pH MODULATING ACTIVITY OF ION CHANNELS OF INFLUENZA A, B, AND C VIRUSES

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Summary. – The BM2 and NB proteins of Influenza B virus (the B virus) and the CM2 protein of Influenza C virus (the C virus) are structural homologs of the M2 protein of Influenza A virus (the A virus). It was shown recently that CM2 *in vitro* forms a voltage-activated ion channel permeable to chloride ion (Hongo *et al*., *Arch. Virol.* **149**, 35–50, 2004). To demonstrate a possible pH modulating activity of BM2, NB and CM2, the latters were co-expressed with a pH-sensitive hemagglutinin (HA) of the A virus. BM2 was able to replace functionally M2 and prevented the A virus HA from adopting its low-pH conformation during transport to the cell surface. In contrast**,** NB had a negative effect on the quality of the co-expressed HA and was unable to modulate the pH in the *trans*-Golgi network (TGN) and to protect HA. A pH modulating activity was also demonstrated for CM2, but it was much lower than that of M2.

Key words: BM2 protein; CM2 protein; influenza viruses; hemagglutinin; M2 protein; NB protein; ion channel

Introduction

The A, B, and C viruses encode the small (97–115 aa) integral membrane proteins M2, BM2 and NB, and CM2, respectively (Lamb and Krug, 1996; Pekosz and Lamb, 1997; Hiebert *et al*., 1986). Each protein has a relatively small extracellular N-terminal domain and a much longer cytoplasmic tail. NB contains two, and CM2 one N-linked carbohydrate chain, and these high-mannose carbohydrates are modified by the addition of polylactosaminoglycan (Pekosz and Lamb, 1997; Williams and Lamb, 1988). These proteins are phosphorylated (Sugrue *et al*., 1990a; Odagiri *et al*., 1999) and contain palmitic acid covalently attached to cytoplasmic cysteine (Sugrue *et al*., 1990b). In native form, these proteins are disulfide-linked homotetramers (Betakova, unpublished data; Hongo *et al*., 1997; Sugrue and Hay, 1991; Paterson *et al*., 2003). They are type III integral membrane proteins (von Heijne, 1988), which are expressed abundantly on the surface of virus-infected cells and are incorporated into virions (Betakova *et al*., 1996; Odagiry *et al*., 1999; Pekosz and Lamb, 1998; Zebedee and Lamb, 1988).

Despite the similarity of M2, NB, BM2, and CM2 in size and topology their coding strategies are completely different. M2 is encoded by a spliced mRNA derived from the RNA segment 7 of the A virus (Lamb *et al*., 1981). NB is encoded by an overlapping ORF in a bicistronic mRNA derived from the B virus RNA segment 6, which also encodes NA (Shaw *et al*., 1983). BM2 is encoded by a bicistronic mRNA derived from the B virus RNA segment 7, which also encodes the matrix M1 protein (Briedis *et al*., 1981). CM2 is encoded by the C virus RNA segment 6, which is initially transcribed into a colinear mRNA transcript that is subsequently predominantly spliced to yield a second mRNA transcript (Yamashita *et al*., 1988). The colinear mRNA contains a 374-aa-long ORF, which is translated into the precursor p42 protein. Proteolytical cleavage of p42 at an internal signal peptidase cleavage site gives rise to the p31 and CM2 proteins (Hongo *et al*., 1997; Pekosz and Lamb, 1998). Their similarity

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Abbreviations: AraC = cytosine arabinoside; HA = hemagglutinin; HRP = horseradish peroxidase; MAbs = monoclonal antibodies; PNG = peptide-N-glycosidase; RNP = ribonucleoprotein; TGN = *trans*-Golgi network; the A, B, and C viruses = Influenza A, B, and C viruses

