Structural Requirements for Interactions between Leucine-sorting Signals and Clathrin-associated Adaptor Protein Complex AP3

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The adaptor protein complexes (APs) AP1, AP2, AP3, and AP4 are important components of the intracellular sorting machinery. They associate with transport vesicles along the secretory and endocytic pathways and can interact with membrane proteins that contain signals for sorting into the appropriate transport vesicles (1, 2). Each AP contains four polypeptides, called adaptins: two large chains of ~100 kDa, a medium chain of ~50 kDa, and a small chain of ~25 kDa. AP1 complexes are associated with transport clathrin-coated vesicles (CCVs) derived from the trans-Golgi network (TGN), AP2 complexes are associated with the endocytic CCVs, whereas the functional localization of AP3 and AP4 is not clear yet. It seems, however, that AP3 mediates sorting to lysosomes and related organelles and mediates sorting on endosomes, whereas AP4 has been implicated in basolateral sorting (3–9).

Two best characterized types of protein-sorting signals are tyrosine- and leucine-based sorting signals (see Refs. 2, 3, and 10). It is believed that interactions between sorting signals and AP complexes is one of the determining steps in protein sorting, namely the sequestration of cargo membrane proteins into a specific type of transport vesicle for delivery to its intermediate or final destination. Interactions between various sorting signals and AP complexes have been demonstrated in a variety of experimental systems (reviewed in Refs. 1 and 11). All APs seem to recognize tyrosine-sorting signals via their respective medium chain, are associated with specific populations of transport vesicles, and confer distinct sorting properties onto these vesicles (1, 3). Interactions between AP complexes and leucine signals were studied less extensively, and different AP subunits have been reported to interact with leucine signals. Kirchhausen and co-workers (12) has reported interactions between several leucine signals and a large (β2) chain of the AP complex in a cross-linking assay. In our laboratories interactions between leucine signals and medium chains of AP1 and AP2 were observed in phage-display assays (13), in protein-protein interaction assays on magnetic beads (14), and in surface plasmon resonance assays (15).

Two molecules containing leucine-sorting signals are of particular interest for this study: the invariant chain (Ii) and lysosomal integral membrane protein II (LIMPII). Ii is a type II transmembrane protein associated with the major histocompatibility complex (MHC) class II in the endoplasmic reticulum (ER). It is believed that Ii has a multitude of functions that aid MHC class II in antigen presentation. Two of the main functions are to prevent endogenous polypeptides from binding to the MHC class II groove and mediate the sorting of the MHC class II to the endosomes either directly or via the plasma membrane (16–18). Ii carries two leucine-sorting signals within its cytoplasmic tail, the membrane-distal signal (LI residues in positions 7 and 8), and the membrane-proximal signal (ML residues at positions 16 and 17). Ii is sorted to endosomes via the plasma membrane, and either signal is independently sufficient for endosomal localization of Ii (19, 20). LIMPII is a type III transmembrane protein, and has one leucine signal in its 20-amino acid cytoplasmic tail. This signal
is necessary and sufficient for direct sorting of LIMPII to endosomes/lysosomes (10, 21). It has previously been shown that sorting signals from α1 interact in vitro with AP1 and AP2, but not to AP3, which is consistent with the in vivo sorting of LIMPII to endosomes indirectly via the plasma membrane (15). On the other hand, the LI-motif of LIMPII preferentially binds to AP3 and in addition to this in vivo observation, LIMPII is known to be sorted to endosomes/lysosomes via a direct transport route, which does not include appearance on the cell surface (10, 21). Because leucine-based motifs of α1 and LIMPII appear to be remarkably similar to each other (leucine pair and double acidic residues in the positions −4 and −5), we investigated the influence of nearby residues on the specificity of interactions between those signals and AP1, AP2, and AP3. We constructed a set of GST fusion proteins containing various chimeric α1-LIMPII constructs, which were then tested for adaptor binding by surface plasmon resonance. To corroborate our in vitro observations, we also analyzed the intracellular fate of the wild type LIMPII and LIMPII with the invariant chain signal, transfected into MDCK cells.

MATERIALS AND METHODS

DNA Constructs—For the in vitro studies, the cytoplasmic tails of the wild type α1 (Met10 to Arg21) and its various mutants and wild type LIMPII (Gly49 to Thr221) and its various mutants (see Fig. 1) were made by PCR from the cytoplasmic tail of both of the proteins. They were then fused in-frame to the C terminus of the GST protein using BamHI and EcoRI sites in pGEX-2T vector (Amersham Biosciences). For the in vivo studies, we received a full-length LIMPII cDNA from I. Sandoval (Madrid), and the construct was subcloned into pMEP4 vector to utilize its inducible promoter and stably transfected into MDCK cells. A LIMPII QRD mutant was constructed by PCR mutagenesis using primers containing mutations in cysteine residues. The construct was subcloned into pMEP4 vector under control of metallothionein promoter (Dr. I. Sandoval, Universidad Autonoma de Madrid). An LIMPII QRD mutant was constructed by PCR mutagenesis using primers containing mutations in cysteine residues (12, 22). The construct was subcloned into pMEP4 vector under control of metallothionein promoter (Dr. I. Sandoval, Universidad Autonoma de Madrid). The LIMPII QRD mutant was used for adaptor binding measurements after dialysis against BIA buffer (20 mM HEPES pH 7, 150 mM NaCl, 10 mM KCl, 2 mM MgCl2, 0.2 mM dithiothreitol).

