ACADEMIC PRESS

Biochemical and Biophysical Research Communications 295 (2002) 149–156

BBRC

www.academicpress.com

# Ile (476), a constituent of di-leucine-based motif of a major lysosomal membrane protein, LGP85/LIMP II, is important for its proper distribution in late endosomes and lysosomes

Norihiko Tabuchi, Kenji Akasaki,* and Hiroshi Tsuji

*Faculty of Pharmacy and Pharmaceutical Sciences, Fukuyama University, Fukuyama 729-0292, Japan*

Received 28 May 2002

**Abstract**

Lysosomal membrane glycoprotein termed LGP85 or LIMP II extends a COOH-terminal cytoplasmic tail of R459GQGSMDEGTADERAPLIRT478, in which an L475 I476 sequence lies as a di-leucine-based motif for lysosomal targeting. In the present study, we explored the role of the I476 residue in the localization of LGP85 to the endocytic organelles using two substitution mutants called I476A and I476L in which alanine and leucine are replaced at I476, respectively, and I476R477T478-deleted LGP85 called Δ 476–478. Immunofluorescence analyses showed that I476A and I476L are largely colocalized in intracellular organelles with an endogenous late endosomal and lysosomal marker, LAMP-1, but there were some granules in which staining for the LGP85 mutants was prominent, while Δ 476–478 is detected in LAMP-1-positive and LAMP-1-negative intracellular organelles, and on the cell surface. The subcellular fractionation studies revealed that I476A, I476L, and Δ 476–478 are different from wild-type LGP85 in the distribution of early endosomes, late endosomes, and lysosomes. I476A and I476L are present more in late endosomes than in the densest lysosomes, whereas wild-type LGP85 is mainly lysosomal. Substitution of I476 for A and L differentially modified the ratios of late endosomal to lysosomal LGP85. A major portion of Δ 476–478 resided in the light buoyant density fraction containing plasma membrane and early endosomes. Taken together, these results indicate that the existence of the 476th amino acid residue is essential for localization of LGP85 to late endocytic compartments. The fact that isoleucine but not leucine is in the 476th position is especially of importance in the proper distribution of LGP85 in late endosomes and lysosomes. 

*Keywords:* Di-leucine sorting motif; Endosome; Lysosome; Lysosomal membrane glycoprotein

Lysosomes are membrane-bound, cytoplasmic organelles involved in intracellular degradation of macromolecules sequestered through endocytosis and autophagocytosis [1]. They contain an assortment of soluble acid-dependent hydrolases and a set of highly glycosylated integral membrane proteins [1–4]. There are complicated intracellular pathways for lysosomal delivery of newly synthesized acid hydrolases and lysosomal membrane proteins [2–8]. Lysosomal proteins are synthesized in endoplasmic reticulum and then transferred to the Golgi apparatus, where these proteins undergo some modifications such as the formation of mannose 6-phosphate residues on sugar chains of lysosomal hydrolases. Then, newly synthesized lysosomal proteins enter the trans-Golgi network (TGN) where mannose 6-phosphate residues on the soluble lysosomal enzymes are recognized by two types of mannose 6-phosphate receptors (MPRs). MPR and lysosomal hydrolase complexes are included into the clathrin-coated vesicles at TGN by interaction of MPRs' cytoplasmic tails with either adaptor protein (AP) complex-1 or Golgi-localized, gamma-ear-containing, ARF binding proteins [6,8]. Lysosomal membrane proteins are recruited into clathrin-coated vesicles at TGN by virtue of AP-1, -3, and -4, which bind to cytoplasmic tails of these membrane proteins [9–17]. The vesicles bearing lysosomal hydrolases and membrane proteins are transported to early and/or late endosomes, and then the

* Corresponding author. Fax: +81-84-936-2024.
*E-mail address:* akasaki@fupharm.fukuyama-u.ac.jp (K. Akasaki).

0006-291X/02/$ - see front matter 
PII: S0006-291X(02)00642-3

proteins become exclusive in localization to lysosomes [3,4,6,35].

It has been shown that certain amino acid sequences in COOH-terminal cytoplasmic tails of membrane proteins are important for their proper localization in the endosomal and lysosomal system [4,18]. Two types of amino acid sequences have thus far been identified in the lysosomal membrane proteins for the lysosomal targeting and MPRs for the late endosomal targeting. One of these is the GYXX Ø motif in which X is any amino acid and Ø is a bulky hydrophobic amino acid [19–22]. The other is a di-leucine-based motif [23–30]. Recent studies have shown that acidic amino acid residues upstream of the di-leucine-based motif are also critical for the targeting to late endosomes and lysosomes [31,32] or trafficking through the endosomal and lysosomal system [33].

LGP85 (also called LIMP II) has a 20 amino acid cytoplasmic tail at the COOH-terminus whose amino acid sequence is R459GQGSMDEGTADERAPLIRT 478. L475 and I476 in its cytoplasmic tail form the di-leucine-based motif [25,26,33]. Substitution mutation at the leucine residue with any amino acid completely abolishes or remarkably reduces the localization of LGP85 to lysosomes, resulting in its appearance on the cell surface [25]. On the other hand, the mutants in which isoleucine is replaced by alanine and leucine are still localized in the intracellular granules. Therefore, it is very likely that the first leucine residue in the motif is more dominant in lysosomal targeting of LGP85 than the second isoleucine; the isoleucine residue seems to play an auxiliary role in the lysosomal targeting. In addition, the COOH-terminal cytoplasmic tail of LGP85 is bound to either AP-1 or AP-3 in vitro [11,14]. L of the LI motif is essential for binding of the LGP85 tail to the two adaptor proteins, whereas little is known about the significance of the isoleucine residue in the binding. Thus, there has been no substantial information concerning the exact role(s) of I of the LI signal in the transport of LGP85 through the biosynthetic and endocytic pathways.

