MUC1 Membrane Trafficking Is Modulated by Multiple Interactions*

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MUC1 is a mucin-like transmembrane protein found on the apical surface of many epithelia. Because aberrant intracellular localization of MUC1 in tumor cells correlates with an aggressive tumor and a poor prognosis for the patient, experiments were designed to characterize the features that modulate MUC1 membrane trafficking. By following [35S]Met/Cys-labeled MUC1 in glycosylation-defective Chinese hamster ovary cells, we found previously that truncation of O-glycans on MUC1 inhibited its surface expression and stimulated its internalization by clathrin-mediated endocytosis. To identify signals for MUC1 internalization that are independent of its glycosylation state, the ectodomain of MUC1 was replaced with that of Tac, and chimera endocytosis was measured by the same protocol. Endocytosis of the chimera was significantly faster than for MUC1, indicating that features of the highly extended ectodomain inhibit MUC1 internalization. Analysis of truncation mutants and tyrosine mutants showed that Tyr20 and Tyr60 were both required for efficient endocytosis. Mutation of Tyr20 significantly blocked coimmunoprecipitation of the chimera with AP-2, indicating that Y20HPM is recognized as a YXX\(^\phi\) motif by the \(\mu_2\) subunit. The tyrosine-phosphorylated Y\(^{60}\)TNP was previously identified as an SH2 site for Grb2 binding, and we found that mutation of Tyr60 blocked coimmunoprecipitation of the chimera with Grb2. This is the first indication that Grb2 plays a significant role in the endocytosis of MUC1.

MUC1 is a mucin-like type 1 transmembrane protein normally expressed on the apical surface of epithelial cells (for review, see Refs. 1 and 2). It is synthesized as a single propeptide and cleaved while in the endoplasmic reticulum to yield the large amino-terminal subunit containing O-glycosylated near-perfect tandem repeats, and the smaller carboxyl-terminal subunit containing the membrane anchor and cytoplasmic tail (3). The resulting subunits remain tightly associated; the heterodimer is SDS-labile but is resistant to boiling, urea, sulfhydryl reduction, peroxide, high salt, or low pH (4). In carcinomas, cells often lose polarity, and MUC1 is found on all surfaces of the plasma membrane. In general, MUC1 expression in tumors from breast, lung, kidney, and thyroid correlates with an aggressive tumor and increased metastasis (5–11). However, a recent immunohistological study of 71 breast carcinomas, coupled with a review of the literature, indicates that it is actually the aberrant localization of intracellular MUC1 or MUC1 in a non-apical pattern that is associated with a worse prognosis for the patient (11).

The reason for the intracellular (cytoplasmic) MUC1 staining in breast carcinomas is not clear. Schroeder et al. (12) found a tumor-specific complex between MUC1 and \(\beta\)-catenin in the cytoplasm and nucleus in metastatic lesions of breast cancer patients; aberrant cytoplasmic and nuclear levels of \(\beta\)-catenin in breast tumors also correlates with a poor prognosis for the patient (13). \(\beta\)-Catenin binding to the cytoplasmic tail of MUC1 at the SXXXXXSSLS\(^{59}\) motif is differentially modulated by phosphorylation at several adjacent sites by Src-family kinases, the epidermal growth factor receptor (EGFR), 1 protein kinase C\(\alpha\), and glycogen synthase kinase 3\(\beta\) (14–18). Although trafficking of the MUC1 cytoplasmic tail and \(\beta\)-catenin (or \(\gamma\)-catenin) to the nucleus is clearly stimulated by either ligand binding to members of the EGF receptor family or stimulation of Src-family kinases, the mechanism for this trafficking is not clear (18–20). The large subunit of MUC1 is not found with \(\beta\)-catenin in the nucleus, but immunohistochemical staining with subunit-specific antibodies indicates that both MUC1 subunits are present in the cytoplasm of breast carcinomas (18, 20, 21). Thus, it is quite likely that trafficking of the MUC1/\(\beta\)-catenin complex to the nucleus involves endocytosis of MUC1 from the cell surface as a first step.

