The novel *Drosophila* Lysosomal Enzyme Receptor Protein Mediates Lysosomal Sorting in Mammalian Cells and Binds Mammalian and *Drosophila* GGA Adaptors

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Biogenesis of lysosomes depends in mammalian cells on the specific recognition and targeting of mannose 6-phosphate-containing lysosomal enzymes by two mammalian 6-phosphate receptors (MPR46, MPR300), key components of the extensively studied receptor-mediated lysosomal sorting system in complex metazoans. In contrast, the biogenesis of lysosomes is poorly investigated in the less complex metazoan *Drosophila melanogaster*. We identified the novel type I transmembrane protein lysosomal enzyme receptor protein (LERP) with partial homology to the mammalian MPR300 encoded by *Drosophila* gene CG31072. LERP contains 5 lumenal repeats that share homology to the 15 lumenal repeats found in all identified MPR300. Four of the repeats display the P-lectin type pattern of conserved cysteine residues. However, the arginine residues identified to be essential for mannose 6-phosphate binding are not conserved. The recombinant LERP protein was expressed in mammalian cells and displayed an intracellular localization pattern similar to the mammalian MPR300. The LERP cytoplasmic domain shows highly conserved interactions with *Drosophila* and mammalian GGA adaptors known to mediate Golgi-endosome traffic of MPRs and other transmembrane cargo. Moreover, LERP rescues missorting of soluble lysosomal enzymes in MPR-deficient cells, giving strong evidence for a function that is equivalent to the mammalian counterpart. However, unlike the mammalian MPRs, LERP did not bind to the multimeric mannose 6-phosphate ligand phosphomannan. Thus ligand recognition by LERP does not depend on mannose 6-phosphate but may depend on a common feature present in mammalian lysosomal enzymes. Our data establish a potential important role for LERP in biogenesis of *Drosophila* lysosomes and suggest a GGA function also in the receptor-mediated lysosomal transport system in the fruit fly.

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1 The abbreviations used are: MPR, mannose 6-phosphate receptor; MPR46, M, 46.000 mannose 6-phosphate receptor; MPR300, M, 300.000 mannose 6-phosphate receptor; GST, glutathione S-transferase; HA, hemagglutinin; IGF, insulin-like growth factor; LERP, lysosomal enzyme receptor protein; MBP, maltose-binding protein; Man6P, mannose 6-phosphate; MEF, mouse embryonic fibroblasts; TGN, trans-Golgi network; UTR, untranslated region; LDL, low density lipoprotein; EST, expressed sequence tag.

Biogenesis of lysosomes plays an important role in many physiological and pathological processes. In mammals, it depends on the transport of soluble lysosomal enzymes to lysosomes by two integral type I membrane proteins, the mannose 6-phosphate receptors (MPR) (1). Mannose 6-phosphate residues (Man6P) are known as a lysosomal sorting signal in mammals and are recognized by MPRs. Two mannose-6-phosphate receptors, MPR46 and MPR300, sort newly synthesized lysosomal enzymes from the trans-Golgi network (TGN) to endosomes in mammalian cells, where enzyme release is triggered by the acidic interior. From there, the enzymes are transported to the lysosomes. MPRs are recycled to the cell surface or carried back to the Golgi complex to undergo multiple rounds of sorting but never reach lysosomes. Defects in the Man6P marker synthesis found in patients with I-cell disease or deletion of the corresponding MPRs results in missorting of lysosomal enzymes to the extracellular lumen and consequently to the formation of storage lysosomes.

The MPR300 is a multifunctional receptor made up of 15 contiguous domains. Besides Man6P-containing lysosomal enzymes, MPR300 binds and endocytoses a variety of ligands involved in embryogenesis, differentiation, immunity, cell proliferation, and cell migration such as insulin-like growth factors 2 (IGF2), proliferin, transforming growth factor-β precursor, leukemia inhibitory factor, renin precursor, granzymes A and B, Herpes simplex virus glycoprotein D, retinoic acid, plasminogen, and urokinase-type plasminogen activator receptor (for a review, see Ref. 2). Conserved MPR sequences have been cloned from several species, including species of mammals, fish, and chicken (Refs. 2 and 3 and references therein).

