L-Type Lectins in ER-Golgi Intermediate Compartment

G.S. Gupta

7.1 L-Type Lectins

7.1.1 Lectins from Leguminous Plants

L-type lectins possess a luminal carbohydrate recognition domain (CRD) that binds to high-mannose-type oligosaccharides in a Ca\(^{2+}\)-dependent manner. The L-type CRD is named after the lectins found in abundance in the seeds of leguminous plants, such as concanavalin A from jack beans. The history of L-type lectins is as old as discovery of plant lectins from seeds of leguminous plants in nineteenth century. The structural motifs of L-type lectins are now known to be present in a variety of glycan-binding proteins from other eukaryotic organisms. The domain is present in plant, fungal, and animal proteins, but plant and animal L-type lectins have divergent sequences and different molecular properties. While plant lectins are secreted-soluble proteins and found at high level in specialised tissues, animal L-type lectins are (often membrane-bound) luminal proteins that are found at low levels in many different cell types. This observation suggests that animal L-type lectins have different functions. The crystal structures of some of the legume seed lectins show structural similarities among these lectins and to some other lectins, including the galectins and a variety of other lectins. Therefore, the term “L-lectins” has been designated as a classification for all lectins with this legume seed lectin-like structure. The L-type lectin-like domain has an overall globular shape composed of a β-sandwich of two major twisted antiparallel β-sheets. The β-sandwich comprises a major concave β-sheet and a minor convex β-sheet, in a variation of the jelly roll fold (Velloso et al. 2002, 2003; Satoh et al. 2006, 2007).

7.1.2 L-Type Lectins in Animals and Other Species

L-type lectins in animal cells are involved in protein sorting in luminal compartments of animal cells. In humans and other mammals there are four L-type lectins: ERGIC-53, ERGIC-53 like (ERGL), vesicular integral membrane protein-36 (VIP36), and VIP36 like (VIPL). ERGIC-53 is found only in mammals and VIP36 is restricted to vertebrates, but ERGIC-53 and VIPL are also found in invertebrates. A protein similar to ERGIC-53 is present in the slime mold Dictyostelium discoideum, a very simple eukaryote. L-type lectins have different intracellular distributions and dynamics in the ER-Golgi system of secretory pathway and interact with N-glycans of glycoproteins in a Ca\(^{2+}\)-dependent manner, suggesting a role in glycoprotein sorting and trafficking. The VIP36 is an intracellular animal lectin that acts as a putative cargo receptor, which recycles between the Golgi and the ER (Hauri et al. 2002).

Proteins more distantly related to ERGIC-53 and VIP36 are present in yeast and other fungi and in protozoa. Emp46p and Emp47p are L-type lectins from S. cerevisiae which cycle between the ER and the Golgi to facilitate the exit of N-linked glycoproteins from the ER. Unlike ERGIC-53, binding of high mannose glycans does not require a Ca\(^{2+}\) ion. Emp46p binds a K\(^{+}\) ion, which is essential for glycoprotein transport, at a different location to that of Ca\(^{2+}\) ion in ERGIC-53, and Emp47p does not bind any metal ions. The differences in metal binding are evident in the primary structure of the proteins. Members of the galectin family of lectins may be considered as members of the L-type lectin family and are the subject of a separate chapter. The pentraxins are a superfamily of plasma proteins that are involved in innate immunity in invertebrates and vertebrates. They contain L-type lectin folds and require Ca\(^{2+}\) ions for ligand binding (Chap. 8). Other carbohydrate-binding proteins that may fit into this category are discussed in this chapter.

VP4 is a monomeric sialic acid–binding domain with an L-type lectin fold. This domain is required for infectivity of most animal rotaviruses. Sialidases or neuraminidases are a superfamily of N-acylneuraminate-releasing (sialic-acid-releasing) exoglycosidases found mainly in higher eukaryotes.
and in some, mostly pathogenic, viruses, bacteria and protozoans and contain L-type lectin domain. Several bacterial toxins such as exotoxin A (ETA) from *Pseudomonas aeruginosa* and Leech intramolecular trans-Sialidase and *Vibrio cholerae* Sialidase are known to possess CRD as observed in many lectins. Calnexin (Cnx) and calreticulin (Crt) are homologous chaperones that mediate quality control of proteins in the ER (see Chap. 2). Both Crt and Cnx are Ca\(^{2+}\)-binding proteins, and their carbohydrate-binding activity is sensitive to changes in Ca\(^{2+}\) concentration. Cnx is a type I membrane protein with its carboxy-terminal end in the cytoplasm. The luminal portion of the protein is divided into three domains: a Ca\(^{2+}\)-binding domain (which is adjacent to the transmembrane domain), a proline-rich long hairpin loop called the P domain, and the amino-terminal L-type lectin domain. The Crt has a similar structure, but it is missing the cytoplasmic and transmembrane regions; it is retained in the ER through its KDEL-retrieval signal at the carboxyl terminus (Fig. 7.1).

### 7.2 ER-Golgi Intermediate Compartment

Protein traffic moving from ER to Golgi complex in mammalian cells passes through the tubulovesicular membrane clusters of the ER-Golgi intermediate compartment (ERGIC), the marker of which is the lectin ERGIC-53. Because the functional borders of the intermediate compartment (IC/ERGIC) are not well defined, the spatial map of the transport machineries operating between the ER and the Golgi apparatus remains incomplete. However, studies showed that the ERGIC consists of interconnected vacuolar and tubular parts with specific roles in pre-Golgi trafficking. The identification of ERGIC-53 has added to the complexity of the exocytic pathway of higher eukaryotic cells. A subcellular fractionation procedure for the isolation of the ERGIC from Vero cells provided a means to study more precisely the compartmentalization of the various enzymic functions along the early secretory pathway. The results suggested that in the secretory pathway of Vero cells O-glycan initiation and sphingomyelin synthesis mainly occur beyond the ERGIC in the Golgi apparatus (Schweizer et al. 1994). The dynamic nature and functional role of the ERGIC have been debated for quite some time. In the most popular current view, the ERGIC clusters are mobile transport complexes that deliver secretory cargo from ER-exit sites to the Golgi. Recent live-cell imaging data revealing the formation of anterograde carriers from stationary ERGIC-53-positive membranes, however, suggest a stable compartment model in which ER-derived cargo is first shuttled from ER-exit sites to stationary ERGIC clusters in a COPII-dependent step and subsequently to the Golgi in a second vesicular transport step. This model can better accommodate previous morphological and functional data on ER-to-Golgi traffic. Such a stationary ERGIC would be a major site of anterograde and retrograde sorting that is controlled by coat proteins, Rab and Arf GTPases, as well as tethering complexes, SNAREs and cytoskeletal networks. The ERGIC also contributes to the concentration, folding, and quality control of newly synthesized proteins (Appenzeller-Herzog and Hauri 2006). Marie et al. (2009) provided novel insight into the compartmental organization of the secretory pathway and Golgi biogenesis, in addition to a direct functional connection between the intermediate compartment and the endosomal system, which evidently contributes to unconventional transport of the cystic fibrosis transmembrane conductance regulator to the cell surface. The ERGIC defined by ERGIC-53 also participates in the maturation of (or is target for) several viruses such as corona virus, cytomegalovirus, flavivirus, poliovirus, Uukuniemi virus, and vaccinia virus. Further analysis of the function of ERGIC-53, and the use of ERGIC-53 as a marker protein, should provide novel results about the mechanisms controlling traffic in the secretory pathway.

### 7.3 Lectins of Secretory Pathway

The most prominent cycling lectin is mannose-binding type I membrane protein ERGIC-53 (ERGIC protein of 53 kDa), a marker for ERGIC, which functions as a cargo receptor to facilitate export of an increasing number of glycoproteins with different characteristics from the ER. The ERGIC-53 is a homo-hexameric transmembrane lectin localized to the ERGIC that exhibits mannose-selective properties in vitro. Two ERGIC-53-related proteins, VIP36 (vesicular integral
membrane protein 36) and a ERGIC-53-like protein (ERGL) are also found in secretory pathway. The homologous lectin VIP36 may operate in quality control of glycosylation in the Golgi. In addition to well-understood role of mannose 6-phosphate receptors in lysosomal protein sorting, the VIP36 functions as a sorting receptor by recognizing high-mannose glycans. Some glycoproteins escape cis-Golgi trimming but may be recognized by VIP36 in the trans-Golgi and recycled to the cis-Golgi for another trimming attempt. Proteins carrying Man-6-P residues are recognized by MPRs in the trans-Golgi and sorted to endosomes via clathrin-coated vesicles. Secreted Man-6-P-bearing glycoproteins can also be internalized from the plasma membrane by the large MPR. N-Glycans also serve as signals for Golgi exit and apical targeting in epithelial cells. These processes may also be mediated by lectins that are, however, unknown. ECV endosomal carrier vesicles, EE early endosome, LE late endosome, TC translocation channel (Reprinted with permission from Hauri et al. 2000a © Elsevier).

