Recycling of MUC1 Is Dependent on Its Palmitoylation*

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MUC1 is a mucin-like transmembrane protein expressed on the apical surface of epithelia, where it protects the cell surface. The cytoplasmic domain has numerous sites for phosphorylation and docking of proteins involved in signal transduction. In a previous study, we showed that the cytoplasmic $YXX\phi$ motif Y^{20}HPM and the tyrosine-phosphorylated Y^{60}TNP motif are required for MUC1 clathrin-mediated endocytosis through binding AP-2 and Grb2, respectively (Kinlough, C. L., Poland, P. A., Bruns, J. B., Harkleroad, K. L., and Hughey, R. P. (2004) *J. Biol. Chem.* 279, 53071–53077). Palmitoylation of transmembrane proteins can affect their membrane trafficking, and the MUC1 sequence CQC³RRK at the boundary of the transmembrane and cytoplasmic domains mimics reported site(s) of *S*-palmitoylation. [³H]Palmitate labeling of Chinese hamster ovary cells expressing MUC1 with mutations in CQC³RRK revealed that MUC1 is dually palmitoylated at the CQC motif independent of RRK. Lack of palmitoylation did not affect the cold detergent solubility profile of a chimera (Tac ectodomain and MUC1 transmembrane and cytoplasmic domains), the rate of chimera delivery to the cell surface, or its half-life. Calculation of rate constants for membrane trafficking of wild-type and mutant Tac-MUC1 indicated that the lack of palmitoylation blocked recycling, but not endocytosis, and caused the chimera to accumulate in a EGFP-Rab11-positive endosomal compartment. Mutations CQC/AQA and Y20N inhibited Tac-MUC1 co-immunoprecipitation with AP-1, although mutant Y20N had reduced rates of both endocytosis and recycling, but a normal subcellular distribution. The double mutant chimera AQA+Y20N had reduced endocytosis and recycling rates and accumulated in EGFP-Rab11-positive endosomes, indicating that palmitoylation is the dominant feature modulating MUC1 recycling from endosomes back to the plasma membrane.

MUC1 is normally expressed on the apical surface of epithelial cells, where its highly extended mucin-like structure serves a protective role by modulating clearance or retention of secreted mucins and by providing a scaffold for the presentation of glycans that are recognized by bacteria and viruses (1–9). In tumors of epithelial origin, cell polarity is lost, and MUC1 expression on all cell surfaces contributes to an aggressive tumor phenotype; the extended peptide core inhibits cell-cell and cell-matrix interactions, whereas the presence of specific glycan structures such as sialyl-Le[X] and sialyl-Le[a] can act as ligands for selectin-like molecules on endothelial cells and thereby enhance metastasis (10–13). The anti-adhesive property of MUC1 is also enhanced by its ability to compete with the cell adhesion molecule E-cadherin for binding of cytoplasmic β-catenin, an important link in the maintenance of actin interactions with the adherins junctions of epithelia (14, 15). In fact, loss of E-cadherin and aberrant localization of both β-catenin and MUC1 correlate with an aggressive tumor phenotype and a poor prognosis for the patient (16, 17). The binding of β-catenin to MUC1 is regulated by phosphorylation at adjacent sites by glycogen synthase kinase-3β, Src family kinases, the epidermal growth factor receptor, or protein kinase Cδ (15, 18–22).

MUC1 is autocatalytically cleaved within the SEA (sea urchin sperm protein, enterokinase, and agrin) domain in the endoplasmic reticulum, but the larger mucin-like subunit remains tightly associated with the small transmembrane subunit (23–25). Using antibodies directed against a peptide corresponding to the MUC1 small subunit C terminus, researchers have reported that (i) treatment of human ZR-75-1 breast cancer cells with the ErbB ligand heregulin targets a complex of γ-catenin and MUC1 to the nucleus; (ii) overexpression of MUC1 in pancreatic cancer cell lines targets β-catenin and MUC1 to the nucleus; (iii) activation of Lyn kinase in multiple myeloma cells with interleukin-7 targets a complex of β-catenin and MUC1 to the nucleus; and (iv) MUC1 is targeted to mitochondria when HCT116 colon carcinoma cells overexpressing MUC1 are stimulated with heregulin (20, 26–28). Although nuclear and cytoplasmic β-catenins were observed by one research group in breast cancer patients (17), others found β-catenin and MUC1 only in the cytoplasm and plasma membrane in both human breast cancer samples and a spontaneous mouse model of breast cancer (29–31).

The mechanism for nuclear or mitochondrial targeting of the MUC1 small subunit is unknown, but delivery of the subunit to any intracellular compartment is likely dependent on its endocytosis from the cell surface. We have reported previously that MUC1 is internalized faster with shorter glycans (32), a feature of MUC1 expressed in several human breast tumor cell lines (33–36). Replacement of the extended ectodomain of MUC1 with that of Tac (interleukin-2 receptor α-subunit) also enhances endocytosis; and using site-specific mutagenesis, we identified two new interactions of the MUC1 tail that are required for its efficient endocytosis (37). Mutation Y20N (numbered from the membrane; see sequence in Fig. 1A) inhibits both AP-2 (adaptor protein complex 2) binding and endocytosis, whereas mutation Y60N inhibits Grb2 binding and endocytosis; maximal inhibition of endocytosis was observed when both Tyr²⁰ and Tyr⁶⁰ were mutated, indicating that both adaptors are needed for clathrin-mediated endocytosis of MUC1 (37). Previous reports indicated that mutation of the sequence CQC to AQA at the boundary of the transmembrane and cytoplasmic domains (see Fig. 1) blocks surface expression of MUC1 in Madin-Darby canine kid-

ney (MDCK)[3] cells (38) and heteromeric cross-linking of the MUC1/Y isoform (lacking tandem repeats) in African green monkey kidney (BSC-1) cells (39), consistent with a functional role(s) for this motif. The context of CQC[3], between the transmembrane domain and a cluster of basic residues (RRK[6]), fits the minimal consensus for protein *S*-palmitoylation (40–42). Inhibition of transmembrane protein *S*-palmitoylation by mutation of target Cys residues has revealed multiple roles for this post-translational modification in regulating homotypic and heterotypic protein-protein interactions, association with lipid microdomains, protein maturation, and membrane trafficking. Therefore, experiments were carried out to determine whether MUC1 is *S*-palmitoylated and, if so, how this affects MUC1 expression and membrane trafficking. The results of our studies indicate that MUC1 exhibits dual palmitoylation of the CQC[3] motif and that blocking palmitoylation by mutation of CQC to AQA inhibits recycling, whereas endocytosis is unaffected. It is interesting that mutations that block palmitoylation also decrease Tac-MUC1 association with AP-1 (adaptor protein complex 1) and cause chimera accumulation in recycling endosomes. Mutation Y20N also inhibits association with AP-1 and blocks both endocytosis and recycling, but this mutant chimera maintains a normal subcellular distribution, indicating that recycling of MUC1 is predominantly regulated by its palmitoylation.

EXPERIMENTAL PROCEDURES

Cell Lines and Recombinant cDNAs—The generation of clonal Chinese hamster ovary (CHO) cells stably expressing human MUC1 with 22 tandem repeats was described previously (32). MDCK1 cells expressing MUC1 with 15 tandem repeats were prepared by transduction with a recombinant retrovirus and cloning by limiting dilution as described previously (6, 43). MUC1 with 22 tandem repeats was also subcloned into pcDNA3-neo (Invitrogen) for transient expression of MUC1 or MUC1 mutants in CHO cells by infection with recombinant vaccinia virus (vT7CP) and transfection with plasmids using Lipofectamine reagent (Invitrogen) as described previously (44). Briefly, cells were infected with vT7CP for 30 min, followed by transfection for 2.5 h before metabolic labeling as described below. The Tac-MUC1 chimera was prepared by replacing the MUC1 ectodomain with the Tac ectodomain as described previously (37). The Cys residue in the MUC1 transmembrane domain (mutant TM-C) or one or both Cys residues in the CQC[3]RRK sequence at the junction of the transmembrane and cytoplasmic domains (mutants CQC/AQC, CQC/CQA, and CQC/AQA) were changed to Ala, or RRK was changed to QQQ (mutant RRK/QQQ) by PCR-based site-directed mutagenesis. A Y20N mutation was also introduced into Tac-MUC1 mutant CQC/AQA (mutant AQA+Y20N). Clonal lines of CHO cells stably transfected with either the Tac-MUC1 chimera or Tac-MUC1 mutants (all in pcDNA3-neo) were selected by growth in G418 (0.5 mg/ml) as described previously (37). All cloned cDNAs were sequenced prior to use.

