Profile-based Data Base Scanning for Animal L-type Lectins and Characterization of VIPL, a Novel VIP36-like Endoplasmic Reticulum Protein*

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Consensus profiles were established to screen data bases for novel animal L-type lectins. The profiles were generated from linear sequence motifs of the human L-type lectin-like membrane proteins ERGIC-53, ERGL, and VIP36 and by optimal alignment of the entire carbohydrate recognition domain of these proteins. The search 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. Sequence analysis suggests that VIPL is a ubiquitously expressed protein and appeared earlier in evolution than VIP36. The cDNA of VIPL was cloned and expressed in cell culture. VIPL is a high-mannose type I membrane glycoprotein with similar domain organization as VIP36. Unlike VIP36 and ERGIC-53 that are predominantly associated with postendoplasmic reticulum (ER) membranes and cycle in the early secretory pathway, VIPL is a non-cycling resident protein of the ER. Mutagenesis experiments indicate that ER retention of VIPL involves a RKR di-arginine signal. Overexpression of VIPL redistributed ERGIC-53 to the ER without affecting the cycling of the KDEL-receptor and the overall morphology of the early secretory pathway. The results suggest that VIPL may function as a regulator of ERGIC-53.

Understanding the molecular basis of secretion requires knowledge of how secretory proteins are correctly folded and assembled in a process known as quality control, and how these itinerant proteins are sorted from resident proteins along the secretory pathway. Evidence is mounting that intracellular membranes are degraded by the proteasome after retrotranslocation to the cytosol. Degradation appears to involve calnexin and an ER α-mannosidase-like protein, termed EDEM in mammalian cells, that enhances degradation of misfolded glycoproteins carrying Man$_n$(GlcNAc)$_m$ glycans (5–7). The strict functional dependence of EDEM on a Man$_n$(GlcNAc)$_m$ structure suggests it may function as a lectin.

Correctly folded glycoproteins leave the ER in COPII-coated vesicles (8) and are transported to the Golgi via the ER-Golgi intermediate compartment (ERGIC, Ref. 9). The exit signals that direct proteins out of the ER are poorly understood with few exceptions (10). The mannose lectin ERGIC-53 (p58 in rat) mediates efficient ER-exit of some secretory glycoproteins by serving as a transport receptor (9, 11–13). ERGIC-53-assisted proteins include blood coagulation factors V and VIII (14), cathepsin C (15), and cathepsin Z (16). ERGIC-53 is a major membrane protein of the tubulovesicular clusters of the ERGIC (17) and efficiently cycles between ERGIC and ER (18–20). A second lectin cycling in the early secretory pathway, VIP36 is related to ERGIC-53 (21). VIP36 is associated with ERGIC and Golgi (22) and to some extent with the plasma membrane (23). VIP36 binds mannose (24) and GalNAc (25) and may operate in glycoprotein transport to the apical plasma membrane of polarized cells (26).

ERGIC-53 and VIP36 are type I membrane proteins. In their luminal segment they carry a single domain of about 200 amino acids that exhibits sequence similarity to the carbohydrate recognition domain (CRD) of soluble lectins of leguminous plants (21). This domain is known as L-type CRD (27). The L-type lectin-like domain of animal lectins is designated LTLD by guest on July 27, 2018http://www.jbc.org/Downloaded from
Reagents—Mouse monoclonal antibodies (mAb): 9E10.2 (IgG1) against c-Myc, 12CA5 (IgG2b) and 16B12 (IgG1, CRP) against the homologue of (HA), A1/182 (IgG1) against BAP31 (20), 2G9 (IgG2a) against CLIMP-63 (29), G1/93 (IgG1) against ERGIC-53 (17), G1/133 (IgG1) against giantin (30), A1/118 (IgG1) against GPP130 (31). Rabbit antibodies against human KDEL receptor and rat Sec31p were kindly provided by H.-D. Soling, University of Göttingen, and F. Goerlich, Yale University, respectively. Secondary goat-anti-rabbit and goat-anti-mouse IgG conjugates (either AlexaFluor 488 or AlexaFluor 568) were from Molecular Probes (The Netherlands). Secondary peroxidase-conjugated goat-anti-rabbit and goat-anti-mouse antibodies were from Jackson Immunoresearch Laboratories Inc. Brefeldin A (BFA) was from Eppendorf (Hamburg, Germany). The 5′-end VIPL primer contained an additional TgoI site and was introduced downstream of the signal sequence cleavage site between amino acids 44 and 45 of full-length VIPL. This construct was substituted by oligonucleotide-directed PCR mutagenesis or sequence-overlapping extension PCR (38), and mutant fragments were recloned via XbaI and I sites into VIPL constructs. For creating membrane association of proteins the cells were collected in ice-cold homogenization buffer (20 mM Hepes/KOH, 300 mM sucrose, 0.2 mM phenylmethylsulfonyl fluoride, 20 mM NEM, pH 7.4), resuspended in 0.1 M sodium carbonate (pH 11.5), passed 10 times through a 22-gauge needle, and kept on ice for 30 min. The sample was then layered onto a small cushion of homogenization buffer, and membranes were pelleted by centrifugation at 100,000 g for 1 h and resuspended in lysis buffer (see immunoprecipitation). The pH of the supernatant was neutralized by adding one-fifth volume of 0.5 M potassium phosphate (pH 8), and Triton X-100 was added to 1% final concentration. All samples were kept on ice for 30 min before centrifugation at 100,000 g for 1 h. The supernatants were subjected to immunoprecipitation.