MUC1 glycosylation is also altered in breast tumor cells (22–26), and our previous studies indicate that changes in MUC1 glycosylation can alter its membrane trafficking. We found that delivery of MUC1 to the cell surface in Chinese hamster ovary (CHO) cells is absolutely dependent on the addition of O-linked glycans to its mucin-like core of tandem repeats (27). In glycosylation-defective CHO cells, only half as much MUC1 was delivered to the cell surface when it was synthesized with shorter glycans (NeuAcGalNAc-) compared with MUC1 with normal glycans (NeuAcGal[NeuAc]GalNAc-), and MUC1 with shorter O-glycans was internalized by clathrin-mediated endocytosis at twice the rate of MUC1 with normal glycans (27). Thus, changes in MUC1 glycosylation can clearly alter its membrane trafficking and potentially its steady state subcellular localization.

We have now carried out experiments to better understand how MUC1 clathrin-mediated endocytosis is regulated independent of its heavily glycosylated ectodomain. Many transmembrane proteins at the plasma membrane use cytoplasmic internalization signals such as YXX\(\phi\) (where X is any amino

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1 The abbreviations used are: EGFR, epidermal growth factor receptor; CHO, Chinese hamster ovary; AP-2, adapter protein complex 2; MESNA, 2-mercaptoethanesulfonic acid sodium salt; HRP, horseradish peroxidase; WT, wild-type; EGF, epidermal growth factor; SH, Src homology.
acid and $\phi$ is a bulky hydrophobic residue) or $[DE]XXXL[LI]$ dileucine motifs to bind the well characterized adaptor protein complex AP-2 (28, 29). AP-2 is a cytosolic heterotetramer with additional binding sites for phosphatidylinositol 4,5-bisphosphate, clathrin heavy chain, and a wide range of other adaptor proteins, including the autosomal recessive hypercholesterolemia protein, $\beta$-arrestin, and epsin family members (for a review, see Refs. 30 and 31). In turn, the autosomal recessive hypercholesterolemia protein recognizes tyrosine-phosphorylated FXNPaY motifs, $\beta$-arrestin recognizes phosphorylated G protein-coupled receptors, and the epsin family members recognize ubiquitylated proteins through their ubiquitin interaction motifs (30, 31). Together these proteins function at the plasma membrane to concentrate cargo in clathrin-coated pits and initiate their invagination.

To focus on identification of endocytosis signals in the cytoplasmic domain of MUC1, we replaced its ectodomain with that of Tac (interleukin 2 receptor $\alpha$-subunit), a protein that has been used previously in chimeric constructs to analyze carboxy-terminal cytoplasmic targeting signals (32). Our initial analysis of cytoplasmic domain truncation-mutants produced complex results. Our subsequent focus on truncations that inhibited MUC1 endocytosis and tyrosine mutants revealed that MUC1 internalization is dependent on at least two tyrosine residues: one within a previously described binding site for the adaptor protein Grb2, and one within a binding site for the adaptor protein complex AP-2.

**EXPERIMENTAL PROCEDURES**

**Recombinant cDNAs and Transfected Cells**—The generation of clonal CHO cells expressing human MUC1 with 22 tandem repeats was described previously (27). The cDNA for full-length Tac (interleukin 2 receptor $\alpha$-subunit) was a gift from Michael S. Marks (University of Pennsylvania, Philadelphia, PA) (32). Nucleotide residues encoding the amino-terminal Tac ectodomain (239 amino acids) and both the carboxy-terminal human MUC1 transmembrane (23 amino acids) and cytoplasmic (72 amino acids) domains were amplified using PCR and Pfu DNA polymerase (Stratagene, La Jolla, CA). Primers for PCR were designed to include nucleotide restriction sites for ligation of Tac and MUC1 cDNA to form the Tac-MUC1 chimera shown in Fig. 1. Mutations of tyrosine residues or placement of stop codons within the Tac-MUC1 cytoplasmic tail was carried out by PCR-based, site-directed mutagenesis using primers with specific nucleotide changes. Clonal lines of CHO cells stably transfected with either the Tac-MUC1 chimera or Tac-MUC1 mutants in pCDNA3 (neo) (Invitrogen) were selected by growth in G-418 (0.5 mg/ml). Cells were cultured as described previously (27).

