JOURNAL OF VIROLOGY, Feb. 1995, p. 1219–1225 Vol. 69, No. 2
0022-538X/95/$04.00+0


# Analysis of the Posttranslational Modifications of the Influenza Virus $M_2$ Protein

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Received 1 September 1994/Accepted 4 November 1994

**The sites of posttranslational modifications of the influenza A virus $M_2$ protein were examined, and the effect of these modifications on the $M_2$ protein ion channel activity was analyzed. Cysteine residues 17 and 19 in the $M_2$ protein ectodomain form disulfide bonds. The cytoplasmic tail is posttranslationally modified by palmitoylation, and mutagenic studies support the view that cysteine residue 50 is the site for fatty acylation. In addition, the cytoplasmic tail of the $M_2$ protein was found to be posttranslationally modified by the addition of phosphate to specific serine residues. Site-directed mutagenesis of serine residues in the $M_2$ protein cytoplasmic tail, combined with phosphoamino acid analysis, indicated that serine residue 64 is the predominant site for phosphorylation but that serine residues 82, 89, and 93 were also phosphorylated but to much lesser extents. Disulfide-bond formation, palmitoylation, and phosphorylation occurred on $M_2$ protein expressed in mammalian cells infected with influenza virus, in mammalian cells in which the $M_2$ protein was expressed from DNA expression vectors, and when the $M_2$ protein was expressed in oocytes of *Xenopus laevis*. The membrane currents of oocytes of *Xenopus laevis* expressing wild-type and site-specifically altered forms of the $M_2$ protein, to ablate posttranslational modifications, indicated that none of the posttranslational modifications significantly affected the ion channel activity of the $M_2$ protein in oocytes. Therefore, these data do not indicate a functional role for posttranslational modifications of the $M_2$ protein in its ion channel activity.**

The influenza A virus $M_2$ protein, which is encoded by a spliced mRNA derived from RNA segment 7, is a small integral membrane protein (97 amino acids) that is expressed at the plasma membrane of virus-infected cells and is oriented such that it has 23 N-terminal extracellular residues, a transmembrane domain of 19 residues, and a 54-residue cytoplasmic tail (20, 24, 26, 46). The $M_2$ protein forms a homotetramer composed of a pair of disulfide-linked dimers or a disulfide-linked tetramer (11, 28, 40, 45). Although the $M_2$ protein is abundantly expressed at the plasma membrane of virus-infected cells, it is a minor component of the virion envelope in comparison with the abundant hemagglutinin and neuraminidase (13, 15, 26, 46).

The $M_2$ protein transmembrane domain was deduced to be the target of the anti-influenza virus drug amantadine, as drug-resistant mutants contained amino acid changes in the $M_2$ protein transmembrane domain (8). From studies on the effect of amantadine on virus replication, it was proposed that the $M_2$ protein functions as an ion channel that permits ions to enter endocytosed virions and to function as a modulator of pH in intracellular compartments (7, 22, 38, 40).

Direct evidence that the $M_2$ protein has ion channel activity was obtained by expressing the $M_2$ protein in oocytes of *Xenopus laevis*. It was found that in response to hyperpolarizing membrane voltages, an inward whole-cell current that is blocked by amantadine and activated by the low pH found in endosomes could be recorded (35). More recently, it has been demonstrated that the $M_2$ protein forms an ion channel in mammalian cells by recording amantadine-sensitive currents that were activated by low pH from CV-1 cells expressing the $M_2$ protein (43). The ion selectivity and amantadine sensitivity of the $M_2$ ion channel is determined by the amino acid sequence of the transmembrane domain which forms the pore of the channel (12, 35, 43). The notion that the transmembrane domain of the $M_2$ protein forms a pore was reinforced by finding that a 25-residue peptide corresponding to this domain inserts into planar lipid bilayers and promotes the formation of channels (at pH 1 to 2) which fail to open upon addition of amantadine (3). In addition, when purified $M_2$ protein was incorporated into planar lipid bilayers, ionic conductances that were blocked by amantadine and activated by lowered pH were observed (41).

The $M_2$ protein is posttranslationally modified by the formation of intermolecular disulfide bonds at cysteine residues 17 and 19 (11). The $M_2$ protein is also posttranslationally modified by palmitoylation through a thioether linkage (39, 42). In addition to acylation, the $M_2$ protein is posttranslationally modified by phosphorylation (40, 44). However, data to demonstrate the site of phosphorylation have not been reported.

We describe here properties of altered $M_2$ molecules to examine the effect of disulfide bond formation and palmitoylation on the $M_2$ protein ion channel activity. In addition, we have determined the sites of phosphorylation at specific serine residues in the cytoplasmic tail and determined the effect of phosphorylation on the ion channel activity of the $M_2$ protein.

## MATERIALS AND METHODS

**Site-specific mutagenesis and construction of recombinant plasmids.** The cDNA to the influenza virus A/Udorn/72 $M_2$ mRNA (14, 46) was cloned into the *Bam*HI site of pGEM3Zf(+) and used as a template for phagemid-based mutagenesis (32). The $M_2$ cDNA was also cloned into the *Bam*HI site of pGEM3 for

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site-directed mutagenesis by the unique-site elimination system (2) performed by the unique-site elimination mutagenesis procedure of Pharmacia-LKB, Inc., Piscataway, N.J. The nucleotide sequences of the altered cDNAs in both the pGEM3Zf(+) phagemid vector and the pGEM3 vector were confirmed by dideoxynucleotide chain-terminating sequencing (36).

**Mammalian cell expression of $M_2$ proteins.** The cysteine-altered $M_2$ cDNAs were expressed in CV-1 cells by using the simian virus 40 (SV40) VP1 replacement vector pSV73E/K (46) such that the $M_2$ cDNA was under the control of the SV40 late-region promoter, splicing, and polyadenylation signals. SV40-$M_2$ recombinant virus stocks were produced as described previously (11, 23). The serine-altered $M_2$ cDNAs were expressed in HeLa T4 cells by a modification of the vaccinia virus-T7 RNA polymerase system (vac-T7) of Fuerst et al. (6) as described previously (30). Only cDNAs cloned in pGEM3 were used, because it was found that the pGEM3 vector yields approximately 50-fold-higher expression levels of protein than does pGEM3Zf(+) for the same cloned $M_2$ cDNA.

