Identification of a Novel Apical Sorting Motif and Mechanism of Targeting of the M2 Muscarinic Acetylcholine Receptor*

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Previous studies have shown that the M2 receptor is localized at steady state to the apical domain in Madin-Darby canine kidney (MDCK) epithelial cells. In this study, we identify the molecular determinants governing the localization and the route of apical delivery of the M2 receptor. First, by confocal analysis of a transiently transfected glycosylation mutant in which the three putative glycosylation sites were mutated, we determined that N-glycans are not necessary for the apical targeting of the M2 receptor. Next, using a chimeric receptor strategy, we found that two independent sequences within the M2 third intracellular loop can confer apical targeting to the basolaterally targeted M4 receptor, Val270-Lys280 and Lys280-Ser350. Experiments using Triton X-100 extraction followed by OptiPrep™ density gradient centrifugation and cholera toxin β-subunit-induced patching demonstrate that apical targeting is not because of association with lipid rafts. 35S-Metabolic labeling experiments with domain-specific surface biotinylation as well as immunocytochemical analysis of the time course of surface appearance of newly transfected confluent MDCK cells expressing FLAG-M2-GFP demonstrate that the M2 receptor achieves its apical localization after first appearing on the basolateral domain. Domain-specific application of tannic acid of newly transfected cells indicates that initial basolateral plasma membrane expression is required for subsequent apical localization. This is the first demonstration that a G-protein-coupled receptor achieves its apical localization in MDCK cells via transcytosis.

The biogenesis of membrane polarity is crucial for the development of multicellular organisms and for the proper functioning of polarized cell types such as neurons and epithelial cells. Biochemically and functionally distinct plasma membrane domains are required for cellular processes such as neuronal transmission, migration, solute uptake and transport, secretion, body water homeostasis, and signal transduction. The mechanism by which cells generate and maintain cellular polarity remains a fundamental question of cell biology.

The renal epithelial Madin-Darby canine kidney (MDCK) cell line provides a model system for the study of protein targeting because of the ability of this cell line, when grown to confluence, to form a polarized monolayer (1). Polarized epithelial cell surface membranes are divided into apical and basolateral domains possessing distinct protein and lipid compositions that are separated by tight junctions. This spatial asymmetry is generated and maintained by one of three pathways. Proteins can be targeted directly to the apical or basolateral membrane from the trans-Golgi network (TGN) via the exocytotic pathway (2). They can also be targeted indirectly by being delivered to one domain, typically the basolateral domain, endocytosed, and then either recycled back to the original membrane or redirected to the opposite domain in a process termed transcytosis (3, 4). Alternatively, proteins can be randomly targeted to both domains and achieve their asymmetric distribution by selective stabilization or retention at one plasma membrane (5–7). Although other epithelial cell types, including hepatocytes and intestinal cells, primarily utilize the indirect pathway for apical protein delivery (8), MDCK cells primarily use the direct pathway (3).

In MDCK cells, newly synthesized apical and basolateral membrane proteins are segregated into separate transport vesicles within the TGN by virtue of sorting signals within the protein (2, 9, 10). The routing of basolaterally targeted proteins expressed in this cell line is mediated by discrete cytosolic amino acid sequences that frequently resemble endocytosis signals. These sequences often contain a critical tyrosine residue within an NPXY or YXXΦ motif (where Φ is a bulky hydrophobic amino acid), a dihydrophobic motif, a cluster of acidic residues, or a combination of these elements (11–13). Recent evidence suggests that in some cases these motifs are recognized by the clathrin adapter proteins AP-1, AP-2, or AP-3 that sequester them into basolateral transport vesicles (3, 14–16). Molecular signals mediating apical transport in MDCK cells are much more diverse. The apical targeting of proteins within MDCK cells can be mediated by the lipid anchor of glycosylphosphatidylinositol (GPI)-anchored proteins, by protein N- or O-linked glycans, or by amino acid residues located in the extracellular, transmembrane, or cytoplasmic domains of proteins (3, 17–19). The prevalent model of apical membrane sort-
ing proposes that apical proteins are sequestered into apical transport vesicles via association with sorting platforms rich in cholesterol and sphingolipid-rich lipid rafts at the level of the trans-Golgi network (20, 21).

The distribution of the mAChR subtypes when exogenously expressed in MDCK cells has been characterized. Although the M1, M3, and M5 receptors appeared to be nontargeted, the M2 and the M4 receptors displayed an apical and a basolateral distribution, respectively (22). The M3 basolateral sorting sequence has been defined in a previous study as a 7-amino acid motif residing in the N-terminal portion of the third intracellular loop (22, 23). In this study, the molecular signals mediating the apical targeting of the M4 mAChR and the mechanism of delivery to the apical membrane were investigated.

In this study we use M2/M4 chimera receptor constructs to show that there is apical targeting information within the third intracellular loop of the M2 mAChR. We use sequences from the M2 third intracellular loop appended to the C terminus of M4 to define two adjacent sequences of amino acids that were able to confer apical targeting to the M4 receptor in a position-independent fashion as follows: an 11-amino acid sequence, Val270–Lys280 and a 71-amino acid sequence, Lys280–Ser350. Furthermore, we demonstrate by using deletion analysis that either of these apical sorting sequences is sufficient to direct the apical sorting of the M2 receptor. To address the mechanism by which the M2 receptor cargo is sequestered into apically targeted vesicles, we determine whether or not the M2 receptor is raft-associated by using both biochemical and confocal analyses. Finally, we use metabolic labeling followed by surface biotinylation, immunocytochemical imaging of the cell-surface staining pattern of newly transfected confluent MDCK cells expressing the M2-GFP, and domain-specific application of the cell-impermeable cross-linking agent tannic acid to demonstrate that the M2 receptor is initially transported to the basolateral domain prior to its subsequent apical localization.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—MDCK (strain II) cells were obtained from Dr. Keith Mostov (University of California, San Francisco). Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, and 0.1 mg/ml streptomycin sulfate at 37 °C in a humidified 10% CO2 environment.

**Transfection of mAChRs and Chimeric Constructs**—To analyze the targeting of mAChR constructs, MDCK cells were seeded at high density (1 × 10⁶ cells/well) on 24.5-mm polycarbonate Transwell filters (0.4-μm pore size; Costar, Cambridge, MA) in DMEM supplemented with 10% fetal bovine serum (1 ml in the apical and 2.5 ml in the basolateral compartments) 24 h before transfection. For each construct to be transfected, 4 μg of DNA was added to a final volume of 100 μl of DMEM (without serum or antibiotics), and 8 μl of Lipofectamine 2000 (1:2 ratio of DNA:Lipofectamine 2000 reagent; Invitrogen) was added to 92 μl of DMEM (without serum or antibiotics). The two solutions were combined and incubated on a bench top for at least 30 min before being added to the medium in the apical compartment of the plated MDCK cells. Twenty four hours after the start of transfection, the wells of transfected cells were replaced with fresh media.

**Immunocytochemical Analysis**—36–48 h after transfection, cells were rinsed three times with phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM NaH2PO4, and 1.5 mM KH2PO4, pH 7.4) and fixed with paraformaldehyde solution (4% (v/v) paraformaldehyde and 4% (v/v) sucrose) in PBS for 30 min at room temperature and processed for immunocytochemistry. For permeabilized conditions, fixed cells were rinsed twice with PBST (PBS containing 0.1% (v/v) Tween 20), permeabilized with 0.25% (v/v) Triton X-100 (in PBS) for 5 min at room temperature, and blocked with 10% (w/v) bovine serum albumin in PBST containing 0.25% Triton X-100 for 2 h at room temperature. After blocking, cells were incubated with anti-FLAG M2 monoclonal antibody (2 μg/ml; Sigma) or affinity-purified subtype-specific polyclonal anti-M2 (1:200) raised to the third intracellular loop of mouse M2 mAChR (24), in PBST containing 3% bovine serum albumin and 0.25% Triton X-100 overnight at 4 °C in a humid chamber or 2 h at room temperature. Following three washes with PBST, cells were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse secondary antibody (1:250; Cappel Research Products, Durham, NC) in PBST containing 3% bovine serum albumin and 0.25% Triton X-100 for 2–3 h at room temperature. After three more washes with PBST, slides were coverslipped with Vectashield (Vector Laboratories, Inc., Burlingame, CA). For nonpermeabilized conditions, Tween and Triton X-100 were omitted from all solutions listed above.