We have recently established separation conditions for endocytic organelles (early endosomes, late endosomes, and lysosomes) from human hepatoma cell lines (HepG2 cells) using the Percoll density centrifugation method [33]. This fractionation procedure enabled us to quantify the steady-state subcellular distributions of wild-type and mutant LGP85 in the endocytic organelles and clarify the roles of D470 and E471 preceding the LI sequence in the LGP85 tail. To elucidate the role(s) of I467, we stably expressed two substitution mutants at I476 and an I476-T478 deletion mutant of LGP85 in HepG2 cells and measured their contents in the endocytic compartments after the subcellular fractionation of the stably expressed cells. Our data here indicate that I476 plays a critical role in the proper distribution of LGP85 in late endosomes and lysosomes.

## Materials and methods

*Materials*. Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were purchased from Osaka Biken (Osaka, Japan) and Boehringer Mannheim GmbH (Mannheim, Germany), respectively. Oligonucleotides for PCR primers were obtained from Takara Shuzo (Kyoto, Japan). Percoll was purchased from Pharmacia Biotec (Uppsala, Sweden). Glycyl-L-phenylalanine 2-naphthylamide (GPN) was from Sigma (St. Louis, MO). All reagents were of analytical grade. Specific rabbit anti-LGP85 polyclonal antibody was prepared as described previously. Anti-human LAMP-1 monoclonal antibody (H4A3) was kindly provided by Dr. J.T. August (Johns Hopkins University).

*Mutagenesis*. Wild-type LGP85 cDNA was cloned into pcDNA3.1 mammalian expression vector (Invitrogen, San Diego, CA) as described previously. cDNAs encoding substitution and deletion mutants of LGP85 were generated by polymerase chain reaction (PCR). A forward primer used commonly for the substitution and deletion mutants was 5′-TTTAAGCTTATCGCACCGGCTCTGCTGCTC-3′. The reverse primers for I476A, I476L, and Δ 476–478 were 5′-TTTTC TAGATTAGGTTCGTGCGAGGGGTGC-3′, 5′-TTTTCTAGATT AGGTTCGTAGGAGGGGTGC-3′, and 5′-TTTTCTAGATTAGA GGGGTGCTCTTTCATC-3′, respectively. The converted and stop codons are underlined. *Hin*dIII and *Xba*I restriction sites were introduced into the forward and reverse primers, respectively. The PCR products were gel-purified, digested with *Hin*dIII and *Xba*I and ligated to pcDNA3.1 vector treated with the same restriction enzymes. The mutations were verified by DNA sequencing using the dideoxy termination method with an ALF automated DNA sequencer (Pharmacia Biotec, Sweden). The sequence analyses confirmed that I476A, I476L, and Δ 476–478 had the cytoplasmic tails of R459GQG SMDEGTADERAPLART478, R459GQGSMDEGTADERAPLLR T478, and R459GQGSMDEGTADERAPL475, respectively.

*Cell culture and transfection*. Human hepatoma-derived HepG2 cell line was cultured in DMEM supplemented with 10% FCS. The expression vectors containing cDNAs encoding I476A, I476L, and Δ 476–478 were transfected into HepG2 cells. Stable transfectants were generated and the cell lines which synthesize these mutants at the same level as wild-type were selected by immunoblotting (data not shown).

*Immunofluorescence microscopy*. The cells expressing mutant LGP85s and wild-type LGP85 were plated on glass coverslips and grown to a confluency of 30%. The cells were fixed, permeabilized with saponin, and immunostained as described previously [33]. An anti-rat LGP85 rabbit antiserum and an anti-human LAMP-1 mouse monoclonal antibody were used as primary antibodies. To determine the cell surface expression of LGP85, these cells were fixed and indirectly immunostained with anti-rat LGP85 antiserum in the absence of saponin. Samples were visualized using a Zeiss LSM 410 confocal microscope system.

*Cell fractionation*. Subcellular fractions were obtained as already described [33]. Briefly, HepG2 cells were homogenized and then centrifuged at 650*g* for 10 min to precipitate the nucleus and cell debris. The postnuclear supernatant (PNS) was diluted with Percoll and centrifuged at 25,000 rpm for 40 min in a Beckman 70.1 Ti rotor. The gradient was divided into 18 fractions by downward displacement before removal of the Percoll particles with ultracentrifugation.

*Selective disruption of lysosomes*. PNS was incubated with 300 μM GPN in an isotonic sucrose solution at 37 °C for 10 min as described elsewhere [34,35] and separated by Percoll density centrifugation as stated above.

*SDS–PAGE and immunoblotting*. SDS–PAGE was performed on slab gel using 10% acrylamide according to the method of Laemmli [36]. Proteins in the SDS-slab gel were electrophoretically transferred to a Zeta-probe nylon membrane (Bio-Rad, Richmond, VA), using a modified procedure of Towbin et al. [37]. The positive bands were visualized by means of ECL Western blotting detection kits (Amersham, Bucks, UK). The quantitative densitometric analysis of

the blots was performed using a Gel-Pro analyzer (Media Cybernetics, Silver Spring, MD) according to the method of Guengerich et al. [38].

