Expression of the Influenza A Virus M2 Protein Is Restricted to Apical Surfaces of Polarized Epithelial Cells

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The M2 protein of influenza A virus is a small, nonglycosylated transmembrane protein that is expressed on surfaces of virus-infected cells. A monoclonal antibody specific for the M2 protein was used to investigate its expression in polarized epithelial cells infected with influenza virus or a recombinant vaccinia virus that expresses M2. The expression of M2 on the surfaces of influenza virus-infected cells was found to be restricted to the apical surface, closely paralleling that of the influenza virus hemagglutinin (HA). Membrane domain-specific immunoprecipitation indicated that the M2 protein was inserted directly into the apical membrane with transport kinetics similar to those of HA. In polarized cells infected with a recombinant vaccinia virus that expresses M2, we found that 88 to 90% of surface M2 was restricted to the apical domain compared with 88 to 90% of HA in a similar assay. These results indicate that the M2 protein undergoes directional transport in the absence of other influenza virus proteins and that M2 contains the structural features required for apical transport in polarized epithelial cells. The ultrastructural localization of the M2 protein in influenza virus-infected MDCK cells was investigated by immunoelectron microscopy using M2 antibody and a gold conjugate. In cells in which extensive virus budding was occurring, the apical cell membrane was labeled with gold particles evenly distributed between microvilli and the surrounding membrane. In addition, a significant fraction of the M2 label was apparently associated with virions. A monoclonal antibody specific for HA demonstrated a similar labeling pattern. These results indicate that M2 is localized in close proximity to budding and assembled virions.

The M2 protein of influenza A virus (for a review, see reference 22) consists of 97 amino acids (24) and is one of the smallest known transmembrane proteins. M2 belongs to the type I signal-anchor protein class and has been used previously to investigate the structural features responsible for this uncommon topology (37, 38). The external domain of M2, which consists of approximately 24 N-terminal amino acids (24), appears to determine the type I signal-anchor topology of M2 and of certain chimeric type II proteins that contain these sequences (37, 38). M2 is cotranslationally inserted into the rough endoplasmic reticulum membrane via a 19-residue hydrophobic signal-anchor domain (17), and 54 C-terminal amino acids form the cytoplasmic domain (24). Recent evidence indicates that the M2 protein forms disulfide-linked dimers, tetramers, and possibly higher-order oligomers and is modified by the addition of palmitate to a cytoplasmic cysteine residue in most influenza A virus strains (15, 48, 54), but a potential N-linked glycosylation site in the external domain is unused (24, 59). M2 is transported through the Golgi complex to the surfaces of cells infected with influenza virus or recombinant expression vectors (24, 59), similar to the pathways traversed by the viral glycoproteins (6). Large numbers of M2 molecules are present on infected-cell surfaces, with a ratio of approximately two M2 molecules per hemagglutinin (HA) trimmer in CV-1 cells (24), but only a few (15 to 60) M2 molecules are estimated to be incorporated into each virion (57). The contrast between the numbers of molecules expressed on the cell surface and in virions suggests that M2 may be partially excluded from budding virus particles.

Polarized epithelial cells are characterized by the presence of apical and basolateral plasma membrane domains that have distinctive protein and lipid compositions (6). Polarity is developed by the directional transport of cellular proteins and lipids (1, 6, 25, 26, 33, 53) and maintained by tight junctions that physically separate apical and basolateral domains (52). Polarized epithelial cells from different tissues, such as hepatocytes (1), kidney cells (26, 28, 31, 40), and intestinal cells (25, 33), utilize distinct pathways of protein sorting and may also differ in the relative amount of signal-mediated and bulk flow (default) transport to the cell surface. Although cellular sorting mechanisms are not yet understood, directional transport apparently involves the recognition of intrinsic structural features of targeted molecules by cellular sorting receptors (6). Newly synthesized proteins are sorted during or immediately after exit from the trans-Golgi network (11), when apical and basolateral proteins are incorporated into distinct populations of transport vesicles that contain sorting and/or targeting receptors (55). Many exogenous proteins, such as viral glycoproteins, are also restricted to specific plasma membrane domains when introduced into polarized cells. The influenza virus glycoproteins, hemagglutinin (HA) and neuraminidase, are restricted to the apical domain of polarized cells infected with influenza virus or virus expression vectors (20, 31, 46). In contrast, the glycoproteins of vesicular stomatitis virus (40, 46) and retroviruses (44, 46) are transported to basolateral membranes, where assembly of these viruses occurs (43, 44). Directional transport of these glycoproteins in the absence of other viral

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proteins indicates that the proteins contain intrinsic structural features recognized by the cellular sorting machinery (20, 46). It is not known whether positive signals are required for both apical and basolateral transport. Proteins that lack sorting signals presumably would be transported to the cell surface by a default pathway, which has not been conclusively identified in kidney cells. The identification of specific sorting signals should be greatly simplified by finding small proteins that are transported directionally to either apical or basolateral membranes.