Antibodies—The mouse monoclonal antibody 29G10 (a kind gift from Dr. I. Sandoval, Universidad Autonoma de Madrid) was used to detect LIMPII as described elsewhere (23). Goat anti-mouse secondary antibodies coupled to Alexa 594 were obtained from Molecular Probes (Eugene, OR). Rabbit anti-mouse secondary antibody was obtained from Dako A/S (Denmark). Expression and Purification of GST Fusion Proteins—All fusion proteins were expressed and purified as recommended by the manufacturer (Amersham Biosciences). Briefly, BL21 cells carrying the constructs of interest were induced with 0.25 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h and collected by centrifugation. The fusion proteins were purified by a series of 15-s sonication steps or by Bug-buster kit (Novagen) and then purified on GST-Sepharose 4B (Amersham Biosciences). The purity and size of the proteins were verified by SDS-PAGE. GST, which served as a negative control, and the chimeric proteins were released by a series of 15-s sonication steps or by Bug-buster kit (Novagen). The purity and size of the proteins were verified by SDS-PAGE. The purity and size of the proteins were verified by SDS-PAGE. The purity and size of the proteins were verified by SDS-PAGE. The purity and size of the proteins were verified by SDS-PAGE. The purity and size of the proteins were verified by SDS-PAGE.

Radiolabeling and Immunoprecipitation—For metabolic labeling, transfected MDCK cells were grown to 80% confluence in full medium supplemented with 25 μM CdCl2. The cells were washed twice and incubated for 40 min at 37 °C in cysteine/methionine-free Dulbecco's modified Eagle's medium (Bio Whittaker) and then incubated for 3 h with 50 μCi/ml of [35S]cysteine/methionine. After metabolic labeling, the cells were washed with ice-cold PBS and lysed in ice-cold lysis buffer (1% Triton X-100, 0.1 M NaCl, 1 mM EDTA, 10 mM Na2HPO4) containing a mixture of protease inhibitors (1 μg/ml leupeptin, 0.25 mM phenylmethylsulfonyl fluoride, 2 μg/ml antipain, 1 μg/ml aprotinin, 1 μg/ml pepstatin A, 1 μg/ml E-64). The lysate was centrifuged at 13,000 × g for 10 min at 4 °C to remove cell debris. LIMPII was precipitated from the supernatant with 29G10 monoclonal antibody overnight at 4 °C. Secondary antibodies, rabbit anti-mouse IgGs coupled to protein A-Sepharose beads (Amersham Biosciences) were added for 2 h at 4 °C. Immunoprecipitates were extensively washed. The proteins were eluted from the beads by incubating in gel-loading buffer (0.5 M Tris/NaOH, pH 7.5, 1% SDS, 10% glycerol, 0.1% bromphenol blue) at 95 °C for 5 min. Immunoprecipitates were resolved by 10% SDS-PAGE. The bands were detected with the Bio-Rad GS-250 PhosphorImager. The intensity of the bands was quantified with the Molecular Analyst 2.0.2 software (Bio-Rad).

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Antibodies against BIA buffer (20 mM HEPES pH 7, 150 mM NaCl, 10 mM KCl, 2 mM MgCl2, 0.2 mM dithiothreitol). For metabolic labeling, transfected MDCK cells expressing various LIMPII constructs were grown on glass cover slips and fixed in 3% paraformaldehyde (PFA) in PBS for 10 min at room temperature. Fixed cells were incubated for 30 min with a primary antibody followed by a 20-min labeling with a secondary antibody to label total LIMPII. Antibodies were diluted in PBS, 0.1% sheep to label total intracellular protein or in PBS containing 0.5% saponin for the experiments where overexpression was used. To monitor internalization of anti-LIMPII antibodies from the cell surface, antibodies were added to the cells for 30 min on ice. The cells were then washed with PBS chased in complete medium for 40 min at different time periods before fixation. Secondary antibodies were then added in PBS with saponin. Fluorescence was detected, and images were acquired using a Leica TCS-NT digital scanning confocal microscope equipped with a 60×1.2 water immersion objective. The pinhole value was kept below 1. The images were processed for presentation with Adobe Photoshop software.