**Microinjection and culture of oocytes.** Ovarian lobules from *Xenopus laevis* females (Nasco, Fort Atkinson, Wis.) were surgically removed and treated with collagenase B (2 mg/ml; Boehringer Mannheim Biochemicals, Indianapolis, Ind.) (35). Selected oocytes (stages V and VI) were given injections of 50 nl of RNA (1.0 μg/μl) via a 20-μm-diameter glass pipette, and the oocytes were maintained in ND96 (35) at 17°C.

**Metabolic labeling of mammalian cells.** CV-1 cells were grown in 10% fetal calf serum in Dulbecco's modified Eagle's medium (DMEM) at 37°C in 5% $CO_2$, infected with recombinant SV40, and labeled 45 to 48 h postinfection (p.i.) with [$^{35}$S]cysteine (100 μCi/ml) in DMEM lacking cysteine (DMEM Cys$^-$) as described previously (11, 21, 26). Recombinant SV40-infected cells were labeled 45 to 48 h p.i. with [$^3$H]palmitic acid (300 μCi/ml; 80 Ci/mmol; New England Nuclear Research Products, Boston, Mass.) in DMEM supplemented with 5 mM sodium pyruvate or were labeled 45 to 48 h p.i. with $^{32}P_i$ (500 μCi/ml; ICN Radiochemicals, Costa Mesa, Calif.) in phosphate-free DMEM. HeLa T4 cells expressing serine-altered proteins, obtained by the vac-T7 expression system at 4 h posttransfection, were incubated for 30 min in DMEM Cys$^-$ and labeled for 1 h with [$^{35}$S]cysteine (100 μCi/ml) in DMEM Cys$^-$ HeLa T4 cells expressing the serine-altered $M_2$ proteins were labeled with phosphate by being incubated at 4 h posttransfection in phosphate-free DMEM for 1 h and then labeled for 2 h with $^{32}P_i$ (500 μCi/ml) in phosphate-free DMEM. For control experiments, CV-1 cells were infected with influenza virus A/Udorn/72 or Sendai virus as described previously (18, 25) and at 6 or 14 h p.i., respectively, were incubated in phosphate-free DMEM for 1 h. They were then labeled for 2 h with $^{32}P_i$ (500 μCi/ml) in phosphate-free DMEM.

**Metabolic labeling of oocytes.** Oocytes were incubated from 24 to 48 h postinjection with ND96 supplemented with either [$^{35}$S]methionine (250 μCi/ml) or $^{32}P_i$ (20 mCi/ml).

**Microsome preparation and proteolysis.** SV40 wild-type (wt) $M_2$ recombinant virus-infected cells were labeled from 45 to 48 h p.i. with [$^{35}$S]cysteine in DMEM Cys$^-$ or with $^{32}P_i$ in phosphate-free DMEM before the preparation of crude microsomal membranes (31, 46). Microsomes were treated with *N*-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin (200 μg/ml; Worthington Biochemical Corp., Freehold, N.J.) and isolated by centrifugation on a sucrose cushion essentially as described previously (29).

**Immunoprecipitation and SDS-PAGE.** Labeled oocytes were homogenized in, per oocyte, 75 μl of radioimmunoprecipitation assay (RIPA) buffer containing 50 mM iodoacetamide and a cocktail of protease inhibitors as described previously (33). Mammalian cells were lysed in RIPA buffer. Extracts were immunoprecipitated (21) with $M_2$-specific 14C2 monoclonal antibody (MAb) ascitic fluid (45). For polypeptides labeled with $^{32}P_i$, 1 μg of RNase A per ml was added to the lysate prior to addition of 14C2. Samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 17.5% polyacrylamide–4 M urea gels and processed for fluorography or autoradiography as described previously (18, 33).

Electrophysiological recordings were made on injected oocytes, and the oocytes were saved for immunoblotting. Individual unlabeled oocytes were lysed in 100 μl of RIPA buffer and extracted once with 1,1,2-trichlorotrifluoroethane to remove yolk and pigment proteins. From 5 to 10 μl of lysates was analyzed on 17.5% polyacrylamide–4 M urea gels and electrotransferred to polyvinylidene difluoride membranes (Millipore, Bedford, Mass.) with a Trans-Blot Semidry transfer cell (Bio-Rad, Richmond, Calif.). Immunoblotting with a 1:3,000 dilution of $M_2$-specific 14C2 ascitic fluid (45) and the ECL system (Amersham International, Arlington Heights, Ill.) was done as described previously (12). Autoradiography was performed with preflashed X-ray film (27) and quantified by laser-scanning densitometry on an LKB Ultroscan XL Densitometer (Pharmacia-LKB, Piscataway, N.J.). For quantification of blots, 50 to 400 pg of purified $M_2$ protein, the precise amount depending on the $M_2$ mutant under analysis, was run with all samples to generate a standard curve. $M_2$ protein was purified from detergent lysates of influenza A/Udorn/72-infected CV-1 cells by immunoaffinity chromatography with $M_2$-specific purified 14C2 immunoglobulin G coupled to CNBr-activated Sepharose 4B as described previously (11).

**Phosphoamino acid analysis.** The method for phosphoamino acid analysis was adapted from that described previously (17, 19). $M_2$ protein was immunoprecipitated and the polypeptide was separated on 17.5%–4 M urea gels under reducing conditions, and phosphate-labeled $M_2$ polypeptides were excised from dried gels with the autoradiogram as a template. Peptides were eluted from the gel pieces by incubation in 50 mM $NH_4HCO_3$–0.1% SDS, incubated with agitation overnight at 37°C, and filtered through glass wool. Peptides were precipitated with 10% trichloroacetic acid, with 20 μg of gamma globulin as the carrier, and lyophilized. Peptides were hydrolyzed in 6 N HCl for 3 h at 110°C and analyzed on 20- by 20-cm cellulose thin-layer plates (100 μM; Brinkman, Westbury, N.Y.) by two-dimensional electrophoresis. In the first dimension, the buffer was formic acid-glacial acetic acid-$H_2O$ (pH 1.9) (50:156:1,794) and electrophoresis was carried out at 1,000 V for 60 min. In the second dimension, the buffer was glacial acetic acid-pyridine-$H_2O$ (pH 3.5) (100:10:1,890) and electrophoresis was carried out at 1,000 V for 75 min essentially as described previously (1). Phosphoserine, phosphothreonine, and phosphotyrosine were used as standards (Sigma Chemical Co., St. Louis, Mo.) and identified by staining with 0.2% ninhydrin dissolved in acetone.

