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Targeting of Wild-Type and Mutated Forms of Lysosome-Associated Membrane Protein-1 (LAMP-1) to Late Endosomes/Lysosomes Depends on Affinities of Their Cytoplasmic Tail Peptides with a Medium Subunit of Adaptor Protein Complex-3 (AP-3)

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Lysosome-associated membrane protein-1 (LAMP-1) is a type I membrane glycoprotein with a COOH-terminal cytoplasmic tail (CT) containing a lysosome-targeting signal of GYQTI^{382}-COOH. This sequence is categorized as a tyrosine-based motif of GYXXΦ where Φ is a bulky hydrophobic amino acid residue. Lysosomal localization of LAMP-1 varies by changing the COOH-terminal amino acid residues. Adaptor protein (AP) complexes are reported to recognize the tyrosine-based signal peptide for efficient lysosomal transport of LAMP-1. In order to better understand the role of APs in lysosomal transport of LAMP-1, we have studied interactions of wild-type (WT) and mutated CTs of LAMP-1 with medium (μ) subunits of the four APs by a yeast two-hybrid (Y2H) system and subsequent computer-based molecular modeling. Among the μ subunits of AP-1, AP-2, AP-3 and AP-4, called μ1, μ2, μ3A, and μ4, respectively, the WT-CT significantly interacted with μ3A in the Y2H system. The degree of interaction of the WT and mutated CTs with μ3A from the Y2H analyses correlated with that of their dissociation constants determined by computer-based molecular modeling, and also with that of the late endosomal and lysosomal amount of WT and the similar mutants of LAMP-1. These results reinforce the notion that AP-3 makes a major contribution to the vesicular transport of LAMP-1 to late endosomes and lysosomes *via* a direct intracellular route.

Key words lysosome, membrane, glycoprotein, adaptor protein complex, tyrosine-based motif

INTRODUCTION

Lysosomes, intracellular digestive organelles, function dynamically at the final stage of endocytic and autophagic processes.[1–3] To degrade various biomacromolecules sequestered into lysosomes, approximately 60 kinds of acid hydrolases are enclosed by the limiting membrane of a lysosome. Lysosome-associated membrane proteins (LAMP)-1 and LAMP-2 represent a major portion of the lysosomal membrane proteins[4,5] and may have an important role in lysosomal integrity.[6] These two proteins share some structural similarities; a large and highly glycosylated luminal domain, a single transmembrane domain and a short cytoplasmic tail (CT) at the COOH-terminus.[7] For targeting of LAMP-1 and LAMP-2 to lysosomes, they have tyrosine-based motifs in their CTs, which conform to GYXXΦ, where Φ is a hydrophobic amino acid residue.[7–11,18] Lysosomal localization of LAMP-1 varies by changing hydrophobic amino acid residues in the Φ position.[6,8,11] The COOH-terminal isoleucine intrinsic to wild-type (WT)-LAMP-1 is optimal for its efficient targeting to dense lysosomes.[6,11]

The tyrosine-based motifs are well known to interact with medium subunits μ1, μ2, μ3 (A or B), and μ4 of adaptor protein (AP) complexes AP-1, AP-2, AP-3, and AP-4, respectively (μ3A and μ3B are ubiquitous and neuron-specific forms, respectively).[10,12–15] The interactions of GYXXΦ to AP complexes cause selective incorporation of the integral membrane proteins such as LAMP-1 into clathrin-coated vesicles that carry these cargo proteins to their own cellular destinations.[16–18] AP-1 mediates the trafficking of membrane proteins from the *trans*-Golgi network (TGN) to the endosomal-lysosomal compartments as well as AP-4 while AP-2 facilitates internalization of endocytic receptors from the plasma membrane. AP-3 is considered to be involved in an alternative protein delivery from the TGN, early endosomes, or tubular sorting endosomes to late endosomes and lysosomes.

