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 YXXø motif Y²⁰HPM and the tyrosine-phosphorylated Y⁶⁰TNP 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 CQC⁴AQA 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 AQA²Y20N 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-LeX and sialyl-Lea 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 Cβ (15, 18–22).

MUC1 is autocatalytically cleaved within the SEA (sea urchin sperm protein, genterokinase, and aggrin) 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) over-expression 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-
nef (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 CQC3 between the transmembrane domain and a cluster of basic residues (RRK)1, 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 CQC3 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 chimeric accumulation in recycling endosomes. Mutation Y20N also inhibits association with AP-1 and blocks both endocytosis and recycling, but this mutant chimaera 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 chimaera 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 CQC3RRK 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 chimaera 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 [1-14C]palmitate (100 Ci/mmol; EasyTag EXPRESSTM Protein Labeling Mix, PerkinElmer Life Sciences). MUC1 was immunoprecipitated 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 Olivia 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 Protein 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 mM hydroxyamine (pH 7.4; Sigma) or 1 mM 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 0.1% 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-dioxaoctanediolamine (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), 0.05% 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 nitrocellulose
Cells were metabolically labeled with [35S]Met/Cys for 30 min and were assayed for endocytosis as described previously (32, 37). In brief, percent soluble on ice with the membrane-impermeant reducing agent MESNA and the indicated times (0–6 min) prior to stripping the cell-surface biotin dithiopropionate (NHS-SS-Biotin). Cells were transferred to 37 °C for times (0–10 min) prior to washing again with MESNA, iodoacetic acid, and Dulbecco’s phosphate-buffered saline (PBS) with calcium and magnesium (Mediatech, Inc.). After one wash with iodoacetic acid. To measure recycling, biotinylated 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 35S-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 35S-labeled Tac-MUC1 or mutant was recovered from anti-Tac immunoprecipitates using avidin-conjugated beads, and 35S-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).

**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 [3H]palmitate, immunoprecipitates revealed metabolic labeling of the wild-type MUC1 small subunit (CQC) and all of the mutants except CQC/AQA (Fig. 1B). 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 [35S]Met/Cys, and 35S-labeled MUC1 and mutants were immunoprecipitated (Fig. 1C). When the levels of 35S-labeled MUC1 and mutants were used to normalize the incorporation of [3H]palmitate, the 3H/35S ratio for labeling wild-type MUC1 (CQC) was 2-fold greater than that for mutants CQC/AQC, CQC/CQA, and TM-C (Fig. 1D). These results are consistent with [3H]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 3H/35S ratio found for mutant TM-C was unexpected and might reflect a secondary role for the trans-
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 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. 2A, 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 reductant, 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. 2B). 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-maleimidopropionamidyl-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 CQC3RRK sequence are required 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-
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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.

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 ImageQuant after SDS-PAGE by comparison with the amount of 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-

4 J. B. Bruns and R. P. Hughey, unpublished data.
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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 \( \text{[35S]} \)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 \( \text{[35S]} \)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.

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 YXX\( \phi \) motif (Y20HPM) 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. 5C, endocytosis of mutant chimera Y20N was noticeably decreased compared with the wild-type chimera (y axes in all panels of

sibility, recycling of Tac-MUC1 from endosomes to the cell surface was measured by first internalizing the biotinylated \( \text{[35S]} \)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 \( (k_1 = 0.2 \pm 0.08 \text{ min}^{-1}) \), 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 \( (k_2 = 0.8 \pm 0.2 \text{ min}^{-1}) \). The rate of movement from endosomes to a non-recycling compartment was also not different for wild-type and mutant Tac-MUC1 \( (k_3 = 0.13 \pm 0.09) \).
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. 5C) and recycling (Fig. 5G) profiles for computer modeling, the best fit indicated that the endocytosis rate constant for Y20N ($k_1 = 0.15 \pm 0.06 \text{ min}^{-1}$) was 25% lower than that for the wild-type chimera, consistent with our previously published data (37), whereas the recycling rate constant for Y20N ($k_2 = 0.4 \pm 0.1 \text{ min}^{-1}$) 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 YXXφ motif Y20HPM 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 YXXφ-like motifs at Tyr8, Tyr20, or Tyr46. 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 Tyr20. The interaction was not blocked by mutation of Tyr8 or Tyr46, indicating that AP-1 and AP-2 bind at the same YXXφ motif at Y20HPM.