**Electrophysiological recordings.** Whole-cell currents were recorded from oocytes with a two-electrode voltage clamp apparatus 48 to 72 h after mRNA injection (35). For all measurements described herein, the small endogenous current exhibited by control oocytes injected with antisense RNA was subtracted from the measured current to yield the current reported.

## RESULTS AND DISCUSSION

**Expression of $M_2$ cysteine-altered mutants in oocytes of *X. laevis*.** The role of $M_2$ cysteine residues in $M_2$ protein disulfide bond formation and their role in forming oligomers were examined previously (11) by converting each of the two extracellular cysteine residues and the single cytoplasmic cysteine residue to serine residues (C-1 [C17S], C-2 [C19S], and C-3 [C50S]) and expressing the altered $M_2$ proteins in eukaryotic cells. It was found that removal of either one of the N-terminal cysteines at residue 17 or 19 resulted in the formation of tetramers that consisted of a pair of noncovalently associated disulfide-linked dimers, indicating that each of the extracellular cysteine residues is equally competent for forming disulfide bonds. When both cysteine residues were removed from the $M_2$ N-terminal domain, no disulfide-linked forms were observed, but chemical cross-linking data of this double-cysteine mutant (C-1,2 [C17S, C19S]) in membranes indicated that the molecule was still a tetramer (11). It has also been shown previously that the $M_2$ protein is modified by covalent attachment of palmitate (39, 42). The palmitate associated with $M_2$ is susceptible to removal by hydroxylamine (39, 42) and by boiling the $M_2$ protein in 5% mercaptoethanol (42), treatments which are indicative of a thioether linkage. Palmitoylation occurs on the cytoplasmic tails of integral membrane proteins (37), and because there is only a single cysteine (residue 50) in the $M_2$ protein cytoplasmic tail, it is presumed this is the site of palmitoylation. In addition, the replacement of cysteine 50 with phenylalanine in some influenza viruses, e.g., A/equine/Fontainebleau/79 (H3N8), results in a concomitant loss of palmitate labeling, a finding which points to cysteine 50 as the site of palmitate attachment (39).

To determine the effect of the cysteine alterations on the $M_2$ protein ion channel activity, synthetic mRNAs were injected into oocytes of *X. laevis*. Synthesis of the $M_2$ protein mutants was examined by metabolically labeling oocytes with [$^{35}$S]cysteine for 24 h. $M_2$ proteins were immunoprecipitated, and polypeptides were analyzed by SDS-PAGE under nonreducing conditions. As shown in Fig. 1, a pattern of polypeptide species consistent with those observed previously in mammalian cells (11) was found. The wt and C-3 $M_2$ proteins were found to form a mixture of disulfide-linked dimers (~30 kDa) and disulfide-linked tetramers (~60 kDa), whereas mutants C-1 and C-2 were found to form disulfide-linked dimers. The replacement of both cysteine residues in the extracellular domain of $M_2$ (C-1,2) eliminated the formation of disulfide bonds, and the protein migrated as a 15-kDa form similar in mobility to that observed under reducing conditions. The accumulation of the C-1,2 mutant was reduced in comparison with the other cysteine-altered $M_2$ proteins. It is possible that the accumula-

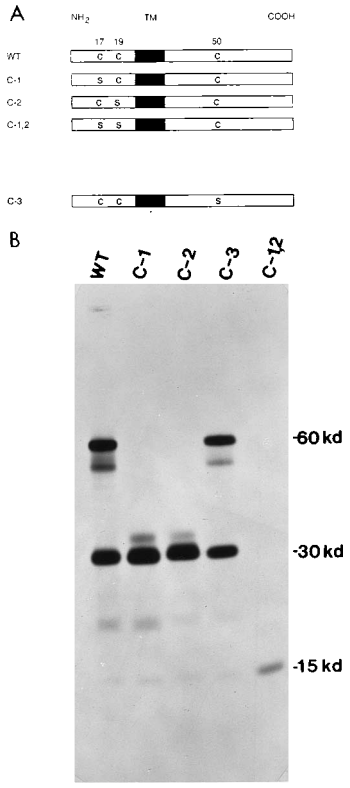



FIG. 1. Expression of the $M_2$ protein cysteine mutants in oocytes of *X. laevis*. (A) Schematic diagram of cysteine mutants which were constructed previously (11). The $M_2$ protein transmembrane domain is represented by a solid box. (Mutant C-1, C17S; mutant C-2, C19S; mutant C-3, C50S; mutant C-1,2, C17S,C19S). (B) Synthetic mRNAs were transcribed from either pGEM3 or pGEM3Zf(+) encoding the wt $M_2$ and cysteine-altered $M_2$ proteins and were microinjected (50 nl of 1-μg/μl RNA) into oocytes of *X. laevis*. At 24 h postinjection, oocytes were labeled with [$^{35}$S]methionine for 16 h and homogenized and detergent lysates were prepared in the presence of 50 mM iodoacetamide. Proteins were immunoprecipitated with an $M_2$-specific MAb (14C2) and analyzed by SDS-PAGE under nonreducing conditions. Positions of the $M_2$ monomer, dimer, and tetramer bands (15, 30, and 60 kDa, respectively) are shown on the right.

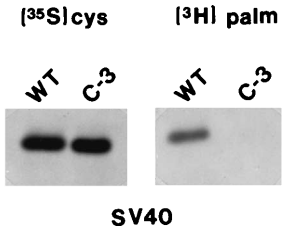



FIG. 2. Lack of addition of palmitate for $M_2$ mutant C-3. SV40-$M_2$ recombinant-infected CV-1 cells were labeled with [$^{35}$S]cysteine ([$^{35}$S]cys) or [$^3$H]palmitic acid ([$^3$H]palm) from 45 to 48 h p.i. Detergent lysates were prepared in the presence of 50 mM iodoacetamide, immunoprecipitated with 14C2 $M_2$-specific ascitic fluid, and analyzed by SDS-PAGE under reducing conditions. Only the relevant portion of the autoradiograph is shown.

tion of the C-1,2 mutant is reduced because it is less stable than its disulfide-bonded counterparts, but this possibility has not been explored.