Preparation of AP1 and AP2—AP1 and AP2 were prepared from pig brain essentially as described elsewhere (26). Briefly, clathrin-coated vesicles were purified from brain after homogenization and differential centrifugation. The adaptor proteins were released from clathrin-coated vesicles with 0.5 M Tris/HCl, pH 7.0 and applied to a Superose-6 column (2.5 × 75 cm, equilibrated in the same buffer) followed by an FPLC (Amersham Biosciences) system at a flow rate of 0.3 ml/min. Fractions containing AP1 and AP2 were identified by SDS-PAGE and separated from each other by subsequent hydroxylapatite chromatography as described elsewhere (27). Fractions containing either AP1 or AP2 were dialyzed against BSA buffer (see above), which was used for all experiments using surface plasmon resonance.

Preparation of AP3—For analyzing AP3 binding, pig brain cytosol was fractionated by gel filtration as described (22) using a 60-cm TSK 3000 SWXL 3000 size-exclusion column connected to a perfusion chromatography pump station (Perseptive Biosystems). The fractions were shown to be devoid of AP1 and AP2 (for details, see Ref. 22).

Surface Plasmon Resonance—The interaction between the different constructs and adaptors was analyzed in real time by surface plasmon resonance (28) using a BIAcore 3000 biosensor (BIAcore AB). Bi-constructs were immobilized via their GST moiety of the GST-li chimera onto the surface of a CM5 sensor chip coated with anti-GST antibodies. The subsequent interaction experiments were performed at a flow-rate of 20 μl/min. Association was recorded for 2 min during which adaptor proteins at different concentrations were injected and followed by recording dissociation for 2 min during which buffer was perfused. A short pulse injection (15 s) of 20 μM NaOH, 0.5% SDS was used to regenerate the surface after each experimental cycle. The biaevaluated its binding capacity for at least 15 cycles of association, dissociation, and regeneration. AP1 and AP2 were used at concentrations ranging from 20 to 200 nM. The cytosolic fractions enriched in AP3 used at three dilutions.

Determination of Kinetic Rate Constants—The association and dissociation rate constants k on and k off for the interactions were calculated by using the evaluation software of the BIAcore 3000. The mathematical models used are described in more detail elsewhere (27) Briefly, the association was determined after 15–20 s following the switch from buffer solution to adaptor solution to avoid distortions due to injection and mixing. The dissociation rate constants were determined after 5–10 s following the switch to buffer solution. After association phase of adaptor from Ii-GST, the dissociation kinetics decreased for that 4 ha that for 20 s following the switch from buffer solution to adaptor solution to avoid distortions due to injection and mixing. The dissociation rate constants were determined after 5–10 s following the switch to buffer solution. After association phase of adaptor from Ii-GST, the dissociation kinetics decreased for that 4 ha that for 20 s following the switch from buffer solution to adaptor solution to avoid distortions due to injection and mixing. The dissociation rate constants were determined after 5–10 s following the switch to buffer solution. After association phase of adaptor from Ii-GST, the dissociation kinetics decreased for that 4 ha that for 20 s following the switch from buffer solution to adaptor solution to avoid distortions due to injection and mixing. The dissociation rate constants were determined after 5–10 s following the switch to buffer solution. After association phase of adaptor from Ii-GST, the dissociation kinetics decreased for that 4 ha that for 20 s following the switch from buffer solution to adaptor solution to avoid distortions due to injection and mixing. The dissociation rate constants were determined after 5–10 s following the switch to buffer solution. After association phase of adaptor from Ii-GST, the dissociation kinetics decreased.
first order kinetic $A + B \rightarrow AB$. Relative binding values were then calculated from the $K_D$ values.

Standard deviations (in percentage of the mean value) for AP1 and AP2 equilibrium constants ($K_D$), or AP3 affinity were measured relative to the wild type LIMPII construct using the same adaptor batch.

**RESULTS**

We have previously demonstrated that AP1 and AP2 adaptor protein complexes could interact with the invariant chain in an *in vitro* assay monitored with surface plasmon resonance technique, and that such interactions were dependent on the intact leucine-sorting signals in the invariant chain (15). We have also shown that two other molecules that contain similar leucine signals, tyrosinase and LIMPII, only poorly interact with AP1 or AP2, but strongly bind to AP3 (15, 22). Furthermore, interactions between LIMPII and AP3 and the invariant chain and AP1 and AP2 were dependent on acidic residues upstream of the critical leucine signals (22, 29). This latter finding suggested that residues in close apposition to the leucine-based motif could play a critical role in determining adaptor binding. We therefore investigated the role of other residues upstream of the leucine signals for their influence on AP1, AP2, and AP3 binding. To this end, we constructed a set of GST fusion constructs containing various chimeric constructs in which one or several amino acids upstream of the leucine signals were swapped between the invariant chain and LIMPII molecules (Fig. 1) and measured their interactions with the adaptor complexes. Since the invariant chain has two independent leucine signals, we made two sets of constructs. Each set had one of the leucine signals knocked out by an independent leucine signals, we made two sets of constructs.