Metabolic Labeling with [³H]Palmitate—CHO cells (35-mm wells) transiently transfected with wild-type MUC1 or Cys mutants were cultured overnight in 2 ml of Dulbecco's modified Eagle's medium and Ham's F-12 medium (1:1) with 1% fetal bovine serum and either 167 mCi of [9,10-³H]palmitic acid (36.3 Ci/mmol; PerkinElmer Life Sciences) or 50 mCi of [³⁵S]Met/Cys (1000 Ci/mmol; EasyTag EXPRE³⁵S³⁵S Protein Labeling Mix, PerkinElmer Life Sciences). MUC1 was immunoprecipi-

tated from cell extracts as described previously (32) using a mixture of rabbit polyclonal antisera prepared against a peptide representing the C-terminal 17 residues of the MUC1 small subunit (C-terminal peptide and antibody prepared by Invitrogen) and mouse monoclonal antibody VU-3C6, which recognizes the tandem repeats in the N-terminal large subunit. Antibody VU-3C6 was prepared by Jo Hilgers (45) and obtained from Olivera J. Finn (University of Pittsburgh, PA). Both proteins A and G immobilized on Sepharose 4B (Sigma) were included in the overnight immunoprecipitation at 4 °C. Immunoprecipitates were analyzed after reducing SDS-PAGE with a Bio-Rad Personal Imager using a Kodak TR screen and Quantity One software.

Assay for Protein S-Palmitoylation—CHO cells (22-mm wells) were transiently transfected with wild-type Tac-MUC1, wild-type MUC1, or the corresponding RRK/QQQ or CQC/AQA mutant and assayed for *S*-palmitoylation using a protocol based on Drisdel and Green (46). The following day, cells were extracted, and Tac-MUC1, MUC1, and mutants were immunoprecipitated as described previously, except that 50 mM *N*-ethylmaleimide (Sigma) was included in the detergent extraction buffer (32, 37). Immunoprecipitates recovered with protein G conjugated to Sepharose 4B were washed as described previously (32, 37), and the beads were further incubated in 0.5 ml of either 1 M hydroxylamine (pH 7.4; Sigma) or 1 M Tris-HCl (pH 7.4; Sigma) by end-over-end rotation at room temperature for 90 min. The beads were washed twice with 1 ml of HEPES-buffered saline (HBS; 10 mM HEPES-NaOH (pH 7.4) and 150 mM NaCl) containing 0.01% SDS (Bio-Rad) and once with 1 ml of HBS prior to incubation in 0.5 ml of freshly prepared EZ-Link® (+)-biotinyl-3-maleimidopropionamidyl-3,6-dioxaoctanediamine (0.2 mg/ml; Pierce) in 50 mM Tris-HCl (pH 7) by end-over-end rotation at room temperature for 2 h. The beads were then washed twice with 1 ml of HBS containing 0.01% SDS, and the immunoprecipitates were released by heating for 2 min at 90 °C in 0.06 ml of HBS containing 1% SDS. After centrifugation in a microcentrifuge, the supernatant was recovered: 10% was retained as "total immunoprecipitate," and 90% was diluted with 0.75 ml of HBS before recovery of biotinylated protein with ImmunoPure immobilized avidin (Pierce) by end-over-end rotation overnight at 4 °C. After washing once with 1 ml of HBS containing 1% Triton X-100 and once with 1 ml of HBS, protein was eluted from the avidin-conjugated beads by heating for 3.5 min at 90 °C in 0.05 ml of SDS sample buffer containing 0.14 M β-mercaptoethanol. Samples were subjected to SDS-PAGE and immunoblotted with Armenian hamster monoclonal antibody CT2, prepared against a peptide representing the C-terminal 17 residues of the MUC1 small subunit (from Sandra J. Gendler, Mayo Clinic, Scottsdale, AZ) (22). Bands on the immunoblot were directly quantified using horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences), and a Bio-Rad VersaDoc with Quantity One software as described previously (37).

MUC1 Solubility in Cold Detergents—MDCK cells expressing MUC1 or CHO cells expressing either wild-type or CQC/AQA mutant Tac-MUC1 were scraped from a tissue culture dish into ice-cold HBS and homogenized by 20 passes through a 25-gauge needle as described by Schuck *et al.* (47). Aliquots of the homogenate (20 μl) were transferred on ice to 1 ml of detergent solution prepared in HBS: 0.5% Brij 58 (Sigma), 0.5% Tween 20 (Sigma), 0.5% Lubrol WX (Serva, Heidelberg, Germany), 20 mM CHAPS (Bio-Rad), 60 mM octyl β-D-glucopyranoside (*n*-octyl glucoside; Sigma), or 0.5% Triton X-100 (Calbiochem). Samples of 40 μl (*n* = 3), obtained before and after centrifugation at 100,000 × *g* at 4 °C (Sorvall RC-M120EX centrifuge with an RP45A rotor), were subjected to SDS-PAGE and analyzed by immunoblotting as described previously (48). For analysis of both subunits of MUC1, the nitrocellu-

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[3] The abbreviations used are: MDCK, Madin-Darby canine kidney; CHO, Chinese hamster ovary; HBS, HEPES-buffered saline; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; NHS-SS-Biotin, sulfosuccinimidyl 2-(biotinamido)ethyl-1,3'-dithiopropionate; MESNA, 2-mercaptoethanesulfonic acid sodium salt; PBS, phosphate-buffered saline; EGFP, enhanced green fluorescent protein; DRMs, detergent-resistant membranes.

lose was cut horizontally at the 66-kDa molecular mass marker (Amersham Biosciences); the top was immunoblotted with mouse monoclonal antibody B27.29 (Fujirebio Diagnostics, Inc., Malvern, PA), which recognizes the immunodominant epitope in the tandem repeat of the MUC1 large subunit from MDCK cells (220,000 Da), and the bottom was immunoblotted with Armenian hamster monoclonal antibody CT2 for the small subunit (25–30 kDa) (22). Extracts of CHO cells expressing Tac-MUC1 were also immunoblotted with antibody CT2. The secondary antibodies used for immunoblotting were horseradish peroxidase-conjugated goat anti-mouse antibody (KPL, Gaithersburg, MD) and horseradish peroxidase-conjugated goat anti-Armenian hamster antibody (Jackson ImmunoResearch Laboratories, Inc.). Reactive bands on the blots were directly quantified with the VersaDoc and Quantity One software. The solubilities of MUC1 subunits and chimeras were calculated from arbitrary VersaDoc units and are presented as means ± S.D. (percent soluble = (after centrifugation/before centrifugation) × 100) from a representative experiment.

Endocytosis and Recycling Assays—CHO cells stably expressing either wild-type or CQC/AQA, Y20N, or AQA+Y20N mutant Tac-MUC1 were assayed for endocytosis as described previously (32, 37). In brief, cells were metabolically labeled with [^{35}S]Met/Cys for 30 min and chased in medium containing Met/Cys for 90 min prior to cell-surface biotinylation on ice with sulfosuccinimidyl 2-(biotinamido)ethyl-1,3′-dithiopropionate (NHS-SS-Biotin). Cells were transferred to 37 °C for the indicated times (0–6 min) prior to stripping the cell-surface biotin on ice with the membrane-impermeant reducing agent MESNA and one wash with iodoacetic acid. To measure recycling, biotinylated ^{35}S-labeled Tac-MUC1 was internalized for 5 min at 37 °C prior to washing with MESNA, iodoacetic acid, and Dulbecco's phosphate-buffered saline (PBS) with calcium and magnesium (Mediatech, Inc., Herndon, VA) on ice. Cells were returned to 37 °C for the indicated times (0–10 min) prior to washing again with MESNA, iodoacetic acid, and Dulbecco's PBS on ice. Biotinylated ^{35}S-labeled Tac-MUC1 or mutant was recovered from anti-Tac immunoprecipitates using avidin-conjugated beads, and ^{35}S-labeled bands were quantified with a Bio-Rad Personal Imager after SDS-PAGE as described previously (37). Data are presented as the percentage of total biotinylated Tac-MUC1 (means ± S.E. from multiple experiments).

