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Shaun Stewart
Washington University in St. Louis

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WASHINGTON UNIVERSITY IN ST. LOUIS

Division of Biology and Biomedical Sciences

Molecular Microbiology and Microbial Pathogenesis

Dissertation Examination Committee:
    Andrew Pekosz, Co-chair
    Michael S. Diamond, Co-chair
    Phyllis Hanson
    Henry V. Huang
    Deborah J. Lenschow
    Lee Ratner
    Dong Yu

THE ROLE OF THE MEMBRANE PROXIMAL REGION
OF THE M2 CYTOPLASMIC TAIL IN VIRUS REPLICATION

by

Shaun Maxwell Stewart

A dissertation presented to the Graduate School of Arts and Sciences of Washington University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

December 2011

Saint Louis, Missouri
Influenza A virus encodes M2, a proton channel that has been shown to be important during virus entry and assembly. The primary aim of this thesis was to investigate the role of the membrane proximal region, residues 46-69, of the M2 cytoplasmic tail during virus replication. A cholesterol recognition/interaction amino consensus (CRAC) motif, previously identified in the membrane proximal region of M2 in some influenza A virus strains, was suggested to play a role in virus replication by mediating incorporation of M2 into budding virus particles. Alteration or completion of the M2 CRAC motif in two different recombinant virus strains caused no changes in virus replication in tissue culture; however, viruses lacking an M2 CRAC motif had decreased morbidity and mortality in the mouse model of infection. In order to further investigate the role of the membrane proximal residues of M2 in basic virus replication, scanning and directed alanine mutants were generated and analyzed in trans-complementation assays and recombinant viruses.
The membrane proximal residues 46-69 tolerated numerous mutations with little, if any, affect on virus replication suggesting that the identity of individual amino acids in this region are less important than the overall protein structure for the M2 protein function. The requirement during virus replication of the ectodomain and the cytoplasmic tail of M2, which includes the membrane proximal region, was further characterized using the influenza C virus CM2 protein and a chimeric influenza A virus M2 protein (MCM) containing the CM2 transmembrane domain. While M2, CM2, and MCM could all alter cytosolic pH to varying degrees when expressed from cDNA, only M2 and MCM could at least partially complement an M2-null virus in a trans-complementation system. This data suggests that while the CM2 ion channel activity is similar to that of M2, sequences in the ectodomain and/or cytoplasmic tail play important roles in infectious virus production. This thesis suggests that the structure of the membrane proximal region of the M2 cytoplasmic tail may stabilize the membrane distal region, which mediates genome incorporation.
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CHAPTER 1

Introduction

Shaun M. Stewart
Influenza Virus Pathogenesis

Influenza A virus is a member of the Orthomyxoviridae family whose genome consists of 8 negative-sense RNA segments which encode 10 or 11 proteins. Influenza A virus remains a major public health burden even with widespread annual vaccination and the availability of antivirals. Each year more than 230,000 hospitalizations and 50,000 deaths in the United States alone are associated with influenza related illness (148, 149). Two types of vaccines are currently on the market, a trivalent inactivated vaccine (TIV) and a trivalent live attenuated influenza vaccine (LAIV) [reviewed in (29)]. However, annual vaccination is indicated for both vaccines because neither generates long term immunity to the ever evolving influenza viruses. Influenza viruses undergo two forms of genetic variation. The immune response of infected individuals, primarily against the viral glycoproteins hemagglutinin (HA) and neuraminidase (NA), provides selective pressure that allows naturally occurring variants with replication advantages, which arise through random mutagenesis, to escape preexisting population immunity, termed genetic drift. A more drastic change in circulating strains can occur when two strains co-infect a single cell and give arise to recombinants that have a mix of the viral RNA from the two viruses, termed genetic shift. Genetic drift is one reason why we require annual vaccination while genetic shift can give rise to potential pandemic strains for which few in the population have immunity against, such as happened in 1918 and 2009 (100). Antivirals also exist that help decrease the severity and duration of disease but they must be administered very early during infection in order to be efficacious and resistance easily arises against these drugs [reviewed in (48)]. The two classes of antiviral
medications are the neuraminidase inhibitors, Oseltamivir and Zanamivir, and M2 inhibitors, Rimantadine and Amantadine.

Due to the threat of future pandemics, the unpredictability of which influenza strains will circulate in a given year, and the existence of resistance to all current influenza antivirals, continued research on the basic viriology of influenza A virus is warranted.

**Assembly of Infectious Virus and Virus-Like Particles**

There are at least four different types of enveloped viral products that can be assembled and released from the plasma membrane of host cells, virus-like particles (VLPs), pseudotyped VLPs, pseudotyped virus particles, and infectious virus particles. Enveloped VLPs are characterized by a cell membrane derived envelope that incorporates at least one viral glycoprotein or internal structural protein (Fig 1A). If any viral genetic material is present, it is either incomplete or artificial; therefore, VLPs are non-replicating. Pseudotyped VLPs are generated by the co-expression of glycoproteins from one or more viruses along with the internal core proteins from a different host virus (Fig 1B). Like VLPs, the genetic material in pseudotyped VLPs is either incomplete or artificial, if present at all, and the particles are non-replicating. Pseudotyped virus particles are generated by the co-expression of glycoproteins from one or more viruses in cells infected by another virus that either has a complete complement of genes or does not encode its own glycoproteins (Fig 1C). The potential infectivity of pseudotyped virus particles depends on whether the host virus used to infect the cells is fully replication competent. Often host viruses are utilized that contain genetic deletions in order to
produce particles that cannot replicate. If pseudotyped virus particles are non-replicating, they might also be termed pseudotyped VLPs. However, for the purpose of this thesis, when the foreign glycoprotein is expressed \textit{in trans} and not from the viral genome of the parent VLP, the particles can never replicate and we term them pseudotyped VLPs. Whereas pseudotyped particles formed when the host viral genome is engineered to express a foreign glycoprotein will be termed pseudotyped virus particles even if they are unable to replicate. Infectious virus particles, unlike VLPs and pseudotyped VLPs, contain a complete viral genome and complement of structural proteins, all from the same virus (Fig 1D). The differences between these four distinct viral particles will be illustrated using vesicular stomatitis virus (VSV), human immunodeficiency virus (HIV), and finally influenza A virus.

**Virus-Like Particles**

The expression of viral glycoproteins or internal proteins is often sufficient to induce the release of enveloped particles from the surface of cells. Despite lacking a complete viral genome and, sometimes, the internal structural proteins, VLPs often retain similar morphology to infectious virus particles. However, the lack of expression of one or more internal proteins can alter packaging organization and result in particles with larger diameters or more pleiotropic morphology than those of infectious virus particles (123). Due to their lack of a complete viral genome, VLPs are non-replicating. They can be generated by transient expression of one or more of the structural proteins. Several techniques used for expression include transfection of mammalian expression vectors, infection with a recombinant vaccinia virus encoding a T7 bacteriophage polymerase
(vvT7) and transfection with a T7 polymerase-driven expression vector, transfection with an alphavirus replicon which encodes one or more structural proteins from another virus, or infection with an unrelated virus, such as an alphavirus or a baculovirus, which has been engineered to express one or more structural proteins from another virus. VLPs are used to study the role of various viral proteins in virus attachment, fusion, and assembly. They have also been safely used as vaccines in animals and humans.

**Vesicular Stomatitis Virus (VSV) VLPs**

One VSV VLP system is characterized by the expression of the vesicular stomatitis virus G glycoprotein (VSV-G) from a Semliki Forest virus (SFV) replicon (123). In this system, VSV-G is sufficient to induce VLP formation and the SFV replicon gets incorporated into the VLPs randomly. However, the envelopes of VLPs are derived from the host, and host proteins may be involved in the release of VLPs. Therefore, the choice of host can greatly affect the production and composition of these particles. VSV-G VLPs are cell system dependent because the system produces VLPs well in BHK, CHO, and C6/36 cells, less well in NRK and COS cells, and not at all in HeLa cells (123). This system, although able to replicate, is considered a VLP here because only the viral glycoprotein is expressed. However, it could, more accurately, be considered a simple manmade virus. A modification of this system, to generate replication incompetent VSV VLPs, expresses the VSV-G from a plasmid that is co-transfected along with the SFV replicon which expresses another gene of interest (24). The latter system avoids the overproduction of the cytotoxic VSV-G protein in cells infected with the VSV VLPs and could enable use of this technology as a transduction vector.
The matrix protein from VSV (VSV-M) has also been shown to be sufficient when expressed alone to induce VLP formation in Sf9 insect cells using a baculovirus expression system (82) and in CV1 cells using a vvT7 infection/transfection system (64).

**HIV VLPs**

The core structure of HIV is encoded by the gag gene which is translated as a precursor polyprotein, Pr55\(^{\text{gag}}\) (MA-CA-NC-p6), which is processed during or after virus particle budding by the protease (PR) protein encoded by the viral pol gene to form, from N-terminus to C-terminus, the major core proteins matrix (MA), capsid (CA), nucleocapsid (NC), and p6. Expression of uncleaved Pr55\(^{\text{gag}}\) alone using a baculovirus expression system is sufficient to form VLPs in Sf9 cells (38). Pr55\(^{\text{gag}}\) is myristylated at the N-terminal glycine residue in MA and induces budding from a type of membrane microdomain enriched in cholesterol, glycosphingolipids, and sphingomyelin [(108), reviewed in (152)], often called lipid rafts [reviewed in (136)]. Pr55\(^{\text{gag}}\) has two modes of membrane binding [reviewed in (20)]. If the myristylation site is mutated (38) or myristylation is chemically inhibited (97), Pr55\(^{\text{gag}}\) fails to bind membranes and induce VLP release, but instead forms circular structures in the cytoplasm and/or nucleus. However, a basic patch on MA of Pr55\(^{\text{gag}}\) is also important for membrane binding and directs interactions with acidic phospholipids, particularly phosphatidylinositol 3,4-bisphosphate [PI(3,4)P\(_2\)] (19, 107, 141, 164). Unlike membrane binding, p6 is not required for virus-like particle formation and release (52, 127). However, expression of a MA-CA construct without NC and p6 results in long tubular structures attached to cells (38, 52) or a vastly decreased number of spherical particles attached to cells (127). CA-NC and CA-NC-p6 can form protein cylinders (13) and MA-CA-NC and MA-CA-NC-p6
(Pr55gag) can form spheres (12) in vitro in the presence of nucleic acid and in the absence of myristylation and phospholipids. This suggests that nucleic acid is important for HIV VLP formation and that it can occur with only expression of Pr55gag. Additionally, Env expression does not increase release of particles (38).

Strategies utilizing HIV VLPs in attempts to generate an HIV vaccine are too numerous to exhaustively describe here [reviewed in (161)]. However, one interesting example that has undergone human clinical trials in various forms includes the expression of Gag, Env, and PR from a canarypox vaccine (28, 91). Infection of mammalian cells with canarypox viruses is abortive and results in only one round of infection. During this one round of infection, expression of the HIV proteins leads to the release of VLPs which are able to generate CTL responses in some individuals [reviewed in (91)].

Influenza Virus VLPs

Like VSV, formation of influenza virus VLPs are affected by the host and expression system utilized. For instance, Cos-1, CV-1, and HeLa cells expressing influenza matrix protein (M1) via a vvT7 infection/transfection system (17, 40) and Sf9 cells expressing M1 using a baculovirus expression system produce VLPs (68, 77, 117, 140) (Fig 2A) while BHK cells expressing M1 via a semliki forest virus (an alphavirus) replicon do not result in VLP production (163). In the latter example, 3 hours post transfection was the latest time point investigated, and it is possible that VLPs might have been detected at later time points. Others have shown that in 293T cells, expression of either M1 or the proton channel matrix 2 protein (M2) alone by mammalian expression vectors results in the production of VLPs (Fig 2A and B), but co-expression of the glycoprotein neuraminidase (NA) or both the glycoproteins hemagglutinin (HA) and NA
together increases the number of VLPs produced (17, 76). Additionally, the level of M1, but not HA or NA, expression, has also been shown to be correlated with the level of VLP production when measured by the incorporation of a reporter gene (102). Correlation of M1 and reporter gene levels may relate to the role of M1 during genome packaging.

It has been reported that expression of HA alone is insufficient to produce VLPs (77). However, these experiments do not take into account that, during both viral entry and egress, HA binds sialic acid and will result in retention or clumping of budded VLPs and virus particles unless sialidase activity is present either in a co-expressed NA or in exogenously added NA (17, 41, 83, 110). In fact, several reports have now shown that expression of HA alone can produce VLPs when an NA is added exogenously (17, 76) (Fig 2C). Additionally, both insect and plant cells, which usually do not produce proteins containing terminal sialic acid residues (90, 134), can also be used to produce HA containing VLPs in the absence of NA (23, 32, 33, 45, 72, 117, 119).

Recently, several groups have investigated the ability of NA to induce VLPs in the absence of the other viral proteins. Expression of some strains of NA alone are able to induce VLP formation while others are not (76, 77, 160) (Fig 2D).

All of these studies taken together show that in various systems, the expression of M1, M2, HA, or NA alone is sufficient to produce influenza A virus VLPs (Fig 2). Differences in the expression systems and cellular hosts may account for why each system shows that a different protein is necessary and sufficient for VLP formation. Assembly and release of VLPs is highly dependent on cellular processes like expression levels and locations, glycosylation, vesicular sorting, and trafficking. Each cell system
may have slight differences in these or other cellular processes which are required for, contribute to, or restrict the production of VLPs.

Influenza virus VLPs have been used to successfully induce protective immunological responses in animals [reviewed in (67)] when expressing M1 and M2 (Fig 2E) (139, 140), HA and M1 (Fig 2F) (32, 33, 68, 72, 117, 119, 138), M1, HA, and NA (Fig 2G) (3, 4, 88, 115, 116, 144), M1, HA, NA, and M2 (Fig 2H) (93, 158), and all viral proteins minus NS2/NEP (154), but not M1 alone (140). Influenza virus VLPs expressing HA, NA, and M1 are currently moving through human clinical trials (70). Influenza virus VLPs and inactivated vaccines can both be multivalent (29, 114, 119). VLPs have also been considered as a supplement to inactivated vaccines in an attempt to broaden the cross protection (139) and have been utilized as a carrier to present foreign glycoproteins which will be discussed below as pseudotyped VLPs.

**Pseudotyped Virus-Like Particles**

Pseudotyped VLPs are produced like VLPs except that one or more glycoproteins are co-expressed from a different virus. There are two advantages to generating pseudotyped VLPs. One is if the glycoprotein of interest does not induce VLP formation, then core proteins from a virus known to induce VLPs can be used to generate pseudotyped VLPs that incorporate the glycoprotein of interest. A second purpose is that VLPs from a characterized system can used to quickly generate new ones that express a glycoprotein from another virus without the development of new characterization or purification protocols. The simultaneous expression of glycoproteins from one or more viruses along with the internal proteins from another virus has been achieved via several
mechanisms. The glycoproteins from one virus can be co-expressed with the internal proteins from another virus using mammalian expression vectors. Pseudotyped virus particles can also be generated by the expression of the glycoproteins using expression vectors followed by infection with a virus that does not encode its own glycoproteins. Like VLPs, pseudotyped virus particles have been used to study the role of various viral proteins during virus attachment, fusion, and assembly and are being utilized to generate vaccines that elicit immune responses to the glycoproteins.

Pseudotyped VSV VLPs

Whereas, the expression of influenza matrix (M1) protein has been used as a means of generating VLPs that can also incorporate glycoproteins from foreign viruses, VSV matrix (VSV-M) VLPs have not been utilized in the same way, perhaps because less research has been conducted on them. Nonetheless, non-replicating VSV particles have been engineered from a recombinant VSV which has its own glycoprotein deleted (VSVΔG) (143). In situations where the glycoprotein is provided in trans from another expression system, VSV pseudotyped VLPs have been formed using glycoproteins from several viruses including influenza C virus (47), measles virus (145), SARS coronavirus (31), Hantaan and Seoul viruses (105), HBV (128), Andes virus (120), Nipah virus (98), Ebola virus (143), and human T-cell leukemia virus 1 (106).

Due to the relative safety and easy creation of pseudotyped VSV VLPs, they have been used in virus entry studies of Andes virus (120), Nipah virus (98), Ebola virus (143), and human T-cell leukemia virus 1 (106), and measles virus (145) as well as effective vaccines in animal models for Hantaan and Seoul viruses (78).

Pseudotyped HIV VLPs
HIV VLPs are easily generated from expression of Gag alone, see above, and these VLPs can also be pseudotyped with foreign glycoproteins. Generation of pseudotyped HIV VLPs has been successfully used to generate protective immunity against influenza virus (51). Additionally, HIV VLPs pseudotyped with influenza HA (159) and VSV-G (74) have been used in attempts to generate immunity against HIV Gag in the absence of the immunodominant HIV Env. HIV VLPs co-expressing both HIV Env and other additional glycoproteins have also been utilized in an attempt to boost the immune response to Env (45).

**Pseudotyped Influenza VLPs**

Influenza VLPs can be pseudotyped when VSV-G is co-expressed with either PB2, PB1, PA, NP, M1, M2, and NS2 (102) or NA, M1, and M2 (77). However, both of these systems are fairly complex and have not been studied further. Simple pseudotyped VLP systems are preferred that only require matrix from one virus and glycoproteins from another. Indeed, influenza VLPs can be generated from the individual expression of M1 protein, see above. Although some debate in the field on whether M1 was sufficient has hindered until recently the generation and use of pseudotyped influenza VLPs. Co-expression of viral glycoproteins from other viruses, along with M1, can result in their incorporation into the VLPs. When the transmembrane domain and cytoplasmic tails of the spike glycoprotein from SARS coronavirus is replaced with that from influenza HA, it can be successfully incorporated into influenza M1 VLPs and administration in the absence of adjuvant protects mice better than purified spike protein (Fig 3A) (84). Recently, respiratory syncytial virus (RSV) F and G glycoproteins were
both successfully pseudotyped onto influenza VLPs and were both able to induce some protective immunity in mice (Fig 3B) (118).

