A di-leucine-based motif in the cytoplasmic tail of LIMP-II and tyrosinase mediates selective binding of AP-3

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Among the various coats involved in vesicular transport, the clathrin associated coats that contain the adaptor complexes AP-1 and AP-2 are the most extensively characterized. The function of the recently described adaptor complex AP-3, which is similar to AP-1 and AP-2 in protein composition but does not associate with clathrin, is not known. By monitoring surface plasmon resonance we observed that AP-3 is able to interact with the tail of the lysosomal integral membrane protein LIMP-II and that this binding depends on a DEXXXLI sequence in the LIMP-II tail. Furthermore, AP-3 bound to the cytoplasmic tail of the melanosome-associated protein tyrosinase which contains a related EEXXXLL sequence. The tails of LIMP-II and tyrosinase either did not interact, or only interacted poorly, with AP-1 or AP-2. In contrast, the cytoplasmic tails of other membrane proteins containing di-leucine and/or tyrosine-based sorting signals did not bind AP-3, but AP-1 and/or AP-2. This points to a function of AP-3 in intracellular sorting to lysosomes and melanosomes of a subset of cargo proteins via di-leucine-based sorting motifs.

Keywords: biosensor/coated vesicles/endosome/membrane traffic/protein sorting

Introduction

The lysosomal integral membrane protein LIMP-II (Barrinocanal *et al.*, 1986; Vega *et al.*, 1991), originally isolated from rat cells, belongs to a group of proteins that is highly enriched in lysosomal membranes. This group also comprises membrane proteins of the lamp-1, lamp-2 and lamp-3 family, and lysosomal acid phosphatase (LAP) (reviewed in Hunziker and Geuze, 1996). LIMP-II is a type-III protein that traverses the membrane twice, with an N-terminal transmembrane anchor as a result of an uncleaved signal peptide and a second membrane spanning region near the C-terminus, followed by a 20 amino acid cytoplasmic tail. Most of the protein loops into the lysosome where five cysteine residues may be involved in disulfide bond formation. This region also contains 11 potential N-glycosylation sites. Glycosylation of the protein results in an apparent molecular weight of 74 kDa for the 477 amino acid mature protein. LIMP-II does not display homology to any of the other lysosomal

membrane proteins and its function is not yet understood (Barrinocanal *et al.*, 1986; Vega *et al.*, 1991). Unlike the proteins of the lamp family, which contain a tyrosine-based sorting signal of the GYXXZ type (Z being either I, F, L or V) mediating intracellular sorting (Höning and Hunziker, 1995; Höning *et al.*, 1996; Gough and Fambrough, 1997), the LIMP-II cytoplasmic tail harbors an LI signal of the di-leucine type. This signal has been shown to be necessary for the intracellular targeting of the protein to lysosomes (Vega *et al.*, 1991; Sandoval *et al.*, 1994). Though LIMP-II has been shown to be packed into an as yet undefined type of coated vesicles at the TGN (Barriocanal *et al.*, 1986), further details of the intracellular pathways of LIMP-II are not known.

Clathrin-coated vesicles (CCVs) were the first type of intracellular vesicles to be identified and characterized. Clathrin is the major structural component of CCVs; the specificity of its function is mediated by the associated adaptor complexes AP-1 and AP-2 (reviewed in Pearse and Robinson, 1990). Coated pits and coated vesicles that are formed during endocytosis only contain the AP-2 complex, while the same type of coated pits and coated vesicles that derive from the TGN contain the AP-1 complex (Robinson, 1987). The four subunits that build up the AP-1 complex are the large γ - and β 1-subunits together with the μ 1-medium and the σ 1-small subunit. The related AP-2 complex consists of the α - and β 2-subunits that assemble together with the μ 2- and σ 2-subunits (Ahle *et al.*, 1988; Schröder and Ungewickell, 1991; Traub, 1997). The two complexes display significant homology to each other and in addition exhibit the similar structure of a large trunk that is separated from appendices ('ears') by a flexible hinge. A major function of both adaptor complexes is to promote clathrin-lattice formation onto the respective membrane (Wilde and Brodsky, 1996). This function seems likely to involve the large subunits γ - and β 1 of AP-1 and α - and β 2 of the AP-2 complex. A second important function of AP-1 and AP-2 is the interaction with sorting signals that are present in the cytoplasmic tails of cargo proteins to be concentrated in CCVs (for review see Kirchhausen *et al.*, 1997; Robinson, 1997). Studies using different experimental systems have revealed that tyrosine-based sorting signals of membrane proteins such as the LDL receptor (Pearse, 1988), LAP (Sosa *et al.*, 1993), mutant hemagglutinin (Heilker *et al.*, 1996) and lamp-1 (Höning *et al.*, 1996), as well as di-leucine-based sorting signals of membrane proteins such as the MPR300 (Glickman *et al.*, 1989), mutant hemagglutinin (Heilker *et al.*, 1996), invariant chain (Salamero *et al.*, 1996) and MPR46 (Mauxion *et al.*, 1996; Höning *et al.*, 1997), can be recognized by adaptor complexes. The situation could be more complex for proteins that contain more than one sorting

signal in their tail, such as the LDL receptor (Matter *et al.*, 1992, 1994) and the MPRs (Denzer *et al.*, 1997; Höning *et al.*, 1997), and it is not clear whether adaptors recognize only a single motif or if they simultaneously interact with multiple sequence motifs. With the yeast two-hybrid system it was possible to show that different types of tyrosine-based sorting signals can be recognized by the μ -chains of AP-1 and AP-2 (Ohno *et al.*, 1995, 1996; Boll *et al.*, 1996). However, it is still not clear if membrane proteins containing di-leucine-based sorting signals also interact with the μ -chain, or if one of the other subunits is involved in the recognition of this type of sorting signal.