**Cell Culture and Transfection—** COS-1 cells were cultured and transfected (DEAE-dextran method) as described (35). HEK293 cells (kindly provided by T. Meier, Myocontract, Switzerland) were cultured in Dulbecco's minimal essential medium (4.5 g/liter glucose) supplemented with 10% fetal calf serum and 100 IU/ml penicillin, 100 μg/ml streptomycin, and 1 μg/ml fungizone. HEK293 cells were transfected by the calcium phosphate precipitation method (32). HepG2 cells were cultured as described (20) and transfected by use of FuGene 6 transfection reagent (Roche Molecular Biochemicals, Switzerland). For immunofluorescence experiments cells were plated in poly-L-lysine-coated 8-well multichambered glass slides (Lab-Tek, Nalgene–Nunc Int’l). All cultures were grown at 37 °C with 5% CO2 in humidified air, and transfection of cells was carried out 1 day after plating.

**Immunoblotting—** Blots were treated with 0.2 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, and 0.5 μg/ml leupeptin in PBS and rinsed twice with lysis buffer, once with 100 mM sodium phosphate (pH 8) and once with 10 μM sodium phosphate (pH 8).

**Endoglycosidase Digestion—** For digestion with endoglycosidase H (endo H) the immunoprecipitates were boiled for 5 min in 50 mM TrisCl, 1% SDS, and 0.1 μg/μl-mercaptoethanol (pH 8.8). An equal volume of 0.15 M sodium citrate (pH 5.3) supplemented with protease inhibitors was added, and digestion with 100 μl of endo H (Roche Molecular Biochemicals) was carried out at 37 °C overnight. For digestion with endoglycosidase F (PNGase F), the immunolysates were boiled for 5 min in 100 mM sodium phosphate, 0.1% SDS, 0.1 μg/μl-mercaptoethanol (pH 8.8). An equal volume of 1% Triton X-100 instead of SDS was added and the sample was incubated with 400 μg of PNGase F (Roche Molecular Biochemicals, Switzerland) at 37 °C overnight.

**Sodium Carbonate Extraction—** Membrane association of proteins was tested by the sodium carbonate procedure (39). After metabolic labeling, the cells were collected in ice-cold homogenization buffer (20 mM Hepes/KOH, 300 mM sucrose, 0.2 mM phenylmethylsulfonyl fluoride, 20 mM NEM, pH 7.4), resuspended in 0.1 M sodium carbonate (pH 11.5), passed 10 times through a 22-gauge needle, and kept on ice for 30 min. The sample was then layered onto a small cushion of homogenization buffer, and membranes were pelleted by centrifugation at 100,000 g for 1 h and resuspended in lysis buffer (see immunoprecipitation). The pH of the supernatant was neutralized by adding one-fifth volume of 0.5 M potassium phosphate (pH 8), and Triton X-100 was added to 1% final concentration. All samples were kept on ice for 30 min before centrifugation at 100,000 g for 1 h. The supernatants were subjected to immunoprecipitation.

**Bioinformatics—** The GCG programs (Madison, WI) were used for sequence analysis (40). Other software used is available at ExPaSy server (www.expasy.org) (41). SwissProt (release 40.7) and TrEMBL/ release 19.1) data bases were searched. MEME/MotifSearch (42) was performed with full-length sequences of human ERGIC-53, ERGL, and VIP36 with default settings. The algorithm automatically generated a profile, based on settings with 6 motifs of 8 amino acids in length each with one or zero occurrence in each sequence. A ProfileSearch (43) against SwissProt and TrEMBL data bases was done with a profile generated of the alignment of ERGL (amino acids 286–292), VIP36 (amino acids 50–286), and ERGIC-53 (amino acids 312–328). For structure predictions we used the GCG program PgettudeStructure and PredictProtein (44). Potential sites for domains and motifs were identified by MOTIF search in the Priteo library (45) and by PSORT (psort.ins.ims.u-tokyo.ac.jp). Coiled coil regions were identified with COILS (46), and signal sequences with SPScan and signalP (47). A putative transmembrane domain was identified using TMHMM (48), and TMpred were applied in combination with structure predictions. The phylogenetic tree was constructed using the GrowTree software of the GCG package, and distance calculation was according to Jukes-Cantor.

**Gel Electrophoresis and Western Blotting—** Samples were separated by gradient SDS-PAGE. Radioactivity was visualized by fluorography using sodium salicylate and BioMax MR-1 films (Rochester). Fluorograms were quantified with a ChemiImager™ and AlphaEase™ software (Alpha Inotech Corporation). For Western blotting, proteins were transferred to nitrocellulose B85 (45 μm, Schleicher & Schuell, Germany) at 100 V for 1 h in the cold using transfer buffer containing 15.6 mM Tris, 120 mM glycine, and 20% (v/v) methanol (pH 8.4). The nitrocellulose was rinsed with 50% methanol and stained with Amido Black (Serva, Germany). All subsequent incubations were in PBS containing 5% nonfat dry milk and 0.05% Tween 20 and 1 h incubation with the first antibody for 60 min, rinsing three times 10 min, incubation with peroxidase-coupled secondary antibody for 60 min, rinsing 10 min, and 10 min incubation with enhanced chemiluminesence (ChemiGlow ECL reagent, Alphainotech Corp.) and exposed to BioMax MR-1 films or directly analyzed in a Chemilumer™.