Fluorescent images were collected in both the x-y and x-z planes using a Leica TCS SP1/NT confocal microscope (Leica Microsystems, Inc., Exton, PA) using ×100, 1.4 N.A. oil immersion lens in the W. M. Keck Imaging Center at the University of Washington. For each x-y image, a projected z-series image was generated by taking images at 0.6-μm intervals from the apical to the basolateral regions of the cells and compressing to a single x-y image. Images were processed using Adobe Photoshop. Quantitation of the apical/basolateral distribution of mAChR deletion constructs was performed using the public domain NIH Image program (developed at the National Institutes of Health). The mean pixel intensity/unit area was determined by manually outlining the areas of interest in the raw (unprocessed) x-z images. Data are expressed as percent basolateral of total staining and are presented as means ± S.E. for the number of experiments indicated. Between 2 and 16 images were quantitated for each experiment. Data were processed using Microsoft Excel.

**Construction of Epitope-tagged Chimeric and C-terminal Fusion mAChRs**—A modified FLAG epitope (DYKDDDDA) was added to the extracellular N termini of the porcine M2 (clone Mc7 (25)) and human M4 (26) mAChR coding sequences immediately after the initiator methionines using PCR as described (22). The M2 and M4 receptors were cloned into the KpnI/EcoRI and EcoRV sites of pcDNA3.1 (Invitrogen), respectively.

M2/M4 chimeric mAChRs were constructed by using sequential PCR using Pfu or Pfu Turbo polymerase (Stratagene) as described previously (27) to replace parts of the M2 coding sequence with the homologous regions of M4 coding sequence.
as described previously, pFM2, pFM4, or M2/M4 chimeric constructs were used as PCR templates for subsequent chimeras. All PCR-amplified constructs were designed with KpnI and EcoRI sites at their 5' and 3' ends, respectively, and ligated into pcDNA3.1. The sequences comprising the M2/M4 chimeras are as follows, with the numbers in parentheses representing the NotI sites of pcDNA3.1 to create M4-C

1–1437 that possessed three C-terminal alanine residues (gcc gcc gcc) in place of the amber stop codon to create a NotI site and a C-terminal XhoI site.

The following M2 sequences were then ligated into the pcDNA3.1 and pFM4 as a template. The above C-terminal fusion constructs were inserted into the BglII/EcoRI site of pCDPS. The sequences comprising the M4 C-terminal M2 appendages are as follows: M2(208–327), coding nt 622–981; M2(280–280), and M4(280–352), in which M2 sequences were fused to the C terminus of M4, were generated by sequential PCR using pFM2 and pFM4 as a template. The above C-terminal fusion constructs were inserted into the BglII/EcoRI site of pCDPS. The sequences comprising the M4 C-terminal M2 appendages are as follows: M2(208–327), coding nt 622–981; M2(280–280), and M4(280–352), in which M2 sequences were fused to the C terminus of M4, were generated by sequential PCR using pFM2 and pFM4 as a template. The above C-terminal fusion constructs were inserted into the BglII/EcoRI site of pCDPS. The sequences comprising the M4 C-terminal M2 appendages are as follows: M2(208–327), coding nt 622–981; M2(280–280), and M4(280–352), in which M2 sequences were fused to the C terminus of M4, were generated by sequential PCR using pFM2 and pFM4 as a template. The above C-terminal fusion constructs were inserted into the BglII/EcoRI site of pCDPS. The sequences comprising the M4 C-terminal M2 appendages are as follows: M2(208–327), coding nt 622–981; M2(280–280), and M4(280–352), in which M2 sequences were fused to the C terminus of M4, were generated by sequential PCR using pFM2 and pFM4 as a template.

C-terminal fusion proteins M4+M2(208–325), M4+M2(280–350), M4+M2(220–260), M4+M2(250–270), and M4+M2(280–280) were constructed by first generating an M4 construct (nt 1–1437) that possessed three C-terminal alanine residues (gcc gcc gcc) in place of the amber stop codon to create a NotI restriction site. This construct was inserted into the EcoRV/NotI sites of pcDNA3.1 to create M4-C’NotI. The following M2 sequences were PCR-amplified with three N-terminal alanines (gcc gcc gcc) to create a NotI site and a C-terminal XhoI site. The following M2 sequences were then ligated into the M4-C’NotI construct in pcDNA3.1 using the NotI/XhoI sites: M2(220–280), coding nt 658–840; M2(280–350), coding nt 838–1050; M2(220–260), coding nt 658–780; M2(250–270), coding nt 748–810, and M2(260–280) and coding nt 778–840. The M4+M2(270–280), coding nt 808–840, was constructed by including the M2 nucleotides to be added to the C terminus of M4 within the 3’-primer, placing the STOP codon of M4 and terminated with a STOP codon followed by repeated XbaI sites. The 5’-primer was designed to anneal to the M4 sequence 6 bp upstream through 9 bp downstream (nt 982–1002) of the unique SacII of M4. The construct was digested with SacII and XbaI and ligated into a similarly digested M4 construct within pCDNA3.1 (+)

A C-terminal M2-GFP fusion protein was constructed by PCR amplifying FLAG-M2 with an N-terminal EcoRI site and a C-terminal XbaI site (which replaced the C-terminal ochre stop codon) and ligating the PCR product into pCS2+ XLT vector (28) that was engineered to produce an in-frame N-terminal GFP fusion protein, pCS2+ XLT-GFP (obtained from R. T. Moon).

Generation of FLAG-M2 mAChR Deletion and Glycosylation-defective Mutants—The following M2 mAChR deletion mutants were generated by sequential PCR as described previously (27) using porcine FLAG-M2 as a template: M2(del 220–380), lacking nt 658–1140; M2(del 227–315), lacking nt 678–945; M2(del 220–280), lacking nt 658–840; M2(del 280–325), lacking nt 838–975; and M2(del 303–380) lacking nt 907–1140. Mutants M2(del 227–315), M2(del 280–325), and M2(del 303–380) were generously provided by Dr. Michael Schlador. M2(del 220–380) and M2(del 220–280) were cloned into the EcoRI/XbaI pcDNA3.1, whereas the M2(del 272–315), M2(del 280–325), and M2(del 303–380) constructs were cloned into pCDPS. The M2 mAChR glycosylation mutant was described previously (29).

Generation of MDCKII Cells Stably Expressing FLAG-tagged M2 mAChR—MDCKII cells stably expressing FLAG-tagged M2 mAChR were generated by transfecting MDCK II cells cultured in a 10-cm plate at 60–80% confluency using the calcium phosphate precipitation method (30) with 10 μg of the pFM2 receptor in pcDNA3.1. Beginning at 48 h after transfection, stable transformants were selected by treatment with 1 mg/ml G418 (Geneticin; Invitrogen) for at least 4 weeks following transfection. Drug-resistant cells were selected and then screened by both sterile fluorescence-assisted cell sorting (FACS) and immunocytochemistry. For FACS analysis, cells were dissociated from plates using trypsin (as performed in cell culturing), pelleted by centrifugation, suspended in PBS with 3% BSA and 2 μg/ml monoclonal anti-FLAG antibody, and incubated on ice for 30 min. Cells were again pelleted by centrifugation, rinsed 2× in PBS, and resuspended in PBS with 3% BSA with 1:250 anti-mouse FITC-conjugated secondary antibody, and incubated for 30 min. Cells were rinsed 2× in PBS, resuspended in PBS with 3% BSA, and subjected to FACS analysis (Coulter Elite; Beckman Coulter). Selected cells (75,000) were plated, grown, and split into a 96-well culture dish (Costar) at a dilution of 1 cell/ml. Clones were expanded and further evaluated for FLAG-M2 receptor expression by immunocytochemistry as described under “Immunocytochemical Analysis.”