7.4 ER-Golgi Intermediate Compartment Marker-53 (ERGIC-53) or LMAN1

7.4.1 ERGIC-53 Is Mannose-Selective Human Homologue of Leguminous Lectins

Secretyory proteins are cotranslationally translocated into lumen of ER, where they interact with ER-resident chaperones such as calnexin, and/or calreticulin. Only secretory proteins
that fold correctly are transported through the Golgi apparatus to their final destinations. Several proteins are known to be transported by specific receptors. Such receptors may include membrane proteins ERGIC-53, VIP36, the p24 family, and Erv29p, which cycle between ER and the Golgi apparatus (Nichols et al. 1998; Appenzeller et al. 1999; Muniz et al. 2000; Belden and Barlowe 2001). Protein ERGIC-53, in humans, is encoded by the LMAN1 gene. The protein encoded by this gene is a type I integral membrane protein localized in the intermediate region between the endoplasmic reticulum and the Golgi, presumably recycling between the two compartments. Also named LMAN1, the protein is a mannose-specific lectin and is a member of L-type lectin family.

ERGIC-53 bears homology to leguminous lectins and binds to mannose (Itin et al. 1996). It is, therefore, proposed to recognize high mannose-type oligosaccharides attached to proteins and to transport these glycoproteins from the ER to Golgi apparatus. The ERGIC-53, a member of a putative new class of animal lectins, is associated with the secretory pathway. It is type I transmembrane lectin, which facilitates the efficient export of a subset of secretory glycoproteins from ER. Indeed, the lack of ERGIC-53 impairs the secretion of procathepsin C and blood coagulation factors V (FV) and VIII (FVIII) glycoproteins (Nichols et al. 1998; Vollenweider et al. 1998). Chemical cross-linking studies revealed that ERGIC-53 interacts with procathepsin Z in a mannose- and calcium-dependent manner (Appenzeller et al. 1999; Appenzeller-Herzog et al. 2005). However, ERGIC-53 and its mutant, which is unable to bind to mannose, both coimmunoprecipitate with FVIII, and treatment with tunicamycin does not reduce the interaction between ERGIC-53 and FVIII (Cunningham et al. 2003), which indicates that protein–protein interactions also contribute to this interaction. It is therefore possible that ERGIC-53 also acts as a molecular chaperone in addition to transporting glycoproteins.

Although ERGIC-53 has selectivity for mannose yet it has a low affinity for glucose and GlcNAc, but not for galactose. Since leguminous family of lectin proteins possesses a highly conserved invariant asparagine essential for carbohydrate binding, the corresponding mutation in ERGIC-53 as well as a mutation affecting a second site in the putative CRD abolished mannose-column binding and co-staining with mannosylated neoglycoprotein. Based on its monosaccharide specificity, domain organization, and recycling properties, it was proposed that ERGIC-53 functions as a sorting receptor for glyco–proteins in the early secretory pathway (Itin et al. 1996). ERGIC-53 is present as reduction-sensitive homo-oligomers, i.e. as a balanced mixture of disulfide-linked hexamers and dimers, with the two cysteine residues located close to the transmembrane domain playing a crucial role in oligomerization. It is present exclusively as a hexameric complex in cells. Beyond its interest as a transport receptor, ERGIC-53 is an attractive probe for studying numerous aspects of protein trafficking in the secretory pathway, including traffic routes, mechanisms of anterograde and retrograde traffic, retention of proteins in the ER, and the function of the ERGIC.

### 7.4.2 Cells of Monocytic Lineage Express MR60: A Homologue of ERGIC-53

Most mammalian macrophages express mannose receptor (membrane lectin of 175 kDa) allowing endocytosis of their ligands, but cells of monocytic lineage (HL60, U937, monocyte) lack this receptor. However, after permeabilization, promyelocytic, promonocytic cells and monocytes bind D-mannose-terminated neoglycoproteins. The intracellular mannose binding protein from the human promyelocytic cell line HL60 is a 60 kDa protein (MR60). Under similar conditions, mouse macrophages express a 175 kDa mannose receptor but not the 60 kDa receptor (Pimpanee et al. 1991). Under non-reducing conditions, MR60 migrates as a 120 kDa protein. MR60 does not contain any N-glycan moiety that could be cleaved by N-glycanase. MR60 induces a sugar selective aggregation of beads coated with α-D-mannosyl residues while beads bearing α-D-glucosyl residues are not.

Independently, a promyelocytic protein (MR60) was purified by mannose-column chromatography, and a cDNA was isolated that matched MR60 peptide sequences. This cDNA was identical to that of ERGIC-53 and homologies with the animal lectin family of the galectins were noticed. Not all peptide sequences of MR60, however, were found in ERGIC-53, raising the possibility that another protein associated with ERGIC-53 might possess lectin activity. This lectin is a type I transmembrane protein which includes a luminal N-terminal domain, a transmembrane domain, and a short C-terminal cytosolic domain (Fig. 7.3). The sequence of MR60 is identical (except for one amino acid) to that of ERGIC-53 (Arar et al. 1995; Schindler et al. 1993). The sequence of MR60/ERGIC-53 from human cells and that of the rat homologue p58 revealed 89% identity at the amino acid level (Lahtinen et al. 1996). Homologous proteins of MR60/ERGIC-53 have been characterized in Caenorhabditis elegans and Xenopus. MR60 is not present at the cell surface and is structurally and immunochemically distinct from 175 kDa mannose receptor of mature macrophages. MR60/ERGIC-53 shares significant homologies with VIP-36 and with leguminous plant lectins (Fiedler and Simons 1994).

The recombinant protein binds mannosides and is oligomeric, up to the hexameric form. Two truncated proteins...
showed that the luminal moiety of MR60/ERGIC-53 contains a device, which allows both its oligomeric pattern and its sugar binding capability (Carriere et al. 1999a, b). ERGIC-53 is present as reduction-sensitive homo-oligomers, i.e. as a balanced mixture of disulfide-linked hexamers and dimers, with two cysteine residues located close to transmembrane domain playing a crucial role in oligomerization. It is present exclusively as a hexameric complex in cells. However, the hexamers exist in two forms, one as a disulfide-linked, Triton X-100, perfluoro-octanic acid, and SDS-resistant complex, and the other as a non-covalent, Triton X-100, perfluoro-octanoic acid-resistant, but SDS-sensitive, complex made up of three disulfide-linked dimers which are likely to interact through the coiled-coil domains present in the luminal part of the protein (Neve et al. 2005).

The cDNA sequence of the monkey homologue of ERGIC-53 revealed a sequence of 2,422 nt with 96.2% identity to the human ERGIC-53 cDNA and 87% and 67% identity to the rat and amphibian cDNA, respectively. The translated CV1 ERGIC-53 protein is 96.47% identical to the human ERGIC-53, 87% identical to rat p58 and 66.98% to *Xenopus laevis* protein. ERGIC-53 is expressed as a major transcript of about 5.5 kb in either monkey CV1 or in human CaCo7. A shorter transcript of 7.3 kb was detected in both cell lines and in mRNAs derived from human pancreas and placenta (Sarnataro et al. 1999).

### 7.4.3 Rat Homologue of ERGIC53/MR60

**7.4.3.1 The 58-kDa Protein in Rat**

The 58-kDa (p58), the type I homodimeric and hexameric microsomal membrane protein, has been characterized and localized to tubulo-vesicular elements at the ER-Golgi interface and the cis-Golgi cisternae in pancreatic acinar cells in rat (Lahtinen et al. 1992). The rat cDNA encodes a 517-amino acid protein having a putative signal sequence, a transmembrane domain close to the C terminus and a short cytoplasmic tail. The C-terminal tail contains a double-lysine motif (KKFF), known to mediate retrieval of proteins from the Golgi back to the endoplasmic reticulum. The rat p58 sequence is 89% identical with those of ERGIC-53 and MR60 and a strong homology with the frog sequence, indicating a high evolutionary conservation. Over-expression of c-Myc-tagged p58 resulted in accumulation of the protein both in the ER and in an apparently enlarged Golgi complex, as well as its leakage to the plasma membrane. The C-terminal tail of p58 located in the ER and transport intermediates is hidden, but becomes exposed when the protein reaches the Golgi complex (Lahtinen et al. 1996).