**Endocytosis Assay**—The protocol used to measure endocytosis of MUC1 in CHO cells was previously published (27). In brief, cells were metabolically labeled with $[35S]$Met/Cys for 30 min and chased in media containing Met/Cys for 90 min before cell surface biotinylation on ice with sulfosuccinimidyl 2-(biotinamido)-ethyl-1,3-dithiopropionate. Cells were moved to 37°C for the indicated times before stripping the cell surface biotin with the membrane-impermeant reducing agent MESNA on ice. Biotinylated MUC1 or Tac-MUC1 was recovered from immunoprecipitates using avidin-conjugated beads before SDS-PAGE and quantifying $[35S]$-labeled bands using the BioRad Personal Molecular Imager FX (Bio-Rad) and Quantity One software. The fraction internalized at each time point was calculated after subtracting background at time zero by using total biotinylated $[35S]$-labeled MUC1 or chimera (without MESNA stripping) as 100%. At least two clonal cell lines expressing either MUC1 with 22 tandem repeats or Tac-MUC1 chimeras were analyzed multiple times as indicated in the figure legends. The mean and S.E.M. for data from each construct are presented in the figures, and the statistical significance of the differences between data obtained with the wild-type Tac-MUC1 and the mutants was calculated using an unpaired Student's $t$ test based on equal variance. Equal variance was determined using Stata Statistical Software (ver. 7.0; Stata Corp., College Station, TX).

**Antibodies and Immunoblotting**—Mouse monoclonal antibody against MUC1 (VU-3-C6) prepared by Jo Hilgers (Free University, Amsterdam, The Netherlands) (33) was obtained from Olivia Finn (University of Pittsburgh, Pittsburgh, PA); Armenian hamster mono-

clonal antibody against a peptide representing the carboxy-terminal 17 amino acids of MUC1 (CT2) was obtained from Sandra Gendler (Mayo Clinic, Scottsdale, AZ) (34); and mouse monoclonal antibody against Tac (human CD25, clone 7G13) was purchased from Ancell Corporation (Bayport, MN). Mouse monoclonal antibody against the AP-2 $\alpha$ subunit (AP-6) was purchased from Affinity BioReagents (Golden, CO), and the rabbit polyclonal antibody (R11–29) against a peptide representing the amino-terminal sequence of the $\mu$2 subunit prepared by Juan Bonifacino (35) was obtained from Linton Traub (University of Pittsburgh). Mouse anti-Grb2 monoclonal antibody was from Research Diagnostics, Inc. (Flanders, NJ). Horseradish peroxidase (HRP)-conjugated anti-Armenian hamster antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA), and HRP-conjugated goat anti-rabbit antibodies were from Kirkegaard and Perry Laboratories (Gaithersburg, MD).

AP-2 was immunoprecipitated from detergent extracts of CHO cells using the anti-AP-2 $\alpha$ subunit mouse monoclonal antibody before immunoblotting with an Armenian hamster anti-MUC1 cytoplasmic tail antibody (CT2) and HRP-conjugated second antibody using our published protocol (36). Phosphatase inhibitors (mixture set II) and protease inhibitors (mixture set III) were added to the detergent solution as directed by the manufacturer (Calbiochem). The blots were subsequently stripped by incubation in 0.1 M glycine, pH 2.3, for 30 min at room temperature before rebloclking and immunoblotting with a rabbit antibody to subunit rabbit polyclonal antibody and HRP-conjugated second antibodies. Grb2 was immunoprecipitated from detergent extracts of CHO cells after 4–6 h in serum-free media followed by 20 min with 25 ng/ml betaestrin (R&D Systems, Minneapolis, MN). Grb2 immunoprecipitates were immunoblotted with an Armenian hamster anti-MUC1 cytoplasmic tail antibody (CT2) followed by HRP-conjugated second antibody using our published protocol (36). Phosphatase inhibitors (mixture sets I and II) and protease inhibitors (mixture set III) were added to the detergent solution as directed by the manufacturer (Calbiochem). Tac-MUC1 expressed in each cell line (1%) was immunoprecipitated as a control with mouse anti-Tac antibody and blotted with hamster CT2 antibody. Bands on film were analyzed with a Mirotek 8700 scanner and Bio-Rad Quantity One software for Fig. 5. Bands in Fig. 6 were directly quantified with a Bio-Rad Versadoc and Quantity One software.