**Immunofluorescence Microscopy—** The procedure has been described (10). Specimens were examined with a Polyvar microscope or a Leica confocal laser scanning microscope.

**Biocomputing—** The GCG programs (Madison, WI) were used for sequence analysis (40). Other software used is available at ExPaSy server (www.expasy.org) (41). SwissProt (release 40.7) and TrEMBL/ release 19.1) data bases were searched. MEME/MotifSearch (42) was performed with full-length sequences of human ERGIC-53, ERGL, and VIP36 with default settings. The algorithm automatically generated a profile, based on settings with 6 motifs of 8 amino acids in length each with one or zero occurrence in each sequence. A ProfileSearch (43) against SwissProt and TrEMBL data bases was done with a profile generated of the alignment of ERGIC-53 (amino acids 286–292), VIP36 (amino acids 50–286), and ERGIC-53 (amino acids 312–328). For structure predictions we used the GCG program PgettudeStructure and PredictProtein (44). Potential sites for domains and motifs were identified by MOTIF search in the Priteo library (45) and by PSORT (psort.ins.ims.u-tokyo.ac.jp). Coiled coil regions were identified with COILS (46), and signal sequences with SPScan and signalP (47). A putative transmembrane domain was identified using TMHMM (48), and TMpred were applied in combination with structure predictions. The phylogenetic tree was constructed using the GrowTree software of the GCG package, and distance calculation was according to Jukes-Cantor.
RESULTS

Consensus Profiles for the Identification of Animal L-type Lectins—Profiles for the identification of lectins have been established in several databases (27). For plant L-type lectins such profiles include PS00307 and PS00308 in PROSITE (45) and PF00138 and PF00139 in Pfam (49). We noticed, however, that these profiles do not identify animal L-type lectins accurately in the existing databases. Therefore, we developed new consensus profiles specific for animal L-type lectins.

In a first approach we used software MEME/MotifSearch of GCG (42) to establish a consensus motif pattern from non-aligned full-length sequences of the known human L-type lectins ERGIC-53, VIP36, and VIPL with selected plant lectins (Favin, Lea I, LSL, Con A, and ECorL), as well as the putative lectins Uip5 of yeast (SwissProt P36137), Qi9GP0 of T. bruci and Qi9GR5 of L. leishmania. Shown are sequences from \(\beta\)-sheet 6–15 referring to the resolved structure of rat ERGIC-53/p58 (51). The alignment performed with ClustalW (69) was edited based on defined or predicted secondary structures of the lectins and formatted by ESPript program (prodes.toulouse.inra.fr/ESPript/cgi-bin/nph-ESPript_exe.cgi). The secondary structures are indicated above the sequences. Boxed arrows indicate \(\beta\)-sheets, cylinders indicate \(\alpha\)-helices. Conserved cysteines are highlighted by \$. Key residues for metal and sugar binding are marked by asterisks. Additional residues important for metal and sugar binding in plants are marked by circles. Black boxes represent identity and white boxes similarity.

Fig. 1. Animal L-type lectin homologs. A, dendrogram constructed with GCG software GrowTree. For protein names and corresponding accession numbers see Table I. B, alignment of partial LTLDs of the human L-type lectins ERGIC-53, VIP36, and VIPL with selected plant lectins (Favin, Lea I, LSL, Con A, and ECorL), as well as the putative lectins Uip5 of yeast (SwissProt P36137), Qi9GP0 of T. bruci and Qi9GR5 of L. leishmania. Shown are sequences from \(\beta\)-sheet 6–15 referring to the resolved structure of rat ERGIC-53/p58 (51). The alignment performed with ClustalW (69) was edited based on defined or predicted secondary structures of the lectins and formatted by ESPript program (prodes.toulouse.inra.fr/ESPript/cgi-bin/nph-ESPript_exe.cgi). The secondary structures are indicated above the sequences. Boxed arrows indicate \(\beta\)-sheets, cylinders indicate \(\alpha\)-helices. Conserved cysteines are highlighted by \$. Key residues for metal and sugar binding are marked by asterisks. Additional residues important for metal and sugar binding in plants are marked by circles. Black boxes represent identity and white boxes similarity.
The scored proteins of both scanning methods were then analyzed by alignment and comparison to animal L-type lectins, and the presence of residues functionally important for metal/sugar binding was investigated. Moreover, structural features of the LTLD were examined by hydrophilicity plots and secondary structure predictions. Finally we analyzed the features of the LTLD were examined by hydrophilicity plots and secondary structure predictions. Finally we analyzed the identified proteins for possible protein motifs and domains.

Identification of Orthologs and New Family Members of Animal L-type Lectins by Profile Scanning—Both profile scans recovered all the known orthologs of ERGIC-53, ERGL, and of VIP36 from the data bases (Table I and Fig. 1A). The scores were higher for ERGIC-53 orthologs since profile characteristics were contributed mostly by ERGIC-53 and ERGL and to a lesser extent by VIP36. Two entries (SpTrEMBL accession numbers Q9H0V9 and Q9BQ14) also appeared with high scores. They stand for the same sequence that encodes a novel VIPL (VIP36-like, see below).