Shortly after synthesis, p58 forms dimers and hexamers, after which they are in equilibrium. The mature p58 contains four cysteine residues in the luminal domain which are capable of forming disulfide bonds. The membrane-proximal half of the luminal domain consists of four predicted alpha-helical domains, one heavily charged and three amphipathic in nature, all candidates for electrostatic or coiled-coil interactions. Using single-stranded mutagenesis, the cysteine residues were individually changed to alanine and the contribution of each of the alpha-helical domains was probed by internal deletions. The N-terminal cysteine to alanine mutants C198A and C238A and the double mutant, C198/238A oligomerized like the wild-type protein. The two membrane-proximal cysteines were found to be necessary for the oligomerization of p58. Mutants lacking one of the membrane proximal cysteines, either C473A or C482A, were unable to form hexamers, while dimers were formed normally. The C473/482A double
mutant formed only monomers. Deletion of any of the individual alpha-helical domains had no effect on oligomerization. The dimeric and hexameric forms bound equally well to D-mannose. The dimeric and monomeric mutants displayed a cellular distribution similar to the wild-type protein, indicating that the oligomerization status played a minimal role in maintaining the sub-cellular distribution of p58 (Lahtinen et al. 1999).

### 7.4.3.2 Crystal Structure

The structures of many of L-type lectins have been thoroughly characterized, and many are employed in a wide range of biomedical and analytical procedures. All soluble L-type lectins found to date are multimeric proteins, although all do not have the same quaternary structure. Thus, these lectins are multivalent with more than one glycan-binding site per lectin molecule. The same multivalent principle applies to the membrane bound L-type lectins glycan-binding site per lectin molecule. The same multivalent situation. Thus, these lectins are multivalent with more than one

L-type lectins found to date are multimeric proteins, although all do not have the same quaternary structure. Thus, these lectins are multivalent with more than one glycan-binding site per lectin molecule. The same multivalent principle applies to the membrane bound L-type lectins because the presence of two or more molecules on a membrane surface essentially presents a multivalent situation. The L-type CRD is a \( \beta \)-sandwich structure with a concave sheet of seven \( \beta \)-strands and a convex sheet of five. The ligand binding site is in a negatively-charged cleft of conserved residues. Most L-type CRDs require metal ions for ligand binding: in concanavalin A, a transition metal is bound at one site and \( \text{Ca}^{2+} \) at the second site. Despite divergent sequences and function, it seems likely that the L-type CRDs have retained similar mechanisms of sugar binding. Certain key residues in four loop regions that contribute to the binding sites in the plant proteins are conserved in all of the animal and plant L-type CRDs. The L-type lectins are distinguished from other lectins primarily on the basis of tertiary structure. In general, either the entire lectin monomer or the CRDs of the more complex lectins are composed of antiparallel \( \beta \)-sheets connected by short loops and \( \beta \)-bends, and they are usually devoid of any \( \alpha \)-helical structure. These sheets form a dome-like structure related to the 'jelly-roll fold,' and it is often called a 'lectin fold'. The carbohydrate-binding site is generally localized toward the apex of this dome (Srinivas et al. 2001).

Velloso et al. (2002) determined the crystal structure of CRD of p58, the rat homologue of human ERGIC-53, to 1.46 Å resolution. The CRD of rat p58 was over-expressed in insect cells and \( E. \ coli \), purified and crystallized using \( \text{Li}_2\text{SO}_4 \) as a precipitant. The crystals belong to space group I222, with unit-cell parameters \( a = 49.6, b = 86.1, c = 128.1 \) Å, and contain one molecule per asymmetric unit. The fold and ligand binding site are most similar to those of leguminous lectins. The structure also resembles that of the CRD of the ER folding chaperone calnexin and the neurexins, a family of non-lectin proteins expressed on neurons. The CRD comprises one concave and one convex \( \beta \)-sheet packed into a \( \beta \)-sandwich. The ligand binding site resides in a negatively charged cleft formed by conserved residues. A large surface patch of conserved residues with a putative role in protein-protein interactions and oligomerization lies on the opposite side of the ligand binding site (Fig. 7.4a). Knowledge of the structure of p58/ERGIC-53 provides a starting model for understanding receptor-mediated glycoprotein sorting between the ER and the Golgi (Velloso et al. 2002).

### 7.4.4 Structure-Function Relations

#### 7.4.4.1 The Recycling of ERGIC-53 in the Early Secretory Pathway

COPII proteins are necessary to generate secretory vesicles at ER. Investigation on the targeting of ERGIC-53 by site-directed mutagenesis revealed that its luminal and transmembrane domains together confer ER retention (Schokman and Orci 1996). In addition the cytoplasmic domain is required for exit from the ER indicating that ERGIC-53 contains determinants for ER retention as well as anterograde transport which, in conjunction with a dilsine ER retrieval signal, control the continuous recycling of ERGIC-53 in the early secretory pathway. In vitro binding studies revealed a specific phenylalanine-dependent interaction between an ERGIC-53 cytosolic tail peptide and the COPII coat component Sec23p. Results suggest that the ER-export of ERGIC-53 is mediated by direct interaction of its cytosolic tail with the Sec23pSec24p complex of COPII and that protein sorting at the level of the ER occurs by a mechanism similar to receptor-mediated endocytosis or Golgi to ER retrograde transport (Kappeler et al. 1997).

Nufer et al. (2003a) suggested that the ER export determinant of ERGIC-53, which cycles in the early secretory pathway, requires a phenylalanine motif at the C-terminus, known to mediate COPII interaction, which is assisted by a glutamine in the cytoplasmic domain. Disulfide bond-stabilized oligomerization is also required. Efficient hexamerization depends on the presence of a polar and two aromatic residues in the transmembrane domain (TMD). ER export is also influenced by TMD length, 21 amino acids being most efficient. Results suggest an ER-export mechanism in which transmembrane and luminal determinants mediate oligomerization required for efficient recruitment of ERGIC-53 into budding vesicles via the C-terminal COPII-binding phenylalanine motif (Nufer et al. 2003a).
7.4.4.2 Cytoplasmic ER-Retrieval Signal in ERGIC-53

Although ERGIC-53 is not a resident protein of the rough ER its cDNA sequence carries a double lysine ER retention motif at cytoplasmically exposed COOH terminus. Cell surface ERGIC-53 is efficiently endocytosed by a mechanism that is disturbed when the two critical lysines of the ER retention motif are replaced by serine residues. Results suggested a similarity between pre-Golgi retention by the double lysine motif and lysine based endocytosis (Kappeler et al. 1994). Although ERGIC-53 contains a cytoplasmic ER-retrieval signal, KKFF, over-expressed ERGIC-53 is transported to the cell surface and rapidly endocytosed. The ERGIC-53 endocytosis signal KKFF and like the ER-retrieval signal require a C-terminal position. In fact, the minimal consensus sequence determined by substitutional mutagenesis was also related to the ER-retrieval consensus (K-K-X-X). Evidence shows that internalization of VIP36, a protein that cycles between plasma membrane and Golgi, is mediated by a signal at its C-terminus that matches the internalization consensus sequence. The relatedness of the two signals suggests that coatamer-mediated retrieval of proteins may be mechanistically more related to clathrin-dependent sorting than previously anticipated (Itin et al. 1995a). Further dissection of the cytoplasmic domain revealed a COOH-terminal di-lysine ER-retrieval signal, KKFF, and an RSQQE targeting determinant adjacent to the transmembrane domain. Surprisingly, the two COOH-terminal phenylalanine residues influence the targeting. They reduce the ER-retrieval capacity of the di-lysine signal and modulate the RSQQE determinant (Itin et al. 1995b).