We also investigated AP1 and AP2 binding to these L17A constructs. As shown in Fig. 2B, AP1 binding did not decrease dramatically in all but two chimeric constructs and stayed around 80% of that of the L17A construct. These two constructs, L17A, QRD -> RAD, and L17A, QRD -> RAP, exhibited AP1 binding of around 20–25% of that of the L17A construct. Interestingly, the construct that lost most of the AP1 binding capacity, L17A, QRD -> RRP, also gained the highest AP3 binding. AP2 binding (Fig. 2C) did not change for all but 2 constructs, L17A, QRD -> QAP and L17A, QRD -> QRP. These two constructs showed AP2 binding of ~60% of the L17A construct. There was no apparent correlation between the loss of AP2 binding and increase in AP3 binding for this set of constructs. Next, we performed swaps of the residues upstream of the leucine pair between LIMPII and the second, membrane-proximal signal of the invariant chain. It is noteworthy, that in this case only two amino acids had to be swapped, since the membrane-proximal signal of the invariant chain also has a proline residue at the −1 position from the critical di-leucine (ML) signal.

**LIMPII constructs (in vitro studies)**

| WT | GST-HDDQRDL3SNHNLQPLMGRPGPESKCSR |
|----|----------------------------------|
| L17A | GST-HDDQRDL3SNHNLQPLMGRPGPESKCSR |
| L17A, QRD -> RAP | GST-HDDRPL3SNHNLQPLMGRPGPESKCSR |
| L17A, QRD -> RAD | GST-HDDRRL3SNHNLQPLMGRPGPESKCSR |
| L17A, QRD -> QAP | GST-HDDRQAP3SNHNLQPLMGRPGPESKCSR |
| L17A, QRD -> RRP | GST-HDDRQPR3SNHNLQPLMGRPGPESKCSR |
| L17A, QRD -> RRD | GST-HDDRQPR3SNHNLQPLMGRPGPESKCSR |
| L17A, QRD -> QAD | GST-HDDRQAP3SNHNLQPLMGRPGPESKCSR |
| L17A, QRD -> QRP | GST-HDDRQPR3SNHNLQPLMGRPGPESKCSR |
| L7A | GST-HDDQRDL3SNHNLQPLMGRPGPESKCSR |
| L7A, QLP -> RAP | GST-HDDQRAP3SNHNLQPLMGRPGPESKCSR |
| L7A, QLP -> RLP | GST-HDDQRAP3SNHNLQPLMGRPGPESKCSR |
| L7A, QLP -> QAP | GST-HDDQRAP3SNHNLQPLMGRPGPESKCSR |
| L7A, QLP -> RRP | GST-HDDQRAP3SNHNLQPLMGRPGPESKCSR |
| L7A, QLP -> DERAP | GST-HDDQRAP3SNHNLQPLMGRPGPESKCSR |

**LIMPII constructs (in vivo studies)**

Wild type LIMPII, full length

LIMPII, full length, R108A/R104P mutated into QRD

**Fig. 1. Constructs used in this study.** Constructs for the *in vivo* and *in vitro* studies were made as described under “Materials and Methods.” Leucine-sorting signals are underlined. Mutations introduced are shown in bold italic font.
leucine pair (DE and DD, respectively), whereas the membrane-proximal signal of the invariant chain has only one acidic residue at the H110024 position. We therefore introduced an extra acidic residue at the H110025 position into the membrane-proximal signal of the invariant chain together with the swap of residues in positions H110022 and H110023. The resulting construct, L7A, NEQLP DERAP possessed much higher AP3 binding (80% of LIMPII) than the L7A, QLP DERAP construct (also labeled as L7A, QLP DERAP) that had only 10% of LIMPII AP3 binding. Therefore, two acidic residues at the H110024 and H110025 positions seem to give rise to a better AP3 binding than a single acidic residue at the H110024 position.

As with the membrane-distal signal, we studied the effect of the amino acid swap upstream of the membrane-proximal signal on AP1 and AP2 binding. As seen from comparing Fig. 3A and Fig. 3B, there is a strong correlation between the loss of AP1 binding and the increase in AP3 binding for all constructs studied. In the case of AP2 binding, a correlation between gain of AP3 binding and loss of AP2 binding was not significant, although two constructs that had the highest AP3 binding (L7A, QLP RRP and L7A, NEQLP DERAP) also showed some reduced AP2 binding (around 65% of the AP2 binding to the L7A construct).