In mammals, the transport of MPRs is operated by sorting signals that are determined in the cytoplasmic domains of the receptors (4–7). At the TGN, the recently identified family of the Golgi-localizing, β-ear-containing, ARF-binding proteins (GGAs) regulates the transport of MPRs (8–10) and other transmembrane cargo proteins such as sortilin (10, 11), LDL receptor-related protein 3 (10), and β-secretase (12). Three different GGAs and additional splice variants exist in mammals that share functional similarities with tetrameric adaptor complexes (AP1–4) but are monomeric cytoplasmic adaptor proteins that are recruited to the TGN by ARF1-GTPase. They
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Consists of four distinct domains: a VHS domain that binds the acidic dileucine sorting signal (DXXLL) found in the MPRs and other transmembrane cargo proteins; the GAT domain, which interacts with the GTP-bound form of ARF1; a hinge region, which recruits clathrin; and a GAE domain, which exhibits sequence similarity to the ear region of γ adaptin and recruits a number of accessory proteins (Ref. 13 and references therein). The interaction with the GGAs leads to the selective incorporation of the MPRs into clathrin-coated vesicles destined for the endosomal/lysosomal sorting route. Mutations in the cytoplasmic domains of the MPRs that cause a loss in binding to the GGAs result in missorting and incomplete processing of lysosomal hydrolases (5, 7, 9, 14, 15). In yeast, two homologous GGA proteins exist that seem also to be involved in the receptor-mediated transport of lysosomal hydrolases to the vacuole, the equivalent of lysosomes (16). The vacuolar yeast-sorting receptor Vps10p represents the functional analog of the mammalian MPRs. However, it sorts its ligands by peptide recognition signals and not by carbohydrate recognition (17). The vesicular sorting system for the intracellular vascular/lysosomal transport pathways is highly conserved between yeast and mammals (18).

Data on the biogenesis of lysosomes in Drosophila are rare, and no components of a receptor-mediated lysosomal transport system have been identified so far (20). Only the tetraspanin sln was recently identified as a lysosomal membrane protein in Drosophila photoreceptor cells (21). With the exception of the aspartic acid proteinase carboxypeptidase 1, which may represent a homologous protein to mammalian cathepsin L, no other lysosomal enzymes, sorting receptors such as MPRs, or homologous proteins have been identified (22).

Here we report on the identification of a novel lysosomal enzyme receptor protein (LERP) from Drosophila melanogaster. Our results show that LERP rescues missorting of lysosomal enzymes in mouse fibroblasts deficient for MPRs and displays highly conserved interactions with the VHS domains of Drosophila GGA homolog and mammalian GGAs. These data establish a potential role for LERP in the biogenesis of Drosophila lysosomes and suggest that the participation of GGA in packaging sorting receptors of the lysosomal system is conserved between mammals and fruit fly.

Experimental Procedures

Chemicals

Chemicals were from Sigma. 4-Methylumbelliferyl substrates for the lysosomal glycosidases were from Calbiochem.

Cell Culture and Transfection

Mouse embryonic fibroblasts (MEF) were grown in Dulbecco’s minimal essential medium supplemented with Glutamax I (Invitrogen) and 10% fetal calf serum. Transfection of MEF cells deficient in MPR46 and MPR300 (mpc−/− MEF) was performed as described (23). Drosophila S2 cells were cultured in Schneider’s Drosophila medium (Invitrogen) containing 10% fetal calf serum, 50 units/ml penicillin, 50 units/ml streptomycin, and 2 mM L-glutamine in the absence of CO2.

mRNA Analysis and Reverse Transcription-PCR

RNA Preparation—Total RNA was prepared from Drosophila embryos and S2 cells using RNA kit (Qiagen, Hilden, Germany) according to the supplier’s manual.

Reverse Transcription-PCR—First strand cDNA synthesis from total RNA of Drosophila embryos and S2 cells was performed with the Omniscript kit (Qiagen) using oligo(dT)16 primer. Generated first strand cDNAs were used as templates for PCR with primers flanking base pairs 2159–2737 of LERP sequence. The resulting 578 bp PCR fragment was cloned into pCR-Blunt II-TOPO vector (Invitrogen) and analyzed by sequencing.

