INFLUENCE OF EXTRACELLULAR AND CYTOPLASMIC DOMAINS OF M2 ION CHANNEL OF INFLUENZA A VIRUS ON ITS ACTIVITY

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Summary. – Co-expression of M2 protein of Influenza A virus (IAV) with pH-sensitive hemagglutinin (HA) reduces the conversion of HA to its low pH conformation during transport to the cell surface. Mutational analysis of extracellular domain of M2 protein showed that single aa substitution W15A did not influence the ion channel activity of M2 protein. Replacement of first 24 aa of M2 protein with first 18 aa of NB protein of Influenza B virus in chimera BAA resulted in the loss of ion channel activity. The chimera B(SSD)AA had only first 21 aa replaced with first 18 aa of NB protein The remaining aa at positions 22, 23, and 24 were preserved from the wtM2 protein. The ion channel activity of B(SSD)AA chimera was fully restored and showed that the aa S22, S23, and D24 from wtM2 were important for the ion channel activity. However, these aa did not contribute to the activity of M2 protein directly, since they could be substituted by A, R, or H without change in ion channel activity. Mutational analysis of cytoplasmic domain of M2 protein showed that substitutions C50S, C50P, and H90S did not change the ion channel activity. The extracellular and cytoplasmic domains of M2 protein were not essential for ion channel activity of M2 protein.

Key words: Influenza A virus; ion channel; M2 protein; mutational analysis

Introduction

The M2 protein of Influenza A virus (IAV) is a type III integral membrane protein, which is incorporated into the virions and transported to the surface of virus-infected cells (von Heijne, 1986; Zebedee, 1988). The M2 protein is 97 aa long with a putative 19 aa transmembrane domain located between short extracellular domain (24 aa) and the cytoplasmic tail (54 aa) (Lamb *et al*., 1985). The native form of M2 protein is a phosphorylated disulfide-linked homotetramer containing palmitic acid covalently attached to C50 of the cytoplasmic tail (Sugrue *et al.*, 1990; Sugrue and Hay, 1991). The M2 protein is supposed to play a role in the uncoating of IAV virions in endosomes (Martin and Helenius 1991; Helenius 1992). In some H7 and H5 subtypes of IAV, the M2 protein also equilibrates pH between the lumen of the *trans* Golgi network (TGN) and the cytoplasm what is important for preventing of acidification of newly synthesized HA polypeptide, which are cleaved intracellularly (Hay *et al*., 1985; Ciampor *et al*., 1992; Grambas *et al*., 1992, Grambas and Hay, 1992). The M2 protein as the ion channel is highly selective for protons and at the same time has a relatively low permeability for other physiological ions (Chizhmakov *et al*., 1996; Mould *et al*., 2000). Its conductance properties were studied by electrophysiological measurements (Pinto *et al*., 1997; Chizhmakov *et al*., 2003; Wang *et al*., 1995). The ion channel activity of M2 protein is specifically blocked by the antiviral drugs amantadine and rimantadine. The extracellular domain of the M2 protein is important for its incorporation into virion and also is able to induce production of antibodies with inhibitory activity against IAV replication (Park *et al*., 1998; Liu *et al*., 2003). The cytoplasmic tail of M2 protein is important for the ion channel activity, mediates binding

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Abbreviations: AraC = cytosine arabinoside; HA = hemagglutinin; IAV = Influenza A virus; MAb(s) = monoclonal antibody (ies); TGN = *trans* Golgi network; wtM2 = wild-type M2 protein of H7N1 Rostock strain

to the M1 protein, and plays an important role in viral assembly (Iwatsuki-Horimoto *et al*., 2006; McCown and Pekosz, 2006; Tobler *et al*., 1999).

The NB protein of influenza B virus is considered to be a structural and functional analogue of M2 protein of IAV, but its ability to modulate pH in TGN has not been proved (Kollerova and Betáková, 2006). The NB protein contains a short extracellular domain (18 aa), a hydrophobic transmembrane domain (22 aa) and a longer cytoplasmic tail (61 aa) (Williams and Lamb, 1986). NB protein is glycosylated and polylactosaminoglycan side chains are attached to the asparagine (N3 and N7) (Williams and Lamb, 1988).

The aim of this work was to study a possible role of extracellular and cytoplasmic domains of M2 protein in their pH modulating activities. For these experiments we used vaccinia co-expression system with a series of plasmids expressing various mutated forms of M2:

1/ M2 mutants possessing single aa substitutions in extracellular or cytoplasmic domains,
2/ M2/NB chimeric proteins with extracellular domain replaced fully or to some extent with extracellular domain of NB protein,
3/ truncated form of M2 protein with 3 additional aa substitutions in the cytoplasmic tail.