With lower scores the scans also identified lectin-like proteins in yeast. These proteins are the ERGIC-53 like Emp47p and Emp46p whereas entry O42707 is related to Emp47p and Emp46p whereas entry O94401 is presumably the ortholog of the novel VIPL (Figs. 1A and 2). While the O94401 protein has all the hallmarks of a functional LTLD, the O42707 protein lacks typical key residues required for metal and sugar binding in its LTLD fold.

The scans identified two putative lectins in the protozoans Trypanosoma cruzi (SpTrEMBL Q9GPB0) and Leishmania major (SpTrEMBL Q9GRK5). These proteins exhibit a LTLD fold similar to that of animal L-type lectins. Examination of the similarity to animal lectins and of the domain organization suggests that entry O42707 is related to Emp47p and Emp46p whereas entry O94401 is presumably the ortholog of the novel VIPL (Figs. 1A and 2). While the O94401 protein has all the hallmarks of a functional LTLD, the O42707 protein lacks typical key residues required for metal and sugar binding in its LTLD fold.

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protease Ulp1 that mediates deconjugation of Smt3 from septin components during G2/M phase transition of the cell cycle (54). A GFP-tagged version of Ulp5 was localized to the nuclear membrane. No specific phenotype was found in the deletion strain.

VIPL, a Novel Gene Related to VIP36—Alignment of the SpTrEMBL entries Q9H0V9 (gene designation DKFZP564L2423) and Q9BQ14 revealed that they encode the same hypothetical protein although the latter lacks a defined N-terminus. The predicted protein is 43% similar (35.2% identity) to human ERGIC-53 and 68% similar (57.8% identity) to human VIP36. Because of its high similarity to VIP36, we named the protein VIPL for VIP36-like. The LTLD fold of VIPL is similar to that of ERGIC-53 and VIP36. It contains all residues required for a functional LTLD (Fig. 1B) including the conserved cysteine residues required for intramolecular disulfide bond formation in ERGIC-53.

VIPL is predicted to be a type 1 membrane protein with a cytoplasmic tail of 12 amino acids. Unlike ERGIC-53, the LTLD of VIPL does not follow a stalk, and no coiled coil is predicted. Thus, VIPL exhibits a domain organization very similar to VIP36. The VIPL protein is expected to be translocated into the ER by a 38-amino acid long N-terminal signal sequence. The mature protein (residues 39–348) has a predicted mass of 35.6 kDa and a pI of 7.51. Moreover, the net charge is almost zero in a pH range of 6.8–8.0.

Detailed examination by gap alignments and evolutionary analysis indicates that the previously found lectins of Drosophila (SpTrEMBL Q9VCC2) and Caenorhabditis elegans (SpTrEMBL Q22170) are orthologs of VIPL rather than VIP36, suggesting that VIP36 appeared later in evolution than VIPL. An alignment of human VIPL and its orthologs in Drosophila (55.1% similarity), C. elegans (53.5% similarity), and the newly discovered S. pombe ortholog (O94401, 44.2% similarity) is shown in Fig. 2. The mouse VIPL (NCBI XP_129848) exhibits 92.5% similarity to the human sequence. Sequence conservation among VIPL orthologs is striking in the LTLD fold, particularly in the functional loops comprising the key residues for metal and sugar binding (Fig. 2). The cysteine residues at positions 200 and 237 of human VIPL are also conserved.

The human gene DKFZP564L2423 (EMBL AL136617) for VIPL is located on chromosome 2 (q11.2) and has a total length of 34.14 kb with 8 exons. For comparison, the gene of human VIP36 with a total length of 14.9 kb also contains 8 exons but is located on chromosome 5 (q35.5). The VIPL gene has an ATG start codon within a proper initiation context (55) and an in-frame termination codon. The 5'-untranslated region showed no other open reading frame of significant length suggesting the defined start site is correct. The correctly spliced cDNA has a total length of 2416 bp with a coding region of 1046 bp. An expression profile obtained from cDNA and expressed sequence tag (EST) sources (UniGene cluster Hs. 18627 for VIPL at NCBI server, and SAGE expression profile of VIPL gene with Ensemble ID ENSG00000114988 at EBI server) suggests that VIPL is a ubiquitous protein expressed in many cell types.

**Fig. 2. VIPL and its orthologs.** Alignment of VIPL proteins of human (HSapiens), fly (DMelanogaster), worm (CElegans) and yeast (ScPombe) based on ClustalW using blosum65 matrix and default settings. The alignment was formatted by the ESPript program. Conserved amino acids are highlighted in black boxes. Partially conserved amino acids are framed. Key residues for metal and sugar binding are indicated by asterisks and circles, and conserved cysteines by $. Predicted transmembrane domains are underlined by a bar. Numbering of amino acids is with respect to human VIPL.