**RESULTS**

Results from our previous studies indicated that MUC1 endocytosis was enhanced 2-fold when it was modified with truncated rather than full-length O-glycans in a glycosylation-defective CHO cell line (27). Because this effect on MUC1 clathrin-mediated endocytosis was most probably caused by a decrease in either steric hindrance or interaction of the extended mucin-like ectodomain with other proteins, a chimera was prepared with the ectodomain of Tac, a cell surface type 1 transmembrane glycoprotein that has been used previously in chimeric constructs to analyze carboxy-terminal cytoplasmic targeting signals (see Tac-MUC1 chimera design in Fig. 1) (32). Replacement of the MUC1 mucin-like ectodomain of 819 amino acids with the Tac ectodomain of 239 amino acids dramatically altered the level of endocytosis (see representative profiles and data in Fig. 2, A–C). Comparison of data from multiple experiments, using levels of $[35S]$-labeled MUC1 or chimera internalized after 10 min, indicates that this 2.7-fold increase in endocytosis that we observe in Fig. 2D, as a result of the exchange of the ectodomains, is statistically significant ($p < 0.001$). Thus, the mucin-like ectodomain of MUC1 severely inhibits internalization of this cell surface molecule.

Because our previous data indicated that MUC1 internalization proceeds by clathrin-mediated endocytosis (27), we predicted that truncation of the MUC1 cytoplasmic tail would block internalization. Binding sites for clathrin adaptor complexes are consistently found in the cytoplasmic domain of cell surface proteins. As shown in Fig. 3, removal of all but 10 amino acids in the cytoplasmic tail of Tac-MUC1 slowed endocytosis by $\sim 40\%$. Comparison of data from multiple experiments indicated that this difference in endocytosis caused by the truncation of the cytoplasmic tail is statistically significant.
(compare Tac-MUC1 mutant X10 to Tac-MUC1 with a full-length tail, WT 72, \( p < 0.01 \)). It is interesting that when endocytosis of WT 72 was compared with other truncation mutants, the results were consistent with the presence of multiple signals for endocytosis within the MUC1 cytoplasmic tail. For example, removal of 13 amino acids (mutant X59) also inhibited Tac-MUC1 endocytosis by \( \sim 40\% \) \( p < 0.05 \), but removal of 23 amino acids (mutant X49) did not alter endocytosis, and removal of 34 amino acids (mutant X38) or 47 amino acids (mutant X25) actually stimulated endocytosis \( p < 0.01 \). Truncation of cytoplasmic domains can reveal cryptic endocytosis signals that are not functional in the full-length protein (37), so the stimulation of endocytosis observed for mutants X38 and X25 is likely to be irrelevant. Because endocytosis was inhibited when residues 11 to 25 or residues 60 to 72 were removed, this is consistent with binding of adaptor proteins within these domains.

The MUC1 cytosolic tail contains seven tyrosine residues.

**FIG. 1. Structure of the Tac-MUC1 chimera.** The amino-terminal ectodomain of Tac was fused to the carboxyl-terminal transmembrane (TM) domain and cytoplasmic tail (CT) of MUC1 as indicated. The amino acid sequence of the cytoplasmic tail is presented in single letter code. The full-length tail of 72 amino acids (WT 72), the truncated mutations within the tail (X10, X25, X38, X49, and X59), and tyrosine residues mutated in the context of the full-length tail (Y8, Y20, Y46, and Y60) are underlined.