mRNA Analysis—Total RNA (25 μg) from Drosophila embryos and S2 cells was transferred to Hybond-N nylon membrane (Amersham Biosciences) and analyzed with an 32P-labeled EcoRI-fragment from pBSK-LD06659 (bp 1–1283 of LERP-cDNA). The cDNA of LERP was isolated from the EST clone pOT2-LD41311 (Drosophila Genomics Resource Center (Bloomington, IN)). An EcoRI/XhoI fragment encoding the Drosophila GGA-VHS domain (amino acid residues 1–147) was obtained by PCR amplification from pOT2-LD41311 and subcloned into the corresponding sites of pGEX4T-1 (Amersham Biosciences). The glutathione S-transferase (GST) fusion protein was expressed in BL21 (pLysS) (Novagen, Schwalbach, Germany) and purified using glutathione 4B-Sepharose (Amersham Biosciences).

pMALc2x Constructs—An EcoRI/Sall fragment encoding the cytoplasmic domain of LERP (amino acid residues 539–886) was obtained by PCR amplification from pBSK-LD06659, digested, and subcloned into the corresponding sites of pMALc2x (New England Biolabs, Frankfurt, Germany). Mutations in the cytoplasmic domain of LERP were introduced using altered reverse primers resulting in the amino acid substitutions of either aspartate to asparagine or leucine to alanine. The maltose-binding protein (MPB) fusion proteins were expressed in BL21 (pLysS) (Novagen) and purified using amylose resin (New England Biolabs). Oligonucleotide sequences used are available on request.

Glycosylation Analysis

Extracts of cell membranes were prepared from the MPR-deficient MEF stably transfected with HA-tagged LERP. Digestion analysis was done with endoglucosaminidase H and peptide-N-glycanase F (New England Biolabs) with the buffers recommended by the supplier.

The sample (homogenate, 72 μg of protein) was incubated in 0.5% SDS, 1% β-mercaptoethanol for 10 min at 100 °C for glycoprotein denaturation. Digestion with 1 unit of endoglucosaminidase H was done in 50 mM sodium citrate, pH 5.5, at 37 °C for 1 h. Digestion with 1 unit of peptide N-glycanase F was done in 50 mM sodium phosphate, pH 7.5, supplemented with 1% Nonidet P-40 at 37 °C for 2 h. The digested samples were run on a SDS-PAGE and analyzed by immunoblotting using an anti-HA-specific antibody for detection of LERP. The apparent molecular weights of the LERP bands were calculated using the scion 3-software (Scion Corp., Frederick, MD).

Assays of Lysosomal Glycosidases

Stable transfected MPR-deficient MEFs were cultured for 24 h in normal growth medium. The medium was changed to Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum in which the lysosomal hydrolases had been inactivated. The cells were then incubated for 10 h. Cells and media were analyzed for the activities of lysosomal enzymes using fluorometric assays as described (23).

Affinity Chromatography

MPR300-Sepharose affinity chromatography of [35S]methionine-labeled supernatants from cultured cells was performed as described (20). MPR300 was purified from human liver as described (26) and coupled to Affi-Gel 10 (Bio-Rad) according to the manufacturer’s manual. Media obtained after labeling were subjected to MPR300-Sepharose affinity chromatography. The material precipitated with 0.5 M ammonium sulfate was dialyzed overnight against buffer A (50 mM imidazole HCl, pH 7.0, 0.15 M NaCl, 5 mM sodium β-glycerophosphate, 0.05% Triton X-100, 10 mM MgCl2, and 2 mM EDTA). Media dialyzed against buffer A were incubated overnight with MPR-Affi-Gel 10 (30 mg of MPR/2 ml of MPR-Affi-HG column). When the material was washed with 6 × 2 ml of buffer A and with 3 × 1 ml of 50 mM glucose 6-phosphate and 3 × 1 ml of 5 mM mannose 6-phosphate in buffer A. The mannose 6-phosphate eluates were precipitated with 10% trichloroacetic acid; pooled and dissolved in 50 μl of 0.4 M Tris; heated for 5 min at 95 °C in 10 mM dithiothreitol, 1% SDS; and analyzed by SDS-PAGE (10%) and fluorography.