**KKAA Retrieval Signal in Yeast:** Studies on ERGIC-53 KKAA as a signal revealed a mechanism for static retention of mammalian proteins in the ER. This mechanism is conserved in yeast. Making use of a genetic assay, this signal was shown to induce COPI-dependent ER retrieval. ER retention of KKAA-tagged proteins was impaired in yeast mutants affected in COPI subunits. Furthermore, post-ER carbohydrate modifications detected on reporter proteins indicated that KKAA-tagged proteins recycle continuously within early compartments of the secretory pathway. Therefore in yeast, the KKAA signal might only function as a classical dilysine ER retrieval signal (Dogic et al. 2001).

**A Single C-Terminal Valine Is Export Signal:** The ERGIC-53 carries a C-terminal diphenylalanine motif that

---

**Fig. 7.4** (a) Overall structure of Rat homologue (p58) of ERGIC53/MR60. Ribbon diagram of p58 monomer shown perpendicular to the β-sheets. Secondary structure elements are labeled. Positions of the N and C termini are indicated. β-Strands belonging to the concave and convex β-sheets are shown in red and dark blue, respectively. Strands that do not take part in the β-sheets and are variable when compared with leguminous lectin structures are shown in light blue. Loops and helices are shown in gray and green, respectively (PDB ID: 1GV9 (Adapted with permission from Velloso et al. 2003 © Elsevier)). (b) Overall structure of exoplasmic/luminal domain of VIP36. Ribbon model of VIP36 (Man2-bound form) (PDB ID: 2DUR) is shown. The secondary structures are highlighted (β-strands belonging to concave β-sheets, yellow; β-strands belonging to convex β-sheets, blue; β-strands belonging to β-hairpin, cyan; β-strands belonging to short β-sheet formed between the stalk domain and one of the loops of the CRD, magenta; helices, red), and the loops of the CRD and stalk domain are colored gray and green, respectively. The bound Ca$^{2+}$ is shown as a pink sphere. The bound oligomannoses are superimposed from the VIP36 complex structures with Man-x,2-Man and Man-x,2-Man-x,1,3-Man and are shown as a green stick model. The reducing-end mannose residue in the Man2-bound form is omitted because its position is almost the same as that of the Man$_3$GlcNAc-bound form. Positions of Loops 1, 2, and 3, which are bound to the oligomannose, are indicated (Adapted by permission from Satoh et al. 2007 © The American Society for Biochemistry and Molecular Biology)
is required for efficient ER export. Replacement of C-terminal diphenylalanine motif by a single C-terminal valine accelerates transport of inefficiently exported reporter constructs and hence operates as an export signal. The valine signal is position dependent. Results suggest that cytoplasmic C-terminal amino-acid motifs, either alone or in conjunction with other transport determinants, accelerate ER export of numerous type I and probably polytopic membrane proteins by mediating interaction with COPII coat components (Nufer et al. 2002).

### 7.4.4.3 Two Distinct Pathways for Golgi-To-ER Transport

The cytosolic COP-I interacts with cytoplasmic ‘retrieval’ signals present in membrane proteins that cycle between ER and the Golgi complex, and is required for both anterograde and retrograde transport in the secretory pathway. The role of COP-I in Golgi-to-ER transport of distinct marker proteins has been described. For example, anti-COP-I antibodies inhibit retrieval of ERGIC-53 and of the KDEL receptor from the Golgi to the ER. Transport to the ER of protein toxins, which contain a sequence that is recognized by the KDEL receptor, is also inhibited. Results indicated the existence of at least two distinct pathways for Golgi-to-ER transport, one COP-I dependent and the other COP-I independent. The COP-I-independent pathway is specifically regulated by Rab6 and is used by Golgi glycosylation enzymes and Shiga toxin/Shiga-like toxin-1 (Girod et al. 1999).

### 7.4.4.4 Site of Transport Arrest of Mitotic Cells

Using ERGIC-53/p58 and plasma membrane protein CD8, Farmaki et al. (1999) established the site of transport arrest between ER and Golgi stack of mitotic animal cells. Recycled ERGIC-53/p58 and newly synthesized CD8 accumulate in ER cisternae but not in COP-II-coated export structures or more distal sites. During mitosis the tubulovesicular ER-related export sites were depleted of the COP-II component Sec13p, which indicated that COP-II budding structures are the target for mitotic inhibition. Findings established that the site of ER-Golgi transport arrest of mitotic cells is COP-II budding structures (Farmaki et al. 1999).

### 7.4.5 Functions of ERGIC-53

#### 7.4.5.1 ERGIC-53 as a Cargo Receptor

Soluble secretory proteins are transported from the ER to ERGIC in vesicles coated with COP-II coat proteins. The sorting of secretory cargo into these vesicles is thought to involve transmembrane cargo-receptor proteins. According to Appenzeller et al. (1999) a cathepsin-Z-related glycoprotein binds to ERGIC-53. Binding that occurs in the ER, is carbohydrate- and calcium-dependent and is affected by untrimmed glucose residues. Binding does not, however, require oligomerization of ERGIC-53, although oligomerization is required for exit of ERGIC-53 from the ER. Dissociation of ERGIC-53 occurs in the ERGIC and is delayed if ERGIC-53 is mislocalized to the ER. These results indicate that ERGIC-53 may function as a receptor facilitating ER-to-ERGIC transport of soluble glycoprotein cargo (Appenzeller et al. 1999).

#### 7.4.5.2 ERGIC-53 in Traffic and in the Secretory Pathway

Functional deficiency in ERGIC-53 leads to a selective defect in secretion of glycoproteins in cultured cells and to hemophilia in humans. Beyond its interest as a transport receptor, ERGIC-53 is an attractive probe for studying numerous aspects of protein trafficking in the secretory pathway (Haurie et al. 2000b). Studies suggest that ERGIC is a dynamic membrane system composed of a constant average number of clusters and that the major recycling pathway of ERGIC-53 bypasses the Golgi apparatus (Klumperman et al. 1998). To investigate if ERGIC-53 is involved in glycoprotein secretion, a mutant form of this protein was generated that is incapable of leaving the ER. If expressed in HeLa cells in a tetracycline-inducible manner, this mutant accumulated in the ER and retained the endogenous ERGIC-53 in this compartment, thus preventing its recycling. It was suggested that recycling of ERGIC-53 is required for efficient intracellular transport of a small subset of glycoproteins, but it does not appear to be essential for the majority of glycoproteins (Vollenweider et al. 1998).

**pH-Induced Conversion of ERGIC-53 Triggers Glycoprotein Release:** Binding of cargo to ERGIC-53 in the ER requires Ca\(^{2+}\). Cargo release occurs in the ERGIC, but the signals required for the cargo-receptor interaction are largely unknown. Though the efficient binding of ERGIC-53 to immobilized mannose occurs at pH 7.4, the pH of the ER, binding fails at slightly lower pH. pH sensitivity of the lectin was more prominent when Ca\(^{2+}\) concentrations were low. A conserved histidine in the center of the CRD was required for lectin activity suggesting it may serve as a molecular pH/Ca\(^{2+}\) sensor. Acidification of cells inhibited the association of ERGIC-53 with the known cargo cathepsin Z-related protein (Appenzeller-Herzog et al. 2004).

#### 7.4.5.3 ERGIC-53 as a Marker of ER-Cargo Exit Site

A two-step reconstitution system for the generation of ER cargo exit sites from starting ER-derived low density microsomes (LDMs) has been described. The first step
involves the hydrolysis of $\text{Mg}^{2+}\text{ATP}$ and $\text{Mg}^{2+}\text{GTP}$, leading to the formation of a transitional ER (tER) with the soluble cargo albumin, transferrin, and the ER-to-Golgi recycling membrane enriched proteins $\alpha 2p24$ and $\beta 58$/ERGIC-53. Upon further incubation (step two) with cytosol and mixed nucleotides, interconnecting smooth ER tubules within tER transform into vesicular tubular clusters (VTCs). The cytosolic domain of $\alpha 2p24$ and cytosolic COPI coat affect VTC formation. This was observed from the effect of Abs to the C-terminal tail of $\alpha 2p24$, but not of Abs to the C-terminal tail of calnexin on this reconstituted system. Therefore, the p24 family member, $\alpha 2p24$, and its cytosolic coat ligand, COPI coatplayer a role in the de novo formation of VTCs and the generation of ER cargo exit sites (Lavoie et al. 1999).

