Multiple Signals Regulate Trafficking of the Mannose 6-Phosphate Uncovering Enzyme

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Running Title: Trafficking of Uncovering enzyme
SUMMARY

“Uncovering enzyme”, which catalyzes the second step in the formation of the mannose 6-phosphate recognition marker on lysosomal enzyme oligosaccharides, resides primarily in the trans-Golgi network and cycles between this compartment and the plasma membrane. Analysis of green fluorescent protein-uncovering enzyme chimeras revealed that the transmembrane segment and the first 11 residues of the 41-residue cytoplasmic tail are sufficient for retention in the trans-Golgi network. The next 8 residues (486YAYHPLQE) facilitate exit from this compartment. Kinetic studies demonstrated that the 488YHPL sequence also mediates rapid internalization at the plasma membrane. This motif binds AP-2 in GST-uncovering enzyme cytoplasmic tail pull-down assays, indicating that “uncovering enzyme” is endocytosed via clathrin-coated vesicles. Consistent with this, endogenous uncovering enzyme was detected in purified clathrin-coated vesicles. Enzyme with a Y486A mutation is internalized normally, but accumulates on the cell surface due to increased recycling to the plasma membrane. This residue is required for efficient return of the enzyme from endosomes to the trans-Golgi network. These findings indicate that the YAYHPLQE motif is recognized at several sorting sites, including the trans-Golgi network, the plasma membrane and the endosome.
INTRODUCTION

N-acetylglucosamine-1-phosphodiester α-N-acetylglucosaminidase, also known as “uncovering” enzyme or UCE\(^1\), catalyzes the second step in the formation of the Man-6-P targeting signal on lysosomal hydrolases. These residues mediate high affinity binding of the hydrolases to mannose 6-phosphate receptors in the trans-Golgi network (TGN), a necessary step in the transport of the hydrolases to lysosomes (1). We have recently shown that UCE itself resides in the TGN and cycles constitutively between this compartment and the plasma membrane (2). This previous study also established that the \(^{488}\) YHPL and C-terminal \(^{511}\) NPFKD motifs present in the cytoplasmic domain of UCE were important in the trafficking of the enzyme. Mouse L cells expressing human UCE with a Y488A mutation had 63% of total enzyme activity on the cell surface at steady state compared to 1% of wild-type enzyme activity. The H510 stop mutant was intermediate with 7% of the enzyme activity at the cell surface. Since this analysis only involved steady state measurements of enzyme activity, it was not possible to distinguish whether the mutations affected the rate of internalization, recycling of internalized UCE to the cell surface, enhanced escape of the enzyme from the TGN or some combination of these processes. Further, the molecular mechanism(s) by which the mutations alter trafficking were not explored.

In the present study we have examined the kinetics of internalization of these and additional mutant forms of UCE. We have also analyzed the ability of the cytoplasmic tail of UCE to interact with the plasma membrane adaptor complex AP-2 and with Eps15, an accessory protein that binds NPF sequences and the α subunit of AP-2. Finally, we have examined the role of the cytoplasmic tail in the exit of UCE from the TGN. Our findings indicate that multiple signals regulate the trafficking of UCE at different compartments along the plasma membrane, endosomal and TGN trafficking pathway.
EXPERIMENTAL PROCEDURES

Distribution of UCE activity

The cellular distribution of UCE activity using the standard assay with $^{3}$H GlcNAc-P-Man$\alpha$Me as substrate was performed on a variety of parental and human UCE expressing mouse L-cells plated in 24 well tissue culture dishes as previously described (2). Assays were performed on cells in buffer that contained either 1% Triton-X-100 (total UCE activity) or no detergent (surface UCE activity).