Detergent Extraction and Flotation in OptiPrep™ Density Gradients—MDCK cells stably expressing FLAG-tagged M2 mAChR were grown 3–5 days post-confluency on three 10-cm culture dishes with daily media changes before use. Cells were washed twice in ice-cold PBS and lysed for 30 min in 3 ml of ice-cold TNE (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA), 5 mM dithiothreitol, with 0.5% Triton X-100 and protease inhibitors (Complete; Roche Applied Science) on ice. The lysate was scraped from the dishes, and the plates were rinsed 25–30 times, on ice. The extract was brought to 40% OptiPrep in 1 cell/ml. Clones were expanded and further evaluated for FLAG-M2 receptor expression by immunocytochemistry as described under “Immunocytochemical Analysis.”

M2 mAChR Apical Sorting Signal
**M<sub>2</sub> mAChR Apical Sorting Signal**

Laboratories, and polyclonal anti-human transferrin antibodies (1:200; Santa Cruz Biotechnology, H-300). Renaissance Western blot chemiluminescent reagent (PerkinElmer Life Sciences) was used to detect immunoreactivity.

Quantification of the distribution of protein in fractions throughout the gradient was performed using the public domain NIH Image program (developed at the National Institutes of Health). The mean pixel intensity/unit area was determined by manually outlining the areas of interest. The percent of total was determined by dividing the pixel intensity of each band by the sum of the intensities of all the bands throughout the gradient. Data were processed using Microsoft Excel; graphing was performed on CA-Cricket Graph III.1.

**Cholera Toxin and Antibody-induced Patching**—MDCK cells stably expressing FLAG-tagged M<sub>2</sub> mAChR were plated on MDCK cells seeded at 3.5 × 10<sup>5</sup> cells/well on 2-well glass chamber slides (4.2 cm<sup>2</sup>/well; Nalge Nunc International) and grown to confluency for 3–4 days. AlexaFluor 594 conjugate of cholera toxin β-subunit (ChTxB; Molecular Probes) was used to label endogenous glycosphingolipids. Cells were incubated with ChTxB (10 μg/ml) in DMEM-HEPES (25 mM HEPES), 0.2% BSA on ice for 30 min and then rinsed three times with ice-cold PBS. For the nonpatched (control) condition, cells were rinsed, fixed, and stained as described under “Immunocytochemical Analysis.” Lipid raft aggregation, or patching of ChTxB, was induced by incubating the cells with anti-ChTxB antibody (1:250 in DMEM-HEPES, 0.2% BSA; Calbiochem-Novabiochem) for 20 min at 37 °C. Cells were removed from the incubator, fixed, rinsed, and stained as described previously. Co-patching of M<sub>2</sub> mAChR and ChTxB was performed by simultaneously incubating cells on ice with ChTxB and anti-FLAG antibody (2.5 μg/ml) in DMEM-HEPES, 0.2% BSA. After rinsing three times with ice-cold PBS, cells were patched with both anti-ChTxB antibody (as described above) and FITC-conjugated goat anti-mouse secondary antibody (1:250; Cappel Research Products, Durham, NC) for 20 min at 37 °C. Cells were then rinsed three times with PBS, fixed, and visualized as described above. As a positive control, cells were labeled simultaneously with BODIPY FL-labeled C5 ganglioside GM1 (500 nM on PBS; Molecular Probes) and ChTxB for 30 min on ice and were either subsequently fixed or patched as described above with anti-ChTxB.

Quantitation of colocalization was performed using the plug-in RG2B_colocalization to ImageJ (version http://rsb.info.nih.gov). The colocalization measurements were taken using a minimum threshold pixel intensity adjusted between 100 and 200 and set equivalently for both channels to correspond to the image intensity. The minimum ratio for pixel intensity between the two channels was set to 0.5. Colocalization pixels were displayed as an image with maximum pixel intensity (255). The RGB output of the plug-in was split into green, red, and blue images, and each image was quantitated by measuring the total pixel area above the threshold intensity used in colocalization determination (see above). Results are displayed as percent colocalization as determined by dividing the area of colocalization pixels by the total pixel area over the threshold of whichever channel was limiting.

**Functional Confirmation of Intact Monolayers Prior to Metabolic Labeling**—For polarity experiments, MDCKII cells were seeded at a density of 1 × 10<sup>6</sup> cells/24.5-mm polycarbonate membrane filter (Transwell chambers, 0.4-μm pore size; Costar, Cambridge, MA) and cultured for 5–8 days with medium changes every day. Prior to each functional or immunocytochemical experiment, the integrity of the monolayer was assessed by adding [<sup>3</sup>H]methoxyinulin (PerkinElmer Life Sciences) to the apical medium and monitoring the leak of [<sup>3</sup>H]methoxyinulin from the apical compartment to the basolateral compartment by sampling and counting the basolateral medium in a scintillation counter after a 1-h incubation at 37 °C. Chambers with greater than 3% leak per h were discarded.

**Metabolic Labeling/Biotinylation Strategy for Determining Surface Delivery of the M<sub>2</sub> mAChR**—MDCK cells were metabolically labeled, surface-biotinylated, and evaluated as described (32). MDCK cells stably expressing the FLAG-M<sub>2</sub> receptor cells grown on Transwells were washed twice with PBS and grown for 45 min in methionine/cysteine-free medium (Invitrogen) supplemented with 4 mM glutamine and 5% fetal calf serum. Following removal of this medium, cells were inverted (cell side down), and 150–200 μl of methionine/cysteine-free medium with 5% fetal bovine serum containing 1 μCi/μl 35S-Express protein labeling mixture (NEG-072; PerkinElmer Life Sciences) was added to the bottom of inverted filters. The cells were incubated at 37 °C for various lengths of time. Each time point reflects a pooled sample from three 24.5-mm wells. At the end of the labeling period, cells were washed three times with PBS/CM (PBS containing 0.5 mM CaCl<sub>2</sub> and 2 mM MgCl<sub>2</sub>). Sulfo-NHS-LC-biotin (Pierce) at 1 mg/ml in PBS buffer was then applied to the apical or basolateral surface and incubated for 30 min on ice in order to covalently label surface proteins. The opposite compartment not being treated with biotin was incubated with PBS/CM containing 1% (w/v) BSA. After the incubation, cells were washed once in 50 ml Tris to quench and three times for 5 min with PBS/CM. Cells were harvested in 1 ml of ice-cold PBS using a rubber policeman and pelleted by centrifugation. Membranes were solubilized by resuspending the pellet in solubilization buffer as follows: 1% digitonin (Calbiochem) and 0.1% cholate (Sigma) in phosphate buffer (10 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.0, 1 mM EDTA, 50 mM NaCl) with protease inhibitors (Complete; Roche Applied Science) and rotating at 4 °C for 6 h. Membranes were pelleted by centrifugation, and the supernatant was rotated overnight at 4 °C with 9.0 μg/ml anti-FLAG M<sub>2</sub> monoclonal antibody (Sigma). 50 μl of a 1:1 slurry of protein G-agarose (Roche Applied Science) was added to the membrane/antibody mixture after preincubating the agarose beads for 1 h in 2.5% BSA in solubilization buffer followed by two 5-min washes with solubilization buffer alone and rotated overnight at 4 °C. The next day the protein G-agarose was pelleted by centrifugation. Pellets were washed three times for 5 min with 0.5% digitonin and 0.1% cholate in phosphate buffer with protease inhibitors (Complete; Roche Applied Science). Following the last wash, proteins were eluted from the protein G-agarose beads by incubating in 100 μl of SDS sample buffer at 90 °C for 20 min and agitated every 5 min. A second elution was performed in 50
µl of sample buffer. Samples were combined and subjected to SDS-PAGE on a 10% polyacrylamide gel and subsequent autoradiography using BioMax MS-1 film and a BioMax TransScreen-LE (PerkinElmer Life Sciences). The identity of the radioactive band as that of the M₂ receptor was confirmed by analyzing membranes of M₂ stably expressing MDCK cells by Western analysis using an affinity-purified subtype-specific polyclonal anti-M₂ (1:200) raised to the third intracellular loop of mouse M₂ mAChR and matching the electrophoretic mobility of the immunoreactive band with that of the radioactive band.