Apart from AP-1 and AP-2, which promote the formation of clathrin-coated pits and vesicles at the plasma membrane and at the TGN, many organelles do not seem to contain a clathrin coat yet vesicular transport to and from all organelles is a prerequisite for the viability of the living cell. This has led to the hypothesis that other protein coats beside clathrin may exist that have a similar function in membrane traffic (Stoorvogel *et al.*, 1996; Robinson, 1997). The COP-I and COP-II coats that are involved in transport between the ER and the Golgi are two examples of recently characterized non-clathrin coats (for review see Cosson and Letourneur, 1997). The identification of novel coats that may have a function in membrane protein sorting along their intracellular traffic routes is a subject of active research (Stoorvogel *et al.*, 1996; Robinson, 1997).

Based on a significant sequence homology to the known AP-1/AP-2 subunits and the search of EST databases, four subunits of a novel adaptor-like complex named AP-3 have recently been cloned and characterized (Newman *et al.*, 1995; Dell'Angelica *et al.*, 1997a; Simpson *et al.*, 1997). Like AP-1 and AP-2, AP-3 is a protein heterotetramer, which is composed of δ -adaptin (160 kDa), β 3-adaptin (140 kDa), the medium chain μ 3 (47 kDa) and the small chain σ 3 (22 kDa). The complex is ubiquitously expressed, with special variants of β 3 (β -NAP) and μ 3 (p47B) being expressed in brain. In contrast to AP-1 and AP-2, the AP-3 complex does not associate with clathrin. Immunofluorescence analysis has revealed a distribution of AP-3 in the Golgi region and in peripheral structures that are thought to represent endosomes (Newman *et al.*, 1995; Simpson *et al.*, 1997). By the use of the yeast two-hybrid system it was demonstrated that μ 3 can interact with tyrosine-based sorting signals similar to the clathrin-associated counterparts μ 1 and μ 2 in AP-1 and AP-2 (Dell'Angelica *et al.*, 1997b).

In an attempt to characterize the adaptor complexes that bind to the cytoplasmic tail of LIMP-II, we observed that LIMP-II interacts with AP-3 but not with AP-1 or AP-2. This interaction is specific and dependent on the LI motif that is critical for sorting of LIMP-II. In addition, the acidic residues DE in positions -4 and -5 to the LI signal appear to modulate this interaction. Furthermore, the cytoplasmic tail of tyrosinase, a membrane protein of lysosome-related melanosomes which contains a similar di-leucine motif, was also found to interact with AP-3. We propose a role of AP-3 in the sorting of a subset of lysosomal and melanosomal membrane proteins.

Results

The LIMP-II tail is not recognized by AP-1 and AP-2

It has recently been shown that the lysosomal membrane protein lamp-1 binds through a tyrosine-based sorting signal in its cytoplasmic tail to AP-1 and AP-2 with high affinity (Höning *et al.*, 1996). In addition, electron microscopic analysis of cells expressing wild-type (wt) or mutant lamp-1 have shown a co-localization of lamp-1 and AP-1, and the appearance of lamp-1 in TGN-derived clathrin-coated vesicles. LIMP-II shares with lamp-1 the direct routing to lysosomes, bypassing the cell surface (Vega *et al.*, 1991; Sandoval *et al.*, 1994). Unlike lamp-1, the cytoplasmic tail of LIMP-II lacks a tyrosine-based sorting signal. Targetting of LIMP-II to lysosomes depends on a pair of LI residues which belongs to the class of di-leucine-based sorting motifs (Sandoval and Bakke, 1994; Sandoval *et al.*, 1994).

To analyze a possible interaction of the LIMP-II cytoplasmic tail with purified AP-1 and AP-2, we utilized a biosensor system monitoring surface plasmon resonance (SPR). This method has been used in several studies to analyze the interaction between purified adaptors and the EGF-receptor (Boll *et al.*, 1995), the hemagglutinin tail (Heilker *et al.*, 1996) and the lamp-1 tail (Höning *et al.*, 1996), and to define the AP-1 and AP-2 binding sites on the MPR46 tail (Höning *et al.*, 1997). A peptide corresponding to the full-length LIMP-II tail (Figure 1) was synthesized, together with a mutant tail peptide where the leucine of the LI-sorting signal of LIMP-II is replaced by glycine (LIMP-II L18G; Figure 1). This substitution was previously shown to disrupt the lysosomal sorting of LIMP-II (Sandoval *et al.*, 1994). As a positive control we used a peptide corresponding to the tail of MPR46, which is known to bind to AP-1 and AP-2 (Sosa *et al.*, 1993; Höning *et al.*, 1997). The LIMP-II peptides were synthesized with an additional N-terminal cysteine residue which allows coupling of the peptides to the sensor chip surface via this residue (see Materials and methods). All peptides were coupled to a CM-5 sensor chip in equal densities (~ 0.2 pmol/mm² per peptide, data not shown) and tested for their ability to bind to purified AP-1 and AP-2. As shown in Figure 2, only the MPR46 tail interacted with AP-1 ($K_D=13$ nM) and AP-2 ($K_D=17$ nM). In contrast, neither the wt LIMP-II peptide nor the LIMP-II L18G peptide interacted detectably with AP-1 or AP-2 at adaptor concentrations of up to 500 nM (shown for 100 nM AP-1 or AP-2, Figure 2).

Brain cytosol contains a component with LIMP-II tail binding activity

Since we did not detect any binding of AP-1 or AP-2 to the LIMP-II tail, we examined whether or not other cytosolic components interact with the LIMP-II cytoplasmic tail in a manner that is dependent on the LI sorting signal. For this purpose, cytosol from pig brain was prepared. When the brain cytosol was passed over the MPR46 tail-derived sensor surface, a strong interaction was observed suggesting the presence of functional adaptors (see below and Figure 5). A similar interaction was monitored for the wt LIMP-II tail, while only a reduced interaction was detected for the mutant LIMP-II L18G tail peptide (Figure 3). In the presence of