Data Analysis—Curve fitting of kinetic data was performed using IGOR Pro 4.19 (WaveMetrics, Lake Oswego, OR). Experimental data from endocytosis and recycling assays were simulated using Scheme 1, in which A represents Tac-MUC1 at the surface, and B and C represent intracellular pools of Tac-MUC1 available and unavailable, respectively, for recycling to the surface.

$$A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} B \overset{k_3}{\longrightarrow} C$$
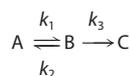
SCHEME 1

Differential equations derived from Scheme 1 (Equations 1–3) were solved using the IntegrateODE function of IGOR Pro 4.19.

$$\frac{dA}{dt} = k_2[B] - k_1[A] \tag{Eq. 1}$$

$$\frac{dB}{dt} = k_1[A] - k_2[B] - k_3[C] \tag{Eq. 2}$$

$$\frac{dC}{dt} = k_3[C] \tag{Eq. 3}$$

Data from endocytosis and recycling assays were fit simultaneously for both wild-type and mutant Tac-MUC1, with kinetic rate constants as global parameters and initial concentrations as local parameters.

Co-immunoprecipitation of Tac-MUC1 with AP-1—CHO cells (10-cm plate) were transiently transfected with Tac-MUC1; Cys mutant CQC/AQA; Tyr mutant Y8N, Y20N, or Y46N; or double mutant AQA+Y20N. Tyr mutants were prepared as described previously (37). The day after transfection, cells were extracted with detergent, and AP-1 was immunoprecipitated with mouse anti-γ-adaptin monoclonal antibody (BD Biosciences) or anti-Tac antibody using the same protocol as described previously for analyzing co-immunoprecipitation of Tac-MUC1 with AP-2 (37). The immunoprecipitates were analyzed by immunoblotting after SDS-PAGE with antibody CT2, stripped, and then reprobed with rabbit anti-μ_1-subunit antibody RY/1 (from Linton M. Traub, University of Pittsburgh) (49). Bands on Kodak MR film were analyzed with a Microtek 8700 scanner and Bio-Rad Quantity One software.

Immunofluorescence Microscopy—CHO cells expressing wild-type or CQC/AQA, Y20N, or AQA+Y20N mutant Tac-MUC1 were seeded onto poly-L-lysine (Sigma)-coated coverslips and transfected with cDNAs encoding enhanced green fluorescent protein (EGFP)-Rab11 (from Richard E. Pagano, Mayo Clinic and Foundation, Rochester, MN) (50). Twenty-four hours post-transfection, cells were rinsed with PBS, fixed with 3.7% paraformaldehyde in PBS, quenched in PBS containing 10 mM glycine, and permeabilized in blocking buffer (PBS and 0.2% fish skin gelatin) containing 0.025% saponin (all reagents from Sigma). Coverslips were incubated with anti-CD25 (Tac) monoclonal antibody (1:500 dilution; Ancell Corp., Bayport, MN) for 1 h, washed with blocking buffer, and incubated with Alexa Fluor-conjugated goat anti-mouse antibody 647 (1:500 dilution; Invitrogen) for 30 min. After extensive washing, coverslips were mounted on sides with Aqua-Poly/Mount (Polysciences, Inc., Warrington, PA). Images were captured using a Leica TCS-SL confocal microscope equipped with argon and with green and red helium neon lasers. Images were taken with a ×40 Plan-Apochromat oil objective. TIFF images were processed using Adobe Photoshop.

RESULTS

MUC1 Is Dually Palmitoylated—Experiments were carried out to determine whether the only three Cys residues present in MUC1 were palmitoylated. One or both Cys residues in the CQC motif at the boundary of the transmembrane domain and the C-terminal cytoplasmic tail (mutants CQC/AQC, CQC/CQA, and CQC/AQA) and the single Cys residue within the MUC1 transmembrane domain (mutant TM-C) were mutated to Ala. When wild-type MUC1 (CQC) or the mutants were transiently expressed in CHO cells and cultured overnight in the presence of [^{3}H]palmitate, immunoprecipitates revealed metabolic labeling of the wild-type MUC1 small subunit (CQC) and all of the mutants except CQC/AQA (Fig. 1*B*). These results indicate that there is no palmitoylation of the transmembrane Cys residue (mutant TM-C), but there is palmitoylation of both Cys residues in the CQC motif. Duplicate wells of transfected CHO cells were also labeled overnight with [^{35}S]Met/Cys, and ^{35}S-labeled MUC1 and mutants were immunoprecipitated (Fig. 1*C*). When the levels of ^{35}S-labeled MUC1 and mutants were used to normalize the incorporation of [^{3}H]palmitate, the ^{3}H/^{35}S ratio for labeling wild-type MUC1 (CQC) was 2-fold greater than that for mutants CQC/AQC, CQC/CQA, and TM-C (Fig. 1*D*). These results are consistent with [^{3}H]palmitate attachment to both Cys residues in the CQC motif and to one Cys residue in mutants CQC/AQC and CQC/CQA. However, the lower ^{3}H/^{35}S ratio found for mutant TM-C was unexpected and might reflect a secondary role for the trans-

A **Cytoplasmic tail of MUC1**

FIGURE 1. **MUC1 exhibits dual palmitoylation of the CQC motif.** The amino acid sequence of the MUC1 cytoplasmic tail indicates the sites for binding AP-2 at Y^{20}HPM, Grb2 at tyrosine-phosphorylated Y^{60}TNP, and β-catenin (*A*). The CQCRRK motif at the boundary of the transmembrane and cytoplasmic domains is *underlined*. Residues are numbered from the transmembrane domain by convention. CHO cells were transiently transfected with MUC1 (CQC) or mutant CQC/AQA, CQC/AQC, CQC/CQA, or TM-C and incubated overnight with either [^{3}H]palmitate (*B*) or [^{35}S]Met/Cys (*C*) in medium with 1% fetal bovine serum. Control cells were transfected without plasmid (*mock*). MUC1 was recovered by immunoprecipitation after SDS-PAGE prior to analysis with a Bio-Rad Personal Imager using a TR screen. *30K* refers to the mobility of the molecular mass marker (Amersham Biosciences). The level of palmitoylation was normalized to the ^{3}H/^{35}S ratio from *B* and *C* (*D*).
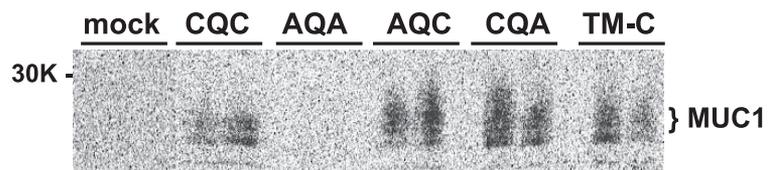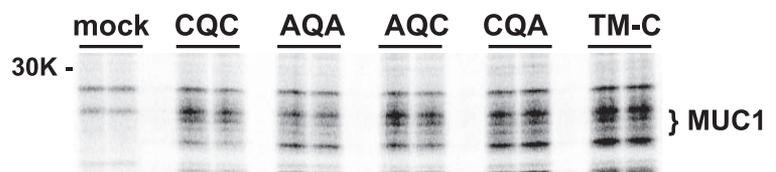
membrane domain Cys residue in normal MUC1 processing and/or membrane trafficking within the biosynthetic pathway.

