Signals Involved in Protein Intracellular Sorting

Inauguraldissertation

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<tr>
<td>ABP-1</td>
<td>actin binding protein-1</td>
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<tr>
<td>AP-1, 2, 3, 4</td>
<td>adaptor protein 1, 2, 3, 4</td>
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<td>ARF-1</td>
<td>ADP-ribosylation factor 1</td>
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<td>ATM</td>
<td>ataxia talangiectasias mutated protein</td>
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<td>BFA</td>
<td>brefeldin A</td>
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<td>CALM</td>
<td>clathrin assembly lymphoid myeloid leukaemia protein</td>
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<td>CCV</td>
<td>clathrin coated vesicle</td>
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<td>CD-MPR</td>
<td>cation-dependent mannose 6-phosphate receptor</td>
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<td>cytoplasmic dynein 1</td>
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<td>early endosome</td>
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<td>epidermal growth factor receptor</td>
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<td>EH</td>
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<td>EGFR pathway substrate 15</td>
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<td>ER</td>
<td>endoplasmic reticulum</td>
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<td>ERGIC</td>
<td>ER-Golgi intermediate compartment</td>
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<td>ESCRT- I, II, III</td>
<td>endosomal complexes required for transport-I, II, III</td>
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<td>GAT</td>
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<tr>
<td>GDF</td>
<td>GDI-displacement factor</td>
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<td>GDI</td>
<td>GDP-dissociation inhibitor</td>
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<td>GED</td>
<td>GTPase effector domain</td>
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<td>GEF</td>
<td>guanine nucleotide exchange factor</td>
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<td>GFP</td>
<td>green fluorescent protein</td>
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<td>GGA</td>
<td>golgi-localising γ-ear containing ARF-binding protein</td>
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<td>GHR</td>
<td>growth hormone receptor</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>N-acetylglucosamine</td>
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<td>GPCR</td>
<td>G-protein coupled receptor</td>
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<td>G protein</td>
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<tr>
<td>Hrs</td>
<td>hepatocyte growth factor regulated tyrosine kinase substrate</td>
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<td>IGF-II</td>
<td>insulin-like growth factor II</td>
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<td>ITAM</td>
<td>immunoreceptor tyrosine-based activation motif</td>
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<td>LAMP</td>
<td>lysosome-associated membrane protein</td>
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<td>lysosomal acid phosphatase</td>
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<tr>
<td>LBPA</td>
<td>lysobisphosphatidic acid</td>
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<tr>
<td>LDL</td>
<td>low-density lipoprotein</td>
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<td>LE</td>
<td>late endosome</td>
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<td>LIMP</td>
<td>lysosomal integral membrane protein</td>
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<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
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<tr>
<td>M6P</td>
<td>mannose 6-phosphate</td>
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<td>MPR</td>
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<tr>
<td>MVB</td>
<td>multivesicular body</td>
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<tr>
<td>Nef</td>
<td>negative factor</td>
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<tr>
<td>NSF</td>
<td>N-ethylmaleimide sensitive factor</td>
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<tr>
<td>PACS-1</td>
<td>phosphofurin acidic cluster sorting protein 1</td>
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<tr>
<td>PDGF</td>
<td>platelet derived growth factor</td>
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<td>PH</td>
<td>pleckstrin homology</td>
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<td>pIgR</td>
<td>polymeric immunoglobulin receptor</td>
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<tr>
<td>phosphotransferase</td>
<td>UDP-GlcNAc:lysosomal enzyme GlcNAc-1-phosphotransferase</td>
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<tr>
<td>PI(3)K</td>
<td>PtdIns(3) kinase</td>
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<tr>
<td>PKC</td>
<td>protein kinase C</td>
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<td>PNS</td>
<td>post-nuclear supernatant</td>
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<td>proline-rich domain</td>
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<td>phosphotyrosine binding domain</td>
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<td>PtdIns(3)P</td>
<td>phosphatidylinositol 3-phosphate</td>
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<td>PtdIns(4,5)P_2</td>
<td>phosphatidylinositol 4,5-bisphosphate</td>
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<td>Rab</td>
<td>Ras-like in rat brain</td>
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<td>Sac1</td>
<td>suppressor of actin</td>
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<td>SH3</td>
<td>Src-homology 3</td>
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<td>SNARE</td>
<td>soluble NSF-attachment protein receptor</td>
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<td>SNX</td>
<td>sorting nexin</td>
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<tr>
<td>STAM</td>
<td>signal transducing adaptor molecule</td>
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<tr>
<td>TGF-β</td>
<td>transforming growth factor-β</td>
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<tr>
<td>TGN</td>
<td>trans-Golgi network</td>
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<tr>
<td>TIP47</td>
<td>Tail Interacting Protein of 47 kDa</td>
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<tr>
<td>TMD</td>
<td>transmembrane domain</td>
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<tr>
<td>TRAM</td>
<td>translocon-associated membrane protein</td>
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<tr>
<td>UCE</td>
<td>uncovering enzyme</td>
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<tr>
<td>UIM</td>
<td>ubiquitin-interacting motif</td>
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<tr>
<td>VHS</td>
<td>conserved in Vps27, Hrs, STAM</td>
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<tr>
<td>VSV-G</td>
<td>vesicular stomatitis virus G</td>
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<tr>
<td>α-SNAP</td>
<td>α-soluble NSF attachment protein</td>
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<tr>
<td>SRP</td>
<td>signal recognition particle</td>
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Summary

“...Confusion appears to occur just after the articulation of a major conceptual advance that served to greatly clarify a problem of exceptional importance.” - Ira Mellman, 1996.

What could be more fitting than the domain of protein trafficking to elucidate the above statement made by one of the several pioneers in the field? Ever since the pioneering groundwork laid down by Blobel and colleagues, emphasising protein translocation across intracellular membranes, the field of protein trafficking has been a playground of debates, dogma-reversals and rediscoveries. The possession of a valid cellular address tag is the basic requirement for the delivery of a given protein at its intracellular destination. However, the complexity involved in the foray of proteins from their site of synthesis to their site of function is within the scope of no comprehensive treatise. In this thesis, the work done on two individual transport steps of two different proteins has been summarised.

In the first part of this thesis, the trafficking of the cation-dependent (CD-) mannose 6-phosphate receptor (MPR) has been studied. The CD-MPR cycles between the TGN and the plasma membrane, through the early and late endosomal compartments. It performs the important function of transport of lysosomal enzymes to lysosomes, a process which ensures the correct biogenesis of lysosomes. However, it is important that the receptor itself be excluded from lysosomes and safely retrieved to the TGN from late endosomes in order to avoid degradation in lysosomes. This is essential to ensure that the CD-MPR is available to support several rounds of lysosomal enzyme transport. This retrieval step has been shown to depend on a pair of aromatic residues F^{18}W^{19} in the cytoplasmic tail of the receptor. Mutation of the residues to alanines has been shown to result in massive mislocalisation of the CD-MPR in lysosomes, the W19 residue being more crucial to this function and the F18 residue playing a contributory role. The retrieval has also been shown to take place in a Rab9 dependent manner using the cytosolic adaptor protein TIP47 (Tail Interacting Protein of 47 kDa). TIP47 specifically interacts with the di-aromatic motif to effect this transport step. In this study, we demonstrated a strict requirement for di-aromaticity at the positions 18 and 19 of the cytosolic tail of the CD-MPR both for correct intracellular sorting in vivo and optimal TIP47 interaction in vitro, thus demonstrating the significance of the di-aromatic motif in endosomal sorting and
establishing the highly specific nature of this interaction. This also established a paradigm for the CD-MPR as a representative member of a generic family of di-aromatic motif containing proteins.

The second part of this thesis deals with the trafficking of the human mannose 6-phosphate uncovering enzyme (UCE). The recognition of the mannose 6-phosphate tag on lysosomal enzymes by the MPRs is facilitated by UCE which exposes the recognition signal on the lysosomal enzymes in a two-step enzymatic reaction: the first starts in the cis-Golgi and is mediated by a phosphotransferase and the second, mediated by UCE, occurs in the TGN. At steady state, UCE is mostly localised to the TGN and it cycles between the TGN and the plasma membrane. It is rapidly internalised from the surface in a clathrin dependent endocytic pathway and the internalisation has been shown to be mediated by a critical tyrosine-488 residue in its cytoplasmic tail. The transmembrane domain and first 11 residues of the cytoplasmic tail of UCE have been shown to be involved in its TGN retention. In this study, we identified the residues involved in TGN exit of UCE using a combination of biochemical and confocal immunofluorescence methods. Using a high dimensional neural network capable of identifying differences between images not visible to the eye, we determined that the residues 492QEMN were involved in TGN exit of UCE. The same method was also used to analyse the individual contribution of each amino acid in the sequence and it was found that residue Q492 is the most important to the exit function while residues M494 and N495 also contribute. The identification of a trans-Golgi network exit signal in its cytoplasmic tail elucidates the trafficking pathway of uncovering enzyme, a crucial player in lysosomal biogenesis.

With these two analyses, we contributed to a better understanding of signal sequences involved in intracellular protein trafficking of two related proteins both involved in lysosomal biogenesis.
1. Intracellular Protein Trafficking.

Animal cells typically contain over 10 billion proteins of 10,000-20,000 different kinds and these have the essential function of catalysing reactions, transporting molecules, marking surfaces and defining borders. Compartmentalisation is the key to the successful functioning of such a complex system which is in a permanent attempt to maintain homeostasis. Intracellular membrane systems help maintain distinct compartments characterised by function- and structure-based segregation and thus provide for more than just increased membrane area. The maintenance of such clear and often not-so-clear boundaries comes at a high price- a complex network of intracellular trafficking pathways in which numerous players are involved, each communicating with several others. These transport processes ensure that distinct biochemical reactions take place in distinct compartments. The nature of these processes and the mediators involved are the mainstay of cell-biologists studying protein trafficking. Intracellular transport can be thought of as being of three different kinds: gated transport, in which protein traffic between the cytosol and nucleus occurs between topologically equivalent spaces, which are in continuity through the nuclear pore complexes, transmembrane transport, in which membrane-bound protein translocators directly transport specific proteins across a membrane from the cytosol into a space that is topologically distinct and vesicular transport, in which membrane-enclosed transport intermediates—which may be small, spherical transport vesicles or larger, irregularly shaped organelle fragments—ferry proteins from one compartment to another. In all these kinds of transport, delivery of cargo at the right destination depends on the possession of a valid cellular address.

Traffic lights, all the way.

Sorting signals direct proteins to the correct intracellular compartment. Typically, sorting signals may be a linear sequence of amino acids or a stretch of residues opening up for interaction in the correct three-dimensional conformation of the cargo protein. The latter type is referred to as signal patches. The odyssey of most proteins begins at their point of synthesis- on ribosomes attached to the labyrinthine maze of membranes surrounding the nucleus, the endoplasmic reticulum (ER).
Figure 1. A simplified "roadmap" of protein traffic. Proteins can move from one compartment to another by gated transport, transmembrane transport or vesicular transport. The signals that direct a given protein's movement through the system, and thereby determine its eventual location in the cell, are contained in each protein's amino acid sequence. The journey begins with the synthesis of a protein on a ribosome in the cytosol and terminates when the final destination is reached. At each intermediate station a decision is made as to whether the protein is to be retained in that compartment or transported further. In principle, a signal could be required for either retention in or exit from a compartment.

1.1 The Secretory Pathway
1.1.1 Transport through the ER, post-translational modifications and exit from the ER

Accounting for more than 50% of the total membrane in a secretory cell, the ER functions as a gateway to the secretory pathway and ensures the correct folding of newly synthesised proteins with the help of resident folding enzymes and chaperones (Chevet, 2001). Translocation of newly synthesised proteins into the ER lumen occurs cotranslationally in mammalian cells through a protein-conducting channel called the translocon. The nascent polypeptide chain contains a signal peptide which is cleaved off
by the signal peptidase and it gets subsequently glycosylated by the oligosaccharyltransferase, both enzymes being located adjacent to the translocon. Besides, the glycosylation machinery is composed of a large family of ER associated glycosyltransferases and ER mannosidases which also function in glycosylation and folding. The translocon is composed of several proteins: TRAM (Translocon-Associated Membrane Protein), Sec61α and two other polypeptides which were purified as a heterotrimer with Sec61α and which were termed Sec61β and Sec61γ. Besides the core components several additional proteins play crucial roles in the translocation. Calnexin, an ER membrane protein has been shown to chaperone the folding of the nascent chains (Chen, 1995). Soluble luminal proteins such as calreticulin, protein disulfide isomerase, BiP and ERp57 are other proteins that interact with the nascent chain co-translationally (Nicchitta, 1990), (Helenius, 1997), (Olivier, 1997). A heterodimeric signal recognition particle (SRP) receptor targets the ribosome-nascent chain-signal sequence-SRP complex to the membrane of the ER. After the completion of ribosome-nascent chain targeting, the BiP protein seals the luminal end of the mammalian ribosome-free translocon (Johnson, 1999). Briefly, after the SRP binds the signal sequence, an interaction between the SRP and the SRP receptor leads to ribosome binding and insertion of nascent chain into the translocon pore. After about 70 amino acids are added to the growing chain, the release of BiP leads to the opening of the luminal end of the pore through which translocation proceeds. After translation is completed the protein gets released into the ER lumen and the pore is sealed on its luminal side by BiP and the ribosome is released into the cytoplasm (Johnson, 1999). The topology of a given membrane protein is established precisely during this process and has been shown to be dependent on charges flanking the transmembrane domain (TMD), length of the TMD, hydrophobicity, folding and glycosylation of the signal sequence. Type I membrane proteins have been shown to be targeted by an N terminal cleavable signal sequence and anchored in the membrane by a stop-transfer-membrane anchor sequence whereas in type II membrane proteins a signal-anchor sequence is responsible for targeting and anchoring. Type III membrane proteins owe their topology to reverse signal anchors which translocate their N terminus across the membrane. For multiple membrane
spanning proteins, the first hydrophobic span decides the fate of translocation and membrane insertion (Goder, 2001).

Certain proteins are retained within the ER whereas certain others are routed for onward transport to the Golgi. The retention of proteins in the ER has been shown to be mediated by two main mechanisms: exclusion from vesicles destined for onward transport to the Golgi or retrieval by retrograde transport from the Golgi to the ER. Both processes have been shown to be mediated by signal sequences in the cytoplasmic tails of the proteins and specific cytoplasmic protein machineries that interact with those signals.

Figure 2. The Translocon. The components of the translocon are depicted approximately to scale in this cross section that is perpendicular to the plane of the membrane. Transmembrane segments are represented by a cylindrical volume with the dimensions of an average \( \alpha \)-helix (12-Å diameter), whereas the cytoplasmic and lumenal domains of each protein are modeled using the dimensions of globular proteins or of portions of proteins with the same number of amino acids (the three-dimensional structures of ubiquitin, phospholipase, and bacteriorhodopsin were used as models for globular domains and \( \alpha \)-helices). The shape of each domain is arbitrary and is shown merely to indicate the relative amounts of space occupied by translocon components on each side of the membrane. From Ann. Rev. Cell Dev. Biol. 15(1); 799.
The former class is referred to as retention signals and the latter, recycling signals. The KDEL tetrapeptide has been shown to be necessary and sufficient for the retention of a family of resident ER proteins and transplantation of the KDEL motif onto the carboxy-terminus of lysozyme led to its ER residency (Teasdale, 1996). Di-lysine motifs have been shown to be involved in the ER retention of several resident proteins. A di-lysine motif DEKKMP of the E19 protein encoded by adenovirus 3 localises the protein to the ER and retains it there in mammalian cells (Nilson, 1989). ERGIC-53, a protein that continually cycles between the ER and the Golgi with a steady-state localisation in the ERGIC (ER Golgi Intermediate Compartment) has been shown to be targeted to the ERGIC by a di-lysine motif (Itin et al., 1995a; Itin et al., 1995b). Furthermore it was shown that the flanking residues to the di-lysine motif also influence ER retention. Lysines positioned at -3 and -4 from the carboxy terminus function in ER targeting when surrounded by serine or alanine but not by glycine or proline residues (Teasdale and Jackson, 1996). The localisation of the di-lysine motif with respect to the membrane also plays a role in the localisation of the protein. Yet another ER retention signal identified in the p33 isoform of the invariant chain is the di-arginine motif which is also found in TRAM and p63 (Schutze, 1994). Furthermore, it has been shown that the amino-terminal 23 amino acids in the cytoplasmic tail of p63, specifically, the residues Arg-7, Gly-8, Lys-10, Gly-11 and Gly-22 are essential for correct ERGIC localisation (Schweizer et al., 1994). A carboxy terminal H/KDEL motif has also been shown to be used by soluble ER resident proteins for retention in the ER (Sweet, 1992).

Signals in cargo proteins are recognised by appropriate cytosolic machinery that interacts with cargo and mediates their onward transport to the next compartment. Cargo from the ER moves in vesicles coated with proteins of the COPII family towards the Golgi. The first step in the formation of a coated vesicle on the ER membrane involves the recruitment of a small GTPase called Sar1p to the target membrane, the nucleotide exchange on Sar1p being catalysed by Sec12p, an integral membrane protein. The formation of the COPII coat occurs in two steps subsequently: the Sec23p complex (comprising of hSec23p and hSec24p) binds, followed by the recruitment of the Sec13p complex (comprising of hSec13p and hSec31p), both being core components of the COPII coat (Wieland and Harter, 1999). The mammalian homologue of Sec31p has been
shown to contain a propeller-shaped WD motif that mediates interaction with the rest of the complex. The sorting of membrane cargo into a COPII prebudding complex has been shown to be mediated by interaction with the Sec23p complex (Aridor et al., 1998). ERGIC53 has been shown to be incorporated in COPII coated vesicles by means of interaction with Sec23p mediated by a di-phenylalanine motif in its cytoplasmic tail (Kappeler et al., 1997). Sorting of soluble cargo into COPII vesicles requires transmembrane cargo receptors with one or more transmembrane domains, a luminal domain able to interact with cargo and a cytoplasmically exposed domain that can interact with coat subunits. Emp24p and Erv25p are two p24 family members in yeast and they have been shown to be localised to ER derived COPII coated vesicles. Their mammalian homologues have been designated p24 and p23 (Schimmoller et al., 1995), (Belden, 1996). Furthermore, v-SNAREs (vesicle soluble N-ethylmaleimide sensitive factor attachment protein receptors) Bet1p and Bos1p interact with the Sec23p complex in the presence of Sar1pGTP (Springer, 1998).

Some resident ER proteins owe their localisation to retrieval mechanisms that return them from the Golgi to the ER in a retrograde fashion. This retrograde transport is mediated by COPII- and COPI-vesicle components. The COPI coat or coatomer is made up of seven subunits: α-COP, β-COP, β'-COP, γ-COP, δ-COP, ε-COP and ζ-COP. ADP-ribosylation factor 1 (ARF1) functions in the membrane recruitment of the COPI system and the guanine nucleotide exchange on ARF-1 is mediated by cytosolic factors. The COPI coated bud formation is initiated by recruitment of a preassembled coat by interaction with ARF-1 GTP followed by a deformation of the membrane into the coated bud (Wieland and Harter, 1999). The retrieval of soluble cargo that has escaped from the ER is mediated by the KDEL-receptor, a multispansing membrane protein that recognises a carboxy terminal KDEL tetrapeptide and retrieves KDEL bearing proteins from the Golgi (Lewis, 1992). Furthermore, it was shown using immunolocalisation studies that retrieval of di-lysine bearing proteins to the ERGIC from the Golgi apparatus is mediated by COPI coated vesicles (Jackson et al., 1993). The delivery of coated vesicles to the target compartment is achieved by fusion of the vesicular intermediate with the target membrane. Fusion of vesicles has been shown to require prior dissociation of the vesicle coat. GTPase activating proteins or GAPs have been
demonstrated to increase the rate of hydrolysis of Sar1p (for COPII coated vesicles) or ARF1 (for COPI coated vesicles). In the COPII system, Sec23p acts as the GAP for Sar1p whereas in the COPI system an ARF1 specific GAP is recruited from the cytosol (Cukierman, 1995). In the presence of non-hydrolysable homologues of GTP, COP proteins have been shown to be retained and thus vesicles tend to accumulate (Wieland and Harter, 1999). Once the GTPase is released, the COP coated vesicles develop into vesiculo-tubular complexes which are ready to fuse with the target membrane.

The regulation of protein exit from the ER in COPII coated vesicles is mediated by cell-division and specific signaling mechanisms. It has been shown that mitotic cells have reduced exit of proteins from the ER owing to increased dissociation of Sec13p, a COPII protein from the ER membrane. Protein phosphorylation has also been shown to control rate of exit from the ER possibly by affecting the Sec13p/Sec31p complex (Gorelick, 2001). Several unanswered questions in the transport of cargo from the ER to the Golgi and the retrograde transport from the Golgi to the ER exist. It is not known what controls the binding of coatamer to di-basic motifs or what provides for anchors for the coat on COPII vesicles.

1.1.2 Transport through the Golgi apparatus and post-translational modifications

The mammalian Golgi complex is comprised of a reticular network of flattened stacks of cisternae punctuated by pores of various sizes through which tubules project and vesicles move (Marsh, 2002). It functions as a central organelle in the secretory pathway and communicates with the ER on both sides of the stack. The formation of the Golgi apparatus is the subject of long-standing fiery debates with controversial experimental data supporting contrasting theories. According to J. Lippincott-Schwartz, the mammalian Golgi forms by the self-organisation of components as they are exported from the ER (Ward, 2001). G. Warren and colleagues proposed in 2000 that the Golgi forms using a persistent matrix that nucleates Golgi assembly (Seemann, 2000). Historically, secretory transport though the Golgi complex has been explained by one of two models. In the vesicular transport model, cargo is transported in the anterograde direction by COPI-coated vesicles. Simultaneously, some intra-Golgi retrograde transport is mediated by COPI-coated vesicles in order to offset leakage of resident
proteins from one compartment to the other. In the cisternal maturation model, the cisternae themselves act as carriers for cargo and COPI-coated vesicles function to transport resident Golgi components in the retrograde direction (Storrie, 2000). The evidence in favour of the cisternal maturation model is that newly synthesised protein complexes too large to fit in transport vesicles are transported through the pathway (Bonfanti, 1998). Furthermore, transport intermediates formed in a COPI-dependent manner transfer medial/trans glycosylation activity preferentially to the cis-most cisternae (Love, 1998). The evidence in favour of the vesicular transport model is that COPI is required for ER-to-Golgi transport in vivo (Pepperkok et al., 1993). Furthermore, two populations of COPI vesicles have been identified in vivo, one of which contains anterograde cargo (Orci et al., 1997).

Figure 3 Model for COP function in the early secretory pathway. COPII (shown in yellow) buds vesicles from transitional zones of the ER. After COP II coats are shed, ER-derived vesicles fuse with or form vesicle tubular clusters (VTCs). As VTCs fuse with or form the cis-Golgi network, COPI coats (shown in orange) bud retrograde directed vesicles that contain recycling factors and resident modifying activities. From Traffic 1(5), 371-377.

Besides serving as a major sorting point in the secretory pathway, the Golgi also contains numerous enzymes that modify nascent polypeptides in a post-translational
manner. These include the glycosidases and glycosyltransferases responsible for synthesising the huge repertoire of complex oligosaccharides attached to proteins in an N-linked or O-linked fashion, and the glycolipids. The Golgi also contains enzymes that synthesise many sphingolipids such as sphingomyelin and glucosylceramide, the precursor of several other glycolipids. Tyrosine sulfation, palmitoylation and proteolytic cleavage are other post-translational modifications that occur in the Golgi (Munro, 1998).