limp-II wt	-CRQGSTDEGTADERAP L IRT
limp-II L18G	-CRQGSTDEGTADERAP G IRT
limp-II Δ17-21	-CRQGSTDEGTADERA
limp-II D13R E14R	-CRQGSTDEGT ARR APLIRT
limp-II D13A	-CRQGSTDEGT AA ERAPLIRT
limp-II E14A	-CRQGSTDEGTAD A RAPLIRT
limp-II S6A	-CRQ Q STDEGTADERAPLIRT
limp-II E9Q	-CRQGSTD Q GTADERAPLIRT
limp-II T11A	-CRQGSTDEG A ADERAPLIRT
lamp-1 wt	-RKRSHAG Y QTI
lamp-1 Y8A	-RKRSHAG A QTI
LAP wt	-RMEAEPPG Y RHVADGGQDHA
LAP Y9A	-RMEAEPPG A RHVADGGQDHA
Tyrosinase wt	-C---KKQPQEERQ PLL MDKDDYH SL YQSHL
Tyrosinase L17A/L18A	-C---KKQPQEERQ AA MDKDDYH SL YQSHL
Tyrosinase L27A/L28A	-C---KKQPQEERQ PLL MDKDDYH SA YQSHL
Tyrosinase LLLL/AAAA	-C---KKQPQEERQ AA MDKDDYH SA YQSHL
MPR46 wt	-QRLVVGAKGMEQ F PHLAFWQDLGNLVADGCDFVCRSKPRNVPA AY RGVGGDQLGEESEERDDH LL PM

Fig. 1. Amino acid sequences of cytoplasmic tail peptides. Peptides corresponding to the cytoplasmic tails of wt and mutant LIMP-II, lamp-1, LAP, tyrosinase and MPR46 are shown in the one letter code with the C-terminal end to the right. The open boxes on the left represent the junction of the membrane spanning region and the cytoplasmic tail. Residues known to be critical for sorting, and the corresponding mutations, are indicated by bold letters. All peptides represent the full-length cytoplasmic tails of the respective proteins except that of tyrosinase. The tyrosinase peptide lacks the membrane proximal residues 1–5. All peptides were immobilized on the sensor chip surface involving primary amino or thiol groups (see Materials and methods).

carboxymethylated dextran, which minimizes unspecific binding to the chip surface which itself contains carboxymethylated dextran, the residual binding to the mutant LIMP-II tail was negligible (Figure 3). In contrast, the signal obtained with the wt LIMP-II tail and the MPR46 tail peptide was only slightly reduced indicating the specificity of the interaction. Thus pig brain cytosol contains one or more components that have the ability to bind to the LIMP-II tail in a manner that is dependent on the LI sorting signal.

AP-3, but not AP-1 or AP-2, binds to the LIMP-II tail

The experiments described above demonstrated that a cytosolic factor(s) exists which can bind specifically to the LIMP-II tail. To further analyze the cytosolic component(s) that binds to the cytoplasmic tails of MPR46 and LIMP-II, we fractionated pig brain cytosol by gel filtration. All fractions obtained were then passed over sensor surfaces derived with the MPR46 tail, with wt LIMP-II or the LIMP-II L18G mutant tail peptide. To compare the binding, the resonance units that remained bound to the chip-surface after a 2 min association/2 min dissociation cycle were plotted. It should be noted that this experiment was performed without quenching non-specific interaction by adding carboxymethylated dextran. As shown in Figure 4, we observed a separation of the components that bind to

Fig. 2. Binding of purified AP-1 and AP-2 to LIMP-II and MPR46 tail peptides. AP-1 and AP-2 preparations were passed over MPR46-, wt LIMP-II- and LIMP-II L18G-derived chip surfaces for 2 min (association) before the chip surface was washed with buffer A for 2 min (dissociation). Only MPR46 was able to interact with both AP-1 and AP-2, whereas wt and mutant LIMP-II did not interact with either adaptor complex.

LIMP-II from that binding to MPR46. Fractions 23–26, while exhibiting an interaction with the MPR46 tail peptide, did not interact at all with the LIMP-II peptides. On the other hand, fractions 28–30 exhibited binding activity for the LIMP-II tail but not for the MPR46 tail. Furthermore, the L18G mutant peptide was only poorly recognized by fractions 28–30. No other fractions besides those plotted in Figure 4 were found to bind to the tail peptides.

As it had already been shown that MPR46 binds to both clathrin adaptors, it was suspected that fractions

Fig. 3. Binding of pig brain cytosol to the LIMP-II cytoplasmic tail. Crude brain cytosol from pig brain was prepared and passed in the presence or absence of 1 mg/ml carboxymethylated dextran over tail-peptide derived sensor chip surfaces to record interaction. MPR46 and wt LIMP-II were able to bind a component(s) of brain cytosol, whereas binding to mutant LIMP-II L18G was of low affinity. Carboxymethylated dextran, which reduces non-specific binding to the chip surface, abolished almost completely the residual binding to LIMP-II L18G.

23–26 may contain AP-1 and AP-2. All fractions obtained from gel filtration were subjected to SDS-PAGE and Western blotting, followed by incubation with antibodies specific for the known adaptor complexes. To detect the AP-1 complex, a monoclonal antibody against the γ -subunit was applied; the AP-2 complex was detected by an α -subunit specific antibody; and the AP-3 complex was identified by antiserum specific for the δ -subunit (Simpson *et al.*, 1997). As shown in Figure 4, the AP-2 specific antibody only reacted with fractions 23–26. An identical pattern was observed with an antibody against AP-1 (data not shown). In contrast, the anti-AP-3 antibody reacted with fractions 28–30, but not with fractions 23–26. Thus, gel filtration of brain cytosol leads to the separation of AP-1/AP-2 from AP-3. The apparent size of AP-3 as compared with a molecular weight standard matched a molecular weight of ~350 kDa. In contrast, the clathrin-associated adaptor complexes appeared to run as a complex slightly larger than expected from their calculated weight, which is comparable with that of AP-3. This unusual behaviour during gel filtration of AP-1 and AP-2 has been noted previously (Keen *et al.*, 1979; Keen 1987). Most interestingly, the LIMP-II tail is only recognized by factors present in the AP-3 enriched fractions but does not show any interaction with AP-1/AP-2-containing fractions (Figure 4). This result is

consistent with the finding that the LIMP-II tail does not interact with purified AP-1 or AP-2 (Figure 2).