Cold Detergent Solubility of MUC1 Is Independent of Its Palmitoylation—Apical delivery of some proteins is dependent on their association with lipid microdomains enriched in cholesterol and glycosphingolipids that form a liquid-ordered phase that is poorly solubilized in cold detergents such as Triton X-100, CHAPS, Brij 58, and Lubrol WX (51–54). Because cold detergent insolubility of some transmembrane proteins is dependent on their palmitoylation (55–60), we performed experiments to determine whether palmitoylation of MUC1 correlates with its association with detergent-resistant membranes (DRMs). MDCK cells expressing MUC1 were scraped and homogenized prior to solubilization of membranes in a variety of cold detergents and centrifugation at $100,000 \times g$ to remove insoluble material. The levels of MUC1 in the homogenate and supernatant were compared using the VersaDoc to directly quantify bands on an immunoblot. As shown in Fig. 2*A*, the small subunit of MUC1 including the transmembrane domain was fully soluble in cold *n*-octyl glucoside and Triton X-100, mostly insoluble in cold Lubrol WX and CHAPS, and almost completely insoluble in cold Brij 58 and Tween 20. The large subunit of MUC1 was more soluble than the small subunit in cold Tween 20, Lubrol WX, and CHAPS. Although Julian and Carson (61) reported that MUC1 heterodimers are SDS-labile but resistant to treatment by boiling, urea, sulfhydryl reduction, peroxide, high salt, or low pH, our results indicate that the subunits of MUC1 also dissociate in the presence of some nonionic detergents. Macao *et al.* (25) recently described exposed hydrophobic residues specific to the SEA domain in MUC1 that could account for this observed effect.

Recombinant MUC1 was previously localized to the apical surface of polarized MDCK cells, but mutation of either one or both Cys residues in the CQC motif blocks surface expression of MUC1 in these cells (38, 48). However, we found that both MUC1 and a chimera prepared with the Tac ectodomain and the MUC1 transmembrane and cytoplasmic domains (37) were present on the surface of CHO cells even when CQC was mutated to AQA (see below). It is interesting that the cold detergent

solubility profile of the Tac-MUC1 chimera in CHO cells was very similar to the profile of the MUC1 small subunit in MDCK cells (Fig. 2*B*). The increased cold CHAPS solubility of Tac-MUC1 in CHO cells compared with the MUC1 small subunit in MDCK cells might reflect a different profile of lipids or a different environment for the chimera in the two cell lines because cold detergent solubility of proteins is determined by the characteristics of the lipid microdomains (62–64). More important, mutation of CQC to AQA did not alter the profile of cold detergent solubility, suggesting that association of Tac-MUC1 with lipid microdomains is not influenced by its palmitoylation.

Tac-MUC1 Palmitoylation Is Not Dependent on the Adjacent Basic Residues—To confirm that Tac-MUC1 is palmitoylated in CHO cells, chimera were immunoprecipitated from transiently transfected cells and assayed for *S*-palmitoylation. Wild-type and mutant Tac-MUC1 (or MUC1 as a control) were immunoprecipitated from extracts of CHO cells in the presence of 50 mM *N*-ethylmaleimide to block all free sulfhydryl groups. Immunoprecipitates were subsequently treated with 1 M hydroxylamine (or 1 M Tris-HCl as a control) to release *S*-linked palmitate and then treated with EZ-Link® (+)-biotinyl-3-maleimidopropi-onamidyl-3,6-dioxaoctanediamine to tag any free sulfhydryl groups for subsequent recovery of the biotinylated protein with avidin-conjugated beads. As shown in Fig. 3, both Tac-MUC1 and MUC1 biotinylation and recovery with avidin-conjugated beads were dependent on hydroxylamine treatment, whereas mutant CQC/AQA was not biotinylated and recovered under the same conditions, consistent with *S*-palmitoylation of both Tac-MUC1 and MUC1. Because reducing reagents were omitted from samples representing the total immunoprecipitates (Fig. 3, *lower panels*), dimers of Tac-MUC1 and the MUC1 small subunit were prevalent and included in the analysis. Under reducing conditions, the level of dimers on the gel is always directly proportional to the level of monomers. To determine whether the adjacent basic residues RRK in the CQC^{3}RRK sequence are required for *S*-palmitoylation as reported for other modified transmembrane proteins, Tac-MUC1 mutant RRK/QQQ was similarly assayed. It is surprising that mutant RRK/QQQ showed the same level of hydroxylamine-dependent biotinylation com-

pared with wild-type Tac-MUC1. By comparison with the total immunoprecipitate (Fig. 3, *lower panels*), we estimated that only 10% of total Tac-MUC1 and MUC1 was modified with palmitate, consistent with transient palmitoylation that regulates homotypic or heterotypic protein-protein interactions.
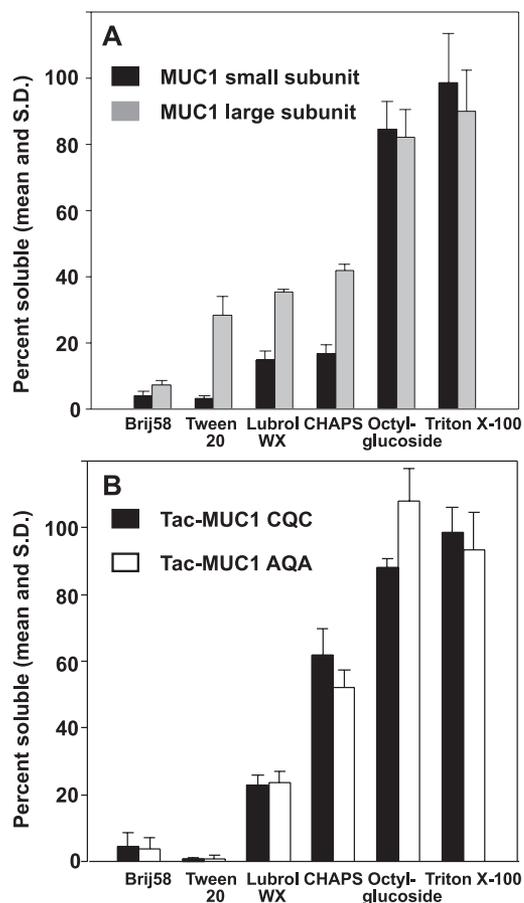
FIGURE 2. **MUC1 association with DRMs is not dependent on palmitoylation.** *A*, MDCK cells stably expressing MUC1 were scraped and homogenized prior to addition of various detergents (Brij 58, Tween 20, Lubrol WX, CHAPS, *n*-octyl glucoside, and Triton X-100) on ice as described under "Experimental Procedures." Solubility was determined by immunoblotting for MUC1 with either monoclonal antibody B27.29 (large subunit) or monoclonal antibody CT2 (small subunit) before and after centrifugation at $100,000 \times g$ using a Bio-Rad VersaDoc. *B*, CHO cells stably expressing either wild-type (CQC) or CQC/AQA mutant Tac-MUC1 were solubilized in the same detergents as described for *A*. Aliquots were analyzed by immunoblotting with antibody CT2 before and after centrifugation using a Bio-Rad VersaDoc. The percent of soluble MUC1 or Tac-MUC1 is presented as the means ± S.D. from representative experiments.

Blocking Tac-MUC1 Palmitoylation Does Not Affect Chimera Synthesis or Degradation in CHO Cells—To determine whether palmitoylation of Tac-MUC1 affects its delivery to the cell surface from the biosynthetic pathway, CHO cells expressing wild-type or CQC/AQA mutant Tac-MUC1 were pulse-labeled for 30 min with [35S]Met/Cys, and surface delivery was monitored after 0–90 min of chase by treatment of cells with membrane-impermeant NHS-SS-Biotin. As shown in Fig. 4 (*A* and *B*), the rates of delivery to the CHO cell surface were similar for the wild-type chimera and mutant CQC/AQA. Similar data were obtained for MUC1 and the corresponding CQC/AQA mutant.[4] Tac-MUC1 stability was assessed with a similar protocol. Cells were pulse-labeled for 30 min, chased for 90 min prior to surface biotinylation, and returned to culture for 0–135 min. The apparent half-life of mutant chimera CQC/AQA (*open circles*) was slightly less than the half-life of the wild-type chimera (*closed circles*) for either the total chimera (Fig. 4C) or the surface chimera (Fig. 4D), but analysis of the data revealed no statistically significant difference. A similar analysis of 35S-labeled wild-type MUC1 (*closed circles*) and the corresponding CQC/AQA mutant (*open circles*) also revealed no statistically significant difference in stability due to the lack of *S*-palmitoylation for either total (Fig. 4E) or surface-biotinylated (Fig. 4F) MUC1.