Surface Biotinylation of UCE and Internalization Assay

The surface biotinylation of UCE was carried out by the method previously described (3) on 12-well plates of confluent mouse L-cells transfected with human UCE. In brief, the plates were chilled on ice, the cells were washed with 1 ml of PBS at 4°C and then twice with 1 ml of PBS++(PBS supplemented with 0.7mM CaCl$_2$ and 0.25mM MgSO$_4$). Then 500µl PBS++ containing 1mg/ml Sulfo-NHS-SS-biotin (Pierce) were added for 30 min on ice. After the biotinylation reagent was removed the cells were quickly washed twice with ice cold Tris-buffered saline (TBS) containing 50mM glycine, pH 7.0, and then twice with TBS, pH 7.0, alone. Control plates were kept on ice to allow measurement of total cell surface biotinylated UCE. The other plates were quickly brought to 37°C with prewarmed $\alpha$-MEM medium containing 10% fetal calf serum and incubated at 37°C for various times to allow uptake of biotinylated UCE, followed by rapid cooling on ice to stop uptake and washing twice with ice-cold TBS. The remaining cell surface biotinylated UCE was stripped of biotin with 2-mercaptoethane sulfonic acid (MESNA from Sigma) as described by Moll et al, (4). The cells were treated with 500µl of 50mM MESNA in 50mM Tris-HCl, pH 8.7, 100mM NaCl and 2.5mM CaCl$_2$ at 4°C two times for 20 min before washing twice with ice-cold TBS, pH 7.0. One well was stripped at zero time (never exposed to 37°C) to correct for the small amount of biotinylated UCE (usually less than 10%) that cannot be stripped. The control wells were never stripped. The cells in all wells were lysed in 250 µl of lysis buffer (50mM Tris, pH 7.0, 1% Triton X-100) containing protease.
inhibitor cocktail tablets (Boehringer Mannheim). The lysed cells were incubated 30 min at 4°C before sedimenting at 260,000x g for 20 min. An aliquot of the supernatant lysate was assayed for total UCE activity and 200µl were added to a Microfuge tube containing 25µl of packed Streptavidin-agarose beads (Pierce) pre-equilibrated with lysis buffer. The biotinylated UCE taken up was allowed to adsorb to the beads for 2.5 hours with turning at 4°C. The beads were removed by centrifugation at 835x g (HERMLE, Z323 K) for 1 min and washed twice with lysis buffer before being assayed directly for UCE activity. The control beads represent the UCE biotinylated at the cell surface initially. The zero time represents the UCE activity that could not be stripped and was used to correct the uptake at 37°C for various times. The uptake is expressed as a % of the total biotinylated UCE. Values for t_{1/2} of endocytosis were derived graphically from semilog plots of % biotinylated UCE remaining at the cell surface versus time using the early time points.

**Exocytosis Assay**

The exocytosis of internalized UCE was measured for the Y486A UCE mutant by allowing biotinylated UCE uptake as described above for 5 minutes, followed by chilling to 4°C and stripping of the cell surface biotin with MESNA. The cells were re-warmed to 37°C for various times to allow exocytosis of internalized biotinylated UCE, chilled at 4°C, stripped, and the biotinylated UCE within the cells was measured after cell lysis as described above.

**Preparation of GST fusion proteins**

Full length cDNAs encoding wild-type, mutant Y488A and H510Stop UCEs, prepared as previously described (2), were used as templates in PCR reactions. The PCR reactions also contained a sense strand primer including an in-frame Bam H1 restriction site at the 5’ end (CCTGTCTGGATCCTGTCCAGAGC) and an antisense strand primer including an Xho I site at the 3’ end (5’ to 3’ CAAGCTTTCTCGAGGTGCCACCCC). The PCR fragments, encoding L473 through the stop codon at 510 or 516, were gel purified, digested with Bam HI
and Xho I and inserted into a similarly digested pGEX-5X-3 plasmid downstream of GST.

Other mutations of the cytoplasmic tail in the GST fusion protein constructs were derived from the GST-UCE fusion constructs by “Quik Change” mutagenesis using the kit from Stratagene and the appropriate mutant primers. All coding sequences created by PCR were verified by sequencing. The fusion proteins were expressed in *E. coli* BL21 cells and purified as described by Traub et al. (5).

**Preparation of additional mutants of full length UCE and their transfection into mouse L-cells**

New mutants in the cytosolic tail of human full length UCE cDNA were prepared as previously described (2) using the new Stratagene “Quik Change XL” mutagenesis kit and the appropriate nucleotide primers encoding the mutant sequence according to the manufacturer’s instructions. The mutant cDNAs were transfected into mouse L-cells and stably transfected cells isolated as previously described (2).

**Preparation of cytosol**

Cytosol from bovine adrenal glands was prepared as described (6). The cytosol was depleted of AP-2 by adsorption with an immobilized anti-AP-2 antibody as previously described (7). Eps15 was depleted from the cytosol by adsorption with immobilized GST-αC (αC is the C-terminal domain of the α subunit of AP-2) as previously described (5). *E. coli* BL21 cells expressing GST-αC were kindly provided by Dr. Linton Traub and the fusion protein was purified as above. GST-αC can effectively remove Eps 15, Epsin, amphiphysin, AP180 and dynamin from cytosol (5).

**GST fusion protein binding assays**

The association of cytosolic proteins with the GST-UCE cytoplasmic tail fusion proteins was carried out essentially as described (5) using bovine adrenal cytosol (5 mg/ml) and 125 μg of
GST fusion proteins adsorbed to 25 µl of packed glutathione-Sepharose in a reaction volume of 300 µl. After incubation at 4°C for 1 hour the glutathione-Sepharose beads were pelleted and washed twice with assay buffer(25mM Hepes-OH, pH 7.2/ 125mM potassium acetate/ 2.5mM magnesium acetate/ 1mM DTT) containing 0.1% Triton X-100 and twice with PBS before boiling in reducing SDS-PAGE sample buffer. An aliquot of the supernatant was also boiled in SDS-PAGE sample buffer and 1% to 4% of the total supernatant and 10% to 40% of the total pellet for each sample were run on an 8% reducing SDS-PAGE gel. The gel was blotted to nitrocellulose and probed with antibodies to Eps 15 and AP-2 α followed by exposure to appropriate HRP secondary antibody and detection with ECL using X-Omat film (Kodak). For quantitation of Eps15 and AP-2 binding, the film was scanned using Adobe Photoshop 6.0 and analyzed using NIH Image 1.61 and SigmaPlot, 1997 software.