Phosphomannan-Sepharose affinity chromatography was performed
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RESULTS

Characterization of Drosophila Lysosomal Enzyme Receptor Protein—We analyzed the Drosophila genome for sequences homologous to the human MPR300 (accession number J03528) by Blast searches of the Drosophila Flybase (flybase.net) and found the annotated gene CG31072. CG31072 has been mapped to the chromosomal region 97D3–D4 of D. melanogaster chromosome arm 3R. Two EST clones have been cited for this gene, LD05154 and LD06659. Only LD06659 was available from Invitrogen. We sequenced the EST clone LD06659; it codes for 3476 bp and contains an open reading frame of 886 amino acids. The sequence data have been submitted to the GenBank data base under accession number AY684632. Protein structure analysis predicted a type I transmembrane glycoprotein with a signal peptide of 40 amino acids, a luminal domain of 775 amino acids, a transmembrane domain of 23 amino acids, and a cytoplasmic domain of 48 amino acids (Fig. 1 and for details, see supplemental data). We entitled it lysosomal enzyme receptor protein because it has structural homology to the MPR300, one of the lysosomal sorting receptors in mammals. The luminal domain displays homology to the carbohydrate recognition sequences of the P-lectins and consists of five contiguous repeats of about 155 amino acids corresponding to repeats 9–13 of the 15 repeats in the human MPR300 (Fig. 1). The homologies of the repeats to the human MPR300 repeats are 23–29.5% (for details, see the alignment in Fig. B of the supplemental data). The overall similarity on the amino acid level is 23.5% to the luminal domain of the human MPR300. A detailed amino acid comparison is shown in Fig. B in the supplemental data. Six cysteine residues are found in each repeat unit of the MPR300 that are known to form three structural important disulfide bonds (Fig. 1) (2). The positions of the cysteine residues are well conserved in the homologous Drosophila LERP, except in repeat 5, where only one of the disulfide bonds appears to be conserved. Residues identified as essential in the Man6P-binding pocket of repeat 9 of the human MPR300 are not conserved in any of the LERP repeats. For the mammalian MPR300, it has been shown that IGF2 binds to repeat 11. There the isoleucine residue 1572 is conserved in LERP (see supplemental data, Fig. B, 1).
Multiple hydrophobic residues identified in the IGF2-binding pocket and core residues for IGF2 binding are also conserved, suggesting a similar function in the Drosophila LERP. However, until now, no gene coding for IGF2 or other IGFs has been identified in Drosophila. Like MPR300, the cytoplasmic domain of LERP contains a classical tyrosine-dependent sorting motif $^{585}YRSV^{661}$ of the type YXXΦ (where Φ is a bulky hydrophobic amino acid). Also found is a potential GGA-binding motif $^{883}DMLL^{886}$ of the type DXXL known to be implicated in packaging sorting receptors such as MPR300 in clathrin-coated vesicles at the TGN (Fig. 1).

By mRNA analysis of Drosophila embryos and S2-culture cells, we detected one band of 3.6 kb, suggesting that the 3476 bp of LD06659 represents the full-length transcript (not shown). For the annotated gene CG31072, two transcripts (CG31072-PA and CG31072-PB) have been predicted as potential exon sequences. The comparison with the LD06659 sequence revealed additional amino acids in the luminal domain in the range of the LERP nucleotides 2350–2584 that were not included in the predictions of the annotated genes. To find out whether more mRNA species for LERP exist, a reverse transcription-PCR was run from Drosophila embryonic mRNA. Primers were placed at nucleotides identical in LD06659 and the predicted transcripts on both sides of the region in question. The only fragment found had exactly the LERP sequence (not shown). This demonstrates that LD06659 represents the transcript of the gene CG31072. A detailed comparison of the intron and exon sequences will be published in the FlyBase data bank (flybase.net).

Expression and Localisation of LERP—The LERP cDNA was cloned into the pcDNA3.1 expression vector tagged with a C-terminal HA tag to allow the biochemical and functional analysis of the resulting protein by anti-HA antibodies. Stable transfections were made in MPR-deficient mouse embryonic fibroblasts (mp300−/−MEF) that missort up to 98% of soluble lysosomal enzymes to the medium due to the lack of sorting MPR. In these cells, the missorting of lysosomal enzymes, causing accumulation of non-degraded material (inclusions), can be rescued by the expression of a receptor protein that re-establishes functional sorting (23). Immunohistochemical detection of HA-tagged LERP in the transfected mp300−/−MEF mouse fibroblasts revealed an intracellular distribution of the Drosophila protein similar to the human MPR300 (Fig. 2, D–F and G–I). Localization of LERP and MPR300 occurs in the same compartment as shown by cotransfection of both cDNAs to mp300−/−MEF (Fig. 2, J–L), and no colocalization with the lysosomal marker lamp-2 was detected (Fig. 2, D–F and G–I). Expression of LERP cDNA resulted in a clear reduction in the appearance of inclusions already visible in the light microscope, indicating proper sorting of the degrading lysosomal hydrolases. This corresponds to the significant reduction in size and amount of lamp-2-positive vesicles, which represents lysosomes (compare Fig. 2, B, E, and H).