The small size and the unusual topology of M2 make it an attractive probe to analyze plasma membrane biogenesis, and the transport and ultrastructural distribution of the M2 protein have not been determined in polarized epithelial cells. Therefore, we wished to determine whether M2 was transported to the surfaces of polarized epithelial cells via a directional pathway. Previous studies have focused on the polarized transport of classical type I or II transmembrane glycoproteins, and the expression of type I signal-anchor proteins in polarized cells has not been reported. In addition, an analysis of M2 expression and localization in polarized cells, which restrict influenza virus glycoprotein expression and virus budding to the apical domain (20, 31, 43, 46), would be of interest in order to determine whether the site of M2 expression is correlated with the site of virus budding. In this study, we used a monoclonal antibody specific for the external domain of M2 (57) in immunofluorescence, immunoelectron microscopy, surface radioimmunoassay, and plasma membrane domain-specific immunoprecipitation assays to investigate the transport and distribution of M2 in polarized epithelial cells.

MATERIALS AND METHODS

Cells and viruses. All cells were obtained from the American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 10% newborn calf serum or 9% newborn calf serum plus 1% fetal calf serum. TK− 143 cells were grown in DMEM containing 25 μg of 5-bromodeoxyuridine per ml. Stocks of influenza virus A/WSN/33 (H1N1) were prepared in MDBK cells and titered by plaque assay in MDCK cells. Vaccinia virus recombinant stocks were grown in CV-1 cells and titered by plaque assay in CV-1 and Vero C1008 cells. Recombinant vaccinia virus VV-HA, which contains the influenza virus A/WSN HA gene, was provided by Y. Matsuoka (32). Recombinant vaccinia virus VVpSC11, which expresses the β-galactosidase gene, was provided by R. Owens. Vaccinia virus was concentrated by centrifugation through a sucrose cushion (35% sucrose in 10 mM Tris [pH 9.1]) for 80 min at 13,500 rpm, resuspended in 1 mM Tris buffer (pH 9.1), and stored at −70°C.

Polarity assays. The polarity of cells grown on 0.45-μm-pore-size nitrocellulose filters (Milli-HA filters; Millipore Corp., Bedford, Mass.) was determined by measuring the transepithelial resistance and the ability to exclude [3H]inulin across monolayers (7) and by examining the polarity of influenza virus budding by electron microscopy. In addition, the distribution of a monoclonal antibody designated D6, which reacts specifically with basolateral membranes of MDCK cells, was assessed by immunofluorescence and 125I-labeled antibody binding.

Antibodies and immunological reagents. Monoclonal antibodies specific for the extracellular domain of M2 (57) or parainfluenza type 3 virus HA-neuraminidase (42) were described previously. A hybridoma line secreting a monoclonal antibody specific for influenza virus A/WSN HA, designated 21J, was derived from BALB/c mice immunized with purified influenza virus. The antibody specificity was determined by immunofluorescence and immunoprecipitation assays using influenza and VV-HA virus-infected MDCK cells. The hybridoma line secreting the monoclonal antibody D6, which reacts with a MDCK cell basolateral antigen, was derived from BALB/c mice immunized with a smooth membrane fraction that was obtained from homogenized influenza virus-infected MDCK cells and separated by centrifugation on a 25 to 60% sucrose step gradient. Hybridoma culture supernatants were screened using indirect immunofluorescence on uninfected MDCK cells. The basolateral polarity of the D6 antigen was confirmed by using 125I-labeled antibody binding to filter-grown MDCK cells. Affinity-purified fluorescein- or rhodamine-conjugated secondary antibodies were obtained from Southern Biotechnology Associates, Inc. (Birmingham, Ala.). Protein A- and protein G-Sepharose were obtained from Pierce (Rockford, Ill.). Goat anti-mouse immunoglobulin (lg) conjugated to colloidal gold was obtained from Probe Tech, Inc. (Pershia, Pa.).

Construction of recombinant vaccinia virus VV-M2. A TK− recombinant vaccinia virus that expresses M2 under the control of an early vaccinia virus promoter was constructed and designated VV-M2. Briefly, the influenza A/Urdom/72 virus M2 cDNA (23) was inserted into the pSC11 vaccinia virus insertion plasmid (9). TK− 143 cells were maintained in DMEM minus 5-bromodeoxyuridine for 18 h and then infected with wild-type vaccinia virus (IHD-J strain; multiplicity of infection [MOI] = 1) for 2 h at 37°C. Ten micrograms of pSC11-M2 plasmid DNA and 25 μg of lipofectin reagent (BRL Life Technologies, Inc., Gaithersburg, Md.) were combined, incubated for 15 min at 20°C, and then added to the infected cells. After 20 h at 37°C, the cells exhibited extensive cytopathology, and virus was harvested by three freeze-thaw cycles followed by brief sonication. Recombinant virus was passaged twice in TK− 143 cells incubated with 25 μg of 5-bromodeoxyuridine per ml and isolated by three rounds of plaque purification in TK− 143 cells incubated with 300 μg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) per ml and screened by indirect immunofluorescence and immunoprecipitation assays using M2 antibody.