*Other procedures*. HRP was assayed by fluorometry using 3-(*p*-hydroxyphenyl)propionic acid and hydrogen peroxide as the substrates according to the method of Zaitsu and Ohkura [39]. Phosphodiesterase I and β-glucuronidase were assayed by the methods of Brightwell et al. [40] and Robins et al. [41], respectively. Protein concentrations were determined by means of the Bio-Rad protein assay kit using bovine serum albumin as the standard.

## Results

### *Immunofluorescence microscopic analysis*

We analyzed the cellular localization of I476A, I476L, Δ 476–478, and wild-type LGP85 in their respective cells by immunofluorescence microscopy (Fig. 1). Wild-type LGP85 was almost completely colocalized with the endogenous late endosomal and lysosomal marker, LAMP-1 (Figs. 1J–L). Double staining experiments for LAMP-1 and I476A or I476L showed a large overlap of the two signals, but there were some granules in which staining for the LGP85 mutants (green) was prominent (Figs. 1A–C for I476A and D–F for I476L). Subsequently, HepG2 cells were stably transfected with the DNA construct encoding LGP85 with a cytoplasmic tail in which the COOH-terminal of three amino acid residues including I476 was truncated (Δ 476–478) and analyzed by immunofluorescence microscopy (Figs. 1G and H). Δ 476–478 was detectable not only in perinuclear LAMP-1-positive structures but also in cell peripheral small vesicles which are devoid of LAMP-1. Δ 476–478 resided significantly on the cell surface as visualized in green outlines (Fig. 1I). The presence of Δ 476–478 on the plasma membrane was also revealed by the fact that a significant fluorescence reaction was observed when the Δ 476–478 was processed for immunomicroscopic analyses without detergents (Fig. 1O).

### *Separation of late endosomes from lysosomes in the Percoll density gradient*

We have previously established a fractionation method to separate three major endocytic compartments from HepG2 cells using Percoll gradient centrifugation [33]. As shown in Figs. 2B and C, wild-type LGP85 is distributed with two peaks. A major portion of wild-type LGP85 was located in the densest lysosomal fraction; it formed a high peak at fraction 17, which had a shoulder over fractions 13–15. Since fractions 13–15 contain late endosomes as assessed by the previous kinetic data of endocytic transport of asialofetuin (Fig. 4 in Ref. [33]), late endosomes were expected to be co-distributed significantly with lysosomes in these fractions. The overlap of late endosomes and lysosomes interferes with the exact quantitative analyses of the late endosomal and lysosomal LGP85 proteins. To overcome this difficulty, we used glycyl-L-phenylalanine 2-naphthylamide (GPN), which disrupts lysosomes but not late endosomes in rat hepatocytes [34,35]. Figs. 2B and C show the distribution of wild-type LGP85 in the Percoll density gradient after incubation of PNS with GPN. After GPN-reatement, wild-type LGP85 was drastically reduced in the densest fraction (fractions 16–18), while wild-type LGP85 remained as a broad peak around fraction 15 (Fig. 2C), indicating that the remaining broad peak around fraction 15 reflects net late endosomal LGP85. A similar decrease in the densest lysosomal fraction was observed for endogenous β-glucuronidase (Fig. 2A). The amounts of late endosomal and lysosomal LGP85s are estimated to be approximately 28% and 45% of the total, respectively (Fig. 4). These values are notably different from those calculated from the distribution profile without the GPN-treatment: lysosomal LGP85 was overestimated by 10% in the previous study [33].

### *Distribution of I476A, I476L, and Δ 476–478 in the Percoll gradient*

We investigated the relative abundance of the three LGP85 mutants in the Percoll density gradient. As shown in Fig. 3A, I476A was distributed with a major peak around fraction 15 and a minor peak around fraction 6, whereas it resides little in the densest fraction. Since late endosomes are recovered predominately in fraction 15, it is likely that the majority of I476A is localized to late endosomes. After GPN-treatment, the distribution profile of I476A was not significantly changed (data not shown). As shown in Fig. 4, late endosomal I476A amounted to 40% of the total, while lysosomal I476A was less than 10%. I476L showed a broader distribution through fractions 4–18 and caused a small lysosomal accumulation (Fig. 3B). Nevertheless, I476L was enriched in the late endosomal fraction approximately 2.5-fold more than in the lysosomal fraction (Fig. 4). The ratios of lysosomal/late endosomal LGP85 were different when I476 was substituted by A476 and L476 (Fig. 4). These data suggest that the distribution of LGP85 between late endosomes and lysosomes is exactly determined by the nature of the amino acid in the 476th position. Similar phenomena have been observed for the COOH-terminal amino acid in GYXX Ø type of lysosomal targeting signal in the cytoplasmic tail of LAMP-2 [15]. Changes of the hydrophobic amino acid at Ø alter the cellular localization of LAMP-2.