To provide evidence that cysteine residue 50 is the site of palmitate addition, an attempt was made to label oocytes expressing wt $M_2$ and $M_2$ mutant C-3 with [$^3$H]palmitic acid. However, when 2 mCi of [$^3$H]palmitic acid per ml was used, it was not possible to detect labeling on wt $M_2$ even after a 2-month autoradiographic exposure. Presumably, the fatty acid pool size in oocytes is too large to obtain detectable radioisotopic incorporation. Indirect evidence that $M_2$ protein synthesized in *X. laevis* oocytes is modified by addition of palmitate was obtained by boiling the immunoprecipitated $M_2$ protein in 5% dithiothreitol and observing a slight shift in mobility (data not shown), as described previously (42). However, when wt $M_2$ and the $M_2$ C-3 mutant were expressed in CV-1 cells with SV40-$M_2$ recombinant virus vectors, wt $M_2$ could be labeled with [$^3$H]palmitic acid whereas the $M_2$ C-3 mutant protein could not (Fig. 2). Thus, these data provide further evidence to suggest that $M_2$ cysteine residue 50 is the site of palmitate addition.

**Ion channel activity of the $M_2$ cysteine-altered mutants.** To examine for the ion channel activity of the cysteine-altered $M_2$ proteins expressed in oocytes of *X. laevis*, a two-electrode voltage clamp procedure was used and total membrane currents were measured. The currents were studied by holding the membrane voltage at −40 mV and hyperpolarizing the membrane with step voltage clamp pulses from −130 to −60 mV in normal Barth's solution (pH 7.5) and in Barth's solution (pH 6.2). The peak amplitudes of the whole-cell currents measured following hyperpolarization at −130 mV were normalized to the amount of accumulated $M_2$ protein (determined by a quantitative immunoblot procedure) in individual oocytes, and the current (microamps) at pH 6.2 per nanogram of $M_2$ protein accumulated was measured. The current, protein, and their ratio varied from batch to batch. Within a single batch, the ratio was found to be spread only over a twofold range for the wt $M_2$ protein and the cysteine-altered mutants (0.124 to 0.273 ± 0.024 μA/ng [mean ± standard error of the mean, $n$ = 6]). Following activation at pH 6.2, the currents of the wt $M_2$ and all the cysteine-altered $M_2$ proteins were blocked by the $M_2$ channel blocker amantadine (data not shown). When the pH sensitivities of the membrane currents of oocytes expressing the wt $M_2$ and cysteine-altered $M_2$ proteins were compared

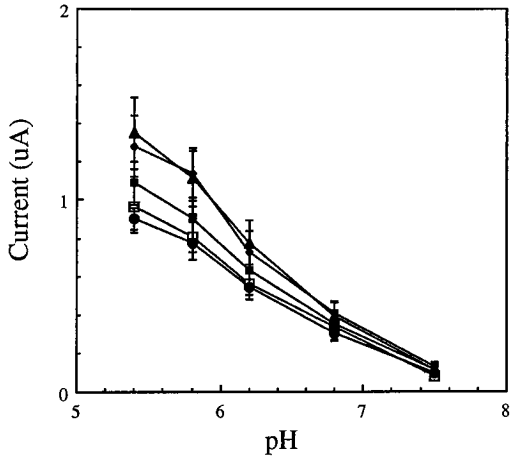



FIG. 3. Modulation of the membrane current of oocytes expressing the wt and cysteine-altered $M_2$ proteins by pH. Oocytes were given microinjections of synthetic RNA transcripts encoding the wt $M_2$ and cysteine-altered $M_2$ proteins. Electrophysiological measurements were performed at 48 to 72 h p.i. The peak amplitude of the amantadine-sensitive inward currents following a hyperpolarization pulse of −130 mV for the indicated $M_2$ protein is plotted against extracellular pH. Background endogenous currents recorded from oocytes given injections of antisense RNA were subtracted from each datum point. For each datum point, $n$ = 5. Symbols: □, wt; ●, C-1; ●, C-2; ▲, C-3; ■, C-1,2.

over the pH range from 7.5 to 5.4, it was found that all the mutants had similar slopes of pH activation (Fig. 3).

The observation that disulfide bond formation is not essential for ion channel activity was not unexpected, because oligomerization of the $M_2$ protein occurs in the absence of cysteine residues 17 and 19 (11). It is possible that oligomerization is specified largely by the transmembrane domain, because deletions in this domain can alter the form of the $M_2$ oligomer (12). The lack of a significant change in the pH activation curve of the ion channel activity of $M_2$ cysteine-altered mutant C-1,2 from wt $M_2$ protein suggests that the presumptive conformation change in the $M_2$ protein on pH activation does not depend on disulfide bond formation. Although modification of the cysteine residues in the $M_2$ protein by disulfide bond formation or fatty acylation appears to have no role in significantly modulating $M_2$ protein ion channel activity, these modifications may play other roles in the life cycle of influenza virus, such as stabilizing the $M_2$ protein in the membrane during virus budding or facilitating the incorporation of $M_2$ protein in the virion particle.

**Nature of the influenza virus $M_2$ protein phosphorylated residue.** It has been shown previously that when influenza virus-infected cells are metabolically labeled with $^{32}P_i$, the $M_2$ protein is phosphorylated (40, 44). To identify the phosphorylated amino acid residue, influenza virus-infected cells were metabolically labeled with $^{32}P_i$, the $M_2$ protein was immunoprecipitated and analyzed by SDS-PAGE, and the $M_2$ protein band was excised and hydrolyzed with 6 N HCl. Amino acids were separated by two-dimensional high-voltage electrophoresis. As shown in Fig. 4, The phosphorylated residue was found to be phosphoserine. To rule out the possibility that phosphothreonine was hydrolyzed to threonine by the procedure used, metabolically $^{32}P$-labeled M protein of Sendai virus, which is known to contain phosphothreonine (19), was hydrolyzed, and phosphothreonine was recovered (Fig. 4). For mutagenesis experiments (see below), it was necessary to express the $M_2$ protein with eukaryotic vectors. To eliminate the possibility that the protein kinase activities present in an influenza virus-infected cell phosphorylate $M_2$ protein differently from vector-expressing cells, the phosphoamino acid(s) of $M_2$ protein expressed by the vac-T7 expression system as well as recombinant SV40 expression was investigated and found to be phosphoserine in all cases (Fig. 4). Therefore, the nature of the $M_2$ protein phosphorylated residues is not changed between influenza virus-infected and vector-expressing cells.