Materials and Methods

Cells and viruses. CV-1 cells were grown in Eagle's minimum medium (MEM) containing 10% calf serum. Recombinant Vaccinia virus vTF7-3, which expresses the bacteriophage T7 RNA polymerase gene (kindly provided by Dr. B. Moss, NIH, USA) was propagated in HeLa cells.

Antibodies. Anti-HA monoclonal antibodies (MAbs) HC2, HC58, H9, and rabbit antiserum against the M2 protein (kindly provided by Dr. A.J. Hay, NIMR, London, UK) were previously described (Sugrue *et al*., 1990). MAb HC2 recognizes all forms of HA, MAb HC58 recognizes only the native form of HA, and MAb H9 recognizes only the low pH-form of HA.

Plasmid construction. Coding sequences for the HA of IAV strain A/Chicken/Germany/34 (H7N1 Rostock strain), the M2 protein of H7N1 Rostock strain, and the NB protein of influenza B virus strain B/Johannesburg/26/94 were inserted into plasmid pVOTE.1 (kindly provided by Dr. B. Moss, NIH, Bethesda, USA) to generate plasmids pVOTE.1-HA, pVOTE.1-M2, and pVOTE.1-NB, respectively (Betáková and Kollerová, 2006). The mutant genes of M2 were prepared by four-primers PCR and cloned into pVOTE.1 (chimera proteins and M2 mutants are listed in Table 1 and Table 2). The oligonucleotide sequences of used primers are available upon request. All of the constructs were sequenced to eliminate unwanted mutations. Plasmid DNA was purified using Wizard Plus SV Minipreps kit (Promega).

Table 1. pH modulating activity of mutated M2 proteins with the aa substitutions in the extracellular domain determined by ELISA

Mutant	Increase of native form HA in %	Decrease in low-pH form HA in %
M2-positive control	25.0 ± 2.509	-23 ± 2.361
NB-negative control	0	0
W15A	26.7 ± 6.6	-19,7 ± 5,8
S22A	24 ± 5.1	-20 ± 4.2
S23A	25.5 ± 5.0	-21± 3.8
S22A, S23A	23 ± 3.6	-22 ± 5.3
D24R	25 ± 3.6	-20 ± 4.0
S22R	22 ± 4.5	-20 ± 4.4
S23R	22.5 ± 3.5	-21 ± 3.7
S22R,S23R	25 ± 3.0	-22 ± 4.7
S22H	21 ± 3.6	-19 ± 4.2
S23H	23 ± 4.2	-19.5 ± 5.6

Average values with SD (n = 6).

Table 2. pH modulating activity of mutated M2 proteins with the aa substitutions in the cytoplasmic tail determined by ELISA

MutMutMutant	Increase of native form HA in %	Decrease in low-pH form HA in %
M2-positive control	25.0 ± 2.509	-23 ± 2.361
NB-negative control	0	0
C50F	23.8 ± 4.7	-18.1 ± 1.5
C50S	24.5 ± 5.0	-19.4 ± 3.6
H90S	30.5 ± 4.2	-20.3 ± 1.7

Average values with SD (n = 6).

Transfection protocol. Confluent CV-1 cells were infected with 10 PFU of recombinant Vaccinia virus vTF7-3 in OPTIMEM containing 40 μg/ml of cytosine arabinose (AraC). After 1 hr incubation, the infected cells were transfected with plasmids coding for various mutated M2 proteins mixed with Lipofectine (Life Technologies).

Western blot analysis. The transfected cells were lyzed in extraction buffer (1% Triton-X-100, 1 mmol/l EDTA, 20 mmol/l Tris-HCl (pH 7.4), and proteinase inhibitor Complete Mini Protease Inhibitor (Roche). After lysis for 10 mins on ice, the cell lysates were cleared by centrifugation for 1 min, and the supernatants were analyzed by PAGE on 12.5% gels. Immunoblotting was done using antiserum against M2 and protein A-HRP conjugate labeled with TMB stabilized substrate for HRP (Promega) to stabilize substrate (Grambas *et al.*, 1992).

ELISA. CV-1 cells grown on 96 well plates were transfected with 0.25 μg of pVOTE.1-HA together with increasing concentration of plasmids encoding mutated M2 proteins. Four hrs after transfection, the cells were overlaid with MEM containing 20% FCS, 40 μg/ml AraC and 5 μg/ml rimantadine. Rimantadine specifically blocks the ion channel activity of M2 protein. Transfected cells were incubated for 20 hrs and fixed with 0.05% glutaraldehyde in *PBS.* ELISA was carried out in duplicate wells using anti-HA MAbs HC2, HC58 and H9. The amount of HA in % determined by MAbs HC58 or H9 recognizing native and low pH-form HA, respectively, was estimated from the ratio of corresponding A_{450} using formulas: HC58(%) = [HC58:HC2 (plus M2)]x100 – [HC58:HC2(no M2)]x100 or H9(%) = [H9:HC2 (plusM2)]x100 – [H9:HC2(no M2)]x100.

exclude the influence of aromatic aa at positions 47, 48, and 52 on activity of the ion channel, these aa were replaced by A to form substitutions F47A, F48A, and W52A.