Δ 476–478 was accumulated in the light buoyant density fraction (Fig. 3C). Our previous fractionation study revealed that this light density fraction contains the plasma membrane and early endosomes [33]. To-

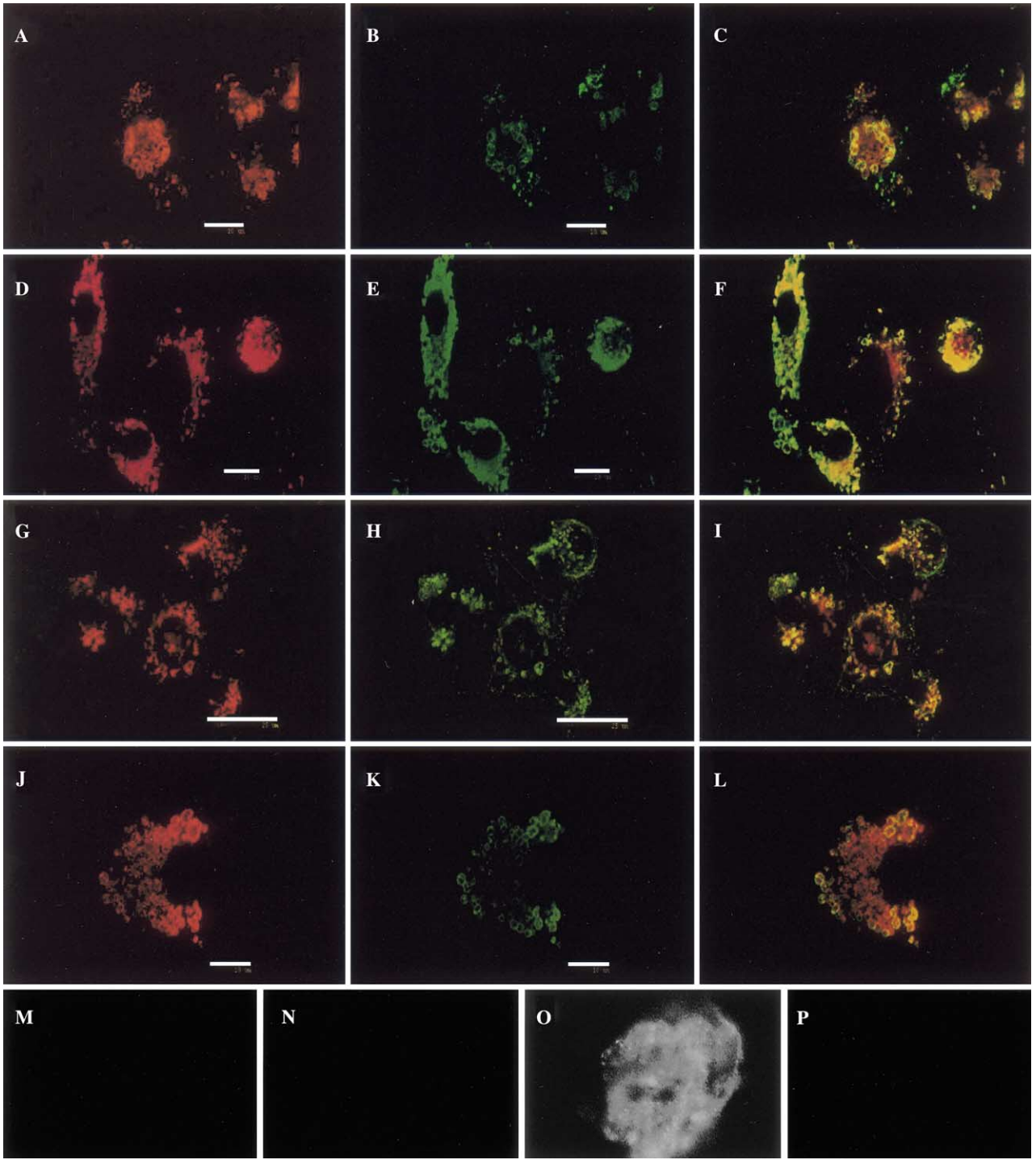


Fig. 1. Subcellular localization of LGP85 following mutation at I476 in the C-terminal LI motif. (A–I) Stable HepG2 cell lines expressing I476A, I476L, Δ 476–478, and wild-type LGP85 were generated as described in Materials and methods. Cells were fixed, permeabilized, and stained by indirect immunofluorescence staining for endogenous LAMP-1 (A, D, G, and J) and transfected LGP85 mutants, I476A (B), I476L (E), Δ 476–478 (H), and wild-type LGP85 (K). Anti-LAMP-1 mouse monoclonal antibody followed by Texas Red-coupled secondary antibody and anti-LGP85 rabbit polyclonal antibody followed by FITC-coupled secondary antibody were used to detect LAMP-1 and LGP85, respectively. The images shown in C, F, I, and L were obtained by overlaying A and B, D and E, G and H, and J and K, respectively. Bars, 10 μm for A, B, D, E, J, and K; 25 μm for G and H. (M–P) The cells expressing I476A (M), I476L (N), Δ 476–478 (O), and wild-type LGP85 (P) were fixed and indirectly labeled with anti-LGP85 antibody without saponin permeabilization.