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**Table:**

| Species     | Accession          | Start    | End      | Length |
|-------------|--------------------|----------|----------|--------|
| HSapiens    | Q9H0V9             | 1        | 348      | 348    |
| DMelanogaster| Q9BQ14             | 1        | 348      | 348    |
| CElegans    | Q9BQ14             | 1        | 348      | 348    |
| ScPombe     | Q9BQ14             | 1        | 348      | 348    |

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VIPL Is an N-Glycosylated Membrane Protein of the ER—To study the VIPL protein the corresponding cDNA was isolated from total RNA of HepG2 cells by RT-PCR. The cloned sequence was identical to the hypothetical cDNA of the gene DKFZP564L2423. VIPL was tagged with a HA tag and expressed in various cell lines. To test for membrane association, homogenates of transfected HEK293 cells metabolically labeled with \[^{35}\text{S}]\)methionine were subjected to the carbonate/pH 11 extraction procedure. Like the integral membrane protein ERGIC-53, VIPL-HA quantitatively distributed to the pellet fraction, while Sec31p, a component of the COPII coat that is peripherally associated with membranes was quantitatively recovered in the soluble fraction as expected (Fig. 3A). The

**Fig. 3.** VIPL is an N-glycosylated type I membrane protein. A, association of VIPL-HA with the lipid bilayer. 42 h after transfection with VIPL-HA, HEK293 cells were labeled for 30 min with \[^{35}\text{S}]\)methionine and subjected to carbonate extraction at pH 11.5 followed by ultracentrifugation. Samples of total homogenate (T), supernatant (S), and pellet (P) were subjected to immunoprecipitation with antibodies against HA, ERGIC-53, or Sec31p. Immunoprecipitates were separated by 7–10% SDS-PAGE and visualized by fluorography. B, glycosylation of VIPL. HEK293 cells were subjected to immunoprecipitation with mAb 12CA5 against HA 42 h after transfection with VIPL-HA. Immunoprecipitates were digested (+) with endo H or PGNase, or left untreated (–), separated by 10% SDS-PAGE, and transferred to nitrocellulose. VIPL-HA was detected with mAb 16B12 against HA and ECL. C, newly synthesized VIPL remains endo H-sensitive. COS-1 cells were transfected with VIPL-HA. After 42 h the cells were pulse-chased with \[^{35}\text{S}]\)methionine, chased for the indicated times, and subjected to immunoprecipitation with anti-HA. Immunoprecipitates were digested with endo H (+) or left untreated (–), separated by 8–12% SDS-PAGE and VIPL-HA was visualized by fluorography.

**Fig. 4.** VIPL is localized in ER. HepG2 cells were permeabilized and processed for confocal scanning immunofluorescence microscopy 42 h after transfection with VIPL-HA. Double staining for VIPL-HA and organelle markers is shown. A, BAP31 (ER); B, ERGIC-53 (ERGIC); C, Sec31p (COPII coat); D, giantin (cis-Golgi); E–H, VIPL-HA.

**Fig. 5.** VIPL co-distributes with ER on Nycodenz gradients. HepG2 cells were transiently transfected VIPL-HA. 42 h after transfection, the cells were treated for 3 h with BFA (panel B) or solvent only (panel A). Postnuclear supernatants were fractionated by Nycodenz density gradient centrifugation, and fractions collected from bottom (fraction 1) to top (fraction 13). The distribution of proteins was determined by Western blotting. Blots were quantified, and total counts in gradient were set to 100%. Closed rectangles, ER marker BAP31; open circles, cis-Golgi marker GPP-130; crosses, ERGIC-53; gray triangles, VIPL-HA. Shown is a representative experiment.
results confirm the prediction that VIPL is an integral membrane protein.

VIPL carries a single consensus site for N-glycosylation at position 181 (Fig. 2). To test if this site is used, VIPL-HA was immunoprecipitated from cells and treated with glycosidases. Fig. 3B shows that VIPL-HA is sensitive to both endo H and PNGase F indicating that VIPL is a glycoprotein. The persistent sensitivity to endo H suggests that the N-glycan of VIPL is of the high-mannose type. This finding was confirmed by pulse-chase experiments in which VIPL remained endo H-sensitive throughout a 3-h chase (Fig. 3C). Similarly, VIPL-HA did not acquire Endo D sensitivity when expressed in Lec-1, a cell line deficient in GlcNAc-transferase (data not shown).

We next studied the localization of transiently transfected VIPL-HA by immunofluorescence microscopy. In HepG2 cells VIPL-HA displayed a reticular pattern typical for ER (Fig. 4, E–H). Double immunofluorescence microscopy with the ER marker BAP31 confirmed this finding (Fig. 4A). A similar localization was found in COS-1, HEK293, Lec-1, and HeLa cells (Fig. 7B and not shown).

To test if VIPL cycles in the early secretory pathway, VIPL-HA transfected cells were treated with BFA. BFA redistributes rapidly cycling proteins of ERGIC and Golgi, such as ERGIC-53 (18), VIP36 (22), and the KDEL receptor (56), to characteristic punctate structures in the cytoplasm that remain separate from the ER. While BFA treatment led to the expected punctate pattern for the KDEL receptor (Fig. 6, E and G) it had no effect on the localization of VIPL-HA (Fig. 6, D, H, L). These results suggest that VIPL-HA does not cycle between ER and post-ER compartments.

We confirmed the ER resident localization of VIPL-HA by subcellular fractionation (Fig. 5). A postnuclear supernatant of HepG2 cells transiently expressing VIPL-HA was separated by Nycodenz density gradient centrifugation, and the position of VIPL-HA and organelle markers was probed by SDS-PAGE followed by immunoblotting. Fig. 5A shows the expected distribution of BAP31 (ER), GPP130 (cis-Golgi), and ERGIC-53 (ER and ERGIC) (20). VIPL co-distributed with the ER. Upon BFA treatment, GPP130 relocalized to ER fractions at the bottom of the gradient (Fig. 5B), while ERGIC-53 accumulated in the ERGIC near the top of the gradient typical for cycling proteins.

The distribution of VIPL-HA was largely unchanged by BFA treatment. The results confirm the IF data that VIPL is a non-cycling ER protein.