Antibodies

Rabbit antiserum raised to a synthetic peptide of Eps15 was kindly provided by Dr. Ernst Ungewickell (MMH, Hannover, Germany). Mouse monoclonal antibody 100/2 against the α adaptin subunit of AP-2 and mouse monoclonal antibody 100/1 against AP-1/2β subunits were purchased from Sigma Chemical Company (St. Louis, Missouri). Mouse monoclonal antibody TD.1 against clathrin heavy chain was kindly provided by Frances Brodsky (UCSF) and rabbit antibody to α-mannosidase II was kindly provided by Kelley Moreman (Univ. of Georgia). The affinity purified rabbit antibody to cation-independent mannose 6-phosphate receptor (CI-MPR) was a gift from Walter Gregory of this laboratory and the affinity purified rabbit anti-human UCE was prepared as previously described (2).

Isolation of clathrin-coated vesicles

Clathrin-coated vesicles were isolated by the method of Woodman and Warren (8) from mouse L-cells that were CI-MPR negative and had been stably transfected with bovine CI-MPR (Cc2 cells [9]). 2 ml fractions were collected from the second density gradient centrifugation
(Ficoll-D₂O) from the bottom to the top. Aliquots of each fraction were submitted to SDS-PAGE on an 8% gel, blotted to nitrocellulose and probed with a mixture of antibodies to: CI-MPR, clathrin heavy chain, α-Mannosidase II and both AP-2 α and AP-1/2 β. The blot was developed with a mixture of HRP anti-rabbit and HRP anti-mouse secondary antibodies and ECL detection. Another aliquot of 0.5 ml from fractions 3-11 was diluted in MES buffer and pelleted at 260,000 x g for 20 min. in a Beckman Optima TL ultracentrifuge. The pellet from each fraction was resuspended in 40 µl of 20 mM Tris HCl pH7 and 20 µl were used for another SDS gel and 20 µl were assayed for UCE activity.

Preparation, transfection, and microscopy of GFP-UCE-TMD-cytosolic tail fusion proteins

Nucleotide sequences encoding the transmembrane domain (TMD) and cytoplasmic tail of UCE were fused to the preprolactin signal sequence-Green fluorescent protein (GFP) construct and transiently expressed in HeLa cells as previously described (2). Confocal fluorescence microscopy on GFP-UCE transfected HeLa cells was carried out using stacks of images that were deconvoluted as previously described (2).
RESULTS

Effect of Cytoplasmic Tail Mutations on UCE Distribution

As the initial approach to further define the determinants in the 41-residue cytoplasmic tail of UCE (Figure 1) that modulate its trafficking, a series of additional mutations in this domain were generated and their effect on cell surface expression of UCE activity analyzed. The non-transfected parental mouse L cells have 1.6% of their endogenous UCE on the cell surface (Table I). Cells transfected with wild-type human UCE have only a slightly greater percentage of activity on the cell surface (2.3-3.2%) in spite of expressing 14-21-fold more total activity. As reported previously, UCE with a Y488A mutation had 63% of total activity on the cell surface (2). The UCE cytoplasmic tail contains a second tyrosine located two residues upstream from the Y^{488}HPL sequence. Interestingly, mutation of this tyrosine to alanine (the Y486A construct) resulted in 26-36% of the enzyme activity being present on the cell surface in different clones. Mutation of both tyrosines to alanines (the Y486A/Y488A construct) had a small additional effect (68% of activity on the cell surface) over mutation of Y^{488} alone.

Deletion of the outer six residues of the tail (the H510Stop construct) resulted in 7.8% of UCE activity being at the plasma membrane whereas deletion of the outer twenty one residues (the M494Stop construct) did not increase cell surface expression (2.2% on cell surface). Furthermore, mutation of Y^{488} to alanine in the context of the M494Stop mutation resulted in 50% cell surface expression (versus 63% with the Y488A mutation in the full-length tail). And mutation of Y^{486} to alanine in the M494Stop background gave rise to only 5.9% of enzyme activity on the cell surface (versus 26-36% when this mutation is made in the full-length tail). When all of the cytoplasmic tail except for eleven residues was deleted (the Y486Stop construct), only 16% of the UCE activity was present on the cell surface even though this mutant lacks both Y^{486} and Y^{488}.