**Pseudotyped Virus Particles**

Pseudotyped (or chimeric) virus particles are produced when a glycoprotein from one virus (glycoprotein donor) is expressed in cells that are infected with another virus (core donor). The two donor viruses can be from completely different viral families or simply different strains of the same virus. The core donor can express its own glycoproteins or can be engineered not to express its own surface proteins. The expression of donor glycoprotein(s) can be accomplished using several techniques including transfection with cDNA expression vectors, co-infection with either the donor virus or an unrelated virus expressing donor glycoprotein, or a recombinant core donor virus can be engineered to express the donor glycoprotein in addition to or in place of its own glycoprotein(s).

**Pseudotyped VSV Virus Particles**

A strain of VSV encoding a temperature sensitive (ts) mutation in the glycoprotein G has been used to study the ability of glycoproteins from various other viruses to incorporate into budding particles (30). Rabies virus glycoprotein G was shown to incorporate into this ts VSV and mediate subsequent infection which could be blocked by antibodies against rabies G but not VSV-G (155). Co-expression of a fusion of HIV Env with the cytoplasmic tail of VSV-G, but not of full length HIV Env, can also be incorporated into this ts VSV when it buds, suggesting that amino acids in the cytoplasmic tail of VSV-G may interact with matrix and drive incorporation (109, 156).
Indeed, native Moloney murine leukemia virus (MuLV) glycoprotein is unable to be incorporated into this ts VSV at non-permissive temperatures (157). The ability to form rabies G pseudotyped VSV VLPs may relate to the fact that rabies virus and VSV are both rhabdoviruses and their glycoproteins might share enough sequence homology that the cytoplasmic tail of rabies G can interact with VSV-M sufficiently to incorporate it into budding virus particles.

VSV pseudotypes that incorporate VSV-G as well as a foreign viral glycoprotein have been generated by the co-infection of VSV and many other viruses including influenza A virus (126), MuLV (157), visna virus (39), lymphocytic choriomeningitis virus (9), HIV (124), and human T-cell leukemia viruses (21). However, distinguishing between the two types of virus particles, VSV genome/core or foreign virus genome/core, can be quite difficult and limits the usefulness of pseudotyped virus particles generated from co-infections.

The genome of VSV can be engineered to encode extra genetic material and this has been utilized as a mechanism to express foreign proteins (132). HIV Gag and Env expressed this way generate their own HIV VLPs but do not get incorporated into the VSV particles, and therefore do not form VSV pseudotyped virus particles (46). The lack of incorporation of Env into VSV virus particles could be due to an inherent incompatibility between the Env and VSV matrix since Env incorporation into VSV can be induced by deleting three residues from its cytoplasmic tail or replacing the tail with the VSV-G cytoplasmic tail (62, 63). Similarly, VSV which encodes core, E1, and E2 from hepatitis C virus (HCV) seems to release HCV VLPs but no HCV-pseudotyped VSV virus particles (2, 26). Like HIV Env, HCV glycoproteins E1 and E2 can be
incorporated into VSV virus particles by alteration of the cytoplasmic tail, which removes the ER retention motifs in E1 and E2 (75, 94). Additionally, other full length viral glycoproteins are able to be incorporated into the wildtype VSV virus particles including cellular CD4 (131), H and F of measles virus (131), F and G from RSV (66), GP from Ebola, Marburg, and Lassa viruses (35), and HA and NA from influenza A virus (73).

More recently, reverse genetics have been used to generate recombinant VSV that does not encode VSV-G (143). This VSVΔG virus can be generated in cells expressing VSV-G and when used to infect cells expressing a foreign glycoprotein in trans, non-replicating pseudotyped VSV VLPs are generated (see above). However, this VSVΔG can also be engineered to express a foreign glycoprotein in cis by cloning the glycoprotein into the VSV genome, which generates pseudotyped VSV virus particles. As long as cells which express the receptor to the foreign glycoproteins are infected, then the pseudotyped virus particles are able to replicate and produce new pseudotyped virus particles. This system has been developed for several diverse viruses including influenza A virus (121), HCV (10), Ebola, Marburg, and Lassa viruses (35), and RSV (65).

Pseudotyped virus particles, have been used as effective vaccines in animal models when core proteins and genetic material from VSV are encased in lipid membranes containing glycoproteins from other viruses, including Ebola, Marburg, and Lassa viruses [(35), reviewed in (27)], as well as Nipah virus (14), HCV (89), and RSV (65).

Pseudotyped Influenza Virus Particles
Before the creation of an influenza A virus reverse genetic system, reassortment of two strains of influenza was used as a mechanism of studying individual genes as well as a way to generate the yearly vaccine candidates. A reassortant virus that expresses HA and/or NA from one strain and the core proteins from another strain is essentially a pseudotyped virus particle. Since influenza A viruses contain 8 genomic segments, a co-infection with two strains could result in \(2^8\) or 256 different possible strains. Reverse genetics has greatly improved the ability of obtaining a virus that encodes glycoproteins one strain and all other segments from another strain, often referred to as a master donor strain (Fig 4).

Pseudotyping influenza virus particles is the primary method used every year to generate both the live attenuated and inactivated annual seasonal influenza vaccines [reviewed in (29)]. The inactivated seasonal vaccine is currently generated using a reverse genetics system in which the HA and NA genes from the strains predicted to circulate in the upcoming influenza season are combined with the 6 other segments from a high growth donor strain adapted to grow to high titers in eggs, A/Puerto Rico/8/34 (PR8). Likewise, the live attenuated seasonal vaccine is generated using similar methodology with the exception that the donor strain which provides all segments other than HA and NA is a cold-adapted, live attenuated strain, A/Ann Arbor/6/60.

**Infectious Virus Particles**

Infectious virus particles can enter a cell and generate infectious, and sometime non-infectious, progeny. They are normally generated by the infection of cells with live virus, but they can also be initially generated several other ways. With many positive
stranded RNA viruses, transfection of RNA or expression of the RNA from a plasmid can result in production of infectious virus particles. Reverse genetic systems also exist for many negative sense RNA viruses where infectious virus can be produced from the expression of the polymerase genes, either by mammalian expression vectors or non-replicating helper viruses, along with negative sense viral RNA, either by RNA transfection or plasmid expression of the viral RNA using polymerase I promoter and terminators in order to generate ribosomal RNA that lacks both 5’ cap and a 4’ poly(A) tail (25, 101, 103).

**Infectious VSV Virus Particles**

Another virus that assembles with a two step process is VSV. However, this process is different from influenza virus in that both steps, RNA incorporation and particle formation/release, are directed by one protein, VSV-M. The assembly and budding of VSV has been studied extensively [some reviewed in (60)]. After transcription and replication of the single segment of VSV vRNA, the vRNA is bound and protected by the VSV-N protein to form a ribonucleoprotein complex (RNP) (44, 147). The VSV-M protein has inherent membrane binding capabilities and exists as two pools in cells, one in the cytoplasm and one at the plasma membrane (79). Membrane binding by VSV-M can induce membrane curvature and is the only viral protein required for particle release, see above, but it is insufficient to induce complete budding in the absence of cellular proteins (137). The binding of VSV-M to the RNPs induces condensation and formation of the skeleton structure that is found in viral particles (69, 87, 104). This VSV-M interaction with RNPs may be seeded by the presence of the glycoprotein G (142). Indeed, cryo-electron microscopy models of N-M complexes
suggest that VSV-N forms the tip of the bullet-shaped viral particle and is only surrounded and strengthened by VSV-M (37). However, this condensation, incorporation, and release of particles has been shown in the absence of the glycoprotein G (143). As was previously noted, expression of M alone can lead to production of VLPs, further suggesting that VSV-G is not required for production of virus. However, in the absence of VSV-G, the budding “bald” particles are unable to bind, enter, and infect subsequent cells since VSV-G is responsible for viral attachment and fusion (143).

VSV buds from microdomains at the plasma membrane which are enriched in the glycoprotein G (6, 85, 86). Influenza microdomains are similar to lipid rafts in that they are in enriched in cholesterol, gangliosides glycosphingolipids, and GPI-anchored proteins and can be extracted due to their low solubility in cold non-ionic detergents such as TX-100 [reviewed in (136)], whereas VSV microdomains are thought to be independent of both lipid rafts and CD4-containing microdomains (5, 7). In fact, VSV-G is often used as a negative control for lipid rafts (162).

VSV-M contains two late motifs [reviewed in (11)], a PPxY late motif, which is important for virus particle release, interacts with WW domain containing proteins like Nedd4 (22, 49, 50, 61) and a PSAP late motif that does not appear to be important for virus particle release (58).

As discussed previously, expression of VSV-G alone, like VSV-M, is sufficient to induce VLP formation; however, non-infectious virus particles can form in the absence of G albeit less efficiently (143). Although there are not specific interactions between the VSV-G cytoplasmic tail and VSV-M (130), VSV-G is nonetheless believed to enhance
the ability of VSV-M to bind and condense RNPs, thereby slightly enhancing particle release in the presence of VSV-G (60, 130, 142).

**Infectious HIV Virus Particles**

HIV assembly and budding occurs in two steps like VSV. However, for HIV, the first step involves RNA packaging, as well as virus assembly and release, and the second step involves maturation of the particles. HIV budding is largely driven by Pr55\(^{gag}\), see above, and the localization of Pr55\(^{gag}\) in particular membrane microdomains drives virus particle assembly and budding from these locations. Therefore, like influenza, VSV, and many other viruses, HIV buds from a type of membrane microdomains often called lipid rafts which are enriched in cholesterol, glycosphingolipids, and sphingomyelin [reviewed in (136, 152)]. Also, similar to influenza, tetraspanins get incorporated into HIV virus particles and but their role in virus budding is unclear [reviewed in (146)].

In a simplified model of HIV budding, MA directs membrane binding, CA assists Gag-Gag interactions, NC binds viral RNA, and p6 recruits cellular factors involved in particle release [reviewed in (34)]. It should be stated that most of these functions of the Gag proteins are occurring while Pr55\(^{gag}\) is uncleaved and, therefore, multiple Gag domains interact in a highly complex way in order to direct and control the various budding processes including membrane and RNA binding, oligomerization, and particle release.

During virus particle release, protease (PR) is activated and cleaves Pr55\(^{gag}\) inside immature particles into several proteins, including matrix (MA), capsid (CA), nucleocapsid (NC), and p6 [reviewed in (34)]. This cleavage causes structural rearrangement inside the virion, often called maturation, resulting in the formation of
infectious, mature particles. After this structural rearrangement there are three distinct layers inside the viral membrane; MA remains bound to the inside leaflet of the viral membrane forming an electron dense ring, CA assembles into a conical capsid that sits inside the MA layer, and NC coats the vRNA and resides in the center of the virions.

Although MA-CA-NC, without p6, can induce VLP formation (see above), p6, in particular a PTAP late domain within the protein, is required for efficient virus particle release in the context of virus infection (42, 53). Particles can be released in the absence of this late domain within p6 if the protease activity of PR is disrupted; however, particles that bud in this fashion never undergo proteolytic maturation of Pr55\textsuperscript{gag} and are non-infectious (53). The late domain in p6 has been shown to mimic the cellular Hrs protein in order to bind TSG101, a cellular component of ESCRT-I complex which normally aids in the cellular vesicle protein sorting pathway, and co-opt it into helping release HIV virus particles [(36, 92, 113), reviewed in (11)].

The release of HIV virus particles can be restricted by a cellular protein, tetherin, which can bind both the plasma membrane and the viral membrane and prevent particles that have undergone scission from being released into the supernatant (99, 151). The activity of tetherin can be modulated by the HIV protein Vpu which downregulates the surface expression level of tetherin (99, 151).

Like influenza virus and VSV, HIV virus particles can bud in the absence of glycoprotein Env expression. However, whereas specific interactions between the matrix and glycoproteins of the former two viruses have been shown to various extents, interactions between HIV Env and Gag have never been conclusively proven [reviewed in (152)]. An interesting model put forth recently based on Ebola pseudotyping of HIV in
the presence of Env co-expression is that microdomains containing Gag interact with distinct microdomains containing different glycoproteins thus producing particles with either HIV Env or Ebola glycoprotein but not both (81). This study suggests that incorporation of glycoproteins into HIV virus particles may rely more on aggregation of membrane microdomains rather than direct interactions between Gag and they glycoproteins.

**Infectious Influenza Virus Particles**

The assembly of influenza virus particles occurs at microdomains in the plasma membrane which are enriched in the viral glycoproteins, HA and NA. These microdomains contain an enrichment of cholesterol and resemble lipid rafts [(80, 129, 162), reviewed in (136)]. M1 interacts with the HA and NA in these sites, potentially through their cytoplasmic tails (1, 162). M2 is then recruited to the budding site and is important for viral RNA incorporation (16, 43, 95, 96). The mechanism of M2 recruitment and incorporation is currently under debate. Several potential methods of M2 incorporation have been suggested including ectodomain binding (112), cholesterol binding (133), and interactions with M1 (16, 95). However, there remain a few issues with these proposed incorporation mechanisms. The work by Park et al. describing that the ectodomain of M2 is sufficient to drive incorporation was based upon chimeras between M2 and the Sendai F protein (112). Because Sendai F has a structure very different from M2 and many of these chimeras were not expressed at the cell surface, complete interpretation of the findings is difficult. No research following up this claim has been published and there are no known interactions between the ectodomains of M2 and the other viral glycoproteins, HA and NA. Schroeder et al. first published that
cholesterol can bind M2 \textit{in vitro} and suggested that binding cholesterol that rims the microdomains where assembly occur could induce M2 incorporation (133). The remaining hypothesis, interactions between M1 and M2 drive the incorporation of M2, has been studied but is not yet definitive. Two M1 binding sites within the M2 cytoplasmic tail have been mapped, one in the membrane proximal region (residues 45-69) and another in the membrane distal region (residues 70-97) (95, 96). Deletion or mutation of amino acids in the membrane distal region lead to decreased vRNA incorporation but do not affect M2 incorporation (16, 43, 95, 96). The mechanism of M2 incorporation and the role the membrane proximal region of the cytoplasmic tail in this process are the focus of this thesis.

The M2 protein and a peptide corresponding to an amphipathic helix in the membrane proximal cytoplasmic tail of the protein, have also been shown to induce membrane curvature and scission in unilamellar vesicles and were suggested to be necessary for membrane scission and virus particle release (125). However, both VLPs (16, 17, 40, 68, 76, 77, 117, 140, 150, 160), as discussed above, and virus particles (18, 43, 57) can be released in the absence of M2.

Despite release of virus particles occurring in the absence of M2, various deletions and mutations in M2, and in particular the cytoplasmic tail, attenuate virus replication significantly (43, 59, 95, 96) and can be complemented with full length M2 expressed \textit{in trans} (16, 43, 95, 96). Thus influenza A virus assembly and budding can be described as two types or steps, similarly to VSV and HIV. Indeed, if M2 is not expressed, virus particles are assembled and released, but they have a defect in vRNA
incorporation (Fig 5A) (43). Infectious virus particles are only produced when functional
M2 is expressed and incorporated into the budding particles (Fig 5B) (16, 43, 95, 96).

During virus budding, viral proteins through late domains have often been shown
to redirect cellular proteins to the assembly site to achieve scission [reviewed in (15)].
The sequence YRKL in M1 was proposed as a late domain, but much of that work has
subsequently been retracted (54-56). Additional support that M1 might not contain a viral
late domain comes from the findings that budding of influenza virus particles, as well as
influenza VLPs, have been shown to be VPS4 independent, unlike other viruses which
utilize late domains (8, 17).

Numerous cellular proteins are known to be required for replication, assembly,
and release of influenza A virus. A genome-wide RNAi screen identified that, among
other cellular proteins, a cellular tetraspanin protein, CD81, was necessary for influenza
virus replication (71). Both CD81 and tetraspanin CD9 have also been identified in
purified influenza virus particles (135). However, further research will be required to
identify how CD81 and possibly other tetraspanins are necessary for virus replication.

Another cellular factor that affects the budding of various viruses is tetherin. However,
the ability of tetherin to restrict influenza budding has not yet been fully elucidated. The
budding of VLPs containing only NA is tetherin restricted in one strain of influenza virus
but not another (160). However, a recent report suggested that infectious influenza virus
particles expressing tetherin-restricted NA are not themselves restricted by tetherin (153).
It may very well turn out that some influenza VLPs but not influenza virus can be
restricted by tetherin, but in the latter paper, the assays used to compare virus and VLP
restriction were completely different, stable expression of tetherin in MDCK cells or
transient over-expression in 293T cells, respectively. Additionally, electron micrographs of infected 293T cells clearly show elongated viral particles when tetherin is transiently expressed, a defect not seen when other viruses are restricted by tetherin. Nonetheless, this suggests that transient tetherin expression in 293Ts can restrict influenza virus and influenza VLPs. However, more work needs to be done to clearly elucidate which, if any, strains of influenza virus are restricted by tetherin during infection or VLP formation. Additional experiments should also determine the average length of influenza virus particles released from 293T cells in the presence and absence of tetherin.