in oligomeric form, size and orientation in the membrane led to a hypothesis that they perform related functions in the virus life cycle. The A virus M2 protein forms a proton-specific transmembrane ion channel, which is activated at acidic pH (Pinto *et al*., 1992; Chizmakov *et al*., 1996). The M2 ion channel plays a role in the uncoating of influenza A virions in endosomes (Martin and Helenius 1991; Helenius 1992), and, in some H7 and H5 subtypes, it functions to equilibrate the pH between the lumen of the TGN and the cytoplasm. The latter fact is important for preventing the acidification of the newly synthesized HA, which is cleaved intracellularly (Hay 1985; Čiampor *et al*., 1992; Grambas *et al*., 1992; Grambas and Hay 1992). The ion channel activity of M2 is specifically blocked by antiviral drugs amantadine and rimantadine. The M2 proteins of different viruses vary in their ability to alter the TGN pH (Grambas *et al*., 1992). The channel activation is associated with the His37-Trp41 interaction (Okada *et al.*, 2001). BM2 possesses an ion channel activity consistent with proton conduction. The transmebrane domain of BM2 contains histidine and tryptophane residues, which are important for the channel function (Mould *et al*., 2003). Some electrophysiological measurements indicate that NB and CM2 form an ion channel (Sunstrom *et al*., 1996; Hongo *et al*., 2004). The NB channel differs from the activated H^+-activated H^+-selective M2 channel in that it possesses 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 a H^+, consistent with an analogous role of M2 in virion disassembly during infection (Chizmakov *et al*., 1998). The CM2-associated ion channel activity is permeable to Cl^- but not to cations (Na^+ or K^+) (Hongo *et al*., 2004). According to electrophysiological measurements M2 is similar to BM2, and NB has some common feature with CM2.

In this study, we investigated the ability of BM2, NB and CM2 to protect pH-sensitive HA of the A virus against low-pH-induced changes.

Materials and Methods

Cells and viruses. CV-1 cells were grown in Eagle's MEM containing 10% of FCS. Recombinant vaccinia vTF7-3 virus expressing T7 RNA polymerase (kindly provided by Dr. B. Moss, NIH, Bethesda, USA) was propagated in HeLa cells (Fuerst *et al*., 1986). The influenza viruses C/Taylor/1233/47, A/Chicken/Germany/34 (H7N1 Rostock strain), and B/Johannesburg/26/94 were grown in 10-day-old fertile hen's eggs.

Antibodies. Anti-HA monoclonal antibodies (MAbs) HC2, HC58, and H9, and a rabbit antiserum against the M2 protein (kindly provided by Dr. A.J. Hay, NIMR, London, UK) were described earlier (Sugrue *et al*., 1990a; Betakova *et al*., 2005). A rabbit polyclonal antibody recognizing the virus HA epitope tag (the anti-Tag HA.11 serum) was obtained from Covance (USA).

Plasmid constructs. The plasmids containing the A virus HA and M2 genes were previously described (Betakova *et al*., 2005). CV-1 cells infected with the C or B virus for 8 hrs were used for extraction and purification of total RNA by means of the Nucleospin Nucleic Acid Purification Kit (Clontech). NB and BM2 cDNAs were synthesized from respective total RNAs by reverse transcription using specific primers. The NB and BM2 genes (their cDNAs) were modified to contain an *Nco*I site at the initiation codon, the A virus HA epitope tag sequence (YPYDVPDYAS), a termination signal, and a *Bam*HI site. The CM2 gene was modified to contain a *Nde*I site at the initiation codon, followed by 24 amino acids of the signal peptide, the A virus HA epitope tag sequence, a termination signal, and a *Bam*HI site. The PCR products of these cDNAs were cut with appropriate restriction enzymes and inserted either into the plasmid pVOTE.1 or pVOTE.2 (both kindly provided by Dr. B. Moss, NIH, Bethesda, USA), so that the constructs pVOTE.2-CM2, pVOTE.1-NB, and pVOTE.1-BM2 were generated. All of the constructs were sequenced to eliminate undesirable mutations. Plasmid DNA was purified using the Plasmid Maxi Kit from Qiagen.

Transfection. Confluent CV-1 cells were infected with the recombinant vaccinia vTF7.3 virus (10 PFU/cell) in the OPTIMEM medium (Gibco, Invitrogen) containing 40 μg/ml cytosine arabinose (AraC). After 1hr, the infected cells were transfected with respective plasmid constructs mixed with Lipofectine (Life Technologies).

Western blot analysis. The transfected cells were lyzed with an extraction buffer (1% Triton-X-100, 1 mmol/l EDTA, and 20 mmol/l Tris-HCl pH 7.4) containing a protease inhibitor (Complete Mini, Roche). After 10 mins on ice, the lysates were clarified by centrifugation for 1 min, and the supernatants were analyzed by electrophoresis in 12.5% agarose gel. Immunoblotting was done as described by Grambas *et al*. (1992), using the rabbit anti-M2 and anti-HA (anti-Tag HA.11) sera, a protein A-horseradish peroxidase (HRP) conjugate, and labeled with TMB stabilized substrate for HRP (Promega).