Endocytosis of Biotinylated UCE from the Plasma Membrane

The steady state level of UCE at the plasma membrane is determined by the rate of
endocytosis of the enzyme, the fraction of internalized enzyme that recycles from endosomes back to the plasma membrane, and the rate at which the enzyme exits the TGN and is delivered to the plasma membrane. This delivery may be direct or occur following an initial transport to an endosomal compartment. Thus mutations in the cytoplasmic tail that increase the steady state level of UCE on the plasma membrane could be influencing one or more of these trafficking events. To begin to distinguish between these possibilities we directly measured the endocytosis rate of wild-type UCE and a number of the mutants. The various cell lines were plated on a series of culture plates and cell surface UCE was tagged with NHS-SS-biotin, a cleavable biotinylation reagent that does not permeate the membrane. Following biotinylation at 4°C the total cell surface biotinylated UCE was measured by lysing the cells from one dish and capturing the biotinylated UCE on streptavidin-agarose beads. To measure uptake of biotinylated UCE, the remaining culture plates were brought to 37°C for various times, then rapidly chilled and residual cell surface biotinylated UCE was stripped by cleavage of the S-S bond with the non-permeant reducing agent MESNA. The internalized biotinylated UCE was captured from lysed cells on streptavidin-agarose beads and UCE activity was assayed directly on the washed beads. Figure 2 shows the results of the uptake experiments expressed as a percent of the total biotinylated UCE at the cell surface and Table I lists the calculated internalization rates.

The rate of uptake of wild-type UCE was very rapid with a t_{1/2} of 0.65 min. Y486A UCE was endocytosed at about the same rate as wild-type UCE (t_{1/2} of 0.4 min). This indicates that the increased amount of this mutant UCE on the cell surface (36%) is not the consequence of impaired internalization. The double mutant Y486A/M494Stop which has much less cell surface enzyme (5.9%) had an endocytosis rate that was modestly slower than that of wild-type UCE (t_{1/2} of 1.5 min). The rate of internalization of H510Stop UCE was also rapid (t_{1/2} of 0.55 min), indicating that the small increase in surface expression of this mutant is not secondary to slow internalization. In contrast, Y486Stop UCE and Y488A UCE were taken up quite slowly (t_{1/2} greater than 30 min). The decreased rate of internalization of Y488A correlates well with its high level of cell surface expression (63%). However Y486Stop accumulates to a much lesser extent on
the cell surface (16%) indicating that it is delivered to the plasma membrane at a slower rate than Y488A UCE.

**Internalized Y486A Rapidly Recycles to the Plasma Membrane**

The rapid uptake of Y486A UCE in the presence of an elevated level of cell surface molecules suggested that the internalized enzyme may be recycling from an endosomal compartment back to the plasma membrane. To estimate the exocytosis rate of Y486A UCE from the recycling endosomal compartment, cells expressing this mutant enzyme were biotinylated at 4°C, warmed to 37°C for 5 minutes to allow endocytosis of the biotinylated UCE and then chilled at 4°C with subsequent stripping of remaining biotin from cell surface UCE. Plates of these cells were then rewarmed to 37°C for 2, 5 or 10 minutes before chilling and stripping the biotin from externalized UCE. The internal biotinylated UCE was captured on streptavidin-Sepharose beads and measured. The result, shown in Figure 3 and expressed as the percent of internal biotinylated UCE that was exocytosed, does indeed indicate a rapid recycling of UCE back to the cell surface. Taking into account the rapid rate of re-endocytosis of externalized biotinylated UCE that is occurring during the experiment, it can be estimated that the \( t_{1/2} \) of exocytosis of Y486A UCE is between 1 and 2 minutes or 2.5 to 5 times slower than the \( t_{1/2} \) of endocytosis. The curve is already approaching steady state by the 10 minute time point. This recycling experiment could not be performed with the wild-type UCE because the low level of surface expression prevented adequate labeling.

**Binding of AP-2 and Eps15 to UCE cytoplasmic tail**

Tyrosine 488 which is essential for rapid endocytosis is part of the YHPL motif that fits the well-known endocytosis motif of YXX\( \phi \) (where \( \phi \) is a bulky hydrophobic residue)(10). This motif has been shown to interact with the \( \mu 2 \) subunit of the plasma membrane adaptor complex AP-2(11). Further, the NPF sequence near the C-terminus of the cytoplasmic tail is a candidate to interact with the cytosolic protein Eps15 which binds NPF sequences by its EH domain (12).
To study these potential interactions directly, various GST-UCE tail fusions (Figure 4) were constructed and expressed and then tested for AP-2 and Eps15 binding using bovine adrenal cytosol as the source of AP-2 and Eps15 in pull-down assays. The GST-wild-type UCE cytoplasmic tail displayed significant binding of both AP-2 and Eps15 whereas GST alone failed to bind either of the proteins (Figure 5). Mutation of $^{488}$Y to alanine marked decreased AP-2 binding (10% of WT) and also impaired Eps15 binding (44% of WT). The Y486A mutation resulted in modest decreases in both AP-2 and Eps15 bindings (43% and 55% of WT, respectively). And the H510Stop fusion protein, which lacks the NPF motif, exhibited almost no binding of both Eps15 and AP-2 (< 1% of WT).