Precisely which AP(s) mediates the vesicular transport of LAMP-1 remains controversial. A small fraction of LAMP-1 exists with mannose 6-phosphate receptors (MPRs) in AP-1-associated clathrin-coated vesicles[19] and LAMP-1's CT strongly binds to AP-1 and AP-2.[20] However, in μ1A-deficient cells, there is no change in the intracellular localization of LAMP-1 while the distribution of MPRs is altered.[21] It has been shown that AP-3 and LAMP-1 are colocalized in endosome-associated tubules,[22] and that dysfunction or defect of AP-3 in mammalian cells leads to altered transport of the lysosomal membrane proteins, LAMP-1(GYQTI), endolyn (NYHTL), and CD63 (GYEVM) whose lysosomal targeting signals are written in parentheses, respectively,[22–27] indicating a role of AP-3 in lysosomal transport of LAMP-1. On the oth-

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er hand, depletion of AP-2 from HeLa cells caused a significant decrease in newly synthesized LAMP-1 transported to lysosomes,[28] suggesting that AP-2 is involved in targeting of LAMP-1 to lysosomes.

The synthetic CT peptide of LAMP-1 binds to both purified AP-1 and AP-2 *in vitro*[19,20] while it binds preferentially to AP-3 on liposomes.[27] Interaction of LAMP-1's tyrosine-based motif with these medium subunits has been extensively studied using the yeast-two hybrid (Y2H) method.[12–15] Ohno *et al.*[12,13] have shown that the LAMP-1's motif as a triple repeat interacts significantly with μ1 and μ2 but not as a single copy at the end of the TGN38's tail peptide. LAMP-1's CT exhibits a clear interaction with μ3A but little with μ1, μ2, and μ4.[15]

As described above, WT and various COOH-terminal mutants of LAMP-1 are different in the late endosomal and lysosomal abundance.[11] The most efficient trafficking was observed for WT (GYQTI) and the second most was I382L (GYQTL). Lower levels of I382F (GYQTF), I382M (GYQM), and I382V (GYQTV) were present in the late endosomal and lysosomal fraction. In order to confirm the role(s) of APs in the lysosomal transport of LAMP-1, we examined the interactions of its WT and mutant CTs with μ1, μ2, μ3A, and μ4 by the Y2H method. The CT of WT-LAMP-1 makes a notable interaction with μ3A but not with the other μ subunits. The binding strengths (K_d) of the WT and mutant CTs with μ3A were calculated on the basis of computer-based molecular modeling using the crystal structures of μ subunits.[29,30] In our investigations, the K_d values well correlated with the Y2H-measured interaction strengths and the order of interaction strengths of the WT and mutant CTs with μ3A agreed well with that of late endosomal and lysosomal amounts of WT and similarly mutated LAMP-1. These results ensure that AP-3 makes a major contribution to vesicular transport of LAMP-1 to late endosomes and lysosomes *via* a direct intracellular route.

MATERIALS AND METHODS

Materials MATCHMAKER Gold Yeast Two-Hybrid System was purchased from Clontech Laboratories Inc. (Palo Alto, CA). pGAD10-μ1, pGAD10-μ2, pGAD10-μ3A, and pGAD10-μ4 were kindly gifted from Prof. Kazuhisa Nakayama (Kyoto University, Kyoto, Japan). WT and mutant LAMP-1 pcDNA3.1 constructs were prepared previously.[11]

Plasmid Construction cDNA fragments corresponding to the cytoplasmic tails (CTs) (amino acids 372-382) of WT and mutant LAMP-1 were amplified by PCR using WT and mutant LAMP-1 pcDNA3.1 aliquots as templates and introduced into the *Eco*RI and *Bam*HI sites of a pGBKT7 vector to produce pGBKT7-WT and mutants (Fig. 1).

Yeast two-hybrid assays pGAD-T7 vector, pGAD10-μ1, pGAD10-μ2, pGAD10-μ3A, or pGAD10-μ4 was introduced into Y187 strain, and pGBKT7-vector, pGBKT7-WT, or one of pGBKT7 mutants was transferred into Y2H gold strain by the lithium acetate method.[31] Then, the Y187 and Y2H gold strains were mated according to the manufacturer's protocol (Clontech Laboratories Inc., Palo Alto, CA). For quantitative growth assays, mated strains were cultured at 30°C in synthetically defined leucine and tryptophan dropout medium (SD-LW), and then suspended at 0.01 OD_{600} in synthetically defined leucine, tryptophan and histidine dropout medium (SD-LWH) and cultured at 30°C up to 6 d. At the indicated time points, OD_{600} was measured using U-1900 Spectrophotometer (Hitachi, Tokyo, Japan).