Immunofluorescent microscopy. CV-1 cells grown on coverslips were transfected with 3 μg of pVOTE.1-HA mixed with 0.5 μg, 1 μg, 2 μg, or 4 μg of pVOTE.2-CM2, pVOTE.1-M2, pVOTE.1-BM2, and pVOTE.1-NB, respectively, in OPTIMEM medium**.** After 4 hrs, the cultures were overlaid with 1 ml of MEM containing 20% of FCS and 40 μg/ml AraC. After additional 20 hrs, the cells were fixed with 3% paraformaldehyde, permeabilized with 0.01% Triton X-100 in PBS, and immunolabeled with the rabbit anti-M2 or anti-HA (anti-Tag HA.11) serum or mouse MAbs HC2, HC58, and H9, respectively, diluted in PBS with 1% BSA. Primary antibodies were visualized using fluorescein- or rhodamine-conjugated secondary antibodies diluted in PBS with 1% BSA. The nuclei were stained for 10 mins with Hoechst 33342. The cells were viewed using a Nikon Eclipse E-400 microscope.

Results and Discussion

Western blot analysis

Since we had no suitable antibodies against NB, BM2, and CM2 proteins, we prepared their HA-tagged versions. The nucleotides encoding an epitope recognized by the anti-

HA.11 rabbit serum (anti-Tag) were inserted into the NB, BM2 and CM2 genes upstream of terminal codons. The expression of M2, NB, BM2, and CM2 was confirmed by transfection of CV-1 cells with plasmids encoding the proper protein followed by Western blot analysis. Multiple bands of M2 (~14 K and ~60 K) were reduced by 2-mercapto-ethanol to only one band of ~14 K (Fig. 1). The expressed CM2 was sensitive to a peptide-N-glycosidase F (PNG) treatment, and, under reducing conditions, one band of ~15 K was detected (data not shown). However, without the PNG treatment, multiple bands of CM2 (15 K, 18 K, and 30 K) could be detected under reducing conditions. NB could be detected in 15 K, 25 K, 30 K, and 40 K bands under non-reducing conditions, but in 15 K, 18 K, and 30 K bands under reducing conditions. As the 25 K and 40 K proteins were sensitive to the PNG treatment, they apparently represented glycosylated forms of NB (data not shown). BM2 appeared in two bands of 15 K and 34 K under non-reducing conditions, but just one band (15 K) could be seen under reducing conditions.

These results indicated that all these proteins were synthesized in a quality comparable with that found in infected cells (Lamb *et al*., 1985; Williams and Lamb, 1986; Watanable *et al*., 2003; Hongo *et al*., 1994).

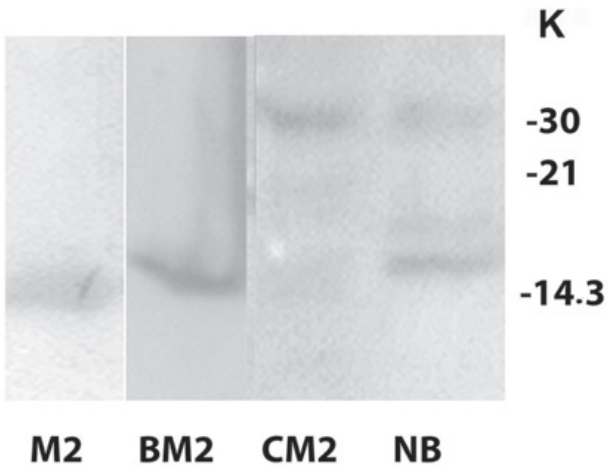

Fig. 1

Western blot analysis of expression of M2, BM2, NB, and CM2 in CV-1 cells

CV-1 cells were infected with vaccinia vTF7.3 virus and transfected with plasmid constructs encoding M2, BM2, NB, and CM2, respectively. The proteins extracted from these cells were subjected to Western blot analysis under reducing conditions. The positions and size (K) of marker proteins are indicated right.