These results indicated that binding of the UCE cytoplasmic tail to the $\mu\,2$ subunit of AP-2 and Eps15 was synergistic in the pull-down assays. This might be expected since Eps15 also binds to the appendage of the $\alpha$ subunit of AP-2 (13). To further explore this possible synergy, two additional experiments were carried out. In the first experiment, Eps15 was depleted from the cytosol by adsorbing it on immobilized $\alpha$ appendage, also referred to as the $\alpha_c$ fragment (5). As shown in Figure 6 this caused a marked decrease in the binding of AP-2 to GST-UCE (27% of WT). In the second experiment, AP-2 was depleted from the cytosol by prior adsorption on immobilized anti-AP-2 antibody. Figure 7 shows that depletion of the AP-2 was complete and that the Eps15 was partially depleted, perhaps by 2-fold, since it binds to AP-2 (see the intensity of the Eps15 in the supernatants). However, the interaction of GST-UCE wild-type with Eps15 was reduced to 23% of the amount bound in normal cytosol. These depletion experiments show that binding of AP-2 and Eps15 to the the cytoplasmic tail of UCE is synergistic under the conditions of the pull-down assays.

**UCE is found in clathrin-coated vesicles**

The GST fusion protein pull-down experiments indicate that UCE is internalized at the plasma membrane via AP-2 containing clathrin-coated vesicles. To directly test this notion, clathrin-coated vesicles were isolated from mouse L-cells which express endogenous mouse UCE.
Following the second density gradient step as detailed in the Experimental Procedures, aliquots of the fractions were subjected to SDS-PAGE, blotted to nitrocellulose and probed with antibodies to the clathrin heavy chain (CHC), AP-2, AP-1, the CI-MPR and Golgi α-mannosidase II as shown in Figure 8. In addition, aliquots of the fractions were concentrated and assayed for UCE activity as well as subjected to SDS-PAGE, blotted and probed with antibody to UCE. It can be seen in Figure 8 that the clathrin-coated vesicles were mostly present in fractions 3, 4 and 5 which stained for clathrin, AP-1, AP-2 and the CI-MPR whereas the remnant of Golgi membranes not removed in the first gradient was concentrated in later fractions as shown by α-mannosidase II staining. Uncovering enzyme fractionated primarily with the clathrin-coated vesicles. The result confirms that endogenous UCE is packaged into clathrin-coated vesicles but cannot distinguish AP-2 clathrin-coated vesicles from AP-1 clathrin-coated vesicles.

**Green fluorescent protein fusion proteins of mutant UCE**

To explore the effects of the various cytoplasmic tail mutations on the intracellular localization of UCE, we turned to visualization of a variety of GFP-UCE-TM-cytoplasmic tail fusion proteins (Figure 9a). These GFP fusion proteins were transiently expressed in HeLa cells and localized by confocal imaging, as shown in Figure 9b. As previously reported, wild-type GFP-UCE is localized primarily in the TGN like the endogenous UCE (2). GFP-UCE M494Stop was also concentrated in the TGN whereas GFP-UCE Y488A/M494Stop showed heavy surface staining with residual labeling in the TGN. The GFP-UCE Y486A/M494Stop exhibited less surface staining, but more than observed with the GFP-UCE wild-type protein. Furthermore, there was much more peripheral staining, suggestive of endosomal localization. A similar pattern was obtained with GFP-UCE Y486A. The GFP-UCE Y488A/Q492A/E493A/M494Stop construct was more concentrated in the TGN region than GFP-UCE Y488A/M494Stop, suggesting that 492QE may play a role in the exit of the fusion protein from the TGN.
DISCUSSION

The data presented in this study demonstrate that the trafficking of UCE between the TGN and the cell surface is regulated by multiple signals. These findings are best discussed in terms of our current understanding of trafficking between these compartments. Proteins that exit the TGN may be delivered directly to the plasma membrane by the constitutive pathway or be routed to an intermediate endosomal compartment and then progress to the cell surface (14). Once at the plasma membrane, recycling proteins are mostly internalized via AP-2-containing clathrin-coated vesicles and delivered to early/sorting endosomes. Some recycling proteins such as the CI-MPR and furin continue on to late endosomes and then return to the TGN (15-18). Other proteins including TGN38 pass from early endosomes to recycling endosomes from which they can go to the TGN or return to the plasma membrane (18,19). Movement from early/sorting endosomes to recycling endosomes and back to the plasma membrane is believed to be a default pathway whereas retention in the endosomal compartment and delivery to late endosomes and the TGN is thought to be signal mediated (20).