Overexpression of VIPL Affects the Localization of ERGIC-53—ERGIC-53 is localized in punctuate structures scattered in the cytoplasm with concentration near the Golgi (17) (Figs. 4, B and 6, D). Surprisingly, overexpression of VIPL led to a striking redistribution of ERGIC-53 to the ER (Figs. 4B and 6I). This effect was observed in all the tested cell lines transiently transfected with VIPL. Interestingly, the relocated ERGIC-53 was insensitive to BFA. It remained in the ER without accumulation in dots (Fig. 6K). To test if the redistribution of ERGIC-53 to the ER required an active LTLD we substituted the conserved asparagine 163 of VIPL by aspartate (N163D). This mutation can be expected to disrupt VIPL's lectin function. VIPL(N163D) still redistributed ERGIC-53 to the ER suggesting that an active LTLD was not required (not shown). Likewise, this redistribution does not involve an active LTLD of ERGIC-53 since the lectin-impaired N156A mutant of ERGIC-53 was also redistributed (not shown).

To test if overexpression of VIPL affects the morphology of the early secretory pathway we studied the localization of Sec31p (a component of COPII coats) and GPP130 by immunofluorescence microscopy. Figs. 4 and 6 show that the distribution of these markers was unchanged suggesting that the general morphology of the secretory pathway is unaffected by overexpression VIPL. Moreover, the normal distribution of the cycling proteins KDEL-receptor (Fig. 6) and VIP36 (not shown) in VIPL-overexpressing cells indicates that VIPL does not just arrest any rapidly cycling protein in the ER.

ER Retention of VIPL Requires an RKR Motif in the Cytoplasmic Segment—The localization of VIPL to the ER is surprising since its cytoplasmic segment possesses a putative ER-exit motif (i.e. an aromatic amino acid in position 2, Ref. 10) but no di-lysine ER localization signal typical for many type I ER membrane proteins (57). ER localization is also unexpected because VIP36, having a similar C-terminal tail sequence (Fig. 7A), is localized to Golgi and ERGIC (22), and the C-terminal four amino acids of VIP36 can mediate efficient ER export when appended to ERGIC-53 (58). To test if ER retention of VIPL is caused by its luminal...
domain we generated a VIPL mutant lacking the transmembrane and cytosolic domains. This soluble mutant was secreted into the cell culture medium (not shown) suggesting that the ER localization signal is not contained in the luminal domain. We noted a conserved RKR motif in the cytoplasmic tail of all orthologs of VIPL that is not present in VIP36 (Fig. 7A). The motif is reminiscent of di-arginine motifs (RR or RXXR) initially characterized as ER retention motifs near the N terminus of type II membrane proteins (57, 59) and near the cytoplasmic C terminus of multispanning membrane proteins (60). To study the role of the RKR motif in ER retention we substituted it by three serines. Furthermore, to prevent interference by a possible lectin activity of VIPL, we also mutated asparagine 163 to aspartate in the putative lectin domain. Like wild-type VIPL-HA, VIPL(N163D)-HA was localized to ER in transfected COS cells (Fig. 7B, panels a and b). By contrast, VIPL lacking the RKR motif was also found at the cell surface in addition to intracellular membranes (Fig. 7B, panel c). This became more obvious when the cells were not permeabilized. While hardly any staining of VIPL-HA was detected in cells transfected with wild-type or VIPL(N163D)-HA cDNA (Fig. 7B, panels d and e), surface staining of VIPL-HA lacking the RKR motif was clearly evident (Fig. 7B, panel f). Despite its transport to the cell surface the SSS mutant of VIPL-HA remained endo H-sensitive as revealed by pulse-chase experiments (Fig. 7C). In addition, no acquisition of Endo D sensitivity was observed when expressing the VIPL constructs in Lec-1 cells (data not shown). The results of this mutagenesis approach indicate that the RKR motif is required for the ER localization of VIPL.

VIPL Lacking the ER Retention Signal Does Not Pull ERGIC-53 to the Cell Surface—Does overexpression of VIPL lacking the RKR retention motif result in surface localization of ERGIC-53? To test this, HepG2 cells were transiently transfected with VIPL-HA carrying a RKR to SSS substitution and probed surface expression of ERGIC-53 by IF. The transfected cells showed intracellular but no apparent cell surface staining of ERGIC-53 (Fig. 8A), while the mutant VIPL exhibiting cell surface staining, as expected (Fig. 8B). The results were confirmed by using non-permeabilized cells. Mutant VIPL but not ERGIC-53 was detectable at the cell surface (Fig. 8, C and D). These results suggest that the interaction of overexpressed VIPL with ERGIC-53 in Lec-1 cells (data not shown). The results of this mutagenesis approach indicate that the RKR motif is required for the ER localization of VIPL.

VIPL Lacking the ER Retention Signal Does Not Pull ERGIC-53 to the Cell Surface—Does overexpression of VIPL lacking the RKR retention motif result in surface localization of ERGIC-53? To test this, HepG2 cells were transiently transfected with VIPL-HA carrying a RKR to SSS substitution and probed surface expression of ERGIC-53 by IF. The transfected cells showed intracellular but no apparent cell surface staining of ERGIC-53 (Fig. 8A), while the mutant VIPL exhibited cell surface in addition to intracellular staining, as expected (Fig. 8B). The results were confirmed by using non-permeabilized cells. Mutant VIPL but not ERGIC-53 was detectable at the cell surface (Fig. 8, C and D). These results suggest that the interaction of overexpressed VIPL with ERGIC-53 does not persist beyond the ER.