Tac-MUC1 Membrane Trafficking at the Cell Surface Is Regulated by Palmitoylation and Adaptor Complex Binding—Because our previous characterization of cytoplasmic signals that direct MUC1 endocytosis was carried out with the Tac-MUC1 chimera, cells stably expressing wild-type or CQC/AQA mutant Tac-MUC1 were similarly assayed. Cells were metabolically labeled with [35S]Met/Cys for 30 min and chased for 90 min to allow the 35S-labeled chimeras to reach the cell surface. Cell-surface proteins were biotinylated on ice with NHS-SS-Biotin, and cells were returned to culture for 0–6 min to allow endocytosis; surface biotin was then stripped from the cells on ice with the membrane-impermeant reducing agent MESNA. Biotinylated 35S-labeled Tac-MUC1 was recovered from immunoprecipitates using avidin-conjugated beads and analyzed with a Bio-Rad Personal Imager after SDS-PAGE by comparison with the amount of the total biotinylated chimera (100%). Internalization of CQC/AQA mutant Tac-MUC1 (Fig. 5B) was notably faster than internalization of wild-type Tac-MUC1 (Fig. 5A); after 6 min, there was 60% more intracellular mutant chimera than wild-type chimera. Because our endocytosis assay measures intracellular biotinylated 35S-labeled chimera that is protected from MESNA stripping, the apparent increase in internalization could also reflect a decrease in chimera recycling. To examine this pos-

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[4] J. B. Bruns and R. P. Hughey, unpublished data.
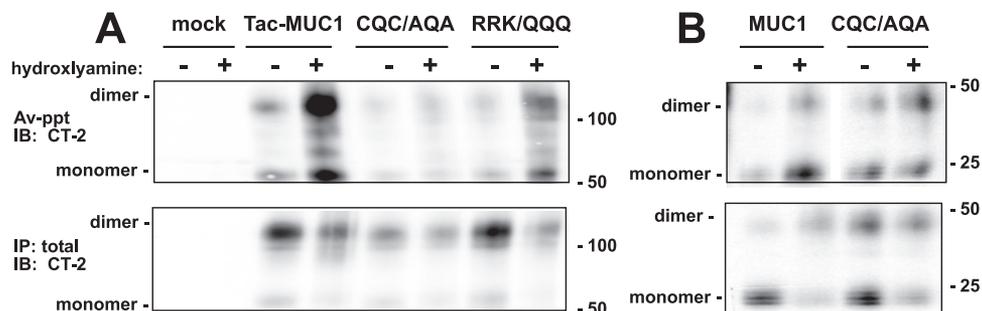
FIGURE 3. **Tac-MUC1 palmitoylation is not dependent on the adjacent basic residues.** CHO cells transiently transfected with wild-type Tac-MUC1 or a chimera with mutation CQC/AQA or RRK/QQQ in the CQC3RRK sequence of the cytoplasmic tail (*A*) or with MUC1 or the corresponding CQC/AQA mutant (*B*) were extracted in detergent containing *N*-ethylmaleimide prior to immunoprecipitation with anti-Tac (*A*) or anti-MUC1 (VU-3C6; *B*) antibody, treatment with (+) or without (−) hydroxylamine to remove *S*-palmitate, and biotinylation of free sulfhydryl groups. A sample of the total immunoprecipitate (*IP*; 10%) was analyzed for comparison with biotinylated protein recovered with avidin-conjugated beads (*Av-ppt*; 90%). Both monomers (55 kDa) and dimers (110 kDa) of Tac-MUC1 (*A*) and monomers (22 kDa) and dimers (45 kDa) of the MUC1 small subunit (*B*) are indicated. *IB*, immunoblot.
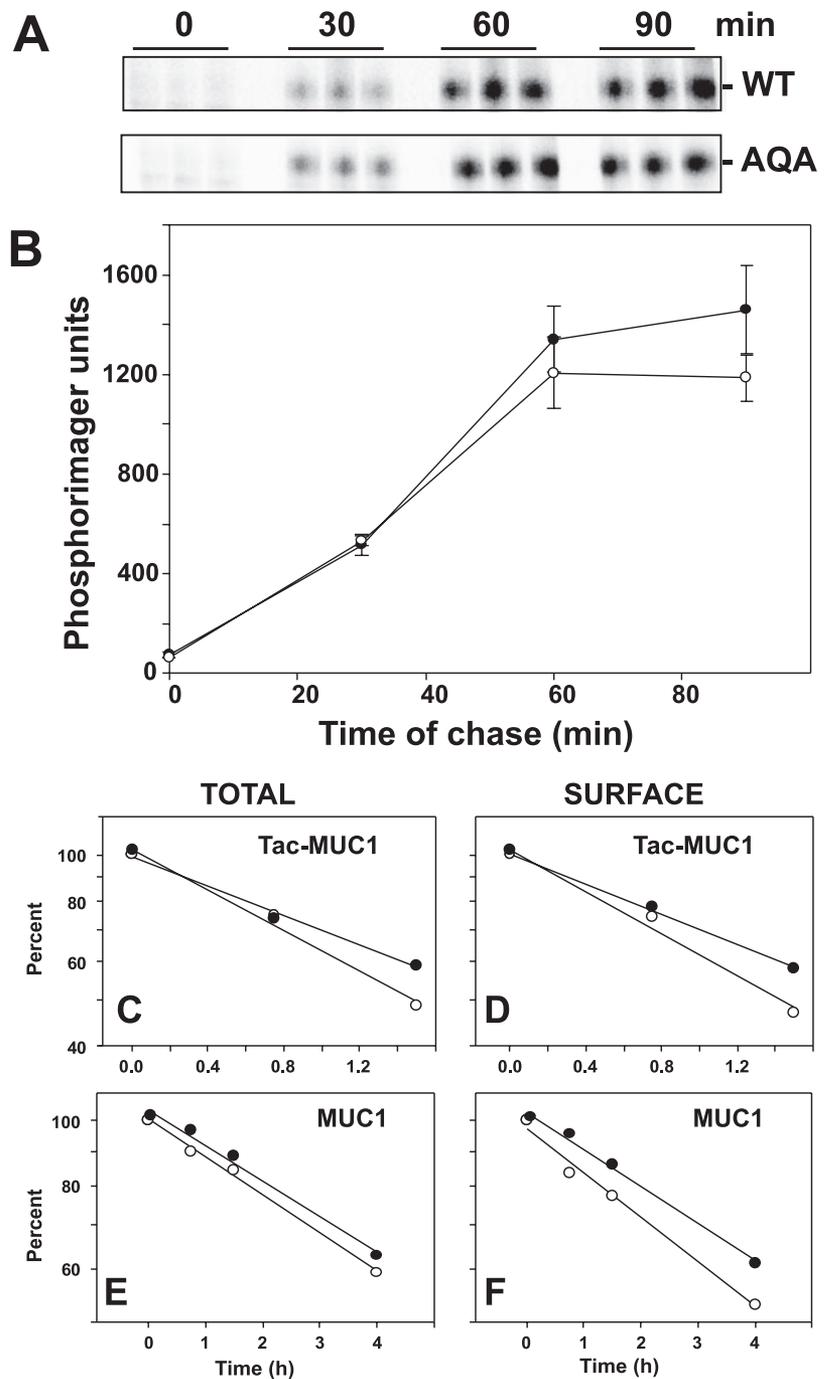
FIGURE 4. **Synthesis and stability of MUC1 are not dependent on its palmitoylation.** CHO cells expressing wild-type (*WT; closed circles*) or CQC/AQA mutant (*open circles*) Tac-MUC1 ($n = 3$) were pulse-labeled for 30 min with [^{35}S]Met/Cys prior to chase for 0–90 min and treatment of the cell surface with NHS-SS-Biotin. The biotinylated chimeras were recovered from anti-Tac immunoprecipitates using avidin-conjugated beads and analyzed with a Bio-Rad Personal Imager after SDS-PAGE and are presented as the means ± S.D. (*A* and *B*). The half-lives of total (*C* and *E*) and surface (*D* and *F*) Tac-MUC1 (*C* and *D*) and MUC1 (*E* and *F*) were estimated after a 30-min pulse with [^{35}S]Met/Cys, a 90-min chase, and treatment of the cell surface with NHS-SS-Biotin prior to returning cells to culture for the indicated times. Although the half-life of mutant CQC/AQA (*open circles*) was consistently shorter than that of wild-type (*closed circles*) Tac-MUC1 or MUC1, there was no statistically significant difference between the wild type and mutant in any case.