Molecular Modeling of the Complexes between GYQTΦ Peptides and μ3A The molecular modeling was performed by a SWISS-MODEL homology modeling server[32] using a rat μ3A structure (PDB ID: 4IKN)[30] as a template. The amino acid sequence of μ3A necessary for homology modeling was obtained from the UniProt[33] database (ID: P84092-AP2M1_RAT).) The range of residues that the SWISS-MODEL server[32] generated was Ile165 to Thr418. The protonation states of these three dimensional models were assigned by the PDB-2PQR server.[34]

Energy Minimization for the Complex Between GYQTΦ Peptide and μ3A All energy minimization was carried out using the AMBER16 suite [35] with combined quantum mechanics/molecular mechanics (QM/MM) potential. The MNDO-PM6 was used for the QM region, which included Tyr180, Phe181, Asp182, Gln381, Val389, Leu392, Phe402, Lys403, Gly404, Val405, Lys406, and Tyr407 residues (within 3.5Å of GYQTΦ). The parm14SB force field was used for the MM region, which included the other residues in the protein.

Prediction of the Dissociation Constant Between the GYQTΦ Peptide and μ3A The dissociation constant (K_d) between GYQTΦ and μ3A was predicted by Neural network[36] following molecular docking.

RESULTS

Interaction of μ Subunits of Four APs with LAMP-1's CT Containing the Lysosome-Targeting Signal of GYQTI The μ subunits of AP-1, AP-2, AP-3, and AP-4 have been shown to interact specifically with YXXΦ sequences. The four μ subunits differ in their relative affinities with different YXXΦ sequences. We examined abilities of the μ subunits of four APs to interact with the GYQTI-containing CT by the Y2H method. The cDNAs encoding μ1, μ2, μ3A, and μ4 subunits were all cloned into the transcriptional activation domain vector pGADT7 AD and cDNA for WT-LAMP-1's CT including GYQTI (Fig. 1) was cloned into the DNA-binding domain vector pGBKT7 DNA-BD. The pGADT7 AD and pGBKT7 DNA-BD constructs were then transferred into haploid yeast strains, Y187 and Y2H gold, respectively. The yeasts trans-

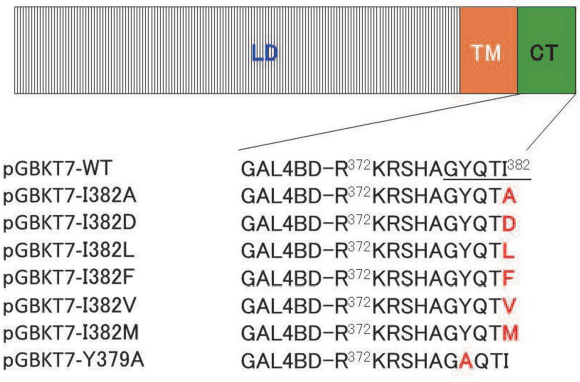

Fig. 1. LAMP-1 CT Constructs Used for the Y2H Assays in This Study

Mouse LAMP-1 is a 382-amino acid polypeptide composed of a large luminal domain (LD), a transmembrane domain (TM), and a short cytoplasmic tail (CT). GYQTI for lysosome targeting is underlined. WT and mutated CT peptides of LAMP-1 were fused with the GAL4BD. The amino acid sequences of CT peptides used for the Y2H were shown.

formed were mated together, and the resulting diploid yeasts were grown according to nutritional requirements for Ade, His, Leu, and Trp.