The expression of M2 protein in CV-1 cells transfected with corresponding plasmids was evaluated in Western blot analysis. The amount of expressed M2 protein depended on the amount of pVOTE.1-X plasmid (where X = M2 or mutants of M2 or M2/NB chimeras) used for transfection of CV-1 cells and increased with increasing amount of input DNA (Fig. 1). Expression of M2 mutants and chimera proteins were similar as expression of M2 (data not shown).

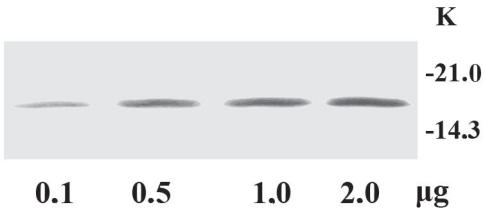

Fig. 1

Expression of wtM2 detected by Western blot analysis

CV-1 cells were transfected with 0.1, 0.5, 1.0, and 2.0 μg of pVOTE.1-wtM2. Size markers are indicated on the right.

Results and Discussion

Expression of M2 mutants with single aa substitutions, M2/NB chimeric proteins, and truncated mutant of M2

To investigate the possible role of extracellular and cytoplasmic domains of M2 protein, we constructed series of M2 mutants with single aa substitutions in extracellular and cytoplasmic domains, two types of M2/NB chimeras, and truncated mutant of M2 (C-truncM2).

1/ M2 mutants possessing single aa substitutions were marked as W15A, S22A, S22R, S22H, S23A, S23R, S23H, D24A, and D24R for extracellular domain and C50F, C50S, and H90S for cytoplasmic tail.
2/ We prepared two types of modified M2/NB chimeric proteins containing extracellular domain of M2 protein replaced by extracellular domain of NB protein. Specifically, the BAA chimera had the extracellular domain of M2 protein replaced by extracellular domain of NB protein and the B(SSD)AA chimera had first 21 aa of M2 protein replaced by extracellular domain of NB protein.
3/ Further, we constructed the 53 aa long truncated protein (C-truncM2) lacking 46 aa from the C-terminus. To

pH modulating activity of M2 mutants

HA co-expressed with M2 underwent conformation changes detected by MAbs HC58 and H9. The degree of HA protection against low pH depended on the ratio of HA and M2 proteins synthesized in transfected cells. This ratio was evaluated by total amount of HA (recognized by MAb HC2), the amount of native form of HA (recognized by MAb HC58) and the amount of low pH-form of HA (recognized by MAb H9). The change in amount of native or low pH-form HA was determined from the A_{450} measurements obtained with these MAbs in ELISA. The maximal protective effect was recorded at the specific HA/M2 ratio, which was found as optimal. The further increase in the expression of M2 reduced the expression of total HA. The maximum values of HA protection from six experiments were analyzed statistically and the average values with standard deviations were used to compare the activities of the various mutant M2 proteins.

Co-expression of HA with no mutated M2 protein strain Rostock (wtM2) resulted in an increase of 25% of native HA and a corresponding decrease of about 23% of the low pH-form of HA, compared to HA expressed without M2

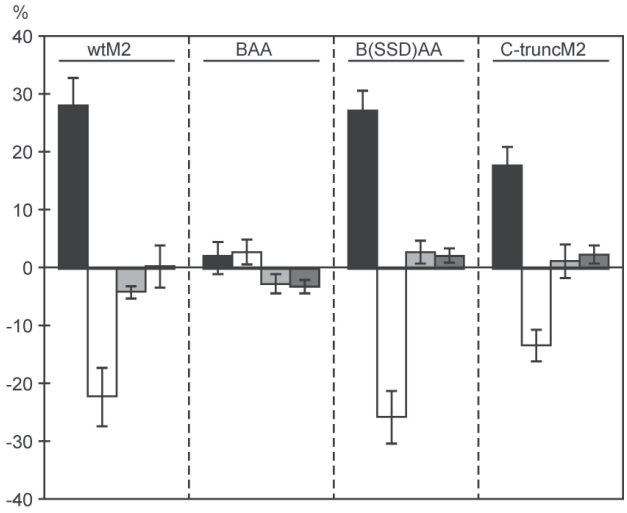

Fig. 2

pH modulating activity of wtM2, chimeric M2/NB, and truncated M2 proteins determined by ELISA

Reactivity with MAb HC58 expressed in % represents the increase of the native form of HA and reactivity with MAb H9 expressed in % represents the decrease of the low pH-form of HA. black – HC58; white – H9; grey – HC58 with rimantadine; dark grey – H9 with rimantadine. The bars indicate standard deviations (n = 6).