**FIG. 7. The RKR motif in the cytoplasmic tail of VIPL is required for ER retention.** A, cytoplasmic C-terminal amino acid sequences of VIP36 and VIPL of different species. The putative ER exit motif (FY) is shown in bold-italic. The conserved RKR motif of VIPL orthologs is bold underlined. B, substitution of the RKR motif to triple serines results in surface localization of VIPL. COS-1 cells were processed for immunofluorescence microscopy with anti-HA 42 h after transfection with wt, N163D, or RKR to SSS VIPL-HA cDNA. a–c, permeabilized cells; d–f, non-permeabilized cells. Bar, 25 μm. C, pulse-chase experiment. COS-1 cells were transiently transfected with the indicated constructs and subjected to pulse-chase/endo H analysis using [35S]methionine. After chase the cells were lysed and subjected to immunoprecipitation with anti-HA followed by endo H treatment, 8–12% SDS-PAGE, and fluorography.

**FIG. 8. Secreted VIPL does not relocalize ERGIC-53 to cell surface.** 42 h post-transfection the effect of overexpressing VIPL-HA with RKR motif substitution by SSS (VIPL(RKR to SSS)-HA) on ERGIC-53 was examined in HepG2 cells by confocal double immunofluorescence microscopy using permeabilized (A and B) or non-permeabilized cells (C and D). A and C, ERGIC-53; B and D, VIPL(RKR to SSS)-HA.
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Fig. 9. The RKR motif mediates retention of a homo-oligomeric reporter protein in absence of an ER exit motif. A, schematic representation of GM constructs used to transfect COS-1 cells. All constructs have an N-glycosylation site (CHO) at position 61 and a c-Myc epitope (34). The transmembrane domain is followed by the amino acid sequence of the cytoplasmic tails in single letter code. The amino acid sequence of the mutant cytoplasmic tails is shown in panel B. GMA, GMA,FF and GMA,FF have been described (10, 35). In chimeric GM-ViTa constructs, the underlined residues in cytoplasmic tail are RKR in wild-type constructs and SSS in mutant (mt) constructs. The most extreme FY motif in GM-ViTa (shown in italic letters in panels A and B) has been additionally mutated to AA in GM-ViTa(AA). C, effect of RKR motif on GM transport. COS-1 cells were transfected with the indicated constructs (panel B) and subjected to pulse-chase/endo H analysis using [35S]methionine. 60 min after chase the cells were lysed and GM constructs were immunoprecipitated with anti-Myc. Immunoprecipitates were digested with endo H (+) or left untreated (−), separated by 7–10% SDS-PAGE and analyzed by fluorography. The upper band represents the endo H-resistant and the lower band the endo H-sensitive form of the GM constructs. D, quantification of fluorograms including that shown in C. Numbers on y-axis refer to constructs in panel B. Mean values ± S.E. of at least three independent experiments. Single and double asterisk symbols indicate statistically significant differences in transport between GM-ViTa and GMA,FF or GM-mtViTa, respectively (p < 0.05, Student’s t test).

Our profile scanning revealed notable differences between animal and plant L-type lectins. The differences include two conserved cysteines, present in the LTLD of animal but not plant lectins, that form an intramolecular disulfide bond in ERGIC-53 and presumably other animal lectins (Fig. 1B). Another characteristic is the unique turn of δ1-helix in animal LTLDs that separates the β-sheets 1a and 1b (51). Further differences became obvious when the (partial) LTLDs of plant and animal L-type lectins were aligned (Fig. 1B). Animal lectins contain a shorter B loop but an elongated loop between the two β-sheets after loop C. The elongated loop forms an α-helix in ERGIC-53 (51) and is predicted to be also present in ERGL, VIP36, and the newly identified protozoan lectins, as well as in the putative yeast lectin Uip5. Uip5 has an additional predicted α-helix in loop B not present in animal and plant lectins (Fig. 1B). Yet another difference between plant and animal LTLDs concerns the residues mediating metal/sugar binding in loop C. While plant lectins possess a glutamate and an aspartate that are conserved and responsible for Mn2+ binding, the animal lectins lack these residues (Fig. 1B). Accordingly, lectin activity of ERGIC-53 and VIP36 does not require Mn2+ binding. Whether or not the protozoan lectins and the putative lectin Uip5 of yeast require Mn2+ for lectin activity is difficult to predict since they lack the glutamate although the aspartate is conserved. Finally, the general domain organization also differs between plant and animal L-type lectins (including the orthologs and homologs in lower organisms, such as yeast). Animal lectins are type I membrane proteins as opposed to plant lectins that are soluble proteins.

The legume lectin fold has evolved independently several times (62). It is not only present in L-type lectins but also in galecins and pentraxins. However, the position of the ligand binding site is not conserved among the lectin families. In the animal L-type-like lectin family, major features of the carbohydrate binding site of legume lectins are conserved. Thus, a divergent evolution from a common ancestor is likely. We propose that Uip5 represents a common ancestor of plant and animal L-type lectins.