To corroborate our findings on the importance of residues neighboring the leucine signal, we then analyzed amino acids swaps between LIMPII and the invariant chain on the ability of LIMPII to bind AP3, AP1, and AP2. As shown in Fig. 4A, when the RAP residues upstream of the leucine pair were swapped with the QRD residues from the invariant chain, the resulting construct exhibited a dramatic loss in AP3 binding (30% of the wild type LIMPII binding), a decrease comparable to that when the critical leucine residue was knocked out with an alanine (21% of the wild-type LIMPII AP3 binding). Therefore, we can conclude that the three residues upstream of the leucine pair are indeed part of the motif that mediates binding to AP3. The introduction of the QRD sequence into LIMPII molecule gave a slight increase in AP1 binding capacity (20%), compared with the wild-type LIMPII, but this was still only 12% of the wild-type Ii binding. When binding to AP2 was analyzed, the QRD introduction did not change the affinity for AP2 binding of the LIMPII mutant as compared with the wild type LIMPII, suggesting that...
FIG. 4. Panel A, binding of AP3 to the invariant chain and various LIMPII constructs. All numbers are shown relative to AP3 binding to the wild-type LIMPII-GST fusion construct. Panel B, binding of AP1 to the invariant chain and various LIMPII constructs. All numbers are shown relative to AP2 binding to the invariant chain-GST fusion construct. For all three panels, the numbers represent the average value obtained from 3–5 independent experiments and S.D. varied from 2 to 8%.

binding of wild-type LIMPII to AP2 require also other structural elements than the amino acid changes we introduced.

Since the introduction of the QRD sequence into the LIMPII molecule significantly reduced its AP3 binding capacity but increased its AP1 binding capacity in vitro, we decided to investigate the in vivo fate of the respective mutant proteins. Wild type LIMPII has been shown to be transported from the TGN to lysosomes via endosomes (presumably via interactions with AP3 complex) and has not been detected on the plasma membrane (30). We speculated that the reduction in AP3 binding activity upon introduction of the QRD sequence into the wild-type LIMPII might result in the transport of LIMPII to the plasma membrane. Since several studies have shown that overexpression of certain proteins can lead to their general missorting and accumulation on the plasma membrane (31, 32), we had to ensure that overexpressing our mutant LIMPII constructs does not cause unspecific cell surface delivery. Fig. 5A shows the immunoprecipitation of LIMPII molecules from the cell lines used for our studies. First, it is notable that expression of the mutant QRD LIMPII molecule was at the same level as the expression of the wild type LIMPII used for control experiments. Fig. 5B shows staining of the cells expressing these levels of the wild type LIMPII. To simplify the detection of endosomal structures, these cells were also transfected with the EEA1-GFP construct, which we have used as an excellent in vivo marker for early endosomes (24, 54). For wild-type LIMPII, only intracellular staining was notable, while staining of LIMPII at the plasma membrane was not detectable, in line with the results from the groups of Sandoval and Hoflack (30, 34). On the other hand, the LIMPII QRD mutant was detectable in significant amounts at the plasma membrane in addition to the intracellular staining (Fig. 5C). Furthermore, anti-LIMPII antibodies could be internalized from the plasma membrane of the cells expressing the LIMPII QRD protein (Fig. 5E), whereas no antibody uptake was observed in wild-type LIMPII-expressing cells (Fig. 5D). Finally, PFA-fixed, non-permeabilized cells expressing either the wild-type LIMPII or the mutant LIMPII QRD construct were labeled with the anti-LIMPII antibody to reveal the surface staining only. As expected, no surface staining was detected in the cells expressing the wild-type LIMPII construct (Fig. 5F), whereas strong membrane staining was observed in cells expressing the mutant LIMPII QRD construct (Fig. 5G). The same results were obtained when these cells expressing the wild-type and the mutant LIMPII constructs were first labeled with the anti-Limpii antibody on ice, and then fixed with PFA (not shown). In conclusion, the introduction of the QRD sequence into wild-type LIMPII indeed leads to its re-routing via the plasma membrane and internalization, most likely due to the loss of efficient AP3 binding whereas the capability to interact with PM adaptors are maintained.

DISCUSSION

In this study, we demonstrated that residues immediately upstream of the leucine pair in leucine-based sorting signals can be critical for sorting of the newly synthesized molecules to endosomal/lysosomal pathway. It has been believed for some time that the structure of the classic leucine sorting signal was (A)XXXLL, where L is leucine, isoleucine, or methionine, A is an acidic residue, and X is any other amino acid residue. This study demonstrates that those "non-critical" residues may also contribute to the determination of adaptor binding, and in fact discriminate between, for example, AP1 and AP3 binding. This would then determine whether the molecules containing various leucine-based signals are sorted directly to endosomes/lysosomes, or sorted via a different route via the plasma membrane.

According to the data presented in this report, the following residues in the proximity of the leucine pair are important for AP3 binding: proline residue at the −1 position from the leucine pair, a positively charged residue such as arginine at position −3, and negatively charged residues at positions −4 and −5. Importantly, effects of these residues on AP3 binding appear to be additive.

