REVIEW

INFLUENZA VIRUSES AND THEIR ION CHANNELS

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Received January 4, 2006; accepted January 18, 2006

Summary. – Influenza is an ancient disease that has infected humans in irregular intervals throughout recorded history. The most infamous pandemic was "Spanish Flu" which affected large parts of the world population and killed in 1918–1919, at a rough estimate, at least 50 million people. More recently, two influenza A pandemics occurred in 1957 ("Asian influenza") and 1968 ("Hong Kong influenza") and caused significant morbidity and mortality globally. Most recently, in 1997 and 2003, limited outbreaks caused by a new influenza A virus subtype H5N1 that was directly transmitted from birds to humans, occurred in the Hong Kong Special Administrative Region of China. Since 2003, the avian H5N1 strain has infected more then 130 persons in Vietnam, Thailand, and Cambodia and has killed more than half of them. To prevent the human and economical losses caused by human and avian influenza viruses, it is necessary to prepare safe universal influenza vaccines. In order to develop a broad-spectrum protection against different influenza virus strains or variants, some recent studies have were aimed at the M2 protein of Influenza A virus. This review compares the influenza A, B, and C viruses by focusing on their ion channels.

Key words: influenza virus; ion channel

Contents:

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Abbreviations: cRNA = complementary RNA; HA = hemagglutinin; M protein = matrix protein; NA = neuraminidase; NEP = nuclear export protein; NP = nucleoprotein; RNP = ribonucleoprotein; the A virus = Influenza A virus; the B virus = Influenza B virus; the C virus = Influenza C virus; vRNA = viral RNA

1. Introduction

The family *Orthomyxoviridae* includes four genera, *Influenza virus A*, *Influenza virus B*, *Influenza virus C* and *Thogotovirus*; the members of the last genus are sometimes called influenza D viruses. Each of the genera *Influenza virus A*, *Influenza virus B*, *Influenza virus C* has only one species (type species), namely *Influenza A virus, Influenza B virus* and *Influenza C virus,* respectively.

Influenza B virus (the B virus) and Influenza C virus (the C virus) are largely restricted to man, whereas Influenza A virus (the A virus) frequently infects avian species and only rarely man and other animals, in particular pigs and horses. Natural transmission of influenza viruses is mediated by aerosol (humans and most non-aquatic hosts) or is waterborne (ducks). The A, B and C viruses can be distinguished on the basis of antigenic differences between their nucleocapsid and matrix (M) proteins. The A virus is further divided into subtypes based on the antigenic nature of their

hemagglutinin (HA, 16 subtypes) and neuraminidase (NA, 9 subtypes) glycoproteins. Both the A and B viruses are important in human disease with their epidemic and pandemic (influenza A) potential. The C virus causes more limited outbreaks in humans and can also infect pigs.

2. The virus genome

The genome of influenza viruses consists of eight (the A and B viruses) or seven (the C virus) molecules (segments) of single-stranded RNA (ssRNA) of negative polarity (i.e. complementary to mRNA). The lengths of the genome segments and the proteins they encode are listed in Table 1. The three largest gene code the components of the RNA polymerase complex: PB1, PB2 and PA proteins. PB1 is responsible for RNA chain elongation, PB2 is essential for transcription and PA is involved in viral RNA (vRNA) synthesis. The segment 4 encodes HA (the A and B viruses) and HEF protein (the C virus). The segment 5 encodes nucleoprotein (NP), the major structural component associated with viral RNA and polymerase complex to form the ribonucleoprotein (RNP). While the segment 6 of the A and B viruses encodes NA, there is no equivalent of this segment in the C virus, since the function of this protein is fulfilled by HEF protein. The segment 6 of the B virus encodes also the NB protein.

The segment 7 of the A and B viruses and the segment 6 of the C virus encode two proteins: M1 and M2 (the A virus), M1 and BM2 (the B virus), and M1 and CM2 (the C virus). The principal product in terms of amount, the M1 protein is the major internal structural component of virus particle (virion). The smallest segment 8 (the A and B viruses) or 7 (the C virus) encodes two products, the non-structural RNA-binding protein NS1 and nuclear export protein (NEP).