To further corroborate these findings, crude brain cytosol was passed over MPR46 and LIMP-II-derived surfaces for a 2 min association cycle. After a 2 min buffer wash (dissociation), antibodies that recognize native AP-2 or AP-3 were passed over the chip surface. As shown in Figure 5, when cytosol had been passed over the MPR46 tail-derived surface a strong signal was obtained with the anti-AP-2 antibody, indicating that AP-2 is recruited to the tail. In contrast, no binding of the anti-AP-3 antibody was observed, in agreement with the observation that AP-3 does not bind to MPR46. On the other hand, when crude brain cytosol was applied to the LIMP-II-derived surface a specific interaction with anti-AP-3 was observed, but not with anti-AP-2 (or anti-AP-1, data not shown). Thus the perfusion of adaptor-specific antibodies over the tail-derived chip surfaces that were first allowed to recruit the adaptors from cytosol confirmed that AP-3 binds to the LIMP-II tail.

AP-3 binding to LIMP-II requires an acidic cluster in addition to the LI sorting motif

It has previously been shown in COS cells transfected with tail-mutants of LIMP-II that the LI motif is critical for correct intracellular sorting. In addition, mutation of the aspartic and glutamic acidic residues at positions –5 and –4 relative to the LI motif also interferes with correct lysosomal targeting of LIMP-II (Sandoval *et al.*, 1994; S.Martinez-Arca and I.V.Sandoval, submitted). In order to define further the sequence determinants in the LIMP-II tail that mediate AP-3 binding, a set of mutant tail peptides of LIMP-II (Figure 1) was synthesized to analyze their binding to AP-3 by SPR (see above and Figure 6). The importance of leucine 475 for AP-3 binding has already been shown since the control peptide of the LIMP-II tail, in which the leucine is replaced by glycine (LIMP-II L18G), does not bind AP-3 (Figures 3 and 4). Furthermore, when a truncated tail-peptide of LIMP-II lacking the C-terminal pentapeptide PLIRT was immobilized, binding of AP-3 was totally abolished (data not shown). This result is in agreement with the data obtained from cells expressing the corresponding truncated LIMP-II protein which is delivered to the cell surface (Sandoval *et al.*, 1994).

When the two amino acid residues DE, in positions –4 and –5 relative to the LI motif, were mutated separately to alanines, a 20- to 60-fold decrease in affinity to AP-3 as compared with the wt LIMP-II tail was observed (Figure 6A). Moreover, the replacement of both residues by arginine led to a >100-fold decrease in binding affinity to AP-3. These results indicate that both of the two acidic residues preceding the LI motif in the LIMP-II tail at positions –4 and –5 are necessary for a high affinity interaction with the AP-3 complex. Another explanation would be that mutations within the tail peptide disrupt the structure of the immobilized peptide, thereby causing loss of adaptor binding. To assess the plausibility of this assumption, three different mutant LIMP-II tail peptides were synthesized (LIMP-II S6A, E9Q and T11A) which correspond to LIMP-II mutants that are known to be correctly sorted *in vivo* (Sandoval *et al.*, 1994). As shown in Figure

Fig. 4. Gel filtration analysis of pig brain cytosol. Cytosol from pig brain was fractionated by gel filtration on Superdex-200. The fractions were analyzed by SDS-PAGE and Western blotting, and probed with antibodies to the α -subunit of AP-2 and the δ -subunit of AP-3 as indicated in the figure. Furthermore, each fraction was tested for interaction with the MPR46, wt LIMP-II and LIMP-II L18G-derived sensor chip surfaces. The bars represent the resonance units that remained associated to the chip after an association (2 min)/dissociation (2 min) cycle. No other fraction, apart from those indicated, exhibited any interaction with the tail peptides. The values presented are corrected for background binding which was below 55 RU in all fractions.

6B, the observed binding of AP-3 to these mutant tail peptides was nearly identical to that observed for wt LIMP-II, indicating that mutations within the tail peptide do not lead *a priori* to loss of adaptor binding. In addition, previous NMR analysis of the LIMP-II tail peptide (Sandoval *et al.*, 1994) has revealed the predominance of random coil conformations indicating a high flexibility of the LIMP-II tail. Taken together, the data obtained with the biosensor confirm the *in vivo* data showing that sorting of LIMP-II is dependent on a di-leucine motif which functions in the context of two acidic residues. The observation that substitutions of several residues known not to interfere with sorting of LIMP-II also do not affect the interaction of the LIMP-II tail with AP-3 underlines the significance of the *in vitro* data.

The specificity of the AP-3 LIMP-II tail interaction was also confirmed by the perfusion of the LIMP-II-derived chip with an anti-LIMP-II tail antiserum prior to the injection of the AP-3 enriched fraction. The antiserum, which specifically recognizes the C-terminal 6 amino acids of the LIMP-II tail, was injected at a low flow-rate to obtain maximal binding. Subsequently, an AP-3-enriched fraction (fraction 29, see Figure 4) was passed over the surface. The antibody perfusion led to a >70% loss in binding of AP-3, while perfusion with a control serum did not interfere with the subsequent binding of AP-3 to the LIMP-II tail (Figure 7). It should be noted that the LIMP-II tail antibody does not cross-react with the mutant tail-peptide L18G (data not shown). Thus binding of the

Fig. 5. AP-3 is the cytosolic component that binds to the LIMP-II tail peptide. A cytosol fraction enriched in AP-3 was passed over a LIMP-II-derived chip surface (left) and an AP-2 enriched cytosol fraction over a MPR46-derived chip surface (right). After an association/dissociation cycle, antibodies specific for AP-3 (δ -subunit) or AP-2 (α -subunit) were injected. Note that LIMP-II is recruiting AP-3 but not AP-2 from the cytosol, as revealed by the positive signal obtained by the anti-AP-3 antibody. On the other hand, the MPR46 recruits AP-2 but not AP-3 from the cytosol, as indicated by the strong signal with the anti-AP-2 antibody.