Fluorescent microscopy

The HA of the Rostock strain of Fowl plague virus that undergoes its conformation change at pH 5,9 requires a highly efficient M2 to keep the intracisternal pH in the TGN above the denaturation threshold. The rescue of HA in biologically active form by co-expressed M2 protein has been already described (Ohuchi *et al*., 1994; Zebedee *et al*., 1985). The pH-sensitive HA co-expressed with/without M2 in CV-1 cells by vaccinia system, underwent conformational changes that were detected by three MAbs: HC2, specific for all pH forms of HA, HC58 specific for the native form of HA, and H9, specific for the low-pH form of HA. When HA was expressed without M2 protein, the native form (detected by HC58) and also the low-pH form (detected by H9) were detected in similar ratio (Fig. 2a). The protective effect of M2 was indicated by corresponding increase in the labeling of the native form and a decrease in the labeling of the low-pH form (Fig. 2b). The labeling with H9 was very rare, observed only in the cytoplasm near the nuclei. On the other hand, HC58 richly labeled the cytoplasm and occasionally also the membrane. A maximum difference in the labeling with HC58 and H9 was recorded at an optimum HA/M2 ratio (Betakova *et al*., 2005). It is known that the M2 ion channel activity is specifically blocked by antiviral drugs amantadine and rimantadine. The pattern of HA decoration with HC58 and H9 in the cells co-transfected with M2 in the presence of 5 μmol/l amantadine was similar to HA expressed in the cells without M2 protein (Fig. 2c). In this case, both HA forms were detected. These data demonstrated that conformational changes of HA depended on the activity of the co-expressed M2.