sibility, recycling of Tac-MUC1 from endosomes to the cell surface was measured by first internalizing the biotinylated ^{35}S-labeled chimeras for 5 min prior to stripping surface biotin with MESNA and then returning the cells to culture for 0–10 min prior to a second stripping with MESNA (Fig. 5, *E* and *F*). When data from the endocytosis and recycling assays were analyzed as described under "Experimental Procedures," the curves that best fit the profiles indicated that the rates of endocytosis of wild-type and CQC/AQA mutant Tac-MUC1 were not different ($k_1 = 0.2 \pm 0.08$ min^{-1}), but that the rate of recycling for the mutant ($k_2 = 0.3 \pm 0.12$) was less than half that for the wild-type chimera ($k_2 = 0.8 \pm 0.2$ min^{-1}). The rate of movement from endosomes to a non-recycling compartment was also not different for wild-type and mutant Tac-MUC1 ($k_3 = 0.13 \pm 0.09$).

The cumulative data indicate that palmitoylation of Tac-MUC1 is required for normal recycling from endosomes to the cell surface and that the apparent increase in mutant CQC/AQA endocytosis is due to a decreased rate of recycling. We reported previously that endocytosis of Tac-MUC1 mutant Y20N was inhibited by 30% compared with the wild-type chimera when internalization was measured after 10 min; this was consistent with the loss of a functional Y$XX\phi$ motif (Y[20]HPM) in the cytoplasmic tail that also blocked co-immunoprecipitation with AP-2 by 75% (37). To more carefully characterize the role of this motif in MUC1 membrane trafficking, time courses of endocytosis (0–6 min) and recycling (0–10 min) were generated as already described. As shown in Fig. 5*C*, endocytosis of mutant chimera Y20N was noticeably decreased compared with the wild-type chimera (*y axes* in all panels of

Endocytosis

Recycling

FIGURE 5. **Endocytosis and recycling profiles for wild-type Tac-MUC1 and mutants.** CHO cells expressing wild-type Tac-MUC1 (*WT*; CQC) (*A* and *E*), mutant CQC/AQA (lacking palmitoylation; *B* and *F*), mutant Y20N (*C* and *G*), and double mutant AQA+Y20N (*D* and *H*) were assayed for endocytosis (*A–D*) and recycling (*E–H*) as described under "Experimental Procedures." Data at each time point are presented as the means ± S.E. from three to six experiments. The curves were computer-generated based on the best fit of the endocytosis and recycling data as described under "Experimental Procedures." The calculated rate constants (k_1 and k_2) are indicated.
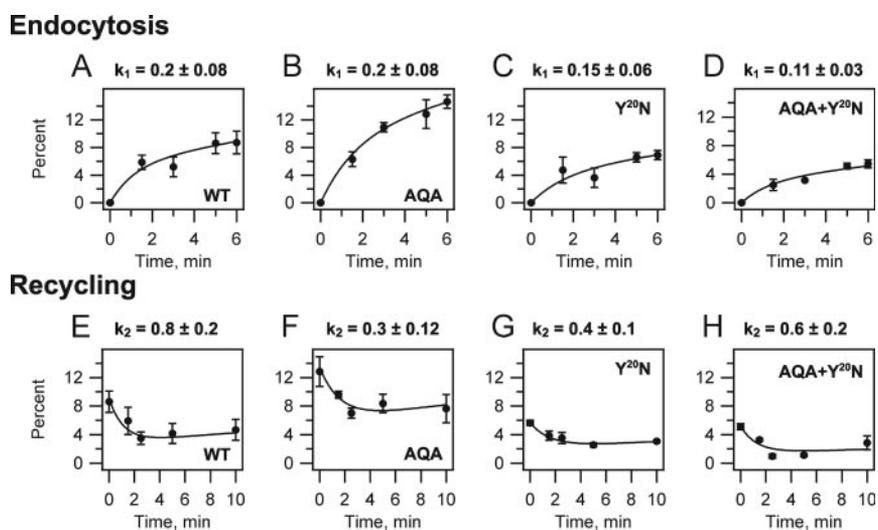
Figs. 5 are the same); after 6 min of internalization, there was 20% less mutant than wild-type chimera internalized. Using data from the endocytosis (Fig. 5*C*) and recycling (Fig. 5*G*) profiles for computer modeling, the best fit indicated that the endocytosis rate constant for Y20N ($k_1 = 0.15 \pm 0.06$ min^{-1}) was 25% lower than that for the wild-type chimera, consistent with our previously published data (37), whereas the recycling rate constant for Y20N ($k_2 = 0.4 \pm 0.1$ min^{-1}) was 50% lower than that for the wild-type chimera (and k_3 is unchanged). Analysis of double mutant AQA+Y20N revealed inhibition of both endocytosis and recycling (Fig. 5, *D* and *H*). Thus, the Y$XX\phi$ motif Y^{20}HPM in MUC1 may play a dual role in membrane trafficking by interacting with AP-2 during endocytosis and by interacting with a different adaptor in endosomes for recycling to the cell surface.

Pagano *et al.* (65) showed recently that formation of endosome-derived vesicles is dependent on AP-1 and clathrin and regulated by the small GTPase Rab4 and the adaptor rabaptin-5, which interacts with both AP-1 and Rab4. To determine whether Tac-MUC1 binds to AP-1 and whether the interaction correlates with Tac-MUC1 recycling or palmitoylation, anti-γ-adaptin immunoprecipitates were prepared from CHO cells expressing either wild-type Tac-MUC1 or a chimera mutated at CQC or one of three Y$XX\phi$-like motifs at Tyr^8, Tyr^{20}, or Tyr^{46}. As shown in Fig. 6, co-immunoprecipitation of chimeras with AP-1 was blocked by 30% by the CQC/AQA mutation and by 25% by mutation of Tyr^{20}. The interaction was not blocked by mutation of Tyr^8 or Tyr^{46}, indicating that AP-1 and AP-2 bind at the same Y$XX\phi$ motif at Y^{20}HPM.