Based on their biochemical and functional characteristics, Golgi proteins have been categorised into 6 major groups. These include: membrane enzymes which are involved in glycan synthesis (most of which are type II integral membrane proteins) such as α2,6 sialyltransferase, β1,4 galactosyltransferases, N-acetylglucosaminyltransferase and α-mannosidase II; multi-membrane spanning nucleotide sugar transporters such as the UDP-GlcNAc transporter; type I viral glycoproteins such as the Coronavirus M protein and the E1 and E2 glycoproteins of Rubella virus; retrieval receptors such as the KDEL receptor and Rer1p; matrix and cytoskeleton-binding proteins such as giantin, GM130, ankyrin and spectrin; peripheral membrane proteins involved in membrane transport such as p115, β-COP, dynamin, heterotrimeric G proteins and SNARE-like molecules and several recycling TGN membrane proteins (Gleeson, 1998). Certain late-acting Golgi enzymes such as sialyltransferase and galactosyltransferase have been shown to be localised in the trans-Golgi and they do not cycle through the cell surface. The retention of some of these enzymes in the Golgi has been proposed to be mediated in part by their TMDs (Teasdale et al., 1994). Two different models have been proposed to explain the mechanism of retention of such enzymes by their TMDs. According to the ‘kin recognition model’, enzymes in a particular cisterna interact to form oligomers that are too large to enter transport vesicles. This model was supported by the observation that two medial Golgi enzymes, N-acetylglucosaminyltransferase I and mannosidase-II are tightly associated in vivo (Nilsson T, 1994). Furthermore, it was also proposed that the immobility of the oligomers formed is facilitated by binding to a putative Golgi matrix between the cisternae (Slusarewicz et al., 1994). Alternatively, the lipid sorting model claims that the bilayer of the Golgi cisternae contains distinct lipid domains between which Golgi enzymes partition differentially. This model was supported by the fact that
the TMDs of Golgi enzymes do not contain any known sequence motifs and that the TMDs of mammalian Golgi enzymes are on average five amino acids shorter than those of plasma membrane proteins and contain more of the bulky hydrophobic phenylalanine residue (Bretscher, 1993). This explains the preferential localisation in the Golgi based on the fact that Golgi proteins with short TMDs would be excluded from cholesterol-enriched domains (lipid rafts) that are incorporated into transport vesicles destined for the plasma membrane (Lundbaek JA, 2003).

Intra-Golgi transport and retrograde transport from the Golgi to the ER have both been shown to be mediated by COPI-coated vesicles. It has been shown that COPI vesicles bud from all levels of the pathway. Functional COPI-derived vesicles or related transport intermediates have been isolated or generated in vitro and they have been shown to contain relatively high levels of Golgi resident enzymes and KDEL receptor (Love, 1998). COPI-derived transport intermediates have been shown to dock and fuse preferentially with early Golgi membranes and Golgi glycosyltransferases have been suggested to bind COPI in vitro (Dominguez et al., 1998). However, mammalian cells also contain COPI-independent mechanisms of transport. Such a recycling pathway has been shown to be the major mechanism of returning bulk lipids to maintain the balance of the secretory pathway. Retrograde transport between the Golgi and the ER has also been shown to be mediated by microtubule-dependent activities such as kinesin-related motor proteins (Storrie, 2000). The positioning of the Golgi apparatus within the cell has been found to be maintained by cytoplasmic dynein 1 (CD1), a microtubule motor protein. CD1 has been localised to the Golgi apparatus and to Golgi-associated vesicles by immunofluorescence and immunoelectron microscopy (Allan and Kreis, 1986). Conventional kinesins have been demonstrated to influence Golgi architecture and vesicular transport between the Golgi and the plasma membrane. Myosin I has been shown to mediate the transport of Golgi-derived vesicles along actin filaments to the apical plasma membrane and within the sub-microvillar region of epithelial cells (Fath, 1993). Myosin VI, a dimeric unconventional myosin, has been shown to be involved in the possible transport of plasma membrane and Golgi-derived vesicles to actin filament minus ends (Buss et al., 1998). The mechanism by which motor proteins facilitate transport through the Golgi has not yet been fully elucidated. However, it has been
postulated that a cytoskeletal filament and its associated motor protein might pull apart a Golgi tubule creating a tension that could subsequently facilitate pinching off of vesicles, mediated by dynamin (Allan, 2002).

At the distal end of the trans-cisternae of the Golgi is situated, the trans-Golgi network (TGN). This is the compartment where the secretory and endocytic pathways converge and where a number of late-Golgi post-translational modifications occur. It is the sorting station of the Golgi from which vesicles are packaged and sent off to different destinations. Transport from the TGN has been dealt with in detail in the following section.

In spite of the wealth of information on transport processes to and from the Golgi, several debates on the structure-function relationships of the Golgi continue to remain the mainstay of biologists. These include questions whether the mammalian Golgi is formed de novo or by a persistent matrix, the main mechanism of intra-Golgi transport relating to cisternal progression versus maturation models and transport via tubules versus via vesicles, the function of the COPI-coated vesicles in anterograde transport, questions where and how cargo is sorted for exit from the Golgi to the constitutive, endosomal-lysosomal and regulated secretory pathways, the definition of the trans-Golgi network and the role of signaling in Golgi function (Marsh, 2002).

1.1.3 Transport through the TGN

Ever since the first description of the TGN by Griffiths and Simons as a ‘specialised organelle on the trans side of the Golgi stack that is responsible for the routing of proteins to lysosomes, secretory vesicles and the plasma membrane from the Golgi complex’, the view of the TGN as a sorting station has greatly expanded to include proteins, lipids and various small molecules that influence the sorting capacity of the TGN (Griffiths, 1986). The TGN was discovered by Novikoff and colleagues who described a membranous compartment which, like lysosomes, was cytidine monophosphatase positive and found to be specialised regions of the smooth ER (Novikoff, 1976). Thus, they named it GERL, for Golgi saccule that is part of the ER and that forms Lysosomes before subsequent evidence argued against the continuity of this compartment with the ER. Subsequently, it came to be known as the TGN.
Morphologically, the TGN was found using electron microscopic analysis of chemically fixed cells to be in the form of saccules attached to an anastomotic network of membrane tubules, some of which were fragmented into smaller tubules, tubular networks and vesicles (Rambourg, 1995). Today it is well known that TGN morphology is dependent on cell-type. Cells which have an extensive lysosomal system and which do not form large secretory granules have a multi-layered TGN. Cells which form medium-sized secretory granules have moderate sized TGN while cells which form huge secretory granules have almost no TGN. Thus, the TGN seems to be a dynamic structure in flux rather than a stable permanent structure and its size and configuration appear to be functions of the nature of the post-TGN intermediates formed by the particular cell type (Gu, 2001). Several integral membrane proteins habitate the TGN and share structural characteristics such as the ability to recycle from post-Golgi compartments, retention signals located within their cytoplasmic tails, a saturable TGN localisation and in several cases, a type I membrane orientation. TGN38 (and the human homolog TGN46), Menkes P type ATPase, furin are some examples (Gleeson, 1998). Although these proteins are located predominantly at the TGN at steady state, they have all been shown to continuously recycle between the TGN, endosomes and the cell surface although the pathway that these resident proteins follow en route to the cell surface is not well characterised (Gleeson, 1998).

Cargo transported from the TGN can be broadly categorised into two main types: secreted proteins that are destined for the plasma membrane and proteins that are targeted to the endocytic pathway components. Lysosomal hydrolases are transported from the TGN to the lysosomes through the early and late endosomal compartments and this pathway has been shown to be mediated by the mannose 6-phosphate receptors (MPRs), TGN associated adaptor protein AP-1, clathrin and several accessory and regulatory proteins. However, a direct route, independent of the MPRs, for transport of lysosomal membrane proteins such as Lamp1 and Limp-II from the TGN to lysosomes has been shown to be mediated by AP-3 (adaptor protein complex-3) (Le Borgne et al., 1998). Transport out of the TGN is mediated not only by cytoplasmic adaptor protein complexes but also by specific sorting signals in the cytoplasmic tail of cargo molecules.
The nature of these signals and their interaction with the adaptors and accessory molecules has been dealt with in detail in the chapter on sorting at the TGN.

The TGN plays a central role in defining the composition of the apical and basolateral surfaces of polarised cells by directing sorting of their membrane proteins resulting in the typical asymmetric distribution of proteins found in such cells. Sorting of proteins to the basolateral surface relies on specific sorting signals such as tyrosine, di-leucine and acidic sequences as well as other unrelated amino acid motifs. To support this, it was shown that the polymeric immunoglobulin receptor pIgR could be co-immunoprecipitated with the AP-1 adaptor complex en route from the TGN to the basolateral membrane (Orzech, 1999). Furthermore, it was shown that an epithelial-specific µ1 adaptor isoform, µ1B is involved in the basolateral sorting of the low density lipoprotein (LDL) receptor and the transferrin receptor in LLC-PK1 cell lines (Fölsch et al., 1999). Apical sorting mechanisms are fundamentally different from those of basolateral sorting. Since apical membranes are usually enriched in glycosphingolipids, apical sorting is based on lipid-lipid and protein-lipid interactions. It has been proposed that segregation of apically-directed cargo into cholesterol-sphingolipid microdomains or lipid rafts might ensure their apical transport (Benting, 1999). Besides, VIP21/caveolin-1, which has a high affinity for cholesterol and which has been proposed to form homo-oligomers that interact with GPI-anchored proteins, no proteinaceous coat or adaptor proteins have been identified for apical sorting (Dupree, 1993).

Endocrine and neuroendocrine cells have evolved pathways for sorting of prohormone molecules into the regulated secretory pathway whence they are processed and packaged into secretory granules (Gu, 2001). Two models have been proposed for sorting in the regulated secretory pathway. These are the ‘sorting by entry’ and ‘sorting by retention’ models. In the former model, sorting occurs at the TGN either by the intrinsic ability of regulated pathway proteins to aggregate or by virtue of possessing a sorting signal or by one or more TGN sorting receptors. The latter model postulates a post-TGN sorting event (Gu, 2001).

Retrograde transport to the TGN is a well studied pathway for a large number of transmembrane proteins. Lysosomal enzyme-MPR complexes dissociate in the endosomal compartments owing to the lowering of the pH in endosomes, following
which the lysosomal enzymes are transported onwards to lysosomes while the MPRs follow the retrograde transport route to the TGN in a pathway that has been shown to be mediated by Rab9 (Diaz et al., 1997). TGN38 and furin are examples of other proteins that follow similar routes from the endosomes to the TGN. Furin has been shown to be retrieved from post-TGN compartments in a phosphorylation-dependent manner in order to maintain its steady-state localisation in the TGN (Jones, 1995). This step was found to be mediated by phosphofurin acidic cluster sorting protein-1 (PACS-1) in a yeast two hybrid screen with a mutant mimicking the phosphorylated form of the furin cytosolic domain (Wan et al., 1998). PACS-1 was also shown to interact with HIV-1 Nef protein and direct its transport to the TGN, where it is required for downregulation of MHC-I molecules (Piguet, 2000). The retrieval of the carboxypeptidase Y receptor Vps10p in yeast from the prevacuolar compartment to the TGN has been shown to be mediated by the ‘retromer complex’, formed from the assembly of two sub-complexes: the Vps35p-Vps29p-Vps26p and the Vps5p-Vps17p complex (Seaman et al., 1997).

Besides proteins involved in vesicle formation at the TGN, several lipids have been implicated in sorting at the TGN. Various forms of phosphoinositides and the hydrolysed products from phosphatidylcholine, such as phosphatidic acid have been shown to play a role in post-Golgi transport. Yeast Sec14p, a phosphatidylinositol transfer protein (PITP) and its mammalian homologue have been shown to play a role in TGN sorting in yeast *Saccharomyces cerevisiae* and mammals respectively (Bankaitis, 1990). Furthermore, PITP has been shown to be involved in mediating Ca\(^{2+}\) stimulated exocytosis of secretory granules from the TGN (Hay, 1993). Mammalian phospholipase D has been shown to mediate post-Golgi secretion in the regulated secretory pathway (Chen, 1997). Phosphatidyinositol (4) phosphate (PI4P) is the major phosphoinositide on Golgi membranes and has been shown to be required for AP-1 binding to Golgi membranes. Furthermore, it was also shown to be involved in maintenance of Golgi structure by recruiting cytoskeleton to Golgi membranes and by maintaining the flux of membrane moving through the organelle (Roth, 1999).

### 1.2 The Endocytic Pathway

#### 1.2.1 Internalisation from the plasma membrane
The plasma membrane is a highly dynamic structure that separates the internal milieu of the cell or the cytoplasm from the extracellular environment and it regulates the entry and exit of small and large molecules. Small molecules gain access to the cell and exit it by exploiting one of several channels or pumps in the plasma membrane or by passive diffusion. Macromolecules, on the other hand, have to be transported by membrane-bound vesicles formed by invagination and pinching off of sections of the plasma membrane in a process termed ‘endocytosis’. Endocytosis is crucial to the maintenance of a large number of activities that control cellular homeostasis. These include the transmission of neuronal, metabolic and proliferative signals; the uptake of essential nutrients; regulated interactions with the exterior and defense against invading microbes (Mellman, 1996). Mechanistically, the different types of endocytosis exhibited by eukaryotic cells can be broadly categorised into three classes: phagocytosis, clathrin-mediated endocytosis and clathrin-independent endocytosis. These have been treated in detail here.

**Phagocytosis** : Macrophages, neutrophils, monocytes and phagocytic protozoa are capable of an active and highly regulated process of uptake of large pathogens such as bacteria or yeast, or large debris such as the remnants of dead cells, involving cell surface receptors and signaling cascades mediated by Rho-family GTPases (Hall, 2000). Fc receptors on macrophages recognise and are activated by antibodies bound to surface antigens on bacteria following which a signaling cascade involving activation of Cdc42 and Rac triggers actin assembly and the formation of cell-surface extensions that zipper-up around the antibody-coated pathogen to engulf it (Conner, 2003). Certain bacteria such as *Yersinia* and *Salmonella* synthesise surface proteins enabling bacterial attachment and stimulation of one or more plasma membrane receptors, which stimulate membrane ruffling and subsequent engulfment of the bound bacteria (Mellman, 1996).

**Clathrin mediated endocytosis** : Receptor-bound ligands and extracellular fluids are taken up in vesicles coated with clathrin. Clathrin consists of three 192-kDa heavy chains each bound to either of two ~ 30-kDa light chains, LCa or LCb. This complex called a triskelion is the assembly unit of the polygonal lattice composed of hexagons and pentagons that demarcates the bud-site and eventually encases the transport vesicle (Schmid, 1997). Each triskelion leg is made up of an extended heavy chain molecule
oriented with its C terminus at the vertex. The central hub of the triskelion consists of a small globular domain at the extreme C terminus, a trimerisation domain that constitutes the vertex and a proximal leg to which the LCs are bound. The distal leg segment and the globular, ~ 50-kDa terminal domain located at the N-terminus of each heavy chain are connected to the hub through a protease-sensitive bend, called the knee. The terminal domain is involved in binding the adaptor protein complex-2 (AP-2) and also in binding accessory proteins involved in endocytosis, such as β-arrestin. The light chain contains heptad repeats for binding the heavy chain, Ca $^{2+}$ ions and Hsc70. It also contains the phosphorylation sites (Schmid, 1997). The non-clathrin component of the clathrin coat is a heterotetrameric adaptor complex which plays a critical role in the attachment of clathrin to membranes. Indeed, it is the adaptor complex that is first recruited to the membrane to provide binding sites for clathrin. AP-2 functions at the plasma membrane and consists of either of two closely related 100 kDa chains (αA, αC), a 100 kDa β chain, μ2 and σ2. Besides mediating clathrin attachment, adaptors also recruit membrane proteins selectively to clathrin-coated regions. The cytoplasmic domains of plasma membrane receptors contain specific sequence information that facilitates coated pit localisation. Tyrosine and di-leucine based internalisation signals are the two well known plasma membrane internalisation signals. Some examples are listed here: the FDNPVY signal of the LDL receptor, the YENPTY sequence of the β-amyloid precursor protein, the YTRF sequence of the transferrin receptor, the YQPL signal of the T-cell receptor CD3, the YSAF signal of the polymeric Ig receptor, the LL signal of the Fc receptor and the LI signal of the MHC class II invariant chain (Mellman, 1996). In all of the above cases, the μ subunit or the β subunit of the adaptor complex has been shown to interact directly with the localisation signal in the cargo. The α-subunit- N-terminus interacts with inositolpolyphosphates and serves in plasma membrane targeting, the core region of the α subunit is involved in self-association and σ2 chain binding and the appendages are involved in dynamin binding. Dynamin is a GTPase that can self-assemble into ring-like structures which form a collar around nascent budding clathrin coated vesicles.
Controversial reports exist on whether dynamin acts as a mechanochemical enzyme which generates the force necessary for vesicle pinching. Dynamin interacts with amphiphysin which in turn interacts with AP-2 and targets dynamin to nascent clathrin coated vesicles (Okamoto, 1998). The clathrin coated vesicle cycle that drives receptor mediated endocytosis can be visualised as taking place in the following steps: AP-2 recruitment, clathrin assembly, dynamin recruitment, coated-pit invagination, coated pit
constriction requiring the redistribution of dynamin from the lattice and its assembly at
the neck, coated vesicle budding and clathrin release (Schmid, 1997).

AP-2 is not the only known clathrin adaptor that functions at the surface. β-arrestin 1
and 2 are two known adaptors which participate in the endocytosis of G-protein coupled
receptors (GPCR) (Santini et al., 2000). A clathrin box and an AP-2 appendage-binding
determinant are arrayed in tandem at the COOH terminus of β-arrestin, an ordered
region that becomes unstructured upon binding activated GPCRs. Disabled-2 (Dab2) and
numb are two phosphotyrosine binding (PTB) domain containing proteins that function
in the endocytosis of the LDL-receptor and Notch1 respectively. Both proteins are
linked to the endocytosis machinery by interaction with AP-2 and Eps15 and both bind
to FXNPXY sequences in cargo (Morris and Cooper, 2001). Eps15 and the related
Eps15R both contain Eps15 Homology (EH) domains, several Ubiquitin Interaction
Motifs (UIMs), a Proline-rich domain (PRD) and 15 DPF sequences for interaction with
AP-2 (Benmerah et al., 1996). Intersectin, a coiled-coil domain containing protein that
interacts with Eps15, dynamin and synaptojanin acts as a molecular scaffold for the
organisation of other endocytic regulatory proteins (Yamabhai et al., 1998). AP180, a
brain specific adaptor, its non-neuronal homologue CALM, and epsin1 are two other
monomeric adaptors which contain the ENTH (Epsin N-Terminal Homology) domain.

AP180 interacts with the α and β ears of AP-2 by binding the FXDXF motif and
facilitates membrane curvature during clathrin coated vesicle formation. It also interacts
with clathrin and PIP2 (Brett et al., 2002). Epsin1 resembles AP180 in molecular
architecture and binds to IP3 through the amino terminus of its ENTH domain. It also
binds AP-2 and clathrin. Together, AP-180 and AP-2 principally drive polymerisation of
the clathrin coat into a polyhedral lattice, while epsin1 facilitates the accompanying
membrane invagination (Wendland et al., 1999). Hip1 and the related Hip1R both
belonging to the Epsin superfamily bind AP-2 and actin and link the budding vesicle
to the cytoskeleton (Engqvist-Goldstein et al., 2001).

**Clathrin-Independent Endocytosis:** In several cell types clathrin-independent
mechanisms of endocytosis become obvious when clathrin-coated vesicle formation is
inhibited. In *Saccharomyces cerevisiae*, fluid-phase uptake of lucifer yellow or receptor
mediated uptake of α factor continue to progress although at a reduced rate in cells
lacking the clathrin heavy chain genes (Mellman, 1996). Actin-dependent mechanisms have been postulated to compensate the deficiency. In *Dictyostelium*, non-conventional myosins have been shown to substitute clathrin-mediated endocytic mechanisms (Mellman, 1996). Furthermore, evidence for clathrin-independent endocytic mechanisms are available from studies of polarised Madine Darby canine kidney cells in which cholera toxin was shown to selectively stimulate the uptake of the protein toxin ricin at the apical plasma membrane (Sandvig and Vandeurs, 1999).

Caveolae are flask-shaped invaginations of the plasma membrane which demarcate cholesterol and sphingolipid rich microdomains in which several signaling molecules and membrane transporters are concentrated. Caveolin, a dimeric protein that binds cholesterol inserts as a loop into the inner leaflet of the plasma membrane and self-associates to form a striated coat on the surface of membrane invaginations. Whether caveolae are static structures at the plasma membrane or structures actively involved in endocytosis, continues to remain debated. Activation of serum albumin receptor gp60 has been shown to trigger caveolae uptake. Opportunistic ligands such as simian virus 40 (SV40) particles are known to activate signaling cascades that involve multivalent crosslinking of caveolae-localised surface receptors to trigger their own uptake (Conner, 2003).

Figure 5 Multiple portals of entry into the mammalian cell. The endocytic pathways differ with regard to the size of the endocytic vesicle, the nature of the cargo (ligands, receptors and lipids) and the mechanism of vesicle formation. From Nature 2003 Mar 6;422(6927):37-44.

Macropinocytosis accompanied by membrane ruffling is another means of fluid uptake triggered by signaling through Rho-family GTPases. Actin-driven formation of
membrane protrusions is followed by collapse and fusion of these protrusions with the plasma membrane resulting in the generation of large endocytic vesicles, called macropinosomes. Platelet-derived growth factor (PDGF)-induced macropinocytosis involving the activation of Rho-GTPase Rac and its downstream kinase PAK has been shown to play a role in directed cell migration (Ridley, 2001). The actin-dependence of the process has been demonstrated by the observation that melanoma cells deficient in actin-binding protein (ABP-1) exhibit defects in constitutive membrane ruffling and macropinocytic activity (Cunningham, 1992).

1.2.2 Transport through early and/or recycling endosomes:
Endocytosed molecules such as recycling receptors with their bound ligands and downregulated receptors are delivered to early endosomes where dissociation of the receptor-ligand complexes occurs owing to the mildly acidic lumenal pH of the early endosomes (6.3-6.8) (Kornfeld and Mellman, 1989). Early endosomes represent the first sorting station in the endocytic pathway where recycling receptors such as the transferrin receptor and asialoglycoprotein receptor are rapidly segregated away from their ligand and transported along the recycling route whereas ligands follow the degradation pathway along with downregulated receptors such as the epidermal growth factor receptor (EGFR) and the growth hormone receptor (Gruenberg, 2001). The early endosome (EE) has been shown to be a dynamic organelle capable of homotypic fusion. It consists of cisternal regions from which thin tubules (~ 60 nm diameter) and large vesicles (300-400 nm diameter) appear to emanate. The vesicles appear to contain membrane invaginations that may or may not detach from the limiting membrane to form free vesicles in the lumen. Early endosomes have been shown to be enriched in Rab5 and the early endosomal antigen EEA1 but depleted of Rab4, annexin II, actin and the t-SNARE syntaxin 13 (Trischler et al., 1999). Recycling of receptors to the plasma membrane occurs in recycling endosomes (RE) or vesicles and appears to function as a default pathway. No recycling motif has yet been described in cargo returned to the plasma membrane. Lysosomal sorting motifs have been identified in proteins such as P-selectin, HIV-1 Nef and EGFR although these bear very little resemblance to each other (Blagoveshchenskaya et al., 1998), (Piguet et al., 1999), (Kil and Carlin, 2000). Certain
endosomal vesicles have been shown to bear a clathrin coat. A prominent clathrin coat was reported on the limiting membrane of premelanosomes, which are specialised EEs involved in pigmentation (Raposo, 2001). Early endosomes have been shown to be reached by endocytic tracers in 1-5 minutes whereas recycling endosomes are reached after 5 minutes (Tooze, 1991). It is not clear whether transport between EEs and REs occurs by vesicles or tubules. In addition to pathways of recycling and degradation, a third exit from EEs leads to the TGN. Minor amounts of endocytosed transferrin are delivered to the TGN and an endocytosed TGN38 chimaeric protein is delivered to the TGN after trafficking through REs (Stoorvogel, 1998), (Ghosh et al., 1998).

Several key components that regulate membrane organization and transport on the EE limiting membrane have been described. Rab4 which is involved in recycling, Rab5 which builds a specific effector platform on the membrane to integrate membrane fusion, budding and interaction with cytoskeletal components, EEA1, the Rab5 effector, the t-SNARE syntaxin-13 which is required for endosome fusion and recycling, v-SNAREs cellulobrevin and endobrevin, transferrin and transferrin receptor are all markers of the early and recycling endosomes (Sachse, 2002). Furthermore, transport along the recycling pathway has been shown to depend on the actin cytoskeleton and unconventional myosin motors which might have a mechanical role in tubule biogenesis and dynamics (Apodaca, 2001). RME-1, a new member of the conserved family of EH-domain proteins has been shown to be associated with recycling endosomes and has been proposed to be involved in the exit of membrane proteins from this compartment (Grant, 2001).