Interactions between the CTs (Fig. 1) and the μ subunits were assayed by the ability of yeast cells coexpressing different combinations to grow in liquid medium lacking His, Leu, and Trp (SD-LWH). The yeast cells coexpressing the WT-LAMP-1's CT and μ3A grew in the SD-LWH liquid (Fig. 2). Growth of the yeast cell was initiated after incubation for 2 d and it reached the maximum after incubation for 4 d (Fig. 2). There was no growth of the yeast clone coexpressing the WT-CT and μ1, μ2, or μ4 in the SD-LWH liquid (Fig. 2). To confirm that the interaction of μ3A and WT-CT depends on a critical tyrosine residue in GYQTI, we measured the growth of yeast cells expressing μ3A and the CT of Y379A containing GAQTI. No growth of this yeast clone was detected in the SD-LWH liquid (Fig. 3; ▲Y379A+μ3A, Table 1), attesting that this binding is specific for the tyrosine residue. Altogether, these results are consistent with the evidence provided by Stephen and Banting[15] that WT-LAMP-1's CT exclusively interacts with μ3A but not with μ1, μ2, and μ4.

The COOH-Terminal Amino Acid Residue Affects the Interaction Strength of LAMP-1's CT with μ3A To test whether the COOH-terminal amino acid residue has an effect on the interaction of the LAMP-1's CT and the μ subunits, we simultaneously measured growth of the yeast cells coexpressing LAMP-1's CT (WT or mutated CTs; Fig. 1) and μ subunit (μ1, μ2, μ3A, or μ4). We found positive interactions of the CTs containing GYQTI, GYQTL, GYQTF, GYQTM, and GYQTV with μ3A (Fig. 3 and Table 1) whereas no significant interaction was detected in the other combinations (Table 1). Taken these data together, the COOH-terminal amino acid residues affect its interaction with μ3A; the relative interaction strengths of WT and mutant sequences of LAMP-1 were WT (GYQTI) = GYQTL > GYQTF » GYQTM > GYQTV.

Molecular Modeling of Complexes Between μ3A and GYQTΦ Peptides We next calculated the binding strengths of GYQTΦ peptides with μ3A using computer-based molecular modeling because the crystal structure of μ3A had been solved.[30] The μ3A protein has a bipartite structure with the NH_2-terminal β3 binding domain (amino acid residues 1-164) and the COOH-terminal domain (amino acid residues 165-418). The COOH-terminal half is associated with interactions with YXXΦ motifs. The COOH-terminal domain bound to a tyrosine based-motif of DYQRL from the TGN38's CT was crystallized,[30] and this crystal structure was used for the GYQTΦ peptide docking simulations. The YXXΦ binding domain of μ3A possesses a banana-shape structure consisting of 16 β-sheets arranged into two parts called subdomains A and B. The DYQRL peptide was caught in parallel sheets β1 and β16 of μ3A subdomain A. Two hydrophobic cavities adapt tyrosine and leucine of DYQRL on either side of sheet β16 as observed for the DYQRL-μ2 complex.[29]

Based on the structural analysis data of the μ3A-DYQRL interface,[30] we performed the molecular modeling of complexes between μ3A and GYQTΦ peptides. The structural models of complexes between GYQTΦ peptides and μ3A are illustrated in Fig. 4. The critical tyrosine residue referred to as Y0 is stabilized by a key hydrogen bond between its hydroxyl group and the carboxylate of Asp182 (β1). Y0 of GYQTΦ makes another side chain hydrogen bond to an εN of Lys406 of β16 (Fig. 4). Hydrophobic interactions of Y0 with Tyr180

Table 1. Interactions of WT and Mutated CT Peptides of LAMP-1 with μ Subunits Measured by Y2H

COOH-terminal sequence	μ1	μ2	μ3A	μ4
GYQTI (WT)	-	-	1.8 ± 0.11	-
GYQTL (I382L)	-	-	2.1 ± 0.34	-
GYQTF (I382F)	-	-	1.1 ± 0.21	-
GYQTM (I382M)	-	-	0.40 ± 0.12	-
GYQTV (I382V)	-	-	0.22 ± 0.11	-
GYQTD (I382A)	-	-	-	-
GYQTD (I382D)	-	-	-	-
GAQTI (Y379A)	-	-	-	-

After the transformed yeasts were grown in the absence of histidine for 4 days, OD_{600} was determined. The OD_{600} values below 0.15 are indicated by the (-) symbols. Each value represents the mean ± SD (at least 3 separate cultures).