Immunofluorescence assays. MDCK cells were grown to confluence on 12-mm glass coverslips and infected with influenza virus (MOI = 10) for 1 h at 37°C, and then the inoculum was replaced with DMEM plus 2% newborn calf serum. At intervals postinfection (p.i.), the infected cells were reacted with either M2- or HA-specific antibody (ascitic fluids diluted 1:1,000 in phosphate-buffered saline [PBS] 0.5% bovine serum albumin [BSA]). Primary antibody was visualized using fluorescein-conjugated secondary antibody diluted to 10 μg/ml in PBS 1% BSA. All antibody reactions were performed at 4°C for 30 min. Cells were mounted and photographed with a Nikon Optiphot microscope.

125I-labeled antibody binding assays of influenza virus-infected MDCK cells. MDCK cells were seeded on 0.45-μm-pore-size nitrocellulose filters at a density of 5 × 104 cells per 12-mm filter and infected with influenza virus (MOI = 10) 2 days after reaching confluence. At intervals from 2 to 10 h p.i., the cells were fixed with 1% paraformaldehyde in PBS containing 0.1 mM Ca2+ and 1.0 mM Mg2+ (PBS-CM) and then quenched with 50 mM NH4Cl in PBS. Nonspecific
background was reduced by adding 3% BSA to wash solutions. Apical or basolateral cell surfaces were reacted with M2 or HA antibodies (diluted 1:1,500 in PBS–3% BSA) for 30 min at 4°C and then washed five times (10 min each) with PBS–3% BSA. Apical or basolateral membranes were reacted with 125I-labeled rabbit anti-mouse IgG (2.5 μg of IgG per ml; 150,000 cpm per filter) for 30 min at 4°C, while the opposite surface of the filter was incubated with 2.5 μg of unlabeled rabbit anti-mouse IgG per ml. The filters were washed five times, allowed to dry, excised from the filter holder, immersed in scintillation fluid, and counted in a Beckman liquid scintillation counter. Nonspecific background was determined by reacting the infected cells with 125I-labeled antibody in the absence of primary antibody and was subtracted from the experimental samples.

Membrane domain-specific cell surface immunoprecipitation assays. MDCK cells were seeded on 0.45-μm-pore-size filters at a density of 0.5 × 10⁶ to 1.0 × 10⁷ cells per 30-mm filter and infected with influenza virus (MOI = 10) 2 to 3 days after reaching confluence. Cells were incubated in DMEM minus cysteine from 3 to 6 h p.i., pulse-labeled for 15 min with [3S] cysteine (200 μCi) applied to the basolateral surface complex of DMEM for 0 to 120 min, and then washed twice with PBS-CM and once with PBS-CM–3% BSA at 4°C. Apical or basolateral surfaces were reacted with M2 or HA antibody (diluted 1:1,000 in PBS–CM–3% BSA) for 60 min at 4°C and then washed extensively with PBS-CM. To block unreacted antibody binding sites, the cells were solubilized with lysis buffer (150 mM NaCl, 10 mM Tris, 5 mM EDTA, 1% Triton X-100, 1% deoxycholate, 0.1% sodium dodecyl sulfate [SDS]; pH 7.8) containing an equivalent number of unlabeled influenza virus-infected cells, and then the lysates were centrifuged to remove insoluble material. The immune complexes were reacted with protein A-Sepharose (precoated with rabbit anti-mouse IgG and preadsorbed with unlabeled virus-infected cells lysates) for 60 min at 4°C, washed four times with lysis buffer, solubilized in reducing buffer, and analyzed on SDS–12.5% polyacrylamide gels. Cell surface immunoprecipitation was also used to detect M2 and HA on the surfaces of polarized Vero C1008 cells infected with recombinant vaccinia viruses. Vero C1008 cells in plastic dishes were infected with purified VV-M2 or VV-HA virus (MOI = 10) and radiolabeled continuously from 5 to 8 h p.i. with [35S] cysteine or [35S] mercaptoine-cysteine, respectively. At 8 h p.i., the cells were washed with PBS, reacted with M2 or HA antibodies for 30 min at 4°C, and solubilized in lysis buffer containing unlabeled recombinant virus-infected cell lysates. Nonspecific background was determined by reacting the surfaces of VVpSC11 virus-infected cells with M2 or HA antibody. The immune complexes were recovered using protein G-Sepharose (preincubated with unlabeled recombinant virus-infected cell lysates) and analyzed on SDS–12.5% polyacrylamide gels.