Stephens and Pepperkok (2002) examined the ER-to-Golgi transport of procollagen, which, when assembled in the lumen of the ER, is thought to be physically too large to fit in classically described 60–80 nm COPI- and COPII-coated transport vesicles. Using ERGIC-53 as a marker, data indicated the existence of an early COPI-dependent, pre-Golgi cargo sorting step in mammalian cells (Stephens and Pepperkok 2002).

**Carbohydrate- and Conformation-Dependent Cargo Capture for ER-Exit:** The targeting motif in ERGIC-53 is composed of a high-mannose type oligosaccharide intimately associated with a surface-exposed peptide $\beta$-hairpin loop. The motif accounts for ERGIC-53-assisted ER-export of the lysosomal enzyme pro-cathepsin Z. The second oligosaccharide chain of pro-cathepsin Z exhibits no binding activity for ERGIC-53, illustrating the selective lectin properties of ERGIC-53. Evidences suggest that the conformation-based motif is only present in fully folded pro-cathepsin Z and that its recognition by ERGIC-53 reflects a quality control mechanism that acts complementary to the primary folding machinery in the ER. A similar oligosaccharide/$\beta$-hairpin loop structure is present in cathepsin C, another cargo of ERGIC-53, suggesting the general nature of this ER-exit signal. Perhaps, the molecular mechanism underlying reversible lectin/cargo interaction involves the ERGIC as the earliest low pH site of the secretory pathway. Possibly, this is a report on an ER-exit signal in soluble cargo in conjunction with its decoding by a transport receptor (Appenzeller-Herzog et al. 2004, 2005).

**Regeneration of Golgi Stacks at Peripheral ER Exit Sites:** After microtubule depolymerization, Golgi membrane components are found to redistribute to a distinct number of peripheral sites that are not randomly distributed, but correspond to sites of protein exit from the ER. Whereas Golgi enzymes redistributed gradually over hours to these peripheral sites, ERGIC-53 redistributed rapidly (within minutes) to these sites after first moving through the ER. It was proposed that a slow but constitutive flux of Golgi resident proteins through the same ER/Golgi cycling pathways as ERGIC-53 underlies Golgi dispersal upon microtubule depolymerization. Both ERGIC-53 and Golgi proteins would accumulate at peripheral ER exit sites due to failure of membranes at these sites to cluster into the centrosomal region. Regeneration of Golgi stacks at these peripheral sites would re-establish secretory flow from the ER into the Golgi complex and result in Golgi dispersal (Cole et al. 1996).

**Heat Shock Affects Translation and Recycling Pathway of ERGIC-53:** ERGIC-53 accumulates during heat shock response. However, at variance with unfolded protein response, which results in enhanced transcription of ERGIC-53 mRNA, heat shock leads only to enhanced translation of ERGIC-53 mRNA. In addition, the half-life of the protein does not change during heat shock. Therefore, distinct pathways of the cell stress modulate the ERGIC-53 protein level. Heat shock also affects the recycling pathway of ERGIC-53. The protein rapidly redistributes in a more peripheral area of the cell, in a vesicular compartment that has a low sedimentation density in comparison to the compartment that contains majority of ERGIC-53 at 37°C. Moreover, the anterograde transport of two unrelated reporter proteins is not affected. Interestingly, MCFD2, which interacts with ERGIC-53 to form a complex required for the ER-to-Golgi transport of specific proteins, is regulated similar to ERGIC-53 in response to cell stress. These results support the view that ERGIC-53 alone, or in association with MCFD2, plays important role during cellular response to various stress conditions (Spatuzza et al. 2004).

ER stress induces transcription of ERGIC-53. The ERGIC-53 promoter contains a single cis-acting element that mediates induction of gene by thapsigargin and other ER stress-causing agents. This ER stress response element is highly conserved in mammalian ERGIC-53 genes. The ER stress response element contains a 5′-end CCAAT sequence that constitutively binds NFY/CBF and, 9 nt away, a 3′-end region (5′-CCCCGGGCCC-3′) that is equally important for ER stress-mediated induction of the gene. This sequence is the binding site for endogenous YY1 at the 5′-CCCTGGTT-3′ part and for undefined factors at the CCATC 3′-end. ATF6 alpha-YY1, but not XBP1, interacted with the ERGIC-53 regulatory region and activated ERGIC-53 ER stress response element-dependent transcription (Renna et al. 2007).
7.4.5.4 ERGIC-53 (LMAN1) and MCFD2 Form a Cargo Receptor Complex

Interaction of ERGIC-53 and MCFD2 with Factor VIII: The ERGIC is the site of segregation of secretory proteins for anterograde transport, via packaging into COPII-coated transport vesicles (Schekman and Orci 1996). Correctly folded proteins destined for secretion are packaged in the ER into COPII-coated vesicles, which subsequently fuse to form the ERGIC. Multiple coagulation factor deficiency 2 (MCFD2) is a soluble EF-hand-containing protein that is retained in ER through its interaction with ERGIC-53. Exit of soluble secretory proteins from ER can occur by receptor-mediated export as exemplified by blood coagulation factors V and VIII. Their efficient secretion requires ERGIC-53 and its soluble luminal interaction partner MCFD2, which form a cargo receptor complex in early secretory pathway. ERGIC-53 also interacts with two lysosomal glycoproteins cathepsin Z and cathepsin C. In absence of ERGIC-53, MCFD2 was secreted, whereas knocking down MCFD2 had no effect on the localization of ERGIC-53. Endogenous LMAN1 and MCFD2 are present primarily in complex with each other with a 1:1 stoichiometry, although MCFD2 is not required for oligomerization of ERGIC-53. Coimmunoprecipitation of ERGIC-53 and FVIII from transfected HeLa and COS-1 cells and results of the crystal structure of CRD of ERGIC-53 demonstrated an interaction between ERGIC-53 and FVIII in vivo, mediated via high mannose-containing asparagine-linked oligosaccharides, which were densely situated within B domain of FVIII, as well as protein-protein interactions (Cunningham et al. 2003). Perhaps ERGIC-53 and MCFD2 form a cargo receptor complex and that the primary sorting signals residing in the B domain direct the binding of factor VIII to ERGIC-53 1-MCFD2 through calcium-dependent protein-protein interactions. MCFD2 may function to specifically recruit factor V and factor VIII to sites of transport vesicle budding within the ER lumen (Zhang et al. 2003, 2005). These findings suggest that MCFD2-ERGIC-53 complex forms a specific cargo receptor for the ER-to-Golgi transport of selected proteins. However, MCFD2 is dispensable for the binding of cathepsin Z and cathepsin C to ERGIC-53, since ERGIC-53 can bind cargo glycoproteins in an MCFD2-independent fashion and that MCFD2 is a recruitment factor for blood coagulation factors V and VIII (Nyfeler et al. 2006).

7.4.5.5 ERGIC-53 in Quality Control

Activating Transcription Factors 6, a Regulator of Mammalian UPR: Newly synthesized secretory and transmembrane proteins are folded and assembled in ER where an efficient quality control system operates so that only correctly folded molecules are allowed to move along the secretory pathway. The productive folding process in ER has been thought to be supported by the unfolded protein response (UPR). The accumulation of unfolded proteins in the ER triggers a signaling response. In yeast the UPR affects several hundred genes that encode ER chaperones and proteins operating at later stages of secretion. In mammalian cells the UPR appears to be more limited to chaperones of the ER and genes assumed to be important after cell recovery from ER stress that are not important for secretion.

The mRNA of ERGIC-53 and its related protein VIP36 is induced by inducers of ER stress, tunicamycin and thapsigargin. The rate of synthesis of the ERGIC-53 protein is also induced by these agents. The response was due to the UPR since it was also triggered by castanospermine, a specific inducer of UPR, and inhibited by genistein. Thapsigargin-induced upregulation of ERGIC-53 could be fully accounted for by the activating transcription factors 6 (ATF6) pathway of UPR. It has been suggested that in mammalian cells the UPR also affects traffic from and beyond the ER (Nyfeler et al. 2003). However, a dilemma has emerged; activation of ATF6, the key regulator of mammalian UPR, requires intracellular transport from the ER to the Golgi apparatus. This suggests that unfolded proteins might be leaked from the ER together with ATF6 in response to ER stress, exhibiting proteotoxicity in the secretory pathway. It has been found that ATF6 and correctly folded proteins are transported to the Golgi apparatus via the same route and by the same mechanism under conditions of ER stress, whereas unfolded proteins are retained in the ER. Thus, the activation of UPR is compatible with the quality control in the ER and the ER possesses a remarkable ability to select proteins to be transported in mammalian cells in marked contrast to yeast cells, which actively utilize intracellular traffic to deal with unfolded proteins accumulated in the ER (Nadanaka et al. 2004).