At steady state the bulk of UCE is present in the TGN with 2% being on the cell surface and a small amount enroute between these two destinations. Since UCE cycles between the TGN and cell surface, this distribution can only occur if the rate of exit of UCE from the TGN is slower than its rate of return. Our data are most compatible with these processes being controlled by four types of signals: a TGN retention signal, a TGN exit signal, a plasma membrane internalization signal, and a signal(s) for return of internalized UCE to the TGN. As previously shown, the GFP-UCE Y486Stop construct expressed in HeLa cells is mostly localized to the TGN, indicating that the transmembrane domain, perhaps in conjunction with the neighboring
amino acids, serves as a TGN retention signal (2). A similar role for the transmembrane domain of TGN38 in TGN localization has been reported (21). Since it is difficult to quantitate the amount of fluorescent protein in subcellular compartments, we expressed the Y486Stop UCE construct in mouse L cells and analyzed its distribution based on enzyme activity. The majority of the mutant enzyme was in an intracellular compartment with only 16% of the molecules being on the cell surface. As this construct lacks an internalization signal, any molecules that traveled to the cell surface would be expected to be trapped there. This indicates that Y486Stop UCE leaves the TGN very slowly, most likely because it lacks a TGN exit signal. In contrast, the Y488A/M494Stop UCE which is also very slowly internalized was found to have 50% of its activity on the cell surface, only slightly less than the 63% surface accumulation of Y488A UCE with a full-length cytoplasmic tail. This indicates that Y488A/M494Stop UCE leaves the TGN faster than Y486Stop UCE and suggests that the sequence 486YAYHPLQE plays a role in exit from the TGN as well as in endocytosis at the plasma membrane. One potential mechanism is that these residues interact with the Golgi adaptor complex AP-1 that is known to bind to tyrosine-based motifs (22). The AP-1 derived clathrin-coated vesicles are believed to transport their cargo to endosomal compartments (14). In this scenario, UCE would traffic from the TGN to an endosomal compartment and then to the plasma membrane. However if UCE does exit the TGN via an AP-1 clathrin-coated vesicle, it is surprising that the Y488A mutation does not have a more marked effect on slowing the movement of UCE from the TGN. Mutation of the 492QE residues to alanines appears to slow the exit of GFP-UCE from the TGN, indicating that these amino acids may be either part of the exit signal or have an indirect effect on the signal. It is also likely that residues distal to the 486YAYHPLQE sequence influence the rate at which UCE leaves the TGN.

The rate of internalization of wild-type UCE at the plasma membrane is quite rapid (t1/2 of less than 1 min), comparable to the t1/2 for internalization of dimeric CI-MPR (0.75 min)(23). In comparison, the t1/2 for internalization of TGN38 is 4.6 min (19) and it is 3 min for the transferrin receptor (24). The fact that UCE is a homotetramer may be significant in this regard.
since the complex would have four internalization signals (25). The rate of internalization of the CI-MPR is enhanced 5-fold when it is dimerized by ligand binding (23). The major determinant for the rapid endocytosis of UCE is the $^{488}\text{YHPL}$ sequence, a typical tyrosine-based internalization signal (10). Mutation of $^{488}\text{Y}$ to alanine dramatically slowed the uptake of UCE, accounting for the striking accumulation of UCE with this mutation at the cell surface. This mutation also markedly decreased AP-2 binding in GST-cytoplasmic tail pull-down experiments, consistent with this interaction being essential for rapid internalization. The role of the C-terminal NPFKD motif in UCE trafficking is not clear. The cytoplasmic tail pull-down experiments showed that the NPF motif binds Eps15 which, in turn, enhances AP-2 binding through its interaction with the $\alpha$-subunit appendage. However the H510Stop UCE that is missing the NPFKD sequence is rapidly endocytosed. Thus even though the UCE cytoplasmic tail domain in the form of a GST fusion protein reacts synergistically with AP-2 and Eps15 in in vitro pull down experiments, this three part complex does not appear to be required for rapid endocytosis. The explanation for this apparent discrepancy may be that AP-2 binding to the tyrosine-based motif in vivo is of much higher avidity than occurs in the in vitro binding assays. As a consequence, synergistic binding with Eps15 may not be required for efficient AP-2 binding inside the cell. In this regard it has been reported that a mutant of VAMP4 that completely fails to bind AP-1 in an in vitro pull-down assay exhibits only a partial phenotype in vivo (26). While the NPFKD deletion did not impair the rate of internalization, it did result in an increase in the percent of total molecules on the cell surface (7.8% versus 2.3% for the wild-type control). This raises the possibility that Eps15 binding may facilitate the return of UCE from an endosomal compartment to the TGN. In the absence of Eps15 binding, UCE may undergo an increase in the frequency of recycling to the plasma membrane. Eps15 has been localized to endosomes as well as the plasma membrane, so it could serve functions in addition to facilitating endocytosis (27).

The most unexpected results came from the studies of Y486A UCE. While 26-36% of these molecules were on the cell surface in different clones, the kinetic studies showed that the mutant
enzyme was internalized at the same rate as wild-type enzyme and then rapidly recycled to the plasma membrane. The intracellular GFP-Y486A chimeric protein was found to be localized in both the TGN and endosomal compartments. These findings suggest that internalized Y486A UCE is not properly sorted in the endosomal system. Rather than being efficiently returned to the TGN as occurs with the wild-type enzyme, a large portion of the mutant molecules appear to enter a recycling pathway that delivers them back to the plasma membrane. The implication is that $^{486}Y$ is part of a signal for returning UCE from endosomes to the TGN.