protein (0%) (Table 1, Fig. 2.) The pH modulating activity of NB protein has never been detected (Betáková and Kollerová, 2006). The replacement of extracellular domain (24 aa) of M2 protein with extracellular domain (18 aa) of NB protein in BAA chimera resulted in complete loss of detectable M2 activity and at the same time no increase in the amount of native HA was detected (Fig. 2). To find out which aa in extracellular domain of M2 were required for pH modulating activity of M2, we constructed the another chimeric protein with only first 21 aa replaced by extracellular domain from NB protein. The remained aa at positions 22, 23, and 24 were preserved from the wtM2 protein. The activity of this B(SSD)AA chimera was fully restored (Fig. 2). The activities of both chimera proteins were inhibited by rimantadine (Fig. 2). To prove the importance of aa S22, S23, and D24 for activity of M2 protein, these aa were single or double substituted by A in wtM2. Neither single substitution of individual S22, S23, and D24 nor double substitution of both S22 and S23 significantly changed the activity of mutant M2 proteins. Alanine, a small aa without charge, could not interact with other aa involved in ion channel activity and in this way could not create a barrier for protons flowing through the channel. Therefore, we constructed M2 mutants with R and H substitutions. Arginine, the negative charged aa with long chain, could alter the interaction with other aa during the transfer of protons and histidine has been implicated in both ion selectivity and in mechanism of proton conductance (Pinto *et al*., 1997; Okada *et al*., 2001). We have supposed that substitutions S22R, S23R, D24R, double substitution (S22R, S23R), S22H and S23H in wtM2 change the conductance of protons through the ion channel and influence the activity of M2. However, no significant changes in ion channel activity of M2 were observed in these mutants (Table 1). The activities of all above mentioned extracellular domain M2 mutants were inhibited by rimantadine (data not shown).

Since the W15 is the only one aromatic aa in the extracellular domain of M2 protein, it was suggested that it could influence the activity of M2. The structural changes induced by W15 mutation in M2 caused changes in the ion channel activity by reduction of pH from 8 to approximately 6. This was not observed for the truncated M2 protein lacking the N-terminal 19 aa what might be associated with absence W15 (Czabotar *et al*., 2004). In co-expression assay we tested mutated M2 protein with W15A substitution to find out effect of this substitution on pH modulating activity. The substitution of W15A in M2 did not change the protection of HA (Table 1). The activity of W15A mutant was inhibited by rimantadine (data not shown).

Our results showed that tested aa substitutions in extracellular domain of M2 protein did not significantly change or influence the ion channel activity. It was not likely that NB extracellular domain affected directly the conductance of protons through the transmembrane domain. Extracellular domain of NB protein presumably influenced the structure of transmembrane domain of M2 protein and in this way abolished the activity of this protein. Preservation of 3 aa of wtM2 in positions 22, 23, and 24 resulted in resulted in the stabilization of the transmembrane domain and restoration of its ion channel activity.

The mutated M2 proteins with substitutions C50S or C50F have altered the proton conductance (A.J. Hay, unpublished data). Co-expression of HA with the C50S and C50F mutants resulted in the same activity as with wtM2 protein (Table 2). We supposed that the H90 in the cytoplasmic domain could interact with the transmembrane domain and in this way influence the conductance of ion channel. To test this hypothesis, the M2 mutant with H90S substitution was prepared and tested. Surprisingly, this mutant retained its ion channel activity without any significant change. The activities of all these mutants were specifically inhibited by rimantadine (data not shown).

Previous electrophysiological measurements have shown that some mutants with truncated cytoplasmic domain significantly decreased conductance of ion channel (Tobler *et al.*, 1999). In the last experiment, we tested the co-expression of native HA and truncated mutant C-truncM2 that besides truncation of 46 aa from C-terminus possessed

also 3 aa substitutions F47A, F48A, and W52A. Co-expression of HA with C-truncM2 resulted in an approx.17% increase in native HA level and corresponding approx.13% decrease of in the low pH-form of HA. These changes were half of those found with control wt M2. The activity of C-truncM2 was inhibited by rimantadine (Fig. 2). The amount of expressed C-truncM2 was checked by Western blot analysis and compared with amount of expressed wtM2 protein. We found that the expression of C-truncM2 protein was comparable with that of wtM2 protein (data not shown). Thus, the decreased activity of C-truncM2 could not be due to a lower amount of expressed protein. However, we do not know if the changes in activity of C-truncM2 were associated with the 3 aa substitutions or with the function of the truncated C-terminal domain. To solve this question, further investigation would be required.

Since none of the aa substitution in M2 resulted in loss or significant decrease of pH modulating activity, we concluded that the extracellular and cytoplasmic domains of M2 protein might not be essential for its ion channel activity.

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