ERGIC-53 in the Formation of Russell Bodies: Owing to the impossibility of reaching the Golgi for secretion or the cytosol for degradation, mutant Ig-micro chains that lack the first constant domain (micro-δCH1) accumulate as detergent-insoluble aggregates in dilated ER cisternae, called Russell bodies. The pathological role(s) of similar structures in ER storage diseases remains obscure. In cells containing smooth Russell bodies, ERGIC-53 co-localizes with micro-δCH1 aggregates in a Ca^{2+}-dependent fashion. Studies suggest that interaction with light chains or ERGIC-53 seed micro-δCH1 condensation in different stations of the early secretory pathway (Mattioli et al. 2006).

7.4.6 Mutations in ERGIC-53 LMAN1 Gene and Deficiency of Coagulation Factors V and VIII lead to bleeding disorder

Mutations in the ERGIC-53 gene are associated with a coagulation defect. Using positional cloning, the gene was
identified as the disease gene leading to combined factor V-factor (FV) and VIII (FVIII) deficiency, a rare, autosomal recessive disorder in which both coagulation factors V and VIII are diminished. ERGIC-53 was mapped to a YAC and BAC contig containing the critical region for the combined factors V and VIII deficiency gene. DNA sequence analysis identified two different mutations, accounting for all affected individuals in nine families studied. Findings indicated that ERGIC-53 may function as a molecular chaperone for the transport from ER to Golgi of a specific subset of secreted proteins, including coagulation factors V and VIII (Nichols et al. 1998). The crystal structure of CRD of ERGIC-53 complements the biochemical and functional characterization of the protein, confirming that a lectin domain is essential for this protein in sorting and transfer of glycoproteins from the ER to the Golgi complex. The lectin domains of calnexin and ERGIC-53 are structurally similar, although there is little primary sequence similarity. By contrast, sequence similarity between ERGIC-53 and VIP36, a Golgi-resident protein, leaves little doubt that a similar lectin domain is central to the transport and/or sorting functions of VIP36. The theme emerging from these studies is that carbohydrate recognition and modification are central to mediation of glycoprotein folding and secretion (Schrag et al. 2003).

Studies suggest that mutations in genes LMAN1 and MCFD2 are responsible for FVFVIII action. The binding of ERGIC-53 to sugar is enhanced by its interaction with MCFD2, and defects in this interaction in FV/FVIII patients may be the cause for reduced secretion of factors V and VIII (Kawasaki et al. 2008). Though clinically indistinguishable, MCFD2 mutations generally exhibit lower levels of FV and FVIII than LMAN1 mutations. The LMAN1 is a mannose-specific lectin which cycles between ER and ER- Golgi intermediate compartment. MCFD2 is an EF-hand domain protein that forms a calcium-dependent heteromeric complex with LMAN1 in cells. Missense mutations in the EF-hand domains of MCFD2 abolish the interaction with LMAN1. The LMAN1-MCFD2 complex may serve as a cargo receptor for the ER-to-Golgi transport of FV and FVIII, and perhaps a number of other glycoproteins. The B domain of FVIII may be important in mediating its interaction with the LMAN1-MCFD2 complex (Zhang 2009). Loss of functional ERGIC-53 leads to a selective defect in secretion of glycoproteins in cultured cells. Studies on the effect of defective ER to Golgi cycling by ERGIC-53 on the secretion of factors V and VIII showed that efficient trafficking of factors V and VIII requires a functional ERGIC-53 cycling pathway and that this trafficking is dependent on post-translational modification of a specific cluster of asparagine (N)-linked oligosaccharides to a fully glucose-trimmed, mannose9 structure (Moussalli et al. 1999).
another family showed a nonsense mutation, i.e., G to T substitution, in exon 2 of the LMAN1 gene, the latter being a novel mutation not previously reported. The third family did not show mutations in either of the two genes, suggesting that a significant subset of FV/FVIII cases may be due to additional genes resulting in a similar phenotype (Mohanty et al. 2005). Immunoprecipitation and Western blot analysis detected a low level of LMAN1-MCFD2 complex in lymphoblasts derived from patients with missense mutations in LMAN1 (C475R) or MCFD2 (I136T), suggesting that complete loss of the complex may not be required for clinically significant reduction in FV and FVIII (Zhang et al. 2006).

7.4.6.2 LMAN1 Expression in MSI-H Tumorigenesis
Roeckel et al. (2009) analyzed mutation frequencies of genes of glycosylation machinery in microsatellite unstable (MSI-H) tumors, focusing on frameshift mutations in coding MNRs (cMNRs). Among 28 candidate genes, LMAN1/ERGIC53 showed high mutation frequency in MSI-H colorectal cancer cell lines (52%; 12 of 23), carcinomas (45%; 72 of 161), and adenomas (40%; 8 of 20). Analysis of LMAN1-mutated carcinomas and adenomas revealed regional loss of LMAN1 expression due to biallelic LMAN1 frameshift mutations. In LMAN1-deficient colorectal cancer cell lines, secretion of high mannose-type glycan(s) (Fiedler and Simons 1994, 1996; Hara-Kuge et al. 2002). Punctate cytoplasmic observations suggest a new role for VIP36 in the quality control of secretory vesicles within the trans-Golgi network by binding specific N-glycans. Results indicated that VIP36 was involved in the transport and sorting of glycoproteins carrying high mannose-type glycan(s) (Fiedler and Simons 1994, 1995, 1996; Hara-Kuge et al. 2002). Punctate cytoplasmic structures co-localize with coatamer and ERGIC-53, labeling ER-Golgi intermediate membrane structures. Cycling of VIP36 is suggested by colocalisation with anterograde cargo trapped in pre-Golgi structures and modification of its carbohydrate structure. Further, after brefeldin A treatment VIP36 is segregated from resident Golgi proteins and co-distributes with ER-Golgi recycling proteins (Fulkekrug et al. 1997, 1999).

7.5 Vesicular Integral Membrane Protein (VIP36) OR LMAN2

7.5.1 The Protein
The vesicular integral membrane lectin (VIP36), also called -Lectin, mannose binding 2 (LMAN2) belongs to a family of lectins, conserved from yeast to mammals, trafficking in secretory pathway and closely related to lectin ERGIC-53 that acts as a cargo receptor, facilitating ER to Golgi transport of certain glycoproteins. VIP36 was originally identified as a component of apical post-Golgi vesicles in virally infected, polarized Madin-Darby canine kidney (MDCK) cells (Fiedler et al. 1994). VIP36 was shown to localize not only to the early secretory pathway but also to the plasma membrane of MDCK and Vero cells. The VIP36 recognizes high-mannose type glycans containing 2 Man residues and amino substituted asparagine. The binding of VIP36 to high-mannose type glycans was independent of at optimum pH 6.0 at 37°C. The association constant between Man7-9GlcNAc2 in porcine thyroglobulin and immobilized VIP36 was 7.1 x 10^8 M^-1. This shows that VIP36 functions as an intracellular lectin recognizing glycoproteins which possess high-mannose type glycans, (Manz1—>2)2—>Man5 GlcNAc2 (Hara-Kuge et al. 1999).

Although VIP36 interacts with glycoproteins carrying high mannose-type oligosaccharides, further analysis using the frontal affinity chromatography (FAC) and the sugar-binding properties of a CRD of VIP36 (VIP36-CRD) have shown that glucosylation and trimming of D1 mannosyl branch (Fig. 7.5) interfere with the binding of VIP36-CRD. VIP36-CRD exhibits an optimal pH value of ~6.5. Examining the specificity and optimal pH of sugar -VIP36 interaction and its subcellular localization, along with organellar pH, it was suggested that VIP36 binds glycoproteins that retain the intact D1 mannosyl branch in cis-Golgi network and recycles to ER where, due to higher pH, it releases its cargos, thereby contributing to quality control of glycoproteins.