Molecular Properties of LERP—To analyze whether LERP sorting is based on mannose 6-phosphate residues, we prepared membrane fractions from HA-tagged LERP expressing mp300−/−MEF by solubilization with Triton X-100. MPR300-expressing mp300−/−MEF were used as control. The membrane extracts were passed over a phosphomannan affinity matrix (27). Unbound material and material specifically eluted with 5 mM Man6P was analyzed by immunoblotting using a HA-specific antibody (3F10) for LERP detection or the human MPR300-specific antibody (2C2). Analysis of binding was performed by systematic variation of pH values (pH 6; 6.5 or 7.0) and by variation of the cations present (10 mM magnesium, manganese, or calcium). Binding of LERP could not be obtained at any of the conditions tested, whereas MPR300 was eluted specifically with Man6P from the affinity matrix (98% of total applied material) (Fig. 3). LERP could only be detected in the unbound fraction, indicating that it did not bind to the phosphomannan affinity matrix.

Biochemical characterization of the HA-tagged LERP expressed in mammalian MPR-deficient cells was done by SDS-PAGE and immunoblotting. The protein displays molecular masses of 150 kDa under reducing conditions and those of 129 kDa under non-reducing conditions. The amino acid sequence encoded by LERP cDNA predicts 13 potential N-glycosylation sites. To determine the N-glycosylation pattern of LERP, we used enzymes for deglycosylation. Digestion with peptide-N-glycanase F that cleaves between the innermost GlcNAc and the asparagine residues of N-linked glycoproteins resulted in a band of 98 kDa. The protein size of 98 kDa is in exact agreement with the predicted protein size resulting in a carbohydrate portion of 52 kDa. LERP was not sensitive to endoglucosaminidase H, which cleaves high mannose type carbohydrates; this indicates the presence of complex oligosaccharides (not shown).

Sorting of Lysosomal Enzymes by LERP—To examine the sorting function of LERP in the stably transfected MPR-deficient mouse cells for a large number of lysosomal proteins at a time, secretions of metabolically [35S]methionine-labeled LERP-expressing mp300−/−MEF were subjected to affinity chromatography on columns substituted with human MPR300 as
described (30). Mock-transfected mpr\textsuperscript{+/−}MEF were analyzed for control. The secretions contain all Man6P-bearing lysosomal proteins that escape sorting to lysosomes. The fraction of polypeptides that bound to the receptor affinity column and eluted with Man6P was quantified and characterized by SDS-polyacrylamide gel electrophoresis (PAGE and autoradiography (not shown). In secretions of mock-transfected mpr\textsuperscript{+/−}MEF, about 16.5% of the labeled polypeptides bound in a Man6P-dependent manner to the column. This fraction was reduced to 11.7% in secretions of LERP-expressing mpr\textsuperscript{+/−}MEF. We conclude from these results that overexpression of Drosophila LERP partially corrects the missorting of lysosomal enzymes in mpr\textsuperscript{+/−}MEF. To assess the sorting function of LERP in more detail, the distribution of selected lysosomal enzymes was determined between cells and medium. When untransfected mpr\textsuperscript{+/−}MEF (control) were incubated for 10 h, about 85% of the total β-hexosaminidase activity contained in the culture was found in the medium, and only 15% was found in the cells. Transfection of mpr\textsuperscript{+/−}MEF with LERP resulted in an increase of the cellular fraction of lysosomal glycosidases. Stable clones A–E expressing different levels of LERP were analyzed, demonstrating that the increase in intracellular lysosomal hydrolases depends on the level of receptor expression (Fig. 4A). The highest LERP expression level was found in clone E, which increases intracellular β-hexosaminidase activity from 15% to about 47% of total activity. Similar values were obtained for the distribution of β-glucuronidase (50%) and β-galactosidase (45%) (Fig. 4B). The level of LERP expression in mpr\textsuperscript{+/−}MEF cannot yet be estimated or compared with the endogenous level in Drosophila cells or even the MPR300 level since no antibodies are available for LERP at present.\(^2\)