1.2.3 Transport through Late Endosomes or Multivesicular Bodies:
Multivesicular bodies (MVBs) have been described as transport intermediates along the degradation pathway from early to late endosomes. They do not contain early endosome specific proteins or recycling receptors nor do they contain the major lipid and protein constituents of late endosomes or lysosomes. The limiting membrane and internal invaginations form the two morphologically visible membrane domains of MVBs (Gruenberg, 2001). EGF and the EGF-receptor (EGFR) have been shown to be delivered to MVBs following which fusion of the limiting membrane of the MVB with the
lysosomal membrane results in the delivery of the luminal MVB vesicles and their contents to lysosomes where they are degraded (Futter et al., 1996). Membrane proteins not destined for degradation remain in the limiting MVB membrane and are transferred to the lysosomal limiting membrane, recycled to the plasma membrane or transported to other destinations (Katzmann et al., 2002). Sorting into MVBs has been shown to be a highly regulated process facilitated by post-translational attachment of ubiquitin to the cytoplasmic domains of cargo. Endocytic cargo such as EGFR and Ste2, a GPCR, is selected this way. Ubiquitylation is carried out by a cascade of enzymes called E1, E2 and E3 which execute the formation of an isopeptide bond between the 76 amino acid polypeptide ubiquitin and the ε-amino acid group of a lysine residue in the protein substrate. Monoubiquitylation has been shown to function as a signal for MVB sorting. It has also been shown as a means of downregulating growth hormone receptor (GHR) tyrosine kinase (Katzmann et al., 2002). Several trans-acting factors have been shown to interact with ubiquitin for MVB sorting. Tsg101, a mammalian homologue of the yeast Class E Vps protein, Vps23, contains a UBC (Ubiquitin Conjugating)-like motif and functions in MVB sorting in complex with Vps28, the two together forming the ESCRT-I (Endosomal Sorting Complex Required for Transport) complex. Hrs (Hepatocyte growth factor-regulated tyrosine kinase substrate), and Epsin, both contain UIMs (Ubiquitin Interaction Motif) and bind to ubiquitin. Hrs also interacts with Eps15 and STAM (Signal Transducing Adaptor Molecule) and localises to clathrin containing microdomains on early endosomes and has been shown to be essential for MVB formation (Raiborg et al., 2002). Lysobisphosphatidic acid, an unconventional phospholipid, has been shown to be located exclusively on the luminal membranes of MVBs. Its inverted cone-shaped structure has been postulated to facilitate inward deformation of the limiting MVB membrane (Kobayashi et al., 1999). Phosphatidylinositol 3-phosphate, which is enriched on endosomal membranes, functions to recruit cytoplasmic transport effectors containing a FYVE (Fab1, YOTB, Vac1, EEA1) domain or Phox homology (PX) domain to the MVB membrane. Hrs is known to be recruited in this way (Burd et al., 1998).

Late endosomes (LE), which have also been thought of as more mature forms of MVBs represent the pre-lysosomal compartment rich in Lamp1, rab7, rab9 and the cation-
dependent and independent MPRs (Mellman, 1996). The limiting membrane is believed to be protected from the degradative milieu owing to its high glycosylation state (Griffiths, 1988). MLN64, a homologue of the mitochondrial steroidogenic acute regulatory protein is another constituent of the LE limiting membrane (Alpy et al., 2001). Members of the tetraspanin family, including CD63/Lamp3 have been shown to accumulate within the internal membranes of LEs (Escola et al., 1998). The LE-like Class II MHC compartment contains luminal vesicles in which class II MHC molecules are sequestered where they provide a very important immune function in antigen-presenting cells. Upon stimulation, these LE compartments fuse with the plasma membrane and release the vesicles termed ‘exosomes’ which have been shown to promote B and T lymphocyte activation (Denzel, 2000). Endocytic tracers reach LEs after 10-15 minutes (Griffiths, 1989). LEs are known to contain some of the hydrolytically active lysosomal enzymes and are also known to initiate the degradative process (Mellman, 1996). LEs and lysosomes are said to be in a constant flux and dynamic interchange. Besides transfer of lysosomal enzymes to their final destination, occurring by content mixing between LEs and lysosomes, recycling also occurs from LEs. MPRs and furin recycle to the TGN from LEs (Mallet and Maxfield, 1999). The case for the MPRs has been dealt with in detail in the section on ‘Endosomal Sorting’.

1.2.4 Transport to and through Lysosomes

Lysosomes, the degradative compartments of the cell are reached by endocytic tracers after about 30 minutes (Kornfeld and Mellman, 1989). First described by de Duve in the 1960s as being ‘lytic bodies’ of the cell, lysosomes have come a long way as organelles capable of sorting in the secretory pathway. They are largely devoid of the MPRs and are rich in acid hydrolases and membrane glycoproteins. Their lumen is electron-dense and together with amorphous material can contain internal vesicles in different stages of degradation (Sachse, 2002). The lysosomal limiting membrane compartmentalises the hydrolytic enzymes and its glycoprotein rich nature confers protection against its contents. A subset of lysosomal membrane glycoproteins have been demonstrated to be protected from proteolytic cleavage by their N-linked oligosaccharides (Kundra and Kornfeld, 1999). Two of the best characterised membrane proteins are Lamp1 and
Lamp2. The two display strikingly conserved primary sequences and have similar
domain structures and biochemical properties. Both are type-I membrane proteins with a
luminal ectodomain connected to a transmembrane region and a short cytosolic tail.
Most of the potential N-glycosylation sites are utilised giving an apparent molecular
mass of 90-120 kDa, three-fourths of the molecular weight being represented by the
sugar residues. Both proteins have a conserved C-terminal lysosomal sorting motif
GYXXZ (where Z corresponds to Ile in Lamp 1, or to Phe, Leu or Val in the different
Lamp 2 isoforms) in their cytoplasmic domain. Limp1 or Lamp3 displays no homology
to Lamp1 or Lamp2 and traverses the lipid bilayer four times. It carries an 11-residue
cytoplasmic domain with a C-terminal lysosomal sorting motif GYXXM. Limp2
traverses the membrane twice with its N-terminal transmembrane domain corresponding
to the uncleaved signal peptide and a second hydrophobic region near the C-terminus
serving as a membrane anchor. Its lysosomal targeting is mediated via a di-leucine based
LI type signal. Lysosomal Acid Phosphatase (LAP), a soluble lysosomal enzyme is
transiently associated with the membrane as an integral membrane protein. The
cytoplasmic domain encodes a GYXXV motif which functions in its targeting (Hunziker
and Geuze, 1996).

The dynamic nature of lysosomes is demonstrated by their ability to fuse even with the
plasma membrane thus contributing to plasma membrane repair in wounded cells and
recycling of the plasma membrane. In macrophages and platelets this feature was long
recognised (Tapper, 1990). More recently, it was shown that calcium-dependent fusion
of lysosomes with the plasma membrane also occurs in fibroblast-like cells (Rodriguez
et al., 1997). The three main well established routes of transport to lysosomes are
endocytosis, biosynthetic transport via endosomes and autophagy. The first two routes
have been described earlier in the Introduction and will be taken up again later. Upon
nutrient starvation, the process of autophagy is stimulated which results in the
envelopment of a portion of cytoplasm by the plasma membrane to form an
autophagosome which fuses with lysosomes (Yamamoto et al., 1998).

Transport to lysosomes is mediated by cytosolic adaptor protein AP-3. Mutations in AP-3
occur naturally in fruit-flies and humans leading to alterations in eye colour in the
former and a rare genetic disease in the latter as a result of defects in delivery of proteins
to lysosome-related organelles i.e., *Drosophila* eye pigment granules and platelet dense core granules, respectively. Eye colour mutants in *Drosophila* have been identified to be mutations in specific AP-3 subunits: δ-garnet, β3-ruby, µ3-carmine and σ3-orange. In mice, coat colour mutants *pearl* and *mocha* are caused due to mutations in the genes encoding AP-3 subunits β3B and δ, respectively. Lamps are invariably transported to the lysosomal limiting membrane using AP-3 dependent pathways (Luzio, 2003). Lysosome-related organelles exist in a wide variety of cell-types and share compositional and physiological characteristics with conventional lysosomes. Depending on the cell-type they may either compose the entire pool of lysosomes or co-exist with *bona fide* lysosomes in the host cell. Melanosomes which function in melanin formation, storage and transfer, platelet dense granules which release ATP, ADP, serotonin and calcium for blood clotting, lamellar bodies of the lung epithelial type II cells which function in surfactant production, lytic granules of cytotoxic T lymphocytes and natural killer cells, Major Histocompatibility Complex Type II compartments of antigen presenting cells, Weibel-Palade bodies of endothelial cells and platelet α granules of platelets and megakaryocytes which function in platelet adhesion and blood clotting are some examples (Raposo, 2002).

The acquisition of lysosomal contents is mediated by fusion and fission with the late endosomal compartment and the fusion is mediated by the common cytosolic fusion machinery comprised of NSF (N-ethylmaleimide sensitive factor) and SNAP (soluble NSF attachment protein). This machinery functions according to the tenets of the SNARE hypothesis according to which pairing between specific v-(vesicle) and t-(target organelle) SNAREs leads to the formation of a functional SNARE complex. In a calcium/calmodulin dependent manner, fusion is mediated by tethering and docking of the vesicular compartments involved (Luzio et al., 2000).
2. Lysosomal Biogenesis

Lysosomes are membrane bound degradative organelles of ~ 0.5 µm diameters and contain a large number of acid hydrolases capable of degrading macromolecules such as proteins, lipids, nucleic acids and sulphated compounds. Since their discovery by de Duve in 1949, the repertoire of functions of lysosomes has increased. Lysosomes are not only involved in the terminal degradation of damaged macromolecules but also play a role in functions as diverse as antigen processing and plasma membrane repair. Furthermore, certain mammalian cell types contain specialised lysosome-like structures which are involved in pigmentation and autophagy. Typically, lysosomes are defined as
organelles devoid of the two mannose 6-phosphate receptors, the CD-MPR and the CI-MPR, and other recycling receptors. The biogenesis of lysosomes is a result of the convergence of the biosynthetic and endocytic pathways. Literature abounds in theories propounded to explain the biogenesis of lysosomes. The most widely acknowledged of these is the ‘kiss and run’ theory of Storrie and Desjardins (1996) according to which lysosomes are in a process of continuous fusion and fission with late endosomes resulting in the exchange of contents (Storrie and Desjardins, 1996). Very little experimental evidence exists for the maturation model of lysosomal biogenesis according to which late endosomes mature into lysosomes resulting in the formation of organelles rich in hydrolytic enzymes and lysosomal membrane glycoproteins and devoid of recycling receptors. Even less evidence supports the classical view of vesicular transport between late endosomes and lysosomes. The biosynthetic pathway comprises of vesicular intermediates that transport free cargo or cargo bound to receptors from the ER, where they are synthesised, through the Golgi apparatus and onwards to the plasma membrane, late endosomes and lysosomes. The classical view of the endocytic pathway is that of a linear transport pathway with a few possible deviations and recycling steps depending on the proteins involved. Proteins that are internalised at the plasma membrane are included in early endosomes where dissociation of ligand-receptor complexes occurs owing to acidification of contents, whence the receptors might take a detour directly or through recycling endosomes back to the plasma membrane or be delivered to late endosomes. In late endosomes, the dissociation is completed and the recycling receptors are retrieved to the TGN in vesicular intermediates while the lysosomal enzymes are delivered to lysosomes where they are activated. The paraphernalia involved in these complex transport processes make an overall description of the trafficking pathways almost impossible, thus necessitating a stepwise treatment of each individual transport step involved in lysosomal biogenesis.

3. Intracellular transport and Disease

An alarming number of human diseases have been mapped to defects in genes and proteins responsible for various steps in protein intracellular transport thus highlighting the importance of correct trafficking in eukaryotic cells. Sequencing of the human
genome has unraveled the molecular mysteries behind a large number of congenital disorders linked to trafficking. Some of them have been described here in some detail, although this list is by no means exhaustive or comprehensive.

**Diseases related to ER transport:** Mutations in the cystic fibrosis transmembrane regulator have been shown to lead to retention and degradation of the protein in the ER resulting in a subsequent loss of chloride regulation (Kopito, 1999). Fabri disease, a lysosomal storage disorder results from mutations in α-D-galactosidase, leading to the production of a thermolabile enzyme that is retained and degraded in the ER thus causing an endocrine defect and neurological symptoms (Ishii, 1996). Tay-Sachs disease, a neurological disease, has the same etiology resulting from mutations in β-hexosaminidase (Lau, 1989). Class 2 mutations in the insulin receptor impair its transport from the ER, markedly reducing its surface expression leading to insulin resistance and Diabetes Mellitus (Taylor, 1992). Mutations in the LDL receptor lead to ER retention and degradation of the receptor thus preventing cell-surface expression resulting in familial hypercholesterolaemia (Yokode et al., 1992). Cargo accumulation in the ER leads to signaling and stress responses leading to various diseased states. Charcot-Marie-Tooth syndrome, a neurological disorder results from mutations in the peripheral myelin protein 22 which accumulates in the ER leading to signaling which results in abnormal growth and differentiation (Tobler, 1999). Alzheimer’s disease, in its early onset form, has been shown to result from mutations in Presenilins. Presenilins are largely localised to the ER and are involved in the proteolytic processing of receptors (Gething, 2000). Combined factors V and VIII deficiency is caused by a lack of ERGIC-53 due to mis-sense mutations leading to a secretion block of coagulation factors V and VIII and development of the bleeding disorder (Nichols, 1992).

**Diseases related to transport though the Golgi and the TGN:** The classical I-cell disease is a defect in the N-acetylglucosamine-1-phosphotransferase protein. In this neurological disorder, the mannose 6-phosphate recognition tag is not correctly exposed on lysosomal enzymes leading to their inefficient transport and secretion (Glickman and Kornfeld, 1993). Ataxia talangiectasias (AT) is a mutation in the AT mutated protein (ATM). ATM is a member of the phosphoinositide 3-kinase family. It has been shown to interact with adaptin and its mutation leads to the syndrome which encompasses cancer,
neurological disease and immunodeficiency (Lim, 1998). Niemann Pick C is a neurological disease due to mutations in NPC1, a sterol sensing protein required for transport of cholesteryl ester (and potentially fluid phase constituents) from the late endosome to other organelles. Defects result in an accumulation of lipid in lysosomes (Liscum, 2000). Scott syndrome is a rare bleeding disorder caused by a platelet coagulant defect that results from mutations in scramblase which is responsible for the bidirectional transport of phospholipids (independently of the head group) across the plasma membrane (Raggers, 2000). Tangier disease is a neurological disease occurring due to mutations in the ATP binding cassette transporter 1 which transports lipid-free high-density lipoprotein and mediates export of cholesterol and phospholipids. Mutations in this protein cause morphological changes in the Golgi and lysosomes of mononuclear phagocytes (Robenek, 1991). Defects in Ocr11 lead to the Oculocerebro-renal syndrome of Lowe, a neurological disease. Ocr11 is homologous to phosphatidylinositol 4, 5-bisphosphatase. PIP₂ is implicated in the regulation of Golgi function through activation of phospholipase D and regulation of actin assembly. Disruption of Golgi function is thought to be the cause of the developmental defects of the lens as well as abnormalities of renal and neurological tissues (Suchy, 1995). Acute lymphoblastic leukaemia or acute myeloid leukaemia is caused by mutations in CALM which is a homologue of a synapse-specific clathrin assembly protein, AP180. CALM binds clathrin, and its overexpression inhibits endocytosis. CALM fused to the transcription factor AF-10 has been shown to be a rare but recurring mutation associated with the two forms of leukaemia (Tebar et al., 1999). Myeloid leukaemia is caused due to mutations in AF1p, the human homologue of murine Eps15. In the leukaemic condition, AF1p has been shown to be fused to the transcription factor ALL1/HRX (Fazioli, 1993).

**Lysosomal Storage and Trafficking Diseases:** Lysosomal storage diseases are the result of defects in specific catabolic enzymes which in turn results in incomplete degradation of macromolecules, causing accumulation of metabolic intermediates in lysosomes. Sphingolipidosis is a neurological disease occurring due to a defective β-galactosidase enzyme which causes inappropriate processing of sphingolipids (Gieselmann, 1995). In Mucolipidosis IV, sphingolipids, phospholipids and acid mucopolysaccharides
accumulate in lysosomes. Due to the redistribution of sphingolipid, cholesterol and the sterol sensing protein NPC1 accumulate in late endosomes and lysosomes (Kornfeld and Sly, 1985). Hermansky-Pudlak syndrome (HPS) type I is a pigmentation defect and an immunodeficiency occurring due to mutations in HPS1 which is thought to transiently associate with the lysosomal membrane and mediate lysosomal biogenesis. Furthermore, immunoelectron microscopy analyses of Hps1p in melanoma-derived cell lines revealed that the protein is associated with membranes of the TGN and has been implicated to be involved in the trafficking of melanosomal proteins. HPS patients are characterised by hypopigmentation, bleeding disorders due to platelet storage pool defects and restrictive lung disease. Mutations in the β3A subunit of the AP-3 adaptor protein complex results in mislocalisation of several lysosomal proteins including the Lamps and HPS type II disease. HPS type III is attributed to four additional genes identified due to their association with HPS in humans. They encode polypeptides that display no significant homology to any protein of known function. Pallidin and the muted protein are two such relatively small polypeptides with a high propensity to form coiled coil structures. A possible role for pallidin in intracellular protein trafficking has been suggested based on its reported interaction with syntaxin 13 (Starcevic, 2002). Chediak-Higashi syndrome is a pigmentation defect in which lysosome and lysosome-related organelle function is disrupted due to mutations in the 429-kDa LYST protein, resulting in albinism and immune deficiency. The mechanism by which LYST regulates lysosome function is unknown. It is predicted to be a cytosolic protein with three to four defined domains including a weak ARM/HEAT repeat containing alpha-helices, a possible perilipin domain, a BEACH domain containing the amino acid sequence WIDL and a series of WD-40 repeats at the carboxy terminus. ARM motifs are suggested to mediate membrane association and HEAT repeats are thought to play a role in vesicle transport. The perilipin domain is a lipid association domain and is also found in TIP47. The WD-40 domain is a protein-protein interaction domain (Ward et al., 2002). Griscelli’s disease appears to be the result of mutations in either of two neighbouring genes of the chromosome locus 15q21. The first is Rab27 and the second is myosin5a. While mutations in either gene results in partial albinism, only those with mutations in Rab27 show uncontrolled T-lymphocyte activation, indicating that Rab27 may be required for
the exocytosis of cytolytic granules (Menasche et al., 2000). Danon disease is a heart disease owing to mutations in Lamp2 which may be required for the conversion of early autophagic vacuoles to vacuoles (Aridor and Hannan, 2000).

4. Sorting at the TGN

The TGN plays a pivotal role in directing proteins to various intracellular destinations. Conventionally, traffic out of the TGN has been regarded as a process with two options. In the constitutive or default pathway, proteins are delivered to the cell surface. In the alternative selective pathway, proteins are routed to the intracellular endosomal membrane system. However, in polarised cells proteins destined for basolateral delivery are differentially sorted and trafficked in carrier intermediates separate from that which shuttle cargo to the apical membrane surface thus rendering the distribution on the surface asymmetric. In secretory cells undergoing regulated exocytosis, mature dense-core secretory granules packed with cargo are poised for secretion (Traub and Kornfeld, 1997). Analysis of sorting at the TGN has been facilitated by two major tools. Incubation of cells at 19 ºC has been shown to induce a reversible block of TGN export, the reverse being brought about by warming the cells to 37 ºC (Matlin and Simons, 1983). Brefeldin A, a fungal metabolite, reversibly inhibits the guanine-nucleotide exchange factor of the ARF protein thus bringing about a block of protein export from the TGN (Miller, 1992).

4.1 Signal Sequences: Sorting at the TGN is mediated by signals usually located in the cytoplasmic tail of cargo. These are made up of short, linear sequences of amino acids which interact with specific cytosolic machinery to effect vesicle transport between specific destinations. The different signals mediating sorting at the TGN can be grouped into 3 major classes: signals of the YXXΦ type (where X represents any amino acid and Φ represents a bulky hydrophobic amino acid), (DE)XXXL(LI) type signals (where X represents any amino acid) and DXXLL type signals (where X is any amino acid). These signal arrays are not exactly conserved sequences but degenerate motifs of four to seven residues out of which two to three are conserved. Certain sorting signals are not linear peptide motifs but folded structures in which the critical residues are not
necessarily collinear. Examples of this type of conformational determinants are discussed in the section on ‘Endosomal Sorting’. Sorting usually occurs at the coated regions of the TGN membrane indicating the existence of interactions between signals in the cytoplasmic domains of cargo and components of the protein coats (Pearse and Robinson, 1990). Besides being present on several endocytic receptors, the YXXΦ signal is found on intracellular sorting receptors such as the CI- and the CD-MPRs, TGN38 and furin, and lysosomal proteins such as Lamp-1 and Lamp-2. All these proteins have distinct steady state localisation although they all share the property of trafficking through the plasma membrane to some extent. A salient feature of YXXΦ signals involved in lysosomal targeting at the TGN is that most have a glycine residue preceding the critical tyrosine residue. Mutation of this glycine to alanine has been shown to decrease lysosomal targeting but not endocytosis (Harter and Mellman, 1992). Furthermore, YXXΦ type lysosomal sorting motifs tend to have acidic residues at the X positions and these have been shown to contribute to sorting efficiency (Rous et al., 2002). YXXΦ type signals active at the TGN are conspicuous for their presence at 6-9 residues from the transmembrane domain and at the carboxy-terminus of the proteins (Rohrer, 1996). In some proteins targeted to the late endosomal or lysosomal compartments such as CD1b or cystinosin, the Φ residue is followed by only one more residue. The (DE)XXXL(LI) type signals also function in endocytosis and at the TGN. An acidic residue at position -4 from the first leucine appears to be important for targeting to late endosomes or lysosomes but not for internalisation (Pond et al., 1995). Similar to YXXΦ signals, (DE)XXXL(LI) signals also have a position dependence. In NPC1 and Limp II, proteins targeted to late endosomes or lysosomes, VMAT1 and VMAT2, proteins targeted to synaptic dense core granules, the signals are very close to the transmembrane domain (6 to 11 residues away) (Geisler et al., 1998). The DXXLL type sorting signals are present in several transmembrane receptors and other proteins that cycle between the TGN and endosomes, such as the CI- and CD-MPRs, sortilin, the LDL-receptor related proteins LRP3 and LRP10 and β-secretase. They mediate incorporation into clathrin-coated vesicles budding from the TGN and headed for the endosomal system, at least for the MPRs (Johnson and Kornfeld, 1992a), (Doray et al., 2002b). The D and LL residues in these signals have been shown to be critical since
mutation of any of these residues to alanine inactivates the signals and results in increased expression of the transmembrane proteins at the surface (Johnson and Kornfeld, 1992a). The D position has been shown not to tolerate even isoelectric or isosteric substitutions without drastic loss of activity (Chen et al., 1997). The X residues or other residues amino-terminal to the critical D have been shown to be less important for function (Chen et al., 1997). Because the D residue is found in the context of a cluster of acidic residues, this type of signal is referred to as the acidic cluster di-leucine based sorting signal. Most of these signals are separated from the carboxy-termini of the proteins by one or two variable residues. For the CD-MPR, this distance is shortened by palmitoylation of one or two cysteine residues in its cytoplasmic domain facilitating its membrane association (Schweizer et al., 1996). Clusters of acidic residues containing sites for phosphorylation by casein kinase II represent the ‘acidic clusters’ and are found in many transmembrane proteins localised to the TGN at steady state, including the prohormone-processing enzymes furin, PC7 and carboxypeptidase D. In the case of the CD-MPR, E58 and E59 residues of the casein kinase-2 site were shown to be important for high-affinity GGA1 binding in vitro to effect sorting at the TGN. However, phosphorylation of residue S57 did not seem to play any role in GGA1 binding. The same was true for AP-1 interaction with the CD-MPR although all the glutamates surrounding the phosphorylation site, namely E55, E56, E58 and E59, were found to be important for optimal AP-1 interaction (Stoeckli, 2004).