**Identification of the $M_2$ protein-phosphorylated serine residues.** The N-terminal domain of the $M_2$ protein expressed at the cell surface is accessible to protease digestion, and the C-terminal cytoplasmic domain of the $M_2$ protein in a preparation of crude microsomes is accessible to protease digestion (26, 46). To determine the domain of the $M_2$ protein modified by phosphorylation, $^{32}P$-labeled $M_2$ protein expressed at the cell surface, $^{32}P$-labeled $M_2$ protein contained in microsomes, and [$^{35}$S]cysteine-labeled $M_2$ protein contained in microsomes were digested with protease and the proteolytic fragments were immunoprecipitated with N-terminal domain-specific MAb 14C2 or C-terminal domain-specific MAb 1D6-tail. The protease-trimmed cell surface $M_2$ protein, which lacks the N-terminal domain, was labeled with $^{32}P_i$ (Fig. 5A). In comparison, the ~7-kDa trypsin-resistant microsomal $M_2$ fragment that lacks the $M_2$ cytoplasmic tail could not be identified when the $M_2$ protein was $^{32}P$-labeled (Fig. 5A), but this fragment could be readily identified when the $M_2$ protein in microsomes was labeled with [$^{35}$S]cysteine (Fig. 5B). These data indicate that the $M_2$ protein cytoplasmic tail is the region modified by phosphorylation.

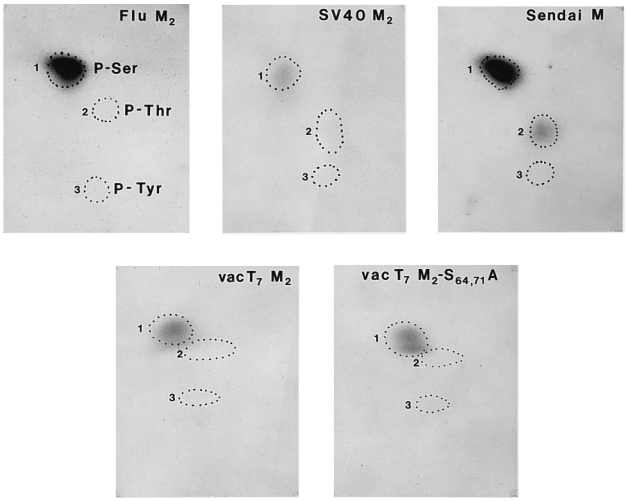



FIG. 4. Phosphoamino acid analysis of wt and serine-altered $M_2$ proteins. The $M_2$ protein was expressed in influenza virus A/Udorn/72-infected CV-1 cells and SV40-$M_2$ recombinant-infected CV-1 cells and from cDNA by the vac-T7 expression system. In addition, a serine-altered $M_2$ protein, $M_2$-S64A,S71A was expressed by the vac-T7 expression system. The $M_2$ proteins were metabolically labeled with $^{32}P_i$. They were immunoprecipitated with 14C2 MAb and analyzed by SDS-PAGE. Gel bands were excised from dried gels with the autoradiograph as a guide. Polypeptides from $^{32}P_i$-labeled Sendai virus-infected CV-1 cells were analyzed directly by SDS-PAGE, and the Sendai virus M protein band was excised from the dried gel. Hydrolysis of polypeptides to amino acids with 6 N HCl and analysis of phosphoaminoacids by two-dimensional high-voltage electrophoresis on cellulose plates were done as described in Materials and Methods. The mobilities of coelectrophoresed phosphoserine (area 1), phosphothreonine (area 2), and phosphotyrosine (area 3) standards were identified by staining with ninhydrin, and their positions were marked. $^{32}P$-labeled amino acids were detected by autoradiography. For the purpose of comparison, panel vac$T_7$ $M_2$-$Ser_{64,71}A$ was exposed for autoradiography ~seven times longer than was panel vac$T_7$ $M_2$.

Each of the five $M_2$ cytoplasmic tail serine residues (Fig. 6D) is in a sequence context such that it could be phosphorylated by a known cellular protein kinase (Table 1). To identify the phosphorylated residue in the $M_2$ cytoplasmic tail, the five serine residues were individually changed to alanine and then combinations of serine-to-alanine changes were made as necessary. To simplify the number of altered $M_2$ proteins that had to be constructed initially, a translational stop codon at residue 89 (S-$89_{STOP}$) was introduced to eliminate serine residues 89 and 93. The serine-altered $M_2$ molecules were expressed in HeLa T4 cells by using the vac-T7 expression system and were metabolically labeled with $^{32}P_i$ or [$^{35}$S]cysteine. As shown in Fig. 6, the mutation S64A resulted in a ~85% reduction in phosphate labeling of $M_2$ protein compared with wt $M_2$ protein, and thus serine 64 is the predominant site for phosphate addition. However, the molar ratio of phosphate addition on $M_2$ residue serine 64 to unphosphorylated serine is not known. Serine 64 is within the recognition motif for both calmodulin-dependent protein kinase II and S6 kinase II (Table 1). To ensure that the residual phosphorylation found on $M_2$-S64A protein occurred on a serine residue and that the mutation had not caused a change to threonine or tyrosine, the phosphoamino acid content of S64A,S71A was determined. The residual phosphorylation was found to remain on the serine residue (Fig. 4, panel $S_{64,71}A$). To investigate which other serine residue was phosphorylated in addition to serine 64 several serine mutants were examined for phosphorylation (S64A,S71A, S64A,S71A,S82A, S64A,S71A,S82A,S89A, S89A, S82A,S89A, S64A,S71A,S82A,S89A, S64A,S71A,S82A, $S89_{STOP}$, S93A). Examples of expression of some of these