Tac-MUC1 Recycling Is Differentially Modulated by Palmitoylation and Interaction with AP-1—To determine the subcellular site(s) where Tac-MUC1 recycling is blocked, cells expressing the wild-type or mutant (CQC/AQA, Y20N, or double mutant AQA+Y20N) chimera were analyzed by indirect immunofluorescence (Fig. 7). It is striking that, whereas the majority of wild-type or Y20N mutant Tac-MUC1 was found on the surface of CHO cells, a significant fraction of mutant CQC/AQA or double mutant AQA+Y20N accumulated in intracellular compartments. We found no co-localization of any chimera with the endosomal marker EEA1, indicating that MUC1 does not accumulate in sorting endosomes at steady state (data not shown). However, when cells were transfected with EGFP-tagged Rab11 (a marker of the recycling endosomal compartment), we found significant co-localization of EGFP-Rab11 with mutant chimeras CQC/AQA and AQA+Y20N.
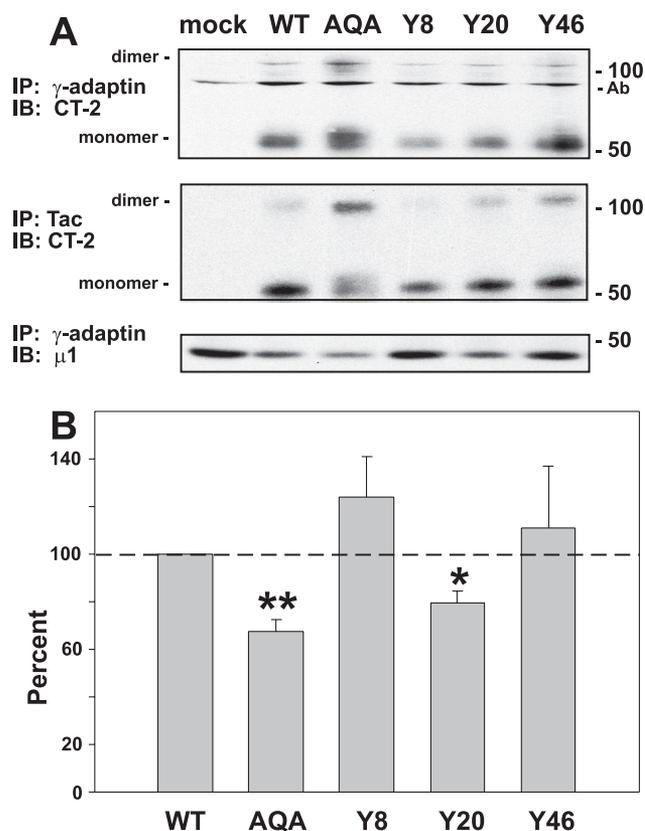
FIGURE 6. **Tac-MUC1 co-immunoprecipitation with AP-1 is blocked by mutation of the CQC or Y^{20}HPM motif.** Extracts from CHO cells transiently expressing wild-type (*WT*) or CQC/AQA mutant Tac-MUC1 or Tac-MUC1 with a tyrosine mutation at Tyr^8, Tyr^{20}, or Tyr^{46} were incubated with either anti-γ-adaptin or anti-Tac antibody prior to SDS-PAGE and immunoblotting (*IB*) with antibody CT2. Blots of anti-γ-adaptin immunoprecipitates (*IP*) were stripped and probed with anti-μ_1-subunit antibody. One representative experiment is shown in *A*, and the cumulative data (means ± S.E.) from four or five experiments are presented in *B*, where data were normalized to the wild-type chimera (100%). Statistically significant inhibition of AP-1 binding was found for mutations CQC/AQA (**, $p < 0.01$) and Y20N (*, $p < 0.05$) using Student's *t* test. Monomers (55 kDa) and dimers (110 kDa) of Tac-MUC1 and a background antibody (*Ab*) band are indicated.

DISCUSSION

MUC1 Exhibits Dual Palmitoylation at the Boundary of the Trans-membrane and Cytoplasmic Domains—There is no clear consensus sequence for palmitate addition to proteins, except that target Cys residues are usually in the vicinity of positive charges and either adjacent
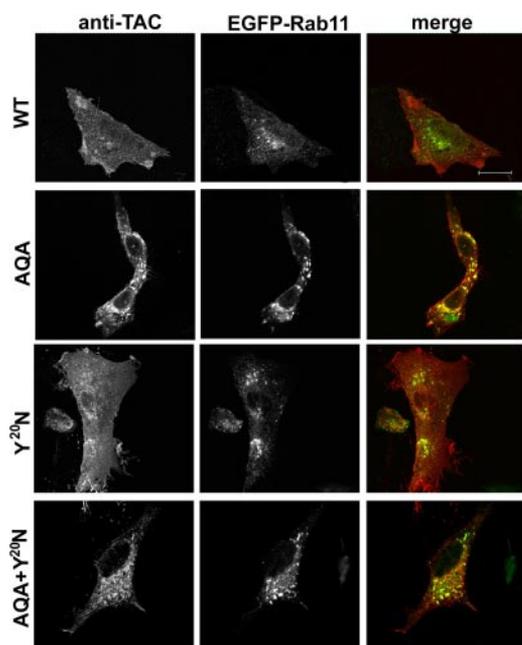
FIGURE 7. **Tac-MUC1 lacking palmitoylation accumulates in Rab11-positive endosomes.** CHO cells expressing wild-type (*WT*) or CQC/AQA, Y20N, or AQA+Y20N mutant Tac-MUC1 were plated on poly-L-lysine-coated coverslips prior to transfection with EGFP-Rab11. The following day, cells were permeabilized, incubated with anti-Tac antibody and Alexa Fluor-conjugated secondary antibodies, and viewed by confocal microscopy to detect Tac-MUC1 (*left panels*) and EGFP-Rab11 (*middle panels*). Merged images are shown (*right panels*). *Scale bar* = 20 μm.

lipid anchors (*N*-myristate or *C*-prenyl groups) or transmembrane domains (41, 42). Identification of palmitoylated Cys residues in proteins generally involves site-directed mutagenesis of target residues and subsequent analysis by metabolic labeling with [3H]palmitate, although a new assay for *S*-palmitate has been reported recently (46). *S*-Palmitoylation has been found on transmembrane proteins at the immediate boundary of the transmembrane and cytoplasmic domains (56, 66–74), in the cytoplasmic tail (40, 75–78), and at both the immediate boundary and within the cytoplasmic tail (79, 80). Cys palmitoylation has also been found within the transmembrane domains of several proteins (81–83), whereas several members of the tetraspanin family exhibit palmitoylation both within transmembrane domains and at the four boundaries of these tetraspanning membrane proteins (84, 85). MUC1 contains only three Cys residues in the entire heterodimer. Using metabolic labeling with [3H]palmitate and site-directed mutagenesis of Cys to Ala, we now report that MUC1 exhibits dual *S*-palmitoylation only at the sequence CQC3RRK found at the immediate boundary of the transmembrane and cytoplasmic domain (Fig. 1). MUC1 palmitoylation at the CQC motif was also confirmed by the assay for *S*-palmitate that is based on removal of *S*-palmitate by treatment with hydroxylamine and subsequent biotinylation of the free sulfhydryl group (46). Both MUC1 and the Tac-MUC1 chimera showed hydroxylamine-dependent biotinylation in the assay, whereas the corresponding CQC/AQA mutants were not biotinylated (Fig. 3). Because palmitoylation of proteins is often dependent on adjacent basic residues, we mutated RRK to QQQ within the CQC3RRK sequence, but found that biotinylation of the mutant was no different from that observed for wild-type Tac-MUC1. It is interesting that Li *et al.* (26) suggested that the MUC1 cytoplasmic sequence CQC3RRKNYGQLD might represent a nuclear localization signal as defined for c-Myc (PAAKRVKLD), where basic residues flanked by neutral residues and the LD motif are all part of the functional signal. They also reported that mutation of RRK6 to AAA6 in MUC1 blocks heregulin-dependent nucleolar targeting of MUC1 and γ-cate-

nin and redirects both proteins to the cytoplasm (26). Future experiments will test the possibility that palmitoylation of MUC1 at the CQC3 motif modulates its trafficking to the nucleolus.

Dual Palmitoylation of MUC1 Does Not Affect Its Synthesis, Turnover, or Association with Membrane Microdomains/Rafts—The function of *S*-palmitate in transmembrane proteins has been characterized for a limited number of examples either by comparison of defined activities for wild-type *versus* Cys mutant proteins or by inhibition of protein palmitoylation with 2-bromopalmitate (86). Mutation of target Cys residues can (i) modulate exit from the endoplasmic reticulum (69, 87), (ii) alter steady-state subcellular localization (70, 78), (iii) alter specific membrane trafficking steps (66, 68, 77, 81, 87), (iv) alter homotypic and heterotypic protein-protein interactions (72, 84, 88), (v) alter the association with lipid microdomains (82, 89), or (vi) alter protein function (71, 87, 90, 91).