Time Course of M₂-GFP Surface Expression and Tannic Acid Treatment—Cells were plated near confluency at 1 × 10⁶ cells/well in Transwell filters (0.4 µm pore size; Costar, Cambridge, MA). 36 h later, cells were transfected with 4 µg of the M₂ C-terminal GFP fusion protein FLAG-M₂-GFP in pCS2⁺XLT using Lipofectamine as described above. 12 h after the start of the transfection, when cell surface staining of the M₂-GFP receptor construct was just detectable by immunocytochemical methods, 0.5% tannic acid (Sigma) in serum-free DMEM was applied to either the apical or basolateral compartments of Transwells for an additional 30, 60, or 120 min. The opposite compartment to the one being treated with tannic acid contained standard growth media. Control wells at each time point were not treated with tannic acid and are displayed in Fig. 10. The integrity of the monolayer and confirmation of polarization was determined by staining with β-catenin antibody after 2 h of tannic acid treatment of either the basolateral or apical compartments. Cells were rinsed three times with PBS at the end of the incubation, fixed, labeled, and stained using goat anti-mouse Alexa-568-conjugated secondary antibody (1:500; Molecular Probes) using nonpermeabilizing conditions, or stained with β-catenin antibody (1:200; Transduction Laboratories) using permeabilized conditions, as described under “Immunocytochemical Analysis.”

RESULTS

Localization of Wild Type M₂ mACHR and a Glycosylation-defective Mutant M₂ Receptor in MDCK Cells—Many studies have indicated that N- or O-glycosylation is critical not only for the exit of newly synthesized proteins from the endoplasmic reticulum (33, 34) and the Golgi (35) but also for targeting of newly synthesized proteins to the correct membrane domain of polarized epithelial cells (35–37). Despite mounting evidence indicating that apical sorting can occur independently of N-glycosylation (18, 38–40), a role for N-glycans in apical sorting continues to be supported by recent evidence that N-glycans alone can mediate apical sorting of the membrane dipeptidase, independent of raft association (41). Furthermore, the addition of glycosylation sites to the basolaterally localized Na,K-ATPase β₁ resulted in the redirection of this subunit to the apical domain of HGT-1 cells (a human gastric adenocarcinoma cell line) and the redistribution of the associated Na,K-ATPase α₁ subunit to the apical domain as well (42).

The M₂ mAChR is a dialglycosylated transmembrane protein (43) that has three potential sites for N-glycosylation. A previous study has shown that N-glycosylation of the M₂ receptor is not required for cell-surface localization or ligand binding and does not confer increased stability against receptor degradation in Chinese hamster ovary cells (29); however, the importance of N-glycans in mediating the membrane targeting of the M₂ receptor within a polarized cell type has not been investigated. To determine whether N-glycosylation plays a role in the steady-state apical localization of the M₂ mAChR in MDCK cells, a glycosylation-defective mutant in which three asparagine codons were substituted with aspartate residues (29) was transfected into MDCK cells, and the steady-state distribution of the mutant within the polarized monolayer was analyzed by immunocytochemistry and confocal microscopy. Similar to the wild type receptor, the glycosylation-defective mutant was localized to the apical domain of MDCK cells (Fig. 1, A and B, respectively). This result demonstrates that the steady-state distribution of the M₂ mAChR to the apical domain of confluent MDCK cells is independent of N-glycosylation.

The Third Intracellular Loop of the M₂ mAChR Contains Apical Sorting Information—Whereas the M₂ receptor displays an apical distribution when expressed in MDCK cells, the M₄ receptor was shown previously to exhibit an apparently nonpolarized, cytoplasmic distribution under permeabilizing staining conditions when transiently expressed in MDCK cells (22). Because of the differential localization of these receptors and their high degree of amino acid identity (56% identity, 78% if the third intracellular loop is excluded), we tested the use of M₂/M₄ receptor chimeric constructs in a gain-of-function approach to determine which domain of M₂ could confer apical targeting to the M₄ receptor. To visualize the plasma membrane distribution of the M₂ and M₄ receptor constructs, we used nonpermeabilizing conditions to stain for cell-surface expression of the extracellular N-terminal FLAG epitope of transiently transfected filter-grown MDCK cells. Under these conditions, the FLAG-M₄ construct was localized to the basolateral domain on the cell surface (Fig. 3B).
Because of the differential distribution of the M2 and M4 receptors on the cell surface, we were able to pursue the approach of defining an M4 apical sorting signal using M2/M4 chimeras. A schematic representation of the chimeric constructs is presented in Fig. 2. Fig. 3 shows the steady-state localizations of the chimeric receptors. The first construct, M4/M2 (208–467), contains the N-terminal portion of M4 through the predicted fifth transmembrane (TM5) region and M2 sequence from the N terminus of the third intracellular loop to the C terminus of the receptor. This chimera displayed an apical distribution in MDCK cells, similar to that of the wild type M2 receptor (Fig. 3, A and C). This result suggested that a region C-terminal to TM5 of the M2 receptor is sufficient to confer apical sorting to the M4 receptor. To test this hypothesis, we examined the surface distribution of a receptor that contained the M4 sequence through the M4 third intracellular loop, and M2 sequence from TM6 through the C terminus of the receptor, M4/M2(391–467). As shown in Fig. 3D, this receptor was basolaterally targeted, suggesting that the M2 apical targeting information was contained in the third intracellular loop of M2. To further examine this possibility, a pair of constructs representing the inverse of the constructs tested above was generated. These constructs consisted of the N-terminal M2 sequence either up to M2(1–207)/M4 or through M2(1–390)/M4, the M2 third intracellular loop within the M4 context. In support of the hypothesis that the M2 third intracellular loop contains apical sorting information, the M2(1–207)/M4, which lacked the M2 third intracellular loop, displayed a basolateral steady-state distribution, whereas the M2(1–390)/M4 construct, which encompassed the M2 third intracellular loop, was apically directed (Fig. 3, E and F). To determine whether the third intracellular loop alone was sufficient to confer apical targeting to the M4 receptor, the M2 third intracellular loop sequence was substituted into the context of the M4 receptor in the construct M4/M2(208–390). The steady-state distribution of M4/M2(208–390) was apical (Fig. 3G). These results suggest that the M2 third intracellular loop contains sufficient sorting information to redirect the basolaterally targeted M4 receptor to the apical domain in MDCK cells.

**The M2 Apical Sorting Signal Is Not Dependent on Its Context within the Third Intracellular Loop**—Results from the chimeric constructs discussed above indicate that the M2 third intracellular loop, when substituted into the M4 receptor context, can redirect the basolaterally targeted M4 receptor to the apical domain of MDCK cells. The redirection of the resulting construct could be due to either the addition of an apical sorting signal in the first construct (M4/M2) or to the use of a novel apical sorting signal within the M2 third intracellular loop when added to the M4 receptor context. As shown in Fig. 4, A–D, the M2 third intracellular loop did not contain a novel apical sorting signal, because substitution of the M2 third intracellular loop into the M4 receptor context, M4/M2(208–390), resulted in an apical distribution of the receptor. This result indicates that the apical sorting signal resides within the N-terminal M2 sequence.
A

M2 3il:

208 220 260 300 340 380 389

M2 C-terminal additions to M4:

208 327

208 280

208 252

B

M2

M4

M4 + M2(208–327)

M4 + M2(208–280)

M4 + M2(208–252)

FIGURE 4. The M2 apical sorting sequence is position-independent and lies within amino acids 208–280 of the M2 third intracellular loop. A, schematic representation of the M2 third intracellular loop and the localization of constructs containing the sequences (bars) of amino acids (numbers) from the M2 third intracellular loop appended to C terminus of the M4 mAChR. B, filter-grown MDCK cells were transfected with FLAG-tagged constructs encoding the following: panel 1, M4 receptor; panel 2, M4 receptor; panel 3, M4 receptor with C-terminal addition of M2(208–327); panel 4, M4 receptor with C-terminal addition of M2(208–280); panel 5, M4 receptor with C-terminal addition of M2(208–252). Cells were fixed, stained, and visualized by confocal microscopy as described under "Experimental Procedures." The upper panels are images taken in the x-y plane (projected z-series), and the lower panels are x-z images of a vertical section taken at or near the midpoint of the image. Each image is representative of at least three independent experiments. Bar = 20 μm.