**FIG. 2. MUC1 endocytosis is affected by features in the ectodomain.** The internalization of \( ^{35} \)S-labeled MUC1 (A) or Tac-MUC1 chimera (B) from the surface of stably transfected CHO cells was followed for the indicated times as described under “Experimental Procedures.” C, triplicate samples on SDS-gels were analyzed with the BioRad Personal Molecular Imager FX, and the mean (± S.D.) levels internalized at each time point are plotted for this representative experiment; the percentage of \( ^{35} \)S-labeled MUC1 (6.9%) or Tac-MUC1 (15.8%) internalized at 10 min was calculated from the total immunoprecipitate (Total) after subtracting the background at time zero. D, the replacement of the MUC1 ectodomain with Tac significantly increased endocytosis when data were analyzed from multiple experiments. *, \( p < 0.0001 \).
Sequences around three of the tyrosines in the MUC1 tail (YGQL, Y20HPM, and Y46EKV) are consistent with the published specificity of binding to the μ2 subunit of AP-2. Because only the tyrosine residue within the YXXφ motif is absolutely essential for μ2 binding, residues Tyr8, Tyr20, and Tyr46 were mutated individually within the context of the full-length Tac-MUC1 chimera tail and stably expressed in CHO cells. No YXXφ, NPY, or dileucine endocytosis motifs are present within the domain between residues 60 and 72, but a tyrosine-phosphorylated motif (pY60TNP) in this domain has been shown previously to be a binding site for the SH2 domain of the adaptor protein Grb2 (38). Therefore, full-length Tac-MUC1 chimera with the Y60N mutation was also stably transfected in CHO cells. As shown in Fig. 4, the Y60N mutation reduced Tac-MUC1 endocytosis by 50% (p < 0.05), consistent with the presence of a YXXφ type endocytosis motif (Y20HPM) in the domain between residues 11 and 25, whereas mutation of either Tyr20 or Tyr46 had no significant affect on chimera endocytosis. However, the Y60N mutation inhibited chimera endocytosis by 50% (p < 0.01), consistent with a role for Grb2 binding in MUC1 internalization. Because neither the Y20N nor the Y60N mutation alone fully blocked Tac-MUC1 endocytosis, both mutations were simultaneously introduced into the full-length Tac-MUC1. As shown in Fig. 4, the Y20,60N double mutation reduced MUC1 endocytosis by 77% (p < 0.001) compared with WT Tac-MUC1.

To determine whether Y20HPM represents a binding site for AP-2, the adaptor protein complex was immunoprecipitated with anti-AP-2 α subunit antibodies and immunoblotted for both the Tac-MUC1 WT chimera and the μ2 subunit of AP-2 (Fig. 5, A and D). Although similar levels of the μ2 subunit appeared in all anti-α subunit immunoprecipitates, as expected, levels of the mutant chimera in the coimmunoprecipitates were decreased compared with the WT chimera. When normalized against the total level of chimera expressed in each cell line, the ratio of chimera associated with AP-2 in two different clonal cell lines expressing the Y20N mutant was decreased by 75% compared with the WT chimera (Fig. 5, B and C). Thus, Tac-MUC1 interaction with the AP-2 adaptor is mediated by the Y20HPM motif. It is interesting that the Y20N mutation reduced chimera co-immunoprecipitation by 33% compared with the WT chimera consistent with the decreased level of endocytosis observed for this mutant in Fig. 4. This indicates that AP-2 can still interact with the chimera even in the absence of Grb2 binding, but Grb2 binding apparently enhances the association of MUC1 with AP-2 by an unknown mechanism.