In this regard it is interesting to note that TGN38, which like UCE cycles between the TGN and the plasma membrane, contains a SDYQRL sequence in its cytoplasmic tail that is required for internalization at the plasma membrane and efficient retrieval from the recycling endosome back to the TGN (28-31). Mutation of the serine residue to alanine or aspartate in full length TGN38 results in missorting of endocytosed TGN38 to late endosomes/lysosomes and, to a lesser extent, back to the plasma membrane (31). Roquemore and Banting have suggested that the hydroxyl group of the serine has a direct or indirect effect on the ability of the cytoplasmic tail of TGN38 to interact with trafficking and/or sorting machinery at the level of the early endosome (31). Perhaps $^{486}\text{Tyr}$ located two residues upstream of the YHPL sequence in the UCE cytoplasmic tail plays a similar role in endosomal trafficking. However we also have evidence that in GFP-UCE transfected HeLa cells the vesicles in the cell periphery that stained for endogenous TGN46 (the human form of rat TGN38) were not colocalized with GFP-UCE vesicles although both markers were perfectly colocalized in the TGN (2). Thus it appears that the route of return of TGN46 and UCE from the plasma membrane to the TGN is not the same. It has also been shown that the cytoplasmic tail of the CI-MPR contains a tyrosine two residues upstream of the YSKV endocytosis motif that enhances the rate of internalization of that receptor (32). A possible role of the upstream tyrosine in endosomal sorting has not been investigated.

In addition to TGN38, tyrosine-based motifs in the cytoplasmic tails of lamp1 (3) and the polymeric immunoglobulin receptor (33) regulate sorting of these proteins in endosomes as well.
as in the TGN and at the plasma membrane. In the cytoplasmic domain of the Gp180/carboxypeptidase D protein which cycles between the TGN and plasma membrane, the FXXL sequence plays the role of YXXL used for sorting in these other proteins (34). The findings with the \(^{486}\text{YAYHPLQE} \) motif in the UCE cytoplasmic tail represent another example of a sorting sequence that operates at multiple sites, including the TGN, plasma membrane and endosomes. It will be important to identify the components of the endosomal sorting machinery that interact with this sorting signal to understand how UCE is returned to the TGN.
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FOOTNOTES

This work was supported by grant CA08759 from the National Institutes of Health (to S.K.) and by a Professor Dr. Max Cloetta fellowship (to J.R.).

1 The abbreviations used are: AP-1 and AP-2, adaptor protein complex 1 and 2; CI-MPR, cation-independent mannose 6-phosphate receptor; Eps15, epidermal growth factor receptor protein substrate clone 15; ECL, enhanced chemiluminescence; GFP, green fluorescent protein; HRP, horseradish peroxidase; Man-6-P, mannose 6-phosphate; mAb, monoclonal antibody; MESNA, 2-mercaptoethane sulfonic acid; PBS, phosphate buffered saline; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; TBS, tris buffered saline; TGN, trans-Golgi network; UCE, “uncovering” enzyme

ACKNOWLEDGMENTS

We thank Dr. Yunxiang Zhu for his kind assistance in isolating the clathrin coated vesicles and Dr. Evan Sadler for his help with the quantitation of the GST-pull down experiments.

FIGURE LEGENDS

Figure 1: Amino acid sequence of the human UCE cytoplasmic tail. The lumenal domain is labeled UCE, the transmembrane domain is shown as the box labeled TM and the single letter abbreviations for the 41 amino acids of the cytoplasmic tail are shown with the putative endocytosis signals underlined.

Figure 2: Rates of endocytosis of wild-type and mutant human UCE expressed in mouse
L-cells. The rate of internalization of surface biotinylated UCE was measured as described in Experimental Procedures, and is expressed as % of total biotinylated UCE endocytosed.

Figure 3: Exocytosis of endocytosed Y486A UCE. The rate of exocytosis of biotinylated Y486A UCE was measured as described in Experimental Procedures and is expressed as % of biotinylated UCE exocytosed. The values are the averages from three separate experiments.

Figure 4: GST-UCE cytoplasmic tail fusion proteins. The fusion proteins were prepared as described in Experimental Procedures. The sequences of the UCE cytoplasmic tail are shown and the bold A show where alanine residues were substituted for the residue in the wild-type UCE.

Figure 5: AP-2 and Eps15 interact with UCE wild-type cytoplasmic tail. GST-UCE cytoplasmic tail fusion proteins, wild-type and mutant as shown in Fig 4, were used in pull-down experiments with cytosol from bovine adrenal glands. GST control consists of glutathione-Sepharose beads with only GST bound. Portions of the blot were probed with either anti-Eps15 rabbit antiserum or anti-α adaptin subunit of AP-2 mAb 100/2. S is supernatant and P is bead pellet. The films were scanned as described in Experimental Procedures and the quantitation of the pellet bands from 5 separate experiments is shown.

Figure 6: Depletion of Eps15 from cytosol decreases binding of AP-2 to GST-UCE cytoplasmic tail fusion protein. Eps15 was depleted from bovine adrenal cytosol as described in Experimental Procedures and the depleted cytosol used in pull-down experiments. Control refers to GST alone whereas WT refers to the GST-
wild-type UCE cytoplasmic tail fusion protein. S is supernatant and P is bead pellet.