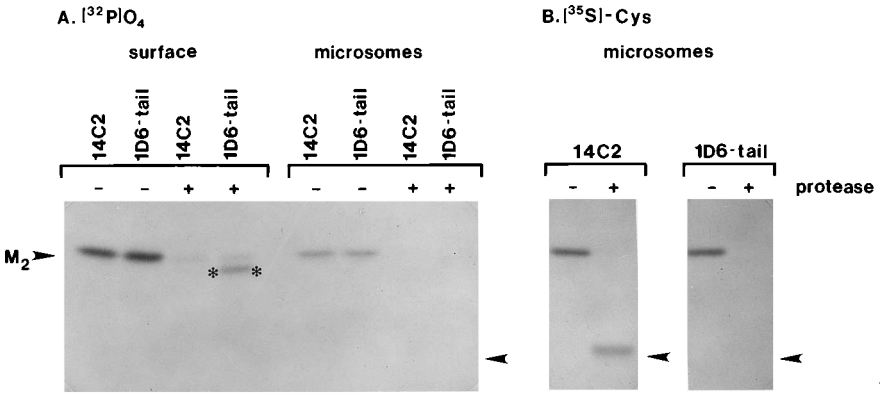
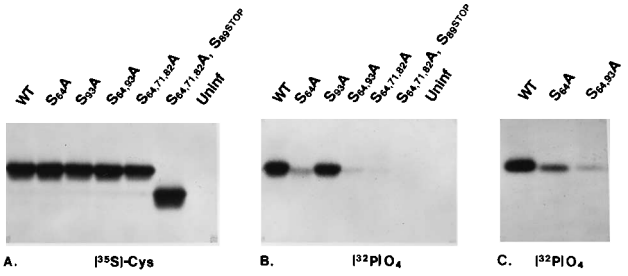



FIG. 5. Localization of the $M_2$ phosphorylation site. SV40-$M_2$ recombinant-infected CV-1 cells at 45 to 48 h p.i. were labeled with [$^{35}$S]cysteine or $^{32}P_i$ as described in Materials and Methods. Where indicated, cell surfaces were treated with (+) or without (−) trypsin to trim the N-terminal domain of $M_2$, as described previously (26). Microsomes were prepared and treated with (+) or without (−) trypsin as described previously (46). Polypeptides were immunoprecipitated with the $M_2$ N-terminal domain-specific MAb 14C2 or with an $M_2$ C-terminal domain-specific MAb 1D6-tail. The position of the $M_2$ protease-protected microsomal fragment, consisting of the N-terminal and transmembrane domains of $M_2$, is indicated by the arrowheads. The stars indicate the cell surface protease-trimmed $M_2$ that lacks the N-terminal domain. The $M_2$ 1D6-tail specific mouse hybridoma was made as described previously (10). The specificity of 1D6-tail for the $M_2$ cytoplasmic tail is demonstrated in this figure. 1D6-tail is reactive with cell surface protease-trimmed $M_2$ (in contrast to the lack of reactivity of 14C2), but it is not reactive with the proteolytic fragment of $M_2$ remaining after protease treatment of microsomes. The remaining ~7-kDa fragment of $M_2$ consists of the $M_2$ N-terminal ectodomain and the transmembrane domain. In contrast to the reactivity of 1D6-tail, MAb 14C2 does recognize this fragment.

serine-altered $M_2$ proteins are shown in Fig. 6. Our data indicate that serine 71 is not modified by addition of phosphate, because the level of phosphate labeling of mutant S64A,S71A is the same as for the single mutant S64A at a comparable level




D.

