. All major viral proteins, NP, HA and M1, except for Ps were synthesized normally, i.e. like those synthesized without the inhibitor (Fig. 3Bi). On the other hand, the synthesis of BM2 and 17 kDa proteins was not observed at all when treated from 2 h p.i. and a trace amount of the BM2 protein was detected when treated from 4 h p.i. (Fig. 3Bii). These results indicate that 17 kDa protein synthesis corresponded well with BM2 protein synthesis.

Localization of BM2 protein in infected cells

To identify the intracellular localization of the BM2 protein throughout the infection cycle, virus-infected cells were fixed at 2 h intervals from 4 h to 10 h p.i. and examined by indirect immunofluorescence using the $\alpha\text{BM2\#3}$ antibody previously adsorbed with lysates of uninfected MDCK cells (Fig. 4). At

4 h p.i., when BM2 protein became detectable in infected cells (Fig. 2), weak fluorescent signals were observed around the nuclear membrane, where the ER/Golgi complex is known to be located (Fig. 4A). The signals became stronger with increased incubation period and a part of the signal was shown to diffuse in the cytoplasm (Fig. 4B). At 8 h p.i., fluorescence was localized along both the nuclear and plasma membranes and at 10 h p.i. strong signals were seen along the periphery of the plasma membrane (Fig. 4C, D). On the other hand, when virus-infected cells were neither fixed nor permeabilized, no specific fluorescent signal was observed, similar to uninfected cells (Fig. 4F–H), indicating that the BM2 protein was not expressed on the cell surface. Although the $\alpha\text{BM2\#3}$ antibody detected both the BM2 and the 17 kDa proteins in infected cells, as shown in Fig. 1(B), the quantitative analysis of these polypeptides detected at 9 h p.i. by scanning of an X-ray film using the Diversity Database software (PDI, USA) indicated that the ratio of BM2 to 17 kDa protein was about 40:1. Therefore, the fluorescent signals detected in infected cells were thought to represent the BM2 protein. From these results, it was most likely that the BM2 protein was transported from the site of synthesis along the nuclear membrane to the site of virion budding on the plasma membrane, but not expressed on the cell surface. It should be emphasized that the BM2 protein was not found in the nucleus throughout infection.

The BM2 and 17 kDa proteins are incorporated into virions

From the profiles of intracellular localization of the BM2 protein, we postulated that it would be incorporated into virions. To examine this, purified virions of B/Yamagata virus were analysed by Western blotting. As shown in Fig. 5(A), both the BM2 and 17 kDa proteins were clearly demonstrated in the virion preparation (lane Y-V), although the amount of 17 kDa protein in virions was slightly decreased compared to that in infected cells (compare lanes Y-V and Y-L). These polypeptides were not detected in A/WSN virions used as a control (Fig. 5A, lane W-V), indicating that they were specific for only influenza B virus. Consistent results were also obtained by the immunoprecipitation of ^{32}P -labelled virions with the $\alpha\text{BM2\#3}$ antibody. In virions two ^{32}P -labelled bands cor-

Fig. 4. Distribution of the BM2 protein in virus-infected cells. Virus-infected cells were incubated for 4 h (A), 6 h (B), 8 h (C) and 10 h (D). At the indicated times, infected cells were fixed and analysed by indirect immunofluorescence using the anti-BM2 antibody adsorbed with the lysates of uninfected MDCK cells. (E) Uninfected cells fixed 10 h after incubation. (F) and (G) show the immunofluorescence of virus-infected cells at 8 h and 10 h p.i. without fix and permeabilization with Triton X-100. (H) Uninfected and non-permeabilized cells at 10 h after incubation.