Co-expression of HA with BM2 of the B virus resulted in an increase in the labeling of native HA form and a corresponding decrease in the low-pH HA form (Fig. 2d).

The decoration of the HA co-expressed with BM2 was similar to that co-expressed with M2. These data are consistent with BM2 equilibrating the pH gradient between the TGN and the cytoplasm. Both the transmembrane domain of BM2 of the B virus and the M2 ion channel protein of the A virus contain a HXXXW motif, and, for both proteins, the His and Trp residues are important for channel function (Mould et al., 2003). Our results confirm the previous finding that BM2, like M2, possesses an ion channel activity. Moreover, BM2 (of the B virus) is able to replace functionally M2 (of the A virus) and protect HA (of the A virus) from undesirable conformational changes.

When NB was co-expressed in the cells, it had no effect on the transport of HA (Fig. 2e). The HA co-localized with NB in the cytoplasm and in the plasma membrane. However, the protection of HA with a co-expressed NB has never been observed. Several HA/NB ratios were tested to find out optimum.

However, the labeling of HA co-expressed with NB completely differed from that of HA expressed alone or with

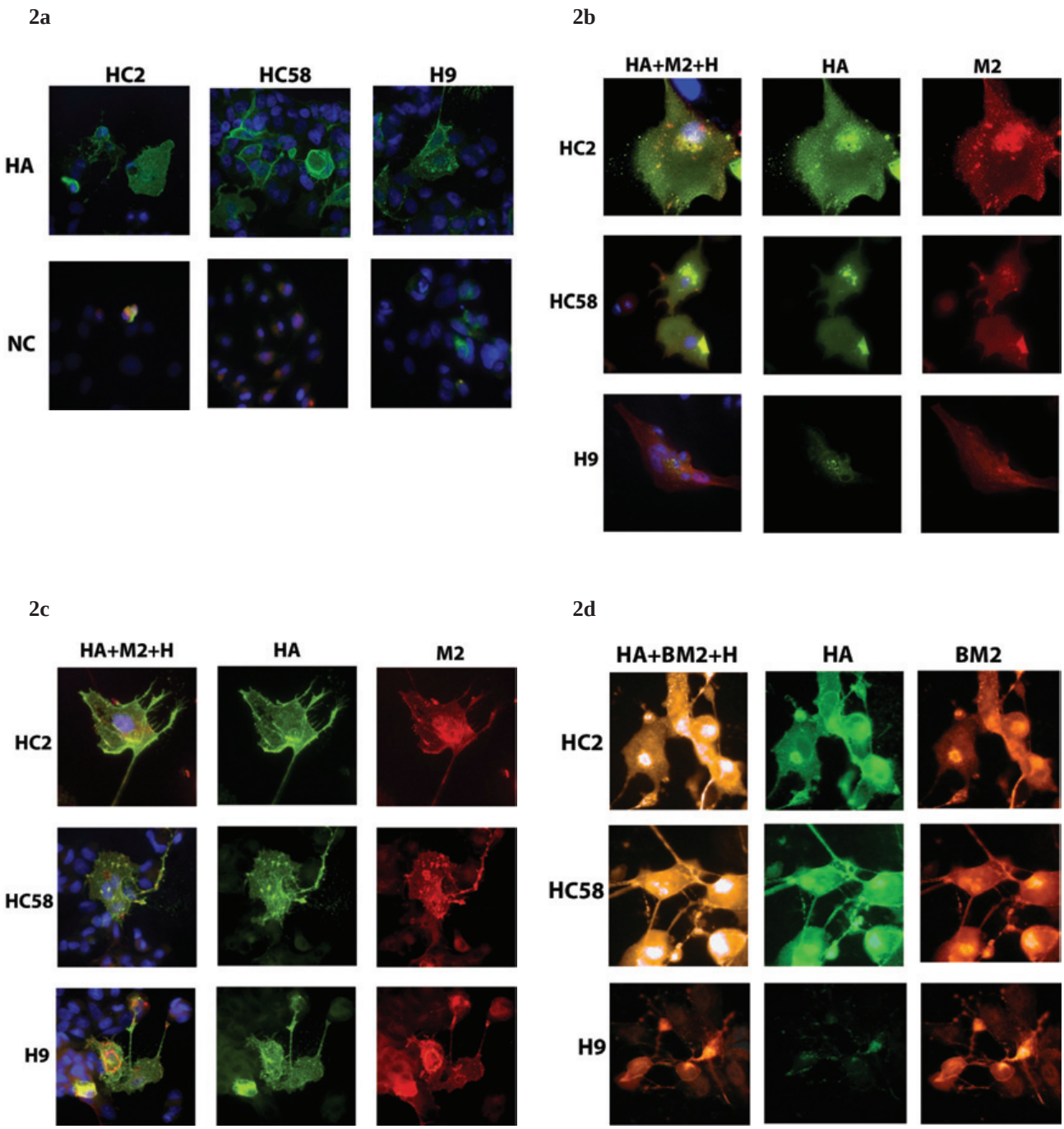
2a
HC2
HC58
H9
HA
NC
2b
HA+M2+H
HA
M2
HC2
HC58
H9
2c
HA+M2+H
HA
M2
HC2
HC58
H9
2d
HA+BM2+H
HA
BM2
HC2
HC58
H9