Figure 7: Depletion of AP-2 from cytosol decreases binding of Eps15 to GST-UCE cytoplasmic tail fusion protein. AP-2 was depleted from bovine adrenal cytosol as described in Experimental Procedures and varying amounts of depleted cytosol and normal cytosol were used in pull-down experiments. S is supernatant and P is bead pellet.

Figure 8: Endogenous UCE in mouse L-cells is present in clathrin-coated vesicles. Clathrin-coated vesicles were isolated on a Ficoll-D_2O density gradient as described in Experimental Procedures and the fractions were (A) submitted to SDS-PAGE, blotted for marker proteins CI-MPR, clathrin heavy chain (CHC), α-mannosidase II (α-Man II) and α2/β1,2 chains of adaptor proteins AP-1 and AP-2 or (B) concentrated and assayed for UCE activity and submitted to SDS-PAGE, blotted and probed with antibody to UCE.

Figure 9: GFP-UCE chimeric proteins. a. The GFP-UCE transmembrane domain-cytoplasmic tail constructs used to transfect HeLa cells. b. Fluorescence micrographs of transfected HeLa cells.
Table I

Distribution of Expressed Human UCE in Transfected Mouse L-Cells

| Cell Line                   | Total UCE* (nmole/hr/mg) | % UCE on Cell Surface | t<sub>1/2</sub> of UCE Endocytosis (min) |
|-----------------------------|--------------------------|-----------------------|----------------------------------------|
| parent L-cells              | 7.3 (4)                  | 1.6 (2)               | ND                                     |
| wild-type UCE               |                          |                       |                                        |
| clone 38                    | 101 (15)                 | 2.3 (8)               |                                        |
| clone 29                    | 148 (2)                  | 3.2 (1)               | 0.65                                   |
| Y486A UCE                   |                          |                       |                                        |
| clone 211                   | 166 (1)                  | 26 (1)                |                                        |
| clone 228                   | 234 (1)                  | 32 (1)                |                                        |
| clone 214                   | 507 (4)                  | 36 (3)                | 0.4                                    |
| Y488A UCE                   |                          |                       |                                        |
| clone 362                  | 173 (7)                  | 63 (6)                | 97                                     |
| Y486A/Y488A UCE             | 368 (2)                  | 68 (2)                | ND                                     |
| H510 stop UCE               | 198 (6)                  | 7.8 (5)               | 0.55                                   |
| M494 stop UCE               | 162 (7)                  | 2.2 (2)               | ND                                     |
| Y486A/M494 stop UCE         | 143 (5)                  | 5.9 (3)               | 1.5                                    |
| Y488A/M494 stop UCE         | 276 (2)                  | 50 (2)                | ND                                     |
| Y486 stop UCE               | 105 (3)                  | 16 (2)                | 39                                     |

*The values are averages of the number of measurements shown in parenthesis.

ND is not determined.
Fig 2

% Biotinylated UCE Internalized

Time (min)
Fig 3
Fig 4.

GST-Wild-type UCE;  GST-L   YAYHPL   AHNPFKD
GST-Y488A UCE;      GST-L   YAAHPL   AHNPFKD
GST-Y486A UCE;      GST-L   AAYHPL   AHNPFKD
GST-H510STOP UCE;   GST-L   YAYHPL   A
Fig 5.

| Protein | Control | WT     | Y486/A | Y488/A | H510/STOP |
|---------|---------|--------|--------|--------|-----------|
| Eps15 (%) | 100 | 55±25 | 44±18 | 0±0.5 |
| AP-2 (%) | 100 | 43±7  | 10±6  | 1±1   |

*The values are relative values ± S.D. with the WT pellet set to 100%.*
Fig 6

|            | Control | wt | Control | wt |
|------------|---------|----|---------|----|
| AP-2 2    |         |    |         |    |
| Eps 15    | S       | P | S       | P |
| Cytosol; depleted normal |         |   |         |   |
FIG 7

AP-2

Eps 1.5

Depleted (mg/ml): 0 1 2.5 5 10

Normal (mg/ml): 5 0 1 2.5 5 10
Fig 9a

GFP-UCE wt

GFP-UCE Y486A

GFP-UCE M494Stop

GFP-UCE Y486A/494Stop

GFP-UCE Y488A/494Stop

GFP-UCE YQE-A/494Stop

ppL | GFP | RAERNRLHGDYA YPLQEM...HNPFKD*

ppL | GFP | RAERNRLHGDAAYHPLQEM...HNPFKD*

ppL | GFP | RAERNRLHGDAYAYHPLQEM...HNPFKD*

ppL | GFP | RAERNRLHGDAAAYHPLQEM...HNPFKD*

ppL | GFP | RAERNRLHGDAAAYHPLQEM...HNPFKD*

ppL | GFP | RAERNRLHGDAAAYHPLQEM...HNPFKD*

ppL | GFP | RAERNRLHGDAAAYHPLQEM...HNPFKD*