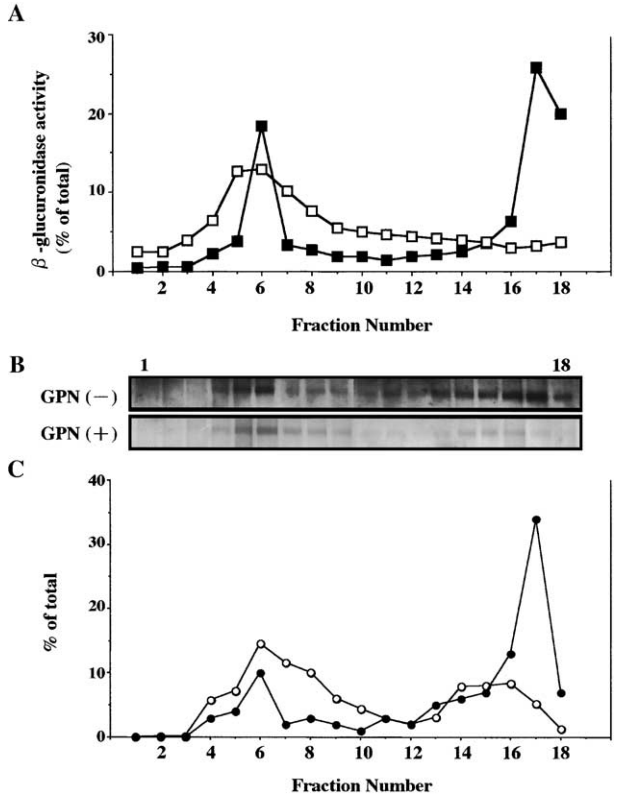


Fig. 2. Selective disruption of lysosomes with glycl-L-phenylalanine 2-naphthylamide (GPN). PNS from HepG2 cells expressing wild-type LGP85 was incubated with 300 μM GPN in an isotonic solution at 37 °C for 10 min and then fractionated by Percoll density centrifugation. (A) Distribution of β-glucuronidase in the fractions obtained from PNS treated with (□) and without (■) GPN. (B) Western blotting of LGP85 in overall fractions obtained from PNS treated with (bottom) and without (top) GPN. Fraction numbers 1 and 18 are written above the top panel. (C) The blots in panel B were quantified by densitometric tracing and the amount of wild-type LGP85 in each fraction is expressed as a percentage of the total. Open (○) and closed (●) circles represent wild-type LGP85 distribution after treatment with and without GPN, respectively.

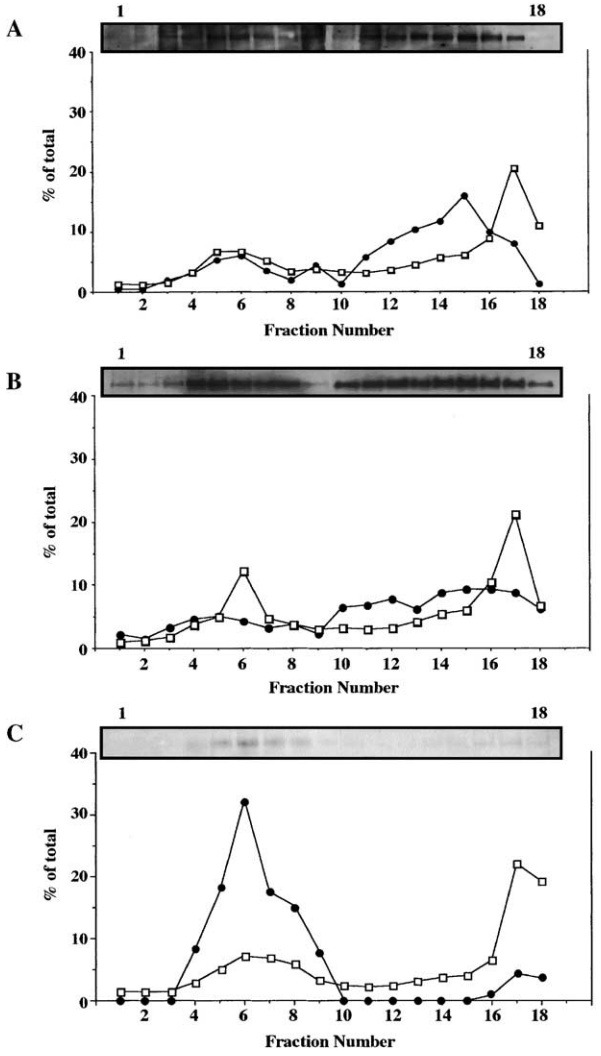


Fig. 3. Distribution of LGP85 mutants (I476A, I476L, and Δ 476–478) in the Percoll gradient. PNSs of HepG2 cells expressing I476A (A), I476L (B), and Δ 476–478 (C) are fractionated by Percoll density gradient centrifugation. Equivalent volumes of the fractions were subjected to Western blotting with anti-LGP85 antibody and β-glucuronidase assay. The resultant blots with fraction numbers 1 and 18 are shown in the upper panels. The lower panels show the quantification of LGP85 blots (●) and β-glucuronidase (○). The amounts of LGP85 mutants and β-glucuronidase in the Percoll density fractions were expressed as % of the total. Similar findings were obtained in three independent experiments.

gether with the immunofluorescence data shown in Fig. 1, Δ 476–478 is present on the cell surface and in intracellular organelles, most of which are early endosomes. A recent study has shown that the plasma membrane and early endosomal fraction in Percoll density gradient of NR6 cells contains multivesicular endosomes that bud from early endosomes [30]. Therefore, we cannot exclude the possibility that Δ 476–478 is also present in the multivesicular endosomes. The localization of β-glucuronidase in I476A-, I476L-, and Δ 476–478-expressing cells (Figs. 3A–C, open squares) is almost the same as that of wild-type LGP85-expressing cells (Fig. 2A), indicating that the differences between wild-type and mutant LGP85s in the Percoll density gradient distribution are not due to changes of lysosomal density of their respective cells.