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Met-Ser-Leu-Leu-Thr-Glu-Val-Glu-Thr-Pro-Ile-Arg-Asn-Glu-Trp-Gly-  (16)
Cys-Arg-Cys-Asn-Asp-Ser-Ser-Asp-Pro-Leu-Val-Val-Ala-Ala-Ser-Ile-  (32)
Ile-Gly-Ile-Leu-His-Leu-Ile-Leu-Trp-Ile-Leu-Asp-Arg-Leu-Phe-Phe-  (48)
Lys-Cys-Ile-Tyr-Arg-Phe-Phe-Glu-His-Gly-Leu-Lys-Arg-Gly-Pro-Ser-  (64)
Thr-Glu-Gly-Val-Pro-Glu-Ser-Met-Arg-Glu-Glu-Tyr-Arg-Lys-Glu-Gln-  (80)
Gln-Ser-Ala-Val-Asp-Ala-Asp-Asp-Ser-His-Phe-Val-Ser-Ile-Glu-Leu-  (96)
Glu.  (97)
```

FIG. 6. Phosphorylation of serine-altered $M_2$ proteins. The serine-altered $M_2$ mutants, S64A,S71A,S82A, and $S89_{STOP}$ (serine residue 89 changed to a translation stop codon, TGA) were constructed as described in Materials and Methods. (A and B) The $M_2$ proteins were expressed in HeLa T4 cells by the vac-T7 expression system and cells labeled with [$^{35}$S]cysteine (A) or $^{32}P_i$ (B). (C) Synthetic mRNAs were transcribed from either pGEM3 or pGEM3Zf(+) and microinjected (50 nl of 1-μg/μl RNA) into oocytes of *X. laevis*. At 24 h postinjection, oocytes were labeled with $^{32}P_i$ for 24 h and homogenized, and detergent lysates were prepared in the presence of 50 mM iodoacetamide. Proteins were immunoprecipitated with the $M_2$-specific 14C2 MAb and analyzed by SDS-PAGE under reducing conditions. Only the relevant portion of the autoradiograph is shown. (D) Amino acid sequence of the $M_2$ protein, showing the five serine residues in the cytoplasmic tail. The $M_2$ transmembrane domain is boxed.

of [$^{35}$S]methionine-labeled $M_2$ protein (data not shown). However, serine residues 82, 89, and 93 are all phosphorylated to minor extents (Fig. 6). Because it is possible that phosphorylation of the residues that are modified to minor extents become moving targets in the serine-altered mutants, the minor phosphorylated residues were not investigated further. Before ion channel activities of the serine-altered $M_2$ proteins were measured, the phosphorylation pattern of the proteins in *X. laevis* oocytes was investigated. As anticipated, a very similar pattern of phosphorylation (examples shown in Fig. 6C) to that observed in mammalian cells was found, with $M_2$ residue serine 64 being the predominant phosphorylated residue.

**Ion channel activity of the $M_2$ serine-altered mutants.** It was of interest to determine a possible effect of phosphorylation of the $M_2$ protein on its ion channel activity, as phosphorylation has been shown to be both a positive and negative regulator of the function of other ion channels (reviewed in reference 9). In addition, the predominant phosphoamino acid, serine 64, is conserved in the $M_2$ protein of all influenza virus subtypes. The ion channel activities of the serine-altered $M_2$ proteins S64A, S64A,S71A,S82A, and S64A,S93A were examined by expressing the proteins in oocytes of *X. laevis* and measuring total membrane currents by a two-electrode voltage clamp procedure as described above. The current, the protein, and their

TABLE 1. Protein kinase phosphorylation site motifs in the influenza virus $M_2$ protein

| $M_2$ residue | $M_2$ Ser within recognition motif[a] | Protein kinase | Reference |
|---|---|---|---|
| Ser-64 | X**R**XX**S***X | Calmodulin-dependent protein kinase II | 34 |
| | | S6 kinase II | 4 |
| Ser-71 | X**S***XX**E**X | Casein kinase II | 16 |
| Ser-82 | X**E**XX**S***X | Casein kinase I | 16 |
| Ser-89 | X**S***XXX**S(P)**X | Glycogen synthase kinase-3 | 5 |
| Ser-93 | X**S***X**E**X | Mammary gland casein kinase | 16 |

[a] *, phosphorylated residue.

ratio varied from batch to batch of oocytes. Within a single batch, the ratio was found to be spread only over a 1.7-fold range for the wt $M_2$ protein and for each of the three serine-altered mutants (0.41 ± 0.3 to 0.69 ± 0.4 μA/ng [mean ± SEM, $n = 6$ for each]), which suggests that the $M_2$ protein ion channel activity is not affected by phosphorylation.

**Summary.** The influenza virus $M_2$ ion channel is one of the smallest regulatable ion channels known to date, and it may be amenable to structural studies. To understand the mechanism of action of the $M_2$ ion channel, it is necessary to examine the effects of known posttranslational modifications on its activity. The influenza virus $M_2$ protein is posttranslationally modified by the formation of disulfide bonds with cysteine residues 17 and 19. The cytoplasmic tail is posttranslationally modified by palmitoylation, and all the available data indicate that cysteine residue 50 is the site for the thio-ether linkage of the fatty acid. The $M_2$ protein is also posttranslationally modified by the addition of phosphate to the cytoplasmic tail on serine residues. The predominant (~85%) phosphoserine residue is serine 64, but serine residues 82, 89, and 93 are also phosphorylated to minor extents. The posttranslational modifications occur on $M_2$ protein expressed in mammalian cells infected with influenza virus, when $M_2$ protein is expressed in mammalian cells by using expression vectors, and on $M_2$ protein expressed in oocytes of *X. laevis*. However, none of the posttranslational modifications dramatically affect the ion channel activity of the $M_2$ protein expressed in oocytes of *X. laevis*. Therefore, if there is a role for the posttranslational modifications of the $M_2$ protein described here in the life cycle of the virus, it would most probably be in the formation of infectious influenza virus virions, and this remains to be determined.

## ACKNOWLEDGMENTS

This research was supported by Public Health Service research grants AI-20201 (R.A.L.) and AI-31882 (L.H.P.) from the National Institute of Allergy and Infectious Diseases. R.A.L. is an Investigator of the Howard Hughes Medical Institute (HHMI). A.M. performed some of the electrophysiological work as a C98 independent studies project at Northwestern University and continued as an HHMI-supported undergraduate summer student.

## REFERENCES

1. **Boyle, W. J., P. van der Geer, and T. Hunter.** 1991. Phosphopeptide mapping and phosphoamino acid analysis by two-dimensional separation on thin-layer cellulose plates. Methods Enzymol. **201:**110–148.
2. **Deng, W. P., and J. A. Nickoloff.** 1992. Site-directed mutagenesis of virtually any plasmid by eliminating a unique site. Anal. Biochem. **200:**81–88.
3. **Duff, K. C., and R. H. Ashley.** 1992. The transmembrane domain of influenza A $M_2$ protein forms amantadine-sensitive proton channels in planar lipid bilayers. Virology **190:**485–489.
4. **Erikson, E., and J. L. Maller.** 1988. Substrate specificity of ribosomal S6 kinase II from *Xenopus* eggs. Second Messengers Phosphoproteins. **12:**135–143.
5. **Fiol, C. J., A. M. Mahrenholz, Y. Wang, R. W. Roeske, and R. J. Roach.** 1987. Formation of protein kinase recognition sites by covalent modification of the substrate. J. Biol. Chem. **262:**14042–14048.
6. **Fuerst, T. R., E. G. Niles, F. W. Studier, and B. Moss.** 1986. Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage $T_7$ RNA polymerase. Proc. Natl. Acad. Sci. USA **83:**8122–8126.