Fig. 5. (A) Identification of the BM2 and the 17 kDa proteins in B/Yamagata virions (lane Y-V) and in A/WSN virions (lane W-V). An equal amount of the purified virions was analysed by Western blotting using the anti-BM2 antibody. Lane Y-L, cell lysate infected with B/Yamagata virus. (B) Identification of ^{32}P -labelled BM2 and 17 kDa proteins in B/Yamagata virions. ^{32}P -labelled virions were immunoprecipitated with the anti-BM2 antibody and the precipitates were analysed by SDS-PAGE followed by autoradiography (9 day exposure) (lane V-IP). The BM2 and 17 kDa proteins in the virion were also detected by Western blotting as markers (lane V).

responding to the 17 kDa and BM2 species were detected, although the lower molecular mass species appeared as a trace amount (Fig. 5B, lane V-IP). These results may imply that the BM2 protein itself was phosphorylated at a low level, although the ^{32}P -labelled BM2 band was not detected in infected cells. Consequently, it can be postulated that both the BM2 and the

17 kDa proteins were phosphorylated to different extents and then incorporated into virions as subviral components.

We next examined whether the BM2 and 17 kDa proteins associate with other viral proteins in virions. Purified virions were disrupted with detergents and fractionated as described in Methods. Fig. 6(A, B) shows the viral components in all fractions collected from the top to the bottom of the CsCl gradient. By a single cycle of the fractionation, most viral components were not sufficiently separated, so that most fractions contained whole viral components in different amounts. However, the virions deficient in the NP and P polypeptides were obtained in fraction 1 (Fig. 6A, lane 1) and the NP component with a small amount of HA protein was obtained in fractions 5 to 7 (Fig. 6A, lanes 5–7). The BM2 and 17 kDa proteins were found in fraction 1, but not in fractions 5 to 7. These results indicated that the BM2 and 17 kDa proteins did not firmly associate with NP, P and HA proteins in virions.

To assess further whether the M1 protein was associated with the BM2 and 17 kDa proteins, the B/Yamagata virions lysed in RIPA buffer were immunoprecipitated with αM1 in addition to αNP antibody. The specificity of these antisera was ensured by immunoprecipitation of ^{35}S -labelled virions (Fig. 6C, lanes αM1 and αNP). Because the BM2 and 17 kDa species were not detected by autoradiography of the ^{35}S -labelled virion preparation (Fig. 6C, lane V), they were detected by Western blotting after the immunoprecipitation. As shown in Fig. 6(D), very small amounts of the BM2 and 17 kDa species were precipitated by the αM1 antibody and only a trace amount of the BM2 protein was precipitated by the αNP antibody. These

Fig. 6. Identification of the BM2 and the 17 kDa proteins in fractionated virions. Purified virions of B/Yamagata virus were disrupted and fractionated by a CsCl/glycerol step-gradient centrifugation as described in Methods. The viral proteins in all fractions collected from the top (fraction 1) to the bottom (fraction 12) were analysed by silver staining (A) and Western blotting using the anti-BM2 antibody (B). Lane V indicates whole virions as a marker lane. (C) Immunoprecipitation of ^{35}S -labelled B/Yamagata virions. The virions labelled with ^{35}S -methionine/cysteine were immunoprecipitated with anti-M1 (lane αM1), anti-NP (lane αNP), anti-BM2 (lane αBM2) or preimmune normal rabbit serum (lane Pre). The precipitates were analysed by SDS-PAGE followed by autoradiography. Lane V indicates ^{35}S -labelled whole virions. (D) Immunoprecipitates of the B/Yamagata virions detected by Western blotting. Virions were immunoprecipitated with anti-NP (lane αNP), anti-M1 (lane αM1) or preimmune normal rabbit serum (lane Pre). The precipitates were then detected by Western blotting using the anti-BM2 antibody. Unlike the anti-NP antibody and preimmune normal serum, the anti-M1 antibody used for immunoprecipitation was mouse serum. The H and L chains of the anti-M1 antibody were not strongly visible by Western blotting using a horseradish peroxidase conjugated anti-rabbit Ig as a second antibody, although the detection the BM2 and 17 kDa proteins was not influenced.

precipitates were evaluated to be less than 3.1% of the BM2 and 17 kDa species present in the intact virion (Fig. 6D, lane V). In addition, neither M1 nor NP in the virion was immunoprecipitated with the $\alpha\text{BM2}\#3$ antibody (Fig. 6C, lane αBM2).