tail-specific antiserum to a hexapeptide comprising the LI-sorting motif leads to the inhibition of AP-3 interaction. Taken together the experiments described above demon-

Fig. 6. Acidic residues preceding the LI motif in the LIMP-II tail are necessary for high affinity binding of AP-3. LIMP-II tail peptides with mutations of the acidic residues D12 and/or E13 (Figure 1) were coupled to the CM-5 sensor chip in equal densities ($0.2 \text{ pmol/mm}^2 \pm 10\%$). The derived surfaces were then tested for their ability to interact with an AP-3 enriched cytosol fraction (A). Tail peptides that correspond to LIMP-II mutants known not to interfere with sorting *in vivo* served as controls (B). The kinetic values for the on-rate (k_a), the off-rate (k_d) and the equilibrium constant K_D (k_a/k_d) represent relative affinities to that of the wt LIMP-II tail set to 1 (C).

strate that a DEXXXLI-sorting motif in the LIMP-II tail mediates a high affinity interaction with AP-3.

AP-3 is the major cytosolic factor that has a LIMP-II binding capacity

The experiments described above provide evidence that the AP-3 complex becomes bound to the cytoplasmic tail of LIMP-II. However, it is possible that additional cytosolic factors contribute to the binding activity detected by SPR and that binding of AP-3 may even be of an indirect nature. To test this possibility, cytosol was immuno-depleted of AP-3. If cytosolic factors apart from AP-3 bind to the LIMP-II tail, immuno-depletion of AP-3 from cytosol should only partially reduce the binding activity. The brain cytosol was depleted from AP-3 by two rounds of incubation with anti-AP-3 antiserum and protein A,

Fig. 7. Binding of AP-3 to the LIMP-II tail is sensitive to the injection of anti-LIMP-II tail antiserum. An anti-LIMP-II tail antiserum or a control serum was passed over the LIMP-II-derived chip. To obtain maximum binding of the antiserum to the tail-peptide the flow-rate was reduced to $5 \mu\text{l/min}$. After antibody washing was extended to 6 min to reach a near constant resonance, an AP-3 enriched cytosol fraction (black curve, see Figure 4) was passed over the chip surface, followed by a wash with buffer. As a control, the dissociation of the antibodies from the LIMP-II tail peptide was recorded separately for up to 10 min (grey curve). Note that AP-3 binding following saturation of the chip surface with the anti-LIMP-II tail antiserum (A) was reduced by $>70\%$ of the control (B).

bearing *Staphylococcus aureus* cells. Control cytosol was treated likewise except that the anti-AP-3 antiserum was omitted (see Materials and methods). Identical aliquots of immuno-depleted and control cytosol were then analyzed for binding to the wt LIMP-II as well as to the mutant LIMP-II tail, and to the MPR46 tail. As shown in Figure 8, after the incubation without antibody the cytosol retained 80% of its original binding activity as revealed by incubation with the LIMP-II and the MPR46 tail peptides. However, if the cytosol was depleted from AP-3 by incubation with the specific antibody prior to SPR analysis, binding of the cytosol to the wt LIMP-II tail was reduced by $>75\%$. The residual binding is in the same range as that observed for the binding of immuno-depleted or control cytosol to the L18G mutant tail-peptide. It should be noted that immuno-depleted cytosol retains its ability to bind to the MPR46 tail, indicating the specific depletion of AP-3. These results indicate that AP-3 accounts for essentially all of the specific binding activity to the LIMP-II tail as detected by SPR.

Fig. 8. Cytosol depleted from AP-3 loses the LIMP-II tail binding activity. Crude brain cytosol was precleared by incubation for 1 h with *S.aureus* followed by centrifugation. The supernatant was divided into three aliquots. One was used directly for SPR analysis (open bars). The other aliquots were subjected to two cycles of immuno-depletion with anti-AP-3 antiserum pre-adsorbed to *S.aureus* (filled bars) or *S.aureus* alone (hatched bars) prior to SPR analysis (see Materials and methods). Binding activity of pre-cleared cytosol to the LIMP-II tail or to the MPR46 tail (determined as in Figure 4) was set to 100% and used as reference for the other binding activities. Immuno-depletion of AP-3 resulted in a reduction of the binding activity to the LIMP-II wt tail that is comparable with that to the mutant tail peptide, indicating that AP-3 is the major cytosolic component accounting for the cytosolic LIMP-II tail-binding activity.

AP-3 binding to lamp-1 and LAP

The lysosomal membrane is enriched in a variety of other membrane proteins that do not belong to the LIMP-II family. These lamp proteins, as well as the transmembrane form of LAP, have in common the fact that their short cytoplasmic tails contain a tyrosine-based sorting motif which is critical for their targeting (Hunziker and Geuze, 1996). LAP has been shown to bind AP-2 but not AP-1 (Sosa *et al.*, 1993), whereas lamp-1 is known to interact with both AP-1 and AP-2 (Höning *et al.*, 1996). Data regarding binding to AP-3 are missing. We therefore compared the binding of adaptors to peptides corresponding with the cytoplasmic tails of LAP and lamp-1. In contrast to LIMP-II (Figure 6), the interaction between AP-3 and the tails of lamp-1 and LAP was of low affinity (Figure 9D). On the other hand, the interaction of lamp-1 and the LAP tail with a cytosolic fraction enriched in AP-1 and AP-2 was of high affinity (Figure 9C). The binding of AP-1/AP-2 to LAP and lamp-1 is dependent on the critical residues of their tyrosine-based sorting motifs, since binding to peptides in which the critical tyrosine residues of the sorting motifs had been substituted by alanines (see Figure 1) was close to background level (data not shown). The sensorgrams shown in Figure 9C and D have been corrected for background binding to the mutant peptides. When purified AP-1 and AP-2 were used, we calculated K_D values of 20 nM (LAP) and 64 nM (lamp-1) (data not shown). As the concentration of AP-3 is unknown, the K_D value cannot be determined. However, the relative affinity of AP-3 to LAP and lamp-1 was 25–1000× lower than that observed for AP-3 binding to LIMP-II. Thus the interaction between AP-3 and lysosomal membrane proteins that contain tyrosine-based sorting signals is of low affinity.