To determine whether Grb2 was binding to the MUC1 cytoplasmic tail when Tac-MUC1 was expressed in CHO cells, Grb2 was immunoprecipitated from CHO cells expressing WT or mutant Tac-MUC1 using anti-Grb2 antibodies and immunoblotted for Tac-MUC1 (Fig. 6). When normalized against the
The Role of Muc1 Cytosolic Tail in Modulating Its Endocytosis—The extended ectodomain of MUC1 clearly has adhesive properties because of its length, as well as adhesive properties as a result of presentation of specific glycan structures (43–53); however, MUC1 functions as far more than a membrane-tethered mucin because of its cytosolic tail-dependent association with members of the EGF receptor family (ErbB1–4) and many of the same cytosolic binding partners that interact with these receptors (14–19, 34, 38). Characterization of MUC1 cytoplasmic interactions by several groups has revealed that MUC1 plays a role in signal transduction and may function as either a cell surface sensor or a modifier of growth factor receptor signaling (for review, see Ref. 54). The MUC1 cytosolic tail can be phosphorylated on 1) Ser\(^{44}\) by glycogen synthase kinase 3\(\beta\), 2) Tyr\(^{46}\) by Src-family kinases or EGFR, 3) Thr\(^{41}\) by protein kinase C\(\delta\), and 4) Tyr\(^{60}\) by an unknown kinase. The STDRS\(^{44}\) sequence is a binding site for glycogen synthase kinase 3\(\beta\), and tyrosine-phosphorylated Y\(^{46}\)EKV and Y\(^{60}\)TNP are binding sites for Src-family kinases and the SH2 binding domain of Grb2, respectively. Phosphorylation at Ser\(^{44}\), Tyr\(^{46}\), and Thr\(^{41}\) differentially regulates \(\beta\)-catenin binding to MUC1 at the SXXXXXXS\(^{59}\) motif and most probably affects any subsequent colocalization of the MUC1 cytosolic tail with \(\beta\)-catenin (or \(\gamma\)-catenin) in the nucleus (18–20). Neither the function nor the mechanism of this nuclear localization of the MUC1 cytosolic tail is presently understood, but it is likely to reflect a new role for MUC1 in transcriptional regulation partly as a result of the stabilization of \(\beta\)-catenin. Wang et al. (55) also found phosphorylation of Tyr\(^{20}\), Tyr\(^{29}\), Tyr\(^{46}\), and Tyr\(^{60}\) when anti-CD8 antibodies were used to cross-link a chimera comprising the CD8 ectodomain and transmembrane domain with the cytosolic tail of MUC1. Phosphorylation of Tyr\(^{20}\) and Tyr\(^{29}\) would produce binding sites for the p85 adaptor of type 1a phosphatidylinositol 3-kinase (p\(^{39}\)HPM, consensus p\(^{YXXM}\)) and for the adaptor Shc (p\(^{39}\)HTH), respectively (56–59). Sites for p85 and Shc binding are also found on EGF receptor family members, and receptor-bound Shc also recruits the adaptor protein Grb2 (56). However, evidence for binding of either p85 or Shc to MUC1 has not been reported.

The results of our present study indicate that two of seven tyrosine residues in the MUC1 cytoplasmic tail are essential for efficient clathrin-mediated endocytosis. Mutation of Tyr\(^{20}\) or Tyr\(^{60}\) partially inhibited chimera endocytosis, whereas mutation of both Tyr\(^{20}\) and Tyr\(^{60}\) blocked 77% of chimera endocytosis. The residual endocytosis of the double mutant is probably caused by constitutive internalization of plasma membrane. It is interesting that truncation of the MUC1 tail to just 10 residues (CQQRRKNYGG\(^{10}\)) blocked endocytosis by only 40%. This anomaly in endocytosis of the mutant X10 could be caused by the loss of a signal for retention at the plasma membrane, such as interactions with ErbB2 or -4 present in CHO cells (60) or association with a specific lipid microdomain, thereby increasing the availability for endocytosis. Lipid microdomains enriched in cholesterol and glycosphingolipids form a liquid-ordered phase that is poorly solubilized in cold detergents; in many cell types, these domains constitute rafts or platforms on the plasma membrane for association of proteins involved in signal transduction (61–64). In fact, Handa et al. (65) recently reported that MUC1 is insoluble in cold Brij 58 and is enriched in a low density membrane fraction with both T cell-specific transducer molecules Lck and CD45, and Src-family kinases Yes and Fyn. Transmembrane proteins in detergent-resistant membranes are often acylated, and in recent studies, we found that MUC1 is palmitoylated at the CQG motif found at the