Discussion

In the present study, using an antiserum raised to the BM2 protein of influenza B/Yamagata/1/73 virus, we showed that the BM2 protein of 12 kDa and its phosphorylated form of

17 kDa were detected in infected cells. These species were synthesized in the late stage of the infection cycle and were found in the cytoplasm throughout infection. Moreover, they were incorporated into virions as subviral components.

In cells infected with B/Yamagata virus, the αBM2 antibody specifically detected two polypeptides with molecular masses of 12 kDa and 17 kDa (Fig. 1B). Since these polypeptides were not detected in either uninfected cells or A/WSN virus-infected cells (Fig. 1B), it was apparent that they were the viral products encoded by B/Yamagata virus. Supporting evidence was obtained with the BM2 expression vector which contained the BM2 ORF of B/Yamagata virus. It predominantly synthesized the 12 kDa species in transfected cells and additionally synthesized the 17 kDa species after a long incubation time (Fig. 1B). These results strongly suggest that both the 12 kDa and the 17 kDa species are translated from the BM2 ORF. However, most influenza B viruses examined so far have shown a single BM2 band (Horvath *et al.*, 1990). It is therefore interesting to know whether our αBM2 antibody reacted with a degradation product derived from another viral protein(s) that was detected as the 17 kDa species or whether the 17 kDa species was found only with B/Yamagata virus. Since we have confirmed that other viral proteins were not detected with the αBM2 antibody (Fig. 1B), the former possibility can be excluded. Examination using other influenza B viruses, B/Aichi/2/82 and B/Aichi/5/88, showed that our αBM2 antibody detected only a single BM2 band which had different electrophoretic mobilities (unpublished data), consistent with the previous observation that the mobility of the BM2 protein was variable among virus strains (Horvath *et al.*, 1990). Therefore, the 17 kDa species was a product specific for B/Yamagata virus.

To obtain further information regarding the origin of the 17 kDa species, we analysed the nucleotide sequences of B/Yamagata virus RNA segment 7. As reported with B/Lee virus (Briedis *et al.*, 1982), there are two ORFs in this segment (Fig. 1A). The M1 protein is believed to be translated from nucleotide residues 25–769 in the +0 frame (Lamb & Krug, 1996). If a small number of polypeptides was additionally translated from the second and third AUG codons located at residues 280 and 331, respectively, in the +0 frame, the deduced molecular mass of the polypeptides should be 17.8 kDa and 16.0 kDa. However, the amino acid identity of these polypeptides to the BM2 protein was less than 15% and no cluster matched to the amino acid alignment of BM2 was found, indicating that the 17 kDa species was not translated from the +0 frame. On the other hand, the second ORF, nucleotide residues 513–1100, in the +2 frame, has a capacity to encode a 195 amino acid polypeptide with a molecular mass of 22.4 kDa. If translation occurred from upstream of the 771AUG initiator codon, the resultant product should possess the same antigenicity as part of the BM2 protein. However, there is no AUG codon between residues 513 and 771. Similarly, there is no GUG triplet, which is known to be used

as an initiator codon about one-thirtieth as frequently as the AUG initiator codon in *E. coli* (Kozak, 1983), in the upstream region. Consequently, consistent with the observation with the BM2 expression vector, the 17 kDa species was thought to be a product translated from the BM2 ORF, but not a product additionally translated from upstream in the +2 ORF independent of the BM2 protein. This conclusion is also sustained by the result that the synthesis of 17 kDa protein was coupled with that of BM2 protein, because their synthesis did not occur in the presence of a protein kinase inhibitor, CKI-7, when added from the early phase of infection, although other viral proteins except for Ps were normally synthesized (Fig. 3B).

The NP and M1 proteins of influenza A virus have been shown to occur in two forms both in the infected cells and in the virion, dependent on the difference in phosphorylation residues (Zhirmov & Bukrinskaya, 1981; Almond & Felsenreich, 1982; Gregoriades *et al.*, 1984). The BM2 protein of B/Yamagata virus may be similar, because it contains three motifs for casein kinase II-mediated phosphorylation and one motif for protein kinase C-mediated phosphorylation (Hardie, 1993; Table 1). In fact, the ³²P-labelled 17 kDa species was detected both in infected cells and in virions (Figs 3A and 5B). Similarly, the ³²P-labelled BM2 band, in extremely low levels, was also detected in virions, although it was not definitely detected in infected cells (Fig. 5B). These results may imply that the number and the site of phosphorylated residues are different between the 17 kDa and the BM2 proteins. The precise phosphorylation site(s) in the BM2 molecule remain to be identified.