## Discussion

Leu, Ile, Met, and Val have been identified as the COOH-terminal second amino acid residues of the di-leucine-based motif [4,32]. However, there have been few studies of the exact role(s) of the second residue in the endocytic transport and subcellular distribution of proteins carrying this motif. The di-leucine-based motif has been regarded to function in the same manner even if any of these bulky amino acids is deposited in the second residue. However, our results indicate stringency

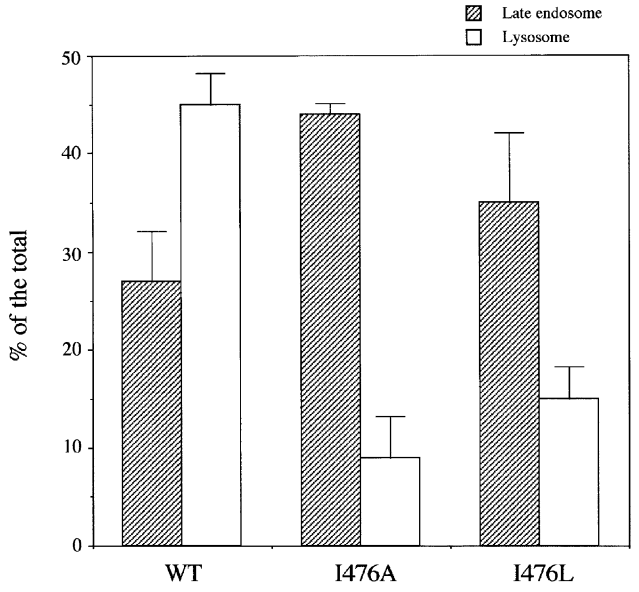


Fig. 4. Relative abundance of wild-type LGP85, I476A, and I476L between late endosomes and lysosomes. Late endosomal and lysosomal LGP85 proteins were accurately measured using GPN-incubation as shown in Fig. 2. Late endosomal LGP85 had a broad peak over fractions 13–17 after GPN-treatment, which accounts for the net late endosomal LGP85 free of lysosomal LGP85. The net lysosomal LGP85 could be obtained by subtracting LGP85 contents in fractions 16–18 after treatment with GPN from those after treatment without GPN. The quantification of I476A and I476L in late endosomes and lysosomes was conducted in the same manner. The amounts of wild-type LGP85 (WT), I476A, and I476L in late endosomes and lysosomes are shown as hatched and open columns, respectively. The data represent the average of the three independent experiments as % of the total.

of the second amino acid residue of this motif in LGP85: replacement of I476 for L476 altered the ratios of late endosomal/lysosomal LGP85, even though these two amino acids have the same size of bulky side chain and similar hydrophobicities (4.2 for Ile and 3.8 for Leu in hydropathy index). Therefore, identity of I476 determines a strict equilibrium of LGP85 with its content being 1.5 times higher in lysosomes than in late endosomes.

It is generally accepted that lysosomal membrane proteins constitutively shuttle between late endosomes and lysosomes [7,35]. The recent in vitro and in vivo studies have shown that trafficking of endocytosed molecules between late endosomes and lysosomes is mediated by hybrid organelles (reviewed in Ref. [7]). Late endosomes and lysosomes fuse into the hybrid organelles where cargo molecules are degraded by lysosomal acid hydrolases. Lysosomal components including lamp and acid hydrolases then move from the hybrid organelles to re-form lysosomes. According to this model, newly synthesized LGP85 (NS-LGP85) seems to be transferred from late endosomes to lysosomes during the recovery of the preexisting lysosomal proteins. I476 would determine the efficiency of NS-LGP85 trafficking from late endosomes to lysosomes at the retrieval step; insufficient inclusion of I476A and I476L into the retrieval flow probably causes their accumulation in late endosomes. Traub et al. [42] have shown that clathrin-coated vesicles containing AP-2 occur on dense lysosomes. These vesicles are considered to mediate retrograde transport from lysosomes. Since the cytoplasmic tail of wild-type LGP85 displays no or little association with AP-2 [11], it could hardly be recruited into the AP-2-containing vesicles. If I476A and I476L are efficiently secluded into the coated vesicles, they would readily return to late endosomes without their retention in lysosomes, but it is unclear whether these mutants could bind to AP-2 with high affinities.

It is of great interest that Δ 476–478 is concentrated in the light buoyant density fraction containing the plasma membrane and early endosomes (Fig. 3C). Combined with the immunofluorescence data (Figs. 1G–I, and O), Δ 476–478 resides mainly in early endosomes and on the cell surface. It is plausible that Δ 476–478 fails to enter clathrin-coated vesicles at TGN, causing its exclusive trafficking to the cell surface, because this deletion mutation impairs its interaction with AP-1 [14]. Although a fraction of Δ 476–478 could be internalized from the plasma membrane, then transferred to early endosomes, this deletion mutation would diminish the transport of LGP85 from early to late endosomes, resulting in its accumulation in early endosomes as observed for an alanine substitution mutant in the di-leucine-based motif of epidermal growth factor receptor [30]. It has been reported that AP-3 is localized mainly in early endosomes and AP-3 deficiency notably affects transport of LGP85 from early to late endosomes [13,16]. Additionally, the LI sequence of LGP85 is shown to be crucial for its binding to AP-3 [11]. Taken together, it seems reasonable that AP-3 is unable to mediate the early to late endosome transport of Δ 476–478, causing its accumulation in early endosomes. Further studies including the biosynthetic transport of wild-type and mutant LGP85s are required to address these speculations.