Fig. 9. Tail peptides that can interact with AP-3 *in vitro*. The wt and mutant tail peptides of tyrosinase (A and B), LAP and lamp-1 (C and D) were immobilized on the sensor chip and analyzed for their binding capacity of AP-1/AP-2 (A and C) and AP-3 (B and D) enriched cytosolic fractions (Figure 4). Note that the sensorgrams shown in (C) and (D) represent the difference in binding to wt and mutant forms of the tail peptides. In the latter, the critical tyrosine or leucine residues of the sorting signals had been substituted by alanine (Figure 1).

Tyrosinase, a melanosomal membrane protein, binds AP-3

It is known that most cells have specialized organelles with features similar to that of lysosomes. One such example is that of melanosomes, which play an important role in pigmentation. A key protein of melanosomes is tyrosinase, a membrane protein that is involved in melanine synthesis (Delmarmol and Beermann, 1996). Tyrosinase, if transfected into COS cells, is targeted to lysosomes. In this context it should be noted that tyrosinase contains two di-leucine motifs in its cytoplasmic tail (Figure 1). However, it is not known which of the signals mediate intracellular targeting of the protein.

Since *Drosophila* AP-3 mutants show a dramatic loss of pigmentation (Ooi *et al.*, 1997; Simpson *et al.*, 1997), it was of interest to analyze whether a tail peptide corresponding to the wt tyrosinase tail binds to adaptors. We therefore analyzed the ability of wt and mutant tyrosinase tail peptides (see Figure 1) to interact with AP-1, AP-2 and AP-3 enriched cytosolic fractions as described above. As shown in Figure 9B, we observed a high affinity interaction between tyrosinase and AP-3. The relative equilibrium constant for the AP-3–tyrosinase interaction was 20% below the value obtained for the LIMP-II tail–AP-3 interaction (arbitrarily set to 1). In contrast to the LIMP-II tail, the tyrosinase tail had a low affinity for AP-1/AP-2, which was >100× slower than the AP-1/AP-2–lamp-1 interaction (Figure 9A and C). In order to test which of the two di-leucine motifs (or both) in the tail of tyrosinase is part of the sorting signal, we also tested tyrosinase tail peptides for adaptor binding in which the proximal and distal di-leucine motifs were mutated individually or in combination (Figure 1). Adaptor binding was totally abolished when both di-leucine motifs were substituted for alanines. The same was observed if the proximal signal was destroyed. The tyrosinase tail

peptide in which the distal di-leucine motif is mutated exhibited a significant residual affinity to AP-3 (Figure 9B). Thus the proximal tyrosinase di-leucine signal is essential for AP-3 binding *in vitro*. This result is consistent with *in vivo* experiments using gp75, a tyrosine-related protein that has a similar tail sequence. For gp75 it was shown that a NQPLLT sequence in the same position as the relevant proximal tyrosinase motif is essential for correct intracellular targeting (Vijayasaradhi *et al.*, 1995). Substitution of either di-leucine motif abolished binding of AP-1/AP-2 (Figure 9A). The experiments described above show, therefore, that the tyrosinase tail harbors a di-leucine-based sorting signal similar to that of LIMP-II, which mediates the high-affinity interaction with AP-3.

Discussion

Binding of AP-3 to the cytoplasmic tails of LIMP-II and tyrosinase

The peptides corresponding to the cytoplasmic tail of LIMP-II or tyrosinase-bound AP-3 with high affinity, while binding of cytosolic fractions enriched in AP-1 and AP-2 was either below the limit of detection (LIMP-II) or of low affinity (tyrosinase). The binding of AP-3 to LIMP-II was characterized in more detail and shown to depend on leucine 18, which is a critical component of the di-leucine-based motif involved in the sorting of LIMP-II to lysosomes (Ogata and Fukuda, 1994; Sandoval *et al.*, 1994). Furthermore, binding was greatly reduced by preincubating the LIMP-II-derived chip with an antibody which recognizes the LI motif within the context of the C-terminal hexapeptide of the LIMP-II tail. For the binding studies, a cytosol fraction enriched in AP-3 and essentially free of AP-1 and AP-2 was used. The complete loss of LIMP-II binding activity that was observed after two rounds of immuno-depletion of crude cytosol from AP-3 showed that AP-3 accounts for essentially all of the cytosolic binding activity to the LIMP-II tail. The lack of purified AP-3 prevented us from calculating the equilibrium constant for binding. Furthermore, we cannot exclude that components complexed to AP-3 contribute to the observed biosensor signal.

The cytoplasmic tails of LIMP-II and tyrosinase share a sorting motif of the di-leucine type (Ogata and Fukuda, 1994; Sandoval *et al.*, 1994; Vijayasaradhi *et al.*, 1995) which in both is directly preceded by a D(E)ERXP sequence. In this context, the acidic residues in positions –4 and –5 are critical for targeting of LIMP-II to lysosomes *in vivo* (Pond *et al.*, 1995; S.Martinez-Arca and I.V.Sandoval, submitted). Here we have shown that the same residues are critical for binding of AP-3. Although the functional significance of the di-leucine motif for sorting of tyrosinase remains to be demonstrated, two observations are of interest with regard to this. First, truncation of the cytoplasmic tail of tyrosinase, as it occurs in mice with the platinum allele of tyrosinase at residue –10 relative to the di-leucine motif, results in misrouting of tyrosinase and severe oculocutaneous albinism (Beermann *et al.*, 1995). Secondly, the EXRQPLL heptamer sequence, which is shared between tyrosinase and several other melanosomal proteins, is critical for the sorting of the tyrosinase-related brown protein gp75 to melanosomes

(Vijayasaradhi *et al.*, 1995). It should be noted that tyrosinase contains a second potential di-leucine motif located nine residues further towards the C-terminus (Figure 1). The contribution of this second motif, which is also preceded by acidic residues in positions –4 and –5, to sorting remains to be determined. However, the observation that a tyrosinase tail peptide, in which the second di-leucine pair is replaced by alanines, retains significant binding for AP-3 makes it likely that the first di-leucine motif is critical for sorting.