125I-labeled antibody binding assays of VV-M2 virus-infected Vero C1008 cells. A variation of the antibody binding assay discussed above was used to quantitate M2 on apical and basolateral surfaces of Vero C1008 cells infected with VV-M2. Vero C1008 cells were infected 5 to 7 days after seeding at a density of ~5 × 10⁵ cells per 12-mm filter. Recombinant vaccinia virus (MOI = 5) was allowed to adsorb for 2 h, and then the inoculum was replaced with DMEM–2% calf serum. At intervals from 4 to 12 h p.i., the samples were removed, washed twice with PBS, and fixed with 1% paraformaldehyde in PBS. The antibody labeling was performed as described above. For comparison, Vero C1008 cells were infected with VV-HA and assayed with HA antibody. Nonspecific background was determined by reacting recombinant virus VVpSC11-infected cells with primary and secondary antibodies and was subtracted from the experimental samples.

Immunoelectron microscopy. MDCK cells were grown in 24-well plastic plates and infected with influenza virus (MOI = 15) for 1 h at 37°C. The cells were maintained in DMEM–2% calf serum until 7 h p.i., rinsed three times with PBS at 4°C, and reacted on apical surfaces with M2 or HA antibody (ascitic fluids diluted 1:1,000 in PBS–0.1% BSA) for 1 h at 4°C. After extensive washings, the cells were reacted with a secondary antibody conjugated to 10-nm-diameter colloidal gold (diluted 1:50 in PBS–0.1% BSA) for 1 h at 4°C. The cells were fixed with 1% glutaraldehyde in PBS and postfixed with 1% osmium tetroxide in PBS for 1 h each at 4°C. Specimens were embedded in epoxy resin for electron microscopy and examined with a Philips EM301 microscope.

Quantitation of immunolabeling by electron microscopy. The distribution of colloidal gold particles on apical cell membranes and virions was quantitated by determining the apparent association of gold particles with cellular and virion membranes in photomicrographs from four separate experiments. Infected influenza virus-infected cells reacted with monoclonal antibody specific for the HA-neuraminidase protein of parainfluenza type 3 virus (42) and the gold probe and uninfected MDCK cells reacted with M2 or HA antibody and the gold probe were examined as negative controls to ensure the specificity of the immunolabeling reagents. No significant binding of influenza virus-specific primary antibody to uninfected cells or of heterologous lgs to infected cells were observed. To compare the specific label density on apical cell membranes and virions, a Bioquant II quantitative morphometry program was used to measure the lengths of cellular and viral membranes in individual electron micrographs of infected MDCK cells. The apparent distribution of gold particles on apical and virion membranes was then determined, and the label density (in gold particles per micrometer) on cellular and viral membranes was calculated.

RESULTS

Expression of M2 in influenza virus-infected MDCK cells. The synthesis of M2 and HA proteins was compared in influenza virus-infected MDCK cells that were pulse-labeled for 30 min at hourly intervals from 3 to 7 h p.i. (Fig. 1). Maximum HA synthesis occurred early in the infection, around 4 to 5 h p.i., whereas M2 protein synthesis occurred at highest levels at approximately 6 h p.i. This result is in agreement with recent work indicating that the ratio of M2 to M1 mRNAs in MDCK cells increases during the course of influenza virus infection, with an abrupt increase in the splicing efficiency to form M2 mRNA predicted to occur around 5 h p.i. (51). When the cells were radiolabeled continuously from 8 to 10 h p.i., we observed two bands corresponding to HA molecules that differ in oligosaccharide composition (12). However, no change in the migration of M2 was observed during the 2-h labeling interval, consistent with previous reports (54).

The time course of appearance and the distribution of the M2 protein on the surfaces of influenza virus-infected MDCK cells were investigated by indirect immunofluorescence and compared with cell surface HA. Polarized MDCK cells were infected with influenza virus (MOI = 10), reacted with monoclonal antibody specific for the extracellular domain of M2 or HA at intervals from 3 to 8 h p.i., and stained...
with a fluorescein-conjugated secondary antibody. Cell surface expression of M2 was detected by 4.5 h p.i. (Fig. 2), while surface HA was detected as early as 3 h p.i. (data not shown). The apical membranes of virus-infected MDCK cells exhibited a punctate fluorescence pattern after labeling with M2 and HA antibodies. However, the M2 fluorescence pattern appeared to be slightly more diffuse than that observed for HA and extended to the cell periphery, giving cells a polygonal appearance, whereas lower levels of HA fluorescence were observed near regions of cell-cell contact. M2 and HA were also colocalized intracellularly (not shown), although HA was detected earlier than M2, consistent with the time course of protein synthesis.