3. Virus entry

The major glycoprotein spike on the virion, the HA of A and B viruses and the HEF protein of the C virus attaches to sialic acid-containing glycoprotein and glycolipid receptors on the cell surface (Fig. 1). The specificity of

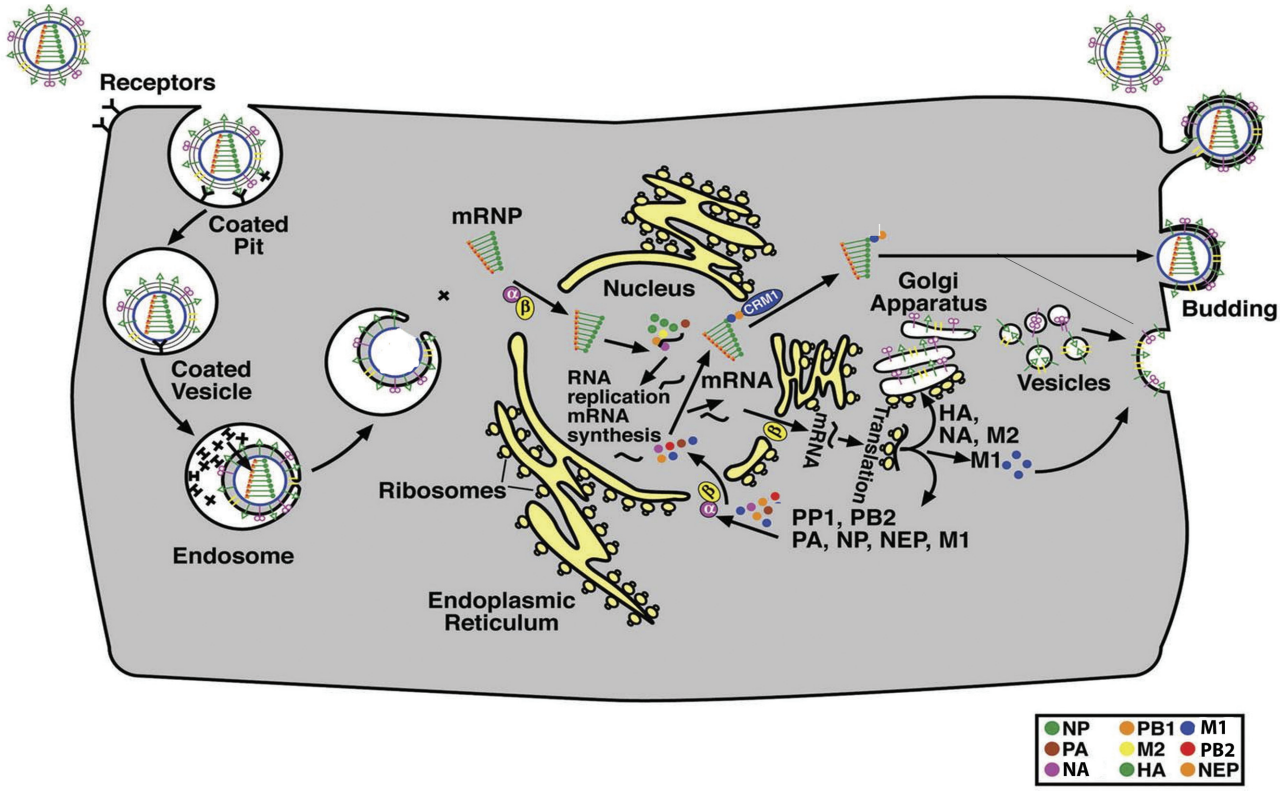

Fig. 1

Life cycle of Influenza A virus

Table 1. Influenza genes and their products

RNA segment	Influenza A virus		Influenza B virus		Influenza C virus	
	RNA segment length (nts)	Polypeptide length (aa)	RNA segment length (nts)	Polypeptide length (aa)	RNA segment length (nts)	Polypeptide length (aa)
PB2	2341	759	2396	770	2365	774
PB1	2341	757	2386	752	2363	754
PA	2233	716	2308	726	2183	704
HA	1778	566	1882	584	2071	654
NP	1565	498	1842	560	1809	565
NA	1413	454	1557	466 (NA) 100 (NB)	–	–
M	1027	252 (M1) 97 (M2)	1188	248 (M1) 109 (BM2)	1180	242 (M1) 139 (CM2)
NS	890	230 (NS1) 121 (NEP)	1098	281 (NS1) 122 (NEP)	934	286 (NS1) 122 (NEP)