The sorting of the lysosomal proteinases cathepsin D and cathepsin L to lysosomes in the LERP-transfected mpr\textsuperscript{+/−}MEF cells was demonstrated by the colocalization of the proteinases with lamp-1 (Fig. 5A for cathepsin D and 5B for cathepsin L). Analysis of the proteinases by immunoblotting revealed that expression of LERP clearly improves normal intracellular steady state concentrations of cathepsin D and cathepsin L (Fig. 5, A and B, respectively). The results were compared with MPR300-transfected mpr\textsuperscript{+/−}MEF. The increase in the intracellular detectable cathepsin D protein was from 54.4% in non-transfected mpr\textsuperscript{+/−}MEF cells to 94.9% by LERP expression and to 95.2% by MPR300 expression. Similar values were obtained for cathepsin L (Fig. 5B), where 93.1% (LERP) and 93.9% (MPR300) were measured intracellularly as compared with 51.8% in non-transfected mpr\textsuperscript{+/−}MEF. Moreover, precursor forms of the proteins were properly processed. The relative distribution of the precursor forms and the intermediate forms of cathepsin D and cathepsin L in LERP- or MPR300-transfected mpr\textsuperscript{+/−}MEF cells is identical.

LERP Binds to Mammalian GGAs and to the Drosophila GGA—The results shown above establish that LERP conveys Golgi-endosome transport in mammalian cells. The cytoplasmic domain of LERP is a candidate for the interaction with adaptor molecules involved in this type of sorting. The recently discovered GGAs play an important role in the Golgi-endosome trafficking of the two MRPs and the Vps10p-related receptor sorting. The C-terminal acidic dileucine motif (DXXLL) essential for the interaction of the GGAs with the receptor domains is found also in the LERP cytoplasmic domain (Fig. 6). The VHS domains of the GGAs have been shown to mediate the interaction with the DXXLL motif in the cytoplasmic domain of the MRPs. One Drosophila counterpart for GGA, CG3002-RB, has been annotated in the FlyBase data base (flybase.net) and by Boehm and Bonifacino (19). We sequenced the corresponding EST clone LD41311, and the sequence data have been submitted to the GenBankTM data base under accession number AY734458. The amino acids 1–147 represent the VHS

\(^2\) A. Dennes, C. Cromme, K. Suresh, N. S. Kumar, J. A. Eble, A. Hahnenkamp, and R. Pohlmann, manuscript in preparation.
domain as deduced from homology comparisons with the human GGAs (Fig. 7).

Experiments were performed to clarify whether LERP could in fact bind to GGAs. The LERP cytoplasmic domain (48 amino acids) was expressed in fusion with MBP and tested for interaction in vitro by pull-down experiments using GST-GGA fusion proteins comprising human GGA1-VHS and GGA2-VHS domains. LERP binding to the VHS domains of human GGA1 was demonstrated in Fig. 8A. In the controls, neither MBP nor the MBP fusion proteins bound to GST fusion proteins, whereas a concentration-dependent co-precipitation of the MBP fusion protein with the LERP cytoplasmic domain was found for both GST-GGA1-VHS and GST-GGA2-VHS (not shown). The interaction of the LERP cytoplasmic domain with GGA1 was clearly stronger than with GGA2 and was comparable with the results obtained with the cytoplasmic domains of receptors known to bind GGAs and to mediate Golgi-lysosome transport (MPR46, MPR300, Vps10p, and sortilin).

We also analyzed the homologous Drosophila GGA product for LERP interaction. We generated a fusion protein with GST comprising the amino acids 1–147 of the homologous Drosophila GGA and tested it for interaction with the LERP cytoplasmic domain as reported above for the mammalian GGAs. The LERP cytoplasmic domain was efficiently co-precipitated with the VHS domain of the homologous Drosophila GGA protein. Moreover, the interaction appeared to be much stronger than that with human GGAs (Fig. 8A). A complete loss of interaction was obtained for the mutation of LRRP at Leu47 or Leu48 to alanine (Fig. 8C, L47-A and L48-A).

