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The Structure, Function, and Pathobiology of the Influenza A and B Virus Ion Channels

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Influenza A virus AM2 protein is an integral membrane protein that is an ion channel (also known as a viroporin). The channel has 24 extracellular residues, 19 residues that span the membrane once and acts as both the channel pore and also the membrane anchoring domain, and a 54-residue cytoplasmic tail. The M2 protein has four identical chains linked via two disulfide bonds that form a four-helix bundle that is 10^7–10^8 more permeable to protons than Na^+ ions. The M2 channel is activated by low pH, His residue 37 is the pH sensor, and Trp residue 41 is the channel gate. The channel is blocked by the antiviral drug amantadine hydrochloride. The influenza B virus BM2 protein does not have homology with the AM2 channel, but BM2 does have the His proton sensor, Trp gate, and is activated by low pH. It is thought that the AM2 and BM2 proteins have common functions in the influenza A and B virus life cycles. Both BM2 and AM2 also facilitate virus budding. The amphipathic helix in the AM2 cytoplasmic tail has an important role in the assembly of the virus, and functional AM2 protein makes the virus independent of the "endosomal sorting complex required for transport" (ESCRT) complex scission.

The influenza A virus genome consists of eight segments of negative-sense viral RNA. Each RNA segment is complexed with the heterotrimeric viral polymerase that consists of three subunits PB1, PB2, and PA. Together with the nucleoprotein (NP) and virion RNA, the complex forms the viral ribonucleoprotein (vRNP) particles. To date, the ~13-kB genome is known to encode 18 different proteins (Inglis et al. 1977; Palese 1977; Lamb et al. 1978, 1980; Lamb and Choppin 1979; Wise et al. 2012; Muramoto et al. 2013). The gene encoding the matrix protein (M1) produces four mRNAs: mRNA 1 is an unspliced transcript, which encodes the M1 protein, the most abundant protein in the virion. mRNA 2 is spliced and encodes the M2 protein (Lamb and Choppin 1981), which is an ion channel. mRNA 4 encodes the M42 protein, which is M2 with a lengthen amino-terminal region and mRNA 3. M2 mRNA 3 is alternatively spliced to M2 RNA 2, does not encode a known protein, and is not required for virus growth in tissue culture (Jackson and Lamb 2008). In 2012, Jagger and colleagues identified a new protein PA-X encoded in an overlapping reading frame on RNA segment 3: PA-X is translated by frameshifting and PA-X represses cellular gene expression in

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tissue culture, and in a mouse model of infection PA-X expression leads to increased inflammatory and apoptotic responses (Jagger et al. 2012).

ARCHITECTURE OF THE M2 ION CHANNEL

The 97-residue M2 protein is expressed at the apical surface of polarized cells (Hughey et al. 1992). The M2 protein consists of 24 extracellular residues, a 19-residue transmembrane domain that spans the membrane once, and a 54-residue cytoplasmic tail. Residues 45–60 form an amphipathic helix that is involved in virus budding and filament formation (Rossman et al. 2010a,b). The presence of an amino-terminal extracellular domain in the absence of a cleavable signal sequence indicates that M2 is a model type III integral membrane protein (von Heijne and Gavel 1988) that is dependent on the signal recognition particle for cotranslational insertion into the endoplasmic reticulum membrane (Hull et al. 1988). The M2 protein is abundantly expressed at the cell surface but is greatly underrepresented in virions with 20–60 molecules present in each virion (Zebedee et al. 1985; Zebedee and Lamb 1988). The M2 protein forms a homotetramer, sometimes called a dimer of dimers, and the M2 protein chains are linked by disulfide bonds (residues 17 and 19).

Oocytes are an ideal model system for electrophysiological experiments and many of the measurements of proton flux were made in oocytes of *Xenopus laevis* expressing the M2 protein. The channel is activated by low pH and is 10^7–10^8 more permeable to protons than Na^+ and K^+ (Chizhmakov et al. 1996; Pinto et al. 1996; Shimbo et al. 1996). The cation permeability of the M2 ion channel allows the ion channel to function as an antiporter facilitating the efflux of Na^+ and K^+ along with proton influx (Leiding et al. 2010). The selective nature of the ion channel is dependent on the four transmembrane domains whose polar and single charged residues face the lipid layer. Each of the four transmembrane domains form an α helix (Fig. 1). Each of the α helices consists of 3.6 residues per turn for residues 26–43. The amino terminus of the ion channel is constricted by the hydrophobic side chain of residue 27. Then the pore of the channel widens until residue 41 where it becomes constricted. Residue 37 is the proton sensor and conducts proteins by protonation/deprotonation of the imidazole (Pinto et al. 1997), and the side chain of Trp residue 41 acts as the gate of the channel (Duong-Ly et al. 2005; Cady et al. 2010).