The data listed are for influenza viruses A/PR/8/34 (all segments), B/AA/1/66 (segments 1,2,3,5,7 and 8; De Borde *et al*., 1988), B/Lee/40 (segments 4 and 6; Krystal *et al*., 1982; Shaw *et al*., 1982), C/JJ/50 (segments 1,2,3 and 6; Yamashita *et al*., 1988, 1989), and C/California/78 (segments 4,5 and 7; Nakada *et al*., 1984a,b; Buonagurio *et al*., 1986).

recognition may differ. In general, human viruses preferentially interact with N-acetylsialic acid attached to galactose by α2,6 linkage (Saα2,6Gal), whereas avian viruses mostly bind to N-acetylsialic acid attached to galactose by α2,3 linkage (Saα2,3Gal) (Connor *et al*., 1994; Matrosovich *et al*., 2001). This difference in binding specificity is reflected in the corresponding specificities of the receptor destroying enzyme, the NA of the A and B viruses and the HEF protein of the C virus. Following the attachment the virus enters the cell by receptor-mediated endocytosis via clathrin-coated pits (Fig. 1). The acid pH (between 5 and 6) of endosomes activates the processes, which promote the uncoating and entry of viral RNP into the cytoplasm. It triggers a structural change in the HA which induces fusion of viral and endosome membranes. Secondly, prior to this fusion event, H^+ flows through the M2 channel in the membrane of the A virus to cause an acid-induced dissociation of the M1 protein from viral RNP, which is essential to permit subsequent migration of free RNPs from the cytoplasm to the nucleus (Bui *et al*., 2000; Whittaker *et al*., 1996).

4. Viral genome transcription and replication

Transcription and replication of the viral genome takes place in the nucleus of infected cells in a RNP complex. Following dissociation of M1 and vRNPs, the latter migrate into the nucleus (Fig. 1). Transport of vRNPs through nuclear pores is mediated by soluble cellular import factors importin (karyopherin) α/ß, Ran and p10 by direct interaction between the viral NP and importin α (O'Neill *et al*., 1995; Wang *et al*., 1997). Replication of vRNA produces a full-length copy of positive-sense RNA (complementary RNA, cRNA), which serves as a template for the synthesis of new vRNA. Initiation of the synthesis of cRNA and vRNA is primer-independent, and an antitermination occurs at the poly(U) sequence during the synthesis of cRNA (Hay *et al*., 1977, 1982). Viral transcription produces mRNA that is shorter than cRNA and is capped at its 5'-end and polyadenylated at its 3'-end. An interaction of the conserved 5'-end of the template vRNA with the polymerase complex is required to activate the cap-binding function of PB2 to 'snatch' a host cell pre-mRNA. An interaction of the conserved 3'-end of the template vRNA is then required to activate endonucleolytic cleavage of the bound cell mRNA to generate the 9–15 nt-long primer which initiates transcription, usually at the penultimate 3'-C residue of the template. An interaction of the common sequence at the 5'-ends of viral mRNAs with the polymerase complex selectively protects viral mRNAs from a similar endonucleolytic cleavage (Shih and Krug, 1996). The transcription terminates at the stretch of 5–7 uridylate residues some 20 nts from the 5'-end and a polyadenylate (poly A) tail is added by a process of reiterative copying of the oligo(U) sequence (Poon *et al*., 1999).

5. Transport and assembly of virus components

The assembly of the virus starts in the nucleus. The vRNA is wrapped around each NP monomer (Martin-Benito *et al*., 2001). The stoichiometry of the interaction is estimated at 1 NP per 24 nts of RNA (Compans *et al*., 1972; Ortega *et al*., 2000). The newly assembled RNPs is exported out of the nucleus by CRM1 export pathway (Fig. 1). In RNPs export, the major role play M1 and NEP proteins (Martin and Helenius, 1991; Bui *et al*., 2000; Paragas *et*