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FIG. 5. Cathepsin D and cathepsin L in mpr(-/-)MEF expressing LERP. Top, cell homogenates (25 μg of protein) (C) and equal amounts of the medium (M) from 10-h cultured LERP-expressing mpr(-/-)MEF were analyzed for cathepsin D and cathepsin L by immunoblotting using polyclonal antisera specific for cathepsin D and cathepsin L. MPR300-transfected mpr(-/-)MEF and non-transfected mpr(-/-)MEF were analyzed for control. P (precursor) and I (intermediate) indicate the positions of the precursor and intermediate forms of cathepsin D (A) and cathepsin L (B), respectively, and M indicates the position of the mature form of cathepsin D (A). The signal intensities were calculated using the scion3-software (Scion Corp.). The relative distribution of cathepsin D and cathepsin L in cells and media, respectively, is given below the lanes. Bottom, the lysosomal localizations of cathepsin D and cathepsin L were analyzed by immunohistochemical analyses using specific polyclonal antisera. The lysosomal compartment was labeled by detection of the lysosomal membrane protein lamp-1 in mock-transfected cells (mpr(-/-)), in LERP-overexpressing cells (mpr(-/-)/LERP), and in MPR300-transfected cells (mpr(-/-)/MPR300). The colocalizations of the cathepsins and lamp-1 were shown by merging of the individual results. The bar indicates 10 μm.

FIG. 6. Comparison of the GGA-binding motif of LERP and transmembrane proteins with DXXLL-sorting motifs. Amino acids are shown in single-letter codes. Leucine residues or big hydrophobic residues (M and V) in position +3 and the aspartic acid residue in position 0 that are essential for the interaction with GGAs are highlighted in borders.
aspartic acid 44 to an asparagine residue (D44-N) as well as for changing both leucine residues simultaneously to alanines (l/48-AA) (Fig. 8C). Since the tested GST and MBP fusion proteins have been expressed in bacteria, we conclude that the interaction between the *Drosophila* LERP cytoplasmic domain and the VHS domain of GGAs functions independent from eucaryotic modifications. The presented data show that the molecular basic mechanism of interaction between acidic dileucine motifs and GGA-VHS domains already exists in lower metazoans.

**DISCUSSION**

**LERP Functions in Lysosomal Enzyme Sorting**—The function of MPRs in mammalian cells is well documented. The receptors are type I integral membrane proteins harboring signals for their intracellular trafficking in their cytoplasmic domains and target newly synthesized lysosomal enzymes to lysosomes. The presence of homologous genes or proteins for MPRs has been documented in non-mammalian vertebrates as well as invertebrates. In the *Drosophila* genome, carbohydrate recognition-like domains have been identified. However, none contains residues known to be essential for Man6P recognition in MPR (31). It is not known whether Man6P residues are synthesized in *Drosophila*, whereas conflicting data were provided for SF9 insect cells (32, 33).

We identified and characterized the novel *Drosophila* LERP. It shares structural and functional homology to the mammalian MPR300. LERP contains five repeating units in its luminal domain that display a homology of 23–29.5% at the amino acid level to those of the human MPR300. It should be mentioned that the homology between the single domains of the human MPR300 to each other and to that of the functional related MPR46 varies from 14 to 38% (2). This is in average not lower than to that the LERP domains. However, the residues known for Man6P binding are lacking in LERP. Thus it was a surprising result that *Drosophila* LERP rescues the missorting of lysosomal enzymes occurring in mammalian MPR-deficient cells. The sorting has been demonstrated for an overall set of Man6P-containing ligands known to bind to MPR300 as well as for selected enzymes, such as β-hexosaminidase, β-glucuronidase, and β-galactosidase. The sorting of the proteases cathepsin D and cathepsin L to lysosomes has been demonstrated by colocalization with lamp-1, a lysosomal membrane protein. These data strongly suggest that *Drosophila* LERP recognizes mammalian lysosomal enzymes. The basis for the recognition of mammalian lysosomal enzymes by LERP is unknown. No binding of LERP to a multimeric Man6P ligand could be detected. The sorting of mammalian lysosomal enzymes by LERP could also not be impaired by the addition of Man6P to the medium (not shown). We conclude from these findings that the mammalian lysosomal enzymes are recognized by the *Drosophila* LERP not via Man6P residues but probably by another common feature that they provide and which may be a glycan or simply a peptide structure. Altogether the data suggest that the Man6P recognition system may be a more recent development of animal cells. The analysis of the interaction of LERP and mammalian lysosomal enzymes might highlight common evolutionary characteristics of mammalian and *Drosophila* lysosomal enzymes.