**Concluding Remarks**

**Non-enveloped VLPs**

Although this discussion has focused on enveloped particles, it is noteworthy that VLPs can also be produced by the expression of proteins from non-enveloped viruses. Two licensed, VLP-based human papillomavirus vaccines are on the market, Cervarix from GlaxoSmithKline and Gardasil from Merck, which are produced from the expression of L1 from baculovirus and yeast cells, respectively [reviewed in (111)]. Expression of some enveloped virus capsid proteins can also generate non-enveloped VLPs. Natural infection with hepatitis B virus (HBV) results in the production of subviral particles (essentially VLPs) which can be used to generate protective immune responses. Recombinant versions of HBV VLPs are currently licensed by several manufacturers [reviewed in (122)].

**Summary of VLPs and Infectious Virus Particles**
The differences between VLPs, pseudotyped VLPs, pseudotyped virus particles, and infectious virus particles have been carefully defined in this review. However, the literature sometimes uses some of the terms interchangeably. Nonetheless, VLPs remain a valuable tool for dissecting virus biology, but results found with them should always be confirmed in the context of infectious virus particles. Pseudotypes, in particular pseudotyped VLPs, remain promising vaccine platforms. Similarities and differences between infectious virus particles will continue to be revealed.

**Goals of the Thesis**

The primary aim of this thesis was to determine the role of the membrane proximal region of the M2 cytoplasmic tail in virus replication. It was known previously that this region was able to bind M1 (95), was able to bind cholesterol *in vitro* (133), and that deletion of the rest of the M2 cytoplasmic tail still allowed incorporation of M2 (96). Through a series of experiments that I proposed, I sought to investigate the various aspects of the membrane proximal region of M2. Mutant proteins were investigated using mammalian expression systems, a trans-complementation system, and recombinant viruses. I determined that the M2 cholesterol binding motif is not required for virus replication in tissue culture but viruses lacking the motif caused less morbidity and mortality in mice. I additionally established that the membrane proximal residues tolerated numerous mutations with little, if any, affect on virus replication suggesting that the protein structure of this region, rather than the identity of individual amino acids, may be critical for M2 protein function. Chimeric proteins between influenza A virus M2 and an M2 homolog in influenza C virus, CM2, were also utilized to compare the ion channel
function of the two proteins. I further confirmed that residues in the ectodomain and cytoplasmic tail of M2 are important in the assembly of infectious influenza A virus.
Figure Legends

Figure 1. Composition of various enveloped particles produced by the expression of viral proteins. A) The expression of viral glycoproteins and/or internal core proteins can induce the formation of virus-like particles (VLPs). B) The co-expression of glycoproteins from one or more distinct viruses during the production of VLPs from another virus can produce pseudotyped virus-like particles which may contain glycoproteins and/or core proteins from one virus along with glycoproteins from other viruses. C) Pseudotyped virus particles are produced by the co-expression of glycoproteins from distinct viruses in cells infected with another virus. D) Infectious virus particles are the product of natural virus infections and are characterized by a complete viral genome and complement of viral proteins. Natural infections can sometimes also produce incomplete virus particles that are non-infectious, not shown. In pseudotypes, foreign glycoproteins are shown in blue and yellow and, in all particles, proteins from the parental virus are shown in red.

Figure 2. Influenza virus-like particles (VLPs). A-D) Depending on the particular expression system and strain of influenza A virus, M1 (A), M2 (B), HA (C), or NA (D) expression alone has been shown to be sufficient to induce VLP budding and release. E-H) Various influenza A VLPs which have been shown to induce various levels of protective immunity in immunized animals include ones generated from the expression of M1 and M2 (A); M1 and HA (B); M1, HA, and NA (C); and M1, HA, NA, and M2 (D).
**Figure 3. Pseudotyped influenza VLPs.** Foreign glycoproteins can be incorporated into influenza VLPs when co-expressed with M1 using a baculovirus expression system. A) The ectodomain of SARS coronavirus spike (S) protein fused to the transmembrane domain and cytoplasmic tail of influenza HA and B) the native glycoproteins G and F from RSV can be incorporated into influenza VLPs.

**Figure 4. Pseudotyped influenza virus particles.** Pseudotyping influenza viruses is utilized to generate influenza vaccines candidates. Either by co-infection or reverse genetics, the HA and NA glycoproteins from a glycoprotein donor strain are combined with all the proteins expressed by the other 6 segments from a core donor strain to generate a pseudotyped influenza virus particle.

**Figure 5. Infectious influenza virus particles.** A) If M2 is not expressed, virus particles assembly and are released, but they have a defect in viral RNA (vRNA) incorporation and viral infectivity. B) If functional M2 is expressed, infectious virus particles are formed that incorporate M2 and vRNA.
Figure 1. Composition of various enveloped particles produced by the expression of viral proteins.
Figure 2. Influenza virus-like particles (VLPs).
Figure 3. Pseudotyped influenza VLPs.
Figure 4. Pseudotyped influenza virus particles.
Figure 5. Infectious influenza virus particles.
References


Tail of the Influenza A Virus M2 Protein Plays a Role in Viral Assembly. J. Virol. 80:5233-5240.


Glycoproteins: RSV Fusion Protein Can Mediate Infection and Cell Fusion.

Virology 254:81-91.


(VLPs) that protect mice against challenge with SARS-CoV. Vaccine 29:6606-6613.


93. **Matassov, D., A. Cupo, and J. M. Galarza.** 2007. A Novel Intranasal Virus-Like Particle (VLP) Vaccine Designed to Protect against the Pandemic 1918 Influenza A Virus (H1N1). Viral Immunology 20:441-452.


multiple subtypes of hemagglutinin and protect from multiple influenza types and subtypes. Vaccine 29:5911-5918.


CHAPTER 2

The cholesterol recognition/interaction amino acid consensus motif of the influenza A virus M2 protein is not required for virus replication but contributes to virulence

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Shaun M. Stewart, Wai-Hong Wu, Erin N. Lalime, Andrew Pekosz.

Abstract

Influenza A virus particles assemble and bud from plasma membrane domains enriched with the viral glycoproteins but only a small fraction of the total M2 protein is incorporated into virus particles when compared to the other viral glycoproteins. A membrane proximal cholesterol recognition/interaction amino acid consensus (CRAC) motif was previously identified in M2 and suggested to play a role in protein function. We investigated the importance of the CRAC motif on virus replication by generating recombinant proteins and viruses containing amino acid substitutions in this motif. Alteration or completion of the M2 CRAC motif in two different virus strains caused no changes in virus replication in vitro. Viruses lacking an M2 CRAC motif had decreased morbidity and mortality in the mouse model of infection, suggesting that this motif is a virulence determinant which may facilitate virus replication in vivo but is not required for basic virus replication in tissue culture.
Introduction

Influenza A virus remains a major public health burden and potential pandemic threat even with widespread annual vaccination and the availability of antivirals. The M2 protein is required for several steps in the viral life cycle (reviewed in (28)). Following hemagglutinin (HA)-mediated virus-cell membrane fusion, the ion-channel activity of M2 is activated in acidified endosomes. M2 translocates protons into the core of the virus particle which mediates the release of viral ribonucleoprotein (vRNP) complexes from their association with matrix (M1) protein and viral membranes. The vRNP are transported to the nucleus where viral transcription and genome replication occurs. The ion-channel activity of M2 also raises the pH of the Golgi compartment, thereby preventing the low pH induced conformational changes in HA proteins which are processed to their fusion-competent forms by intracellular proteases. Virus particle assembly occurs at the plasma membrane and the cytoplasmic tail of M2 is required for efficient incorporation of vRNP into infectious virus particles (19, 20).

Influenza A virus particles assemble and bud from plasma membrane domains enriched with the viral glycoproteins, HA and neuraminidase (NA). These domains may also reflect lipid rafts (15, 32, 45). The cytoplasmic tails of HA and NA bind and recruit M1 to membranes (1). Even though M2 is not found at these sites of glycoprotein enrichment, a small amount of M2 is incorporated into virus particles (15, 45). Additionally, the amount of M2 incorporation can be increased if the glycoproteins are targeted away from lipid microdomains by the deletion of their cytoplasmic tails (3).

Several mechanisms have been hypothesized to contribute to M2 virion incorporation, including random incorporation and incorporation via interaction with
cholesterol (34). A membrane proximal cholesterol recognition/interaction amino acid consensus (CRAC) motif has been identified in the peripheral-type benzodiazepine receptor and other proteins known to bind cholesterol (16), including caveolin-1 (6) and human immunodeficiency virus (HIV) gp41 (8, 39, 40). The CRAC motif in gp41 is adjacent to a transmembrane helix and has been shown to bind cholesteryl-hemisuccinate agarose (39). Mutation of the motif decreased cholesterol binding but also altered fusogenic activity when introduced into HIV (4, 40). Schroeder et al. identified a putative CRAC motif in M2 and determined that M2 protein purified from a baculovirus expression system binds cholesterol (34). A second CRAC motif immediately downstream of the first one is present in a limited number of influenza virus strains. They suggest that cholesterol-bound M2 protein may be able to either rim or unite lipid microdomains, thereby facilitating M2 incorporation into virions. Because M2 is required during several distinct steps in the virus life cycle, we investigated the importance of the CRAC motif on virus replication by generating recombinant proteins and viruses containing alterations in this motif.

Results

Oligomerization and expression of mutant M2 proteins in stable cell lines.

Influenza A/WSN/33 (rWSN) encodes an M2 protein with a consensus (L/V-X_{1-5}-Y-X_{1-5}-R/K) CRAC motif ((13, 14, 16, 17), reviewed in (5)) while A/Udorn/72 (rUd) encodes a protein with an R at position 54 which disrupts the consensus (Figure 1). These influenza virus strains do not possess a second putative CRAC domain (34). Mutations were made in rWSN M2 to alter the CRAC motif by either mutating residue 54 from
arginine to phenylalanine (rWSN M2 R54F), the amino acid found in the rUd M2 protein, or changing all of the critical CRAC motif residues to alanine (rWSN M2 delCRAC) in order to eliminate the consensus sequence. Additionally, the CRAC motif in rUd M2 was completed by mutating residue 54 from phenylalanine to arginine (rUd M2 F54R). The WSN M2 proteins also contain an asparagine to serine mutation at residue 31 which conveys amantadine sensitivity (9, 36) so that the potentially toxic effects of ion channel activity could be inhibited during routine cell culture.

Stably transfected MDCK cell lines were generated which constitutively express the wildtype or mutant M2 proteins. Mutation of the CRAC motif does not affect M2 protein oligomerization as determined by non-reducing SDS-PAGE and Western blotting (Fig 2A and D). To determine if the mutant M2 proteins were expressed at the cell surface, flow cytometry was performed on live cells using an antibody which recognizes the extracellular domain of M2. Greater than 90% of the cells express M2 at the cell surface (Fig 2B and E). The total amount of mutant M2 expressed in the stable cell lines was comparable to a control cell line expressing wildtype M2 protein (Fig1A and D) as was the amount of M2 expressed at the cell surface (data not shown).

**M2 proteins with altered CRAC motifs are able to complement M2 deficient viruses.** The M2 protein plays several roles in the virus life cycle and altering any of these functions can drastically reduce virus replication and fitness (12, 19, 20, 36). The ability of stably expressed M2 to complement M2 deficient viruses has been previously used to study mutations in M2 which may prevent the rescue of recombinant viruses (2, 19, 20, 37). To determine if M2 proteins with mutations in the CRAC motif are able to complement M2 deficient viruses, stable cell lines expressing M2 were infected with
viruses which contain a stop codon in their M2 gene (M2Stop viruses). Neither alteration of the CRAC motif (Fig 2C) nor completion of the CRAC motif (Fig 2F) resulted in a statistically significant change in the production of infectious virus when compared to wt M2, indicating all the mutated M2 proteins are fully functional in this assay.

**Alteration of M2 CRAC motif does not affect the replication of recombinant influenza A viruses in tissue culture.** In order to confirm that mutation of the CRAC motif in the M2 protein does not affect virus replication, recombinant viruses were generated which encode M2 proteins with mutations in the CRAC motif (Figure 1). Infection of MDCK cells with these viruses results in a similar level of cell surface M2 as determined by flow cytometry (Fig 3A and D). Multistep growth curves in MDCK (Fig 3B and E) and the human lung adenocarcinoma, CaLu-3 cells (Fig 3C and F) results in no statistically significant changes in either replication kinetics or peak infectious virus titers, indicating the recombinant viruses with altered M2 CRAC motifs maintain the ability to replicate in these cell lines.

Many primary and some laboratory-adapted influenza A virus strains produce filamentous particles, including rUd, while most laboratory-adapted influenza A virus strains, such as rWSN, produce spherical particles (22, 29, 30). In order to determine if completion of the CRAC motif in Ud M2 affects the ability of recombinant virus to produce filamentous particles, confocal microscopy was performed to compare the number of infected cells which produce filaments. Filaments formed by both rUd M2 F54R and wildtype were similar in appearance (Fig 4A and B) and the number of infected cells showing filaments was not significantly different (Fig 4C). Additionally, like wildtype rWSN, rWSN M2 R54F and rWSN M2 delCRAC failed to formed
filaments on the surface of infected cells (data not shown). In order to determine if the filamentous virus particles were similar in size and structure, transmission electron micrographs were taken of infected MDCK cells. The rUd virus encoding M2 F54R was able to produce filamentous particles like wildtype virus (Fig 4D and E). Together, this data indicates that completion of the CRAC motif does not alter the ability of influenza virus to produce filamentous virus particles.

**Virion protein composition of recombinant viruses encoding M2 CRAC mutants.** In order to investigate the requirement of the CRAC motif for incorporation of viral proteins into virions, virus particles were collected and concentrated through a 20% sucrose cushion by ultracentrifugation. Virus pellets were resuspended and the amount of incorporated viral proteins was determined by Western blot (Fig 5A and B). Relative amounts of full length M2 and total M2 were quantified from replicate experiments. Total M2 incorporated into either CRAC altered or CRAC completed recombinant viruses was not statistically different from wildtype (Fig 5C and D). There was a statistically significant decrease in the amount of truncated M2 protein incorporated into rWSN M2 R54F virions. The truncated form of M2 is thought to be generated via cleavage by caspases at the C-terminus of M2 but this cleavage does not appear to affect viral replication (46, 47). Additionally, HA, NP, and M1 were incorporated into virus particles at levels indistinguishable from wildtype M2 when the CRAC motif was altered or completed (Fig 5A and B; data not shown).

**Decreased morbidity and mortality in mice infected with recombinant viruses expressing M2 CRAC mutants.** Some M2 mutations have been shown to have no effect *in vitro* but display decreased *in vivo* pathogenesis (7, 41, 42). In order to
determine if mutation of the M2 CRAC motif affects *in vivo* pathogenesis, mice were infected with rWSN viruses expressing the wildtype M2, M2 R54F, or M2 delCRAC. When intranasally inoculated with $10^3$ TCID$_{50}$, mice infected with all viruses displayed similar survival (Fig 6A). When mice were infected with $10^5$ TCID$_{50}$ of virus, rWSN caused significantly more mortality compared to rWSN M2 R54F and rWSN M2 delCRAC (Fig 6B). The median time of death for rWSN (7 days) and rWSN M2 R54F (11 days) differed as well. Change in body mass induced by infection with $10^3$ TCID$_{50}$ of either rWSN or rWSN M2 R54F was more significant than that induced by rWSN M2 delCRAC (Fig 6C). Mice infected $10^5$ TCID$_{50}$ of the viruses did not display significant differences in loss of body mass over the first 6 days of infection, despite the decreased mortality of mice infected with the CRAC altered recombinant viruses (Fig 6D).

Infection of mice with $10^5$ TCID$_{50}$ of rUd M2F54R did not result in a significant increase in virus replication or morbidity when compared to rUd, suggesting that restoration of the complete CRAC domain did not enhance in vivo replication of rUd (data not shown). This data indicates that elimination of the M2 CRAC motif leads to a modest attenuation of virus virulence in the mouse model of infection despite no obvious defects in *in vitro* virus replication.

**Alteration of the M2 CRAC motif does not affect replication of recombinant influenza A viruses in mTEC cultures.** Given the discrepancy between the ability of the recombinant viruses to replicate in tissue culture cells and their attenuation in the mouse model of infection, we investigated whether virus infection of mTEC cultures would better reflect the *in vivo* virus phenotypes. These primary cell cultures are differentiated into cell types normally found in the mouse trachea and therefore represent a faithful
tissue culture surrogate for virus infection of the airways (11, 24, 27). There was no statistically significant difference in the replication of the mutant viruses in mTEC cultures as compared to the corresponding wildtype virus (Fig 7), suggesting that replication in mTEC cultures was not compromised by altering the CRAC motif.

Discussion

This study sought to investigate the role of the CRAC motif found in the M2 protein of two influenza A virus strains. The R54F mutation in the WSN M2 protein was made to mimic the amino acid found at that residue in Ud M2. The rWSN M2 R54F mutant virus still contains a putative consensus CRAC sequence because it has another basic amino acid at residue 56. This could explain why this virus has an intermediate change in virulence when compared to rWSN and rWSN delCRAC. Mutation of WSN M2 to either R54F or delCRAC leads to no changes in virus replication \textit{in vitro}. Likewise, completion of the CRAC motif in the Ud M2 protein also has no effect on virus replication in MDCK cells, CaLu-3 cells, or in mTEC cultures. Together, this suggests that the CRAC motif is not required for \textit{in vitro} replication of influenza A virus.