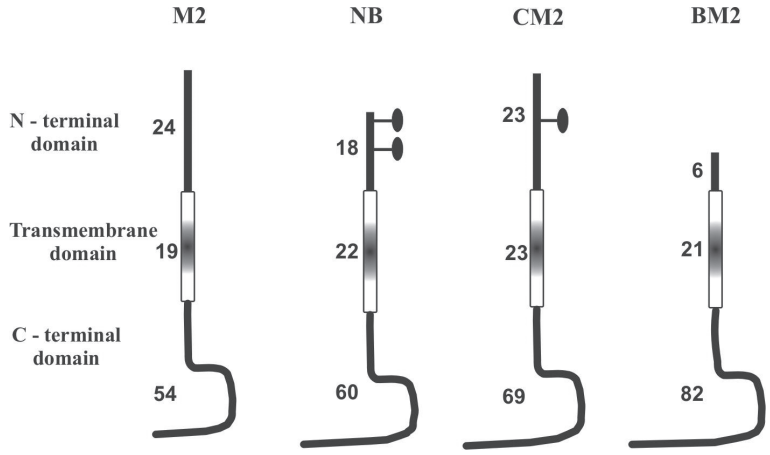

Fig. 2

Scheme of ion channels of influenza viruses

M2, NB, BM2, and CM2 proteins have a relatively small N-terminal extracellular domain, transmembrane domain and a long cytoplasmic tail. NB contains two, and CM2 one N-linked carbohydrate chain, and high mannose carbohydrates are modified by the addition of polylactosaminoglycan. These proteins are phosphorylated and contain palmitic acid covalently attached to a cytoplasmic cysteine residue.

al., 2001). The newly synthesized M1 protein migrates to the nucleus of the infected cell where it binds to RNP (Ye *et al*., 1999). The binding of M1 to RNP leads to the transport of M1/RNP complex from the nucleus to the cytoplasm (Huang *et al*., 2001; Martin and Helenius, 1991). The NEP (Yasuda *et al*., 1993) binds M1 that is already bound to RNP and mediates the contact between the M1/RNP complex and the cellular exportin, CRM1 (Akarsu *et al*., 2003; Neumann *et al.*, 2000). Specific interaction of M1 with the nucleosomes during viral replication could play a role in the release of vRNP from the chromatin (Garcia-Robles *et al*., 2005). Membrane proteins of the virus envelope, the HA, NA, M2 and NB proteins of the A and B viruses and the HEF and CM2 proteins of the C virus are synthesized in association with the endoplasmic reticulum and inserted into the plasma membrane by a signal recognition particle-dependent mechanism. During transport to the apical surface of epithelial cells via the Golgi apparatus, the proteins assemble into their mature multimeric structures and are modified by the addition of carbohydrate side chains and fatty acyl groups. The virus membrane proteins are incorporated into the areas of the plasma membrane from which cellular proteins become largely excluded. The A virus selects cholesterol-sphingomyelin-rich raft domains for the site of assembly of budding virions at the plasma membrane (Scheiffele *et al*., 1999; Leser and Lamb, 2005). Rafts then function as platforms for intracellular sorting and virus budding (Brown and Rose, 1992; Simons and Ikonen, 1997).

6. The ion channels

The A, B, and C viruses encode the small (97–115 aa) integral membrane proteins M2, NB, BM2, and CM2 (Lamb and Choppin, 1981; Lamb *et al*., 1981; Shaw *et al*., 1983; Horvath *et al*., 1990). Each protein has a relatively small N-terminal extracellular domain (M2 – 24 aa, NB – 18 aa, BM2 – 6 aa, CM2 – 23 aa), transmembrane domain (M2 – 19 aa, NB – 22 aa, BM2 – 21 aa, CM2 – 23 aa) and a much longer cytoplasmatic tail (M2 – 54 aa, NB – 60 aa, BM2 – 82 aa, CM2 – 69 aa) (Fig. 2). NB contains two (Williams and Lamb, 1989), and CM2 one (Pekosz and Lamb, 2003) N-linked carbohydrate chain, and the high mannose carbohydrates are modified by the addition of polylactosaminoglycan (Williams and Lamb, 1988). These proteins are phosphorylated (Holsinger *et al*., 1995; Hatta and Kawaoka, 2003; Hongo *et al*., 1997) and contain palmitic acid covalently attached to a cytoplasmic cysteine residue (Sugrue *et al*., 1990). The native form of these proteins is disulfide-link homotetramers (Holsinger and Lamb, 1991; Paterson *et al*., 2003; Li *et al*., 2001). All the proteins are type III integral membrane proteins (nomenclature of von Heijne, 1988), expressed abundantly on the surface of virus-infected cells, and are incorporated into virions (Betakova *et al*., 1996; Brassard *et al*., 1996; Odagiri *et al*., 1997; Pekosz and Lamb, 1998; Zebedee and Lamb, 1988). Despite the similarity of size and topology of the M2, NB, BM2, and CM2 proteins their coding strategies are completely different.