In the plasma membrane, VIP36 exhibited an apical-predominant distribution, the apical/basolateral ratio being approximately 7. Localization of over-expressed VIP36 to plasma membrane, endosomes, and Golgi structures, together with evidence for lectin activity lead to the hypothesis that it functions to segregate apical cargo into distinct vesicles within the trans-Golgi network by binding specific N-glycans. Results indicated that VIP36 was involved in the transport and sorting of glycoproteins carrying high mannose-type glycan(s) (Fiedler and Simons 1994, 1995, 1996; Hara-Kuge et al. 2002). Punctate cytoplasmic structures co-localize with coatamer and ERGIC-53, labeling ER-Golgi intermediate membrane structures. Cycling of VIP36 is suggested by colocalisation with anterograde cargo trapped in pre-Golgi structures and modification of its carbohydrate structure. Further, after brefeldin A treatment VIP36 is segregated from resident Golgi proteins and co-distributes with ER-Golgi recycling proteins (Fulkekrug et al. 1997, 1999).

VIP36 shares significant homology with leguminous lectins as well as with ERGIC-53. Its ability to recognize high-mannose type glycans (Hara-Kuge et al. 1999; Kamiya et al. 2005, 2008) and its broad localization from ER to cis-Golgi (Fulkekrug et al. 1999; Shimada et al. 2003a, 2003b) indicates that VIP36 also functions as a cargo receptor that facilitates the transport of various glycoproteins. Proteins that interact with VIP36 during quality control of secretory proteins have been identified. An 80 kDa immunoglobulin-binding protein (BiP), a major protein of Hsp70 chaperone family, binds VIP36. The interaction between VIP36 and BiP is not due to chaperone-substrate complex. The interaction depends on divalent cations but not on ATP. These observations suggest a new role for VIP36 in the quality
control of secretory proteins (Nawa et al. 2007; Kamiya et al. 2008). It was speculated that VIP36 binds to sugar residues of glycosphingolipids and/or glycosylphosphatidylinositol anchors and might provide a link between the extracellular/luminal face of glycolipid rafts and the cytoplasmic protein segregation machinery (Fiedler et al. 1994).

VIP36 is highly expressed in salivary glands, especially the parotid gland, which secretes α-amylase in large quantities. Endogenous VIP36 is localized in trans-Golgi network, on immature granules, and on mature secretory granules in acinar cells and co-localized with amylase. VIP36 is involved in the post-Golgi secretory pathway, suggesting that VIP36 plays a role in trafficking and sorting of secretory and/or membrane proteins during granule formation. EM demonstrated that VIP36 was primarily localized to secretory vesicles in glandula parotis of the rat, where α-amylase also resided. It was suggested that VIP36 is involved in the secretion of α-amylase in the rat parotid gland (Hara-Kuge et al. 2004; Shimada et al. 2003a). In GH3 cells endogenous VIP36 is localized mainly in 70–100-nm-diameter uncoated transport vesicles between the exit site on ER and the neighboring cis-Golgi cisterna. The thyrotropin-releasing hormone (TRH) stimulation and treatment with actin filament-perturbing agents, cytochalasin D or B or latrunculin-B, caused marked aggregation of the VIP36-positive vesicles and the appearance of a VIP36-positive clustering structure located near cis-Golgi cisterna. The size of this structure, which comprised conspicuous clusters of VIP36, depended on the TRH concentration. Furthermore, VIP36 colocalized with filamentous actin in the paranuclear Golgi area and its vicinity. It suggests that actin filaments are involved in glycoprotein transport between the ER and cis-Golgi cisterna by using the lectin VIP36 (Shimada et al. 2003b).

### 7.5.2 VIP36-SP-FP as Cargo Receptor

To investigate the trafficking of transmembrane lectin VIP36 and its relation to cargo-containing transport carriers (TCs), Dahm et al. (2001) analyzed a C-terminal fluorescent-protein (FP) fusion, VIP36-SP-FP. At moderate levels of expression, VIP36-SP-FP is localized to the ER, Golgi apparatus, and intermediate transport structures, and colocalized with epitope-tagged VIP36. VIP36-SP-FP recycles in the early secretory pathway, exhibiting trafficking representative of a class of transmembrane cargo receptors, including the closely related lectin ERGIC-53. The VIP36-SP-FP trafficking structures comprised tubules and globular elements, which translocated in a salutatory manner. Simultaneous visualization of anterograde secretory cargo and VIP36-SP-FP indicated that the globular structures were pre-Golgi carriers, and that VIP36-SP-FP segregated from cargo within the Golgi and was not included in post-Golgi TCs (Dahm et al. 2001).

### 7.5.3 Structure for Recognition of High Mannose Type Glycoproteins by VIP36

It has been shown that ERGIC-53 interacts with glycoproteins carrying high mannose type glycan by endo-β-N acetylgalactosaminidase H treatment (Appenzeller 1999; Moussalli 1999) and binds glycoproteins in a Ca2+- and pH-dependent manner (Appenzeller-Herzog et al. 2004, 2005). VIP36 has high affinity for high mannose type glycans containing Man-α,1,2-Man residues in Man7–9(GlcNAc)2-Asn peptides (Hara-Kuge et al. 1999). Kamiya et al. (2005) reported carbohydrate binding properties of VIP36 by frontal affinity chromatography and suggested Ca2+-dependence of carbohydrate binding and the specificity for D1 arm, Man-α,1,2-Man-α,1,2-Man residues, of high mannose type glycans (corresponding to Man(D1)-Man(C)-Man(4)) (Fig. 7.5).

The exoplasmic/luminal domain of VIP36 as well as the luminal domain of ERGIC-53 and Emp46/47p share homology with L (leguminous)-type lectins and are thus identified as CRDs. It has been shown that ERGIC-53 interacts with glycoproteins carrying high mannose type glycan by endo-β-N-acetylgalactosaminidase H treatment (Appenzeller et al. 1999; Moussalli, et al. 1999; Appenzeller-Herzog et al. 2005) and binds glycoproteins in a Ca2+- and pH-dependent manner (Appenzeller-Herzog et al. 2004). It has been found that VIP36 has high affinity for high mannose type glycans containing Man-α,1,2-Man residues in Man7–9(GlcNAc)2-Asn peptides (Hara-Kuge et al. 1999). Kamiya et al. (2005) reported the carbohydrate binding properties of VIP36 by frontal affinity chromatography. This study suggested Ca2+-dependence of carbohydrate binding and the specificity for D1 arm, Man-α,1,2-Man-α,1,2-Man residues, of high mannose type glycans (corresponding to Man(D1)-Man(C)-Man(4); (Fig. 7.5). In addition, using a VIP36 binds glycoproteins carrying high mannose type glycans (Kawasaki et al. 2007). These observations suggested that VIP36 is involved in the transport of glycoproteins via high mannose type glycans.

Crystal structures of the CRD of rat ERGIC-53 in the absence and presence of Ca2+ have been determined, confirming its structural similarity to the L-type lectins (Velloso et al. 2002, 2003). In these reports, it was shown that the putative ligand-binding site of ERGIC-53 is similar to the mannose-binding site of the L-type lectins. The crystal structures of the CRD of Ca2+-independent K+-bound Emp46p and the metalfree form of Emp47p have also been reported (Satoh et al. 2006). Satoh et al. (2007) determined structures of transport lectin in complex with high mannose type glycans, and determined crystal structures of the exoplasmic/luminal domain of VIP36 alone and in complex
with Ca\(^{2+}\) and mannose, Man-\(\alpha\)-1,2-Man (termed Man2, which corresponds to Man(D1)-Man(C), Man(C)-Man(4), Man(D2)-Man(A), or Man(D3)-Man(B) of Man\(_9\)(GlcNAc)\(_2\); and Man-\(\alpha\)-1,2-Man-\(\alpha\)-1,2-Man-\(\alpha\)-1,2-Man, which corresponds to Man3GlcNAc, which corresponds to Man(C)-Man(4)-Man(3)-GlcNAc(2) (Fig. 7.5).

Satoh et al. (2007) reported the crystal structure of VIP36 exoplasmic/luminal domain comprising a CRD and a stalk domain and in complexed form with Ca\(^{2+}\) and mannosyl ligands. The CRD is composed of a 17-stranded antiparallel \(\beta\)-sandwich and binds one Ca\(^{2+}\) adjoining the carbohydrate-binding site. The structure reveals that a coordinated Ca\(^{2+}\) ion orients the side chains of Asp131, Asn166, and His190 for carbohydrate binding. This result explains the Ca\(^{2+}\)-dependent carbohydrate binding of this protein. The Man-\(\alpha\)-1,2-Man-\(\alpha\)-1,2-Man, which corresponds to the D1 arm of high mannose type glycan, is recognized by eight residues through extensive hydrogen bonds. The complex structures reveal the structural basis for high mannose type glycoprotein recognition by VIP36 in a Ca\(^{2+}\)-dependent and D1 arm-specific manner (Fig. 7.4b).