FIG. 6. Mutation of Tyr\(^{60}\) inhibits binding of Grb2. A, Grb2 was immunoprecipitated (IP) from detergent extracts of CHO cells stably expressing either Tac-MUC1 (WT) or Tac-MUC1 with either the Tyr\(^{20}\) or Tyr\(^{60}\) mutated, using the anti-Grb2 mouse monoclonal antibody before immunoblotting (IB) with an Armenia hamster anti-MUC1 cytoplasmic tail antibody (CT2). B, detergent cell extract was incubated with anti-Tac monoclonal antibodies as a control to compare expression levels. The ratio of chimera in the co-IP (A) compared with that in the total IP (B) is expressed as a ratio (C). The immunoblots and data are representative of two separate experiments. Note that two different clones of the Tyr\(^{60}\) mutant (Y60a and Y60b) expressing very different levels of chimera gave similar ratios of co-IP to total IP.
junction of the transmembrane and cytoplasmic domains.\(^2\) Thus, inefficient palmitoylation of the mutant chimera X10 or its association with other proteins in lipid microdomains could explain its anomalous rate of endocytosis in the absence of the two critical tyrosines at Tyr\(^{120}\) and Tyr\(^{130}\).

Although there are three potential tyrosine-based YXX\(\phi\) internalization motifs in the MUC1 cytosolic tail (Y\(\Phi\)GQL, Y\(\Phi\)HPM, and Y\(\Phi\)EKV), we found that only Tyr\(^{130}\) was essential for efficient endocytosis of MUC1. It is interesting that the context of Tyr\(^{130}\) (RDY\(\Phi\)HPM) is also optimal for \(\mu\)2 binding; Arg at Y-3, Asp at Y-2, Thr at Y-1, His at Y+1, and Pro at Y+2 are all favored residues for \(\mu\)2 binding, whereas the context of Tyr\(^{98}\) (RKNY\(\Phi\)GQL) and Tyr\(^{46}\) (RSPTY\(\Phi\)EKV) are less optimal (see Fig. 1) (66). Ohno et al. (67) also found that shortening the distance to eight residues between the endocytosis motif (SDYQRL) and the transmembrane domain in TGN38 caused a decrease of more than 10-fold in interactions with the clathrin heavy chain, dynamin II, or subunits of AP-2.\(^{(66)}\)

The interaction with its SH3 binding site (31, 75–77). Stang et al. (78) recently showed that a Grb2-Cbl complex binding to EGF-stimulated EGFR caused receptor recruitment to the edge of clathrin-coated pits along with the adaptor Eps-15. However, entry into the clathrin-coated pit required ubiquitination by Cbl because entry was blocked by overexpression of either dominant-negative ubiquitin (lacking two carboxyl-terminal Gly and thereby blocking ubiquitin-interacting domains on adaptor proteins) or the Cbl-binding/regulatory protein hSpry2 (78, 79). Because MUC1 exhibits sites on its cytoplasmic tail for many of the same binding partners as the EGFR, it is highly probable that Cbl will be recruited through Grb2 to MUC1.

In conclusion, we report that replacement of the highly extended ectodomain of MUC1 with that of Tac stimulated endocytosis, confirming our previous hypothesis that the ectodomain interferes with clathrin-mediated endocytosis of MUC1. We have also identified two new interactions of the complex cytoplasmic tail of MUC1 that are required for its efficient endocytosis. Although AP-2 is probably the major link between MUC1 and the clathrin heavy chain, Grb2 binding to MUC1 clearly plays a major role in incorporation of MUC1 into clathrin-coated vesicles. The most likely role for Grb2 is in the recruitment of Cbl to MUC1, and future experiments will test this possibility.

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\(^2\) R. P. Hughey, unpublished data.