Fig. 2

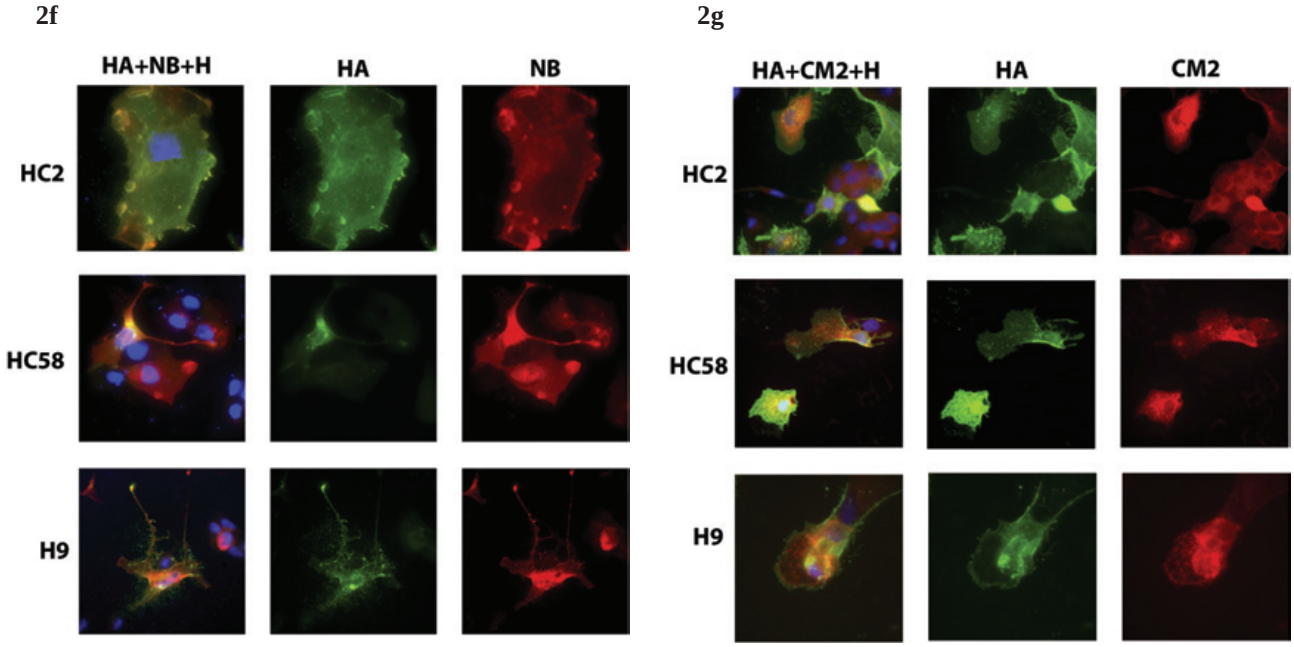

Fig. 2

Specific immunofluorescent labeling of HA, M2, BM2, NB, and CM2

CV-1 cells were transfected with pVOTE.1-HA (a), pVOTE.1-HA/pVOTE.1-M2 (b), pVOTE.1- HA/pVOTE.1-M2 in the presence of amantadine (c), pVOTE.1- HA/pVOTE.1-BM2 (d), pVOTE.1-HA/pVOTE.1-NB (e), and pVOTE.1-HA/pVOTE.2-CM2 (f). HA was labeled green with the following MAbs: HC2 recognizing all HA forms, HC58 recognizing the native HA form, and H9 recognizing the low-pH HA form. M2 was labeled with the rabbit anti-M2 serum, and BM2, NB, and CM2 with the anti-Tag HA11 serum. The nuclei were stained blue with Hoechst 33342 (H). Negative control (NC).

M2. Whereas the HC58 labeling vanished, the H9 labeling was intensive and comparable with that when HA was expressed alone. HC58 labeled HA in the cytoplasm close to the nuclei. That implied that the native HA form was present in virus factories but not in the cytoplasm. The labeling with HC2, which recognized all HA forms, was unchanged and the cytoplasm and the membrane were intensively decorated. Thereby we assume that the low-pH HA form predominated in these cells. It has been reported that NB may form some kind of channel in the membrane, which is permeable to sodium and chloride ions (Sunstrom *et al*., 1996; Premkumar *et al*., 2004). However, the biological activity of NB as an ion channel has never been demonstrated *in vivo*. Since the B virus HA is not intracellularly cleaved and its fusion does not depend on pH, the presence of a protein with ion channel activity should not be required in this case. Nevertheless, a low-pH step is required to release the matrix protein M1 from RNP (Zhirnov, 1992). It has been published recently, that the B virus BM2 protein has an ion channel activity and conducts protons across membranes (Mould *et al*., 2003). Furthermore, NB was not found essential for the B virus replication in cell culture but promoted the virus growth in mice (Hatta *et al*., 2003). The function of NB should be further investigated.

To demonstrate the pH modulating activity of the C virus CM2 protein, we co-expressed it with HA of the A virus. CM2 was detected mostly in the cytoplasm, in same cases it co-localized with HA (Fig. 2f). Expression and localization of CM2 and HA were indistinguishable from those of M2 and HA (Fig. 2B). The co-expression of CM2 and HA resulted in an increase in the native HA form in the cytoplasm (recognized by HC58) and some decrease in the low-pH HA form (recognized by H9). The labeling with H9 was not as intensive as that with the HA expressed alone but it was much more intensive than that with HA co-expressed with M2.

The effectiveness of CM2 in elevating the TGN pH and protecting HA against low-pH-induced changes depended on the ratio of the expressed HA and CM2. However, the protective effect of CM2 was much lower and never reached that of M2.

Only little is known about the transport of the C virus HEF through the TGN. HEF is not cleaved intracellularly but pH equilibration between the lumen of the TGN and the cytoplasm may be important (Pleschka *et al*., 1995;

Szepanski *et al*., 1994). The channel activity of CM2 may play an important role in facilitating the interaction between M1 and RNP, which enhances the virion assembly. Hongo *et al*. (2004) have suggested that CM2 forms a voltage-activated ion channel permeable to chloride ion. However, the ion channel activity of CM2 has never been demonstrated *in vivo*. Our results suggest that CM2 is able to modify the pH in the TGN and protect the A virus HA to some degree. But this activity is much lower than that of M2.

The data reported here have implications for understanding the properties of ion channels. In conclusion, we showed that (i) NB did not have an ion channel activity like M2, and (ii) CM2 was able to change the pH in the TGN, however, its ion channel activity reached only half of that of M2. Therefore it will be important to further characterize the ion channels activities of these proteins and investigate their role in the life cycle of respective viruses.

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