### 7.5.4 Emp47p of \textit{S. cerevisiae}: A Homologue to VIP36 and ERGIC-53

Whereas mannose 6-phosphate receptor functions as a cargo receptor for lysosomal proteins in the trans-Golgi network, ERGIC-53 (Hauri et al. 2000a, b; Zhang et al. 2003) and its yeast orthologs Emp46/47p (Sato and Nakano 2003; Schroder et al. 1995) are transport lectins for glycoproteins that are transported out of ER. The \textit{S. cerevisiae} EMP47 gene encodes a type-I transmembrane protein with sequence homology to ERGIC-53 and VIP36. The 12-amino acid COOH-terminal cytoplasmic tail of Emp47p ends in the sequence KTKLL, which agrees with the consensus for di-lysine-based signals in ER. Despite the presence of this motif, Emp47p is known as a Golgi protein at steady-state. The di-lysine motif of Emp47p was functional when transplanted onto Ste2p, a plasma membrane protein, conferring ER localization. Emp47p cycles between the Golgi apparatus and the ER and requires a di-lysine motif for its \(\alpha\)-COP-independent, steady state localization in Golgi (Schröder et al. 1995).

### 7.6 VIP36-Like (VIPL) L-Type Lectin

The profiles of human L-type lectin-like membrane proteins ERGIC-53, ERGL, and VIP36 and optimal alignment of entire CRD of these proteins revealed numerous orthologous and homologous L-type lectin-like proteins in animals, protozoans, and yeast, as well as the sequence of a novel family member related to VIP36, named VIPL for VIP36-like. VIPL has 43% similarity to ERGIC-53 and 68% similarity to VIP36. Its orthologues are broadly distributed in
many eukaryotes from human to fission yeast, whereas VIP36 is restricted to higher organisms. This phylogenetic finding suggests that VIP36 evolved from VIPL by gene duplication. VIPL is also predicted to have type I transmembrane topology with a putative CRD homologous to L-type lectins (Neve et al. 2003; Nufer et al. 2003b). Although the structural similarity between VIP36 and VIPL is very high, their characteristics are quite different. VIP36 and VIPL have the same cytoplasmic ER exit motif (KRKY) on their C-termini, but VIPL has an additional arginine at the fifth position from the C-terminus, creating the ER localization motif RKY. As a result, VIPL is a resident of ER (Neve et al. 2003; Nufer et al. 2003b), unlike ERGIC-53 and VIP36, which cycles in the secretory pathway. The sugar-binding activity of VIPL is also thought to be different from those of ERGIC-53 and VIP36; for example, HA-tagged VIPL does not bind to immobilized Man or BSA conjugated to Glc, Man, or GlcNAc as ERGIC-53 does (Nufer et al. 2003b). Moreover, VIPL does not compete with ERGIC-53 for binding to immobilized Man (Nufer et al. 2003b). Based on these observations, VIPL is thought to be an L-type lectin without sugar-binding activity. On the other hand, another group reported that knockdown of VIPL mRNA using short interfering RNA in HeLa cells significantly slowed down the secretion of two glycoproteins (35 and 250 kDa) into the medium (Neve et al. 2003), suggesting that VIPL does function in the secretion of glycoproteins, possibly by serving as an ER export receptor, similarly to ERGIC-53 (c/r Yamaguchi et al. 2007).

Although VIPL is structurally similar to VIP36, VIPL was thought not to be a lectin, because its sugar-binding activity had not been detected in several experiments. Yamaguchi et al. (2007) examined the sugar-binding activity and specificity of recombinant soluble VIPL (sVIPL) using cells with modified cell surface carbohydrates as ligands. Competition experiments with several high-mannose-type N-glycans indicated that VIPL recognizes the Man1–2Man1–2Man sequence and that the glucosylation of the outer mannose residue of this portion decreased the binding. Although the biochemical characteristics of VIPL are similar to those of VIP36, the sugar-binding activity of VIPL was stronger at neutral pH, corresponding to the pH in the lumen of the ER, than under acidic conditions. Unlike VIP36 and ERGIC-53 that are predominantly associated with ER membranes and cycle in the early secretory pathway, VIPL is a non-cycling resident protein of the ER. The results suggest that VIPL may function as a regulator of ERGIC-53 (Nufer et al. 2003a).

VIPL has been conserved through evolution from zebra fish to man. The 7.4-kb VIPL mRNA was widely expressed to varying levels in different tissues. The 32-kDa VIPL protein was detected in various cell lines. VIPL localized primarily to the ER and partly to the Golgi complex. Like VIP36, the cytoplasmic tail of VIPL terminates in the sequence KRFY, a motif characteristic for proteins recycling between the ER and ERGIC/cis-Golgi. Mutating the retrograde transport signal KR to AA resulted in transport of VIPL to the cell surface. Knockdown of VIPL mRNA using siRNA slowed down the secretion of two glycoproteins (M, 35 and 250 kDa) to the medium, suggesting that VIPL may also function as an ER export receptor (Neve et al. 2003).

References
Appenzeller C, Andersson H, Kappeler F, Hauri H-P (1999) The lectin ERGIC-53 is a cargo transport receptor for glycoproteins. Nat Cell Biol 1:330–334
Appenzeller-Herzog C, Hauri HP (2006) The ER-Golgi intermediate compartment (ERGIC): in search of its identity and function. J Cell Sci 119(Pt 11):2173–2183
Appenzeller-Herzog C, Nyfeler B, Burkhard P et al (2005) Carbohydrate- and conformation-dependent cargo capture for ER-exit. Mol Biol Cell 16:1258–1267
Appenzeller-Herzog C, Roche AC, Nufer O, Hauri HP (2004) pH-induced conversion of the transport lectin ERGIC-53 triggers glycoprotein release. J Biol Chem 279:12943–12950
Arar C, Carpentier V, Le Caer JP et al (1995) ERGIC-53, a membrane protein of the endoplasmic reticulum-Golgi intermediate compartment, is identical to MR60, an intracellular mannose-specific lectin of myelomonocytic cells. J Biol Chem 270:3551–3553
Belden WJ, Barlowe C (2001) Role of Erv29p in collecting soluble secretory proteins into ER-derived transport vesicles. Science 294:1528–1531
Carriere V, Landemarre L, Altemayer V, Motta G, Monsigny M, Roche AC (1999a) Intradermal DNA immunization: antisera specific for the membrane lectin MR60/ERGIC-53. Biosci Rep 19:559–569
Carriere V, Pillier V, Legrand A et al (1999b) The sugar binding activity of MR60, a mannose-specific shuttling lectin, requires a dimeric state. Glycobiology 9:995–1002
Cole NB, Sciaky N, Marotta A et al (1996) Golgi dispersion during microtubule disruption: regeneration of Golgi stacks at peripheral endoplasmic reticulum exit sites. Mol Biol Cell 7:631–650
Cunningham MA, Pipe SW, Zhang B et al (2003) LMAN1 is a molecular chaperone for the secretion of coagulation factor VIII. J Thromb Haemost 1:2360–2367
Dahm T, White J, Grill S, Fullekrug J, Stelzer EH (2001) Quantitative ER<<->Golgi transport kinetics and protein separation upon Golgi exit revealed by vesicular integral membrane protein 36 dynamics in live cells. Mol Biol Cell 12:1481–1498
Dogic D, Dubois A, de Chassey B, Lefkir Y, Letourneur F (2001) ERGIC-53 KAAA signal mediates endoplasmic reticulum retrieval in yeast. Eur J Cell Biol 80:151–155
Farmaki T, Ponnambalam S, Prescott AR et al (1999) Forward and retrograde trafficking in mitotic animal cells. ER-Golgi transport arrest restricts protein export from the ER into COPII-coated structures. J Cell Sci 112:589–600
Fiedler K, Parton RG, Kellner R et al (1994) VIP36, a novel component of glycolipid rafts and exocytic carrier vesicles in epithelial cells. EMBO J 13:1729–1740
Fiedler K, Simons K (1994) A putative novel class of animal lectins related to those of leguminous lectins. Cell 77:625–626