4.2 Interacting Proteins: AP-1: YXXΦ type signals have been shown to interact with heterotetrameric adaptor protein complexes, specifically AP-1 at the TGN. AP-1 is made up of 4 adaptin chains: two large chains, γ1 and β1, one medium chain μ1 and one small chain σ1. The large chains are ~100 kDa in size whereas the medium and small chains are ~50 kDa and ~20 kDa, respectively (Kirchhausen, 1999). Yeast two hybrid systems and in vitro binding studies revealed that signal recognition is a property of the μ subunit of the adaptor. Binding to the μ subunit is known to occur with apparent affinities in the range of 0.05-100 μM and has been shown to be strictly dependent on the Y and Φ subunits (Ohno et al., 1995). Furthermore, it has been shown that for proteins trafficking from the TGN, the positions Y+1 and Y+2 are occupied by
negatively charged or polar residues (Boll et al., 1996). By crystallographic analysis it has been shown that the \( \mu \) subunit is organised into an amino-terminal domain comprising one-third of the protein and a carboxy-terminal domain comprising the remaining two-thirds. The amino-terminal domain mediates assembly with the \( \beta \) subunit whereas the carboxy-terminal domain harbours the binding site for YXX\( \Phi \) type signals (Bonifacino, 2003). Residues 132-331 in the amino-terminal end of the \( \gamma \) chain have been shown to be involved in TGN-targeting of AP-1 and in \( \sigma \)1 chain interaction. Residues 616-633 in the hinge region of the \( \beta \) chain have been shown to bind clathrin whereas the core region binds the \( \mu \) chain. Residues 704-822 of the appendage of the \( \beta \) chain have also been implicated to a minor extent in TGN-targeting of the adaptor (Schmid, 1997). AP-1 recruitment to the TGN is shown to be mediated by ARF1 and has also been found to be sensitive to Brefeldin A. Cargo molecules are thought to define the docking sites for adaptors on the TGN membrane. To ensure targeting specificity in AP-1 assembly on the TGN, components in addition to the transmembrane cargo molecules are involved. Using chemical crosslinking and metabolic labeling experiments, a 75-80 kDa protein was found to be the predominant AP-1 interacting species (Schmid, 1997). Regulation of AP-1 function has been shown to be mediated by phosphoinositide binding. Phosphinositides phosphorylated at the D-3 position of the inositol ring enhance recognition of YXX\( \Phi \) signals by AP-1 and diminish the recognition of LL motifs thereby skewing the transport machinery toward collection of YXX\( \Phi \)-containing cargo and away from LL-containing cargo (Kirchhausen, 1999). Furthermore, cytosolic and to a lesser extent membrane-bound AP-1 is phosphorylated, particularly in several serine residues located close to the clathrin-binding box in the \( \beta \)-hinge region. These phosphorylations have been shown to prevent binding of AP-1 to clathrin cages \textit{in vitro} (Kirchhausen, 1999). AP-1 has also been shown to interact with the (DE)XXXL(LI) type signals in cargo such as the invariant chain Ii. The fine specificity of the interaction has been shown to be mediated by the X residues and contextual factors (Bonifacino, 2003).

AP-1 has also been shown to be involved in retrograde transport from endosomes to the TGN in studies using fibroblasts from AP-1 knockout mice in which CD-MPR was
found to exit the TGN, get transported to the surface, reendocytosed at the surface and accumulate in an early endosomal compartment positive for EEA1 (Meyer et al., 2000). AP-1 has also been shown to interact with a large number of accessory proteins involved in traffic out of the TGN. γ-ear binding ENTH and ANTH domain proteins have been identified and shown to mediate sorting at the TGN. Enthoprotein (also called epsinR or Clint) associates with elements of the TGN and endosomes that contain clathrin and AP-1 (Duncan, 2003). The ENTH domain has been proposed to direct enthoprotein to the TGN membrane. Other γ-ear binding accessory proteins include Eps15, γ-synergin, auxilin-2, ARFGap-1 and Rabaptin-5 (Duncan, 2003). Most of these accessory proteins function in the assembly of clathrin-coated vesicles containing the adaptor and cargo at the TGN membrane and therefore are capable of multiple interactions afforded by their domain architecture.

**Golgi-localised, γ-ear containing, Arf-binding (GGA) proteins:** GGAs are ubiquitously expressed, monomeric proteins that contain a carboxy-terminal domain homologous to the carboxy-terminal domain of the γ-1 and γ-2 adaptin chains of the AP-1 isoforms. They are associated with the TGN and endosomes and interact with Arf proteins, clathrin and the cytosolic tails of intracellular transport receptors. There are 3 GGAs in humans (GGA1, 2 and 3), 2 in the yeast *Saccharomyces cerevisiae* and one each in *Caenorhabditis elegans* and *Drosophila melanogaster*. They all have a modular organisation consisting of a tandem arrangement of three folded domains: the VHS domain (found in Vps27, Hrs and Stam), the GAT domain (found in GGA and TOM) and a GAE (gamma adaptin ear) domain (Bonifacino, 2004). These are separated by two linker sequences. The VHS domain functions as a recognition module for sorting signals in the cytosolic tails of sortilin and the MPRs (Puertollano et al., 2001a). The signal recognised has been shown to be the acidic cluster di-leucine sorting signal (Doray et al., 2002a). The acidic cluster in the casein kinase II site of the CD-MPR but not its phosphorylation was shown to mediate GGA binding, recently (Stoeckli, 2004). The GAT domain mediates interaction with Arf1 and Arf3 and thus enables TGN recruitment of the GGAs (DellAngelica et al., 2000). The GAT domain also interacts with Rabaptin-5, a Rab4/Rab5 effector that is present with the Rab5 GEF, Rabex-5 (Horiuchi et al., 1997) The GAT domain consists of an elongated, all α-helical fold that
comprises two subdomains- an amino-terminal hook that binds Arf and a carboxy-terminal triple-α-helical-bundle that binds Rabaptin-5 (Bonifacino, 2004). The hinge segment of the GGAs consists of a long polypeptide sequence that is predicted to be largely devoid of secondary structure. It contains variants of the clathrin-box motif that mediate in vitro clathrin binding. It also contains sequences that bind to the γ1-ear domain indicating that they interact with one another and may co-operate in sorting of cargo at the TGN (Doray et al., 2002b). The GAE domain interacts with accessory proteins such as γ-synergin, p56, Rabaptin-5 and enthoprotein (Bonifacino, 2004). This interaction has been shown to be mediated by a canonical DFGXΦ motif in the interacting proteins (Mattera et al., 2004). The GAE domain has also been shown to interact with clathrin although the nature of this interaction has not been clearly elucidated (Puertollano et al., 2001b). GGAs have been proposed to act in conjunction with clathrin, AP-1, cargo and accessory proteins to effect budding at the TGN as visualised by immunoelectron microscopy (Doray et al., 2002b). This hypothesis has been strengthened by the observation that the steady state localisation of cargo proteins that cycle between the TGN, endosomes and the plasma membrane in cells overexpressing GGA3 displays a dramatic shift from the TGN to the plasma membrane (Boman et al., 2000).

**AP-1 μ1B and AP-4:** An alternate AP-1 adaptor protein called μ1B was found to be expressed in epithelial cells. This isoform of AP-1 has been shown to mediate basolateral sorting from the TGN by interacting with specific sequences in the cytoplasmic tail of cargo. Mutational analysis of the 103-amino acid cytoplasmic tail of the polymeric immunoglobulin receptor (pIgR) has shown that a 17-amino acid segment in its tail is necessary and sufficient for basolateral targeting. This segment however does not contain the tyrosine residues critical for endocytosis of the receptor suggesting that the two signals are mutually exclusive (Casanova et al., 1991). Proteins not included in vesicles containing AP-1B and clathrin are included in glycolipid rafts and reach the apical surface in polarised cells whereas vesicles containing AP-1B and clathrin are competent for fusion with the basolateral plasma membrane. The selective inclusion into such vesicles depends on the presence of the adaptor isoform in the given cell-line and the presence of specific sorting signals in the cargo (Fölsch, 1999). Apical targeting
from the TGN has also been shown to be directed by the presence of a glycosylphosphatidyl (GPI) anchor on the cargo molecule (Simons, 1990).

Figure 7 Schematic representation of the assembly of GGA-containing coats. Membrane-tethered Arf (ADP-ribosylation factor)–GTP (blue) binds to the GAT (GGA and TOM (target of myb); yellow) domain, which results in the recruitment of the GGA (Golgi-localized, ψ-ear-containing, Arf-binding protein) to the membrane. The VHS (Vps27, Hrs, Stam; pink) domain binds DXXLL-type signals (where X is any amino acid) in the tails of mannose-6-phosphate receptors and other transmembrane cargo. An autoinhibitory, internal DXXLL sequence (that is, pSLLDFLM in GGA1, where pS indicates phosphoserine) in the hinge segment regulates signal recognition. The hinge segment binds through clathrin-box-like sequences (for example, LLDDE in GGA1) to the terminal domain (TD) of clathrin, and the GAE domain (ψ-adaptin ear; green) binds through DFGXØ-like sequences (where Ø is a bulky hydrophobic residue) to accessory proteins (Rabaptin-5 is shown in this figure, which also interacts with the GGA GAT domain). The position of the Rabaptin-5 binding partner Rabex-5 is not known and is therefore not shown in the figure. The order of these different steps has not been established. Other proteins such as adaptor protein (AP)-1 and enthoprotin/Ent5 might intercalate into these coats and might also participate in the recruitment of clathrin, cargo and accessory proteins. The red dashed lines represent the unstructured sequences in the GGA, and the black dashed lines represent the unstructured sequences in Rabaptin-5. From Nature Reviews Molecular Cell Biology 5; 23-32 (2004)
Last but not least, AP-4 has been shown to interact with tyrosine-based sorting signals at the TGN. The AP-4 complex is a novel heterotetrameric adaptor protein comprised of ε, β4, σ4 and μ4 subunits. AP-4 has been shown to be largely associated with the TGN and has been co-localised with the TGN markers TGN38 and furin (Dell'Angelica et al., 1999). Recruitment of AP-4 to the membrane of the TGN has been shown to be dependent on ARF1 (Hirst et al., 1999). The hinge domains of the ε and β4 subunits of AP-4 lack consensus clathrin-binding motifs suggesting that AP-4 is probably not part of a clathrin coat. In support of this, immunoelectron microscopy revealed that AP-4 was localised to non-clathrin-coated vesicle areas of the TGN (Hirst et al., 1999). The μ4 subunit has been shown to bind the YXXΦ type signals in TGN38, Lamp1, Lamp2 and CD63 (Stephens and Banting, 1998), (Aguilar et al., 2001). The μ4 subunit has been shown to have a preference for residues surrounding the critical tyrosine, notable being the phenylalanine at positions -1 and +3 relative to the tyrosine (Aguilar et al., 2001). The physiological significance of the preferences, however, has not been postulated.

5. Endosomal Sorting:

5.1 Signal Sequences: Sorting in endosomes occurs following internalisation from the plasma membrane and delivery from the recycling pathway. Internalised molecules rapidly enter early sorting endosomes where they are segregated into tubular membrane extensions or remain within the central portion which matures into the late endosome or multivesicular body by involution of the limiting membrane. Recycling vesicles or endosomes are thought to arise from the tubular extensions of early endosomes although the mechanism of their production is not clearly known. Some recycling endosomes fuse with the plasma membrane while some others translocate to the perinuclear cytoplasm on microtubule tracks where they accumulate as a distinct population of vesicles. This has been shown to be true for the transferrin receptor which transits through early endosomes during 2-3 minutes while it is seen in the recycling endosomes for 5-10 minutes (Schmid et al., 1988). Rab4 and Rab5 GTPases are characteristic of early endosomes although Rab4 appears to be depleted from recycling endosomes.
Figure 8 Schematic diagrams of the four AP complexes. All four complexes consist of two large subunits and a small subunit. The carboxy-terminal domains of the two large subunits project as ‘ears’, connected to the ‘head’ of the complex by flexible hinges. From Curr.Opinion.Cell Biol. 1 August 2001, Pages 444-453

However, recycling endosomes have been shown to be enriched in Rab11 (Daro et al., 1996). From endosomes, proteins may move to the lysosome or recycle to the TGN or cell surface for further rounds of transport. Retrograde transport to the TGN has been shown to be mediated by signal sequences directing TGN localisation for cargo whose predominant steady-state localisation is at the TGN. Localisation of furin to clathrin-coated regions of the TGN has been shown to require a motif within its cytosolic tail consisting of an acidic cluster of amino acids (EECP\textsubscript{773}DS\textsubscript{775}EEDE) and containing a pair of casein kinase II phosphorylatable serine residues (Schafer et al., 1995). TGN localisation of the varicella zoster virus gE has been shown to require phosphorylation of an acidic cluster sequence containing a casein kinase II site (Alconada et al., 1996). TGN38 has been shown to be localised to the TGN using a tyrosine based motif (Wong and Hong, 1993). Other proteins which contain phosphorylatable acidic cluster motifs are sortilin, the MPRs, the polymeric immunoglobulin receptor and synaptotagmin. The sorting of these proteins has been shown to be mediated by PACS1 and this has been
dealt with in detail in the section on *Interacting Proteins*. Retrograde transport of proteins from endosomes to the TGN takes place in a variety of ways depending on the cargo. The MPRs have been shown to be returned from the late endosomes to the TGN in a TIP47 and Rab9 dependent manner (Diaz and Pfeffer, 1998). This sorting has been dealt with in detail in the section on MPR trafficking. In yeast, the secretory v-SNARE Snc1p recycles to the TGN from early endosomes whereas Vps10p returns via the late endosome/prevacuole.

### 5.2 Interacting Proteins: PACS-1

Member of a novel gene family of cytosolic connector proteins, PACS-1 (Phosphofurin acidic cluster sorting-1), a cytosolic protein, has been shown to direct the TGN localisation of TGN38. It has also been shown to be involved in the endosomal retrieval of the CI-MPR, carboxypeptidase D and the HIV-1 Nef protein (Wan et al., 1998), (Piguet et al., 2000). It has been shown to directly bind the localisation signal in cargo and link the cargo to the clathrin-sorting machinery (Wan et al., 1998). PACS homologues have been shown to be expressed in a wide variety of metazoans such as mammals, nematodes, insects and plants. It binds to membrane proteins phosphorylated on serine residues and connects them to AP-1 and is itself phosphorylated *in vivo* (Lin et al., 1997). The formation of a ternary complex with AP-1 correlates well with its role in retrieval pathways to the TGN. Furthermore, PACS-1 has also been shown to interact with AP-3 but not AP-2 from cytosol. A short sequence of 18 amino acids was identified in PACS-1 to be necessary and sufficient for adaptor binding. Mutation of eight consecutive residues within this sequence was shown to abolish PACS-1 adaptor binding activity but showed no effect on the interaction with acidic cluster motifs. Furthermore, the binding of PACS-1 to AP-3 was shown not to have an effect on AP-3 dependent lysosomal targeting of proteins such as Lamp1 (Crump et al., 2001). The model proposed to explain the role of PACS-1 in endosomal sorting envisages a scenario in which budding of cargo from the TGN is mediated by direct interaction of the tyrosine motif with AP-1. At this stage, the phosphorylated acidic cluster is silent and the silence has been proposed to be owing to an absence of PACS-1 from the membrane of the TGN. During the actual retrieval event, PACS-1 binding to the phosphorylated acidic cluster enables AP-1 recruitment, thus silencing the
tyrosine motif. The silencing mechanism has been proposed to result from steric interference by PACS-1, compartment specific modification of AP-1 or from the exclusion of accessory proteins that mobilise the tyrosine-bound AP-1 molecules into clathrin coats (Wan et al., 1998), (Seaman et al., 1996).

**The Retromer Complex and Sorting Nexins:** Genetic analysis in yeast identified a complex capable of retrieving protein selectively from the prevacuolar compartment and transporting them to the Golgi. In yeast, a receptor encoded by the VPS10p gene has a function very similar to that of the CD-MPR i.e. the delivery of certain proteins to the yeast equivalent of the lysosome, the vacuole (Cooper and Stevens, 1996). Vps10p releases its bound ligands in the prevacuolar compartments and gets recycled to the TGN to carry out further rounds of enzyme transport. Three key genes that were identified to be required for the transport of the sorting receptor Vps10p were VPS29, VPS30 and VPS35 (Seaman et al., 1997). Further analysis showed that Vps35p and Vps29p interact with each other as part of a macromolecular retromer complex containing Vps35p, Vps29p, Vps26p, Vps5p and Vps17p (Seaman et al., 1998). The membrane association and assembly properties of Vps35p and Vps29p indicated that these subunits were involved in cargo selection while the others functioned in coat assembly and budding (Seaman et al., 1998). Binding of Vps35p to the cytoplasmic domain of cargo was shown to be mediated by two phenylalanine residues in the cytoplasmic domain of the cargo (Notwehr, 1993). Furthermore, the retromer complex has been implicated in the retrieval of the CI-MPR from endosomes to the TGN. In support of this finding, it was also shown that lysosomal degradation of CI-MPR occurs upon depletion of retromer subunits from mammalian cells (Mullins and Bonifacino, 2001). The sorting nexin (SNX) family consists of a diverse group of cytoplasmic and membrane associated proteins that are involved in endosomal sorting. The hallmark of this group of proteins is the presence of a PX domain- a sequence of approximately 100-130 amino acids that has been shown to bind phosphatidylinositol phosphates thereby potentially targeting these proteins to specific cellular membranes enriched in these phospholipids (Worby and Dixon, 2002).
Figure 9 Multiple pathways operate in retrograde transport from endosomal compartments to the TGN. Retrograde transport of STB (red circle) and TGN38 (green bar) occurs from early endosomes, either directly to the TGN or via Rab11-positive recycling endosomes. Transport in this pathway might depend on AP-1/clathrin coats or the formation of specific lipid microdomains in the early endosomal membrane, and is regulated by the Rab6A GTPase and the Golgi-localised Syn6–Syn16–Vti1A t-SNARE complex (lilac bars). Retrograde transport from late endosomes to TGN involves different mechanisms; namely, the recycling of M6PRs (blue bars) is regulated by Rab9 (Rab9 domain), and has been suggested to require an adaptor-like protein TIP47; whereas the transport of furin (yellow bars) involves another cytosolic factor, PACS-1. Possible Rab/lipid domains are indicated as thick membrane areas. From Curr Opin Cell Biol. 2003 Aug;15(4):438-45.

Based on their domain architecture three different subgroups of the family can be recognised: SNX1, 2, 4, 5, 6, 7, 8, 15 and 16 all have long carboxy-terminal extensions containing 1-3 coiled-coil domains for oligomerisation or interaction with other nexins. SNX1 was the first mammalian nexin to be characterised. It interacts with the lysosomal targeting motif of the EGF-receptor, a YLVI sequence and functions as a candidate endosomal retention protein (Kurten et al., 1996). It is both membrane associated and cytosolic and has been proposed to exist as a tetramer in large complexes (Teasdale et al., 2001).
Figure 10 Protein and membrane trafficking. In this simplified scheme, binding of ligand to plasma-membrane receptors results in their internalization in clathrin-coated pits. Internalized clathrin-coated pits shed their clathrin coat and fuse with early endosomal vesicles that have various possible sorting pathways. The transferrin receptor (TFR) is often sorted into recycling endosomes for its return to the plasma membrane (1). Tyrosine kinase receptors (TKRs) and seven-transmembrane G-protein-coupled receptors (GPCRs) are partly recycled through pathway 1, but they also accumulate in late endosomes (2) for transportation to the lysosome for their subsequent degradation (2). The third pathway involves transportation to and from the trans-Golgi network (TGN) to the late endosome and involves proteins such as hydrolases that are destined for the lysosome. Proteins are also sorted in the TGN for delivery to the plasma membrane or for secretion from the cell (4). The positions where the sorting nexins (SNXs) are thought to function are indicated by the pink (mammalian) and purple (yeast) boxes. AP2, adaptor protein complex 2; EEA1, early endosome autoantigen 1; PtdIns(3)P, phosphatidylinositol-3-phosphate. PtdIns(4,5)P_2, phosphatidylinositol-4,5-bisphosphate; PtdIns(3,4,5)P_3, phosphatidylinositol-3,4,5-triphosphate. From Nature Reviews Molecular Cell Biology 3; 919-931 (2002)

It associates with proteins that make up the retromer complex- Vps26p, Vps29p and Vps35p(Haft et al., 2000). It has also been suggested that SNX1 forms complexes with SNX2 and the two have been colocalised with EEA1 (Haft et al., 2000). SNX1 also interacts with Hrs and several members of the receptor tyrosine kinase family, including the platelet-derived growth factor receptor and the insulin receptor (Chin et al., 2001).
SNX2 is also capable of oligomerisation with itself and with SNX1. Although SNX2 interacts with EGFR, PDGFR and IR it does not interact with TfR showing a certain binding specificity. Despite the extensive identity that is shared between their PX domains, the SNX2 PX domain binds preferentially PtdIns(3)P, but not PtdIns(3,4,5)P₃ (Worby and Dixon, 2002). SNX3 and related nexins are small hydrophilic molecules that mainly contain a PX domain. SNX3 almost exclusively interacts with PtdIns(3)P, a fact which supports its cellular distribution in early and perinuclear endosomes where it has been shown to colocalise with EEA1 and TfR, respectively (Xu et al., 2001). SNX13 colocalises with EEA1 in early endosomes and has been shown to be involved in the endosomal sorting of the β₂-adrenergic receptor. It has been proposed to be involved in attenuating the signaling activity of the receptor in endosomes (Zheng et al., 2001). SNX15 has been shown to contain a PX domain capable of interaction with the PDGFR, the interaction being dependent on the activation state of the receptor. Overexpression of SNX15 has been found to negatively affect the internalisation and degradation of the PDGF and transferrin receptors and has also been shown to impair the post-translational processing of the IR and the hepatocyte growth factor receptor precursor. Furthermore, the overexpression of SNX15 has been shown to alter the morphology of several endosomal compartments, thereby affecting the steady state localisation of a large number of TGN localized proteins (Ghosh et al., 1998). SNX17 has a mainly cytoplasmic localization with some localisation to punctate membrane structures and has mainly been shown to play a role in endocytosis of signaling receptors such as the LDL-receptor (Worby and Dixon, 2002). Sorting nexins thus play an important role in sorting and vesicle formation not only at the plasma membrane but also along the endosomal pathway.

**The Ubiquitin Sorting Signal and Multivesicular Body Sorting:** In mammalian cells, ubiquitin has been shown to play a role in the post-internalisation sorting of the G-protein coupled chemokine receptor CXCR4 (Katzmann et al., 2002). It has also been shown to modify Eps15 and Hrs, the latter having an effect on sorting at the multivesicular endosomes.
Ubiquitin (Ub) functions as a positive sorting signal for selection as multivesicular-body (MVB) cargo. The following model is consistent with the published data. First, ESCRT-I (which is composed of Vps23, Vps28 and Vps37) seems to have a crucial role in the selection of ubiquitylated MVB cargoes, such as carboxypeptidase S (CPS). As Vps27 binds both ubiquitin and phosphatidylinositol-3-phosphate (PtdIns(3)P; a phosphoinositide species that is enriched at the endosome), it is tempting to speculate that it activates the MVB sorting reaction and directs cargo selection together with ESCRT-I. Next, the ESCRT-II and ESCRT-III complexes are recruited and direct the continued sorting of cargoes into invaginating vesicles during MVB formation. ESCRT-II (which is composed of Vps22, Vps25 and Vps36) seems to direct the ESCRT-III complex to the appropriate endosomal membrane. ESCRT-III (which is composed of Vps20, Snf7, Vps2 and Vps24) seems to have a role in the concentration and sequestration of MVB cargo. Removal of ubiquitin from MVB cargoes, which occurs before the cargo enters the luminal vesicles of an MVB, requires the enzymatic activity of the de-ubiquitylating enzyme Doa4 (degradation of alpha-2), and Doa4 recruitment to the endosome requires the correct assembly of ESCRT-III. After ubiquitin removal, cargoes are sorted into invaginating vesicles that eventually bud into the lumen. This requires the function of class E Vps proteins, but the precise players and mechanism are not yet clear. Finally, the AAA-ATPase Vps4 is required for the disassembly and release of the entire MVB sorting machinery, which allows the ESCRT machinery to recycle back into the cytoplasm for further rounds of MVB sorting. Recruitment of Vps4 to the endosomal membrane also requires ESCRT-III function. ESCRT, endosomal sorting complex required for transport; GPCR, G-protein-coupled receptor; Vps, vacuolar protein sortin. From Nature Reviews Molecular Cell Biology 3, 893-905 (2002);
Vps23, Vps28 and Vps37 plays a crucial role in the selection of ubiquitylated MVB cargo. Next, ESCRT-II and ESCRT-III complexes are recruited and direct the continued sorting of cargo into invaginating vesicles. ESCRT-II composed of Vps22, Vps25 and Vps36 directs the ESCRT-III complex to the appropriate endosomal membrane. ESCRT-III comprised of Vps20, Snf7, Vps2 and Vps24 concentrates and sequesters the cargo. The ubiquitin signal is removed before the cargo enters the luminal vesicles of an MVB and is thought to be mediated by the de-ubiquitylating enzyme Doa4. Subsequently, cargo is sorted into invaginating vesicles that eventually bud into the lumen. Finally, Vps4 is required for the disassembly and release of the entire MVB sorting machinery allowing the ESCRT machinery to recycle back into the cytoplasm for further rounds of MVB sorting. Recruitment of Vps4 to the endosomal membrane has been shown to require ESCRT-III function (Katzmann et al., 2002). Phospholipids such as cholesterol and lysobisphosphatidic acid (LBPA) which is exclusively found in the membrane of late endosomes, have been implicated in MVB sorting. The inverted cone-shape structure of LBPA has led to the proposal that its conformation favours the inward deformation of the limiting MVB membrane. Phosphoinositides have also been proposed to act as regulators of sorting at this stage. PtdIns(3)P has been predicted to have a direct role in membrane invagination during vesicle formation by recruiting effector proteins that participate in vesicle formation. MVB sorting is implicated in a large number of physiological processes such as receptor and agonist downregulation, delivery of hydrolases, retroviral budding, antigen presentation, exosome production and degradation of lipids and membrane proteins.