Because Handa *et al.* (92) and Mukherjee *et al.* (93) reported that MUC1 in T-lymphocyte cell lines is insoluble in cold Brij 58 and Triton X-100, respectively, and is enriched in low density membranes, we first surveyed the cold detergent solubility profiles for wild-type and CQC/AQA mutant Tac-MUC1 to determine whether palmitoylation is required for MUC1 association with lipid microdomains/rafts, which are also termed DRMs. Insolubility of proteins and lipids (*i.e.* DRM association) has usually been characterized with cold Triton X-100 and cold CHAPS (94, 95), but the apical pentaspan protein prominin shows 90–95% solubility in cold Triton X-100; ~50% solubility in cold Brij 58, Lubrol WX, and CHAPS; but only 5–10% solubility in cold Tween 20, indicating that there are several types of DRMs (53). Schuck *et al.* (47) reported that pelleting from membrane detergent extracts and flotation gradient protocols are equally accurate in the evaluation of DRM association, so we evaluated MUC1 and Tac-MUC1 solubility by sampling membrane detergent extracts before and after high speed centrifugation. We found that MUC1 from MDCK cells and wild-type and CQC/AQA mutant Tac-MUC1 from CHO cells showed similar profiles of detergent solubility, being insoluble in cold Brij 58 and Tween 20, partially soluble in cold Lubrol WX and CHAPS, and fully soluble in cold *n*-octyl glucoside and Triton X-100 (Fig. 2). Thus, palmitoylation of MUC1 does not affect its association with lipid microdomains. Although palmitoylation of many transmembrane proteins is required for their association with DRMs (55–60, 96), there are many examples where palmitoylated transmembrane proteins are not associated with DRMs (75, 81, 97, 98).

When we used metabolic labeling to follow the synthesis of Tac-MUC1, we found that mutation of CQC to AQA did not alter the initial rate of Tac-MUC1 delivery to the cell surface (Fig. 4). Similar data were obtained for the delivery of MUC1 and the corresponding Cys mutants TM-C, CQC/AQC, CQC/CQA, and CQC/AQA to the cell surface, indicating that MUC1 palmitoylation does not play a role in MUC1 transit of the biosynthetic pathway.4 When the half-lives of Tac-MUC1 and MUC1 were estimated by following the loss of 35S-labeled Tac-MUC1 and MUC1 with time, we consistently observed a slightly shorter half-life for mutant CQC/AQA compared with the wild-type protein, but the difference was not statistically significant (Fig. 4).

Membrane Trafficking of Some Transmembrane Proteins Is Palmitoylation-dependent—There are only a few examples from the literature in which specific steps in membrane trafficking of transmembrane proteins are affected by their own palmitoylation; in these cases, palmitoylation of the protein is likely to be transient and regulated by the activity and localization of transferases and esterases. Information on what regulates palmitate addition or removal is limited (40–42). Identification of palmitoyltransferases has been hampered by the fact that many target

Recycling of MUC1 Is Dependent on Its Palmitoylation

Cys residues in proteins can be spontaneously acylated in the presence of palmitoyl-CoA and in the absence of enzyme (for review, see Ref. 99). However, three proteins that promote palmitoylation of cytosolic proteins have been identified in *Saccharomyces cerevisiae*, and these have been localized to the endoplasmic reticulum, the Golgi complex, and the yeast vacuole (100–102). Thus far, only the rat Golgi-specific DHHC zinc finger protein has been implicated in palmitoylation of a transmembrane protein (103), although a membrane-bound palmitoyltransferase activity that modifies the transmembrane cation-dependent mannose 6-phosphate receptor has been described recently (104). Only three mammalian thioesterases that remove palmitate from proteins have been identified (41). The two protein palmitoyl thioesterases are apparently involved in protein degradation, whereas cytoplasmic acylprotein thioesterase-1 can act on intact palmitoylated soluble and transmembrane proteins (105, 106). The existence of palmitoyl thioesterases and palmitoyltransferases throughout cellular compartments is consistent with the rapid turnover of palmitate both on cytosolic proteins, where turnover regulates protein association with membranes and membrane proteins (40), and on transmembrane proteins, where turnover seems to modulate membrane trafficking of the proteins and thereby their associated activities (75, 87, 104).

The mechanism by which palmitoylation at or very near the boundary of the transmembrane and cytoplasmic domains directs membrane trafficking is not as clear. For example, mutation of palmitoylated Cys residues in the human transferrin receptor increases the rate of ^{125}I-labeled apotransferrin endocytosis in CHO cells by 46%, whereas the rate of recycling is unchanged (66). However, no difference in iron uptake was observed in chick embryo fibroblasts expressing either the wild-type or mutant human receptor, suggesting that this could represent a cell-specific function (107). Conversely, mutation of palmitoylated Cys residues in the asialoglycoprotein receptor inhibits endocytosis of ^{125}I-labeled asialo-orosomucoid in Hep-1 cells; internalization became insensitive to hyperosmotic media, indicating that uptake had shifted from clathrin-mediated endocytosis to an alternative pathway of endocytosis (68).

Trafficking of MUC1 from Endosomes Is Dependent on Palmitoylation—Computer modeling of the endocytosis and recycling profiles of wild-type and CQC/AQA mutant Tac-MUC1 indicated that recycling of MUC1 to the cell surface is regulated by its palmitoylation. Although the overall profile of Tac-MUC1 endocytosis was enhanced by blocking palmitoylation, simultaneous computer modeling of the endocytosis and recycling data showed that the rate constant k_2 for recycling was reduced by more than half for the CQC/AQA mutant, whereas the rate constant k_1 for endocytosis (and k_3 for trafficking to other intracellular compartments) was unchanged compared with those for the wild-type chimera (Fig. 5). Similar conclusions were reached when we compared endocytosis and recycling at the earliest time point in each profile. For example, endocytosis at the earliest 1.5-min point was similar for Tac-MUC1 (5.87 ± 1.03%) and the CQC/AQA mutant (6.28 ± 1.06%). However, recycling at the earliest 1.5-min point was notably slower for the CQC/AQA mutant (25 ± 3%) compared with wild-type Tac-MUC1 (31 ± 8%).

Because we found that Tac-MUC1 without palmitoylation recycles poorly, it is possible that transient palmitoylation alters the conformation of the cytoplasmic tail and its affinity for adaptor proteins or binding partners required for endocytosis or recycling. We reported previously that the Y20N mutation in the cytoplasmic tail of MUC1 inhibits both endocytosis and binding to AP-2 (37). In the present study, we found that recycling was also inhibited by the Y20N mutation, sug-

gesting that the Y^{20}HPM motif may bind AP-1 in endosomes for recycling to the plasma membrane. Although the role of AP-1 and clathrin in budding from endosomes is not yet fully appreciated (108–112), Pagano *et al.* (65) showed recently that formation of endosome-derived vesicles is dependent on AP-1 and clathrin. It is interesting that these experiments followed internalized biotinylated asialoglycoprotein receptor H1, which exhibits palmitoylation near the boundary of the transmembrane and cytoplasmic domains much like MUC1. In fact, we did find that either mutation of tyrosine in the Y^{20}HPM motif or mutation of CQC to AQA significantly inhibited AP-1 binding to Tac-MUC1, indicating a potential correlation between palmitoylation of MUC1 and its association with AP-1. Future experiments will be designed to determine whether AP-1 binding is directly affected by MUC1 palmitoylation. Moreover, we observed that the lack of palmitoylation resulted in the steady-state redistribution of a significant fraction of Tac-MUC1 to an EGFP-Rab11-positive compartment that was likely recycling endosomes. Because double mutant AQA+Y20N also accumulated in this compartment, whereas mutant Y20N had a normal steady-state distribution, it is clear that MUC1 recycling is predominantly dependent on palmitoylation rather than on AP-1 binding. The simplest explanation for our results is that palmitoylation is required for efficient cell-surface retrieval of Tac-MUC1 directly from recycling endosomes. Alternatively, palmitoylation may be required for rapid recycling of Tac-MUC1 from sorting endosomes, and lack of palmitoylation may divert the chimera to recycling endosomes.

We reported recently that MUC1 glycosylation continues during recycling, most likely by transit of the *trans*-Golgi network, where new *O*-glycans continue to be added (113). Thus, MUC1 trafficking is clearly complex and could involve several coincident pathways leading from endosomes. Future studies will be designed to dissect the complex signals that modulate these myriad pathways.

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