There Are Redundant M2 Apical Targeting Determinants within the M2 Third Intracellular Loop—The observation that M2 amino acids Glu253–Lys280 within the larger context of amino acids 208–280 was sufficient to confer apical targeting to the basolaterally targeted M4 receptor led us to ask if a minimal sequence resided within this larger context that was sufficient to confer apical targeting information. Smaller overlapping M2 third intracellular loop sequences were appended to the full-length M4 receptor and tested as described above. Additionally, because there is evidence for redundancy of targeting sequences within proteins such as the transforming growth factor-α precursor (47), the low density lipoprotein receptor (11), and the α2A-adrenergic receptor (48), a longer M2 sequence further downstream from the area in question, Lys280–Ser350, was appended to M4. As shown in Fig. 5B, M4 sequences 220–280 and 280–350, when appended to the N terminus of M4, could each redirect this protein to the apical domain. The shorter M4 sequences M4(220–260) and M4(250–270), when appended to full-length M4, could not confer apical targeting to the M4 receptor (Fig. 5B). In contrast, M2 amino acids 260–280, when appended to the M4 C terminus, did result in an apical distribution similar to wild type M4. In light of these results, an additional construct was made to address the question of whether the M4 residues 270–280 held sufficient apical targeting information to confer apical targeting to the M4 receptor. As shown in Fig. 5B, panel 6, the M4 + M2(270–280) was apically targeted indicating that 11 amino acids from the third intracellular loop of M2 can indeed confer apical targeting to the full-length M4 receptor.

Attempts at further defining the apical sorting sequence within Lys280–Ser350 by dividing it into smaller sequences and appending these to the C terminus of the M4 receptor resulted in constructs that did not express well. It was therefore not possible to determine whether a smaller region of this sequence was sufficient to confer apical targeting. Nonetheless, these results indicate that there are redundant apical targeting sequences within the third intracellular loop of M2 and that M2 amino acids 270–280 and 280–350 each contain sufficient sorting information to confer apical targeting to the M4 receptor. Both apical sequences are indicated in Fig. 5C.

Disruption of Both M2 Apical Sorting Sequences 270–280 and 280–350 Results in Basolateral Accumulation of the M2 Receptor—Several constructs containing the M2 third intracellular loop deletions (diagrammed in Fig. 6A) were evaluated to determine whether sequences localized to this region were also

3 R. S. Chmela and N. M. Nathanson, unpublished observations.
**M2 mAChR Apical Sorting Signal**

A

M2 3 il:

208

220 260 300 340 380 389

M2 C-terminal addition to M4:

220 260 280

250 260 270 270 280

B

M4 + M2(220–280) M2(280–350) M2(220–260) M2(250–270) M2(260–280) M2(270–280)

270 VTENCVOQGEK 280

280 KESSNDSTSVSAVSNMRDDEITQDVSTSLGH SKDENSKCTCIIVKTQKSDSTCPTANTTVELVGS 350

FIGURE 5. M2 amino acids 270–280 and 280–350 contain autonomous apical sorting signals. A, schematic representation of the M4 third intracellular loop and the localization of constructs containing the sequences (bars) of amino acids (numbers) from the M2 third intracellular loop appended to the C terminus of the M4 mAChR. B, filter-grown MDCK cells were transfected with FLAG-tagged constructs encoding the following: panel 1, M4 + C-terminal addition of M2(220–280); panel 2, M4 + C-terminal addition of M2(280–350); panel 3, M4 + C-terminal addition of M2(220–260); panel 4, M4 + C-terminal addition of M2(250–270); panel 5, M4 + C-terminal addition of M2(260–280); and panel 6, M4 + C-terminal addition of M2(270–280). Cells were fixed, stained, and visualized by confocal microscopy as described under "Experimental Procedures." The upper panels are images taken in the x-y plane (projected z-series), and the lower panels are x-z images of a vertical section taken at or near the midpoint of the image. Each image is representative of at least three independent experiments. Bar = 20 μm. C, apical sorting sequences of the M2 mAChR, amino acids 270–280 and 280–350.

necessary for the apical targeting of the M2 receptor. First, to determine whether deletion of the M2 third intracellular loop was sufficient to cause mis-localization of the receptor, amino acids 220–380 (comprising 89% of the third intracellular loop) were deleted. Although less than 10% (8.0 ± 4.4%, n = 6, where n is the number of independent transfections, between 2 and 14 cells were quantitated in each experiment; mean ± S.E.) of the total staining for the wild type receptor was present in the basolateral domain, the deletion mutant receptor M2(del 220–380) displayed a significant increase in basolateral staining (30.8 ± 10.1%, n = 5). A series of mutants containing smaller overlapping deletions was generated to determine the necessity of those sequences for the apical targeting of the M2 receptor. Although deletion mutants M2(del 220–280), M2(del 280–325), and M2(del 303–380) displayed little or no increase in the amount of basolateral staining of the receptor when compared with wild type (10.4 ± 4.6%, n = 2; 10.5 ± 4.1%, n = 5; 23.0 ± 5.7, n = 6), deletion of amino acids 227–315, which disrupts both apical sorting sequences, resulted in the largest degree of basolateral accumulation (40.5 ± 6.4%, n = 5). These results support the finding that the M2 receptor contains redundant sorting information because either sorting sequence was sufficient to direct the receptor apically, and only when both were deleted was there significant mis-localization of the receptor.

The M2 Receptor Is Not Found in Detergent-resistant Membranes—Sphingolipid- and cholesterol-rich microdomains called rafts are postulated to form sorting platforms within the TGN that concentrate apically targeted proteins into transport vesicles (49, 50). Association with rafts has been shown to be crucial for the correct targeting of a number of apically directed proteins in MDCK cells, as disruption of rafts by cholesterol depletion often results in the mistargeting of these proteins (51–53). To determine whether the M2 mAChR receptor achieves its apical localization in MDCK cells through association with rafts, confluent MDCK cells stably expressing FLAG-M2 were extracted with the nonionic detergent Triton X-100 on ice followed by density gradient centrifugation using a multitstep gradient. As shown in Fig. 7B, the M2 receptor was not found in appreciable amounts in the detergent-resistant buoyant fractions of the gradient that contained the well-characterized raft marker caveolin-1. It was, however, found in highest concentration (94% of total) in the high density detergent-soluble fractions together with the nonraft marker transferrin at the bottom of the gradient. Quantitation of the immunoreactivity throughout the density gradient of each of the proteins is provided in Fig. 7B. These results indicate that by the method of detergent extraction, the M2 mAChR is not significantly associated with detergent-resistant lipid rafts.
FIGURE 6. Deletion of M₂ amino acids 227–316 compromises the ability of M₂ to achieve its apical localization. A, schematic representation of the M₂ third intracellular loop and the deletions (indicated by lines) of the amino acids (numbers) of each M₂ deletion construct. B, filter-grown MDCK cells were transfected with FLAG-tagged constructs encoding the following: panel 1, M₂; panel 2, M₂(del 220–380); panel 3, M₂(del 220–280); panel 4, M₂(del 227–316); panel 5, M₂(del 303–380); and panel 6, M₂(del 280–325). Cells were fixed, stained, and visualized by confocal microscopy as described under “Experimental Procedures.” The upper panels are images taken in the x-y plane (projected z-series), and the lower panels are x-z images of a vertical section taken at or near the midpoint of the image. Each image is representative of at least three independent experiments. Bar = 20 μm.