6. Uncovering Enzyme

6.1 Structure and Function: Lysosomal enzymes are transported from the site of their synthesis to the lysosomes where they function, by means of a series of a transport steps involving receptor-mediated transport through different subcellular compartments. Following core glycosylation occurring in the ER, the folded polypeptide chains of the enzyme are transported to the Golgi apparatus where they acquire a phosphomannnosyl recognition marker which mediates interaction with one of the two MPRs. The recognition marker which mediates their translocation to lysosomes has been shown to
be synthesised in two steps. First, N-acetylyglucosamine 1-phosphate is transferred to an acceptor mannose by UDP-N-acetylglucosamine: lysosomal enzyme N-acetylglucosamine-1-phosphotransferase, resulting in a phosphate group in diester linkage between the outer N-acetylglucosamine and the inner mannose. Next, an N-acetylglucosaminidase removes the N-acetylglucosamine, leaving the phosphate in monoester linkage with the underlying mannose residue. This exposed phosphomannosyl residue serves as the essential component of the recognition marker which leads to binding to high-affinity MPRs and subsequent translocation to lysosomes. The first enzyme in this scheme, the phosphotransferase catalyses the determining step by which newly synthesised acid hydrolases are distinguished from other newly synthesised glycoproteins and are thus eventually targeted to lysosomes (Kornfeld et al., 1982).

This enzyme has been shown to be localised in the ER-Golgi intermediate compartment and the cis-Golgi where it is active (Pohlmann et al., 1982). N-acetylglucosamine-1-phosphodiester alpha N-acetylglucosaminidase (EC 3.1.4.45), also known as ‘uncovering enzyme’ (UCE) has been cloned, purified and characterised extensively using biochemical methods. Bovine pancreatic UCE was found to be a 272 kDa complex of four identical 68 kDa glycoprotein subunits arranged as two disulfide-linked homodimers. A soluble form of the enzyme isolated from fetal bovine serum was found to have the same subunit structure (Kornfeld et al., 1998). Characterisation studies on UCE purified from Epstein-Barr virus-transformed human lymphoblast cells showed that the catalytic activity of UCE was maximal at pH 6.95 and that the enzyme retained full activity following incubation for 10 minutes at 60 ºC. Furthermore, no requirement was found for a divalent cation, but Zn+2, Hg+2 and Cu+2 were found to reduce the enzyme activity by 30-40% (Page et al., 1996). Human UCE is a type I membrane protein and is composed of 515 amino acids containing a 24-amino acid signal sequence, a luminal domain of 423 residues, a predicted 27 residue transmembrane region and a 41 residue cytoplasmic tail (Kornfeld et al., 1999). UCE purified from bovine liver has been shown to lack the signal peptide. Upstream of the signal sequence is located an RARLPR sequence which serves as a furin cleavage site. Furin which is a calcium-dependent subtilisin-like serine endoprotease and a type I membrane glycoprotein, is also localised to the TGN like the UCE and proteolytically activates UCE in the TGN
(Do et al., 2002). Further, it has been shown that pro-UCE has little or no enzymatic activity and the excision of the propeptide has been shown to be essential for the generation of the active enzyme. Such a mechanism of activation has been proposed to be central to avoiding an active form of the UCE in the Golgi stack. This is important in order to prevent the hydrolysis of UDP-N-acetylglucosamine, a sugar donor for the many N-acetylglucosaminytransferases located in the lumen of the Golgi cisternae (Do et al., 2002). Therefore, if UCE were active as it moved through the Golgi on its way to the TGN (or if it resided in the Golgi), it is likely to interfere with the assembly of complex-type N-linked glycans and O-glycans. Human UCE expressed in mouse L cells has been shown to be sialylated in the TGN further confirming its localisation in the TGN. Furthermore, the individual subunits were shown to be sialylated to the same extent (Rohrer and Kornfeld, 2001).

The absence of a functional UCE in the TGN has obvious implications for normal protein trafficking. Lack of recognition of lysosomal enzymes by the MPRs would eventually lead to accumulation of the enzymes in the TGN and subsequent secretion of the enzymes into the exterior milieu by the default secretory pathway, thus diseases resembling the I-cell disease. The correct trafficking of the UCE is thus crucial to the normal transport of several lysosomal enzymes. Although very few publications have appeared on the UCE in the past, a good deal is known about functional and potential signal sequences in its cytoplasmic tail and certain steps of its trafficking itinerary have been characterised. In the following section, a brief summary of the background information on the trafficking pathways of the UCE and its interacting proteins is discussed.

6.2 Trafficking Itinerary: UCE contains both a tyrosine based internalisation motif $^{488}$YHPL and an NPFXD sequence that can bind to Eps15 and has been found to promote endocytosis in yeast (Tan et al., 1996). It has been shown to cycle between the plasma membrane and the TGN where it is localised at steady state thus confirming the hypothesis that, even though the phosphotransferase acts in the ERGIC and cis-Golgi compartments, the mannose 6-phosphate (M6P) recognition signal is probably generated by UCE in the final compartment of the Golgi where the MPRs reside (Rohrer and
UCE has been co-localised with TGN38 in Madine Darby Bovine Kidney (MDBK) cells and TGN46 in HeLa cells. Furthermore, a GFP-UCE fusion construct where the luminal domain of UCE was replaced by GFP was found to redistribute into a distinct network after BFA treatment, differently from the redistribution of galactosyltransferase, a marker of the trans-Golgi, thus confirming its distinct TGN localisation (Rohrer and Kornfeld, 2001). 0.8-1.6% of UCE was found to be present at the cell-surface at steady state and this cell-surface enzyme was shown to recycle to the TGN as demonstrated by the ability of MDBK cells to internalise anti-UCE antibodies from the cell-surface and deliver them to the TGN. The YHPL motif was shown to be crucial for internalisation and 63% of the enzyme activity of a Y488A mutant was found on the cell-surface (Rohrer and Kornfeld, 2001). The motif was shown to bind AP-2 in glutathione-S-transferase-uncovering enzyme-cytoplasmic tail pulldown assays, indicating that the uncovering enzyme is endocytosed via clathrin-coated vesicles. Endogeneous UCE was also detected in clathrin-coated vesicles. The NPF motif was shown to bind Eps15 in vitro using mammalian cell extracts, such an interaction further enhancing AP-2 interaction through the α-subunit appendage. It has been postulated that Eps15 binding may facilitate the return of UCE from an endosomal compartment to the TGN and that the absence of Eps15 binding may cause UCE to undergo an increase in the frequency of recycling to the plasma membrane (Lee et al., 2002). Consistent with this, Eps15 has been localised to endosomes as well as the plasma membrane indicating that it could serve functions in addition to facilitating endocytosis (Torrisi et al., 1999). Furthermore, it was also shown that the first 11 residues of the 41 residue cytoplasmic tail of UCE were sufficient for retention in the TGN and a tyrosine residue at position 486 was shown to be involved in endosomal recycling to the TGN (Rohrer and Kornfeld, 2001). Tyrosine residues have been in previous instances implicated in endosomal recycling. TGN38, which like UCE cycles between the TGN and the surface, contains a SDYQRL sequence in its cytoplasmic tail that has been shown to be required for efficient internalisation from the plasma membrane and retrieval from recycling endosomes back to the TGN (Bos et al., 1993). Mutation of the serine residue to aspartate or alanine has been shown to result in
mis-sorting of endocytosed TGN38 to late endosomes and/or lysosomes and to a smaller extent, back to the plasma membrane (Roquemore and Banting, 1998).

7. The mannose 6-phosphate receptors

The two sole members of the P type lectin family, the cation dependent- and cation-independent mannose 6-phosphate receptors function in lysosomal biogenesis. They transport lysosomal enzymes from the TGN through a series of endosomal compartments to the lysosomes. Central to this process is the M6P recognition signal on lysosomal enzymes the function and generation mechanism of which have been dealt with in detail in the previous section. In this section, the structure, properties and trafficking itineraries of the two receptors have been outlined in some detail.

7.1 CD-MPR: The 46 kDa isoform of the MPR is a glycosylated type I integral membrane protein which spans the membrane once and exposes its N-terminal ligand binding domain into the lumen of membrane vesicles or the cell surface. It is highly conserved from mouse to man with 93% overall homology and has completely identical amino acid sequences within the cytoplasmic tail across the different species with the exception of chicken. It has 5 potential N-glycosylation sites, two of which have been reported to be occupied by high mannose residues and two others by complex oligosaccharides. Three pairs of disulfide bonds between conserved cysteine residues exist in the extracytoplasmic domain. The CD-MPR contains a single M6P binding site and is present primarily as a noncovalent homodimer at the membrane (Dahms and Hancock, 2002). Receptor dimerisation has been shown to promote high-affinity ligand binding in a multivalent fashion (Byrd and MacDonald, 2000). X-ray crystallographic analysis revealed that the extracellular domain of the CD-MPR crystallises as a homodimer with approximately 20% of the entire surface area of each monomer contacting one another predominantly through hydrophobic interactions. A single alpha helix near the amino terminus followed by nine primarily anti-parallel beta strands that form two beta sheets positioned orthogonally to each other makes up the tertiary structure. Each monomer forms a flattened beta barrel structure afforded by extensive hydrophobic interactions between the two beta sheets (Roberts et al., 1998). The carbohydrate recognition domain (CRD) lies relatively deep in the protein thus allowing
a conformation in which the terminal M6P residue and the penultimate sugar ring of bound pentamannosyl phosphate are buried in the receptor (Olson et al., 1999). Such a binding pocket has been postulated to allow numerous interactions between receptor and ligand. Comparison of the X-ray structures of the receptor in liganded and unliganded conformations revealed that the receptor undergoes considerable scissoring and twisting movements to open up for ligand binding (Olson et al., 2002).

The cytoplasmic tail of the CD-MPR contains 67 amino acids and a wealth of sorting information therein. The identical nature of the sequence across most of the species examined highlights the importance of the signals and their contexts. The CD-MPR is found in the TGN, early endosomes, recycling endosomes, late endosomes and the plasma membrane, but is absent from lysosomes at steady state. Its trafficking itinerary involves constitutive cycling between these organelles and a concerted signal recognition mechanism that essentially avoids receptor delivery to lysosomes. Three different internalisation signals have been identified in the receptor tail- the F_{13}-XXXX-F_{18}, non-classical motif in which the F_{18} (18 amino acids away from the TMD) has been shown to play the key role, the Y_{45}-XXΦ motif and the L_{64}-L_{65}, di-leucine motif. Controversial reports exist as to the functionality of these individual signals notwithstanding which, the mechanism of internalisation has been undisputedly shown to be clathrin and AP-2 mediated (Denzer et al., 1997). Using yeast two-hybrid analysis and surface plasmon resonance experiments AP-2 has been shown to mediate internalisation from the surface (Ricotta et al., 2002; Storch and Braulke, 2001).

Baselateral sorting of the CD-MPR in polarised cells has been shown to be mediated by two residues E_{11} and A_{17}, mutations of which lead to apical targeting of the receptor (Bresciani, 1997). Endosomal sorting of the CD-MPR has been shown to be mediated by a pair of aromatic residues F_{18}W_{19} in the cytoplasmic tail, mutations of which lead to lysosomal delivery of the receptor (Schweizer et al., 1997). Furthermore, the spacing of the diaromatic motif with respect to the transmembrane domain has also been shown to be important for avoidance of lysosomal delivery (Schweizer et al., 1997). Additionally, the requirement for diaromaticity at the indicated positions was shown to be strict as hydrophobic residues failed to replace the diaromatic motif in mediating endosomal sorting of the receptor. Consistent with this finding, mutations of the diaromatic motif
also resulted in decreased binding of the receptor to Tail-Interacting Protein of 47 kDa (TIP47), a cytosolic adaptor protein that was shown to bind the tails of the CD- and CI-MPR for transport from late endosomes to the TGN (Nair et al., 2003). TIP47 was identified as a cargo selection device that is predominantly cytosolic although a portion of it is associated with endosomes. It has been shown to selectively bind the MPR tails and fails to interact with the cytosolic tails of TGN38 or the LDL-receptor. Anti-TIP47 antibodies have been shown to significantly inhibit an in vitro transport assay reconstituting endosome-to-TGN transport. Furthermore, the binding of TIP47 to the tails is directly dependent on the diaromatic motif in the CD-MPR and a proline rich putative loop PPAPRPG and other hydrophobic residues in the membrane proximal domain of the CI-MPR. TIP47 interaction with the CI-MPR has been shown to be highly conformation dependent and also depends on the residues 48-74 in the cytosolic tail of the CI-MPR (Orsel et al., 2000). TIP47 does not have significant homology to any of the known clathrin adaptor proteins. It is now known that TIP47 triggers the efficient inclusion of MPRs into newly forming transport vesicles departing from endosomes (Diaz and Pfeffer, 1998). It was also shown that TIP47 functions in conjunction with Rab9 which is localised predominantly to late endosomes. Rab9 GTPase activity was shown to be required for MPR transport from endosomes to the TGN (Lombardi et al., 1993). TIP47 was also shown to bind Rab9 directly in its active GTP-bound conformation. Furthermore, it was found to increase the affinity of TIP47 for cargo. Residues 167SVV in TIP47 were shown to be involved in Rab9 binding. Thus TIP47 was postulated to be a Rab9 effector protein which enhances interaction of TIP47 with the MPR cytoplasmic domains. It has also been postulated to enhance the endosome-recruitment of TIP47 in addition to the cargo capture process (Carroll et al., 2001). Previous evidence indicates that Rab9-GTP can recruit cytosolic docking factors to the surfaces of transport vesicles to facilitate SNARE pairing before fusion indicating that Rabs could function in vesicle formation and docking (Pfeffer, 1999). Furthermore, TIP47 has been shown to occur as an oligomer in the cytosol and its oligomerisation has been shown to be important for TIP47 stimulation of MPR transport from endosomes to the TGN but not for recognition of the MPRs (Sincock PM, 2003).
One of the important post-translational modifications undergone by the CD-MPR is a reversible palmitoylation of one and possibly two cysteine residues, at positions 34 and possibly 30 in the cytoplasmic tail. It has been shown that the reversible palmitoylation of cysteine-34 but not cysteine-30 is important for avoidance of lysosomal delivery of the receptor (Schweizer et al., 1996). Furthermore, it was also shown that the palmitoylation is essential to maintain efficient sorting of lysosomal enzyme cathepsin D in the Golgi (Schweizer et al., 1996). The location of the palmitoylated cysteine in several receptors such as the transferrin receptor and the CD4 cell surface glycoprotein is usually in the transmembrane domain or close to the membrane. The location of the modification in the CD-MPR, 34 amino acids away from the membrane, led to the proposal that the palmitoylation acts as a regulatory switch exposing the internalisation, endosomal and TGN-sorting signals of the tail to control the trafficking of the receptor in a conformation dependent manner. Furthermore, the half-life of palmitoylation of the receptor is in the order of a couple of hours compared to the half-life of the receptor itself which exceeds 48 hours. This strengthens the view of the modification being a regulatory switch to control the normal recycling of the receptor (Schweizer et al., 1996). It was recently shown using a combination of \textit{in vivo} labeling and an \textit{in vitro} assay based on cell fractionation methods that the palmitoyltransferase of the CD-MPR cycles between the endosomes and the plasma membrane, a fact which is in keeping with the proposed model of the reversible palmitoylation acting as a regulator of the endosomal sorting of the receptor (Stockli and Rohrer, 2004).

Another important modification of the receptor is the phosphorylation of a serine-57 residue in the cytoplasmic tail. Serine-57 has been shown to be phosphorylated by casein-kinase 2 in a reversible manner (Hemer et al., 1993; Körner et al., 1994). The phosphorylated serine is in the context of a series of acidic residues, the role of which in ensuring the normal trafficking of the receptor has been the topic of fierce parley. Pairs of glutamic acid residues on either side of the serine residue have been shown to be both important and not important for sorting of cathepsin D, by opposing groups (Breuer et al., 1997; Johnson and Kornfeld, 1992b). Phosphorylation of the receptor by casein kinase-2 has been shown to be important for AP-1 binding at the TGN (Ghosh and Kornfeld, 2003), although it was also shown using surface plasmon resonance
experiments that AP-1 binds to non-phosphorylated peptides of the cytoplasmic tail of the receptor (Stockli et al., 2004). Thus, the functional significance of phosphorylation of the receptor is as yet in the realm of murky waters.

At the TGN and also probably at the endosomes, the CD-MPR interacts with AP-1. The signal mediating AP-1 binding is essentially bipartite. Residues 49-67 in the cytoplasmic tail were implicated in AP-1 binding. This stretch includes the acidic cluster casein kinase 2 site and the DXXLL motif, although it has been shown that neither the phosphorylation of the serine within nor the dileucine motif is required for AP-1 binding in vitro (Stockli et al., 2004). Residues 28-42 in the tail have also been implicated in binding AP-1 in vitro and the crucial residues include the palmitoylated cysteines and the basic residues arginine-35, lysine-37 and arginine-39 (Honing, 1997). The role of palmitoylation in this binding has not been elucidated beyond doubt as the peptides used in the surface plasmon resonance analysis were not palmitoylated. Binding has been shown to take place with low nanomolar affinities- a fact which corroborates with our own experience of difficulties in pulling down AP-1 from cell extracts using the receptor cytoplasmic tail.

At the TGN, the CD-MPR also interacts with another player, the GGA. Two known variants of the GGA protein have been shown to interact with the CD-MPR. GGA1 and to a much smaller extent GGA3 bind the cytoplasmic tail of the CD-MPR. It has been shown that the GGAs bind to the crucial acidic cluster di-leucine motif through their VHS domains (Puertollano et al., 2001a). Furthermore, it was also shown that sorting-defective MPRs with mutations in this motif failed to bind GGAs indicating that the interaction is important in the targeting pathway (Zhu et al., 2001). Recently, it was demonstrated that the acidic cluster of the casein kinase 2 site of the CD-MPR but not its phosphorylation was important for GGA binding using GST- pulldown, surface plasmon resonance and in vivo analysis (Stockli et al., 2004).

The dileucine motif of the CD-MPR has been shown to be involved in retrograde transport from the endosomes to the TGN. A mutant CD-MPR in which the dileucine motif was replaced by alanines failed to return from endosomes back to the TGN and was found to accumulate in early endosomes were it could be localised with internalised BSA-gold and Rab5 thus indicating the role of the dileucine in retrograde transport to
the TGN. This motif however, has not been implicated in binding AP-1 (Tikkanen et al., 2000).

7.2 CI-MPR: The 300 kDa CI-MPR functions in two distinct biological processes—protein trafficking and transmembrane signal transduction. The CI-MPR consists of four structural domains: a 44-residue amino-terminal signal sequence, a 2269-residue extracytoplasmic domain, a single 23-residue transmembrane region, and a 163-residue carboxy-terminal cytoplasmic domain. The lumenal domain contains 19 potential N-linked glycosylation sites and a repetitive structure consisting of 15 contiguous repeating segments of approximately 147 amino acids each. The location of the cysteine residues is highly conserved in the repeats. The CI-MPR seems to be a dimer in the membrane although it has been shown to behave as a monomer in detergent solutions under most circumstances (York et al., 1999). The short linker segment between the CI-MPR repeating segments (5-12 residues) places considerable constraint on the possible arrangements of the domains in the intact receptor to account for which Brown et al proposed a model in which even-numbered domains face in one direction, while odd-numbered domains face in another direction thus giving rise to a receptor conformation with two functional sides— one side involved in ligand interactions and the other involved in dimerisation (Brown et al., 1999). The extracytoplasmic domain also contains two distinct M6P binding sites (repeating segments 3 and 9) and a single IGF-II binding site (segment 11). The overall structure of the IGF-II binding domain 11 of the CI-MPR is similar to that of the CD-MPR. However, the molecule contains a surface hydrophobic patch that equates spatially to the hydrophilic M6P binding pocket on the CD-MPR. This hydrophobic patch has been postulated to bind IGF-II as it contains isoleucine 1572, which is required for this interaction (Garmroudi et al., 1996). Certain effects of IGF-II have been shown to be due to the interaction of the ligand with its receptor. The CI-MPR has been shown to positively or negatively regulate the activity of $G_{1-2a}$ in response to IGF-II and Man 6-P binding respectively (Körner et al., 1995). Furthermore, the clearance of IGF-II and TGF-β-1 from circulation is another function attributed to the CI-MPR (Kornfeld, 1992). CI-MPR has also been shown to play a role in the degradation of the extracellular matrix. CI-MPR has been postulated to bind acid hydrolases that degrade cell surface and substratum-attached proteoglycans at the
The cytoplasmic domain of the receptor is highly conserved among bovine, human and rat species. Overall, the amino acid sequences of the bovine and human receptors are 80% identical. Several sorting signals are clustered in the cytoplasmic tail as is the case for the CD-MPR. At steady state the receptor is primarily localised to the TGN, endosomes and the plasma membrane and is excluded from lysosomes. The CI-MPR is palmitoylated at cysteines 15 and or 16 in its cytoplasmic tail although the precise function of this modification is not known yet (Westcott and Rome, 1988). The internalisation of CI-MPR from the surface has been shown to be exclusively mediated by a YSKV motif in its cytoplasmic tail (Jadot et al., 1992). The rate of internalisation of the CI-MPR has been shown to be considerably increased on binding of the multivalent ligand β-glucuronidase owing to a possible conformational change that results in optimal presentation of the internalisation signal in the cytoplasmic tail (York et al., 1999). Both AP-1 and GGA1 have been shown to interact with the CI-MPR for sorting at the TGN through the acidic cluster dileucine motif in the cytosplasmic tail of the CI-MPR (Puertollano et al., 2001a). The motif involved was found to be the aspartate\textsuperscript{157-}\text{x-x-}\text{leucine-leucine and the phosphorylation of serine-156 was found to have a positive effect on the binding of GGA1 and GGA3 (Kato et al., 2002). The AP-1 binding sites were mapped to residues 26-29, 39-44, 84-88 and 154-160. Two of these sites have been shown to be casein kinase 2 phosphorylation sites (Le Borgne et al., 1993). At the level of the endosomes, TIP47 binds to a putative loop formed by residues \textsuperscript{45}PPAPRPG in the cytoplasmic tail of the CI-MPR which lacks a diaromatic motif (Orsel et al., 2000). This binding takes place in the presence of Rab9 which increases the affinity of cargo selection device for cargo by three-fold. PACS-1 has been shown to bind the acidic cluster in the CI-MPR cytoplasmic tail to effect retrograde transport to the Golgi (Wan et al., 1998). CI-MPR also undergoes phosphorylation \textit{in vivo} and \textit{in vitro} at serine residues 85 and 156, the functional significance of which becomes evident for GGA1 and PACS-1 binding (Meresse et al., 1990).

\textbf{Sorting independent of the MPRs:} Cell type specific mechanisms of lysosomal enzyme sorting, independent of the MPR’s has been proposed in B lymphocytes, spleen, liver, kidney and brain cells of patients of I cell disease, a congenital disorder occurring due to deficiency of the phosphotransferase enzyme that generates the M6P tag.
lysosomal enzyme targeting occurs in a manner mediated by AP-1 and clathrin but no MPRs have been discovered yet. Although evidence for modification by N-acetylglucosamine-1-phosphate exists, mannose 6-phosphate has not been shown to decorate the oligosaccharides of lysosomal enzymes in *Dictyostelium* (Mehta DP, 1996).

**8. Aim of the thesis:**

The importance of signal sequences in intracellular trafficking cannot be overemphasised, given the wealth of information available on the different signal-mediated transport steps along the secretory and endocytic pathways. The aim of this thesis was to characterise the signal sequences involved in two individual steps of the pathway—endosomal sorting and exit from the TGN. To this end, the trafficking of two different proteins involved in lysosomal biogenesis was studied: the CD-MPR in its retrieval step from late endosomes to the TGN and the human UCE in its exit step from the TGN.