It was reported previously that influenza virus budding as well as expression of the viral glycoproteins, HA and neuraminidase, are restricted to apical membranes of polarized epithelial cells (20, 31, 43, 46). Therefore, we wished to examine the kinetics of M2 accumulation on apical and basolateral membranes of influenza virus-infected MDCK cells that were grown on permeable nitrocellulose filters to allow antibody access to either membrane surface. For comparison, we quantitated the binding of the D6 monoclonal antibody, which recognizes an antigen present on basolateral membranes of MDCK cells, to apical and basolateral surfaces and found that 94% of the total D6 binding was restricted to the basolateral domain of MDCK cells. Surface expression of M2 and HA on apical and basolateral surfaces of influenza virus-infected MDCK cells was assayed at intervals from 2 to 10 h p.i. using M2 and HA primary antibodies and 125I-labeled secondary antibody. We found that the expression of HA on apical surfaces preceded that of M2 by 2 h (Fig. 3). M2 and HA cell surface expression increased at approximately similar rates from 6 to 10 h p.i., while the ratio of M2 to HA antibodies bound to apical membranes increased from 1:6 at 6 h p.i. to 1:2 at 10 h p.i. M2 was detected on the apical surfaces of MDCK cells grown on glass coverslips somewhat earlier, around 4 to 5 h p.i., but the rate of surface accumulation was not found to be appreciably different from that observed with the filter-grown cells (data not shown). Both M2 and HA were predominantly expressed on apical membranes of influenza virus-infected MDCK cells throughout the infection time course, and approximately 84 to 86% of the total M2 antibody and 90 to 93% of the HA antibody were bound to apical surfaces at 8 to 10 h p.i. Thus, the M2 and HA proteins exhibit similar patterns of polarized expression on apical membranes.

Different transport pathways have been reported for targeting of proteins to apical plasma membranes (6). Apical proteins in hepatocytes are transported exclusively via an indirect pathway, appearing first on basolateral membranes before being endocyotted and redirected to the apical surface (1). In contrast, intestinal cells appear to use both direct and indirect pathways to the apical surface, depending upon the protein in question (25, 33). The majority of apical membrane proteins in kidney cells appear to be transported exclusively by a direct pathway that does not involve the basolateral surface (26-28, 31), but certain membrane proteins, including the polymeric Ig receptor, are transported to apical surfaces by an indirect pathway when expressed in MDCK cells (3, 35). Therefore, to examine the kinetics of transport and the initial site of M2 plasma membrane insertion in influenza virus-infected MDCK cells, we used pulse-chase metabolic labeling in conjunction with plasma membrane domain-specific immunoprecipitation and SDS-polyacrylamide gel electrophoresis. For comparison, the transport of the HA protein to apical or basolateral surfaces of infected cells was determined. M2 was first detected on the apical surfaces of influenza virus-infected MDCK cells after a 15-min pulse and 60-min chase (Fig. 4). The level of apical M2 increased after 2 h, while levels of M2 protein on basolateral membranes were very low at both time points, consistent with the low levels of basolateral M2 detected by 125I-labeled antibody binding. A fraction of the HA protein was cleaved to HA1 and HA2 during transport, and significant levels of both cleaved and uncleaved HA were detected on apical surfaces after 1 h chase. Similar results were obtained using domain-specific biotinylation and streptavidin precipitation (data not shown). These results strongly suggest that M2 is targeted directly to the apical domain without appearing transiently on the basolateral surface, similar to the direct apical transport of HA. The M2 protein appears on the plasma membrane at a slightly slower rate than HA, which could be due to a difference in the oligomerization rate of M2 (15, 48) and HA (12) or in the rate of their transport to the plasma membrane.