The binding of AP-3 to tyrosinase and its likely involvement in targeting of tyrosinase to melanosomes is in agreement with the observation that altered expression of *garnet*, the *Drosophila* ortholog of the mammalian δ -subunit of AP-3 (Ooi *et al.*, 1997; Simpson *et al.*, 1997), results in defects in eye pigmentation. This suggests that the pigmentation defect is produced by mis-sorting of components of the biosynthetic machinery for pigments, including tyrosinase, due to defective AP-3.

Structural requirements for AP-3 binding

The μ 3A and μ 3B chains of AP-3 have been shown to interact with tyrosine-based sorting signals in a yeast two-hybrid approach, in which the μ -chains were expressed as fusions with the Gal-4 activation domain and the YQRL sorting signal contained in the itinerant Golgi protein TGN38 in the context of the Gal-4 DNA binding domains (Dell'Angelica *et al.*, 1997b). In our system we failed to observe any interaction of AP-3 with tail peptides containing tyrosine-based sorting signals known to bind AP-1 and/or AP-2 (lamp-1, LAP and MPR46).

The only peptides with AP-3 binding activity were found to share a di-leucine-based sorting motif. It is, however, of interest that the wt MPR46 tail peptide, which bears a di-leucine-based signal that is critical for lysosomal enzyme sorting (Johnson and Kornfeld, 1992), did not bind to AP-3. This observation suggests that the pairs of LL- or LI- are not sufficient for binding. Neighboring acidic residues in position –4 and –5 were of critical importance for the binding of the LIMP-II tail to AP-3. A similar pair of acidic residues, in positions –4 and –5 relative to the di-leucine motif, is found also in the tyrosinase tail (EEXXXLL) and the tail of invariant chain (DDXXXLI). Since AP-3 does not bind to invariant chain (D.Rodionov, S.Höning, K.V.Figura and O.Bakke, unpublished), the sequence D/E EEXXXLL/I is necessary, but not sufficient alone, for AP-3 binding. The specificity of the AP-3–tail interaction must be determined by additional structural features or unknown *in vivo* factors. It should be noted that in the MPR46-, MPR300- and CD3- γ chain cytoplasmic tails, acidic residues (single or pairs) are found neighboring the di-leucine motifs. Indeed, it has recently been shown for the CD3- γ chain that an aspartic acid tail residue resembling a DXXXLL motif is important for the interaction with AP-1 and AP-2 (Dietrich *et al.*, 1997). This points to a general role of acidic residues in adaptor binding to di-leucine motifs.

Role of AP-3 in sorting to lysosomes and related melanosomes

There is ample evidence that lysosomes and melanosomes are related organelles; they share the internal acidic pH and membrane proteins such as lamp-1, LAP and other

acidic hydrolases (Orlow *et al.*, 1993; Schraermeyer, 1995). Moreover, the similarities extend to the protein targeting machinery since melanosomal proteins, when expressed in fibroblasts, are transported to lysosomes which together with the other observations has led to the view that melanosomes are specialized lysosomes (Orlow *et al.*, 1995; Schraermeyer, 1995). The observation that LIMP-II and tyrosinase share the interaction with AP-3 lends strong support to the view that AP-3 is involved in sorting steps common to the biogenesis of lysosomes and melanosomes.

The involvement of AP-3 in the transport of membrane proteins to lysosomes gains further support from a recent report about the functional role of AP-3 in yeast (Cowles *et al.*, 1997a). The authors have first identified the four yeast subunit homologs of AP-3 as APS3 (σ 3A), APM3 (μ 3A), APL5 (δ 3) and APL6 (β 3A), and show that yeast mutants lacking any of the four AP-3 subunits exhibit a defect in the vacuolar sorting of alkaline phosphatase and the vacuolar t-SNARE Vam3p. In addition, the transport defect in the AP-3 mutants is selective, as transport of other vacuolar proteins such as carboxypeptidase S and carboxypeptidase Y was not affected. In this context it should be noted that sorting of alkaline phosphatase to the yeast vacuole depends on a di-leucine-type signal in its cytoplasmic tail (Cowles *et al.*, 1997b).

The question remains as to where in the cell the interaction between AP-3 and cargo proteins such as LIMP-II and tyrosinase occurs. Recent studies using immunofluorescence and immunogold labeling of NRK cells have localized AP-3 to a late Golgi compartment and to endosomal membranes (Newman *et al.*, 1995; Dell'Angelica *et al.*, 1997b; Simpson *et al.*, 1997). It is therefore likely that AP-3 function can be localized to the TGN, or an endosomal compartment or both.

We also performed double immunofluorescence to co-localize AP-3 with different intracellular marker proteins, including lamp-1, LIMP-II, TGN38, AP-1 and clathrin. However, we failed to detect a significant overlap with any of the markers used. It was evident that AP-3 is distributed in very small vesicular structures throughout the entire cell and not concentrated to a perinuclear compartment (data not shown). It should be noted, however, that the steady-state distribution of proteins does not disclose their site of function.

A functional role of AP-3 at the level of the TGN is supported by the observation that the binding of AP-3 to membranes is ARF1-dependent like that of the TGN-localized AP-1 (Simpson *et al.*, 1997). Membrane proteins that have been shown to interact with AP-1 include the MPRs (Sosa *et al.*, 1993; Mauxion *et al.*, 1996; Glickman *et al.*, 1989), lamp-1 (Höning *et al.*, 1996) and invariant chain (Salamero *et al.*, 1996). All these proteins are detected in early endosomes, and also to some extent at the plasma membrane. AP-3-mediated packaging of cargo proteins at the TGN may, in contrast, initiate a pathway which bypasses the endosomal target of AP-1 dependent vesicles, resulting in a more direct delivery of proteins to lysosomes (LIMP-II) or storage vesicles (tyrosinase). With regard to this, the observation that LIMP-II is packed into coated vesicles at the TGN may be significant (Barriocanal *et al.*, 1986). An alternative model would allocate AP-3 function to an endosomal compartment to function in the

segregation of proteins that enter the late endosomal/lysosomal pathway from proteins such as invariant chain and MPR46 which are targeted to the plasma membrane or other intracellular organelles. The failure of lamp-1 and LAP, which share with LIMP-II the lysosome as the final destination, to interact with AP-3 points to additional routes from endosomes to lysosomes that are independent of AP-3. Only further detailed biochemical and morphological studies will help us to identify the intracellular location(s) at which AP-3 is functional in the pathways to lysosomes/melanosomes.