FIGURE 7. Lack of lipid raft association of the M₂ mAChR as determined by Triton X-100 extraction followed by OptiPrep™ gradient centrifugation. A, MDCK cells stably expressing the M₂ mAChR were extracted with Triton X-100 on ice, and extracted membranes were subjected to an OptiPrep™ multistep gradient. Fractions were collected from the top and were analyzed by Western blotting as described under “Experimental Procedures.” Each image is representative of two other experiments. B, quantitative analyses of the protein present in each fraction of the gradient. The intensity of each band was quantitated using NIH Image and expressed as a percentage of the total. % Opti-prep

MD₄ mAChR Caveolin-1 Transferrin

A

Fraction #: 1 2 3 4 5 6 7 8 9 10 11 12

M₂ mAChR Caveolin-1 Transferrin

B

Fraction #: 1 2 3 4 5 6 7 8 9 10 11 12

Lack of lipid raft association of the M₂ mAChR as determined by Triton X-100 extraction followed by OptiPrep™ gradient centrifugation. A, MDCK cells stably expressing the M₂ mAChR were extracted with Triton X-100 on ice, and extracted membranes were subjected to an OptiPrep™ multistep gradient. Fractions were collected from the top and were analyzed by Western blotting as described under “Experimental Procedures.” Each image is representative of two other experiments. B, quantitative analyses of the protein present in each fraction of the gradient. The intensity of each band was quantitated using NIH Image and expressed as a percentage of the total.

M₂ mAChR Apical Sorting Signal

sphingolipids and exhibits a particularly strong affinity for the GM1 glycosphingolipid, a widely used indicator for the distribution of lipid rafts (56). Cholera toxin binding to five cell surface GM1 molecules leads to co-clustering of various glycosylated raft-associated proteins that are further aggregated, or “patched,” by exposure to anti-ChToxβ antibody, which brings entire lipid microdomains together into patches (21, 57).

To verify the ability of patched ChToxβ-594 to aggregate the cell-surface raft marker GM1, live MDCK cells were labeled with a fluorescent-conjugated GM1 lipid, GM1-BODIPY, treated with ChToxβ-594, and then either fixed or patched with anti-ChToxβ antibody and then fixed. As shown in Fig. 8A, panel 1, and quantitated in Fig. 8C, unpatched GM1-BODIPY displayed a homogeneous distribution and minimally colocalized with ChToxβ-594 (23.5 ± 1.7% colocalization, mean ± S.D., average of three cells from a single experiment). After patching with anti-ChToxβ antibody (Fig. 8A, panel 2), both the ChToxβ-594 and the GM1-BODIPY labeling appeared more punctate, and there was a significant increase in colocalization between anti-ChToxβ-induced patches and aggregated GM1-BODIPY (67.6 ± 19.9% colocalization, three cells from a single experiment).

We next investigated whether or not the M₂ receptor was associated with ChToxβ-induced patches. When live cells were treated with ChToxβ-594, fixed, and stained with anti-FLAG antibody, a homogeneous distribution of both FLAG-M₂ and ChToxβ-594 was detected, and the overlay of the two images (Fig. 8B, panel 3) as well as quantitation indicate a low level of colocalization (15.3 ± 3.2% colocalization, three cells from two independent experiments; Fig. 8C). When ChToxβ-594-labeled cells were patched using anti-ChToxβ antibody, a more punctate distribution of ChToxβ-594 labeling was apparent (Fig. 8B, panel 4); however, there was minimal effect on the distribution of either the M₂ receptor or on colocalization of the M₂ recep-
$M_2$ mAChR Apical Sorting Signal

A

| GM1 | ChTx-β | overlay | 5X |
|-----|--------|---------|----|
| 1   |        |         |    |

unpatched

| patched ChTx-β |
|----------------|
| 2              |

B

| M$_2$ mAChR | ChTx-β | overlay |
|-------------|--------|---------|
| 3           |        |         |

unpatched

| patched ChTx-β |
|----------------|
| 4              |

| patched ChTx-β and patched M$_2$ mAChR |
|----------------------------------------|
| 5                                       |

Bar = 20 μm

C

![Graph showing percent co-localization](image)

- unpatched
- ChTx patched
- $M_2$-ChTx patched
- patched
- patched ChTx

$M_2$ and ChTx

GM1 and ChTx
tor with ChTxβ-594 patches (14.6 ± 8.0% colocalization, five cells from three independent experiments; Fig. 8C). To increase the resolution of the staining for the M2 receptor, and to enhance the visualization of colocalization with lipid rafts, both the M2 receptor and ChTx were co-patched by simultaneously treating cells with mouse anti-FLAG antibody and ChTxβ-594 on ice, and then patching with anti-mouse FITC-conjugated secondary antibody and anti-ChTxβ antibody (Fig. 8B, panel 5). Under these conditions, staining for the M2 receptor indeed appeared more punctate; however, there was little increase in colocalization between M2 receptor and ChTxβ-594 labeling (24.2 ± 3.5% colocalization, eight cells from two independent experiments; Fig. 8C), as the vast majority of receptor labeling was found outside the ChTxβ-induced patches.

35S-Metabolic Labeling and Surface Biotinylation of MDCK Cells Stably Expressing Epitope-tagged M2 mAChR—Although in most epithelial cells, including MDCK cells, newly synthesized apical and basolateral proteins are targeted directly from the TGN to their final surface localization, the Na+/K+-ATPase (58) and the α2-adrenergic receptor (6) achieve their final basolateral localization after random surface delivery and selective stabilization at the basolateral domain, whereas the polymeric immunoglobulin A receptor (plgA-R) (59, 60) is targeted directly to the basolateral surface of MDCK cells and subsequently achieves its apical localization via transcytosis. To determine the pathway by which the M2mAChR achieves its steady-state apical distribution when expressed in MDCK cells, FLAG-M2 stably expressing cells were metabolically labeled and surface-biotinylated, and proteins were immunoprecipitated with anti-FLAG antibody, followed by streptavidin precipitation, and analyzed by SDS-PAGE, followed by autoradiography. As shown in Fig. 9, at 15 min, newly synthesized epitope-tagged M2 mAChR was first detected on the basolateral surface, whereas the amount of receptor present on the apical domain was similar to that of control (representing nonbiotinylated samples). At 30 min, the majority of newly synthesized epitope-tagged M2 mAChR remained localized at the basolateral surface (83.6 ± 13.7%, n = 2 independent experiments); however, significant apical accumulation was observed. By 60 min the receptor showed a greater accumulation on the apical domain when compared with the basolateral domain (60.0 ± 3.8%, n = 2). The identity of the band as the M2 receptor was ensured by concurrently subjecting a membrane sample of the same M2 stably expressing MDCK cells to SDS-PAGE and matching the migration of the immunoreactive M2 band (using a monoclonal anti-M2 antibody) with that of the radioactive signal.3 These results suggest that the mobility of M2 mAChR accumulates initially on the basolateral surface of MDCK cells, and within an hour the majority of the receptor is present on the apical domain.

Time Course of Expression of the M2 mAChR in Newly Transfected MDCK Cells as Monitored by Confocal Microscopy—As discussed above, the metabolic labeling studies indicate that the M2 mAChR accumulates initially within the basolateral domain of MDCK cells. As a further test of this result, the fusion protein FLAG-tagged M2-GFP was transiently transfected into MDCK cells. The surface expression of the newly synthesized M2 receptor was analyzed by staining (red) for the FLAG epitope under nonpermeabilizing conditions, whereas GFP fluorescence allows visualization of total cellular M2 expression. Because preliminary experiments indicated that the receptor started to appear on the cell surface 12 h after the start of transfection, the time course of receptor expression was determined using 12 h as the initial time point. Although the expression level varied among cells, only the highest expressing cells were analyzed at the stated time points. Twelve hours after transfection (t = 0; Fig. 10), the surface staining of very faint M2-GFP expressing cells was undetectable above background staining. Within 30 min (t = 30; Fig. 10), the surface distribution of the receptor in the most highly expressing cells appeared basolateral. By 60 min, the apical domain was enriched with M2 receptor compared with expression within the basolateral domain (t = 60; Fig. 10). At 120 min, the M2 distribution appeared apical in highly expressing MDCK cells (t = 120; Fig. 10). However, if the gain at which the confocal images were collected was unaltered from that which was used at earlier time points, the image corresponding to t = 120 min did indeed display M2 immunoreactivity basolaterally as well.3 The gain was lowered for collection at these later time points in order to avoid over-