Polarized expression of M2 from a recombinant vaccinia virus. The observation that M2 expression is predominantly restricted to apical surfaces of influenza virus-infected cells raised the question whether M2 is directionally transported in the absence of other viral proteins, as has been shown for the influenza virus glycoproteins, HA and neuraminidase (20, 46). Alternatively, the polarized transport of M2 might be due to an interaction between M2 and other viral proteins, such as the glycoproteins. To address this question, we constructed VV-M2, a recombinant vaccinia virus that expresses the M2 protein. The polarized monkey kidney cell line Vero C1008 (45) was used to analyze M2 transport in the absence of other influenza virus proteins, as we obtained higher levels of M2 expression with the recombinant virus in these cells than in MDCK cells. The M2 protein was detected on the surfaces of VV-M2 virus-infected Vero C1008 cells by indirect immunofluorescence (data not shown) and by cell surface immunoprecipitation (Fig. 5) using M2 antibody. Similarly, HA expressed from recombinant vaccinia virus VV-HA was detected on the cell surface using surface immunoprecipitation (Fig. 5). The site of M2...
FIG. 2. Cell surface expression of M2 in influenza virus-infected MDCK cells. MDCK cells were infected with influenza A/WSN virus (MOI = 10) and reacted with monoclonal antibody specific for M2 (A to C) or HA (D to F) at 4.5 (A and D), 6.0 (B and E), or 7.5 (C and F) h p.i. Primary antibody was visualized using a fluorescein-conjugated secondary antibody. The exposure times of photographs were adjusted to remain constant throughout the experiment.
FIG. 3. M2 accumulation on apical and basolateral surfaces of influenza virus-infected MDCK cells. Filter-grown MDCK cells were infected with influenza A/WSN virus and assayed for M2 (squares) or HA (triangles) on apical (closed symbols) or basolateral (open symbols) surfaces using monoclonal antibodies and 125I-labeled secondary antibody. Values represent the 125I-labeled antibody (Ab) (in kilocounts per minute [kcpm]) bound to infected cells after subtracting nonspecific background and were derived from the mean values of quadruplicate samples (standard error of the mean, <15%). Similar results were obtained in two independent experiments.

Cell surface expression was investigated in filter-grown monkey kidney cells infected with the VV-M2 virus using 125I-labeled antibody binding (Fig. 6). The expression of HA on apical and basolateral membranes was compared using Vero C1008 cells infected with VV-HA, a recombinant virus that expresses HA (32). The M2 protein was detected on apical membranes of the recombinant virus-infected cells 2 h later in the infection time course than HA, even though immunofluorescence assays indicated that a similar percentage of cells was infected with either virus (data not shown). Significant levels of HA were detected on the apical surface by 6 h p.i., while M2 was expressed at low levels at 8 h p.i. and

FIG. 4. Kinetics of M2 cell surface appearance in influenza virus-infected MDCK cells. Influenza virus-infected MDCK cells on nitrocellulose filters were pulse-labeled for 15 min at ~6 h p.i. and chased for 0 to 2 h, as indicated above the lanes. Apical (A) or basolateral (B) cell surfaces were reacted at 4°C with M2 (A) or HA (B) antibody, the cells were lysed, and the immune complexes were recovered and analyzed on 12.5% polyacrylamide gels.

FIG. 5. M2 is expressed on the surfaces of Vero C1008 cells infected with VV-M2 recombinant virus. Polarized Vero C1008 cells were grown in plastic dishes, infected with recombinant virus VV-M2 (M), VV-HA (H), or VVpSC11 (S), and metabolically labeled from 5 to 8 h p.i. At 8 h p.i., the plasma membranes were reacted with M2 or HA antibody for 30 min at 4°C. After excess antibodies were removed the cells were solubilized in lysis buffer containing unlabeled infected cell lysates. The immune complexes were recovered using protein G-Sepharose and analyzed on 12.5% polyacrylamide gels. Contaminating vaccinia virus bands that migrate considerably faster than HA were also precipitated from cells reacted with HA antibody. Lane V, which contains marker proteins from purified influenza virions, was overexposed to visualize M2.

FIG. 6. M2 is expressed on apical membranes of polarized Vero C1008 cells infected with VV-M2 virus. Filter-grown Vero C1008 cells were infected with VV-M2 (squares) or VV-HA (triangles) and assayed for M2 or HA on apical (closed symbols) or basolateral (open symbols) surfaces using monoclonal antibodies and 125I-labeled secondary antibody. Values represent the 125I-labeled antibody (Ab) (in kilocounts per minute [kcpm]) bound to infected cells after subtracting nonspecific background and were derived from the mean values of triplicate samples (standard error of the mean, <15%). Similar results were obtained in three independent experiments.
accumulated at higher levels between 8 and 12 h.p.i. Both M2 and HA were predominantly restricted to the apical surfaces of polarized monkey kidney cells infected with the respective viruses. Approximately 88 to 90% of the total surface HA was expressed on the apical domain between 8 and 12 h.p.i. compared with 86 to 93% of the surface M2 expressed at 10 to 12 h.p.i. Therefore, the degree of apical polarity was very similar for M2 and HA expressed using vaccinia virus recombinants. The basolateral expression of HIV gp160 has been demonstrated previously by a similar assay in polarized epithelial cells infected with a recombinant vaccinia virus (36). These results demonstrate that the localization of M2 and HA is similar in polarized epithelial cells infected with influenza virus or the recombinant vaccinia viruses and indicate that the M2 protein contains the information necessary for directional transport to apical membranes in the absence of other influenza virus proteins.