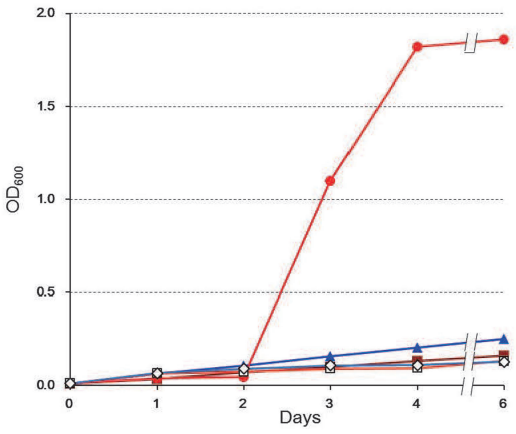

Fig. 2. The Growth Kinetics of Yeast Clones Coexpressing WT-LAMP-1's CT and μ1, μ2, μ3A or μ4

The yeast cells synchronously expressing LAMP-1's CT peptide and μ1 (▲), μ2 (■), μ3A (●), μ4 (□), or empty vector (◇) were incubated in SD-LWH (-His) over 6 d as described under 'MATERIALS AND METHODS.' OD_{600} readings were taken at the time indicated. The kinetic data were reproducible in three separate experiments. The representative growth kinetics was shown.

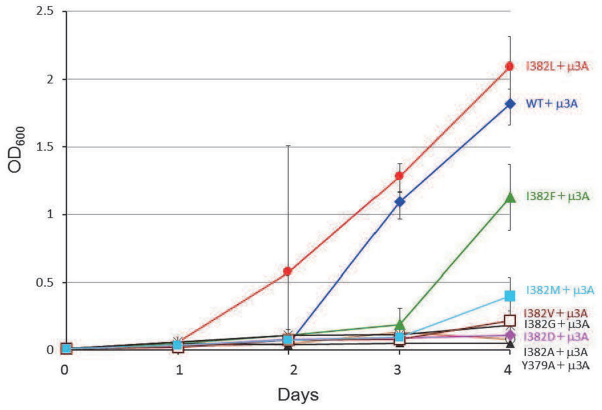

Fig. 3. Interaction of the WT and Mutated CTs of LAMP-1 with μ3A in the Y2H System

SD-LWH liquid cultures containing yeast transformants coexpressing μ3A and one of the WT and mutated CTs (I382L, I382F, I382M, I382V, I382A, I382G, I382D, and Y379A) of LAMP-1 were adjusted to be 0.01 OD_{600} units and were incubated at 37°C for 4 d. OD_{600} readings were taken at the time indicated. The control culture was carried out using the yeast cells containing LAMP-1's CT and empty pGAD-T7, giving the maximum OD_{600} value of 0.15 after 4 d-incubation. Each value represents the mean ± SD (at least 3 separate cultures).

(β1), Phe402 (β16) and the aliphatic portion of Lys406 (β16) exist in the pocket. The binding pocket for the Φ amino acid is formed by the hydrophobic side chains of Phe181 (β16), Val389 (β15), Leu392 (β15), Val405 (β16), and the aliphatic portion of Lys403 (β16). Since the COOH-terminal variants have almost the same tertiary structures regarding the GYQT moiety, it is likely that differential affinities of GYQTΦ with μ3A are closely related to different hydrophobic interactions of the Φ side chains with these amino acid residues that form the pocket.

K_d was calculated from the molecular docking model by NNScore2.0, a computation program developed for scoring the receptor-ligand association. The K_d values of WT (GYQTI), I382L (GYQTL), I382F (GYQTF), I382M (GYQTM), and I382V (GYQTV) for binding with μ3A were calculated to be 0.52, 1.13, 1.50, 2.47, and 2.71 μM, respectively, and were plotted against the Y2H interaction strengths (Fig. 5), providing a strong correlation ($r = 0.95$) between them. The structural simulations of GYQTΦ complexed with μ3A in its binding pockets rationalized the experimentally observed interaction strengths.