7. **Hay, A. J.** 1992. The action of adamantanamines against influenza A viruses: inhibition of the $M_2$ ion channel protein. Semin. Virol. **3:**21–30.
8. **Hay, A. J., A. J. Wolstenholme, J. J. Skehel, and M. H. Smith.** 1985. The molecular basis of the specific anti-influenza action of amantadine. EMBO J. **4:**3021–3024.
9. **Hille, B.** 1992. Ionic channels of excitable membranes. Sinauer Associates Inc., Sunderland, Mass.
10. **Holsinger, L. J.** 1993. A structural and functional analysis of the influenza A virus $M_2$ protein: an ion channel essential for virus entry. Ph.D. dissertation. Northwestern University, Evanston, Ill.
11. **Holsinger, L. J., and R. A. Lamb.** 1991. Influenza virus $M_2$ integral membrane protein is a homotetramer stabilized by formation of disulfide bonds. Virology **183:**32–43.
12. **Holsinger, L. J., D. Nichani, L. H. Pinto, and R. A. Lamb.** 1994. Influenza A virus $M_2$ ion channel protein: a structure-function analysis. J. Virol. **68:**1551–1563.
13. **Hughey, P. G., R. W. Compans, S. L. Zebedee, and R. A. Lamb.** 1992. Expression of the influenza A virus $M_2$ protein is restricted to apical surfaces of polarized epithelial cells. J. Virol. **66:**5542–5552.
14. **Hull, J. D., R. Gilmore, and R. A. Lamb.** 1988. Integration of a small integral membrane protein, $M_2$, of influenza virus into the endoplasmic reticulum: analysis of the internal signal-anchor domain of a protein with an ectoplasmic $NH_2$ terminus. J. Cell Biol. **106:**1489–1498.
15. **Jackson, D. C., X. L. Tang, K. G. Murti, R. G. Webster, G. W. Tregear, and W. J. Bean.** 1991. Electron microscopic evidence for the association of $M_2$ protein with the influenza virion. Arch. Virol. **118:**199–207.
16. **Kemp, B. E.** 1990. Peptides and protein phosphorylation. Uniscience CRC Press, Inc., Boca Raton, Fla.
17. **Lamb, R. A.** 1975. The phosphorylation of Sendai virus proteins by a virus particle-associated protein kinase. J. Gen. Virol. **26:**249–263.
18. **Lamb, R. A., and P. W. Choppin.** 1976. Synthesis of influenza virus proteins in infected cells: translation of viral polypeptides, including three P polypeptides, from RNA produced by primary transcription. Virology **74:**504–519.
19. **Lamb, R. A., and P. W. Choppin.** 1977. The synthesis of Sendai virus polypeptides in infected cells. III. Phosphorylation of polypeptides. Virology **81:**382–397.
20. **Lamb, R. A., and P. W. Choppin.** 1981. Identification of a second protein ($M_2$) encoded by RNA segment 7 of influenza virus. Virology **112:**729–737.
21. **Lamb, R. A., P. R. Etkind, and P. W. Choppin.** 1978. Evidence for a ninth influenza viral polypeptide. Virology **91:**60–78.
22. **Lamb, R. A., L. J. Holsinger, and L. H. Pinto.** 1994. The influenza A virus $M_2$ ion channel protein and its role in the influenza virus life cycle. *In* E. Wimmer (ed.), Receptor-mediated virus entry into cells, in press. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
23. **Lamb, R. A., and C.-J. Lai.** 1982. Spliced and unspliced messenger RNAs synthesized from cloned influenza virus M DNA in an SV40 vector: expression of the influenza virus membrane protein ($M_1$). Virology **123:**237–256.
24. **Lamb, R. A., C.-J. Lai, and P. W. Choppin.** 1981. Sequences of mRNAs derived from genome RNA segment 7 of influenza virus: colinear and interrupted mRNAs code for overlapping proteins. Proc. Natl. Acad. Sci. USA **78:**4170–4174.
25. **Lamb, R. A., B. W. J. Mahy, and P. W. Choppin.** 1976. The synthesis of Sendai virus polypeptides in infected cells. Virology **69:**116–131.
26. **Lamb, R. A., S. L. Zebedee, and C. D. Richardson.** 1985. Influenza virus $M_2$ protein is an integral membrane protein expressed on the infected-cell surface. Cell **40:**627–633.
27. **Laskey, R. A., and A. D. Mills.** 1975. Quantitative film detection of $^3H$ and $^{14}C$ in polyacrylamide gels by fluorography. Eur. J. Biochem. **56:**335–341.
28. **Panayotov, P. P., and R. W. Schlesinger.** 1992. Oligomeric organization and strain-specific proteolytic modification of the virion $M_2$ protein of influenza A H1N1 viruses. Virology **186:**352–355.
29. **Parks, G. D., J. D. Hull, and R. A. Lamb.** 1989. Transposition of domains between the $M_2$ and HN viral membrane proteins results in polypeptides which can adopt more than one membrane orientation. J. Cell Biol. **109:**2023–2032.
30. **Parks, G. D., and R. A. Lamb.** 1991. Topology of eukaryotic type II membrane proteins: importance of N-terminal positively charged residues flanking the hydrophobic domain. Cell **64:**777–787.
31. **Paterson, R. G., and R. A. Lamb.** 1987. Ability of the hydrophobic fusion related external domain of a paramyxovirus F protein to act as a membrane anchor. Cell **48:**441–452.
32. **Paterson, R. G., and R. A. Lamb.** 1990. RNA editing by G-nucleotide insertion in mumps virus P-gene mRNA transcripts. J. Virol. **64:**4137–4145.
33. **Paterson, R. G., and R. A. Lamb.** 1993. The molecular biology of influenza viruses and paramyxoviruses, p. 35–73. *In* A. Davidson and R. M. Elliott (ed.), Molecular virology: a practical approach. IRL Oxford University Press, Oxford.
34. **Pearson, R. B., J. R. Woodgett, P. Cohen, and B. E. Kemp.** 1985. Substrate specificity of a multifunctional calmodulin-dependent protein kinase. J. Biol. Chem. **260:**14471–14476.
35. **Pinto, L. H., L. J. Holsinger, and R. A. Lamb.** 1992. Influenza virus $M_2$ protein has ion channel activity. Cell **69:**517–528.
36. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
37. **Schmidt, M. F. G.** 1989. Fatty acylation of proteins. Biochim. Biophys. Acta **988:**411–426.
38. **Sugrue, R. J., G. Bahadur, M. C. Zambon, S. M. Hall, A. R. Douglas, and A. J. Hay.** 1990. Specific structural alteration of the influenza haemagglutinin by amantadine. EMBO J. **9:**3469–3476.
39. **Sugrue, R. J., R. B. Belshe, and A. J. Hay.** 1990. Palmitoylation of the influenza A virus $M_2$ protein. Virology **179:**51–56.
40. **Sugrue, R. J., and A. J. Hay.** 1991. Structural characteristics of the $M_2$ protein of the influenza A viruses: evidence that it forms a tetrameric channel. Virology **180:**617–624.
41. **Tosteson, M. T., L. H. Pinto, L. J. Holsinger, and R. A. Lamb.** 1994. Recon-

stitution of the influenza virus $M_2$ ion channel in lipid bilayers. J. Membr. Biol. **142:**117–126.
42. **Veit, M., H. D. Klenk, A. Kendal, and R. Rott.** 1991. The $M_2$ protein of influenza A virus is acylated. Virology **184:**227–234.
43. **Wang, C., R. A. Lamb, and L. H. Pinto.** 1994. Measurement of the influenza virus $M_2$ ion channel activity in mammalian cells. Virology **205:**133–140.
44. **Zebedee, S. L.** 1988. Structure and function analysis of the influenza A virus $M_2$ protein. Ph.D. dissertation. Northwestern University, Evanston, Ill.
45. **Zebedee, S. L., and R. A. Lamb.** 1988. Influenza A virus $M_2$ protein: monoclonal antibody restriction of virus growth and detection of $M_2$ in virions. J. Virol. **62:**2762–2772.
46. **Zebedee, S. L., C. D. Richardson, and R. A. Lamb.** 1985. Characterization of the influenza virus $M_2$ integral membrane protein and expression at the infected-cell surface from cloned cDNA. J. Virol. **56:**502–511.