Even though the CRAC motif is not essential for \textit{in vitro} virus replication, mutation of the motif resulted in decreased morbidity and mortality when mice were infected with a virus lacking the CRAC motif. The decreased pathogenesis of mutants may suggest that the CRAC motif of the M2 protein may be a virulence determinant in that it is not required for \textit{in vitro} virus replication but is critical for efficient virus infection in animal models. It is also possible that presence of a complete CRAC motif in M2 protein contributes to virus infection of other cell types such as alveolar epithelial
cells or macrophages. Rossman et al. recently demonstrated that amino acids outside of
the CRAC domain contribute to the cholesterol binding of M2 and mutation of these
amino acid sequences leads to decreased in vitro replication (31). The M2 protein only
showed cholesterol binding activity in the context of a virus infection and changes at
multiple amino acids that line the hydrophobic face of a membrane proximal alpha helix
of the protein were demonstrated to be critical for in vitro virus replication. This suggests
that the CRAC motif in and of itself, is not required for influenza virus replication in
vitro, a fact supported by our data.

The cytoplasmic tail of M2 has been implicated in the stabilization of the open
state of the M2 ion channel (33, 38), virion incorporation of M1 and vRNP (12, 19, 20),
virus morphology (12), and virus infectivity (2, 12, 19, 20, 36). Although the
transmembrane domain of M2 has been studied by NMR and crystallography, the
structure of the cytoplasmic domain is less well understood (33, 35). The structure of
residues 45-60 of the M2 cytoplasmic tail has been determined by NMR in concert with
the transmembrane domain (33), but the structure of the entire cytoplasmic tail has yet to
be determined. Residues 47-50 form a short, flexible loop linking the transmembrane
domain to a C-terminal amphiphatic helix (residues 51-59) that forms a stable “base”
important for holding the tetramers together during the conformational changes
associated with ion channel activation. The CRAC domain and a site for palmitoylation
(Ser 50) fall into these regions and it has been speculated that these modifications may
stabilize the interaction of the M2 cytoplasmic tail with lipid membranes (33).
Elimination of the CRAC motif or the M2 palmitoylation site (7) does not alter virus
replication \textit{in vitro}, but does yield viruses that are attenuated in the mouse model of
infection. This data argues against a critical role for these two sequences in virus replication, though both sequences are essential for maintaining virus virulence in vivo. While these sequences may be dispensable for virus replication, this does not imply that this region of the M2 protein is not important for M2 function either as a structural motif, or through properties as yet undefined.

The CRAC motif in the M2 protein is not required for incorporation of M2 into virions, nor does it affect the incorporation of other viral proteins. Alternate methods mediating the incorporation of M2 into virions must therefore exist. One study suggests that the ectodomain of M2 can drive a foreign protein to be incorporated into influenza A virus particles (26) but there are no known interactions between the M2 ectodomain and the ectodomains of the other viral glycoproteins. The mechanism by which a limited amount of M2 is incorporated into virus particles despite the high amounts of M2 located in the plasma membrane but not in the same membrane microdomains as the glycoproteins remains to be determined.

**Materials & Methods**

**Plasmids.** The plasmid pCAGGS (25) was used for M2 expression in mammalian cells. A plasmid expressing the M2 cDNA from influenza A/Udorn/72 (Ud M2) has been described previously (20). The M2 coding region from influenza A/WSN/33 (Genebank Accession number ABF21317) was amplified by RT-PCR and cloned as described previously for M2 Ud (18). A WSN M2 cDNA encoding a protein sensitive to the antiviral drug amantadine was constructed by changing the amino acid at position 31 from arginine to serine (N31S) (36). All M2 mutations were introduced into the
expression plasmids by overlap extension PCR and the sequences of the mutated plasmids were confirmed. Primer sequences are available upon request.

The pBABE plasmid, which expresses puromycin N-acetyltransferase, was used to generate stable cell lines expressing mutant M2 proteins as previously described (21).

In order to generate recombinant influenza viruses expressing M2 proteins with altered CRAC motifs, mutations were introduced into the pHH21 M segment plasmids via site-directed mutagenesis (Stratagene). The sequence of the entire M segment of the resulting plasmid was confirmed.

**Cells.** Madin-Darby canine kidney (MDCK) cells, human lung adenocarcinoma (CaLu-3) cells (ATCC HTB-55), and human embryonic kidney (293T) cells (36) were cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals), 100U/mL penicillin (Invitrogen), 100μg/mL streptomycin (Invitrogen), 1mM sodium pyruvate (Sigma), and 2mM GlutaMAX (Invitrogen). Cells were maintained at 37°C in a humidified 5% CO₂ environment.

**Generation of stable cell lines.** MDCK cells stably expressing M2 proteins were generated as described previously (20). Briefly, MDCK cells were cotransfected in suspension with 1μg pCAGGS M2 expression plasmid and 0.5μg pBABE puromycin expression plasmid using 9μL of Lipofectamine 2000 (Invitrogen) as per the manufacturer’s protocol. Cells were plated into 6 well plates, selected using 7.5μg/mL puromycin dihydrochloride (Sigma), and single colonies were isolated and expanded. Expression of M2 was confirmed by indirect immunofluorescence staining of surface M2 using monoclonal antibody (MAb) 14C2. M2 expressing cells were maintained with 5μM amantadine hydrochloride (Sigma).
Viruses. The viruses used in this study were rWSN and rUd (recombinant versions of A/WSN/33 and A/Udorn/72, respectively). Viruses were generated using a 12-plasmid rescue system described previously (19, 20, 23, 36). The entire coding region of the M segment sequence of all rescued viruses was confirmed. There were no discernable differences in plaque size or morphology between any of the recombinant viruses (data not shown).

Virus growth curves were performed at a multiplicity-of-infection (MOI) of ~0.001. For complementation assays, MDCK cells expressing mutant M2 proteins were infected with recombinant viruses that were functionally deleted for the expression of M2 (M2stop viruses) as described previously (19). For recombinant viruses, mutant M2-expressing viruses were used to infect MDCK or CaLu-3 cells. Cells were infected by twice washing confluent 6 well plates of MDCKs with phosphate buffered saline (PBS, Invitrogen) then infecting with the indicated viruses in 500μL infectious media (DMEM supplemented with 5% bovine serum albumin (BSA, Calbiochem), 100U/mL penicillin, 100μg/mL streptomycin, 2mM GlutaMAX, and 4μg/mL N-acetyl trypsin (NAT, Sigma)) at room temperature with rocking for 1hr. Cells were then washed twice with PBS and incubated with 500μL infectious media. Media was removed and replaced with fresh media at the indicated timepoints. Infectious virus titers were quantified by determining the 50% tissue culture infectious dose (TCID50) on MDCK cells (for M2-expressing viruses) or MDCK cells expressing WSN M2 N31S (for M2Stop viruses). Media lacking trypsin was used for infection of CaLu-3 cells. Standard error of the mean is graphed from infections done in duplicate or triplicate. The experiments were repeated at least twice and one representative example is graphed.
For microscopy, MDCK cells were grown to confluency on tissue culture treated glass coverslips and media changed every 2 days. Four days after confluence, cells were washed twice with PBS, infected with 500,000 TCID$_{50}$ (~0.9 MOI) of the indicated virus in 500μL medium for 1 hr at room temperature. Cells were then washed, the media was replaced and the cells incubated at 37°C for 15 hours.

**Virus purification.** Virus particles were isolated from the supernatants of MDCK cells infected at an MOI of 5 for 15 hr. Cell debris was removed by centrifugation at 1,900 g for 10 min at 4°C. Virus particles were then concentrated through a 20% sucrose cushion with centrifugation at 118,000 g in a TH641 rotor (Sorval) for 1 hr at 4°C. Virus pellets were resuspended in 100μL PBS and mixed 1:1 in 2x SDS-PAGE sample buffer.

**SDS-PAGE and Western Blotting.** MDCK cells were lysed in 1% SDS (Fisher Scientific) in PBS and mixed 1:1 in 2X SDS-PAGE sample buffer. Polypeptides were resolved on 17.5% polyacrylamide gel with 4M urea and transferred to polyvinylidene difluoride membranes (PVDF, Millipore). Membranes were blocked with PBS containing 0.3% Tween-20 (Sigma) and 5% dry milk, incubated 1 hr RT with primary antibody, washed three times with PBS with 0.3% Tween-20, incubated 1 hr RT with secondary antibody, and washed four times with PBS containing 0.3% Tween-20. The primary antibodies used were mouse α-M2 14C2 MAb (1:1,000 dilution) (44), mouse α-M1 HB-64 MAb (1:100 dilution) (20, 43), mouse α-NP HB-65 MAb (1:100 dilution) (20, 43), goat α-A/Udorn/72 serum (1:500 dilution) (45), or goat α-HA0 A/PR/8/34 (1:500 dilution, V-314-511-157; National Institute of Allergy and Infectious Diseases). Secondary antibodies were goat α-mouse immunoglobulin G (IgG) conjugated to AlexaFluor 647 (4μg/mL, Invitrogen), donkey α-mouse IgG conjugated to AlexaFluor
647 (4μg/mL, Invitrogen), donkey α-goat IgG conjugated to AlexaFluor 647 (4μg/mL, Invitrogen). Membranes were then scanned using an FLA-5000 (FujiFilm), samples were normalized to total M1 and M2 expression relative to wild type protein was determined. Structural proteins and oligomeric forms of M2 are indicated.

**Flow Cytometry.** Cells were removed from the tissue culture plates with trypsin treatment. The trypsin was inactivated with serum containing media and cells were washed once with PBS and incubated on ice for 30min, in the presence of blocking buffer (PBS supplemented with 0.5% BSA and 3% normal goat serum (NGS, Sigma)). Cells were then incubated for 1hr on ice with an antibody that recognizes the M2 ectodomain (14C2 MAb, 1:1000 dilution), washed three times with PBS, incubated with goat α-mouse IgG conjugated to AlexaFluor 488 (4μg/mL, Invitrogen) for 1hr on ice, and washed three times with PBS. Cells were then fixed with 1% formaldehyde (Fisher Scientific) in PBS for 15min at room temperature. The cells were analyzed on a FACSCalibur (Beckton Dickinson) and quantified using FlowJo software (Tree Star). All antibody dilutions were made in blocking buffer.

**Microscopy.** The cells were treated as for flow cytometry except antibodies were goat α-H3 sera raised against A/Aichi/2/68 (1:500 dilution, V-314-591-157; National Institute of Allergy and Infectious Diseases) and donkey α-goat IgG conjugated to AlexaFluor 555 (4μg/mL, Invitrogen). After incubation with antibodies, the cells were fixed with 2% paraformaldehyde (Sigma) in PBS for 15min at room temperature, washed twice with PBS, then mounted on slides using ProLong Gold anti-fade (Invitrogen). Samples were imaged on a Nikon Eclipse 90i microscope. For quantification, 10 or 20 images of random fields of view were taken with a 20x objective in two separate
experiments. For each image, the number of infected cells and cells with filaments were counted and used to determine the percentage of infected cells expressing filaments. Data from one representative experiment is shown. For high magnification images, Z-sections were taken at 0.3μm intervals on a Leica 510 Meta LSM confocal microscope using a 100x oil-immersion lens. Volocity 3D imaging software (Improvision) was used to analyze and flatten Z-sections.

**Transmission Electron Microscopy.** MDCK cells were grown to confluency in 3.5cm dishes and infected at an MOI of ~5 with either rUd or rUd M2 F54R. At 10hpi, cells were washed twice with PBS and fixed with 2mL of fresh fixative (2% glutaraldehyde, 0.1M cacodylate, 3% sucrose, and 3mM CaCl₂ in PBS pH7.4) overnight at 4°C. Cells were post-fixed with 1% osmium tetroxide reduced in potassium ferrocyanide for 1 hr at 4°C. After fixation, cells were stained en bloc with a 2% aqueous solution of uranyl acetate and dehydrated in graded ethanol. Embedding was done in Eponate 12 Resin (Ted Pella). Thin sections (70-90nm) were cut on a Reichert-Jung Ultracut E and placed on 200 mesh copper grids. The sections were stained with uranyl acetate and lead citrate and viewed on a Hitachi 7600 TEM with an AMT digital camera.

**Infection of BALB/c mice.** Six to 8-week old female BALB/c mice (Charles River) were used as described previously (24, 42). Mice were anesthetized and administered an intranasal inoculation of 10³ or 10⁵ TCID₅₀ of rWSN, rWSN M2 R54F, or rWSN M2 delCRAC virus diluted in 20μL DMEM supplemented with 100U/mL penicillin, 100μg/mL streptomycin, and 4μg/mL NAT. Animals were monitored for 14 dpi for morbidity and mortality (42). Changes in body mass are graphed as percent of starting mass.
**Infection of mTEC cultures.** Mouse tracheal epithelial cells (mTECs) were harvested, isolated, and differentiated as described previously (10, 24). Cultures were infected via the apical chamber with 3,300 TCID₅₀ in 100μL of media, ~0.01 MOI assuming all cells are susceptible to infection. After 1hr, apical supernatants were removed and replaced with fresh media. At the indicated hpi, apical and basolateral supernatants were removed and replaced with fresh media. Throughout the infection, infectious media without NAT was used. Infectious virus titers were quantified at indicated times as above. Standard error of the mean is graphed from infections done in duplicate.

**Statistical Analysis.** Infectious virus production and body mass changes were analyzed using mixed ANOVAs with time and virus as the independent variables. Protein concentrations and differences in the percentage of cells with filaments were calculated using t-tests. Mean day of death was determined by logrank test. Significant interactions were further evaluated using the Tukey method for pairwise multiple comparisons. Statistically significant differences of p<0.05 (*) or p<0.01 (**) are indicated and all analyses were done with Prism 4.0 (GraphPad Software Inc.).

**Acknowledgements**

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This study was supported by T32 GM007067 (SMS), AI053629 (AP), the Eliasberg Foundation (AP), and the Marjorie Gilbert Foundation (AP).
Figure Legends

Figure 1. Sequence alignment of M2 residues 46-56. The amino acids that define the CRAC motif are in bold and substitutions that disrupt or complete the consensus are indicated. An asterisk indicates no change in sequence. The rWSN M2 R54F mutant still contains a potential CRAC consensus sequence due to the presence of another basic amino acid at position 56.

Figure 2. Expression and function of M2 proteins with modified CRAC motifs. (A and D) MDCK cells expressing the indicated M2 proteins were analyzed by Western blotting to detect disulfide linked oligomeric forms of the M2 protein. Monomers, dimers and trimers are indicated and some higher order oligomers can be detected. (B and E) The number of cells expressing M2 at the cell surface was quantified by flow cytometry from the indicated stably transfected MDCK cell lines. (C and F) The ability of the indicated stably transfected MDCK cells to complement infection with a recombinant influenza virus that does not encode the full length M2 protein was assessed by infecting the cells with the indicated recombinant virus and quantifying infectious virus production at various hours post infection (hpi). The average and standard error of the mean are graphed. The standard error is smaller than the size of the individual points. The limit of detection is marked by a dotted line. Proteins or recombinant viruses based on the rWSN (A-C) and rUd virus strains (D-F) were used.
Figure 3. The effects of altering the CRAC motif on replication in tissue culture. (A and D) MDCK cells were infected with the indicated viruses at an MOI of ~3 and the cell surface expression of M2 protein was measured by flow cytometry at 15 hours post infection (hpi). The relative expression of M2 represents the mean channel fluorescence of the indicated infected cells divided by the mean channel fluorescence of the wildtype infected cells. MDCK (B and E) or CaLu-3 (C and F) cells were infected at an MOI of ~0.001 with the indicated recombinant viruses. At the indicated hpi, infected cell supernatants were harvested and the number of infectious virus particles determined by TCID$_{50}$ assay. Data points represent the average and standard error of the mean. The horizontal dotted line is the limit of detection.

Figure 4. The effects of completing the CRAC motif on formation of filamentous virus particles. MDCK cells were infected with 500,000 TCID$_{50}$ (~0.9 MOI). At 15hpi, immunofluorescence staining was performed for HA and visualized by confocal microscopy. Confocal Z-sections (taken with a 100x objective) of rUd (A) or rUd M2 F54R (B) infected cells were flattened and show viral filaments for both viruses. The percentage of infected cells showing filaments was determined from 10-20 images taken with a 20x objective on an epifluorescence microscope (C). One representative experiment is shown. For TEM, MDCK cells were infected at an MOI of ~5. Samples were processed for transmission electron microscopy at 10 hpi. Cells were infected with rUd (D) or rUd M2 F54R (E). Arrows indicate microvilli, solid arrowheads indicate filamentous virus particles, and empty arrowheads mark either spherical virus particles or cross-sections of filamentous virus particles.
Figure 5. The effects of altering the CRAC motif on incorporation of structural proteins. MDCK cells were infected with the indicated viruses at an MOI of 5. At 15hpi, virus particles were collected and concentrated through a 20% sucrose cushion at 118,000 g for 1hr. Virus pellets were resuspended and incorporation of viral proteins was determined by Western blot with antibodies which detect the structural proteins HA, NP, M1, and M2. (A and B) Representative Western blots showing incorporation of viral proteins into virions. (C and D) Quantification of full length M2 and total M2 from Western blot analysis of replicate virion incorporation assays. Increased contrast was used on the Western blot of virion-associated proteins to allow for detection of low intensity bands. Relative expression was determined by the negative log of M1 normalized data. Recombinant viruses based on the rWSN (A and C) or rUd virus strains (B and D) were used. * = p<0.05.