The Interaction Strengths of LAMP-1's CT Variants with μ3A Correlate with Late Endosomal and Lysosomal Amounts of WT and Similarly Mutated LAMP-1 We have previously determined that the amounts of WT and the COOH-terminal mutants of LAMP-1 in late endosomal and lysosomal fractions are depending on the COOH-amino acid residues,[11] raising the possibility that affinities of the GYQTΦ peptides with the μ3A subunit are related to abundance of LAMP-1 and its COOH-terminal mutants in late endosomes and lysosomes. To test this possibility, we plotted the interaction strengths *versus* the late endosomal and lysosomal amounts (Fig. 6). These plots showed a good linearity with a correlation coefficient of 0.94, supporting the idea that targeting of LAMP-1 to late endosomes and lysosomes is largely dependent on its interaction with μ3A.

DISCUSSION

LAMP-1 has a typical tyrosine-based motif of GYQTI in its COOH-terminal CT. The substitution of isoleucine at the COOH-terminus for other hydrophobic amino acid affects lys-

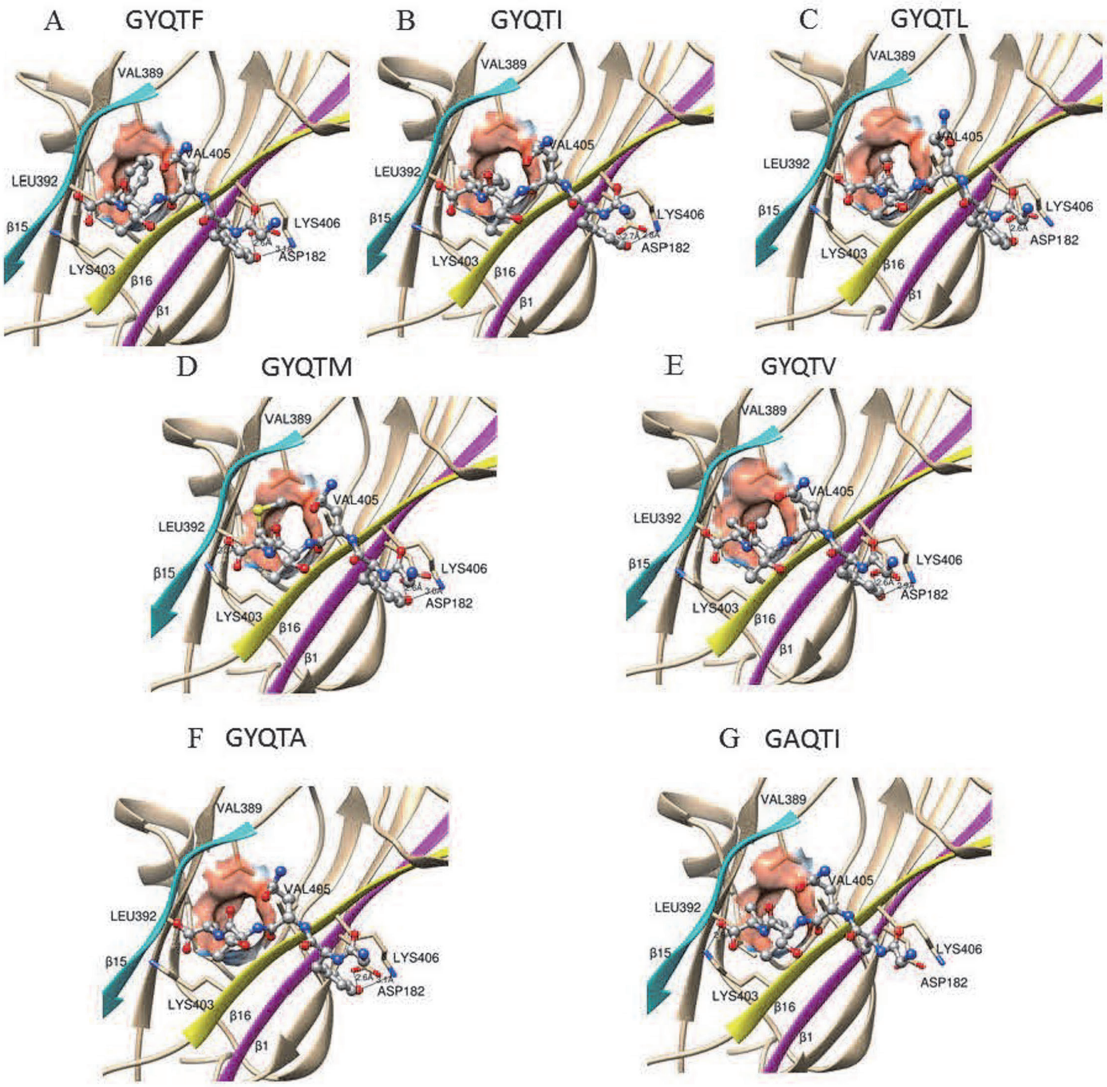

Fig. 4. Structures of GYQTΦ Peptides Docking with the μ3A Subunit

Complexes of μ3A and GYQTΦ peptide were shown as a stick and ball model. The hydrogen bonds were indicated by gray lines. A, GYQTF (I382F); B, GYQTI (WT); C, GYQTL (I382L); D, GYQTM (I382M); E, GYQTV (I382V); F, GYQTA (I382A), and G, GAQTI (Y379A).

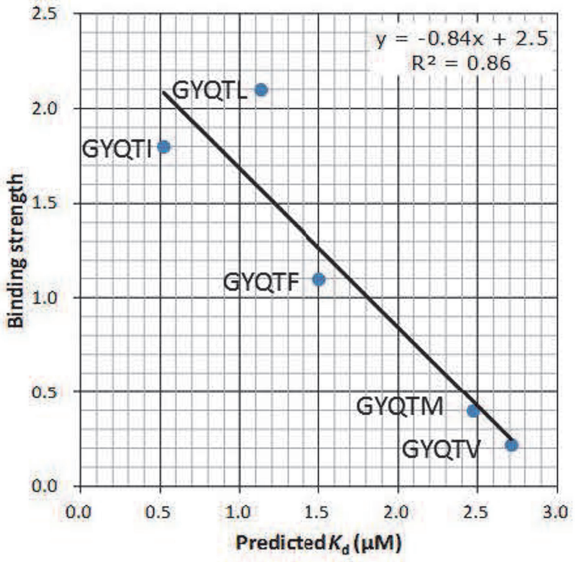

Fig. 5. Correlation Between the Y2H Interaction and Computational Binding Strengths of LAMP-1 and Its Mutants with μ3A

The dissociation constants (K_d) between GYQTΦ and μ3A were calculated by NNScore2.0 developed for computational characterization of small molecule docking. The resultant K_d values were plotted against the Y2H interaction strengths (Table 1).

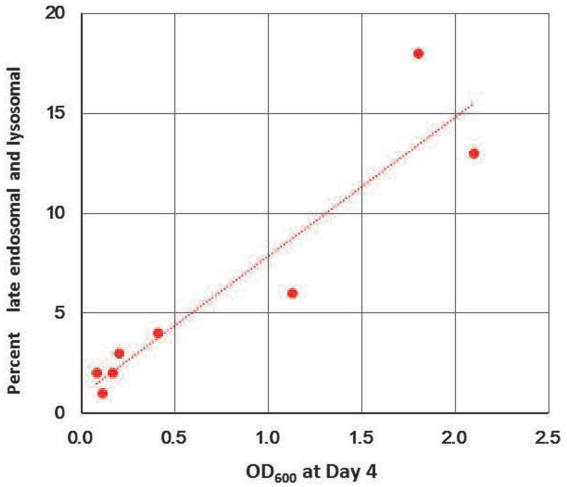

Fig. 6. The Interaction Strengths of LAMP-1 and Its Mutants with μ3A Correlate with Their Localization in Late Endosomes and Lysosomes