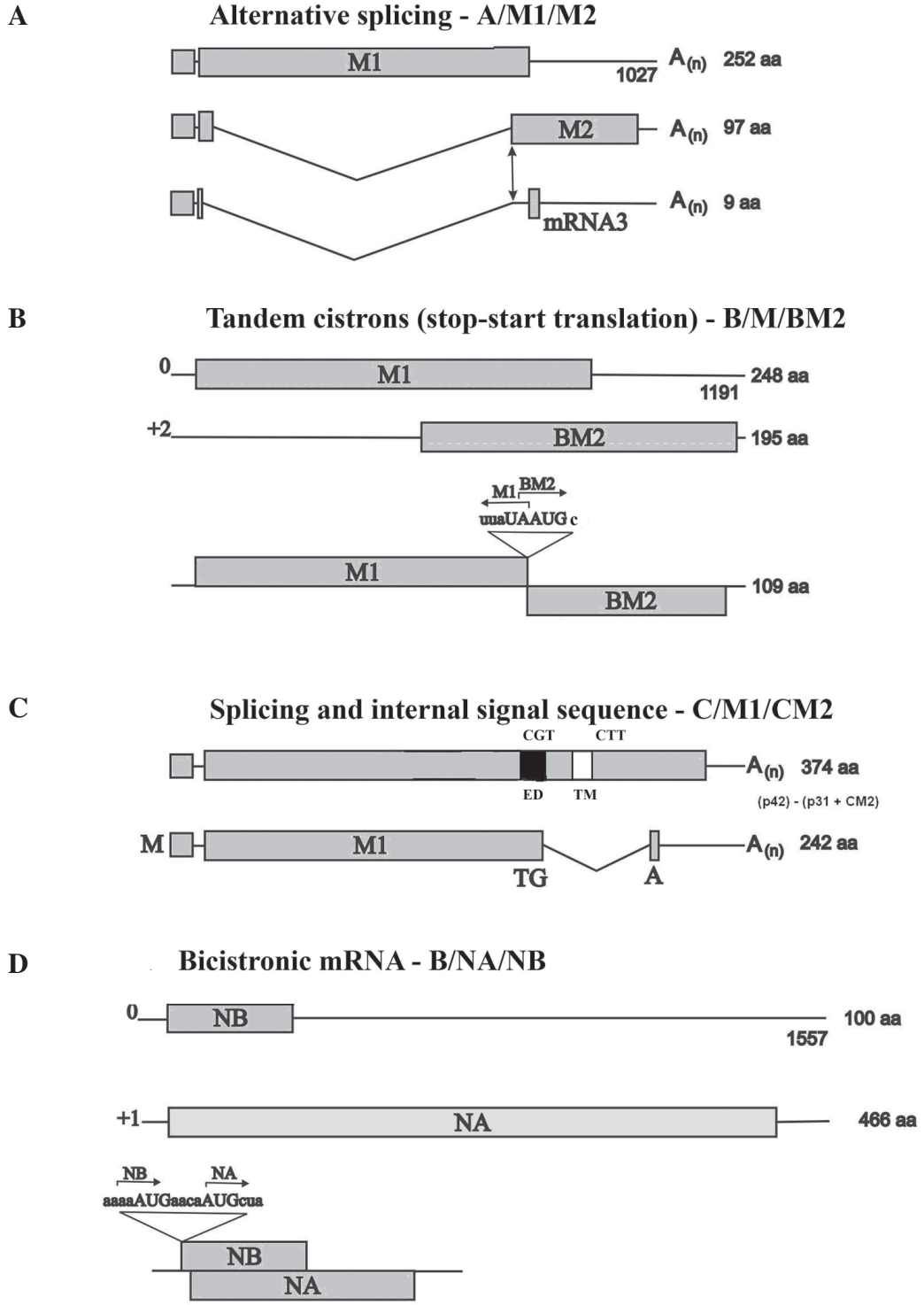

Fig. 3

The genome organization of some segments of influenza viruses (Lamb and Takeda, 2001)