Figure 6. Mortality and morbidity of mice infected with recombinant influenza A/WSN/33 viruses encoding M2 proteins with altered CRAC motifs. Mice were administered an intranasal dose of $10^3$ (A and C) or $10^5$ (B and D) TCID$_{50}$ of the indicated viruses and monitored for 14 days post infection (dpi). (A and B) Mortality and (C and D) morbidity associated with infection, as judged by loss of starting weight. Data points indicate the average and standard deviation. Significant differences in (B) are between the CRAC altered viruses and rWSN while in (C) the differences are between rWSN M2 delCRAC and the other two viruses. * = p<0.05 and ** = P<0.01.
Figure 7. The effects of altering the CRAC motif on replication in mouse tracheal epithelial cell (mTEC) cultures. mTEC cultures were infected with 3300 TCID$_{50}$ (~0.01 MOI) of the indicated recombinant viruses in the rWSN (A) or rUdorn (B) genetic backgrounds. At the indicated hpi, infected cell supernatants were harvested and the number of infectious virus particles determined by TCID$_{50}$ assay in MDCK cells. Data points represent the average and standard error of the mean. The horizontal dotted line is the limit of detection.
<table>
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<th>CRAC Motif</th>
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**Figure 1. Sequence alignment of M2 residues 46-56.**
Figure 2. Expression and function of M2 proteins with modified CRAC motifs.
Figure 3. The effects of altering the CRAC motif on replication in tissue culture.
Figure 4. The effects of completing the CRAC motif on formation of filamentous virus particles.
Figure 5. The effects of altering the CRAC motif on incorporation of structural proteins.
Figure 6. Mortality and morbidity of mice infected with recombinant influenza A/WSN/33 viruses encoding M2 proteins with altered CRAC motifs.

A. $10^3$ TCID50

B. $10^5$ TCID50

C.

D.

- rWSN n=10
- rWSN M2 R54F n=15
- rWSN M2 delCRAC n=15
Figure 7. The effects of altering the CRAC motif on replication in mouse tracheal epithelial cell (mTEC) cultures.
References


Structural basis for the function and inhibition of an influenza virus proton channel. Nature **451**:596-599.


CHAPTER 3

Mutations in the membrane proximal region of the influenza A virus M2 protein cytoplasmic tail have modest effects on virus replication

This chapter is reprinted here essentially as published.

Shaun M. Stewart and Andrew Pekosz.

Abstract

Influenza A virus encodes M2, a proton channel that has been shown to be important during virus entry and assembly. In order to systematically investigate the role of the membrane proximal residues in the M2 cytoplasmic tail on virus replication, we utilized scanning and directed alanine mutagenesis in combination with trans-complementation assays and recombinant viruses. The membrane proximal residues 46-69 tolerated numerous mutations with little, if any, affect on virus replication suggesting that protein structure, rather than individual amino acid identity in this region, may be critical for M2 protein function.
Introduction

Influenza A virus is a member of the Orthomyxoviridae family whose genome consists of 8 negative-sense RNA segments which encode 10 or 11 proteins. The highly conserved integral membrane protein M2 is encoded by segment 7 via a spliced mRNA, consists of 97 amino acids, and forms disulfide-linked tetramers which have a pH-gated, proton-selective ion channel activity. M2 is a type III integral membrane protein with an extracellular amino-terminus and intracellular carboxy-terminus. M2 is required during virus entry, where it translocates protons into the virion interior which allows for the dissociation of viral ribonucleoprotein complexes (vRNPs) from the site of virus-cell membrane fusion, thereby allowing vRNP transport to the nucleus (12, 18, 26). M2 is also required during virus assembly where its cytoplasmic tail is required for proper incorporation of vRNPs into budding virions (9, 19, 20). The M2 protein and a peptide corresponding to an amphipathic helix in the membrane proximal cytoplasmic tail of the protein, have been shown to induce membrane curvature and scission in unilamellar vesicles and were suggested to be necessary for membrane scission and virion release (31). However, virus-like particles (VLPs) (3, 4, 8, 15, 17, 39) and virions (6, 9, 13) can be released in the absence of M2.

Various deletions and mutations in the M2 protein, and in particular the cytoplasmic tail, attenuate influenza A virus replication significantly (9, 14, 19, 20) and can be complemented with full length M2 expressed in trans (3, 9, 19, 20, 35). Residues within the cytoplasmic tail form a canonical cholesterol-binding motif (CRAC) and have been shown to mediate cholesterol binding in purified bacterially expressed protein and during virus infection but not when expressed alone in mammalian cells (30, 33, 37).
Mutation of residues within the CRAC motif caused no defect in virus replication in tissue culture but a slight attenuation of virus in vivo (35). Although structural studies have not been performed on the full-length M2 sequence, NMR studies on peptides corresponding to the transmembrane domain and portions of the cytoplasmic tail reveal that residues adjacent to the transmembrane domain form an amphipathic helix (25, 32, 34).

To systematically investigate the role of the membrane proximal residues (amino acids 46-69) in the M2 cytoplasmic tail on virus replication, we substituted alanine residues at a number of positions and assessed M2 protein function with a trans-complementation assay and reverse genetics. We show that the residues 46-69 tolerate numerous mutations with little, if any, attenuation of virus replication in both complementation assays and growth curves of recombinant viruses. Therefore, despite being highly conserved and forming a stable structure, this region can tolerate a high number of amino acid substitutions without significantly affecting influenza A virus replication.

Materials and Methods

Plasmids. The plasmid pCAGGS (24) M2Ud expressing the M2 cDNA from influenza A/Udorn/72 has been described previously (20). All M2 mutations were introduced into the expression plasmid by overlap extension PCR (35). The pHH21 M segment plasmid, which encodes the entire M segment used for generating recombinant viruses (20), was mutated via QuikChange Site-Directed Mutagenesis (Stratagene) (35).
All inserts in mutant plasmids were confirmed by sequencing. Primer sequences are available upon request.

**Cells.** Madin-Darby canine kidney (MDCK) cells were cultured in Dulbecco’s modified Eagle medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals), 100U/mL penicillin (Invitrogen), 100μg/mL streptomycin (Invitrogen), 1mM sodium pyruvate (Sigma), and 2mM GlutaMAX (Invitrogen). Cells were maintained at 37°C in a humidified 5% CO₂ environment.

MDCK cells stably expressing wildtype M2Ud or M2WSN N31S have been described previously (9, 35). The N31S mutation conveys amantadine sensitivity to the M2 protein encoded by influenza A/WSN/33 (11, 36). The pBABE plasmid, which expresses puromycin N-acetyltransferase (21) was used to generate MDCK cells stably expressing mutant M2 proteins as described previously (35). Cells expressing M2 proteins were maintained with 5µM amantadine hydrochloride (Sigma) and 7.5µg/mL puromycin dihydrochloride (Sigma).

**Viruses.** The wildtype viruses used in this study, rUd and rWSN (recombinant versions of A/Udorn/72 [H3N2] and A/WSN/33 [H1N1]), as well as viruses encoding functional deletions of the M2 open reading frame, have been described (20, 22, 36). Recombinant viruses were generated using a 12-plasmid rescue system described previously (19, 20, 22, 36). Recombinant viruses expressing mutant M2 proteins were generated as described (9), by replacing the pHH21 M segment plasmid with one encoding the indicated mutant M2 open reading frame. Viruses expressing mutant M2 proteins were plaque purified and grown in MDCK cells stably expressing M2WSN N31S in order to alleviate any selective pressure on the virus to revert the M2 mutations.
The entire coding region of the M segment of all rescued viruses was confirmed by sequencing.

**Virus infections.** Multi-step virus growth curves were performed at a multiplicity of infection (MOI) of 0.001 50% tissue culture infectious doses (TCID$_{50}$) per cell. For complementation assays, MDCK cells expressing mutant M2 proteins were infected with recombinant viruses that do not encode the full-length M2 protein (M2Stop viruses) as described (19). For recombinant viruses, mutant M2-expressing viruses were used to infect MDCK cells. Cells were infected by twice washing confluent 12-well plates with phosphate-buffered saline with calcium and magnesium (PBS+, Invitrogen) then infecting with the indicated viruses in 250µL infectious media (DMEM supplemented with 0.5% bovine serum albumin (BSA, Sigma), 100U/mL penicillin, 100µg/mL streptomycin, 1mM sodium pyruvate, 2mM GlutaMAX, and 4µg/mL N-acetyl trypsin (NAT, Sigma)) at room temperature with rocking for 1hr. Cells were then washed twice with PBS+ and incubated with 500µL infectious media at 37°C. At indicated times, media was removed, stored at -80°C, and replaced with fresh media. Infectious virus titers were determined by TCID$_{50}$ assay on MDCK cells expressing M2WSN N31S.

High MOI infections (MOI 0.5 or MOI 5) were performed using the protocol for multi-step growth curves with the exception that both NAT and BSA were omitted during all steps of infection. For protein expression studies, the media was removed at 16hpi and cells were processed for Western blot analysis. For virion composition, the cell lysates and supernatants were collected at 12hpi. Cell debris was removed from supernatants by centrifugation at 1300g for 10 min at 4 °C. Virus particles were then concentrated through a 35% sucrose cushion with centrifugation at 182,000g in a Sorvall TH-641 rotor.
for 1 h at 4 °C. Virus pellets were resuspended in 200 μL PBS, mixed 3:1 in 4xSDS–PAGE sample buffer, and analyzed along with the cell lysates by Western blot analysis.

**Plaque assay.** Plaque assays were carried out by infecting confluent 6-well plates of MDCK cells with indicated viruses serially diluted in infectious media. Cells were washed twice with PBS+, infected with 250μL of virus dilutions for 1hr at room temperature with rocking, the inoculums were aspirated, and cells were overlaid with DMEM (Invitrogen) containing 1% agarose (Invitrogen), 0.5% BSA, 100U/mL penicilillin, 100μg/mL streptomycin, 2mM GlutaMAX, and 4μg/mL NAT. After overlays had solidified, the cells were incubated at 37°C for 3 days. Cells were then fixed with 4% formaldehyde (Fisher Scientific) in PBS for 1hr at room temperature and stained with a Naphthol Blue Black solution overnight. Individual plaque diameters were measured from scanned images using ImageJ (NIH).

**Microscopy.** MDCK cells were grown to confluency on tissue culture treated glass coverslips (Fisher Scientific) in 12-well plates and media was changed every 2 days. Four days after confluence, cells were infected with 500,000 TCID₅₀ per well (MOI ~0.9) in 500μL of infectious media as per high MOI infections above and incubated at 37°C. At 15hpi, the cells were incubated on ice for 15min, washed twice with cold PBS+, and blocked for 30min on ice in PBS with 0.5% BSA and 3% normal goat serum (Sigma). Surface staining was performed for 1hr on ice in blocking solution containing goat anti-H3 sera raised against A/Aichi/2/68 (1:500 dilution, V-314-591-157; National Institute of Allergy and Infectious Diseases). Cells were then washed 3 times with cold PBS+, fixed for 10min with 2% paraformaldehyde (Sigma) in PBS at room temperature (RT), and incubated 1hr at RT with blocking solution containing donkey anti-goat IgG
conjugated to AlexaFluor 555 (4µg/mL, Invitrogen). Cells were then washed twice with PBS+ and mounted on slides using ProLong Gold anti-fade (Invitrogen).

Samples were imaged on a Nikon Eclipse 90i epifluorescence microscope. Twenty non-overlapping images were taken of each sample using a 20x objective. For each image, the total number of infected cells and cells expressing filaments were counted and used to determine the percentage of infected cells expressing filaments.

**TCID$_{50}$ assay.** MDCK cells expressing M2WSN N31S were plated in 96-well plates. When confluent, cells were washed twice with PBS+, infected with 100µL of virus serially diluted in infectious media, and incubated for 4 days at 37°C. Cells were then fixed by adding 50µL of 4% formaldehyde in PBS, stained with a Naphthol Blue Black solution, and scored for cytopathic effect. The 50% tissue culture infectious dose (TCID$_{50}$) was calculated by the method of Reed and Muench (27).

**SDS-PAGE and Western blotting.** Cells were lysed in 1% SDS (Fisher Scientific) in PBS and mixed 1:1 in 2x SDS-PAGE sample buffer. Purified virus particles were mixed 3:1 with 4x SDS-PAGE sample buffer. Sample buffer for samples analyzed for total expression contained the reducing agent DTT while samples analyzed for oligomerization did not. Proteins were resolved on 17.5% polyacrylamide gel with 4M urea and transferred to polyvinylidene difluoride membranes (PVDF-FL, Millipore). Wash buffer contained PBS with 0.3% Tween-20 (Sigma) and block buffer was wash buffer with 5% dry milk added. Membranes were blocked for 30min at RT, incubated 2hr RT with primary antibody, washed three times each for 5min, incubated 1hr RT with secondary antibody, and washed four times each for 5min. Primary and secondary antibodies were diluted in block buffer. The primary antibodies used were a mouse anti-
M2 14C2 MAb (1:500 dilution) (40), goat anti-A/Udorn/72 (1:500 dilution) (41), and a mouse anti-β-actin AC-15 MAb (1:10,000 dilution, Abcam). The AlexaFluor 647 conjugated secondary antibodies used were a goat anti-mouse immunoglobulin G (IgG), a donkey anti-goat IgG, and a donkey anti-mouse IgG (all 1:500 dilution, Invitrogen). For visualization, membranes were scanned using an FLA-5000 phosphorimager (FujiFilm).

**Sequence alignments.** All M2 protein sequences from H1N1 and H3N2 influenza A virus strains (excluding pandemic 2009 H1N1 and laboratory strains) were obtained from the NCBI Influenza Virus Sequence Database (1). Sequences were aligned using ClustalW 2.0.10 (16). The percent of the sequences which encode the most conserved residue at each amino acid was determined using WebLogo 3 (7).

**Statistical analysis.** Plaque diameters and percentage of infected cells expressing filaments were compared using student $t$-tests. Growth curves were analyzed using mixed ANOVAs and Bonferroni post-tests with time and virus titer as independent variables in trans-complementation assays or in growth assays of recombinant viruses. Statistically significant differences of $^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$, are indicated. All analyses were done with Prism 4.0 (GraphPad Software Inc.).

**Results**

**Oligomerization and expression of mutant M2 proteins in stable cell lines.**

The membrane proximal region of the M2 protein cytoplasmic tail consists of a region that displays some sequence variability (amino acids 47-57) and a region that shows very high conservation (amino acids 58-69) among seasonal influenza A virus strains (Fig 1A). In order to determine if residues 46-69 of the M2 protein cytoplasmic tail are
required for protein function, two types of alanine-substitution mutations were generated (Fig 1B). First, triple-scanning alanine mutations were generated across the region. Second, an NMR structure (34) of the M2 protein was utilized to select amino acids corresponding to two separate faces (M2UdCYTO and M2UdPORE) of the amphipathic helix within the membrane proximal region cytoplasmic tail. Figure 1C shows the residues 52, 53, 56, 57 and 60 of the helices which face the cytoplasm and were mutated to alanines in the M2UdAlaCYTO mutant. Figure 1D shows the residues 51, 54, and 55 of the helix which face inward toward the amphipathic helixes of the other M2 peptides and were mutated to alanines in the M2UdAlaPORE mutant. Alanine substitutions were utilized in order to minimize structural perturbations on the amphipathic alpha helix while still assessing the role of amino acid side chains on interactions with viral or cellular factors.

Stably transfected MDCK cells were generated which express the mutant M2 proteins. All cell lines expressed M2 above the level required to complement M2-deficient viruses (Fig 2A) (20). Additionally, the mutations do not alter the oligomerization potential of M2 as determined by SDS-PAGE and Western blot analysis under non-reducing conditions (Fig 2B).

**M2 proteins are able to complement M2-null viruses.** To study mutations which may be deleterious to M2 function, a complementation assay was utilized (20). Stably transfected MDCK cells were infected with two strains of M2-null virus, rUd M2Stop (Fig 3A and B) or rWSN M2Stop (Fig 3C and D) (19). Neither rUd M2Stop nor rWSN M2Stop are able to produce infectious virus on MDCK cells that do not express M2 (Fig 3). The replication of rUd M2Stop in all mutant M2-expressing cell lines was
not different from what was observed in wildtype M2-expressing cells (Fig 3A and B), indicating that all of the mutated M2 proteins were capable of supporting rUdorn virus replication.

rWSN M2Stop showed a minor, but statistically significant increase in virus replication on cell lines expressing M2UdAla49-51 and M2UdAla67-69, while slightly reduced replication was observed on cell lines expressing M2UdAla46-48 and M2UdAlaPore (Fig 3 C and D). However, no differences at 24hpi or 48hpi were greater than 1 log compared to the titer of rWSN M2Stop on cells expressing wildtype M2 protein. Despite these minor differences in replication, all cell lines expressing mutant M2 proteins supported high levels of replication of rWSN M2Stop, consistent with the data utilizing the rUd M2Stop virus (Fig 3A and B). Taken together, this suggests that the region of M2 from residues 46-69 is highly amenable to mutation despite being highly conserved.

**Replication of recombinant influenza A viruses expressing M2 alanine mutations.** In order to further investigate the importance of M2 residues 46-69 on virus replication, several recombinant viruses were generated in both the rUd and rWSN background. Because expression of M2UdAla49-51 supported slightly greater replication of rWSN M2Stop at two timepoints, a recombinant virus expressing this mutant were generated. A virus expressing M2Ala67-69 were generated because its expression supported higher levels of rWSN M2Stop at 48hpi (p<0.001). Lastly, because M2 has been shown to interact with M1 (3, 19) and the cytosolically exposed face of the M2 amphipathic helix could mediate interactions with M1 or other proteins, viruses were also generated which express M2AlaCYTO.
Plaque assays were performed on MDCK cells to determine the plaque diameters of each recombinant virus. Recombinant viruses expressing M2Ala49-51 or M2Ala67-69 formed smaller plaques in the rUd background but larger plaques in the rWSN background compared to the respective wildtype viruses (Fig 4A and C). Expression of M2AlaCYTO resulted in smaller plaques in rWSN but no difference in plaque diameter in rUd as compared to wildtype viruses.