Properties of the Ion Channel

Many studies on M2 function and activity have benefited from inhibition of the channel by the drug amantadine (Davies et al. 1964). As shown by solution nuclear magnetic resonance (NMR), solid phase NMR, and X-ray crystallography, amantadine blocks the M2 ion channel pore (Wang et al. 2001; Schnell and Chou 2008; Stouffer et al. 2008; Thomaston et al. 2015). Initial studies by Schnell and Chou (2008) placed amantadine at a low-affinity binding site on the outside of the four-helix bundle but this low-affinity site is probably irrelevant biologically (Pielak et al. 2009). The tilt angle of the four-helix bundle varies depending on the tilt angle of the helices. For a micelle of 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), a tilt angle of 37° was found, whereas in 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), an angle of 33° was found (Nishimura et al. 2002; Duong-Ly et al. 2005; Yi et al. 2009; Hu et al. 2011; Gu et al. 2013). The M2 protein is post-translationally modified by addition of palmitate to the M2 cytoplasmic tail at residue 50 and phosphorylation, predominantly at M2 residue 63.

Biological Function of the M2 Ion Channel

Several mutations in the cytoplasmic tail of M2 tail have been identified that have been shown to inhibit virus assembly and budding (McCown and Pekosz 2005; Grantham et al. 2010; Liu et al. 2018). The cytoplasmic tail is modified in its cytoplasmic domain by the addition of ubiquitination at residue K78 and this posttranslational modification is crucial for production of infectious virus (Su et al. 2018). The cytoplasmic tail contains an α-helical region (residues 45–61) that is essential for budding from the

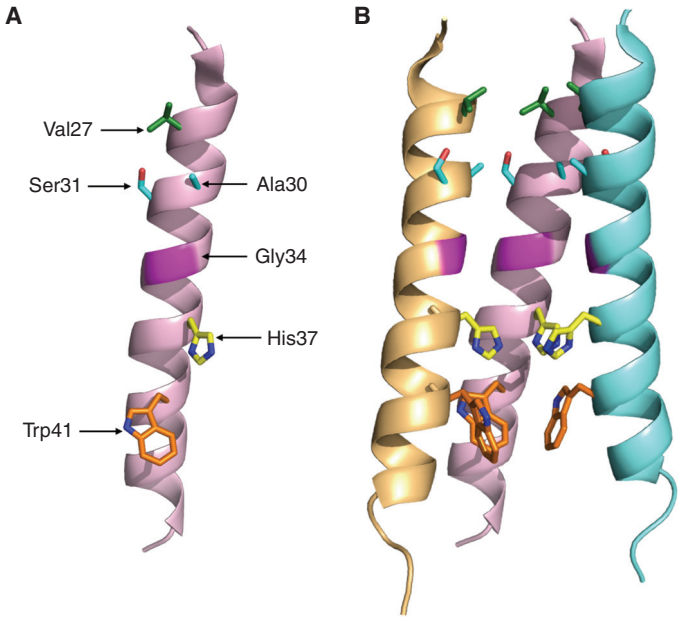

Figure 1. The three-dimensional structure of the influenza A virus M2 ion channel. (*A*) A monomer of the influenza A virus M2 transmembrane domain showing the amino acid residues that face the ion channel pore. (*B*) The nuclear magnetic resonance (NMR) structure (PDB ID: 2RLF) (Schnell and Chou 2008) was used to create the diagram. The structure of the M2 channel determined by X-ray crystallography had the four α helices at a larger degree of tilt to each other (Stouffer et al. 2008).

plasma membrane. This region of M2 also contains a cholesterol recognition/interaction amino acid consensus (CRAC) domain and a LC3-interacting region (residues 91–94) required to subvert autophagy and maintain virion stability (Beale et al. 2014). The AM2 cytoplasmic tail is highly conserved in sequence among strains and tyrosine resides in the cytoplasmic tail are required for the production of infectious particles (Grantham et al. 2010).