(A) A/M1/M2: alternative splicing showing M1 and M2 mRNAs and mRNA3 and their coding regions. M1 and M2 share 9 amino-terminal residues including the initiating metionine; however, the M2 ORF differs from M1 ORF. A peptide that could be translated from mRNA3 has not yet been found *in vivo*. **(B)** B/M/BM2: tandem cistrons (stop-start translation) in the segment 7 ORFs and the organization of the ORFs used to translate M1 and BM2 proteins. The stop-start pentanucleotide is illustrated. **(C)** C/M1/CM2: splicing, internal signal sequence, mRNAs derived from the segment 6 ORF and the CM2 coding region. **(D)** B/NA/NB: bicistronic mRNA and overlapping ORFs of NB and NA in the segment 6.

6.1. M2 ion channel

The M2 protein is encoded by the A virus RNA segment 7. Three mRNAs are transcribed from this segment (Fig. 3): a colinear transcript encoding the M1 protein, a spliced mRNA encoding the M2 protein, and an alternatively spliced mRNA (mRNA3), which has the potential to encode a 9-aa peptide, but its translation has not been proved yet . The M2 protein shares 9 aa with the M1 protein and the bulk of the molecule is translated in a +1 reading frame from a sequence of 263 nts close to the 3'-end of the transcript and overlaps the sequence encoding the M1 product.

The M2 protein has an ion channel activity. It plays important roles in two stages of virus replication: in uncoating of virus in endosomes and in equilibration of the intralumenal pH of the *trans* Golgi Network (Duff and Ashley, 1992; Chizhmakov *et al*., 1996). This low-pH gated channel has a high selectivity for protons. Essential structure responsible for the channel is a parallel homo-tetrameric α-helix bundle having a left-handed twist with each helix tilted with respect to the membrane normal.

Several lines of evidence have suggested that highly conservative residues His37 and Trp41 play a crucial role in activity of the M2 protein. Direct evidence that the M2 protein has an ion channel activity was obtained by electrophysiological measurements (Pinto *et al*., 1992). Replacing His37 with Ala, Glu or Gly results in a large increase in proton conductance and loss of pH-induced gating behavior through the current-voltage relationship (Tang *et al*., 2002; Wang *et al*., 1995). Replacing it with Glu also results in a reduced selectivity (Wang *et al*., 1995), while mutating it to Cys completely abolishes its channel function (Shuck *et al*., 2000). Electro-physiological measurements yield relatively little information on how differences in the M2 conductance correlate with biological activity. In co-expression assay with pH-sensitive HA and several M2 mutants in CV-1 cells, the His37Ala mutant showed some activity, which was blocked by rimantadine (Betakova *et al*., 2004). However, its severe toxicity, due to permeability to other cations, did not allow expression of the protein at sufficient levels for accurate estimation of the activity. Mutants with Gly and Glu substitutions might behave similarly, but were not tested. In contrast, mutants with His 37 substituted by Gln, Phe or Trp, gave no detectable pH-modulating activity and showed little evidence of toxicity (Betakova *et al*., 2004). Because His37 may be located at the "narrowest" point in the channel (Pinto *et al*., 1997), it seems likely that replacement of His with Trp or Phe would change the channel structure and occlude the channel.

For the closed state of M2, most proposed models orient four His37 residues in such way that their imidazole side chains are directed towards the lumen, thereby occluding the pore and forming a channel gate. Four histidines in the pore are the only ionizable groups and the system with two or more charged histidenes is appreciably conductive. The structural switch from uniprotonated to a biprotonated channel causes an electrostatic repulsion between the charged histidines that pushes the helices apart. This results in the formation of a continuous water file that conducts proton via a H (+) wire. pKa calculations place this transition at a pH of 5.6 in remarkable agreement with the experimental value (Kass and Arkin, 2005; Grambas and Hay, 1992).