Indeed, the proline residue at −1 position confers some AP3 binding to the L7A set of constructs (Fig. 3A), and a single Asp to Pro substitution in the −1 position of the membrane-distal signal in the context of L17A grants significant (14% of the wild-type LIMPII) AP3 binding to the invariant chain construct. For comparison, the swap of 3 residues at positions −1, −2, and −3 (L17A QRD → RAP construct) grants the resulting chimera only 25% of wild-type LIMPII AP3 binding (Fig. 2A).

A positively charged residue such as arginine at position −3 has an additive effect on AP3 binding when residues at positions −1 and −2 are also swapped, but may not grant AP3 binding properties by itself. Indeed, when arginine is introduced at position −3 in the membrane-distal signal of the invariant chain (L17A QRD → RRD), AP3 binding is not detectable (Fig. 2A). However, when arginine is introduced in the −3 position of the membrane-proximal signal (L7A, QLP →
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RDP construct, Fig. 3A) significant AP3 binding is observed. We believe that this could be due to the additive effect of arginine at the -3 position and proline at the -1 position that is present in the native membrane-proximal signal of the invariant chain. This is further corroborated by introducing both proline at the -1 position and arginine at the -3 position of the membrane-distal signal (L17A, QRD → RRP construct, Fig. 2A). This construct exhibits much higher AP3 binding than the one in which only the proline residue is introduced at position -1 (L17A, QRD → RQF construct, Fig. 2A).

Interestingly, the invariant chain constructs that contain arginine at positions -3 and -2 together with proline at the position -1 in both membrane-proximal and membrane-distal signals bind AP3 significantly stronger than constructs containing original residues from LIMPII (Figs. 2A and 3A). At the same time, when proline is not present at the -1 position, double arginines at the -2 and the -3 positions do not confer AP3 binding properties to the invariant chain construct (Fig. 2A). It is thus possible that the double positive charge together with proline at the -1 position (which presumably gives the leucine pair flexibility to fit in the recognition site of AP3) is the preferred motif for AP3 recognition.

AP3 binding to the invariant chain chimeras seems to be significantly stronger when double negatively charged residues are present at positions -4 and -5 than if there is only one charged residue in the position -4 (Fig. 3A, compare constructs L7A, QLP → RAP and L7A, NEQLP → DERAP). It is also of notice that glutamic acid residue at the position -4 seems to cause stronger AP3 binding than an aspartic acid residue in the same position (compare constructs L17A, QRD → RAP, 25% relative AP3 binding and L7A, NEQLP → DERAP, 80% relative AP3 binding).

We compared the structure of other molecules containing leucine signals that are known to interact with AP3 complex, or are transported directly to endosomes/lysosomes/melanosomes/vacuoles, presumably, via interactions with AP3 (Fig. 6, data for the figure are taken from Refs. 35 and 36–39). The overwhelming majority of these molecules contain proline at position -1, such as tyrosinase and TRP1 families. A noticeable exception is yeast protein ALP that contains arginine at this position. Yeast adaptor protein complexes are somewhat different from mammalian AP3s, and it is possible that yeast AP3 recognition site differs from that of mammalian AP3s, although another yeast vacuolar protein, Vamp3p has a proline residue in its -1 position.

Interestingly, the majority of the depicted proteins contain a charged residue (in most cases arginine) at position -3 and a glutamic acid residue (rather than aspartic acid residue) at position -4. TRP1 does not have an acidic residue in position -4, but has glutamic acid residue at position -5 and another acidic residue at position -6 (Fig. 6). The general structure of a leucine signal that is recognized by mammalian AP3 is therefore likely to be (D/E)E(R/K)XPLL.

This study also revealed a strong correlation between the loss of AP1 binding and acquisition of AP3 binding in the invariant chain/LIMPII chimeric constructs (compare Figs. 2, A with B, and 3, A with B). We therefore believe that the following may be the basis for sorting at the TGN: some signals with higher affinity for AP3 get sorted in the cargo vesicles destined for direct delivery to endosomes/lysosomes, and other signals have higher affinity for AP1 and are sorted to their destination in AP1-containing cargo vesicles. Previous studies (22) have also shown lack of correlation between the loss of AP2 binding and acquisition of AP3 binding for tyrosinase, and this agrees well with the results of this study. The overall picture of sorting at TGN is likely to be more complicated, as another set of
molecules, so-called GGAs (40–43) have recently been demonstrated to be involved in this sorting step as well. Importantly, GGAs have been recently demonstrated to interact with leucine signals (44–48). AP1B and AP4 complexes may also be involved in sorting from the TGN, as they have been implicated in basolateral sorting of some molecules to polarized cells (5, 49). Further studies are required to determine which molecules are sorted via interactions with AP1, AP3, AP4, or GGAs.