The trafficking of the CD-MPR has been shown to depend on a variety of motifs located in its 67 amino-acid cytoplasmic tail. One of the steps in the trafficking pathway, the retrieval of the receptor from late endosomes to the TGN is crucial to the function of the receptor. Failure of this transport step would lead to an accumulation of the receptor in lysosomes and degradation therein. This transport step has been shown to depend on a pair of aromatic residues, F18,W19 in the cytoplasmic tail of the receptor. Mutation of the W19 residue to an alanine has been shown to cause mislocalisation of the receptor to lysosomes. The F18 residue plays a supporting role in the endosomal sorting of the receptor. The mechanism of retrieval has been shown to depend on a cytosolic machinery involving TIP47, a cytosolic adaptor protein and the Rab9 GTPase. At the level of late endosomes, TIP47 was shown to directly bind the diaromatic motif in the cytosolic tail of the CD-MPR to mediate its inclusion into vesicles destined for the TGN as opposed to vesicles shunted by default to lysosomes, thus ensuring the safe return of the receptor for further rounds of transport of lysosomal enzymes. The diaromatic motif thus gained a central position in receptor sorting. Database analyses revealed the presence of the motif in other transmembrane proteins. Following similar analysis, it
was shown that the human mannose receptor also depends on its diaromatic motif for correct endosomal sorting. The CI-MPR also follows the same trafficking itinerary from late endosomes to the TGN. However, it lacks a diaromatic motif. In the CI-MPR, interaction with TIP47 was shown to be mediated by a stretch of amino acids constituting a putative loop formed by residues PPAPRPG in the cytosolic tail. Furthermore, the interaction of both the CD- and the CI-MPR with TIP47 is completely abolished in the presence of Triton-X 100. This gave rise to the notion that the interaction could be hydrophobic and thus not strictly requiring a diaromatic motif. This, however, would be in opposition with the demonstrable role of the motif in avoidance of lysosomal delivery of the CD-MPR. Therefore, a series of receptor constructs were made to characterise the diaromatic motif in detail. A series of amino acid replacements of the crucial W19 residue were made, comprising of bulky hydrophobic, polar, charged or uncharged residues. The effect of these replacements on the trafficking of the receptor was analysed using two readouts:

a. The lysosomal delivery of wild-type versus mutant receptors was analysed using Percoll density gradient separation of lysosomes from the other fractions.

b. The in vitro TIP47 interaction ability of wild-type versus mutant receptors was analysed using an in vitro pulldown assay using His-tagged TIP47.

The second part of this thesis deals with the exit of human UCE from the TGN. UCE cycles constitutively between the TGN and the plasma membrane with a predominant steady state localisation in the TGN. The steady state levels of UCE can be thought of as being regulated by four different trafficking steps: exit from the TGN, internalisation from the surface, return from endosomes to the TGN and the possible recycling from endosomes to the surface. The signal sequence involved in TGN exit was determined using a combination of immunofluorescence and pattern recognition methods:

a. Analysis of high resolution confocal images using a high dimensional neural network capable of discriminating differences not visible to a visual examiner. Alanine mutants of the putative signal sequence were analysed in a quantitative manner to reveal differences in the localisation of wild-type versus mutant constructs.
b. The individual contribution of each amino acid in the putative exit signal to the efficacy of TGN exit of UCE was analysed using the same method.
Part I

CHARACTERISATION OF THE ENDOSONAL
SORTING SIGNAL OF THE CD-MPR

**Summary**

Intracellular cycling of the cation-dependent mannose 6-phosphate receptor (CD-MPR) between different compartments is directed by signals localized in its cytoplasmic tail. A di-aromatic motif (Phe\textsuperscript{18}Trp\textsuperscript{19} with Trp\textsuperscript{19} as the key residue) in its cytoplasmic tail is required for the sorting of the receptor from late endosomes back to the Golgi apparatus. However, the cation-independent mannose 6-phosphate receptor (CI-MPR) lacks such a di-aromatic motif. Therefore the ability of amino acids other than aromatic residues to replace Trp\textsuperscript{19} in the CD-MPR cytoplasmic tail was tested. Mutant constructs with bulky hydrophobic residues (valine, isoleucine or leucine) instead of Trp\textsuperscript{19} exhibited 30-60% decreases in binding to tail interacting protein of 47 kDa (Tip47), a protein mediating this transport step, and partially prevented receptor delivery to lysosomes. Decreasing hydrophobicity of residues at position 19 resulted in further impairment of Tip47 binding and an increase of receptor accumulation in lysosomes. Intriguingly, mutants mislocalized to lysosomes did not completely co-localize with a lysosomal membrane protein, which might suggest the presence of subdomains within lysosomes. These data indicate that sorting of the CD-MPR in late endosomes requires a distinct di-aromatic motif with only limited possibilities for variations, in contrast to the CI-MPR which seems to require a putative loop (P\textsuperscript{49}PAPRPG\textsuperscript{55}) along with additional hydrophobic residues in the cytoplasmic tail. This raises the possibility of two separate binding sites on Tip47 since both receptors require binding to Tip47 for endosomal sorting.
**Introduction**

The biogenesis of lysosomes depends on the correct sorting of acid hydrolases from their place of synthesis in the endoplasmic reticulum to their final destination in lysosomes. Newly synthesized enzymes are tagged with a mannose 6-phosphate recognition marker by the concerted action of two enzymes: one which transfers a GlcNAc-1-phosphate to specific mannose residues on the acid hydrolases and the other which removes the covering GlcNAc to generate the mannose 6-phosphate tag (Bao et al., 1996; Kornfeld et al., 1998). This tag is required for the subsequent binding of the lysosomal hydrolases to one of two mannose 6-phosphate receptors (MPRs) in the trans-Golgi network (TGN). The receptor ligand complexes then exit the TGN in clathrin coated vesicles and fuse with acidified endosomes. Following the pH-induced dissociation of the complexes, the lysosomal enzymes are further packaged into the lysosomes whereas the receptors are recycled back to the TGN to mediate another round of sorting (Hille-Rehfeld, 1995; Kornfeld, 1992).

The cation-dependent mannose 6-phosphate receptor (CD-MPR) is a type I integral membrane protein with a C-terminal cytoplasmic tail of 67 residues. Within this cytoplasmic tail, several signals have been identified that direct the intracellular trafficking of the receptor. Internalization from the plasma membrane is mediated by 3 independent signals, a pair of phenylalanine residues (Phe$^{13}$XXXXPhe$^{18}$), a classical tyrosine motif (Tyr$^{45}$XXVal$^{48}$) and as indicated by *in vitro* data by a di-leucine based motif (Leu$^{64}$Leu$^{65}$) (Denzer et al., 1997; Johnson et al., 1990). The di-leucine motif has been shown in addition to mediate the transport of the CD-MPR from the TGN to the endosomes by interaction with Golgi-localized, gamma-ear-containing, adenosine diphosphate ribosylation factor-binding protein (GGA) 1 and adaptor protein complex 1 (AP1) and possibly also a sorting event within endosomes (Johnson and Kornfeld, 1992b; Tikkanen et al., 2000). An acidic cluster as part of a casein kinase II site was implicated to be important for sorting in endosomes (Wan et al., 1998). Furthermore, overlapping with one of the internalization motifs (Phe$^{13}$XXXXPhe$^{18}$) is a di-aromatic motif (Phe$^{18}$Trp$^{19}$) which is required for the sorting of the receptor from late endosomes back to the TGN and therefore preventing degradation of the receptor in lysosomes (Schweizer et al., 1997). The sorting efficiency of the di-aromatic motif seems to be
regulated by a posttranslational modification, the reversible palmitoylation of Cys\(^{34}\) (Schweizer et al., 1996; Schweizer et al., 1997).

Sorting of the receptor requires the interaction of the signals in the cytoplasmic tail with components of the cytoplasmic sorting machinery at sites of vesicle formation. Two such factors are the clathrin associated adaptor complexes AP1 and adaptor protein complex 2 (AP2). AP1 mediates the sorting step from the TGN to endosomes and in addition recent data suggest that AP1 might also play a role in recycling MPRs from early endosomes to the TGN (Meyer et al., 2000) whereas AP2 is required for rapid internalization of the receptor from the plasma membrane (Höning et al., 1997). Another protein, phosphofurin acidic cluster sorting protein 1 (PACS-1), was shown to interact with the receptor tail and AP1 \textit{in vitro} suggesting that PACS-1 and AP1 may cooperate to sort MPRs (Crump et al., 2001). Most recently, the novel family of GGA proteins have been shown to mediate the sorting of MPRs from the TGN to endosomes (Puertollano et al., 2001a; Zhu et al., 2001). Yet another sorting event, from late endosomes back to the TGN, is mediated by tail interacting protein of 47 kD (Tip47) which interacts with the di-aromatic motif (Phe\(^{18}\)Trp\(^{19}\)) of the CD-MPR (Diaz and Pfeffer, 1998). Optimal Tip47 function depends on binding to Rab9, a late endosomal GTPase previously implicated in MPR retrieval, further confirming the role of Tip47 in this particular sorting event (Carroll et al., 2001). Interestingly Tip47 binds to both the receptors, the CD-MPR and the cation-independent mannose 6-phosphate receptor (CI-MPR) but the CI-MPR does not contain a di-aromatic motif. A mutational analysis revealed that Tip47 seems to bind a putative loop generated by the sequence P\(^{49}\)PAPRPG\(^{55}\) and other hydrophobic residues in the cytoplasmic tail of the CI-MPR (Orsel et al., 2000). This raises the question whether the binding of Tip47 to the CD-MPR is restricted to a di-aromatic motif or if other residues could replace the aromatic residues.

In this study we have analyzed the effect of mutations that change the key residue of the di-aromatic motif (Trp\(^{19}\)) either to bulky hydrophobic residues, non-polar residues, polar residues or charged residues. Initial screening for the intracellular localization of CD-MPR constructs containing those mutations by immunofluorescence revealed some differences in the extent of co-localization with Lamp1, a lysosomal marker. To obtain a
more quantitative measure for the effect of various mutations we tested their ability to interact with N-terminally His-tagged Tip47 in an *in vitro* pulldown assay. Constructs with Trp$^{19}$ replaced by hydrophobic residues such as leucine and valine exhibited a modest impairment of binding to Tip47. However, correct endosomal sorting of the full-length CD-MPR *in vivo* was only achieved by wild-type receptors whereas the mutants showed an increased missorting to lysosomes by a factor of 5 for replacements of Trp$^{19}$ by hydrophobic residues and up to a factor of 12 for replacements by charged residues.

**Experimental Procedures**

**Materials** Enzymes used in molecular cloning were obtained from Boehringer Mannheim, New England Biolabs, or Promega; α-minimal essential medium (α-MEM), foetal calf serum, and Lipofectin were from GIBCO/BRL; Percoll was from Pharmacia; nitrocellulose from Schleicher & Schuell; protease inhibitors from Sigma; ECL Western blotting reagents from NEN Life Science Products (Boston, MA); and cell culture dishes from Falcon. Oligonucleotides were synthesized either by the DNA synthesis facility of the Friedrich Miescher Institute or Microsynth GmBH (Galbach, Switzerland). Goat anti- rabbit Alexa 488 antibodies and Goat anti-mouse Alexa 568 antibodies were obtained from Molecular Probes (Eugene, OR). The mAb 22D4 specific for the bovine CD-MPR and 931A polyclonal antibodies specific for Lamp1 were generously provided by Drs. D. Messner and M. Fukuda, respectively.

**Recombinant DNA** All basic DNA procedures were as described (Sambrook et al., 1998). The PCR procedure of Ho *et al.* (Ho et al., 1989) was used to generate the mutant constructs with pBSK-MPR TMD/tail (Rohrer *et al.*, 1995) serving as a template together with bp 170–193 and 1260–1341 of pBSK as the down- and upstream primers, respectively. Appropriate partial complementary pairs of oligonucleotides in which the desired amino acid replacement had been incorporated were chosen as internal primers. The final PCR products were subcloned into pSFFV-neo as described (Rohrer *et al.*, 1995). Human Tip47 was cloned by PCR from a Marathon Human Placental cDNA library (Clontech, Palo Alto, CA) using Tip47.down (5’TTTGAATTTCGCGCCGCTGTTTCTGCTGGACG3’) and Tip47.up (5’GAGACGCGGTCCCGCTGAGTCCTCTCCTCTCCTCCCC3’) oligos into the
pcDNA3.1(+) vector (Invitrogen, Carlsbad, CA). 6xHis-Tip47 was created using this pcDNA3.1 (+)-Tip47 vector as a template for a PCR reaction with the oligos BamH1pQE31-TIP47.down (5’CACGGATCCATGTCTGCCGAC3’) and Sac1pQE31-TIP47.up (5’GCGGAGCTCCCCCTATTCTTCTCCTCCGCGGCG3’). The PCR product was cut with BamHI and SacI and ligated into pQE31 (Qiagen AG, Basel). All coding sequences created by PCR were verified by sequencing.

**Cell Culture and Transfection**

HeLa cells were grown in α-MEM to 30% confluency before transfection with 1µg of DNA using FuGene according to the manufacturer’s directions. The cells were incubated with 100 µM each of pepstatin A and leupeptin 24 h before the experiment and analysed 48 h posttransfection by confocal microscopy. A mannose-6-P/insulin-like growth factor-II receptor-deficient mouse L cell line designated D9 (LRec-) was maintained in α-MEM. The cells were transfected with XbaI-linearized DNA with Lipofectin according to the manufacturer’s directions. Selection for resistance to neomycin (G418) was carried out using 500 µg/ml G418 as the final concentration. Resistant colonies were picked individually and screened for the expression of bovine CD-MPR by immunoblotting. Clones expressing similar amounts of receptor were expanded for further study and maintained in selective medium.

**Confocal Immunofluorescence Microscopy and Deconvolution**

Confocal immunofluorescence microscopy was carried out as described (Rohrer and Kornfeld, 2001). Paraformaldehyde-fixed and saponin-permeabilized cells were incubated with a 1:200 dilution of mAb 22D4 and a 1:400 dilution of Ab 931A. The secondary antibodies were goat anti-rabbit Alexa 488 antibodies and goat anti-mouse Alexa 568 conjugates, diluted 1:1000. The coverslips were mounted on glass slides with ProLong Antifade (Molecular Probes) for viewing with an Olympus Fluoview FV500 confocal laser-scanning microscope. Serial sections in the z-axis through the entire cells were taken, and the resulting stacks of images were processed on a multiprocessor SGI UNIX computer using the Huygens program (Scientific Volume imaging, Hilversum, The Netherlands). A maximum likelihood estimation (MLE)-based algorithm was used for image reconstruction. Z-stacks were exported as Tiff files and analyzed with the use of the Imaris program (Bitplane AG, Zürich, Switzerland).
**In vitro Pulldown Experiments** N-terminally His-tagged Tip47 was expressed in the *E.coli* strain M15. A saturated over night culture was diluted 1:10 in 200 ml growth medium and incubated for 1h at 37°C before induction with 1.0mM isopropyl-1-thio-D-galactopyranoside for 2h. The cells were then harvested by centrifugation, washed with ice cold PBS and lysed by sonication in 20 ml binding buffer (20mM NaH₂PO₄.H₂O pH7.8, 500mM NaCl) containing a 1:500 dilution of a protease inhibitor cocktail (5mg/ml benzamidine, and 1µg/ml each of pepstatin A, leupeptin, antipain, and chymostatin in 40% dimethyl sulfoxide-60% ethanol) (PIC) and PMSF (40µg/ml) (final concentration). Insoluble material was removed by centrifugation at 12000 rpm for 10 min at 4°C in a Sorvall GSA centrifuge. 4 ml of the supernatant was incubated for 30 min at room temperature on a rotating shaker with 400 µl Ni-CAM HC resin (50% suspension) (Sigma, St. Louis, MO) which was prewashed with binding buffer and blocked with binding buffer containing 0.1% BSA. The beads were washed 2 times with wash buffer (binding buffer at pH6.0) and once with assay buffer (50 mM HEPES, 150 mM KCl, 8 mM Imidazole, 1 mM MgCl₂ pH7.5) by spinning them at 1000 rpm for 1 min in a Heraeus centrifuge. Extracts from mouse D9 (L Rec-) cells expressing wild-type or mutant CD-MPR constructs were prepared from cells grown on 15 cm Falcon tissue culture dishes in the following way. Confluent 15 cm dishes of cells were put on ice and washed once with 10 ml ice cold PBS and scraped in 5 ml of assay buffer containing PIC and PMSF. The cells were pelleted at 1000 rpm for 5 min at 4°C in a Heraeus centrifuge and resuspended in 1 ml assay buffer containing PIC and PMSF. The cells were homogenized in a ball bearing homogenizer of 51.2 µm clearance using 12 strokes and spun at 700xg at 4°C for 10 min. The supernatant was collected and centrifugation was repeated until no pellet appeared anymore. The protein concentration of the post nuclear supernatant (PNS) was measured using a Bio-Rad protein assay. The appropriate amounts of PNS from each of the mutant cell lines and wild-type cell line expressing the same amount of CD-MPR were determined by western blotting with anti CD-MPR monoclonal antibody 22D4 followed by densitometric scanning and quantification using the ImageQuant5.0 software (Molecular Dynamics, Sunnyvale, CA). These PNS amounts adjusted for expression levels were used for the assay.
Beads incubated with 6xHis-Tip47 were resuspended in 200 µl assay buffer containing PIC and PMSF. 50 µl of resuspended beads were incubated with 200 µg PNS from wild-type cell line or an appropriate amount of PNS from mutant cell lines according to the expression level adjustment described above for 2h at 4ºC on a rotating shaker. Beads were spun at 2500 rpm for 4 min at room temperature in an Eppendorf tabletop centrifuge. The supernatant was collected and stored. The beads were washed three times with assay buffer. Sample buffer was added to the beads to a total volume of 40 µl, boiled and analyzed by SDS-PAGE and western blotting using anti CD-MPR monoclonal antibody 22D4. An aliquot of each of the supernatants was also analyzed similarly to determine the amount of unbound receptor. The amount of CD-MPR bound to 6xHis-Tip47 was determined by densitometric scanning using the ImageQuant5.0 software.

**Percoll Gradient Fractionation** Confluent D9 (LRec-) cells stably expressing wild-type or mutant forms of the CD-MPR were grown in a 100-mm Petri dish and incubated for 24 h in growth medium supplemented with 100 µM each of pepstatin A and leupeptin. The cells were then harvested, ruptured with a ball bearing homogenizer, and fractionated on 17.5% Percoll density gradients as described previously (Rohrer et al., 1995). The specificity of the lysosomal fractions was tested using a β-hexosaminidase assay as described previously (Rohrer et al., 1995) (data not shown).

**SDS-PAGE and Immunoblotting** Proteins were separated on 10% SDS-polyacrylamide minigels (Bio-Rad) by using the Laemmli system (Laemmli, 1970). After electrophoresis gels were transferred onto nitrocellulose membranes according to the method of Towbin et al. (Towbin et al., 1979). The immunoblotting was performed as previously described (Rohrer et al., 1995). The autoradiographs were quantitated by using a personal densitometer (Molecular Dynamics).

**Assays and Miscellaneous Methods** β-hexosaminidase activity was determined as described (Rohrer et al., 1995). Protein concentration was determined with the Bio-Rad protein assay kit by using protein standard I.
Results

Intracellular location of CD-MPR wild-type vs. mutant forms.

To determine whether the replacement of Trp\textsuperscript{19} in the cytoplasmic tail of the CD-MPR by various amino acids affects its ability to avoid delivery to lysosomes, wild-type and mutant constructs were prepared and expressed in HeLa cells. The cells were incubated for 24h with pepstatin A and leupeptin to prevent degradation of receptor that was mislocalized to the lysosomes. After fixation with paraformaldehyde and permeabilization with saponin the cells were stained with antibodies against the CD-MPR and Lamp1, followed by the appropriate secondary antibodies. As expected, Lamp1 (Figure 1, green) was localized in doughnut-shaped structures typical for the staining of a lysosomal membrane protein. In contrast, the CD-MPR wild-type (Figure 1A, red) showed a predominant perinuclear staining representing localization in the TGN and late endosomes and, to a lesser extent, some peripheral staining that might represent early endosomes. There was very little colocalization of the two proteins indicated by the almost complete absence of yellow staining. The construct MPR-W19A was localized partially in lysosomes as indicated by an increased colabeling with Lamp1 compared to the CD-MPR wild-type (Figure 1B). The amount of colabeling with Lamp1 was even further increased for the mutant MPR-W19D, demonstrating that a large percentage of this construct was delivered from endosomes to lysosomes instead of being recycled back to the TGN. Interestingly, although residing within the same structures the fluorescent signal for the CD-MPR mutant constructs was sometimes not filling the entire structure (Figure 1B, C insets; yellow and green staining). Such a partial segregation would suggest distinct domains on continuous membranes.

These results demonstrate that the mutant receptors are not correctly sorted from the late endosomes back to the TGN and therefore accumulate in lysosomes. It also seems that the extent of missorting to lysosomes depends on the properties of the amino acid replacing Trp\textsuperscript{19} but this has to be characterized in more detail using a biochemical assay.

Hydrophobic residues poorly replace aromatic residues at position 19 in the CD-MPR cytoplasmic tail to bind Tip47 in vitro.
The immunofluorescence experiments suggested that the physical properties of the amino acid replacing Trp$^{19}$ determine the sorting efficiency of the CD-MPR mutants in late endosomes. Tip47 was shown to interact specifically with the di-aromatic motif of the MPRs and to mediate the transport from endosomes back to the TGN (Diaz and Pfeffer, 1998). Therefore we wanted to test the interaction of various CD-MPR mutants with Tip47 using an \textit{in vitro} binding assay. Due to the potential effect of post translational modifications we used the full length receptor expressed in mammalian cells in contrast to previously published assays with truncated fusion proteins produced in \textit{E.coli}. For this purpose we created a series of receptor constructs that consist of the full length CD-MPR with various mutations of the residue Trp$^{19}$ (Figure 2). Besides the replacements of Trp$^{19}$ with the bulky hydrophobic residues (Val, Ile, Leu) we also used a less hydrophobic amino acid with a nonpolar side chain (Ala) as well as an amino acid with an uncharged polar side chain (Ser) or charged polar side chains (Arg, Asp). Post nuclear supernatant from wild-type and mutant receptors expressed in mouse D9 (L Rec-) cells were incubated with His-tagged Tip47 bound to Nickel beads and bound receptor was subsequently detected by SDS-PAGE and Western blotting. The mouse

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\textbf{Figure 1: Mutant CD-MPR constructs have an increased colocalization with Lamp1.} HeLa cells transfected with wild type CD-MPR (A), MPR-W19A (B) or MPR-W19D (C) were preincubated for 24 h with pepstatin A and leupeptin before fixation and permeabilization. To detect the endogenous Lamp1 a polyclonal antibody (931A, (Carlsson et al., 1988)) was used followed by goat anti-rabbit Alexa 488. Wild-type and mutant CD-MPRs were detected using the monoclonal antibody 22D4 followed by goat anti-mouse Alexa 568.
monoclonal antibody 22D4 used to detect the bound CD-MPR does not recognize the endogenous receptor in mouse D9 cells and reacts only with transfected bovine receptor

Figure 2

Figure 2: Schematic illustration of the wild-type and mutant CD-MPR constructs. The bovine CD-MPR lumenal domain in each construct is indicated by a striated bar followed by an open bar (transmembrane domain) and a solid line (cytoplasmic tail). The di-aromatic signal is shown in single letter code and residues replacing Trp\(^{19}\) are indicated by bold letters.

(Kornfeld et al., 2001). The antibody recognizes two bands of the CD-MPR, the upper one at 67 kDa being a dimeric form of the receptor as shown previously (Kornfeld et al., 2001). Tip47 bound the wild-type receptor with an affinity greater than all the mutant constructs not containing a di-aromatic motif (Figure 3A). The di-aromatic constructs MPR-FW-YF and MPR-W19Y bound Tip47 as well as the wild-type receptor. There was a modest decrease in binding of Tip47 to the constructs MPR-W19V, MPR-W19I and MPR-W19L indicating that hydrophobic residues replacing Trp\(^{19}\) do not completely abolish the interaction with Tip47. Binding barely above background was seen when Trp\(^{19}\) was replaced either by alanine (MPR-W19A) or serine (MPR-W19S). Similarly,
binding barely above background was detected when charged residues like aspartic acid or arginine were used to replace Trp\textsuperscript{19}.

**Figure 3A:** Interaction \textit{in vitro} between wild-type and mutant CD-MPR constructs with Tip47. Ni-CAM beads blocked with 0.1% BSA and incubated with 6xHis-Tip47 were incubated with either wild-type or mutant receptors in post nuclear supernatant of mouse D\textsubscript{9} cells for 2 h at 4°C and washed three times to remove unbound proteins. Beads alone represents the negative control for the assay done by incubating wild-type receptor with beads without Tip47. Bound receptor was detected using SDS-PAGE and Western blotting with anti CD-MPR antibody. Protein standard shows molecular weight in kD (Amersham Pharmacia).