![FIGURE 8. Lack of lipid raft association of the M2 mAChR as determined by cholera toxin patching. A, patching of BODIPY GM1 ganglioside. MDCK cells stably expressing FLAG-tagged M2 were simultaneously labeled with ChTxβ-594 and BODIPY fluoroescently labeled ganglioside. Cells were then either fixed (panel 1) or patched (panel 2) with anti-ChTxβ antibody, fixed, and then visualized by confocal microscopy as described under “Experimental Procedures.” The 1st column indicates BODIPY fluorescence; the 2nd column indicates ChTxβ-594 fluorescence, and the 3rd column is an overlay of the first two columns. B, MDCK cells stably expressing FLAG-tagged M2 were labeled with the AlexaFluor 594-conjugated cholera toxin β (ChTxβ-594) subunit. Cells were then either fixed, stained with mouse anti-FLAG antibody, and labeled with FITC-conjugated anti-mouse antibody without patching (control) (panel 3); patched with anti-ChTxβ antibody, fixed, stained with mouse anti-FLAG antibody, and labeled with FITC-conjugated anti-mouse antibody (panel 4), or treated with mouse anti-FLAG antibody and co-patched with both anti-ChTxβ and FITC-conjugated anti-mouse antibodies (panel 5). Cells were then visualized by confocal microscopy as described under “Experimental Procedures.” The 1st column indicates FITC fluorescence, the 2nd column indicates ChTxβ-594 fluorescence, and the 3rd column is an overlay of the first two columns. Further ×5 magnifications of the white squares present in each of the merged images are presented to the right. Each image is representative of at least two other experiments. Bar = 20 μm. C, colocalization of fluorophores for each experimental condition was quantitated as described under “Experimental Procedures” and expressed as percent colocalization.](image-url)
M2 mAChR Apical Sorting Signal

### FIGURE 10. Time course of surface expression of M2-GFP

Filter-grown MDCK cells were transfected with FLAG-tagged M2-GFP, fixed at t = 0 (panel 1) when surface expression was first observed (8 h after transfection), t = 30 min (panel 2), t = 60 min (panel 3), and t = 120 min (panel 4). Cells were stained with mouse anti-FLAG antibody, labeled with Alexa-568 conjugated secondary antibody, and visualized using confocal microscopy as described under “Experimental Procedures.” The 1st column indicates GFP fluorescence; the 2nd column indicates Alexa-568 fluorescence, and the 3rd column is an overlay of the first two columns. The upper panels are images taken in the x-y plane (projected z-series), and the lower panels are x-z images of a vertical section taken at or near the midpoint of the image. Each image is representative of at least two other experiments. Bar = 20 μm.

Inhibition of Surface Delivery of Vesicles Containing Newly Synthesized M2 mAChR by Tannic Acid Treatment—To confirm that the M2 mAChR was first delivered to the basolateral domain before its subsequent appearance at the apical membrane, MDCK cells were transfected with FLAG-M2-GFP, and 12 h after the start of transfection cells were treated for varying lengths of time with tannic acid, a cell-impermeable fixative that cross-links carbohydrate groups and does not diffuse across the tight junctions (61, 62), in order to block the fusion of vesicles bearing newly synthesized proteins to either the apical or basolateral domain. As described above, 12 h after the start of transfection, the surface staining of cells expressing FLAG-M2-GFP was not detectable above background staining (t = 0; Fig. 11A). Cells treated on the apical domain with tannic acid for 30 min displayed a basolateral distribution similar to that which was observed at the t = 30 control sample in the previous section (t = 30; Fig. 11A, comparable with Fig. 10, panel 2). After 60 and 120 min of apical tannic acid treatment, the lateral staining intensity increased compared with the previous time points, whereas the delivery of M2 to the apical domain remained blocked (t = 60 and 120; Fig. 11A). When the basolateral domain of filter-grown MDCK cells was treated with tannic acid for 30, 60, and 120 min, M2 surface immunoreactivity was not apparent on either the basolateral or the apical domain but did accumulate intracellularly (t = 30, 60, 120; Fig. 11B). These results show that the M2 mAChR accumulates basolaterally when vesicle fusion to the apical domain is blocked by tannic acid treatment, and that apical surface expression is inhibited by blocking the initial delivery of the M2 mAChR to the basolateral domain of MDCK cells. The latter finding confirms that the M2 receptor must first be targeted to the basolateral domain before undergoing transcytosis to the apical domain of MDCK cells.

The physical integrity and polarization of the MDCK monolayer following treatment with tannic acid were confirmed by staining for basolaterally targeted β-catenin. The x-z view indicates that in MDCK cells transfected with M2-GFP (green), treated for 2 h with tannic acid on the apical domain and stained for β-catenin (red), the integrity of the monolayer is maintained and the basolateral distribution of β-catenin remains unperturbed (Fig. 11C). Similar results were obtained for cells treated with tannic acid on the basolateral domain.3

### DISCUSSION

The goal of this study was to characterize the molecular signals involved in the apical targeting of the M2 mAChR in MDCK cells and address the mechanism by which it achieves its steady-state distribution. The apical targeting of a transmembrane protein in MDCK cells can be mediated by a variety of signals, including glycosylation motifs and amino acid sequences located within the extracellular, transmembrane, or intracellular domain (18, 35, 63, 64). Both N- and O-glycans are postulated to mediate apical targeting in a number of proteins (35, 36, 65, 66) via incorporation into lipid rafts via putative raft-associated carbohydrate-binding lectins localized to the TGN (20, 67). However, there are examples where apical targeting may depend on glycosylation, yet be independent of raft exposure. Images were collected at similar gains for both the green and red channels to reflect what was observed using the microscope alone. The lack of yellow in the overlay projection at earlier time points is because of the intensity differences between the two labels. To ensure that surface staining was not artifactual, an analogous experiment was performed by transiently transfecting FLAG-M2 in MDCK cells and monitoring the time course of appearance of cell surface staining as described above using a fluorescein-conjugated secondary antibody, which yielded similar results.3 These results corroborate the results obtained by metabolic labeling and surface biotinylation discussed above, and again indicate basolateral surface expression of the M2 receptor prior to appearance in the apical membrane.
A combination of M2/M4 receptor chimeras implicated the M2 receptor is not required for its targeting in MDCK cells. While investigating the possibility of utilizing M2/M4 chimeric receptors to determine the location of the M2 receptor apical sorting signal, we determined that the cell surface distribution of the M4 receptor is basolateral when transiently expressed in MDCK cells. The differential steady-state distribution of the M4 receptor allowed us to use a gain-of-function approach to identify apical targeting determinants within the M4 mAChR. The high degree of sequence identity between these two receptors and their similar ability to functionally couple to the Gs class of G-proteins reduced the likelihood that chimeric proteins made by substituting portions of the M4 receptor into the M2 receptor would result in misfolded proteins. A combination of M2/M4 receptor chimeras implicated the M2 third intracellular loop as containing apical targeting information. Appending M2 third intracellular loop sequences to the C terminus of the M4 receptor confirmed this hypothesis and illustrated that M2 sorting information is functional when placed outside of the context of the third intracellular loop. Addition of 11 amino acids (Val270–Lys280) from the M2 third intracellular loop was sufficient to confer apical targeting to the M4 receptor, but appending an adjacent 71-amino acid sequence (Lys280–Ser350) to the C terminus of M4 also resulted in apical targeting. Attempts to express M4 receptors with smaller C-terminal appendages from this larger 71-amino acid sequence were unfortunately unsuccessful.

The apical targeting determinants of the M2 receptor were able to override the basolateral sorting information contained in the M4 receptor. Although a hierarchy of strength in targeting signals has been demonstrated for a number transmembrane proteins in which deletion of a basolateral targeting sequence reveals the existence of a recessive apical targeting determinant (45, 70, 71), there are studies demonstrating that an apical sorting sequence can override a basolateral targeting sequence as demonstrated here and by others (19, 72–74). Interestingly, in a previous study,
**M₂ mACHR Apical Sorting Signal**

the M₂ targeting sequence was not able to override the M₁ basolateral targeting determinant when this M₃ determinant was appended to the C-terminus of the M₂ receptor (22). These results suggest that there is no specific hierarchy in the strength of basolateral compared with apical sorting sequences in MDCK cells, but rather that the strength of the signal may be an inherent property of the signal itself.