## References

[1] E. Holtzman, Lysosomes, Plenum Press, New York, 1989.
[2] E.C. Dell'Angelica, C. Mullins, S. Caplan, J.S. Bonifacino, Lysosome-related organelles, FASEB J. 14 (2000) 1265–1278.
[3] S. Kornfeld, I. Mellman, The biogenesis of lysosomes, Annu. Rev. Cell Biol. 5 (1989) 483–525.
[4] I.V. Sandoval, O. Bakke, Targeting of membrane proteins to endosomes and lysosomes, Trends Cell Biol. 4 (1994) 292–297.
[5] F. Gu, J. Gruenberg, Biogenesis of transport intermediates in the endocytic pathway, FEBS Lett. 452 (1999) 61–66.
[6] Y. RouillŽ, W. Rohn, B. Hoflack, Targeting of lysosomal proteins, Semin. Cell Dev. Biol. 11 (2000) 165–171.

[7] J.P. Luzio, B.A. Rous, N.A. Bright, P.R. Pryor, B.M. Mullock, R.C. Piper, Lysosome-endosome fusion and lysosome biogenesis, J. Cell Sci. 113 (2000) 1515–1524.
[8] E.C. Dell'Angelica, G.S. Payne, Intracellular cycling of lysosomal enzyme receptors, cytoplasmic tails' tales, Cell 106 (2001) 395–398.
[9] H. Ohno, J. Stewart, M.C. Fournier, H. Bosshart, I. Rhee, S. Miyatake, T. Saito, A. Gallusser, T. Kirchhausen, J.S. Bonifacino, Interaction of tyrosine-based sorting signals with clathrin-associated proteins, Science 269 (1995) 1872–1875.
[10] S. Höning, J. Griffith, H.J. Geuze, W. Hunziker, The tyrosine-based lysosomal targeting signal in lamp-1 mediates sorting into Golgi-derived clathrin-coated vesicles, EMBO J. 15 (1996) 5230–5239.
[11] S. Höning, I.V. Sandoval, K. von Figura, A di-leucine-based motif in the cytoplasmic tail of LIMP-II and tyrosinase mediates selective binding of AP-3, EMBO J. 17 (1998) 1304–1314.
[12] I. Rapoport, Y.C. Chen, P. Cupers, S.E. Shoelson, T. Kirchhausen, Dileucine-based sorting signals bind to the β chain of AP-1 at the site distinct and regulated differently from the tyrosine-based motif-binding site, EMBO J. 17 (1998) 2148–2155.
[13] R. Le Borgne, A. Alconada, U. Bauer, B. Hoflack, The mammalian AP-3 adaptor-like complex mediates the intracellular transport of lysosomal membrane glycoproteins, J. Biol. Chem. 273 (1998) 29451–29461.
[14] H. Fujita, M. Saeki, K. Yasunaga, T. Ueda, T. Imoto, M. Himeno, In vitro binding study of adaptor protein complex AP-1 to lysosomal targeting motif (LI-motif), Biochem. Biophys. Res. Commun. 255 (1999) 54–58.
[15] N.R. Gough, M.E. Zweifel, O. Martinez-Augustin, R.C. Aguilar, J.S. Bonifacino, D.M. Fambrough, Utilization of the indirect lysosome targeting pathway by lysosome-associated membrane proteins (LAMPs) is influenced largely by the C-terminal residue of their GYXX Ø targeting signals, J. Cell Sci. 112 (1999) 4257–4269.
[16] E.C. Dell'Angelica, V. Shotelersuk, R.C. Aguilar, W.A. Gahl, J.S. Bonifacino, Altered trafficking of lysosomal proteins in Hermansky–Pudlak syndrome due to mutations in the μ3A subunit of the AP-3 adaptor, Mol. Cell 3 (1999) 11–21.
[17] R.C. Aguilar, M. Boehm, I. Gorshkova, R.J. Crouch, K. Tomita, T. Saito, H. Ohno, J.S. Bonifacino, Signal-binding specificity of the μ4 subunit of the adaptor protein complex AP-4, J. Biol. Chem. 276 (2001) 13145–13152.
[18] I.S. Trowbridge, J.F. Collawn, C.R. Hopkins, Signal-dependent membrane protein trafficking in the endocytic pathway, Annu. Rev. Cell Biol. 9 (1993) 129–161.
[19] M.A. Williams, M. Fukuda, Accumulation of membrane glycoproteins in lysosomes requires a tyrosine residue at a particular position in the cytoplasmic tail, J. Cell Biol. 111 (1990) 955–966.
[20] C. Harter, I. Mellman, Transport of the lysosomal membrane glycoprotein lgp120 (lgp-A) to lysosomes does not require appearance on the plasma membrane, J. Cell Biol. 117 (1992) 311–325.
[21] S. Höning, W. Hunziker, Cytoplasmic determinants involved in direct lysosomal sorting, endocytosis, and basolateral targeting rat lgp120 (lamp-I) in MDCK cells, J. Cell Biol. 128 (1995) 321–332.
[22] F.G. Guarnieri, L.M. Arterburn, M.B. Penno, Y. Cha, J.T. August, The motif Tyr-X-X-hydrophobic residue mediates lysosomal membrane targeting of lysosome-associated-membrane protein 1, J. Biol. Chem. 268 (1993) 1941–1946.
[23] F. Letourneur, R.D. Klausner, A novel di-leucine motif and a tyrosine-based motif independently mediate lysosomal targeting and endocytosis of CD3 chains, Cell 69 (1992) 1143–1157.