**Figure 3B:** Quantitation of binding of mutant receptors to 6xHis-Tip47 relative to wild-type receptor. Results shown are the standard error of the mean values from 8 independent experiments. Binding of wild-type receptor was normalized to 100 %. The binding efficiency of the assay was determined to be more than 20%. Quantitation was performed by densitometric scanning followed by ImageQuant5.0.
These data indicate that the hydrophobic residues, leucine, isoleucine and valine alone are partially able to replace Trp\textsuperscript{19} in an \textit{in vitro} Tip47 binding assay.

**Replacement of Trp\textsuperscript{19} by hydrophobic residues prevents moderately the delivery of the mutant receptors to lysosomes.**

Since the Tip47 binding assay was performed \textit{in vitro} with Tip47 in the context of a His-tagged fusion protein isolated from \textit{E.coli}, an additional assay was performed to analyze the efficiency of mutant constructs to avoid lysosomal delivery \textit{in vivo}. Mouse L cells stably expressing the wild-type and mutant receptors were preincubated for 24h in the presence of pepstatin A and leupeptin to inhibit degradation of receptors that had entered lysosomes (Rohrer et al., 1995). The cells were then harvested, homogenized and the lysosomes were separated on 17.5\% isoosmotic Percoll density gradients (Green, 1987; Rohrer et al., 1995). Under these conditions, dense lysosomes are recovered at the bottom of the gradient (pool I) whereas low density membranes including endosomes, Golgi apparatus, plasma membrane and endoplasmic reticulum are found near the top of the gradient (pool III). Intermediate density membranes are recovered in pool II. The distribution of the receptor in the three pools was determined by SDS-PAGE followed by Western blotting (Figure 4 and Table 1 for quantitation of multiple experiments). As reported previously, the wild-type CD-MPR was almost completely excluded from dense lysosomes (5\% recovered in pool I) and replacement of the di-aromatic motif (Phe\textsuperscript{18}Trp\textsuperscript{19}) with other aromatic residues did not affect the distribution significantly (MPR-FF, 5\%; MPR-FY, 4\%; MPR-YF, 8\% and MPR YW, 3\%, (Schweizer et al., 1997). Substitution of Trp\textsuperscript{19} with bulky hydrophobic residues led to an accumulation of the mutant receptors in dense lysosomes to about 30\% (MPR-W19V, 27\%; MPR-W19I, 27\%; and MPR-W19L, 29\%). A slightly larger accumulation of mutant receptors in lysosomes was found with the mutants that contained less hydrophobic, nonpolar side chains as replacements for Trp\textsuperscript{19} (MPR-W19A, 38\% and MPR-W19M, 40\%). To our surprise we found that the mutant MPR-W19C with the polar side chain of a cysteine residue accumulated only to about 34\% in dense lysosomes. However, according to Kyte and Doolittle (Kyte and Doolittle, 1982) the cysteine residue with its polar side chain is more hydrophobic than an alanine or a methionine residue. We next examined the effect
of mutating Trp<sup>19</sup> to a hydrophilic residue (MPR-W19S) or even charged residues (MPR-W19R and MPR-W19D). All three mutant receptors accumulated more than 50% in dense lysosomes (MPR-W19S, 52%; W19R, 56% and MPR-W19D, 59%) compatible

Figure 4: Receptors with Trp<sup>19</sup> replaced by hydrophobic residues are partially functional for endosomal sorting. (A) Mouse L cells stably expressing MPR wild-type and mutant constructs were preincubated with pepstatin A and leupeptin for 24 h. The cells were then homogenized, and postnuclear supernatants were subjected to Percoll density gradient centrifugation (17.5% Percoll). The collected fractions were combined into pools of three fractions each: I dense lysosomes, II intermediate membranes, and III low density membranes and then further analyzed by SDS-PAGE and immunoblotting with monoclonal antibody 22D4. (B) Quantitation of the immunobLOTS shown in (A) and those from additional experiments; mean values are shown. For each construct the value of pool I (dense lysosomes) is expressed as a percentage of the sum of all three pools. Protein standard shows molecular weight in kDa (Amersham Pharmacia).

with the hypothesis that only hydrophobic residues can replace to a small extent an aromatic residue of the di-aromatic motif.
Discussion

The results presented in this study demonstrate that replacing the key residue of the di-aromatic motif within the cytoplasmic tail of the CD-MPR, Trp\(^19\), by hydrophobic residues maintains endosomal sorting only to a minor extent. Mutant constructs with hydrophilic or even charged residues instead of Trp\(^19\) were highly missorted to lysosomes, indicating that these replacements were not functional at all.

To accomplish their biological function, sorting of lysosomal enzymes from the TGN to endosomes, it is essential that the MPRs avoid delivery to lysosomes where they would be degraded. Therefore the sorting step in late endosomes, which is mediated by the di-aromatic motif (Schweizer et al., 1997) is crucial and needs to be well characterized. Pfeffer and colleagues first demonstrated that Tip47 interacts with the CD-MPR through the di-aromatic motif and with the CI-MPR which is lacking a di-aromatic motif (Diaz and Pfeffer, 1998) and subsequently identified the sequence PPAPRP within the cytoplasmic tail of the CI-MPR as part of a complex three dimensional binding domain for Tip47 (Orsel et al., 2000). This raised the question if the interaction between Tip47 and the CD-MPR is strictly limited to aromatic residues or if other amino acids can replace Trp\(^19\), the key residue of the di-aromatic motif.

Using immunofluorescence we found that there is an increasing colocalization of mutant receptors with the lysosomal membrane protein Lamp1 upon decreasing hydrophobicity of the amino acid replacing Trp\(^19\). Interestingly, there was even less overlap than expected between the wild-type CD-MPR and Lamp1 as both proteins are present to some extent in late endosomes (Griffiths, 1988). This might indicate that Lamp1 and the CD-MPR are in different subdomains within late endosomes. It would make perfect sense that proteins destined for the lysosomes would be separated from proteins that recycle back to the Golgi thus increasing the sorting efficiency of such a complex structure as the multivesicular late endosomes. In fact, it has been observed that even the CD-MPR and the CI-MPR are localised in separate subdomains within late endosomes (Klumperman et al., 1993). Mutant receptors that were delivered partially to lysosomes like MPR-W19A (38% in lysosomes) and MPR-W19D (59% in lysosomes) did show an increasingly overlapping staining with Lamp1, validating this method as a screening procedure to test for the sorting efficiency of the mutant receptors. Intriguingly, there
seemed to be subdomains of lysosomes that were labeled with either one or both proteins. Actually the majority of the structures that were colabeled for CD-MPR mutants and Lamp1 did not show a uniform yellow labeling which would indicate an equal distribution of the proteins but rather an overlap with separate labeling. Such a pattern of segregation of proteins indicates the existence of subdomains on the lysosomal membrane. These subdomains may be specialized for degradation as in the case of CD-MPR mutants or for export as in the case of molecules such as Lamp1 (Akasaki et al., 1993; Furuno et al., 1989; Lippincott-Schwartz and Fambrough, 1987; Reaves et al., 1996). It has been shown by Traub et al., that AP-2 and clathrin could be recruited to lysosomes in permeabilised cells in vitro indicating that clathrin coated vesicles mediate this transport step. Contrary to the existing notion of lysosomes as terminal organelles in the secretory pathway our observation suggests in addition that a functional specialization of its subdomains might exist. To follow up on this would require a method with a higher resolution such as immuno electron microscopy.

To determine the sorting efficiency in a more quantitative way we first analyzed the ability of CD-MPR to bind His-tagged Tip47 in an in vitro assay. As with the immunofluorescence experiments the binding efficiency decreased if Trp\(^{19}\) was replaced by decreasing hydrophobic residues. However, even the mutants with Trp\(^{19}\) substituted by bulky hydrophobic residues such as leucine, valine and isoleucine were moderately reduced in their capacity to bind Tip47. Mutants with a less hydrophobic residue at position 19 like MPR-W19A displayed only marginal Tip47 binding whereas charged residues prevent binding almost completely. Binding was almost not affected when the di-aromatic mutants MPR-FW-YF and MPR-W19Y were tested in the assay. This clearly demonstrates that optimal interaction of the CD-MPR cytoplasmic tail with Tip47 depends on the di-aromatic motif with only limited possibilities for variations. The strict requirement for individual amino acids is further emphasized by the fact that the cytosolic tail of CD-MPR is 100% identical in all mammalian species cloned so far with only two homologous substitutions (Val\(^{5}\) – Ile\(^{5}\) and Asp\(^{55}\) – Glu\(^{55}\)) in lower vertebrates such as chicken (Hille-Rehfeld, 1995; Matzner et al., 1996).

When we analyzed the mutants in vivo in the context of the full length receptor for their efficiency to avoid delivery to lysosomes we found that there were roughly three
categories. Substitution of Trp\textsuperscript{19} with either one of the bulky hydrophobic residues resulted in mislocalization of the mutant construct to lysosomes just below 30\%. This number was slightly increased (34-40\%) when less hydrophobic residues like cysteine, alanine and methionine were used to replace Trp\textsuperscript{19}. Hydrophilic (serine) or charged residues (arginine and aspartic acid) had the greatest effect on endosomal sorting with more than 50\% of the mutants accumulating in dense lysosomes (Figure 4, Pools III). It is important to note that sorting of the mutant CD-MPRs in endosomes was not good even with the bulky hydrophobic residues as substitutions for Trp\textsuperscript{19} when compared to mutants with aromatic residues as replacements for Trp\textsuperscript{19} (table 1) that were sorted like wild-type receptor.

It is interesting that the CD-MPR has a clearly defined signal, the di-aromatic motif that is required for the interaction with Tip47 and the sorting in late endosomes, which seems to be in contrast to the CI-MPR that relies on a domain comprising residues 48-75 in its cytoplasmic tail, with additional residues required for the proper presentation (Orsel et al., 2000). Nevertheless, proper presentation of the di-aromatic signal is also required as mutations that prevent the reversible palmitoylation of the CD-MPR lead to a missorting to lysosomes (Schweizer et al., 1996). Clearly, further studies are required to determine if both receptors have the same binding site on Tip47 or if there are two different binding sites. However, these experiments will be technically demanding since the receptors are membrane proteins that undergo important posttranslational modifications such as palmitoylation.

**Acknowledgements**

We thank Drs. D. Messner and M. Fukuda for kindly providing antibodies against the CD-MPR and Lamp1, respectively. Special thanks also to Dean Flanders from the IT group of the Friedrich Miescher Institute who provided us with enough computer power to run the deconvolution software for the immunofluorescence pictures. We acknowledge the continuous support of Prof. Eric G. Berger and thank him for critical reading of the manuscript.
<table>
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†Percentage of the various mutant receptors recovered in dense lysosomes on Percoll density gradients as shown in Figure 4. The values are expressed as mean ± SE.

*Values taken from ref (Schweizer et al., 1997)
References


10. Schweizer, A., Kornfeld, S., and Rohrer, J. 1997. Proper sorting of the cation-dependent mannose 6-phosphate receptor in endosomes depends on a pair of


Part II

CHARACTERISATION OF THE TGN EXIT SIGNAL
OF UCE
Summary

The human mannose 6-phosphate uncovering enzyme participates in the uncovering of the mannose 6-phosphate recognition tag on lysosomal enzymes, a process which facilitates recognition of those enzymes by mannose 6-phosphate receptors to ensure delivery to lysosomes. Uncovering enzyme has been localized to the trans-Golgi network at steady state. It has been shown to traffic to the plasma membrane from where it is rapidly internalized via endosomal structures, the process being mediated by a tyrosine-based internalization motif, Y^{488}HPL, in its cytoplasmic tail. Using immunogold electron microscopy a GFP-uncovering enzyme fusion construct was found to be co-localized with the CD-MPR in regions of the trans-Golgi network suggesting that uncovering enzyme might follow a similar pathway of exit from the trans-Golgi network as that of the CD-MPR. In this study, we identified the signal sequence in the cytoplasmic tail of uncovering enzyme responsible for its exit from the trans-Golgi network. Using GFP fusion constructs of the transmembrane and cytoplasmic domains of uncovering enzyme, we could show that residues Q^{492}EMN in the cytoplasmic tail of uncovering enzyme are involved in its exit from the trans-Golgi network by automated analysis of confocal immunofluorescence images. Detailed characterization of the exit signal revealed that residue Q^{492} is the most important to the exit function while M^{494} and N^{495} also contribute. The cytoplasmic tail of the uncovering enzyme does not possess any of the known canonical signal sequences for interaction with Golgi associated gamma ear-containing adaptor proteins. The identification of a trans-Golgi network exit signal in its cytoplasmic tail elucidates the trafficking pathway of uncovering enzyme, a crucial player in the process of lysosomal biogenesis.
**Introduction**

Lysosomes are one of the major degradative compartments of mammalian cells and their biogenesis depends on the concerted transport of acid hydrolases from their site of origin to their site of action through a series of intermediate compartments. Lysosomal enzymes are recognized by one of the two known mannose 6-phosphate receptors (MPRs) in the trans-Golgi network (TGN) for transport onward to lysosomes. This crucial recognition event directly depends on the acquisition of mannose 6-phosphate (M6P) tags by the lysosomal enzymes. The tag is generated in a two-step enzymatic reaction which occurs in the Golgi. The first step, the addition of an N-acetyl glucosamine phosphate to selected mannose residues of the lysosomal enzyme, is catalyzed by UDP-N-acetylglucosamine lysosomal enzyme N-acetylglucosamine phosphotransferase. The second step, mediated by N-acetylglucosamine-1-phosphodiester α-N-acetylglucosaminidase, leads to the actual uncovering of the M6P tag by cleaving the GlcNAc residue. The α-N-acetylglucosaminidase is therefore termed ‘uncovering enzyme’ (UCE).

UCE is a type I membrane glycoprotein of 515 amino acids. It has a single transmembrane domain of 27 residues and a 41 residue cytoplasmic tail which contains several established and potential signal sequences ensuring its correct intracellular sorting (Kornfeld et al., 1999; Lee et al., 2002). UCE has been shown to reside in the TGN at steady state and its constitutive recycling between that organelle and the plasma membrane has been established using a combination of steady state measurements of enzyme activity and localization of GFP fusion constructs of the enzyme cytoplasmic tail (Rohrer and Kornfeld, 2001). It was shown that the classical endocytosis motif Y\(^488\)HPL and the C-terminal N\(^511\)PFKD\(^515\) motif are involved in the trafficking of UCE (Rohrer and Kornfeld, 2001). However, the dissection of the trafficking itinerary of UCE to elucidate the individual transport steps involved has not yet been reported. Three different types of signals in the cytoplasmic tail of UCE have been described. First, the transmembrane domain and the first 11 residues of the cytoplasmic tail of UCE were shown to be involved in TGN retention of the enzyme, thereby indicating that the TGN exit signal is excluded from this region of the enzyme. Secondly, the Y\(^488\) residue has been shown to be functional in internalization from the surface (Lee et al., 2002). Third,
the Y^{486} residue was implicated in the return of internalized UCE to the TGN (Lee et al., 2002). The signal(s) leading to TGN exit have been studied in this paper. Signals involved in localization of membrane proteins at specific intracellular locations may be of the retention or the recycling type. A number of transmembrane proteins and receptors are known to cycle between the TGN and the plasma membrane of which TGN38, furin, the envelope glycoprotein gp1 of the Varicella Zoster virus and the human Menkes disease protein are notable. The SDYQRL sequence in the cytoplasmic tail of TGN38 has been shown to be involved in its TGN localization (Wong and Hong, 1993). An acidic TGN retention signal and a YKGL TGN retrieval motif have been identified in furin (Schafer et al., 1995). A tyrosine-based motif and a casein kinase II phosphorylation site were shown to be responsible for the TGN localization of the Varicella Zoster envelope glycoprotein gp1 (Alconada et al., 1996). A C-terminal di-leucine motif has been shown to be involved in the correct localization of the human Menkes disease protein in the TGN (Petris MJ, 1998). A FHRL sequence in the cytoplasmic tail of carboxypeptidase D has been shown to be important for the TGN retention and retrieval of the protein from the surface (Eng, 1999). Although several instances of signals involved in TGN localization exist in the literature, there have been few reports of signal sequences involved in the exit from the TGN of proteins cycling between the TGN and the plasma membrane. Anterograde transport of vesicles from the TGN has been established to be a process mediated by clathrin coated vesicles and Golgi associated adaptor proteins. Cargo binding to such adaptors for selective inclusion in transport vesicles depends on crucial signal sequences in its cytoplasmic tail. For instance, it has been shown that the cytoplasmic signals YKGL^{765}, LI^{760}, F^{790} and clustered acidic amino acids in furin are crucial for interaction with adaptor protein-1 (AP1) (Teuchert et al., 1999).

Using immunoelectron microscopy we have previously found that a green fluorescent protein (GFP) – UCE fusion construct was localized to the TGN in close proximity to the cation dependent MPR (CD-MPR) suggesting that the two proteins might have a similar pathway of exit from the TGN. However, unlike the CD-MPR, which has been shown to interact with AP-1 and GGAs, UCE does not contain any of the known motifs for interaction with GGAs. The localization of the GFP fusion construct of the
transmembrane and cytosolic domains of the human UCE has been previously shown to be identical to that of the endogenous enzyme in HeLa cells (Rohrer and Kornfeld, 2001). Furthermore, it was shown that replacement of the Y^{488} residue with an alanine led to a cell surface accumulation of the GFP fusion constructs thus demonstrating the role of the residue in internalization of the enzyme (Rohrer and Kornfeld, 2001). Additionally, it was reported that a construct truncated at position Y^{486} was mostly trapped in an intracellular compartment. This result was surprising in view of the finding that only 16% of the molecules were found on the cell surface despite the lack of an internalization signal (Lee et al., 2002). This indicated that the construct lacks a TGN exit signal causing it to leave the TGN very slowly.

In this study, we used a series of GFP fusion constructs, confocal immunofluorescence microscopy, and automated image interpretation to identify the signal sequence in the cytoplasmic tail of UCE involved in TGN exit of the enzyme. The identification of the TGN exit signal in the cytoplasmic tail of UCE is an important step in elucidating its trafficking itinerary. It is as yet unknown if UCE is packaged into the same or different vesicles from that of the MPR at the TGN. Given that UCE is crucial to the process of lysosomal biogenesis, the potential interaction of the TGN exit signal with specific Golgi associated adaptor proteins might explain its traffic out of the TGN.
**Experimental Procedures**

**Materials.** Enzymes used in molecular cloning were obtained from Boehringer Mannheim, New England Biolabs, Sigma or Promega; α-minimal essential medium (α-MEM), and fetal calf serum were from GIBCO BRL (Basel, Switzerland). Polyethylenimine (PEI), linear, MW-25,000 was from Polysciences Inc., (Warrington, PA) and cell culture dishes from Falcon. 4’, 6-diamidino-2-phenylindole (DAPI) was from Boehringer Mannheim. Lissamine rhodamine sulfonl chloride (LRSC) was from Molecular Probes (Eugene, OR). Oligonucleotides were synthesized by Microsynth GmBH (Galbach, Switzerland).

**Recombinant DNA.** All basic DNA procedures were as described (Sambrook et al., 1998). The GFP-UCE transmembrane domain-cytoplasmic tail construct was prepared using a PCR procedure (Ho et al., 1989) as described (Rohrer and Kornfeld, 2001). The GFP-UCE 502 stop construct, the double mutant, the single and combined mutant constructs were all made using the pBill-neo GFP-UCE Y488A construct as template for a two step PCR using appropriate partial complementary pairs of oligonucleotides in which the desired amino acid replacements had been incorporated. The PCR products were put together with fragments from the vector in a triple ligation following digestion with Bgl II, MluI and EcoRI. The final PCR products were subcloned into pSFFV-neo as described (Rohrer et al., 1995). All coding sequences created by PCR were verified by sequencing.

**Cell Culture and Transfection.** HeLa cells were grown in α-MEM to 30% confluency before transfection with 2.2 µg of DNA using 7.6 µg PEI according to the manufacturer’s directions. Selection for resistance to neomycin (G418) was carried out using 1 mg/ml G418 as the final concentration. Resistant colonies were picked individually and screened for the expression of GFP-UCE by immunofluorescence analysis. Two or three different clones expressing similar or varying amounts of each of the fusion constructs were expanded for further study and maintained in selective medium.

**Immunoelectron Microscopy** For immunoelectron microscopy, HeLa cells expressing GFP-UCE were fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer at pH 7.4, washed in buffer, and embedded in 10% gelatin. Gelatin blocks with cells were
infused with 2.3 M sucrose and frozen in liquid nitrogen. Cryosectioning and immunogold labeling have been described before (Geuze, 1981), (Raposo, 1997). Double-immunogold labeling of GFP and CD-MPR was done with 15 nm and 10 nm protein A-gold particles, respectively.

**High Resolution Confocal Immunofluorescence Microscopy**

Confocal immunofluorescence microscopy was carried out using a variation on a three-color scheme previously developed to facilitate automated image interpretation (Velliste and Murphy, 2002) as follows. 3 % Paraformaldehyde-fixed and saponin-permeabilized (final concentration of 1 mg/ml) cells were incubated with 1 µg/ml DAPI (to label total DNA) and a 1:40 dilution of a 1 mg/ml stock solution of LRSC (to label total protein) for 10 minutes before being washed four times with PBS-Glycine-Saponin. The coverslips were mounted on glass slides with ProLong Antifade (Molecular Probes) for viewing with a Leica TCS SP2 (AOBS) confocal laser-scanning microscope. Serial sections in the z-axis through entire cells (23 - 110 sections per image stack) were taken with a step size of 0.1628 µm and a pixel size of 0.0976 µm in the x and the y dimensions (1024 X 1024 pixels per section). At least 80 cells were analyzed for every clone of the mutant constructs and two or three clones were analyzed for each construct. The resulting stacks of images were exported as Tiff files and visually analyzed with the Imaris program (Bitplane AG, Zurich, Switzerland).

**Automated image analysis**

Automated segmentation of the GFP images using the DNA and total protein (LRSC) channels were carried out using a seeded watershed approach (Velliste and Murphy, in preparation). For automated analysis of the GFP images, a new feature set, SLF19, was created by combining the set SLF11 (Chen et al., 2003) with the DNA associated features SLF9.9-9.14 and SLF9.21-9.28 (Huang and Murphy, 2004). This feature set was calculated for all images of the various UCE constructs, and a set of 52 features (defined as SLF20) was selected from it by Stepwise Discriminant Analysis (SDA) in order to choose only those features that are useful for distinguishing the constructs (Huang and Murphy, 2004). Dendrograms using the Mahalanobis distance were then constructed using SLF20 (Chen et al., 2003).
**Results**

**CD-MPR co-localizes with GFP-UCE in HeLa cells**

Using immunogold electron microscopy, we observed that the GFP-UCE fusion construct co-localizes with the CD-MPR in tubulovesicular regions of the TGN, suggesting that the two proteins might have the same exit pathway out of the TGN (Figure 1). Furthermore, the two proteins have been co-localized in clathrin-coated buds at the TGN (data not shown). However, UCE does not contain any known motifs for interaction with GGA proteins. Therefore, we sought to identify the TGN exit signal of UCE.

**Figure 1**

![Double immunogold labeling of UCE and CD-MPR with gold sizes as indicated on the figure in nm. Most of UCE localizes to the TGN and only few gold particles are present over cisternae of the Golgi stacks. In the TGN, UCE colocalizes with CD-MPR. Scale bars are 100 nm.](image-url)
Intracellular location of GFP-UCE wild-type vs. mutant forms.