At the same time, no correlation was apparent between the loss of AP2 binding and acquisition of AP3 binding in the same set of constructs (compare Figs. 2, A and B, with 3, A and B).

Previous studies from our laboratories have also demonstrated relative non-specificity of AP2 for interactions with various sorting signals (29, 50). A possible biological role of such relative non-specificity of AP2 for interactions with various sorting signals could be to ensure an efficient internalization of all molecules containing leucine-sorting signals that are known to interact with AP3 or be targeted directly to endosomes/lysosomes/melanosomes/vacuoles. Leucine signals from molecules that have been shown to interact with AP3 are shown in bold. Residues that may be preferred for AP3, but not for AP1 or AP2 binding, are shown in bold italic. Signals that have been shown to bind AP3 are marked with * (based on data from Refs. 35 and 36–39). The other constructs have not, to our knowledge, been tested for AP3 interactions. NT, not tested.

References

1. Kirchhausen, T. (1999) Annu. Rev. Cell Dev. Biol. 15, 705–732
2. Kirchhausen, T., Bonifacino, J. S., and Riezman, H. (1997) Curr. Opin. Cell Biol. 9, 488–495
3. Robinson, M. S., and Bonifacino, J. S. (2001) Curr Opin Cell Biol. 13, 444–453
4. Faundez, V., Horn, J. T., and Kelly, R. B. (1998) Cell 93, 423–432
5. Simmen, T., Honing, S., Icking, A., Tikkanen, R., and Hunkeler, W. (2002) Nat Cell Biol.
6. Simpson, F., Peden, A. A., Christopoulou, L., and Robinson, M. S. (1997) J. Cell Biol. 137, 835–845
7. Shi, G. Y., Faundez, V., Roos, J., Dell’Angelica, E. C., and Kelly, R. B. (1998) J. Cell Biol. 143, 947–955
8. Dell’Angelica, E. C., Klummann, J., Stoortwegen, V., and Bonifacino, J. S. (1998) Science 280, 431–434
9. Dell’Angelica, E. C., Mullins, C., and Bonifacino, J. S. (1999) J. Biol. Chem. 274, 7276–7285
10. Sandoval, I., and Bakke, O. (1999) Trends Cell Biol. 9, 292–296
11. Kirchhausen, T. (2000) Annu. Rev. Biochem. 69, 699–727
12. Rappoport, I., Chen, Y. C., Cuppers, P., Shoelson, S. E., and Kirchhausen, T. (1998) EMBO J. 17, 2148–2155
13. Brennes, T., Lauvrak, V., Lindqvist, B., and Bakke, O. (1998) Immunotechnology 4, 21–28
14. Rodionov, D. G., and Bakke, O. (1998) J. Biol. Chem. 273, 6005–6008
15. Hofmann, M. W., Honing, S., Rodionov, D., Dobberstein, B., von Figura, K., and Bakke, O. (1999) J. Biol. Chem. 274, 36153–36158
16. Cresswell, P. (1996) Cell 84, 505–507
17. Germain, R. N., Castellino, F., Han, R., Reis, E. S., Romagnoli, P., Sadegh-Nasseri, S., and Zhong, G. M. (1996) Immunol. Rev. 151, 5–30
18. Bakke, O., and Nordeng, T. W. (1999) Immunol. Rev. 172, 171–187
19. Brennes, B., Madsen, T., Gedde-Dahl, M., and Bakke, O. (1994) J. Cell Sci. 107, 2021–2032
20. Kang, S., Liang, L., Parker, C. D., and Collawn, J. F. (1998) J. Biol. Chem. 273, 26154–26162
21. Vega, M. A., Rodriguez, F., Segui, B., Cales, C., Alcalde, J., and Sandalov, I. V. (1991) J. Biol. Chem. 266, 16269–16272
22. Honing, S., Sandalov, I. V., and von Figura, K. (1998) EMBO J. 17, 1304–1314
23. Barriocanal, J. G., Bonifacino, J. S., Yuan, L., and Sandalov, I. V. (1986) J. Biol. Chem. 261, 16755–16763
24. Nordeng, T. W., Gregers, T. F., Kongsvik, T. L., Mersere, S., Gorel, J.-P., Jourdan, F., Motta, A., and Bakke, O. (2002) Mol. Cell. Biol. 01, 10-0478
25. Wigler, M., Sweet, R., Sim, G. K., Wold, B., Pellicer, A., Lacy, E., Maniatis, T., Silverston, S., and Axel, R. (1979) Cell 16, 777–185
26. Lindner, R. (1994) in Cell biology: A Laboratory Handbook (Celsius, J. E., ed) p. 1, Academic Press Inc., New York
27. Manfredi, J. J., and Bazzar, W. L. (1997) J. Biol. Chem. 272, 12182–12188
28. Jonsson, U. F., L. (1991) BioTechniques 11, 620–627
29. Kongsvik, T. L., Honing, S., Bakke, O., and Rodionov, D. G. (2002) J. Biol. Chem. 277, 16484–16488
30. Sandalov, I. V., Arredondo, J. J., Alcalde, J., Gonzalez Noriega, A., Vandekerckhove, J., Jimenez, M. A., and Rico, M. (1994)-J. Biol. Chem. 269, 6622–6631
31. Honing, S., and Hunkeler, W. (1995) J. Cell Biol. 128, 321–332
32. Dietel, B., Bauer, U., Le Borgne, R., and Hoflack, B. (1998) J. Cell Biol. 173, 186–193
33. Honing, S., Griffith, J., Geuze, H. J., and Hunkeler, W. (1996) EMBO J. 15, 5230–5239
34. Le Borgne, R., Alonoma, A., Bauer, U., and Hoflack, B. (1998) J. Biol. Chem. 273, 29451–29461
35. Sandalov, I. V., Martinez-Arca, S., Valdueza, J., Palacios, S., and Holman, G. D. (2000) J. Biol. Chem. 275, 38874–38885
36. Le Borgne, R., Planque, N., Martin, P., Dewitte, F., Saulle, S., and Hoflack, B. (2001) J. Cell Sci. 114, 2581–2591
37. Vowels, J. J., and Payne, G. S. (1998) EMBO J. 17, 2482–2493
38. Darsow, T., Burd, C. G., and Emr, S. D. (1998) J. Cell Biol. 142, 913–922
39. Sterner, S., and Brunke, T. (2001) J. Biol. Chem. 276, 4298–4303
40. Hirst, J., Lui, W. Y., Bright, N. A., Totty, N., Seaman, M. N., and Robinson, M. S. (2000) J. Cell Biol. 149, 67–80
41. Dell’Angelica, E. C., Puertollano, R., Mullins, C., Aguilar, R. C., Vargas, J. D., Hartnell, L. M., and Bonifacino, J. S. (2000) J. Cell Biol. 149, 81–94
42. Boman, A. L., Zhang, C., Zhu, X., and Kahn, R. A. (2000) Mol. Biol. Cell 11, 1241–1255