The decreased plaque diameter in mutant M2-expressing rUd viruses correlated with the production of infectious virus at 24hpi in a low MOI growth curve (Fig 4B) but by 48hpi there were no differences in infectious virus production between any of the viruses. The ability of mutant M2-expressing rWSN viruses to form large or small plaques did not fully correlate with their ability to replicate in a low MOI growth curve (Fig 4D). rWSN expressing M2Ala49-51 formed larger plaques and had higher infectious virus titers at 24hpi in the multi-step growth curve as compared to wildtype virus. However, rWSN expressing M2Ala67-69 formed slightly larger plaques compared to wildtype virus but resulted in similar levels of infectious virus at both 24hpi and 48hpi. Additionally, expression of M2AlaCYTO in rWSN resulted in smaller plaques but more infectious virus at 24hpi. Interestingly, despite some statistically significant changes at 24hpi, all mutant M2-expressing rUd and rWSN viruses formed similar amounts of infectious virus at 48hpi compared to their respective wildtype recombinant viruses suggesting that the membrane proximal region 46-69 of M2 can tolerate significant mutations without eliminating the ability of influenza A virus to replicate.

**Expression of M2UdAla67-69 is able to complement a recombinant virus expressing M2Ala67-69.** Because rUd M2Ala67-69 produced less infectious virus
particles at 24hpi but rUd M2Stop replicated to similar levels on cells expressing M2UdAla67-69, the growth of rUd M2Ala67-69 was compared to wildtype rUd in MDCK, M2Ud-expressing, and M2UdAla67-69-expressing cells (Fig 5). In MDCK cells, rUd M2Ala67-69 replicate to lower titers at 12hpi and 24hpi but had formed similar amounts of infectious virus at 48hpi (Fig 5A). In M2Ud- or M2UdAla67-69-expressing cells, both viruses produced similar amounts of infectious virus particles at all times measured (Fig 5B). This indicates that expression of wildtype or or M2UdAla67-69 M2 is able to rescue any potential defect in production of infectious virus at early times post infection. In order to determine if the level of M2 expressed by rUd M2Ala67-69 is equivalent to that of wt virus, high MOI infections were performed on MDCK cells. Expression of M2 was similar in cells infected by both viruses (Fig 5D), indicating that the small defect in the growth of rUd M2Ala67-69 at 12hpi and 24hpi is not due to decreased expression of M2 and suggests that perhaps a greater amount of M2 (either wt or mutated protein) is needed for this virus to replicate efficiently.

**M2 protein with alanine mutations at F47, F48, I51, Y52, and F55 is partially able to complement M2-deficient viruses.** Rossman et al. determined that alanine substitution of residues F47, F48, I51, Y52, and F55 in the rUd M2 protein (M2AlaHelix, Fig 1B & E) resulted in a recombinant virus severely debilitated in growth (30). Given the limited effect of the mutations we introduced into the M2 protein on virus replication, we sought to compare this mutant to our M2 mutants. Stably transfected MDCK cells were generated which express the M2UdAlaHelix protein. The MDCK cell line expressing M2UdAlaHelix expressed a similar level of M2 as compared to a cell line expressing wildtype M2Ud (Fig 6A). Additionally, the M2UdAlaHelix mutant formed
oligomers as determined by SDS-PAGE and Western blot analysis under non-reducing conditions (Fig 6B).

In order to determine if constitutive expression of M2UdAlaHelix was able to complement M2-null viruses, MDCK or M2-expressing MDCK cells were infected at a low MOI and the amounts of infectious virus was determined at the indicated time post infection. There was a reduction in the ability of M2UdAlaHelix to complement either rUd M2Stop (Fig 6C) or rWSN M2Stop (Fig 6D). Both M2-null viruses grew to infectious virus titers approximately 1-2 logs lower on M2UdAlaHelix expressing cells than wild type M2-expressing cells at both 24hpi and 48hpi, which was a greater attenuation than that seen on any other M2-expressing cell lines analyzed.

**Recombinant influenza A virus expressing the M2UdAlaHelix protein is attenuated at early time points but grows to similar peak titers.** Because the attenuation of rUd M2Stop on cells expressing M2UdAlaHelix was less than what was expected based on data with a recombinant virus expressing this mutant M2 protein (30), we generated a recombinant Ud virus expressing the M2UdAlaHelix protein to further characterize this mutation. Plaque assays of this recombinant, rUd M2AlaHelix, on MDCK cells showed significantly smaller plaques when compared to wildtype rUd (Fig 7A). Low MOI growth curves revealed a decrease in production of infectious virus particles at 24hpi, but by 48hpi, rUd M2AlaHelix had produced as much infectious virus as recombinant wildtype rUd. Sequence analysis of the rUd M2AlaHelix virus produced at this timepoint demonstrated that no changes in the M-segment sequence had accumulated, indicating that a revertant virus had not emerged (data not shown). The ability of rUd M2AlaHelix to form filaments in virus-infected cells was not significantly
different when compared to rUd (Fig 7C). The expression levels of HA, NP, and M1 proteins were similar in cells infected with either wildtype rUd or rUd M2AlaHelix (Fig 7D). Western blot analysis of pelleted rUd M2AlaHelix virus particles showed no major defective in the secretion of virus particles as well as no defect in the incorporation of HA, NP, or M1 (Fig 7D). The modest level of attenuation and lack of significant changes in structural protein packaging seen with rUd M2AlaHelix was not consistent with what was observed in previous reports (30). However, this data is consistent with the data on other M2 mutations generated in this region of the protein (Figures 3 and 4), which indicate a limited role for membrane proximal amino acids 46-69 of M2 in supporting influenza A virus infectious virus production.

**Discussion**

The influenza A virus M2 protein plays a crucial role during several steps of the viral life cycle. During virus entry, the ion channel activity of M2 mediates the release of vRNPs into the cytoplasm after HA-induced fusion of the viral and cellular membranes. Additionally, the M2 cytoplasmic tail is critical for proper incorporation of vRNPs into budding particles. The exact mechanism of the incorporation might occur through binding of NP and/or M1. Indeed there is support for the latter in that M1 and M2 have been shown to interact with each other (3, 19). Two separate M1 binding sites have been identified within M2, one within the amino acids 70-77 (3, 19) and another within residues 45-69 (19).

The membrane proximal region consisting of amino acids 46-69 is believed to mediate association of M2 with sites of virus budding. This region consists of a
palmitoylation site, a cholesterol binding motif, and an amphipathic helix that can insert into the lipid bilayer (23, 25, 32, 34, 38). Although palmitoylation was expendable for growth in vitro, mutation of the palmitoylated cysteine at residue 50 led to a reduction in pathogenesis in mice (10) and was required for the partial raft partitioning of full-length M2 in giant plasma membrane vesicles (37). Mutation of the critical residues within the cholesterol binding motif caused no effect on in vitro growth but a small reduction of morbidity and mortality associated with infection in mice (35). This study sought to investigate which residues in the membrane proximal region 46-69 of the M2 protein cytoplasmic tail were required for the function of M2 during virus replication.

Two distinct recombinant strains of influenza A virus, A/Udorn/72 and A/WSN/33, were utilized. rUd is an H3N2 strain that forms both spherical and filamentous virus particles while rWSN is an H1N1 mouse-adapted strain that almost exclusively forms spherical virus particles (2, 28, 29). While some M2 mutations have been shown to be deleterious to virus replication in both virus strains, others have been shown to more deleterious in rUd (9, 19). During infection with rUd, less M2 protein is expressed in cells and, therefore, less M2 protein is incorporated into virus particles compared to infection with rWSN (10, 35).

Triple-scanning alanines were made across the residues 46-69 to systematically test the necessity of these highly conserved residues for the formation of infectious virus particles. Furthermore, two separate faces of the amphipathic helix found in the M2 protein cytoplasmic tail were also separately mutated to alanines, M2UdAlaCYTO and M2UdAlaPORE. All of these M2 mutants were able to complement two strains of influenza A viruses lacking functional M2, rUd M2Stop and rWSN M2Stop (Fig 3).
Moreover, recombinant viruses expressing several of these M2 mutants were generated and grew to similar peak titers at 48hpi (Fig 4). Taken together, these data show that the membrane proximal residues 46-69 of the M2 protein can tolerate significant mutations without eliminating the ability of virus to replicate. While none of these mutated M2 proteins had any major effect on the replication of rUdorn viruses, it is interesting to note that two mutations, Ala49-51 and Ala67-69, caused a slight but significant increase in replication in rWSN. This demonstrates the importance of assessing M2 mutations in different influenza A virus strains. The variable results of these mutations may map to other viral proteins, as was demonstrated for virus budding (5) and for infectious virus production (9, 19).

Analysis of a recent NMR structure revealed that some of the nonpolar residues within the amphipathic helix from one monomer interact with residues in the loop between the transmembrane domain and the amphipathic helix of another monomer (34). L46 was shown to interact with F54 and F48 was shown to interact with both F55 and L59. Interestingly, one of the large hydrophobic residues in each of those pairs is mutated in the M2AlaPORE mutant, F54 and F55. Despite disruption of these interactions, these mutations did not abrogate the functions of the M2 protein during virus replication (Fig 3B and D). However, it is conceivable that mutating these residues from phenylalanines to alanines was insufficient to fully disrupt these hydrophobic interactions. Additionally, the NMR structure revealed that the charged residues K49, R53, H57, K60, and R61 project outward and could interact with the negatively charged phospholipids. The M2AlaCYTO mutant has several of these amino acids altered from charged residues to alanines (R53A, H57A, and K60A), yet this mutant retains its ability
to complement M2-null viruses (Fig 3B and D) and recombinant viruses expressing it show no defect in replication (Fig 4B and D).

These data contrast somewhat with a study by Rossman et al., that demonstrated a critical role for the membrane proximal, cytoplasmic region of the M2 protein in virus budding and membrane scission (30). In that study, while single point mutations at positions 47, 48, 51, 52 and 55 did not affect virus assembly and budding, combining all those mutations led to a greater than 5 log reduction in infectious virus production and a decrease in NP incorporated relative to the other viral proteins (30). These mutations abrogated the ability of a peptide corresponding to this region of the M2 protein to induce membrane scission in unilamellar vesicles and also reduced the ability of full length M2 to induce VLPs (31). The significance of this is not completely clear since VLPs are formed in the absence of M2 expression (3, 4, 8, 15, 17, 39) and viruses encoding either a mutation in the M2 5’ splice site or a nonfunctional M2 both express undetectable levels of M2 protein yet produce virus particles with decreased infectivity (6, 9, 13), thus demonstrating that M2 is not absolutely necessary for membrane scission of influenza A virus particles. We also generated an M2 protein with alanine substitutions at positions 47, 48, 51, 52, and 55 and demonstrated a 1-2 log decrease in infectious virus production from cells expressing M2UdAlaHelix compared to cells expressing wildtype M2 in a trans-complementation assay. The defect in infectious virus production was more pronounced in infections with rUd M2Stop as compared to rWSN M2Stop. A recombinant rUd virus encoding the M2AlaHelix was able to form filaments and had no defect in NP incorporation (Fig 7C and D) whereas the same virus reported by Rossman et al was unable to form filaments and had a defect in NP incorporation (30). The rUd
M2AlaHelix that we generated also showed reduced replication kinetics which were not as large (1-2 log reduction at 24 hpi and identical titers at 48 hpi, Fig 7B) as that reported by Rossman et al, (~3 logs at 24hpi and ~5 logs at 48hpi) when compared to wildtype rUd (30). Although both growth curves were performed at the same 0.001 MOI, the amount of infectious virus was measured differently, plaque assay versus TCID\textsubscript{50} assay. However, titration of stocks of rUd M2AlaHelix by the two assays showed comparable titers (data not shown) indicating the method of detecting infectious virus was not responsible for the discrepancy in attenuation.

This study sought to investigate whether the membrane proximal residues 46-69 of the M2 protein cytoplasmic tail were required for the function of M2 during virus replication. Given the fact that numerous mutations were made in this region of the M2 protein and in both complementation assays and recombinant viruses these mutations caused little, if any, attenuation in virus replication, we conclude that the region is tolerable of numerous amino acid substitutions before protein function is compromised. Protein structure, rather than individual amino acid identity, may be critical for the function of this region of the M2 protein. It will be important to utilize a structural biology approach in order to gain further knowledge about the role of this region on M2 function.

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Figure Legends

Figure 1. Sequence and structural location of M2 cytoplasmic tail amino acids. A) Conservation of residues 46-69 of the M2 protein among H1N1 (virus sequences prior to 2009) and H3N2 influenza A virus strains. B) Sequence of residues 46-69 of wildtype M2 protein and alanine substitutions. The amphipathic helix is highlighted in yellow (34). C-E) Structures of M2 proteins (residues 22-62) with mutated amino acids in green. Residue 22 appears at the top of the structures on the left. The structures on the right are rotated 90° as indicated in order to view the cytosolic face of the M2 tetramer. C) M2UdCYTO - Y52, R53, E56, H57, and K60; D) M2UdPORE - I51, F54, and F55; E) M2UdHelix - F47, F48, I51, Y52, and F55. Structures were generated using PyMol from PDB code 2LOJ.

Figure 2. Expression and oligomerization of mutant M2 proteins. A) MDCK cells stably expressing the indicated M2 proteins were analyzed by Western blotting in order to determine (A) the overall expression under reducing conditions and (B) the presence of disulfide linked oligomers under non-reducing conditions. Monomeric M2 and the cell protein loading control, β-actin, are indicated in (A) and monomers, dimers, and tetramers of M2 are indicated in (B).

Figure 3. Complementation of M2-deficient viruses by expression of mutant M2 proteins. MDCK cells stably expressing the indicated M2 protein were infected at an MOI of 0.001 with a recombinant influenza A virus that does not encode the full-length
M2 protein. The amount of infectious virus at each time point was determined by TCID$_{50}$ assay on cells expressing wildtype M2. Complementation of rUd M2Stop virus (A and B) or rWSN M2Stop virus (C and D) infectivity on cells expressing the indicated M2 protein. The mean and standard error of the mean of triplicate samples are graphed from a representative experiment. The limit of detection is marked by a horizontal dotted line.

**Figure 4. Plaque size and replication kinetics of recombinant viruses expressing mutated M2 proteins.** (A and C) Plaque diameter of MDCK cells infected with the indicated recombinant viruses at 3 days post infection (dpi). The cells were fixed and stained with a Naphthol Blue Black solution and individual plaque diameters were measured from scanned images using ImageJ. A representative example is shown from triplicate experiments. Student $t$-tests were performed to determine significant differences as compared to wild type virus. (B and C) MDCK cells were infected at an MOI of 0.001 with the indicated recombinant virus. At the indicated hours post infection (hpi), cell supernatants were collected and the numbers of infectious virus particles were determined by TCID$_{50}$ assay on M2-expressing cells. The mean and standard error of the mean of triplicate samples are graphed from a representative experiment. The limit of detection is marked by a horizontal dotted line.

**Figure 5. Complementation of a recombinant virus expressing M2Ala67-79.** (A-C) MDCK (A), M2Ud-expressing (B), and M2UdAla67-69-expressing cells (C) were infected at an MOI of 0.001 with the indicated recombinant virus. At the indicated hpi, cell supernatants were collected and the numbers of infectious virus particles were
determined by TCID$_{50}$ assay on cells expressing wildtype M2. The mean and the standard error of the mean of triplicate samples are graphed from a representative experiment. The limit of detection is marked by a horizontal dotted line. (D) MDCK cells were infected at an MOI of 0.5 with the indicated recombinant virus. At 16hpi, expression of M2 and the cell protein loading control, β-actin, were compared in cell lysates by Western blot.

**Figure 6. Expression and function of M2UdAlaHelix protein.** Western blot analysis of MDCK cells stably expressing either wildtype M2Ud or M2UdAlaHelix. (A) The total expression of M2 protein under reducing conditions and (B) the presence of disulfide linked oligomers under non-reducing conditions. Monomeric M2 and the cell protein loading control, β-actin, are indicated in (A) and monomers, dimers, and tetramers of M2 are indicated in (B). (C and D) MDCK cells stably expressing the indicated M2 protein were infected with the indicated recombinant virus that does not encode the full-length M2 protein at an MOI of 0.001. The amount of infectious virus at each time point was determined by TCID$_{50}$ assay on cells expressing wildtype M2. The mean and standard error of the mean of triplicate samples are graphed from a representative experiment. The limit of detection is marked by a horizontal dotted line.

**Figure 7. Characterization of a recombinant virus expressing M2AlaHelix.** (A) Plaque diameter of MDCK cells infected with recombinant viruses expressing either wildtype M2 or M2AlaHelix at 3dpi. The individual and average diameters of plaques were determined. A representative example is shown from triplicate experiments. (B) MDCK cells were infected at an MOI of 0.001 with the indicated viruses. At the
indicated hpi, cell supernatants were collected and the numbers of infectious virus particles were determined by TCID$_{50}$ assay on M2-expressing cells. The mean and standard error of the mean of triplicate samples are graphed from a representative experiment. The limit of detection is marked by a horizontal dotted line. (C) MDCK cells, infected with 500,000 TCID$_{50}$ of indicated virus, were stained for immunofluorescence at 15hpi and visualized by microscopy. The percent and average percent of infected cells showing filaments were determined from 20 non-overlapping images taken with an epifluorescence microscope. One representative experiment is shown. (D) MDCK cells were infected at an MOI of 5 with the indicated viruses. At 12hpi, supernatants were collected and virus particles were concentrated by ultracentrifugation through 35% sucrose in PBS, resuspended in PBS, and equal volumes were analyzed along with the cell lysates by Western blotting under reducing conditions.
Figure 1. Sequence and structural location of M2 cytoplasmic tail amino acids.
Figure 2. Expression and oligomerization of mutant M2 proteins.
Figure 3. Complementation of M2-deficient viruses by expression of mutant M2 proteins.
Figure 4. Plaque size and replication kinetics of recombinant viruses expressing mutated M2 proteins.
Figure 5. Complementation of a recombinant virus expressing M2Ala67-79.
Figure 6. Expression and function of M2UdAlaHelix protein.
Figure 7. Characterization of a recombinant virus expressing M2AlaHelix.
References


CHAPTER 4

The influenza C virus CM2 protein can alter intracellular pH and its transmembrane domain can substitute for that of the influenza A virus M2 protein and support infectious virus production

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Shaun M. Stewart and Andrew Pekosz.