In order to determine the TGN exit signal in the cytoplasmic tail of the human UCE, GFP fusion constructs of the cytoplasmic tail of the UCE were made in which the luminal domain of UCE was replaced by GFP. The localization of this fusion construct has been shown to be the same as that of the wild-type UCE in HeLa cells (Rohrer and Kornfeld, 2001). The cytoplasmic tail of UCE has been shown to contain a tyrosine based internalization signal, residue Y^{488}HPL, which plays a role in rapid internalization from the plasma membrane (Rohrer and Kornfeld, 2001). Previous studies revealed that truncating the cytoplasmic tail at residue 486 resulted in a mutant enzyme trapped in an intracellular compartment with only 16% of the molecules being on the cell surface (Lee et al., 2002). This construct lacks an internalization signal but its low surface levels indicate that delivery to the surface is strongly reduced, possibly due to a slow exit out of the TGN. In contrast, the construct Y^{488}-A/M^{494} Stop, which also lacks a functional internalization signal, was found to be 50% at the cell surface. This suggests that it leaves the TGN faster than the Y^{486} Stop construct because it contains a TGN exit signal (Lee et al., 2002). In order to characterize a TGN exit signal we made an alanine scan of the residues Q^{492}-EMNGEPLAA^{501} two at a time and analyzed the effect of the mutations on the localization of the GFP fusion constructs. All the constructs were made in the background of the Y^{488}-A mutation to prevent internalization if they would reach the plasma membrane. If a TGN exit signal was located in the scanned region, these constructs would be localized to the TGN and not at the surface despite the Y488A internalization defect, as they would be expected to have a reduced rate of exit from the TGN. The rationale for screening the cytoplasmic tail up to residue 502 was based on the fact that signal sequences are usually about six to eight amino acids long and furthermore, it was important to exclude redundant internalization signals such as the N^{511}PFK^{515} signal in order to study the effect of the mutations in the background of an internalization defective UCE (Figure 2A). Conventional techniques of image analysis depend on the investigator’s ability to differentiate localization patterns based on a visual examination of confocal immunofluorescence images. The inherent disadvantage of such a method is the lack of objectivity and inability to obtain quantitative data on the localization pattern. In order to obtain precise and quantitative data on the localization
pattern, we used well-characterized numerical features (SLFs) that have been demonstrated to be capable of discriminating all major subcellular location patterns (Chen and Murphy, 2004).

**Figure 2**

A) Schematic illustration of the wild-type and mutant GFP-UCE constructs. The luminal domain of UCE was replaced with monomeric GFP fused to an N-terminal cleavable preprolactin leader sequence represented by striated bars. The transmembrane domain of UCE is indicated by shaded bars and the amino acids in the cytoplasmic tail are shown in single letter code. Mutations of the Y488 residue and the potential exit signal residues are shown in bold letters. The 502 Stop mutation is indicated by an asterisk.

B) Schematic illustration of the single and combined mutant GFP-UCE constructs. The constructs have the same organization as those in Figure 1A. In addition to the Y488 and the 502 Stop mutation, residues Q492, E493, M494 and N494 are either singly mutated or mutated simultaneously and the mutant residues are indicated by bold letters.

Using these features, we studied the relationship among UCE constructs by dendrogram analysis, in which wild-type and mutant constructs were grouped according to their relative similarity. The “distance” (or inverse of similarity) between the images of each pair of constructs was calculated using feature set SLF20 as described in the methods. The distance was expressed as a Mahalanobis distance, which measures the distance weighted by the statistical variation in each feature and any correlation between features. A clear pattern that emerged from the analysis was the similarity in localization of the
wild-type construct and that of the 502 Stop construct clearly indicating that any information for TGN exit is contained before residue 502 of the cytoplasmic tail (Figure 3). Both constructs were localized to the TGN and could be co-labeled with a known TGN marker protein TGN46 (data not shown). Furthermore, as was clearly discernible from a visual examination of the images, the Y488-A/502 Stop construct was to a great extent localized to the cell surface (Figure 3). The image clustering program separated the different constructs analyzed into three groups – one comprising the wild-type UCE cytosolic tail and the 502 Stop construct, one comprising the Y488; Q492E-A/502 Stop and the Y488; M494N-A/502 Stop constructs, and the last comprising the Y488-A/502 Stop, Y488; G496E-A/502 Stop and Y488; P498L-A/502 Stop constructs (Figure 6A). The predominant surface localization of the Y488; G496E-A/502 Stop and Y488; P498L-A/502 Stop constructs (similar to Y488-A/502 Stop construct) suggests that those mutations do not interfere with the TGN exit signal (allowing the UCE to exit the TGN and then become trapped at the cell surface). The intermediate phenotype of the Y488; Q492E-A/502 Stop and Y488; M494N-A/502 Stop constructs indicates that those mutations have compromised the TGN exit signal (Figure 4).

Figure 3: High resolution confocal immunofluorescence images of HeLa cells expressing wild-type or mutant GFP-UCE constructs. HeLa cells were stably transfected with wild-type GFP-UCE (A), GFP-UCE 502 Stop mutant (B) or GFP-UCE Y488A/502 Stop mutant (C). The localization of the constructs was analyzed using high resolution three-dimensional confocal immunofluorescence microscopy followed by projection of z-stacks using the Imaris 4.0 software. Scale bars are 20 µm.
Analysis of single and combined mutations of the Q\textsuperscript{492}EMN sequence

In order to further characterize the signal and to determine the contribution of the individual residues we made single and combined alanine substitution mutants of the Q\textsuperscript{492}, E\textsuperscript{493}, M\textsuperscript{494} and N\textsuperscript{495} residues (all in the Y\textsuperscript{488}-A/502 Stop background) and analyzed the localization of the constructs by dendrogram analysis as had been done for the double mutants. The dendrogram (Figure 6B) contained the previous three groups plus a new group consisting of Y\textsuperscript{488}, M\textsuperscript{494}-A/502 Stop, Y\textsuperscript{488}, N\textsuperscript{493}-A/502 Stop, and Y\textsuperscript{488}, Q\textsuperscript{492}-EMN-A/502 Stop. This group has a pattern resembling endosomes, suggesting that those constructs can exit the TGN but then may be altered so that they are trapped elsewhere. The most important finding is the placement of Y\textsuperscript{488}, Q\textsuperscript{492}-A/502 Stop within the wild-type group, showing that this residue is the most important part of the TGN exit signal. The grouping of Y\textsuperscript{488}, E\textsuperscript{493}-A/502 Stop with the Y\textsuperscript{488}-A/502 Stop group indicates that E\textsuperscript{493} is not required for the exit signal. Combining results from the single and double mutants leads to the conclusion that the exit signal resides in the Q\textsuperscript{492}EMN residues with Q\textsuperscript{492} being most important but the M\textsuperscript{494}N residues being able to confer a partial exit signal even when Q\textsuperscript{492} is mutated. Our own visual observation of the localization of the constructs confirmed the image analysis results as shown by the representative images of the single and combined mutants (Figure 5). The dendrograms in Figure 6 are available at http://murphylab.web.cmu.edu/services/PSLID/HeLa_UCE/ with a browser interface that allows the underlying images for any branch to be displayed interactively.

Discussion

The results presented in this study demonstrate that the residues Q\textsuperscript{492}EMN in the cytoplasmic tail of UCE direct its exit from the TGN. Furthermore, the single alanine substitutions revealed that the residue Q\textsuperscript{492} is more important to the TGN exit function than residues M\textsuperscript{494} and N\textsuperscript{495} which play a contributory role to the TGN exit of UCE. UCE has been shown to reside in the TGN at steady state and it constitutively cycles between the TGN and the plasma membrane (Rohrer and Kornfeld, 2001). UCE is synthesized as an inactive proenzyme and gets activated by furin in the TGN (Do et al., 2002). It has been previously shown that UCE is internalized rapidly from the plasma
membrane with a half-time of 0.65 minutes using a tyrosine based internalization signal,

**Figure 4**

![Immunofluorescence images of HeLa cells expressing double mutant GFP-UCE constructs of the potential TGN exit signal.](image)

**Figure 4:** High resolution confocal immunofluorescence images of HeLa cells expressing double mutant GFP-UCE constructs of the potential TGN exit signal. HeLa cells were stably transfected with GFP-UCE Y^{488}; Q^{492}E-A/502 Stop (A), GFP-UCE Y^{488}; M^{494}N-A/502 Stop (B), GFP-UCE Y^{488}; G^{496}E-A/502 Stop (C) or GFP-UCE Y^{488}; P^{498}L-A/502 Stop (D). Scale bars are 20 µm.

Y^{488}HPL, by means of clathrin mediated endocytosis (Lee et al., 2002). The trafficking itinerary of proteins that cycle between the TGN and the plasma membrane is either direct or involves an intermediate endosomal step (Lemmon and Traub, 2000). Proteins that reach the recycling or sorting endosomes are retrieved by transport to the TGN before reaching the surface or go directly to the plasma membrane. The steady state localization of UCE at the TGN thus mainly depends on the rate at which the enzyme exits the TGN given the fact that it is rapidly internalized from the surface.

The localization and cycling pathway of UCE is important for the correct transport of lysosomal enzymes to the lysosomes considering its function in the generation of the M6P recognition tag, a process which is completed in the TGN. The TGN exit step of its trafficking pathway is thus crucial to the process of lysosomal biogenesis. Using immunogold electron microscopy we found that a GFP-UCE fusion construct co-
localizes with the CD-MPR at the TGN. This raised the question of whether the two proteins might have similar pathways of exit from the TGN. While the exit of the CD-MPR from the TGN has been extensively characterized, little is known about the TGN exit step in the trafficking itinerary of UCE. Using high resolution confocal immunofluorescence microscopy and automated image analysis we could show that the residues Q\textsuperscript{492}EMN are involved in the TGN exit of UCE. The GFP-UCE transmembrane domain-cytoplasmic tail fusion construct has been previously localized to the TGN (Rohrer and Kornfeld, 2001). Truncating the construct at position 502 did not affect the TGN localization of the enzyme, however, an additional Y\textsuperscript{488}-A mutation led to an accumulation of the construct at the surface. Therefore any construct with the Y\textsuperscript{488}-A mutation which can exit the TGN (and is not otherwise perturbed) must accumulate at the surface. The images made at high resolution were analyzed using feature based machine learning techniques. The advantages of using these approaches to analyze the images are that a. it is possible to obtain quantitative data on the localization pattern and b. the resolution achieved by the program far outweighs that obtained by visual examination of images as previously demonstrated (Murphy et al., 2003). The Y\textsuperscript{488}; Q\textsuperscript{492}E-A/502 Stop and Y\textsuperscript{488}; M\textsuperscript{494}N-A/502 Stop constructs were mainly localized to the TGN as opposed to the Y\textsuperscript{488}; G\textsuperscript{496}E-A/502 Stop and Y\textsuperscript{488}; P\textsuperscript{498}L-A/502 Stop constructs which were mainly localized to the plasma membrane. This indicates the role of the residues Q\textsuperscript{492}EMN in TGN exit. The localization of the Y488A/502 Stop construct was interesting in that although most of the construct was localized at the surface, a significant amount was still found in the trans-Golgi / TGN area indicating that part of the Y\textsuperscript{488}HPL motif might also be involved in the TGN exit step of the enzyme trafficking pathway. Thus, in combination with the residues Q\textsuperscript{492}EMN, the Y\textsuperscript{488}HPL sequence functions in ensuring the proper cycling of UCE between the TGN and the plasma membrane.

In order to determine the relative contribution of each individual residue of the Q\textsuperscript{492}EMN TGN exit signal, single and combined mutants were made and their localization was analyzed using the image clustering program. The closeness of the mutant Y\textsuperscript{488}; Q\textsuperscript{492}-A/502 Stop to the wild-type suggested that Q\textsuperscript{492} plays an important
role in the TGN exit. A single mutation of Q\textsuperscript{492} is sufficient to block the exit function.

**Figure 5**

Figure 5: High resolution confocal immunofluorescence images of HeLa cells expressing combined and single mutant GFP-UCE constructs. HeLa cells were stably transfected with GFP-UCE Y\textsuperscript{488}; Q\textsuperscript{492}-A/502 Stop (A), GFP-UCE Y\textsuperscript{488}; E\textsuperscript{493}-A/502 Stop (B), GFP-UCE Y\textsuperscript{488}; M\textsuperscript{494}-A/502 Stop (C), GFP-UCE Y\textsuperscript{488}; N\textsuperscript{495}-A/502 Stop (D) or GFP-UCE Y\textsuperscript{488}; Q\textsuperscript{492}EMN-A/502 Stop mutant (E). Scale bars are 20 µm.

On the contrary, the mutant Y\textsuperscript{488}; E\textsuperscript{493} A/502 Stop showed a similar pattern to the mutant Y488A/502 Stop, suggesting that mutation of E\textsuperscript{493} does not block the TGN exit. Surprisingly, the Y\textsuperscript{488}; M\textsuperscript{494}-A/502 Stop and Y\textsuperscript{488}; N\textsuperscript{495}-A/502 Stop mutants displayed a discrete localization pattern from the wild-type and Y488A/502 Stop mutant. Inspection of the images from these mutants revealed that they may form an endosome pattern, which suggests that the protein exits the TGN but does not enter the plasma membrane properly. It suggests that the protein could enter the plasma membrane properly when both M\textsuperscript{494} and N\textsuperscript{495} residues are intact while either one of the two residues is sufficient in assisting Q\textsuperscript{492} in the TGN exit. The combined mutant had a mixture of Golgi and endosome patterns which suggests that both the exit from the TGN and plasma membrane delivery are impaired.
Figure 6: Dendrograms showing similarity in localization between A). the GFP-UCE wild-type, GFP-UCE 502 Stop, GFP-UCE Y488A/502 Stop and double mutant constructs of the Q^{EMNGEPL} sequence in the cytoplasmic tail of UCE and B). The wild-type and all 11 mutant constructs. The constructs are clustered based on Mahalonobis distance units calculated on a set of subcellular localization features.
Membrane proteins such as the MPRs and Lamp1 exit the TGN for onward transport to the late endosomes or the plasma membrane either by default or using a signal sequence which interacts specifically with adaptor proteins. The rate of TGN exit of UCE and therefore the rate of its surface delivery is reduced in the absence of its TGN exit signal. This leads to the conclusion that UCE requires the TGN exit signal for optimal trafficking out of the TGN. The nature of its signal sequence however does not resemble the canonical motifs found in other proteins such as the acidic cluster di-leucine motif found in MPRs or the tyrosine-based motifs present in Lamp-1, leading to the speculation that a novel interacting protein might act in the packaging of UCE at the TGN.
Abbreviations

UCE, uncovering enzyme; CD-MPR, cation-dependent mannose 6-phosphate receptor; CI-MPR, cation-independent mannose 6-phosphate receptor; TGN, trans-Golgi network; GGA, Golgi-localized, gamma-ear-containing, adenosine diphosphate ribosylation factor-binding protein; AP, adaptor protein complex; DAPI, 4’-6-diamidino-2-phenylindole; LRSC, lissamine–rhodamine sulfonyle chloride.

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Keywords

Lysosomal biogenesis, mannose 6-phosphate receptors, uncovering enzyme, trans-Golgi network, TGN exit, sncellular location features, protein distribution comparison.
References


11. General Discussion

This work was dedicated to studying two different sorting signals involved in two steps in intracellular sorting- sorting at late endosomes and exit out of the TGN. Analysis of the endosomal sorting signal of the CD-MPR revealed that a diaromatic motif is required for correct retrieval of the receptor from the late endosomal compartment to the TGN. This requirement was shown to be strict, not allowing viable replacements of the crucial W^{19} residue of the diaromatic motif. Failure of this trafficking step would invariably result in receptor mislocalisation to lysosomes and degradation therein. The \textit{in vivo} analysis of receptor mislocalisation upon mutation of the W^{19} residue revealed that bulky hydrophobic amino acids such as valine, leucine and isoleucine at position 19 resulted in about 30% receptor mislocalisation to lysosomes in contrast to the wild-type receptor of which only about 5% was found in lysosomes. All the other substitutions analysed resulted in greater mislocalisation. Interestingly, replacement by other diaromatic motifs resulted in normal sorting of the receptor. The half-life of CD-MPR is about 48 hours and within this time it performs several rounds of transport of lysosomal enzymes. The mislocalisation observed using the mutant constructs was studied 24 hours after addition of protease inhibitors to cells. Thus, a 30% mislocalisation within 24 hours, of a receptor whose life cycle is little over 48 hours is clearly indicative of a physiologically non-viable condition. This highlights the essential and irreplaceable nature of the diaromatic motif. Not surprisingly, the motif is found in several other transmembrane receptors and proteins. The functionality of the motif in trafficking has already been demonstrated for the human mannose receptor. However, several other proteins harbour the motif and its role in the trafficking of those proteins is yet to be explored. The human phospholipase receptor has a Y^{18}Y^{19} motif in its cytoplasmic tail (the numbering starting at the cytosolic tail), the diaromatic motif overlapping with a functional internalisation signal (Zvaritch E, 1996). The Rhesus blood group associated glycoprotein carries a Y^{19}W^{20} motif in its 26 amino acid cytoplasmic tail in addition to a YWKV internalisation signal. The ionotropic glutamate receptor has a F^{19}Y^{20} motif in its 63 residue tail and has been demonstrated to undergo dynamin dependent endocytosis at synaptic membranes by clathrin coated pit formation. It has also been colocalised with AP-2 at synaptic membranes and interacts directly with NSF in the central nervous
system. The human T cell glycoprotein CD8b has a F₁₇Y₁₈ motif in its cytoplasmic tail of 19 amino acids besides an FXXXFY type internalisation sequence. The insulin receptor related receptor contains a 60 amino acid cytoplasmic tail harbouring a Y₁₆F₁₇ motif and an internalisation signal of the NPXYF type. The trafficking itineraries of these proteins are poorly characterised and the question of the role of the diaromatic motif in their endosomal sorting remains open.

**Why is there such a strict requirement for a diaromatic motif in the CD-MPR?**

Signal sequences see the light of day, so to say, when they interact with adaptor proteins. Much has already been said in this thesis on the nature, specificity and affinity of different adaptors for various signal sequences in cargo. The CD-MPR interacts with a multitude of adaptors along the secretory and biosynthetic pathways. At the level of late endosomes, the retrieval of the receptor to the TGN to prevent its fatal misrouting to lysosomes by default mechanisms has been shown to be mediated by a tripartite complex involving the receptor cytosolic tail, TIP47 and the Rab9-GTPase. TIP47 functions as the cargo selection device and Rab9, in its GTP bound form, positively regulates the interaction thus implicating TIP47 as its effector. TIP47 has been shown to directly bind the diaromatic motif in the CD-MPR and our studies of this interaction revealed that the diaromatic nature of the motif is an essential determinant of optimal interaction. Interestingly, the *in vitro* interaction pattern of CD-MPR wild-type and mutant constructs correlated with the *in vivo* sorting efficiency of the same constructs. Thus, it appears that the signal mediates interaction with the adaptor to ensure proper endosomal sorting. Small wonder! However, the diaromatic motif is one among several sorting signals in the 67 residue tail of the receptor. The mutation of reversibly palmitoylated cysteine-34 in the cytoplasmic tail shows a similar mislocalisation to lysosomes. This fact does not come as a surprise, given that the palmitoylation of the receptor might function as a regulatory switch which see-saws the receptor into favourable and restrictive conformations for interaction of the various sorting signals with their respective adaptors. Thus the correlation in the phenotypes observed with the mutation of the cysteine residue and the diaromatic motif. Furthermore, the palmitoyltransferase of the CD-MPR has recently been shown to cycle between the surface and endosomes further reiterating the role of palmitoylation as a conformation-
altering post-translational modification. If the diaromatic motif is so crucial to endosomal sorting, how does the related CI-MPR manage without it? Obviously, other sorting mechanisms exist. TIP47 is still the adaptor involved, however, in this case, it seems to interact with a proline rich region defined by a PPAPRPG loop in the cytoplasmic tail. Binding sites in TIP47 for the two receptors could be independent and/or overlapping thus explaining the discrepancy. An in-depth analysis of the binding sites on TIP47 for the two receptors would help uncover the high degree of specificity in this adaptor-cargo interaction.

The second trafficking step studied in this thesis is the exit of human UCE from the TGN. UCE has been shown to reside in the TGN and cycles constitutively between the TGN and the plasma membrane at steady state. The nature of the intermediate compartments or transport steps involved has not been analysed in detail. It has previously been shown that UCE is rapidly internalised from the surface with a $t_{1/2}$ of internalisation of 0.65 minutes. It has also been shown that UCE is internalised by means of clathrin mediated endocytosis though the interaction of AP-2 with its tyrosine-based internalisation motif Y^{488}HPL in the cytoplasmic tail. Mutation of the critical tyrosine-488 residue leads to accumulation of UCE at the surface. Given that UCE cycles through a yet-to-be-identified endosomal compartment, the steady state levels of the enzyme depend on the rate of several individual transport steps including internalisation from the surface, endosomal recycling to the TGN, surface delivery and exit from the TGN. The transmembrane domain and the first 11 amino acids of the 41 residue cytoplasmic tail of UCE have been shown to be sufficient for TGN retention. In this analysis, we identified the residues that determine TGN exit of UCE. The design of constructs to scan the cytoplasmic tail for an exit signal was based on the hypothesis that these residues must lie distal to the first 11 amino acids. The most prominent signal in this region is the internalisation motif Y^{488}HPL, followed by amino acids Q^{492}EMNGEPLAA until residue 502. Upon truncation of the tail at residue 502, we observed that there was no change in the steady state localisation of a GFP-UCE fusion construct in which the luminal domain of UCE was replaced by monomeric GFP. This construct, like the wild-type construct, was localised mostly to the TGN. In order to identify the TGN exit signal, we did pairwise alanine substitutions of residues QE, MN,
GE and PL all in the background of the 502 stop mutation. The stretch distal to residue 502 in the cytoplasmic tail of UCE contains the NPFXD type internalisation motif. The rationale used to scan the tail for the exit signal required that only one internalisation motif be present in the construct and therefore the 502 stop mutation was included in all the constructs. In addition, the tyrosine-488 residue was mutated to alanine in all the constructs analysed. Thus, the expected localisation of all of these constructs would be at the surface owing to an internalisation defect. However, if an additional mutation affecting TGN exit was introduced these constructs would be detected at the TGN rather than at the surface notwithstanding the tyrosine-488 mutation. This would lead to the identification of potential residues involved in TGN exit.

The localisation of the constructs was analysed using a high dimensional neural network capable of identifying differences not apparent to a visual examiner in high resolution confocal immunofluorescence images of stably transfected HeLa cell-lines. The validity of the neural network has been previously documented owing to its ability to distinguish the localisation patterns of giantin and GPP130, two Golgi marker proteins which are very difficult to resolve by visual observation. The images were compared for morphological, geometrical and texture-based features, among others and subsequently classified into the same or distinct groups. At least 2, if not 3 different clones of each construct were analysed and atleast 100 individual cells of each clone were studied before classifying the constructs. This accounted for differences arising due to clonality and statistical variability. The image recognition program could clearly distinguish between the GFP-UCE wild-type fusion construct and the GFP-UCE Y488A mutant, the former having a TGN localisation at steady state (and completely lacking any surface staining) and the latter, a predominant surface localisation. Furthermore, it could also recognise the GFP-UCE 502 Stop construct as being similar in localisation to the wild-type construct confirming what was already obvious in visual examination. This provided a window of localisation patterns. Constructs which did not include a mutation of the TGN exit signal would have a surface localisation and thus group with the GFP-UCE Y488A construct while constructs harbouring mutations in the TGN exit signal would cluster with the wild-type and GFP-UCE 502 Stop constructs. Based on this rationale, the constructs GFP-UCE Y488; Q492E-A/502 Stop and GFP-UCE Y488; M494N-
A/502 Stop were found to have defects in TGN exit in contrast to the constructs GFP-UC-E Y^{488}; G^{496}E-A/502 Stop and GFP-UC-E Y^{488}; P^{498}L-A/502 Stop which had no significant defects in TGN exit but were stuck at the surface owing to the internalisation defect. This implicated the residues Q^{492}EMN in the TGN exit of UCE. In order to further analyse the signal and to determine the contribution of each individual amino acid to the TGN exit step of the trafficking itinerary of UCE, we made single mutants of the residues Q^{492}EMN, all in the background of the Y488A and 502 Stop mutations. The confocal images of cells stably transfected with these constructs were subjected to similar analysis by the neural network and the localisation of each construct was determined. The image recognition program identified residues Q^{492} to be the most crucial to TGN exit while residues N^{495} and M^{494} were also found to contribute. As expected, the combined mutant, 492QEMN-A; 502 Stop resembled the wild-type although bearing a Golgi and endosomal localization pattern.

Several questions on the trafficking of UCE are yet to be addressed. The observation that a tyrosine-486 residue mutated to alanine leads to most of a GFP-UC-E fusion construct to be trapped in endosomal structures suggests a role for tyrosine-486 in endosomal sorting of UCE. Very little is known about the recycling of UCE through endosomes. Even less is known about potential interacting proteins that might participate in the vesicular trafficking of UCE. A detailed analysis of the cytoplasmic tail of UCE reveals that besides potential internalisation signals, it does not possess any of the canonical motifs for interaction with known adaptor proteins, such as dileucine motifs or acidic clusters. We tested the interaction of wild-type UCE cytoplasmic tail with the adaptor protein complex AP-1 in a yeast three hybrid system but could not find any stable interaction. This was done in collaborative effort with Juan Bonifacino at the NIH. This could simply reflect the recalcitrancy for interaction in this particular in vitro system and does not rule out AP-1 as a potential interaction partner.
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