The Y2H interaction strengths of WT and mutant LAMP-1 CTs (Table 1) with μ3A were plotted against their contents in late endosomal and lysosomal fractions determined by the cell fractionation previously.[11]

osome-targeting of LAMP-1. It is well known that the tyrosine-based motifs of transmembrane proteins are recognized by μ subunits of hetero-tetrameric AP complexes, leading to selective incorporation into vesicles for efficient organelle-destined delivery. Ohno *et al*.[12] initially showed significant interactions of a TGN38-derived tyrosine-based motif, SDYQRL, with μ1 and μ2. A LAMP-1-derived AGYQTI sequence as a triple repeat remarkably interacts with μ2 but not as a single copy at the end of the TGN38's tail peptide.[12,13] Stephens and Banting have later reported that the TGN38's CT but not the LAMP-1's CT displays a high affinity with μ2 in the Y2H system.[15] They have also examined in detail the interactions of these CT peptides with medium subunits of other APs. Interaction profiles reported by Stephens and Banting closely resemble ours; the CT peptide of LAMP-1 significantly interacts with μ3A but not with μ1, μ2, or μ4.

Furthermore, we systematically investigated the interactions of WT and the COOH-terminal mutant peptides of LAMP-1 with μ1, μ2, μ3A, and μ4 subunits of the four major AP complexes. There is the highest interaction between μ3A and WT or I382L. The second highest is between μ3A and I382F whereas I382M and I382V weakly interact with μ3A. These profiles are in good agreement with its interaction preference for isoleucine and leucine at the Φ position.[14] Gough *et al*.[10] carried out the systematic study on interactions of WT and COOH-terminal mutants (GYQSΦ) of LAMP-2 subtype and acquired the similar results to ours in the CT-μ subunit interaction. The relatively strong interaction occurs between μ3A and GYQSI, GYQSL, or GYQSF. GYQSL and GYQSF exert high affinities with μ2 while our analogous GYQTL and GYQTF peptides do not in this study. The different results would be obtained probably due to the experimental difference; i.e. the GYQSΦ-containing TGN38's CT peptide linked to GAL4BD is used for the Y2H assays in contrast to our GYQTΦ-containing LAMP-1's CT peptide linked to GAL-4BD. Thus, the binding strength is likely to be sensitive to the CT context in which the motif lies.

Newly synthesized (NS) lysosomal membrane proteins follow two different routes called 'direct' and 'indirect' pathways.[2,3,17,18] In the direct pathway, they are delivered intracellularly from the *trans*-Golgi network (TGN) to either early or late endosomes and finally to lysosomes. In the indirect pathway, NS lysosomal membrane proteins moves from the TGN to the cell surface like secretory proteins. After reaching the plasma membrane, they follow the endocytic pathway along early endosomes, late endosomes, and lysosomes. The biosynthetic transport of lysosomal membrane proteins is regulated by the sorting machineries including four well known APs which recognize the tyrosine-motifs.[17,18] AP-2 is localized on the cytosolic side of the plasma membrane and help the tyrosine-motif containing membrane proteins assemble into clathrin-coated pits for the efficient endocytosis. AP-1 is associated with clathrin-coated-vesicles from the TGN to late endosomes. Immunocytochemical studies have been undertaken to define intracellular localization of AP-3. Localization of AP-3 differs from one experiment to another. AP-3 is partially colocalized with the TGN marker in the perinuclear region[37] while it is localized in transferrin-positive endosomes.[38] In our previous study,[39] almost all NS-LAMP-1 take the direct intracellular route to late endosomes and subsequently to lysosomes, indicating that the critical role of AP-3 at the TGN. Peden *et al*.[22] have shown by detailed immuno-electron microscopy that AP-3 hardly resides in the TGN but is consistently present in endosome-associated tubular vesicles containing LAMP-1. If AP-3 scarcely functioned in the sorting at the TGN stage, another sorting molecule that recognizes the GYQTI would occur at the TGN because the other μ subunits show no or little affinity with the LAMP-1's CT. Hirst *et al*.[40] have identified a novel AP complex called AP-5. However, AP-5 exclusively localizes to late endocytic compartments and its μ subunit has no binding structure for the tyrosine-based motif. So, it is unlikely that AP-5 plays a role in the sorting of LAMP-1 in the

TGN stage. Further studies are necessary to clarify the molecular machinery for sorting of LAMP-1.

Conflict of interest The authors declare no conflict of interest.

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