Synthesis of the BM2 protein of B/Yamagata virus occurred at least 1 h later than that of the M1 protein and was substantial in the late stage of virus infection (Fig. 2). It has been shown that BM2 protein synthesis is highly controlled by the termination of the upstream M1 protein synthesis (Horvath *et al.*, 1990). This was confirmed by our expression vector, which contained both the M1 ORF and the BM2 ORF of B/Yamagata virus, in that maximum synthesis of the BM2 protein was delayed until at least 6 h after that of the M1 protein (unpublished data). Taken together, these findings strongly suggest that the BM2 protein mainly functions in the late stage of virus infection. In the viral proteins acting in the late phase of infection, the M1 and the nuclear export protein of influenza A virus have been well characterized. These proteins accumulate in the nucleus and regulate the nucleocytoplasmic transport of the vRNP by direct or indirect binding with the vRNP (Enami *et al.*, 1993; Martin & Helenius, 1991; Neumann *et al.*, 1997; O'Neill *et al.*, 1998; Ye *et al.*, 1989; Ward *et al.*, 1995; Yasuda *et al.*, 1993). In contrast, the BM2 protein was not detected in the nucleus throughout infection (Fig. 4). This is consistent with the fact that the BM2 protein did not contain a conventional nuclear localization signal (NLS) like the classical mono and bipartite NLS as found with simian virus 40 T antigen and nucleoplasmin (Nigg, 1997)

or the NLS motifs found with the influenza A virus NP protein (Wang *et al.*, 1997). Consequently, it is clear that the BM2 protein did not contribute to the processes occurring in the nucleus, i.e. transcription and replication of virus genome RNA and nuclear export of vRNP.

It was demonstrated that B/Yamagata virions contained both the BM2 and the 17 kDa proteins (Fig. 5). It was also deduced from the immunofluorescence study that in the late phase of infection the BM2 protein was transported to the virion budding site on the plasma membrane (Fig. 4). However, it is unlikely that the packaging of the BM2 and the 17 kDa proteins was caused by direct association with other viral protein(s) in the cytoplasm. By the fractionation and immunoprecipitation of virion with the antibodies to NP, M1 and BM2 proteins, we could not precisely identify a viral protein which firmly bound to the BM2 protein, although a small amount of the BM2 protein coprecipitated with the M1 and NP (Fig. 6). Similarly, no BM2-interactive viral protein was identified by a protein-protein interaction assay using the GST-BM2 fusion protein and the yeast two-hybrid system (unpublished data). Presumably, the BM2 and the 17 kDa proteins may move to the virion budding site independently of other viral protein(s) and thereafter be incorporated into virions as free molecules.

The role of the BM2 protein in the life-cycle of influenza B virus is not known at present. However, a number of the BM2 molecules were phosphorylated to become the 17 kDa species and these species were present in virions. Although no counterpart to these species has been found so far in influenza A virus, analysis of the function of the BM2 protein and the role of phosphorylation of the BM2 protein should be clarified for understanding and possible identification of such protein(s) in influenza A virus.

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References

- Almond, J. W. & Felsenreich, V. (1982). Phosphorylation of the nucleoprotein of an avian influenza virus. *Journal of General Virology* **60**, 295–305.
- Betakova, T., Nermut, M. V. & Hay, A. J. (1996). The NB protein is an integral component of the membrane of influenza B virus. *Journal of General Virology* **77**, 2689–2694.
- Brassard, D. L., Leser, G. P. & Lamb, R. A. (1996). Influenza B virus NB glycoprotein is a component of the virion. *Virology* **220**, 350–360.