EARLY EFFECTS OF AMANTADINE ON INFLUENZA VIRUS REPLICATION

The M2 protein has an essential role in the life cycle of the virus. Mutants resistant to the effects of amantadine contained changes in the M2 transmembrane domain (Hay et al. 1985), and the effect of the drug was found to be at an early stage between the steps of penetration and uncoating. Later, it was found that amantadine treatment caused the M1 protein to fail to dissociate from the RNPs during uncoating and prevented the transport of the RNP complex to the nucleus (Hay et al. 1985; Zhirnov 1990; Martin and Heleniust 1991).

LATE EFFECTS OF AMANTADINE ON INFLUENZA VIRUS REPLICATION

In addition to the "early" effects of amantadine, the drug has a second "late" effect on the replication of some subtypes of avian influenza virus. These subtypes have a hemagglutinin that is cleaved intracellularly and have a high pH optimum of fusion (e.g., pH 5.7–6.0). In cells infected with highly pathogenic avian influenza viruses (e.g., Fowl Plague Virus, H7N9), amantadine causes a premature conformation change with hemagglutinin (HA) being converted to the low-pH postfusion form (Sugrue et al. 1990; Ciampor et al. 1992a,b; Grambas and Hay 1992; Grambas et al. 1992). By using immunological and biochemical criteria, amantadine caused the form of HA to be indistinguishable from the low pH–induced form of HA. Taken together, the

data led to the hypothesis that the function of the M2 protein is to act as a proton-selective ion channel that modulates the pH of intracellular compartments, principally the *trans*-Golgi network (TGN). When the channel is blocked by amantadine, the TGN is not acidified and uncoating of virus does not occur.

INFLUENZA VIRUS M2 PROTEIN MEDIATES ESCRT-INDEPENDENT MEMBRANE SCISSION

The budding of enveloped viruses is a complex multistep process, the completion of which requires alterations in membrane curvature at the neck of the budding virion. Many viruses use host "endosomal sorting complex required for transport" (ESCRT) proteins. However, influenza virus is thought to be ESCRT-independent and the AM2 protein has been shown to mediate virus budding. The highly conserved AM2 amphipathic helix that residues within its cytoplasmic tail has been shown to cause budding into giant unilamellar vesicles (Rossman et al. 2010b).

BM2 Ion Channel

At the biochemical level, the influenza B virus RNA segment 7 is similar to the influenza A virus RNA segment in coding for two proteins. However, influenza B virus encodes two proteins in RNA segment 7 by using a bicistronic mRNA encoding BM1 and BM2 and using a stop–start of translation and two cistrons in tandem (Horvath et al. 1990). BM2 encodes 109 amino acids. Successful uncoating of the influenza B virus in endosomes requires acidification of the influenza B virus particle (Paterson et al. 2003). When the BM2 protein was expressed in *Xenopus* oocytes or in mammalian cells, acidification of the cells occurred and the BM2 protein has properties consistent with it being a proton selective ion channel. The transmembrane domain of BM2 and influenza A virus M2 protein both contain the motif HXXXW, and for both proteins the His and Trp residues are important for channel function (Mould et al. 2003). Like the A/M2 protein, B/M2 is a four-helix bundle and the atomic structure was determined by NMR (Wang et al. 2009). However, BM2 is not blocked by amantadine because overall its pore has a different amino acid sequence from A/M2. Thus, influenza A virus and influenza B virus both encode proton-selective ion channels using the same ion mechanism, but the two viruses encode the two proteins in different RNA segments and by different strategies (Mould et al. 2003).

INFLUENZA C VIRUS ION CHANNEL

There is some evidence that influenza C virus has an ion channel activity. It has been found that the influenza C virus CM2 protein, which is a small integral membrane protein that spans the membrane once, can alter intracellular pH, and its transmembrane domain can substitute for that of influenza A virus AM2 protein and can support infectious virus production (Stewart and Pekosz 2012). However, electrophysiological recordings have not been made to verify ion channel activity.

CONCLUDING REMARKS

Influenza A and B virus AM2 and BM2 proteins are two of the smallest ion channels identified to date, and influenza A virus AM2 protein is the target for inhibition by the antiviral drug amantadine hydrochloride. The channels are proton-selective, and a histidine and a tryptophan are the proton sensor and the channel gate, respectively. The influenza A virus AM2 protein forms an amantadine-sensitive channel, but the influenza B virus BM2 ion channel is not sensitive to amantadine. The structure of structure of AM2 has been determined by X-ray crystallography, by solution NMR, and by solid phase NMR, and for BM2 by solution NMR, and thus the AM2 and BM2 ion channels have become among the most studied ion channels in biology.

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