[24] K.F. Johnson, S. Kornfeld, A His-Leu-Leu sequence near the carboxyl terminus of the cytoplasmic domain of the cation-dependent mannose 6-phosphate receptor is necessary for the lysosomal enzyme sorting function, J. Biol. Chem. 267 (1992) 17110–17115.
[25] I.V. Sandoval, J.J. Arredondo, J. Alcalde, A. Gonzalez Noriega, J. Vandekerckhove, M.A. Jimenez, M. Rico, The residues Leu(Ile)475-Ile(Leu, Val, Ala)476, contained in the extended carboxyl cytoplasmic tail, are critical for targeting of the resident lysosomal membrane protein LIMP II to lysosomes, J. Biol. Chem. 269 (1994) 6622–6631.
[26] S. Ogata, M. Fukuda, Lysosomal targeting of Limp II membrane glycoprotein requires a novel Leu-Ile motif at a particular position in its cytoplasmic tail, J. Biol. Chem. 269 (1994) 5210–5217.
[27] S. Kang, L. Liang, C.D. Parker, J.F. Collawn, Structural requirements for major histocompatibility complex class II invariant chain endocytosis and lysosomal targeting, J. Biol. Chem. 273 (1998) 20644–20652.
[28] P.A. Calvo, D.W. Frank, B.M. Bieler, J.F. Berson, M.S. Marks, A cytoplasmic sequence in human tyrosinase defines a second class of di-leucine-based sorting signals for late endosomal and lysosomal delivery, J. Biol. Chem. 274 (1999) 12780–12789.
[29] T. Simmen, A. Schmidt, W. Hunziker, F. Beermann, The tyrosinase tail mediates sorting to the lysosomal compartment in MDCK cells via a di-leucine and a tyrosine-based signal, J. Cell Sci. 112 (1999) 45–53.
[30] S.J. Kil, C. Carlin, EGF receptor residues Leu679, Leu680 mediate selective sorting of ligand-receptor complexes in early endosomal compartments, J. Cell Physiol. 185 (2000) 47–60.
[31] L. Pond, L.A. Kuhn, L. Teyton, M.P. Schutze, J.A. Tainer, M.R. Jackson, P.A. Peterson, A role for acidic residues in di-leucine motif-based targeting to the endocytic pathway, J. Biol. Chem. 270 (1995) 19989–19997.
[32] I.V. Sandoval, S. Martinez-Arca, J. Valdueza, S. Palacios, G.D. Holman, Distinct reading of different structural determinants modulates the di-leucine-mediated transport steps of the lysosomal membrane protein LIMPII and the insulin-sensitive glucose transporter GLUT4, J. Biol. Chem. 275 (2000) 39874–39885.
[33] N. Tabuchi, K. Akasaki, H. Tsuji, Two acidic amino acid residues, Asp(470) and Glu(471), contained in the carboxyl cytoplasmic tail of a major lysosomal membrane protein, LGP85/LIMP II, are important for its accumulation in secondary lysosomes, Biochem. Biophys. Res. Commun. 270 (2000) 557–563.
[34] T.O. Berg, P.E. StrØmhaug, T. LØvdal, P.O. Seglen, T. Berg, Use of glycyl-L-phenylalanine-2-naphthylamide, a lysosome-disrupting cathepsin C substrate, to distinguish between lysosomes and prelysosomal endocytic vacuoles, Biochem. J. 300 (1994) 229–236.
[35] K. Akasaki, A. Michihara, K. Mibuka, Y. Fujiwara, H. Tsuji, Biosynthetic transport of a major lysosomal membrane glycoprotein, lamp-1: convergence of biosynthetic and endocytic pathways occurs at three distinctive points, Exp. Cell Res. 220 (1995) 464–473.
[36] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[37] H. Towbin, T. Staehelin, J. Gordon, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications, Proc. Natl. Acad. Sci. USA 76 (1979) 4350–4354.
[38] F.P. Guengerich, P. Wang, N.K. Davidson, Estimation of isozymes of microsomal cytochrome P-450 in rats, rabbits, and humans using immunochemical staining coupled with sodium dodecyl sulfate–polyacrylamide gel electrophoresis, Biochemistry 21 (1982) 1698–1706.
[39] K. Zaitsu, Y. Ohkura, New fluorogenic substrates for horseradish peroxidase: rapid and sensitive assays for hydrogen peroxide and the peroxidase, Anal. Biochem. 109 (1980) 109–113.

[40] R. Brightwell, A.L. Tappel, Subcellular distributions and properties of rat liver phosphodiesterases, Arch. Biochem. Biophys. 124 (1968) 325–332.
[41] E. Robins, H.E. Hirsch, S.S. Emmons, Glycosidases in the nervous system. I. Assay, some properties, and distribution of β-galactosidase, β-glucoronidase, and β-glucosidase, J. Biol. Chem. 243 (1968) 4246–4252.
[42] L.M. Traub, S.I. Bannykh, J.E. Rodel, M. Aridor, W.E. Balch, S. Kornfeld, AP-2-containing clathrin coats assemble on mature lysosomes, J. Cell Biol. 135 (1996) 1801–1814.