- Briedis, D. J., Lamb, R. A. & Choppin, P. W. (1982). Sequence of RNA segment 7 of the influenza B virus genome: partial amino acid identity between the membrane proteins (M1) of influenza A and B viruses and conservation of a second open reading frame. *Virology* **116**, 581–588.
- Chijiwa, T., Hagiwara, M. & Hidaka, H. (1989). A newly synthesized selective casein kinase I inhibitor, *N*-(2-aminoethyl)-5-chloroisoquinoline-8-sulfonamide, and affinity purification of casein kinase I from bovine testis. *Journal of Biological Chemistry* **264**, 4924–4927.
- Enami, K., Qiao, Y., Fukuda, R. & Enami, M. (1993). An influenza virus temperature-sensitive mutant defective in the nuclear–cytoplasmic transport of the negative-sense viral RNAs. *Virology* **194**, 822–827.
- Gregoriades, A., Christie, T. & Markarian, K. (1984). The membrane (M1) protein of influenza virus occurs in two forms and is a phosphoprotein. *Journal of Virology* **49**, 229–235.
- Hardie, D. G. (1993). *Protein Phosphorylation: A Practical Approach*, 1st edn. Edited by D. G. Hardie. Oxford: Oxford University Press.
- Hiebert, S. W., Williams, M. A. & Lamb, R. A. (1986). Nucleotide sequence of RNA segment 7 of influenza B/Singapore/222/79: maintenance of a second large open reading frame. *Virology* **155**, 747–751.
- Holsinger, L. J., Nichani, D., Pinto, L. H. & Lamb, R. A. (1994). Influenza A virus M2 ion channel protein: a structure–function analysis. *Journal of Virology* **68**, 1551–1563.
- Horvath, C. M., Williams, M. A. & Lamb, R. A. (1990). Eukaryotic coupled translation of tandem cistrons: identification of the influenza B virus BM2 polypeptide. *EMBO Journal* **9**, 2639–2647.
- Kozak, M. (1983). Comparison of initiation of protein synthesis in procaryotes, eucaryotes, and organelles. *Microbiological Review* **47**, 1–45.
- Krantz, D. E., Peter, D., Liu, Y. & Edwards, R. H. (1997). Phosphorylation of a vesicular monoamine transporter by casein kinase II. *Journal of Biological Chemistry* **272**, 6752–6759.
- Lamb, R. A. (1989). The influenza viruses. In *Genes and Proteins of the Influenza Viruses*, pp. 1–87. Edited by R. M. Krug. NY: Plenum Press.
- Lamb, R. A. & Krug, R. M. (1996). *Orthomyxoviridae: the viruses and their replication*. *Fields Virology*, 3rd edn, pp. 1353–1395. Edited by B. N. Fields, D. M. Knipe & P. M. Howley. Philadelphia & New York: Lippincott–Raven.
- Lamb, R. A., Lai, C. J. & Choppin, P. W. (1981). Sequences of mRNAs derived from genome RNA segment 7 of influenza virus: colinear and interrupted mRNAs code for overlapping proteins. *Proceedings of the National Academy of Sciences, USA* **78**, 4170–4174.
- Martin, K. & Helenius, A. (1991). Nuclear transport of influenza virus ribonucleoproteins: the viral matrix protein (M1) promotes export and inhibits import. *Cell* **67**, 117–130.
- Nakagawa, N., Maeda, A., Kase, T., Kubota, R. & Okuno, Y. (1999). Rapid detection and identification of two lineages of influenza B strains with monoclonal antibodies. *Journal of Virological Methods* **79**, 113–120.
- Neumann, G., Castrucci, M. R. & Kawaoka, Y. (1997). Nuclear import and export of influenza virus nucleoprotein. *Journal of Virology* **71**, 9690–9700.
- Nigg, E. A. (1997). Nucleocytoplasmic transport: signals, mechanisms and regulation. *Nature* **386**, 779–787.
- Niwa, H., Yamamura, K. & Miyazaki, J. (1991). Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* **108**, 193–199.