The indole ring of Trp41 is also important in maintaining the proper channel activity of the M2 protein. The His37 side chains occlude the channel pore and the Trp41 side chains seem to play a role in stabilizing the tetramer through hydrophobic interactions with Leu and Ile side chains of the adjacent helix. The indole ring of Trp41 is located far away from the imidazole ring of His37 (Okada *et al*., 2001). A possible role of the cation-π interaction between His37 and Trp41 is to stabilize the open form by partially neutralizing the positive charge on the imidazole ring of His37 with negative charge of the π electrons on the indole ring of Trp41. It is known that Trp is the best suited amino acid for the cation-π interaction (Mecozzi *et al*., 1996). This may be one of the reasons why Trp41 is a strictly conserved amino acid in naturally occurring mutants of the M2 protein (Holsinger *et al*., 1994). Replacement of Trp41 with Ala, Cys, or Phe causes a greater decrease of openness of the channel at high pH; that is why has Trp41 been proposed as the gate of the M2 channel (Tang *et al*., 2002). Replacement of Trp41 with Ala resulted in the absence of detectable pH-modulating activity. On the other hand, the Trp41Leu mutant was functional but with reduced pH modulating activity, showing that the proposed role of Trp41 in activation is not essential for functional activity, and that for some mutant viruses, may be disadvantageous (Betakova *et al*., 2004).

Two steps in the life cycle of the A virus are inhibited by amantadine and its derivates: virus uncoating in endosomes and M2 protein-mediated equilibration of the intralumenal pH of the *trans* Golgi Network (Chizhmakov *et al*., 1996; Duff and Ashley, 1992). Mutations in the M2 membrane protein, which confer resistance to the antiviral drug amantadine are exclusively located within the transmembrane region of the molecule. A number of amino acid substitutions, e.g., L26H, A30T, S31N and G34E reduced the activity of the M2 protein of A/chicken/Germany/34 (Rostock). In contrast, 127T and 127S mutations caused an increase in the M2 activity. Furthermore, in double mutants, the 127T mutation suppressed the attenuating effects of the A30T and S31N mutations on the M2 activity. The G34E mutation caused an increase in the activity of the M2 protein of A/chicken/Germany/27 (Weybridge) as opposed by the decrease in the activity of that of A/chicken/Germany/34 (Rostock) (Grambas *et al*., 1992). These two closely related

strains of the avian H7 subtype were found to differ in their pH-modulating activities and activation characteristics. There are three amino-acid differences at the positions 27, 38, and 44 within the membrane-spanning domain (V27I, F38L a D44N). Substitution of all three residues was required to transform the Weybridge phenotype of M2 into that of the Rostock, and conversely, single substitution in Rostock M2 were sufficient to effect the opposite phenotypic change (Chizhmakov *et al*., 2003). However, these mutagenesis experiments did not resolve which of the amino acid differences affected the ion flux through the channel. To answer this question, Betakova *et al*. (2005) have used a semi-quantitative HA-M2 co-expression assay. Only a substitution at the residue 44 was necessary and sufficient to account for differences in *trans* Golgi pH-modulating activity.

The ion channel activity of the M2 protein can be inhibited also by Cu^{2+}. Inhibition and recovery from inhibition can be explained by a model with two binding sites, each capable of inhibiting the current upon binding a ligand. One site of low affinity and low specificity for Cu^{2+} is located near the outside of the electric field of the membrane and only partially impedes ionic current through the channel (Gandhi *et al*., 1999). Another site has higher affinity for Cu^{2+}, is located inside the applied electric field, and more completely blocks current flow (Gandhi *et al.*, 1999). The M2 protein can be partially inhibited by Ni^{2+}, Pt^{2+} and Zn^{2+} (Gandhi *et al*., 1999).

6.2. BM2 ion channel

The BM2 protein of the B virus is translated from a bicistronic mRNA derived from the RNA segment 7 (Fig. 3). There is a pentanucleotide 7 (nt 69–773), in which the initiation AUG codon for the BM2 protein overlaps with the termination codon for the M1 protein. The translation of the BM2 protein depends upon the initiation and termination of the upstream M1 protein (Horvath, 1990).