Ultrastructural localization of M2 in influenza virus-infected cells. The localization of the M2 protein in influenza virus-infected MDCK cells was examined at the ultrastructural level using immunoelectron microscopy. At 7 h.p.i., a time when extensive virus budding was occurring, the cells were labeled at 4°C with M2-specific monoclonal antibody and a gold probe (Fig. 7). For comparison, virus-infected cells were also labeled with monoclonal antibodies specific for HA or an unrelated protein and the gold probe. M2-specific labeling was observed on apical membranes, including microvilli of influenza virus-infected MDCK cells. In addition, gold particles were associated with fully assembled and released virions and were close to both cell membranes and budding virions. Infected cells labeled with HA-specific antibody exhibited similar distributions of gold particles (Fig. 7). No labeling was observed on basolateral surfaces of filter-grown MDCK cells reacted with M2 antibody and secondary antibody conjugated with gold or ferritin (data not shown). The specificity of the immunolabeling reagents was demonstrated by the lack of significant binding of M2 antibody to uninfected-cell surfaces or of the gold probe to infected cells in the absence of influenza virus-specific antibody or in the presence of an unrelated antibody specific for the HA-neuraminidase protein (42) of parainfluenza type 3 virus (data not shown).

The M2 label was determined by quantitating the number of gold particles associated with either cell membranes or budding influenza virus particles on MDCK cells labeled with M2 antibody at 7 h.p.i. (Table 1). A significant proportion of the M2 label was found to be associated with partially and completely assembled virions. Electron micrographs of virus-infected MDCK cells labeled with M2 antibody and the gold probe were analyzed further by quantitative morphometry to estimate the relative concentrations of M2 label on virion and apical cell membranes. These calculations indicate that the density of M2-specific label associated with viral or cellular membranes was roughly equivalent, suggesting that M2 was neither specifically enriched in virion membranes compared with cell membranes nor excluded from budding virus particles.

**DISCUSSION**

We observed that the M2 protein accumulates predominantly on apical surfaces of influenza virus-infected polarized epithelial cells with kinetics similar to those of HA. The delay in M2 surface expression compared with that of HA is apparently due to the time course of M2 protein synthesis, which is delayed in part because of the inefficient splicing of segment 7 mRNA (51). The finding that M2 surface expression was restricted to the apical domain of polarized epithelial cells infected with recombinant virus VV-M2 suggests that M2 contains intrinsic structural features that are recognized by the cellular transport machinery, despite the small size and unusual topology of the molecule. Further analysis of signals for M2 transport using site-directed mutagenesis will be of interest for the identification of the structural requirements for apical transport. The determinants of signal-mediated and default transport pathways to the surfaces of kidney cells remain unresolved, and it is uncertain whether specific sorting signals are required for transport to apical or basolateral membranes or both membranes. Non-directional default transport would indicate that proteins lacking sorting information are incorporated nonspecifically into transport vesicles departing from the trans-Golgi network. Conversely, directional transport by a default mechanism would suggest that proteins that lack sorting signals are excluded from the vesicles involved in signal-mediated transport. Therefore, if M2 transport occurs by default, our findings would support the idea that the default pathway for membrane protein transport is restricted to apical surfaces of kidney cells.

Specific structural features which correlate with directional transport have been recently identified in other protein molecules. A subset of membrane proteins that contain a covalently attached glycosphatidylinositol residue is restricted to the apical domain of polarized epithelial cells (28). A signal directing basolateral transport in MDCK cells is formed by a 14-residue, arginine-rich sequence located adjacent to the membrane in the polymeric Ig receptor cytoplasmic domain (8). A mutated protein lacking this sequence was transported directly to the apical surface, while a chimeric protein containing the signal was redirected from the apical to basolateral surface. A different signal for basolateral transport was identified in a mutated influenza virus HA which was targeted directly to the basolateral domain of MDCK cells after a single cytoplasmic cysteine residue was changed to tyrosine (4). Specific signals in the cytoplasmic domains of several other proteins, including the Fc receptor, lysosomal glycoprotein Igpl20, and low-density lipoprotein receptor, are required for basolateral expression in MDCK cells (18, 19). The finding that mutated proteins lacking putative basolateral targeting signals are predominantly expressed on apical surfaces of MDCK cells suggests that basolateral signals are dominant over apical signals in kidney cells or that proteins which lack basolateral targeting signals are transported to apical surfaces by default (4, 8, 16, 18, 19). The available evidence suggests a correlation between basolateral polarity and endocytosis of certain proteins (4, 8, 16, 18, 19, 35) that contain a critical tyrosine and endocytosis of certain proteins (4, 18, 19). The finding thatcytoplasmic tyrosine residues profoundly alters the polarity and endocytosis of certain proteins (4, 18, 19). It is now evident that cytoplasmic tyrosine residues are not sufficient for basolateral targeting, since we found that the M2 protein was predominantly restricted to the apical domain of polarized cells despite the presence of two or three cytoplasmic tyrosine residues (23, 29) in sequences that are similar to proposed internalization signals (10, 21). Both the position of tyrosine in the cytoplasmic domain and the specific amino acids that surround tyrosine residues apparently influence the ability of these sequences to serve as targeting signals for endocytosis or basolateral transport (16, 18, 19, 21, 27). Therefore, it is likely that the cytoplasmic
FIG. 7. Distribution of M2 and HA on the surfaces of influenza virus-infected MDCK cells. MDCK cells were grown on plastic dishes, infected with influenza A/WSN virus (MOI = 15) and labeled with monoclonal antibody specific for M2 (A and B) or HA (C and D) at 7 h p.i. Primary antibody was visualized using a secondary antibody bound to 10-nm-diameter gold particles. Bar, 0.2 μm.
tyrosine residues in M2 are not in the proper environment to function as basolateral targeting signals. We have observed that M2 is not excluded from coated pits when influenza virus-infected cells are incubated at 37°C with M2 antibody (16a), but it has not been determined whether M2 is internalized to a significant extent in the absence of antibody. Further analysis of the polarity and endocytosis of M2 and other proteins containing cytoplasmic tyrosines will be required to clarify relationships between these events.