- Odagiri, T. & Tashiro, M. (1997). Segment-specific noncoding sequences of the influenza virus genome RNA are involved in the specific competition between defective interfering RNA and its progenitor RNA segment at the virion assembly step. *Journal of Virology* **71**, 2138–2145.
- Odagiri, T. & Tobita, K. (1990). Mutation in NS2, a nonstructural protein of influenza A virus, extragenically causes aberrant replication and expression of the PA gene and leads to generation of defective interfering particles. *Proceedings of the National Academy of Sciences, USA* **87**, 5988–5992.
- Odagiri, T., DeBorde, D. C. & Maassab, H. F. (1982). Cold-adapted recombinants of influenza A virus in MDCK cells. I. Development and characterization of A/Ann Arbor/6/60 × A/Alaska/6/77 recombinant viruses. *Virology* **119**, 82–95.
- O'Neill, R. E., Talon, J. & Palese, P. (1998). The influenza virus NEP (NS2 protein) mediates the nuclear export of viral ribonucleoproteins. *EMBO Journal* **17**, 288–296.
- Parvin, J. D., Palese, P., Honda, A., Ishihama, A. & Krystal, M. (1989). Promoter analysis of influenza virus RNA polymerase. *Journal of Virology* **63**, 5142–5152.
- Peluso, R. W., Lamb, R. A. & Choppin, P. W. (1977). Polypeptide synthesis in simian virus 5-infected cells. *Journal of Virology* **23**, 177–187.
- Pinto, L. H., Holsinger, L. J. & Lamb, R. A. (1992). Influenza virus M2 protein has ion channel activity. *Cell* **69**, 517–528.
- Shaw, M. W., Choppin, P. W. & Lamb, R. A. (1983). A previously unrecognized influenza B virus glycoprotein from a bicistronic mRNA that also encodes the viral neuraminidase. *Proceedings of the National Academy of Sciences, USA* **80**, 4879–4883.
- Steinhauer, D. A., Wharton, S. A., Skehel, J. J., Wiley, D. C. & Hay, A. J. (1991). Amantadine selection of a mutant influenza virus containing an acid-stable hemagglutinin glycoprotein: evidence for virus-specific regulation of the pH of glycoprotein transport vesicles. *Proceedings of the National Academy of Sciences, USA* **88**, 11525–11529.
- Sunstrom, N. A., Premkumar, L. S., Premkumar, A., Ewart, G., Cox, G. B. & Gage, P. W. (1996). Ion channels formed by NB, an influenza B virus protein. *Journal of Membrane Biology* **150**, 127–132.
- Wang, P., Palese, P. & O'Neill, R. E. (1997). The NPI-1/NPI-3 (karyopherin alpha) binding site on the influenza A virus nucleoprotein NP is a nonconventional nuclear localization signal. *Journal of Virology* **71**, 1850–1856.
- Ward, A. C., Castelli, L. A., Lucantoni, A. C., White, J. F., Azad, A. A. & Macreadie, I. G. (1995). Expression and analysis of the NS2 protein of influenza A virus. *Archives of Virology* **140**, 2067–2073.
- Whittaker, G., Kemler, I. & Helenius, A. (1995). Hyperphosphorylation of mutant influenza virus matrix protein, M1, causes its retention in the nucleus. *Journal of Virology* **69**, 439–445.
- Williams, M. A. & Lamb, R. A. (1989). Effect of mutations and deletions in a bicistronic mRNA on the synthesis of influenza B virus NB and NA glycoproteins. *Journal of Virology* **63**, 28–35.
- Yasuda, J., Nakada, S., Kato, A., Toyoda, T. & Ishihama, A. (1993). Molecular assembly of influenza virus: association of the NS2 protein with virion matrix. *Virology* **196**, 249–255.
- Ye, Z., Baylor, N. W. & Wagner, R. R. (1989). Transcription-inhibition and RNA-binding domains of influenza A virus matrix protein mapped with anti-idiotypic antibodies and synthetic peptides. *Journal of Virology* **63**, 3586–3594.
- Zhirnov, O. P. & Bukrinskaya, A. G. (1981). Two forms of influenza virus nucleoprotein in infected cells and virions. *Virology* **109**, 174–179.

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