A series of M₂ third intracellular loop deletion mutants suggested that there are multiple regions within the M₂ third intracellular loop that contribute to its apical targeting. Because deletion of nearly the entire third intracellular loop did not result in a fully unpolarized distribution of the receptor, other sequences besides those investigated here may contribute to the apical targeting of the M₂ receptor. The notion that a protein carries multiple autonomous and redundant targeting motifs is not novel. Examples include the transforming growth factor-α precursor (47), the low density lipoprotein receptor (11), and the kidney AE1 anion exchanger (75). Consistent with this idea, the M₁ mACHR receptor also displays similar redundancy of targeting signals as deletion of the primary basolateral targeting sequence identified in the M₃ receptor did not perturb its steady-state basolateral distribution (22).

It is particularly interesting that the sorting sequences of the two muscarinic receptors examined to date contain sorting determinants localized to their third intracellular loops. The majority of polytopic membrane proteins, including the G-protein-coupled receptors rhodopsin, the metabotropic glutamate receptor, and the serotonin 5-HT₁B receptor, whose expression pattern has been characterized in MDCK cells, is targeted by motifs localized to their C-terminal domains (46, 72, 76–84). A few multipass transmembrane proteins with cytosolic targeting sequences present exceptions to this paradigm. The calcium ATPase type 2 splice form contains a 45-amino acid insert within the first intracellular loop that confers apical targeting to an otherwise basolaterally targeted protein (85), the multidrug resistance protein 1 contains basolateral targeting information in its third cytoplasmic loop (86), and the liver Na⁺-taurocholate cotransporting polypeptide and prominin retain their basolateral and apical distribution, respectively, despite C-terminal truncation (73, 87). To date, none of the previously identified apical targeting sequences demonstrate homology or amino acid similarity to the targeting sequences identified within the M₁ mACHR. Whether structural similarity is conserved between apical sorting determinants has yet to be determined.

The apical sorting of many transmembrane and GPI-anchored proteins in MDCK cells is mediated by their incorporation into rafts (49, 51). Raft incorporation can occur via the association of GPI anchors with the exoplasmic leaflet of the plasma membrane (88), via N- or O-glycans present on the extracellular domains of proteins (35, 37), or via amino acids within the transmembrane domain or membrane-proximal regions of integral membrane proteins (89, 90). The relevance of raft association for apical targeting depends on the protein being investigated. In the case of the p75 neurotrophin receptor, the presence and location of membrane-proximal O-glycosylation is critical for both raft association and apical delivery (91). In some proteins, such as hemagglutinin, neuraminidase, and megalin, sequences mediating apical sorting are distinct from amino acids involved in raft association, and raft association alone is not sufficient to mediate apical targeting of these proteins (89, 90). Still other apically targeted membrane proteins such as CD3ε (63), the dipeptidyl peptidase IV (92), the glycine transporter GLYT2 (69), the glutamate transporter excitatory amino acid transporter (93), the metabotropic glutamate receptor 1b (78), and the guanylyl cyclase C (76) are not raft-associated as determined by Triton X-100 extraction. In this study, by both Triton X-100 extraction and antibody cross-linking of cholera toxin-induced patches, the M₂ mACHR was determined to be minimally associated with lipid rafts. This finding suggests that the mechanism by which the M₂ mACHR receptor is sequestered into apically targeted vesicles at the level of the TGN is not via direct association with rafts. For this protein and other nonraft associated polytopic membrane proteins possessing cytosolic apical targeting domains, the mechanism by which the protein is concentrated in apically targeted cargo vesicles has yet to be determined. Given the evidence presented here, it is indeed possible that this subset of proteins is packaged with basolaterally targeted proteins and redirected apically after achieving a basolateral membrane distribution.

Proteins can achieve their final membrane localization through one of three pathways in epithelial cells: direct delivery, selective stabilization after random delivery, and via transcytosis to one plasma membrane domain following delivery to the opposite domain (3). The data presented here demonstrate that by metabolic ³⁵S labeling and surface immunoprecipitation, as well as confocal analysis of newly transfected confluent MDCK cells, that the M₂ mACHR is first directed to the basolateral domain before it accumulates on the apical domain. This represents the first report of a seven-transmembrane receptor undergoing transcytosis in MDCK cells. For some epithelial cell types other than MDCK cells, transcytosis is a predominant mechanism by which proteins achieve their apical localization (94). Although in MDCK cells most newly synthesized apical and basolateral proteins are targeted directly from the TGN to their final surface localization, the Na⁺/K⁺-ATPase (58) and the α₁b-adrenergic receptor (6) achieve their final basolateral localization after random surface delivery and selective stabilization at the basolateral domain, and the polymeric immunoglobulin A receptor (59, 60) is targeted directly to the basolateral surface of MDCK cells and subsequently achieves its apical localization via transcytosis. Indeed, there are a number of other proteins utilizing the transcytotic pathway in MDCK cells (40, 95). Whether the initial basolateral localization of these proteins is because of infidelity in apical targeting or is reflective of a physiological role for transcytosis is not known.

The data presented here show that the apical accumulation of the M₂ mACHR occurs subsequent to its basolateral localization. ³⁵S labeling and surface biotinylation assays of stably transfected MDCK cells demonstrate that the M₁ receptor accumulates at the basolateral surface starting 15 min after radioactive label is incorporated into newly synthesized receptor. The M₂ receptor only starts to appreciably accumulate over background levels at the apical surface ~30 min after receptors are synthesized, with the majority achieving apical localization 1 h after incorporation of the radiolabel. By immunocytochem-
ical methods, the expression of newly transfected FLAG-tagged M2-GFP in MDCK cells is also first detected in the basolateral domain. Only at subsequent time points does apical surface expression of the M2 receptor become detectable.

Polishchuk et al. (62) used domain-specific treatment with tannic acid to selectively block vesicle fusion and protein delivery to either the apical or basolateral domain of MDCK cells. Blocking fusion of transport vesicles to the apical domain resulted in the basolateral accumulation of the M2 receptor, whereas blocking fusion of transport vesicles to the basolateral domain blocked the appearance of the receptor at both the basolateral and apical domains. These results are consistent with the results of both the metabolic labeling and time course analyses shown in Fig. 9 and provide further support for a requirement for basolateral transport and transcytosis prior to the ultimate transport of the M2 mAChR to the apical membrane. Blocking basolateral fusion of cargo vesicles by tannic acid resulted in the lack of any surface expression of the M2 receptor. These results suggest that basolateral surface expression is prerequisite to subsequent apical surface accumulation of the receptor and that there is a post-plasma membrane sorting mechanism for at least some apically targeted proteins in MDCK cells. We also attempted to determine whether the apical targeting determinant located within amino acids 270–280 contains solely apical targeting information or whether this sequence was required for the basolateral transit of the receptor. Unfortunately, receptor expression of the M2 C-terminal appendages to the M4 mAChR was not sufficiently strong to observe pre-steady-state accumulation at either membrane domain at time points earlier than 24 h post-transfection.3

The conclusions of the work of Polishchuk et al. (62) have recently been criticized by Paladino et al. (96), who suggested that membrane polarity is not fully achieved at 3 days in culture and that treatment with tannic acid can interfere with segregation of the apical and basolateral domains. However, our experiments reveal that polarization is achieved within the limits of our time course by the apical and basolateral localization of the M2 receptor and β-catenin, respectively, as shown in Fig. 11C. Furthermore, the metabolic labeling and domain-specific biotinylation experiments were performed on stably transfected cells ≥5 days after initiation of polarization, and provide independent confirmation of the transcytosis of the M2 receptor.

In conclusion, the results presented here demonstrate the M2 mAChR is an apically targeted protein that achieves its localization independent of raft association and that basolateral localization is prerequisite to apical accumulation. This is the first reported example of a seven-transmembrane receptor that is predominantly localized to the apical surface by undergoing transcytosis from the basolateral domain in MDCK cells. This may represent a model mechanism utilized by other proteins for their localization in MDCK cells as well as other polarized cell types.

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