Abstract

The influenza C virus CM2 protein and a chimeric influenza A virus M2 protein (MCM) containing the CM2 transmembrane domain were assessed for their ability to functionally replace the M2 protein. While all three proteins could alter cytosolic pH to varying degrees when expressed from cDNA, only M2 and MCM could at least partially restore infectious virus production to M2-deficient influenza A viruses. The data suggest that while the CM2 ion channel activity is similar to that of M2, sequences in the extracellular and/or cytoplasmic domains play important roles in infectious virus production.
The influenza A virus M2 protein is a homotetrameric, type III integral membrane protein that functions at several stages of the viral life cycle (24). The proton-selective ion channel activity of M2 (4, 18) mediates the release of viral ribonucleoprotein complexes (vRNPs) from matrix (M1) and viral membranes, allowing migration of vRNPs to the nucleus (9, 15, 23) and also raises the pH of the exocytic pathway, thereby preventing premature low pH-induced conformational changes in hemagglutinin (HA) (5, 26). The cytoplasmic tail of M2 is also required for efficient genome packaging into budding virus particles (8, 16, 17). M2 has also been proposed to be mediate membrane scission, however, in the absence of M2, infected and transfected cells release virus particles and virus like particles, respectively (2, 3, 8, 14, 25).

The influenza C virus CM2 protein is an integral membrane protein generated after proteolytic cleavage of an internal signal peptide of the p42 protein (10, 13, 22). CM2 forms disulfide-linked homotetramers (12, 21) and has been shown to conduct Cl⁻ ions (11) or perhaps protons (19). CM2 has been shown to be involved in the release of vRNPs during virus uncoating and in packaging of vRNPs during virus assembly (7). CM2 is also able to raise the pH of the exocytic pathway; however, the role of this activity during influenza C virus replication is currently unclear (1). This study sought to investigate whether full length CM2 or a chimeric M2 protein containing the CM2 transmembrane domain could functionally substitute for the influenza A virus M2 protein.

CM2, normally generated from proteolytic cleavage from p42, can be expressed efficiently from cDNA using the vaccinia virus-bacteriophage T7 polymerase expression system (21) but expression levels are low from plasmids that rely on host cell
transcription machinery. Efficient plasmid-based expression of CM2 was achieved by deleting the p42 internal signal peptide from the expression plasmid (20), most likely because of the elimination of a mRNA splice donor site present in the RNA corresponding to the signal peptide. The glycosylation site was eliminated by mutating Thr at residue 37 to Ala and epitope tags for the antibodies 3F10 (anti-hemagglutinin epitope) and 14C2 (anti-M2 epitope) were added at the carboxy-terminus in order to facilitate detection of the protein (Fig 1A). In order to determine if the ion channel activity of CM2 can substitute for that of M2, a construct was generated with the extracellular and cytoplasmic tail of M2 and the TM of CM2 (MCM), Fig 1A.

Because some reports show that M2 and CM2 have differential ion channel activity yet both are able to raise the pH of the secretory pathway (1, 5, 6, 11, 19), the ion channel activity of the two proteins was compared in an assay that measures pH-dependent changes in eYFP fluorescence (8). Whereas low-pH treatment of 293T cells co-transfected with eYFP and an empty vector yielded no change in mean fluorescence intensity (MFI) over time, low-pH treatment of cells co-transfected with eYFP and M2 from A/Udorn/72 (M2Ud) resulted in ~40% decrease in MFI (Fig 1B-C). Low-pH treatment of cells co-transfected with eYFP and CM2 only resulted in ~25% decrease in MFI (Fig 1D), indicating the CM2 protein was able to modulate the cytoplasmic pH of transfected cells but not to the same extent as the M2 protein. The MCM protein induced a reduction in eYFP fluorescence which resembled that of CM2 (Fig 1E), suggesting that substitution of the CM2 TM for that of M2 conferred ion channel activity that resembled that of CM2. The level of expression of M2, CM2 and MCM in transfected 293T cells was comparable (Fig 1C- E), indicating that the decreased pH changes induced by CM2
and MCM compared to M2 were not attributable to differential protein levels but most likely reflected altered ion channel activity.

In order to determine if CM2 and MCM were able to complement a M2-null influenza A virus, clonal MDCK cell lines stably-expressing the proteins were generated. Total expression of CM2 and MCM are shown relative to cell lines expressing M2Ud or M2 from A/WSN/33 with a mutation conveying amantadine resistance (M2WSN N31S) (Fig 2A) (27, 28). The expression level of MCM was similar to that of M2, while the CM2 protein expression level was only 2% of the levels of M2. However, even trace amounts of M2 protein can functionally complement an M2-null influenza A virus (17) so the levels of CM2 expression are expected to be sufficient for the complementation assay. MCM was able to form disulfide-linked oligomers, similar to the M2 and CM2 proteins (Fig 2B). In order to determine if constitutive expression of CM2 or MCM prevented replication of influenza A virus, recombinant A/Udorn/72 (H3N2, rUd) and recombinant A/WSN/33 (H1N1, rWSN) were titered on the cell lines (Fig 2C). Both rUd and rWSN had similar titers on all cell lines tested, suggesting that expression of CM2 or MCM does not inhibit influenza A virus infection. To assess the ability of CM2 and MCM to substitute for M2, the recombinant M2-null viruses rUd M2Stop & rWSN M2Stop (8, 27) were titered on the cell lines (Fig 2C). Both rUd M2Stop and rWSN M2Stop showed decreased titers on CM2-expressing cells compared to M2-expressing cells, indicating CM2 cannot functionally replace M2. When comparing MCM to M2-expressing cells, rUd M2Stop had similar titers while rWSN M2Stop showed a decrease in titer, demonstrating that the MCM protein can complement rUd M2Stop but not rWSN M2Stop, which suggests that the CM2 ion channel activity can functionally substitute for
that of M2 in some influenza A virus strains. However, given the low level of CM2 expression, it remains to be determined if overexpression of the CM2 protein to levels much higher than those needed for M2 complementation, can partially complement an M2-null influenza virus.

The ability of MCM to substitute for M2 was also tested in multi-step growth curves (Fig 2D-E). Neither rUd M2Stop nor rWSN M2Stop were able to grow in CM2-expressing cells despite the fact that the level of CM2 expression was higher than that needed to complement these viruses with M2 protein (8, 16, 17). MCM-expressing cells did support growth of both viruses, but the growth of rWSN M2Stop was more attenuated than rUd M2Stop. Since CM2 expression could not complement while MCM expression could partially complement two different M2-null viruses, we conclude that that the ion channel activity of CM2 can replace that of M2; however, the ectodomain and/or the cytoplasmic tail of M2 are required during influenza A virus replication.

M2 mutations that cause strain specific defects in growth of either rUd or rWSN have previously mapped to the M1 protein (2, 8, 16). The ability of MCM expression to complement rUd M-WSN M2Stop (expressing 7 segments from Ud, M1 from WSN, and no M2) growth was comparable to its ability to complement rUd M2Stop (Fig 2D&F). Likewise, the ability of MCM expression to complement rWSN M-Ud M2Stop (expressing 7 segments from WSN, M1 from Ud, and no M2) growth was comparable to its ability to complement rWSN M2Stop (Fig 2E&G). Together, these data indicate that the decreased ability of MCM to complement rWSN M2Stop maps to viral sequences outside of the M1 protein.
The inability of CM2 to complement M2-null viruses could be due to a defect in particle budding. Virus particles from high MOI infections were concentrated by ultracentrifugation through 35% sucrose and analyzed by Western blot and TCID$_{50}$ assay (Fig 3). As previously published, rUd M2Stop virus particles from MDCK cells are released but are defective in the incorporation of full length NP (NPa) and have lower infectious virus titers relative to total HA (Fig 3A-C) (8). rWSN M2Stop virus particles grown on MDCK cells also have decreased titer relative to total HA but increased release and cleavage of HA (Fig 3D-E). Both M2-null viruses grown on CM2-expressing cells showed similar phenotypes compared to those grown on MDCK cells (Fig 3). However, rUd M2Stop virus particles grown on MCM-expressing cells incorporated MCM and did not show the decreased incorporation of NPa observed in virus particles isolated from MDCK and CM2-expressing cells (Fig 3A-C). rWSN M2Stop virus particles grown on MCM-expressing cells also had similar structural protein incorporation compared to particles from M2-expressing cells. The ratio of titer to HA (a measure of the infectivity of the particles) of viruses grown on MCM-expressing cells was intermediate of that from those grown on MDCK or CM2-expressing cells versus M2-expressing cells (Fig 3C&E). Taken together this data demonstrates that expression of MCM, but not CM2, is able to complement the defect of NPa incorporation present in viruses grown on MDCK cells but that the particles from MCM-expressing cells still have a defect in infectivity which is more apparent in rWSN M2Stop versus rUd M2Stop.

Negative stain electron microscopy was performed on the purified rUd M2Stop virus particles in order to assess any morphological differences in virus particles grown on the various cell lines. rUd M2Stop particles grown on MDCK or CM2-expressing
cells had a smaller average diameter and higher particle/TCID$_{50}$ ratios than those grown on M2-expressing cells (Fig 4). Virus particles isolated from MCM-expressing cells were of similar size to those grown on M2-expressing cells, but had slightly higher particle/TCID$_{50}$ ratios (Fig 4F). All cell lines produced comparable numbers of particles (Fig. 4F). These data indicate that the infectivity of the virus particles correlated with the size of the particles, and that MCM, but not CM2, could partially complement the rUd M2stop virus.

These studies suggest that influenza A virus produced in the absence of M2 or in the presence of CM2 has a defect in the incorporation of NPa (and most likely, vRNA), smaller particles, an increased ratio of total to infectious particles, and absence of growth in multi-step growth curves. The level of CM2 expression was higher than the level of M2 expression needed for complementation, however we cannot rule out the fact that higher levels of CM2 expression might lead to some degree of complementation or that the addition of C-terminal epitope tags may be interfering with CM2 protein function. Virus particles produced in the presence of MCM incorporate a normal level of NPa and are of normal size but have a slightly reduced infectivity. We hypothesize that the defect in infectivity of virus particles grown on MCM-expressing cells is due to the differential ion channel activity of the MCM protein versus the M2 protein. The ion channel activity (Fig 1) shows that MCM does not lower the pH of the cytoplasm as efficiently as M2, most likely leading to a decreased ability to release vRNPs during virus entry.

Acknowledgements
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Figure Legends

Figure 1. Schematic and ion channel activity of M2, CM2, and MCM. A) Schematic of the ectodomain (Ecto), transmembrane domain (TM), and cytoplasmic tail (Cyto) of wildtype M2, CM2, and MCM. Amino acid numbers are indicated. Sequences recognized by the HA (black box) and M2 (hatched box) antibodies are also shown. B-E) The ability of M2, CM2, and MCM to conduct ions was indirectly measured using the pH-sensitive fluorescent protein eYFP. 293T cells were transiently transfected with plasmids encoding eYFP and either an empty pCAGGS expression plasmid or a pCAGGS plasmid expressing the indicated protein. The next day, cells were detached and resuspended in media at either pH 7.4 or pH 5.8 and analyzed by flow cytometry. The mean fluorescence intensity (MFI) of 10,000 cells was determined every 15 sec. The change in MFI versus time is plotted from a representative experiment performed at least twice. Solid lines represent curve fits to one phase exponential decay models and dotted lines represent no change in MFI. The expression levels of M2, CM2, and MCM in eYFP positive cells was determined by flow cytometry using the 14C2 antibody and a goat anti-mouse antibody conjugated to AlexaFluor 647. The MFI and standard deviation are presented in each panel.

Figure 2. Expression, oligomerization, and complementation ability of M2, CM2, and MCM. A and B) MDCK cells or MDCK cells stably expressing the indicated proteins were analyzed by Western blotting in order to determine (A) the total expression under reducing conditions and (B) the presence of disulfide linked oligomers under non-
reducing conditions. Antibodies were α-M2 MAb 14C2 (1:500), α-β-actin MAb (1:10,000, Abcam AC-15), and goat α-mouse IgG conjugated to AlexaFluor 647 (1:500, Invitrogen). Monomeric proteins and the cell protein loading control, β-actin, are indicated in (A) and monomers, dimers, and tetramers are indicated in (B). Relative expression levels of M2, CM2, and MCM, normalized to M2WSN N31S, are listed below the corresponding lane. C) Titers of the indicated recombinant virus were determined by TCID$_{50}$ assay on MDCK cells or MDCK cells stably expressing the indicated protein. The mean and the standard error of the mean are graphed from two independent experiments. D-G) MDCK cells or MDCK cells stably expressing the indicated protein were infected at an MOI of 0.001 with the indicated recombinant influenza A viruses. The amount of infectious virus at each time point was determined by TCID$_{50}$ assay on cells expressing wildtype M2. The mean and the standard error of the mean are graphed. The limit of detection is marked by a horizontal dotted line. Infectious virus production from 24-72 hr was analyzed using mixed ANOVAs with time and virus as independent variables using Prism 4.0. Statistical differences between viruses grown on M2-expressing cells and MCM-expressing cells are indicated, ***(p<0.001.

**Figure 3. Analysis of structural proteins incorporated into M2-null viruses.** rUd M2Stop (A-C) or rWSN M2Stop (D-E) virus particles grown on the indicated cell lines were concentrated by ultracentrifugation through 35% sucrose in PBS, resuspended in PBS, and analyzed by Western blotting under reducing conditions and by TCID$_{50}$ assay on wildtype M2-expressing cells expressing. A and D) Incorporation of various structural proteins into M2-null viruses are indicated from images scanned using an FLA-5000. B)
The relative amount of NP\textsubscript{a} incorporated into rUd M2\textsubscript{Stop} virus particles was quantified from Western blots and is indicated as a ratio to total HA\textsubscript{0}. C and E) The relative titer of each sample is indicated as a ratio of the titer versus total HA\textsubscript{0}, as determined by Western blot analysis. The mean and the standard error of the mean are graphed from at least two independent experiments and the Western blots represent one of the quantified replicates.

**Figure 4. Morphology of M2-null virus grown on cell lines expressing M2, CM2, or MCM.** rUd M2\textsubscript{Stop} virus particles grown on MDCK cells or MDCK cells stably expressing the indicated protein were concentrated through PBS containing 35\% sucrose as in Fig 3. Samples were mixed 1:1 with a 1:500 dilution of 100nm Nanosphere beads (Thermo Scientific). 2\muL was allowed to dry onto a 400 mesh carbon and parlodion coated copper grid, negatively stained with filtered 1\% aqueous phosphotungstic acid pH 7.0, and blot dried with filter paper. Samples were viewed on a Hitachi H-7600 TEM operating at 80 kV and digitally captured with an AMT CCD at 1k x 1k resolution. A-E) Representative electron micrographs of virus particles grown on various cell lines, including the average and standard deviation of the particle diameter as measured on the longest axis (n\geq17). Scale bar represents 100nm. F) The total particles (determined from the known concentration of Nanosphere beads (29)) and the ratio of total particles to TCID\textsubscript{50} titer are graphed from three separate experiments.
Figure 1. Schematic and ion channel activity of M2, CM2, and MCM.
Figure 2. Expression, oligomerization, and complementation ability of M2, CM2, and MCM.
Figure 3. Analysis of structural proteins incorporated into M2-null viruses.
Figure 4. Morphology of M2-null virus grown on cell lines expressing M2, CM2, or MCM.
References

1. **Betakova, T., and A. J. Hay.** 2007. Evidence that the CM2 protein of influenza C virus can modify the pH of the exocytic pathway of transfected cells. J Gen Virol **88:**2291-2296.


CHAPTER 5

Conclusions and Future Directions

Shaun M. Stewart
Conclusions

The highly conserved type III integral membrane protein M2 consists of 97 amino acids and forms disulfide-linked tetramers which have a pH-gated, proton-selective ion channel activity (10, 32, 35). M2 is required during virus entry, where it translocates protons into the virion interior which allows for the dissociation of viral ribonucleoprotein complexes (vRNPs) from the site of virus-cell membrane fusion, thereby allowing nuclear transport of the vRNPs (18, 28, 34). M2 is also required during virus assembly where it is important for proper incorporation of vRNPs into budding virus particles (7, 16, 21, 29, 30).

M2 contains several domains, an N-terminal ectodomain, a transmembrane domain, and a C-terminal cytoplasmic tail. The cytoplasmic tail can be divided into two regions based upon deletion mutants and function mapping, the membrane proximal region, residues 46-69, and the membrane distal region, residues 70-97 (Fig 1). The membrane distal region has been studied extensively and is required for proper budding and vRNP incorporation (7, 16, 21, 29, 30). However, much less is known about the role of the membrane proximal region in virus replication. The NMR structure of the transmembrane domain and most of membrane proximal region (residues 22-62) was recently determined by two groups (38, 40). These structures confirm that residues 48-58 form an amphipathic helix. This amphipathic helix also seems to stabilize the tetrameric structure of M2, particularly at low pH when the ion channel is open.