Fig. 6. Molecules containing leucine-sorting signals that are known to interact with AP3 or be targeted directly to endosomes/lysosomes/melanosomes/vacuoles. Leucine signals from molecules that have been shown to interact with AP3 are shown in bold. Residues that may be preferred for AP3, but not for AP1 or AP2 binding, are shown in bold italic. Signals that have been shown to bind AP3 are marked with * (based on data from Refs. 35 and 36–39). The other constructs have not, to our knowledge, been tested for AP3 interactions. NT, not tested.
43. Poussu, A., Lohi, O., and Lehto, V. P. (2000) *J. Biol. Chem.* **275,** 7176–7183
44. Misra, S., Puertollano, R., Kato, Y., Bonifacino, J. S., and Hurley, J. H. (2002) *Nature* **415,** 933–937
45. Shiba, T., Takatsu, H., Nogi, T., Matsugaki, N., Kawasaki, M., Igarashi, N., Suzuki, M., Kato, R., Earnest, T., Nakayama, K., and Wakatsuki, S. (2002) *Nature* **415,** 937–941
46. Puertollano, R., Aguilar, R. C., Gorshkova, I., Crouch, R. J., and Bonifacino, J. S. (2001) *Science* **292,** 1712–1716
47. Nielsen, M. S., Madsen, P., Christensen, E. I., Nykjaer, A., Gliemann, J., Kasper, D., Pohlmann, R., and Petersen, C. M. (2001) *EMBO J.* **20,** 2180–2190
48. Zhu, Y., Doray, B., Poussu, A., Lehto, V. P., and Kornfeld, S. (2001) *Science* **292,** 1716–1718
49. Folsch, H., Ohno, H., Bonifacino, J. S., and Mellman, I. (1999) *Cell* **99,** 189–198
50. Obermuller, S., Kiecke, C., von Figura, K., and Honing, S. (2002) *J. Cell Sci.* **115,** 185–194
51. Ohno, H., Aguilar, R. C., Yeh, D., Taura, D., Saito, T., and Bonifacino, J. S. (1998) *J. Biol. Chem.* **273,** 25915–25921
52. Aguilar, R. C., Boehm, M., Gorshkova, I., Crouch, R. J., Tomita, K., Saito, T., Ohno, H., and Bonifacino, J. S. (2001) *J. Biol. Chem.* **276,** 3
53. Sosa, M. A., Schmidt, B., von Figura, K., and Hille-Rehfeld, A. (1993) *J. Biol. Chem.* **268,** 12537–12543
54. Bergeland, T., Widerberg, J., Bakke, O., and Nordeng, T. W. (2001) *Curr. Biol.* **11,** 644–651
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