The punctate immunofluorescence pattern observed for M2 on apical surfaces of influenza virus-infected MDCK cells was similar to that observed for HA. However, the slightly more diffuse fluorescence pattern observed for M2 appeared to reflect subtle differences in the ultrastructural localization of the two proteins with respect to budding virions, presumably due to differences in M2 and HA incorporation into virions (57). Immuno electron microscopy further localized M2 to microvilli and the surrounding apical cell membrane of virus-infected MDCK cells and to completely assembled and budding virions, which did not exhibit obvious differences in the frequency or density of M2 label. It was surprising that a large percentage of the M2 label was found to be virion-associated and that the apparent density of M2 label on viral and cellular membranes was roughly equivalent, considering the low level of M2 incorporation into virions (57) compared with the large number of molecules on the infected-cell membrane (24). The restriction of M2 to the apical domain of polarized cells, which is the site of influenza virus budding, and the association of M2 with both cellular membranes and nascent virions are consistent with a possible role for M2 in virus assembly. However, the mechanism responsible for excluding the majority of M2 molecules from virions such that only a few molecules are incorporated during virus assembly is not known. As treatment of virions with the drug amantadine hydrochloride inhibits virus uncoating (5), and the antiviral effect is exerted on M2 function (2, 14), it is likely that the M2 molecules incorporated into virions play a role in viral infectivity.

It has been suggested that the M2 protein may be associated with an ion channel activity (47, 49), and direct evidence for a pH-activated ion channel has been obtained by electrophysiological measurements of Xenopus laevis oocytes injected with M2 mRNA (41). Alterations in the intracellular environment induced by M2 might affect virus assembly indirectly, as the late stages of protein transport are sensitive to ionic conditions (30, 47). On the basis of the HA conformational changes that can occur in the presence of amantadine (47), it was suggested that M2 influences the stability of HA trimers during transport and that M2 might function to offset intracellular pH changes induced by the shutdown of host protein synthesis during virus infection (34, 47). Inhibition by amantadine might be due to an interaction between the drug and M2 in virion or cell membranes that alter the ion channel function of M2 (41) and may disrupt either early or late stages of replication (5, 14, 47, 49). Current evidence suggests that M2 might function to influence interactions among virion components during virus entry or budding. A monoclonal antibody to the external domain of M2 inhibited influenza virus replication in a strain-specific manner (50, 57, 58), and the mutation of specific residues in the cytoplasmic domain of M2 or in the M1 protein conferred resistance to the M2 antibody (58). The mechanism that targets M1 to sites of virus budding is not yet known, but immunoelectron microscopic studies indicated that once M1 and the nucleocapsid associate with the plasma membrane, virus budding is rapidly completed (39). The M1 protein is reported to associate spontaneously with lipids by inserting specific regions of the molecule into the bilayer (13, 56), and the association of M1 with the virus envelope appears to be pH sensitive (60). The present finding that M2 label was frequently associated with nascent virions is consistent with the hypothesis that the affinity of the M1 protein for the plasma membrane may be influenced by M2 ion channels or by a targeting signal in the M2 molecule. In addition, virus-associated M2 may facilitate uncoating in secondary endosomes by permitting a flux of ions into virions and altering interactions between M1 and the viral envelope or ribonucleoprotein complexes (5, 41, 61). Therefore, it is conceivable that M2 may function, perhaps in a pH-sensitive mechanism, to regulate the association between internal virion components and the viral envelope during virus uncoating and assembly stages of replication.

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