The primary aim of this thesis was to determine the role of the membrane proximal region of the M2 cytoplasmic tail in M2 incorporation and virus replication. Several potential methods of M2 incorporation were previously suggested that
encompassed the membrane proximal region including cholesterol binding (39) and interactions with M1 (7, 29). I attempted unsuccessfully to co-immunoprecipitate cholesterol and full length M2 transiently expressed from cDNA. Later, others showed that cholesterol could only be pulled down by anti-M2 immunoprecipitations from cellular extracts in context of virus infection, ie the co-expression of other viral proteins (36). Since this cholesterol co-immunoprecipitation requires other viral proteins, some of which, HA and NA, are located at sites enriched in cholesterol, and M2, NA, and HA all get incorporated into virus particles, it is probable that this cholesterol co-immunoprecipitation may be mediated by indirect interactions with other viral proteins. Indeed, I found that the cholesterol binding motif in M2 is not required for M2 incorporation or virus replication in tissue culture in either a trans-complementation system or in recombinant viruses expressing mutant M2 proteins (43). Alteration of the cholesterol binding motif did modestly attenuated the morbidity and mortality induced by recombinant virus in mice, suggesting that this region may be a virulence factor (43).

I also studied the other hypothesis, that interactions between M1 and M2 drive the incorporation of M2. Two M1 binding sites within the M2 cytoplasmic tail have been previously mapped, one in the membrane proximal region and another in the membrane distal region (29, 30). Deletion or mutation of amino acids in the membrane distal region lead to decreased vRNA incorporation but do not affect M1 or M2 incorporation (7, 16, 29, 30). I used scanning and directed alanine mutagenesis in trans-complementation assays and recombinant viruses to determine if the membrane proximal region of M2 was important for M2 incorporation and virus replication. I determined that this region can
sustain numerous linear and structure based mutations with little, if any, effect on virus replication and M2 incorporation (42).

My last aim started as an attempt to use chimeric proteins between influenza A virus M2 and an M2 homolog in influenza C virus, CM2, to determine which regions of M2 were most important for M2 function, incorporation, and virus replication. Unfortunately, several of the chimeric constructs were not expressed at the cell surface correctly, possibly due to differences in the normal way in which M2 and CM2 undergo membrane insertion during translation. This issue prevented a complete analysis of which domains of M2 were necessary and sufficient for M2 incorporation and virus replication. However, one construct, an M2 chimera expressing the CM2 transmembrane domain (MCM), was expressed properly and utilized, along with M2 and CM2, in order to compare the ion channel function and the ability of the transmembrane of CM2 to replace that of M2 during virus infection. I determined that CM2 and MCM both had similar abilities to alter the cytosolic pH when expressed at the cell surface, but that only MCM was able to rescue the infectivity of an M2-null virus (41). This further confirmed that residues in the ectodomain and cytoplasmic tail of M2 are important in the assembly of infectious influenza A virus. This also demonstrated for the first time that the ion channel activity of CM2, although different from M2, is sufficient to induce the release of influenza A vRNPs during virus entry.

**Current Budding Model**

Combining the data presented in this thesis along with other recently published data, I propose the following model for virus budding (Fig 2). After viral transcription
and translation, the major structural proteins and vRNPs traffic to the plasma membrane. The glycoproteins HA and NA traffic with detergent-resistant membrane microdomains to the cell surface (11, 26, 47). At early times after infection, M2 has a more random distribution across the cell surface than HA and NA, does not co-localize in one dimension with HA in immunogold labeled thin-section electron micrographs (TEM), and is susceptible to cold detergent extraction (26, 39, 47). However, at later times after infection, M2 is seen to co-localize two-dimensionally with HA in immunogold labeled electron micrographs of planar sheets of plasma membrane (7). This suggests that as viral proteins begin to accumulate at the cell surface late during infection, M2 and HA begin to localize to similar regions despite remaining in unique microdomains of the plasma membrane (Fig 2A).

Some evidence suggests that Rab11, a small GTP-binding protein involved in trafficking of non-raft proteins to the apical recycling endosome (ARE) en route to the plasma membrane, is required for M2 but not HA trafficking to the plasma membrane (11, 37). M2 has been shown previously to be important for vRNP incorporation (7, 16, 29, 30) and my studies show that in the absence of M2, budded virus particles are indeed smaller, probably reflecting a defect in vRNP incorporation (41). I propose that M2 and vRNPs directly interact while in transport to or after M2 arrives at the plasma membrane. Like M2 trafficking, correct trafficking of vRNPs also require Rab11 (2, 12, 31). This suggests, but does not definitely prove, that M2 and vRNPs interact at the ARE during transport and arrive at the plasma membrane together. Either way, interaction and localization together at the plasma membrane (Fig 2B) may occur before, after, or at the same time as M1 begins to bind to the membrane (Fig 2C) and oligomerize (Fig 2D).
M1 has the inherent ability to bind membranes, oligomerize, and induce particle formation in the absence of the other viral proteins (15, 17, 22, 46). However, studies also show that M1 interacts with the HA and NA cytoplasmic tails and that this interaction directs M1 to detergent-resistant membrane microdomains, where HA and NA are enriched (1). Therefore, this proposed budding model emphasizes that these interactions between M1 and the glycoproteins may assist M1 oligomerization and increase viral budding (Fig 2D).

TEM and cryo-electron microscopy (cryo-EM) reveal that in filamentous influenza virus particles, the vRNPs are located at the distal end of the budding particles (6, 33). Given that M2 is important in vRNP incorporation and that vRNPs, when incorporated, are located at the distal end of particles, I propose that M2 interacting with vRNPs at the plasma membrane becomes the seed with which M1 interacts to drive budding (Fig 2E-F). However, in the absence of M2, virus particles are still produced demonstrating M2 is not required for budding (9, 16, 20, 41), and in the presence of M2, empty particles are formed in addition to infectious particles (33). Together these data suggest that either some particles are seeded by M2 which do not have bound vRNPs or, even when M2 is present, M1 induces some particles to form without the M2/vRNP seed. These particles, without vRNPs would be non-infectious and be in agreement with why few particles are detected that do not contain a partial complement of eight vRNPs (33).

M1 forms a helical lattice around the vRNPs just inside the viral membrane and the cytoplasmic tails of HA, NA, and M2 protrude through holes in this lattice given the glycoproteins regular order on the surface of the virus (6).
This model does not address how particles undergo membrane scission and are released. There is little data on cellular factors that might be involved in this step. M2 and a peptide corresponding to the M2 amphiphatic helix, have been shown to induce membrane curvature and scission in unilamellar vesicles and were suggested to mediate membrane scission and virion release (37). However, virus-like particles (VLPs) (7, 8, 15, 24, 25, 44) and virions (9, 16, 20) can be released in the absence of M2. Perhaps, like respiratory syncytial virus (RSV), influenza membrane scission is mediated by cellular factors which bind Rab11 (5, 45).

**Future Directions**

These studies provide evidence that the cholesterol binding motif is not important during replication in tissue culture but partially modulates pathogenesis in mice. Determining why viruses with an alteration of this motif are attenuated in mice will be important. Perhaps cholesterol binding by M2 can act to sequester cholesterol thereby modulating normal cellular functions. However, the fact that the type of cholesterol binding motif found in M2 is generally not one identified as cholesterol sequestering argues against this hypothesis. Nonetheless, it should be evaluated by comparing the effect of cholesterol metabolism in cells expressing either wildtype M2 or the cholesterol binding motif mutant. It would also be interesting to study the effect of cholesterol levels on virus replication; however, cholesterol depletion has been shown to have multiple non-M2 associated affects on influenza virus replication including disruption of HA and NA containing membrane microdomains, increased particle budding, and decreased particle stability (3).
Given that cholesterol can only be immunoprecipitated in the presence of the expression of the other viral proteins (36), it is also possible that the role of this region as a virulence factor is not related to cholesterol binding. Indeed the first 60 amino acids of M2, which include the cholesterol binding motif, are able to bind Beclin-1 and inhibit the maturation of autophagosomes (14). Inhibition of this pathway by HIV blocks autophagy mediated virion degradation and could also do so for influenza A virus (23). As such, it should be determined if mutation of the residues in the cholesterol binding motif prevents the inhibition of autophagosome maturation.

Although the alanine mutagenesis of the membrane proximal region showed, little or no effect on replication of virus, it will be interesting to see if these mutants, like the virus expressing the cholesterol binding mutant, are attenuated in mice. If some are attenuated and others are not, it would serve to further define the region of M2 that may be a virulence factor in mice. Along with morbidity and mortality, the levels of viral replication and virus-induced cytokine production should be determined since both are associated with severe influenza pathogenesis.

Experiments also showed that a recombinant rUd (H3N2) influenza virus (rUd M2AlaHelix) expressing five alanine substitutions in the membrane proximal region of M2, at residues F47, F48, I51, Y52, and F55, grew to lower infectious titers at 24hpi but produced similar levels of infectious virus at 48hpi compared to wildtype (42). Furthermore, there was no apparent defect in incorporation of HA, M1, or NP in this virus. These findings differed from a similar recombinant virus previously published that produced 4-5 logs less infectious virus at all times hpi tested and had decreased incorporation of NP (36). In order to confirm the results of the recombinant virus, I also
used a trans-complementation system to test the requirement of these five amino acids on virus replication. Although complementation of M2-null viruses with the expression of this mutant did decrease production of infectious virus at all times hpi, it was only decreased by ~1-2 logs (42). These findings together contradict the suggestion that these five amino acids are required in tissue culture as reported (36). It will be important to test, side-by-side, the rUd M2AlaHelix generated previously with the one described here in order to reconcile their phenotypic differences.

All mutations to date in the M2 amphipathic helix have focused on maintaining the structure of the helix while altering the identities of individual amino acids or altering charge faces. It would be beneficial in future studies to either make mutations that are predicted to interrupt the helical structure, perhaps by inserting prolines and/or glycines, or to insert a sequence between the transmembrane and amphipathic helix that is unable to form a helix.

Another finding in this dissertation that should be followed up relates to the ion channel activity of CM2. It has previously been suggested that CM2 is not required to pump ions inside the uncoating virion in influenza C virus, like it is in influenza A virus, because low pH did not separate M1 from vRNPs (48). However, two lines of evidence suggest that a low pH burst may in fact also be required for influenza C virus. First, CM2 has ion channel activity and can result in lowered pH (4, 19, 41). Second, similar to M2, CM2 is required for nuclear migration of vRNPs during virus uncoating (13). In light of the data presented in this thesis, I hypothesize that for influenza C virus low pH is required to separate M1/vRNPs from membranes but not each other. Alternatively, since CM2 may be able pump chloride ions (19), the separation of M1/vRNPs might occur at
different relative salt concentrations compared to influenza A virus M1/vRNPs. Future work should reevaluate the ability of low pH to separate influenza C virus M1/vRNPs from membranes and M1 from vRNPs at various pH levels and salt concentrations.

Related to the ability of CM2 to lower cytosolic pH, it was interesting that expression of MCM but not CM2 was able to complement M2-null viruses in a strain specific manner. MCM complemented rUd strains better than rWSN (H1N1) M2-null strains and this phenotype did not map to M1 as have so many other strain specific effects of M2 have in the past (7, 16, 29). One hypothesis about why MCM is able to complement rUd strains better than rWSN strains of influenza is that the M2-mediated release of vRNPs during entry requires different pH in the two strains. This is in agreement with the findings that M2 incorporation is much lower in wildtype rUd compared to rWSN and that MCM has decreased abilities to alter pH compared to M2 (41). It will be important to test this hypothesis by purifying M1/vRNP complexes from either cells or virus particles and determine the pH that is required for separation of M1 and vRNPs from both strains of viruses, as has been done previously to compare other influenza reassortants (27).

**Implications**

The M2 protein is required for virus entry and assembly. First generation adamantane pharmaceuticals, amantadine and rimantadine, have been available for a number of years and work by blocking the ion channel activity of M2. However, resistance to these drugs easily arises and most circulating H3N2 strains are already resistant to both drugs. Mutations that convey resistance to adamantanes so readily
appear in the population probably due to several reasons. One, M2 is required for virus replication. Two, very high levels of drug are required in vitro in order to completely block ion channel activity and are potentially not achievable in vivo. Lastly, different strains may require more or less M2 ion channel activity for replication. Together this suggests that M2 is a great drug target but that better drugs must be discovered. Many resources are being invested in optimizing adamantanes, but little to date has provided promise. This thesis increases our knowledge of M2 and suggests that new drugs should target functions of M2 in the ectodomain and cytoplasmic tails of the protein.
**Figure 1. Schematic of functional domains of M2.** M2 contains three domains, an N-terminal ectodomain, a transmembrane domain, and a C-terminal cytoplasmic tail. The cytoplasmic tail can be artificially divided into two parts based upon function. The membrane distal region includes residues 70-97 and has been shown to be important for virus budding and vRNP incorporation. The membrane proximal region also contains several important functions and was further studied in this thesis. The schematic shows an image of the crystal structure of residues 22-62 (PDB 2LOJ) generated with PyMol along with lines representing the ectodomain and remainder of the cytoplasmic tail (residues 63-97). Extra is the extracellular space, PM is the plasma membrane, and Cyto is the cytosol.

**Figure 2. Model of infectious influenza A virus assembly.** A) After viral transcription and translation, the major structural proteins and viral ribonucleoprotein complexes (vRNP) accumulate at the plasma membrane. The glycoproteins HA and NA traffic to similar membrane microdomains (purple), which resemble lipid rafts. M2 has a more random distribution across the cell surface and does not accumulate in HA and NA-containing microdomains (grey). B) M2, which is known to interact with vRNPs, may do so at this time. C) M1 has the inherent ability to bind the inner leaflet of the plasma membrane. D) M1, either already bound to the membrane or from a cytoplasmic pool, binds the cytoplasmic tails of HA and NA and undergoes oligomerization. The order of B-D may be different or the steps may occur simultaneously. E) M1 interacts with M2
and initiates membrane curvature and viral budding around the vRNPs. In the absence of M2, M1 still induces budding, but vRNP incorporation is impaired. F) After budding is initiated, M2 and the vRNPs are located at the distal end of filamentous particles. M1-M1 interactions direct the continued growth of virus particles by the aggregation and incorporation of HA and NA-containing microdomains. At some point, unknown cellular factors are probably recruited to the bud and induce membrane scission, releasing the virus particle. M1-M1 structure around the distal vRNPs has been omitted for clarity.
Figure 1. Schematic of functional domains of M2.
Figure 2. Model of infectious influenza A virus assembly.
References


4. **Betakova, T., and A. J. Hay.** 2007. Evidence that the CM2 protein of influenza C virus can modify the pH of the exocytic pathway of transfected cells. J Gen Virol **88:**2291-2296.


CURRICULUM VITAE

SHAUN STEWART

Work Address: Johns Hopkins University
Bloomberg School of Public Health
615 N Wolfe St, Suite 5132
Baltimore, MD 21205
Telephone: (443) 287-8750

Home Address: 6503 Copper Ridge Dr. Apt T1
Baltimore, MD 21209
Cell: (314) 623-6486
Email: stewarts@go.wustl.edu

EDUCATION

Washington University in St. Louis
• Ph.D. Candidate in Molecular Microbiology and Microbial Pathogenesis
• Laboratory of Dr. Andrew Pekosz

University of Pennsylvania
• Bachelor of Arts with Honors in Biology

DOCTORAL THESIS

The Role of the Membrane Proximal Region of the M2 Cytoplasmic Tail in Virus Replication

RESEARCH EXPERIENCE

Ph.D. Candidate, Washington University in St. Louis 2005 – Present
• Cloned and rescued recombinant influenza A viruses
• Purified virus particles by sucrose cushion and density gradient ultracentrifugation
• Characterized purified viruses by plaque assay, TCID₃₀ assay, and real time PCR
• Purified recombinant proteins by GST pulldown
• Analyzed virus infected cells by Western blot and flow cytometry
• Trained collaborating faculty and laboratory personnel to characterize influenza viruses

Research Specialist, University of Pennsylvania 2004 – 2005
• Engineered and cloned HIV envelope genes with loop deletions
• Constructed chimeric Bunyavirus genes for alphavirus replicon expression system
• Performed expressions studies of glycoprotein fusion protein

Research Assistant, University of Pennsylvania 2002 – 2003
• Performed ELISA and WB analysis of cells stimulated with HIV proteins
• Harvested and maintained primary human cells in cell culture

Research Assistant, Centers for Disease Control and Prevention 2001

Laboratory Assistant, University of North Texas 2000 – 2001

Teaching Experience

Teaching Assistant, Washington University in St. Louis 2007
• Laboratory Experiments with Eukaryotic Microbes
HONORS & AWARDS

- American Society for Virology Student Travel Award 2010
- Division of Biological and Biomedical Sciences Scholarship 2005
- University Scholar Honors Induction (research honors program) 2001
- University of Pennsylvania Leadership Scholarship 2001 – 2004
- Barry M. Goldwater Scholarship 2001 – 2003
- Howard Hughes Summer Research Fellowship 2001
- Intel Science Talent Search Semifinalist 2001
- Siemens Westinghouse Regional Finalist 2001
- Welsh Undergraduate Research Fellowship 2000

PUBLICATIONS


PRESENTATIONS


**AFFILIATIONS**

- American Society of Virology
- American Scientific Association

**RESEARCH INTERESTS**

- Viral Replication and Assembly
- Host-Virus Interactions
- Emerging Infectious Diseases
- Vaccine Development