With the discovery of VIPL the family of L-type lectins in humans appears to be complete although some uncertainty remains since the annotation of the human genome has not been completed. The animal L-type lectin family comprises the 4 members ERGIC-53, ERGL, VIP36, and VIPL. The predicted overall structure and domain organization of VIPL most closely resembles that of VIP36. Different from ERGIC-53, secondary structure predictions could not define an α-helix between the β-sheets after loop C in the VIP36 LTLD, although, similar to ERGIC-53, the corresponding region is longer than in the LTLD of plant lectins (Fig. 1B). Interestingly, orthologs of VIPL are found in mouse, fly, worm, and yeast (S. pombe) but not S. cerevisiae whereas VIP36 is restricted to higher organisms (Fig. 1A). It appears, therefore, that VIP36 has evolved from VIPL, presumably by gene duplication.

Unlike ERGIC-53 and VIP36 that cycle in the early secretory pathway, VIPL is a resident of the ER when expressed in cultured cells. The following considerations argue against the possibility that the ER localization is due to misfolding. First, we have no evidence for aggregation of transfected VIPL, both biochemically and by immunofluorescence microscopy. Second, ER retention was abolished by mutating the conserved RKR motif in the cytoplasmic domain of the molecule or by deleting both the transmembrane and cytoplasmic domain. These observa-

[^2]: O. Nufer and H.-P. Hauri, manuscript in preparation.
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VIPL also possesses a C-terminal ER-exit motif (10) that appears to be dominated by the di-arginine ER retention signal. However, RKR was unable to retain a chimeric ERGIC-53 reporter protein carrying the same ER-exit motif. In contrast, the RKR motif in the cytoplasmic tail of Kir6.2, that lacks an ER exit motif, fully retains a CD4 reporter in the ER (63). This discrepancy is most likely due to the fact that the ERGIC-53 reporter is homo-oligomeric, while VIPL, like CD4, is not.2 Our studies on transport motifs in ERGIC-53 revealed that ER-exit motifs are more efficient when presented in an oligomeric protein (Ref. 10).2 Accordingly, inactivation of the ER-exit motif in the ERGIC-53 reporter led to ER retention of the construct in the present study. The results indicate that the RKR motif can indeed operate as an ER targeting signal in type I proteins.

An unexpected finding was the arrest of endogenous ERGIC-53 in the ER upon overexpression of VIPL. The effect was selective since other rapidly cycling proteins, such as KDEL receptor and VIP36, were not affected by the overexpression of VIPL, and the secretory pathway was apparently unaffected. Since ER arrest of ERGIC-53 was also observed in cells expressing low apparent levels of VIPL, we assume that endogenous VIPL must be expressed at rather low levels. The redistribution of ERGIC-53 by VIPL overexpression was still observed when the lectin function of either protein was abolished by mutagenesis. This suggests that VIPL and ERGIC-53 interact in a lectin-independent manner, either directly or indirectly via a third component. Since overexpression of both proteins gave the same phenotype, the latter possibility appears less likely to us, unless a third interacting component is present in large excess. However, we could not detect any interaction by co-immunoprecipitation or by chemical cross-linking. The mechanism by which VIPL and ERGIC-53 interact remains to be elucidated. A hydrophobic surface patch opposite to the carbohydrate binding site of the LTLD of ERGIC-53 (51) may mediate this interaction given the fact that VIPL is uncharged at physiological pH. We speculate that VIPL may control the function of ERGIC-53 by modulating its exit from the ER.

Is VIPL a lectin? The presence of key residues for metal and sugar binding in the LTLD of VIPL would suggest lectin activity. However, HA-tagged VIPL failed to bind to immobilized mannos or fluorescein isothiocyanate-labeled bovine serum albumin conjugated with glucose, mannose, or GlcNAc under conditions used to successfully establish the mannoe specificity of ERGIC-53 (12). Moreover, binding of ERGIC-53 to immobilized mannos was not affected by the presence of VIPL. The metal/sugar binding site of VIPL differs slightly from that of ERGIC-53 and VIP36 in loop C (Fig. 1B). VIPL contains an elongation by one amino acid after the key binding site. A similar elongation is found in Con A but not other plant lectins (Fig. 1B). This variance in length correlates with different affinities of plant lectins for different mannos/glucose derivatives (50). The slightly elongated C loop in VIPL may explain why experiments that demonstrated lectin activity of ERGIC-53 or VIP36 failed for VIPL. Nevertheless, we speculate that VIPL possesses binding preference for mannose based on findings by Sharma and Surolila (50) who reported that legume lectins with a short D loop, such as Favin, LOL I, LSL, or Con A, preferentially bind mannose and glucose while lectins with a long D loop, such as EcoR L, prefer GalNAc (see Fig. 1B). Like ERGIC-53 and VIP36, VIPL has a short D loop.

An ortholog of VIPL was recently identified in zebra fish (EMBL Aam29497) that exhibits 71% similarity to human VIPL and is required for early development (65). Embryos lacking VIPL are touch insensitive and insensitive to tapping on the dish, but they are capable of spontaneous movements. We speculate that this defect may be caused by inefficient processing or transport of some secretory molecules in the absence of VIPL. A case in point is combined deficiency of coagulation factors V and VIII in humans, an inherited disease in which mutations in ERGIC-53 lead to inefficient secretion of coagulation factors V and VIII (14). Interestingly, some patients suffering from this disease have entirely normal ERGIC-53 (66–68). It is conceivable that such patients synthesize excessive levels of VIPL that would render ERGIC-53 non-functional by retaining it in the ER. The findings of the present study provide a basis to test this notion.

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