Prerequisite for the analysis of the recognition mechanism of mammalian and *Drosophila* lysosomal enzymes by LERP will be the cloning of lysosomal enzymes from *Drosophila* and the analysis of their sorting mechanism. The availability of the RNA interference and knock-out technique for *Drosophila* will allow us to analyze the impact of LERP in *Drosophila*, i.e. whether the knock-out phenotype of the flies and the appearance of lysosomes will correlate to the mammalian counterparts. An open question is also which repeat of LERP mediates the binding of mammalian lysosomal enzymes and whether LERP is a multifunctional receptor like the mammalian MPR300, which by the diversity of its ligands is connected to processes like cell growth, cell migration, immunity, and apoptosis (2).

Multiple residues that build up the IGF2-binding pocket in domain 11 of MPR300 as well the essential isoleucine residue requested for IGF2 binding are highly conserved in domain 3 of LERP (see supplemental data, Fig. B). Therefore a further interesting aspect of investigation will be the analysis of the binding of IGF2 from mammalian species and homologs from *Drosophila* by LERP. However, in *D. melanogaster*, an IGF-like gene has to be identified.

The LERP Cytoplasmic Domain Interacts with the Cytoplasmic GGA Adaptors—The mammalian MPR300 is predominantly localized in perinuclear areas representing TGN-associated structures. From there, it cycles to endosomal compartments to transport lysosomal enzymes from their place of biogenesis to their site of function, the lysosomes (see Ref. 2 and references therein). Heterologous expression of a HA-tagged LERP in mammalian cells revealed an intracellular distribution similar to the MPR300 (Fig. 2). This distribution fits very well to the demonstrated lysosomal sorting function of LERP shown in this work. Together, the common distribution and the common sorting function, both provide evidence that LERP is, like MPR300, recognized by key organizers responsible for this specific vesicular sorting pathway.
In mammals, GGAs have been shown to be essential for transport of MPRs and other cargo from the TGN to endosomes. Sequences homologous to the GGA proteins have been identified in Dro sophila and other non-mammalian species, but no analyses have been performed on their expression pattern, localization, or function (19). Acidic dileucine motifs resembling a DXXLL consensus sequence are anticipated as key residues for the binding of cytoplasmic domains of transmembrane cargo to mammalian GGAs via their VHS domains (8–11, 34). A corresponding acidic dileucine motif is also present in the Drosophila LERP (Fig. 6, aligned motifs). Here we have shown for the first time that a homologous GGA from a non-mammalian species recognizes the same elements, the acidic dileucine motifs known to be involved in the regulation of transmembrane cargo proteins. In more detail, we confirmed the importance of the aspartic acid residue and the dileucine residues in the DXXLL motif of LERP for binding to the VHS domain of Drosophila GGA.

The overall binding features and the specific interactions of the three essential residues were the same as in the transmembrane cargo proteins: MPR, β-secretase, LDL receptor-related protein 3 (LRP3), sortilin, and sorLA, in which the interaction with human GGAs has been analyzed (8–12, 35). These residues mediate the interaction with all three identified human GGAs (see references above). Three groups have reported on the crystal structure of the VHS domains of human GGA1, GGA2, and GGA3 complexed with the acidic dileucine motifs of the MPRs and β-secretase (34, 36–38). The VHS domain is a right-handed superhelix of eight helices. Helices 6 and 8 form a surface that makes extensive contacts with amino acids of the C-terminal acidic dileucine motif in the cytoplasmic domains of transmembrane cargo. Among these residues, the dileucine residues and the aspartic acid residue at position 0 have the most extensive interactions with the VHS-binding site, whereas the other residues appear to contribute to a lesser extent to the binding. Briefly, it was shown that the aspartic acid residue (position 0) interacts with three basic groups of GGA-VHS Lys175, Arg180, and Lys191. One of the essential leucine residues at position +3 is interacting with Phe195, Met198, and Ile206, whereas the other at position +4 interacts with Lys175 and Tyr192 (34, 36). VHS residues involved in binding of the DXXLL motif are conserved in mammalian GGAs, and comparison of the amino acid sequence of the VHS domain from those predicted in Drosophila GGA homolog revealed that all of these residues are conserved (Fig. 6). Altogether the presented data demonstrate that the basic mechanism of sorting receptors by interaction with cytoplasmic GGA adaptors has already been established in these lower metazoans and that the same motifs and similar proteins are mediating the interaction in the novel LERP sorting receptor from Drosophila.

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