The BM2 protein of the B virus is essential for its replication (Hatta *et al*., 2004). The role of BM2 protein is to capture the M1-vRNP complex at the virion budding site during virus assembly (Imai *et al*., 2004). The BM2 protein has an ion channel activity, which may be important for virus uncoating in endosomes of virus-infected cell. When expressed in *Xenopus* oocytes or mammalian cells it causes acidification of the cells and possesses ion channel activity consistent with proton conduction (Mould *et al*., 2003). Co-expression of the BM2 protein with pH-sensitive HA prevented HA from undergoing its conformational change to the low-pH-induced form in the Golgi apparatus (Mould *et al*., 2003). The BM2 expression at high levels also causes a delay in intracellular transport in the exocytic pathway and morphological changes in the Golgi apparatus. Both the BM2 and M2 proteins have transmembrane domains that contain a conserved HXXXW motif. His19 and Trp23 are critical for ion selectivity/activation and channel gating (Paterson *et al*., 2003). In contrary to the M2 channel, the BM2 channel is not inhibited by amantadine. One likely reason is that the pore-lining residues facing the ectodomain differ for the two proteins (Mould *et al*., 2003). Another functional difference between BM2 and M2 channels resides in the ability of the former to conduct ions while its ectodomain is exposed to high pH (Mould *et al*., 2003).

6.3. NB ion channel

The NB protein is encoded in an overlapping reading frame on a bicistronic mRNAs derived from the B virus segment 6 (Fig. 3), which also encodes NA (Shaw *et al*., 1983). The NB protein is initiated at the first AUG, four nucleotides upstream of the AUG which initiates translation of NA in a +1 reading frame (Shaw *et al.*, 1983). Only 300 nucleotides encode the 100 amino acids-long NB protein, whereas the sequence of 1398 nucleotides encoding the 466 amino acids-long NA protein occupies most of the mRNA.

The NB protein forms a cation-permeable channel at physiological pH. It may well be permeable to protons but the proton conductance is much lower (Sunstrom *et al*., 1996). The NB channel possess two permeabilities when expressed in MEL cells, a Na^+-activated H^+ permeability and a H^+-activated Cl^- permeability, which together promote electroneutral inward flow of H^+, consistent with the analogous role to that of M2 protein in the virion (Chizhmakov *et al*., 1998). Co-expression of the NB protein with pH-sensitive HA showed that, in contrast to M2, the NB channel did not modify the pH of the trans Golgi Network (T. Betakova, unpublished data). The NB protein is not essential for replication of the B virus in cell culture but promotes its efficient replication *in vivo* (Hatta and Kawaoka, 2003). The limited dependency of the B virus on the NB function may suggest either that the B virus does not depend as much on the ion channel activity as the A virus does or that the NB protein has functions other than that connected with the ion channel. Since the NB protein is highly conserved among the B virus strains, such functions must be important for viral replication in natural conditions (Hatta and Kawaoka, 2003).

10. CM2 ion channel

The CM2 protein is encoded by the C virus segment 6. Two mRNA species are derived from this segment: a colinear transcript containing a 374 aa-long ORF, which is translated to p42 protein, and a spliced mRNA which encodes the M1 (CM1) protein). The p42 protein is cotranslationally trans-

located into the endoplasmic reticulum membrane that requires hydrophobic internal signal peptide (aa 239–259) and predicted transmembrane domain of the CM2 protein (aa 285–308). Then the p42 protein undergoes proteolytic cleavage at a consensus signal peptidase cleavage site downstream of the amino acid 259, yielding the p31 and CM2 proteins. The p31 protein undergoes rapid degradation. The CM2 protein is assumed to be structural and functional analog of the M2 protein of the A virus (Pekosz and Lamb, 2000).

The CM2 protein forms a voltage-activated ion channel. The pH-dependence of the currents of the channel is much smaller than that of the M2 protein (Hongo *et al*., 2004). The CM2 protein ion channel activity is permeable to chloride anions (Cl^-) but not to Na^+ and K^+ cations, and the membrane current cannot be blocked by amantadine (Hongo *et al*., 2004). The role of the chloride channel activity associated with the CM2 protein in virus replication remains to be determined. It is suggested that the CM2 protein transported to the cell surface reduces the ionic strength just beneath the viral budding site by inducing the efflux of chloride ions. The lowered ionic strength may facilite the M1-RNP interaction; it has been reported that a slight increase in salt concentration starts dissociation of the M1-RNP complex of the C virus (Zhirnov and Grigoriev, 1994).

Acknowledgement. This work was supported by the European Social Fund, No. JPD BA Ciel 3 2004/4-047, 1312020050.

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