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**Endocytosis**

![Endocytosis and recycling profiles for wild-type Tac-MUC1 and mutants.](image)

**Recycling**

![Recycling profiles for wild-type Tac-MUC1 and mutants.](image)

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**DISCUSSION**

**MUC1 Exhibits Dual Palmitoylation at the Boundary of the Transmembrane 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...
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 tetranspanning 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 QQK 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 (PAAKRKVLKD), where basic residues flanked by neutral residues and the LD motif are all part of the functional signal. They also reported that mutation of RRK to AAA in MUC1 blocks heregulin-dependent nuclear targeting of MUC1 and γ-cate-
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 125I-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 CQC/AQA mutant, whereas the rate constant for 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 k3 for recycling was reduced by more than half for the CQC/AQA mutant, whereas the rate constant k1 for endocytosis (and k3 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, suggesting that the Y20N 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 asialo-glycoprotein 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 Y20N 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 chimeric MUC1 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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REFERENCES
1. Littenberg, M. J. L., Buijs, F., Vos, H. L., and Hilken, J. (1992) Cancer Res. 52, 2318–2323
2. Wesseling, J., van der Valk, S. W., Vos, H. L., Sonnenberg, A., and Hilken, J. (1995) J. Cell Biol. 129, 255–265
3. Hudson, M. J. H., Stamp, G. W. H., Hollingsworth, M. A., Pignattelli, M., and Lalani, E. (1996) Am. J. Pathol. 148, 951–960
4. van de Wiel-van Kemenade, E., Littenberg, M. J. L., de Boer, A. J., Buijs, F., Vos, H. L., Mielief, C. J. M., Hilken, J., and Figdor, C. G. (1993) Immunol. 15, 767–776
5. Wesseling, J., van der Valk, S. W., and Hilken, J. (1996) Mol. Biol. Cell 7, 565–577
6. Muller, L., Barret, A., Picart, R., and Touguer, C. (1997) J. Biol. Chem. 272, 3669–3673
7. Arcasoy, S. M., Desai, S. S., Latoche, J. L., and Filewski, J. M. (1998) Am. J. Respir. Crit. Care Med. 157, A582 (abstr.)
8. Lillehoj, E. P., Hyan, S. W., Kim, B. T., Zhang, X. G., Lee, D. I., Rowland, S., and Kim, K. C. (2001) Am. J. Physiol. 280, L181–L187
9. Lillehoj, E. P., Kim, B. T., and Kim, K. C. (2002) Am. J. Physiol. 282, L751–L756
10. Zhang, K., Baeckstro¨m, D., Brevinge, H., and Hansson, G. C. (1996) J. Cell Biol. 60, 538–549
11. Hanski, C., Haukioja, E., Zimmer, T., Oggorek, D., Devine, P., and Riecken, E. O. (1995) Cancer Res. 55, 928–933
12. Zhang, K., Baeckstro¨m, D., Brevinge, H., and Hansson, G. C. (1997) Tumour Biol. 18, 175–187
13. Marroni, G., Crottet, P., Cecconi, O., Hanasaki, K., Arufo, A., Nelson, R. M., Varki, A., and Bevilacqua, M. P. (1995) Cancer Res. 55, 4425–4431
14. Yamamoto, M., Bharti, A., Li, Y., and Kufe, D. (1997) J. Biol. Chem. 272, 12492–12494
15. Li, Y., Bharti, A., Chen, D., Gong, J., and Kufe, D. (1998) Mol. Cell. Biol. 18, 5077–5083

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104. Biscardi, J. S., Maa, M. C., Tice, D. A., Cox, M. E., Leu, T. H., and Parsons, S. J. (1999) J. Biol. Chem. 274, 8335–8343
105. Duncan, J. A., and Gilman, A. G. (2002) J. Biol. Chem. 277, 31740–31752
106. Veit, M., and Schmidt, M. F. (2001) Virology 288, 89–95
107. Jing, S. Q., and Trowbridge, I. S. (1990) J. Biol. Chem. 265, 11555–11559
108. Stoorvogel, W., Oorschot, V., and Geuze, H. J. (1996) J. Cell Biol. 132, 21–33
109. Futter, C. E., Collinson, L. M., Backer, J. M., and Hopkins, C. R. (2001) J. Cell Biol. 155, 1251–1264
110. van Dam, E. M., and Stoorvogel, W. (2002) Mol. Biol. Cell 13, 169–182
111. van Dam, E. M., ten Broeke, T., Jansen, K., Spijkers, P., and Stoorvogel, W. (2002) J. Biol. Chem. 277, 48876–48883
112. Deneka, M., Neef, M., Popa, L., van Oort, M., Sprong, H., Oorschot, V., Klumperman, J., Schu, P., and van der Sluijs, P. (2003) EMBO J. 22, 2645–2657
113. Engelmann, K., Kinleigh, C. L., Muller, S., Razawi, H., Baldus, S. E., Hughey, R. P., and Hanisch, F.-G. (2005) Glycobiology 15, 1111–1124