Materials and methods

Preparation of AP-1 and AP-2

The purification and separation of the clathrin-associated protein complexes AP-1 and AP-2 were performed as described (Höning *et al.* 1997).

Antibodies

The monoclonal antibodies against α -adaptin (clone 100/2) and γ -adaptin (clone 100/3) were purchased from Sigma. The antiserum against the tail of LIMP-II was raised by the immunization of rabbits with a peptide corresponding to the last six tail amino acid residues (APLIRT) coupled to hemocyanine-KLH. The antiserum obtained after six immunizations recognized wt LIMP-II, but not a mutant form of LIMP-II in which Leu18 of the cytoplasmic tail was replaced by Gly. The rabbit antibodies specific for the δ -subunit and the μ -subunit of the AP-3 complex were generously provided by Margaret Robinson and are described elsewhere (Simpson *et al.* 1997).

Preparation and fractionation of brain cytosol

Pig brain obtained from a local slaughterhouse was rinsed in 0.9% NaCl; surrounding tissue and blood vessels were removed before the brain was cut into small pieces. 1 g brain was homogenized in 2 ml buffer H (25 mM HEPES-KOH, pH 7.0, 125 mM K-acetate, 2.5 mM Mg-acetate, 1 mg/ml glucose, 0.1 mM EGTA, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin) 20 times using a dounce homogenizer. Subsequently, the homogenate was centrifuged at 4°C for 30 min at 100 000 g. The supernatant was collected and dialyzed twice for 4 h each time against buffer H. After ultracentrifugation as before, the supernatant was aliquoted and used in interaction analysis or for gel filtration. Alternatively, the brain cytosol was shock-frozen in liquid nitrogen for storage.

Gel filtration

For gel filtration experiments the brain cytosol (50 μ l aliquots) was passed over a Superdex-200 column connected to a SMART system (Pharmacia), equilibrated and eluted with buffer A (20 mM HEPES-NaOH, pH 7.0, 150 mM NaCl, 10 mM KCl, 2 mM MgCl₂, 0.2 mM DTT) at a flow-rate of 40 μ l/min. Fractions of 50 μ l were collected and analyzed by Western blot analysis. The proteins thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa) and aldolase (158 kDa) from a gel filtration calibration kit (Pharmacia) were used as standards.

Production of brain cytosol depleted from AP-3

100 μ l of pig brain cytosol (5 mg/ml) were precleared with 20 μ l *S.aureus* (Sigma) for 1 h at 4°C. Subsequently equal aliquots were incubated for 12 h with the antibody against the δ -subunit of AP-3, which was immobilized to *S.aureus*, or with *S.aureus* without antibody. After pelleting the *S.aureus*, the depletion step was repeated a second time. The final supernatant was used for interaction analysis (see below).

Peptide synthesis

All peptides used in this study were synthesized and purified as described (Höning *et al.*, 1997). Their purity was confirmed by HPLC, UV-spectrometry and mass-spectrometry.

SPR interaction analysis

The interaction between the different cytoplasmic tails and AP-1/AP-2 or pig brain cytosolic fractions was analyzed in real-time by SPR (Jonsson *et al.*, 1991) using a BIAcore-2000 biosensor (Biacore AB). The peptides were coupled to a CM5 sensor chip via their primary amino groups exactly according to the manufacturer's instructions.

Peptides that were available with an N-terminal cysteine residue were coupled to the sensor chip using the thiol-coupling procedure. The sensor chip surface is first activated and derivatized to generate an active disulfide on the chip surface. This group is then exchanged for the intrinsic thiol group of the peptide during peptide immobilization. Briefly, the activation of the chip was carried out by the injection of 0.05 M *N*-hydroxysuccinimide/0.2 M *N*-ethyl-*N'*-dimethyl-aminopropyl-carbodiimide for 4 min using a flow-rate of 5 μ l/min. The active disulfide was then introduced by a subsequent injection of 80 mM 2,2-pyridinyldithio-ethane-amine in 0.1 M borate buffer, pH 8.5, for 5 min. The immobilization was carried out by injecting the peptides at 0.1 mg/ml in 10 mM Na-acetate, pH 4.0, for 7 min. Excess reactive disulfides and non-covalently bound peptide were removed by a 5 min injection of 50 mM cysteine/1 M NaCl in 100 mM formate buffer, pH 4.3.

All interaction experiments were performed with buffer A (see above) at a flow-rate of 20 μ l/min unless otherwise stated. Association for 2 min was followed by dissociation for 2 min, during which buffer A was perfused. A short pulse injection (15 s) of 20 mM NaOH/0.5% SDS was used to regenerate the sensor chip surface after each experimental cycle. The peptide-derived sensor chips remained stable and retained their specific binding capacity for >100 experimental cycles of association/dissociation and regeneration. AP-1 and AP-2 were used at 100 nM; crude brain cytosol and the cytosolic fractions derived from gel filtration (see above) were used at a final protein concentration of ~50 μ g/ml.

Determination of kinetic rate constants

The rate constants (k_a for association and k_d for dissociation) of the interaction between tail peptides and purified AP-1 or AP-2 were calculated by using the evaluation software of the BIAcore 2000. The mathematical models that are used are described in more detail elsewhere (O'Shannessy *et al.*, 1993; Schuck and Minton 1996; Schuck, 1997). Association was determined 15–20 s after switching from buffer flow to adaptor solution to avoid distortions due to injection and mixing. The dissociation rate constants were determined 5–10 s after switching to buffer flow. After a first dissociation phase for ~30 s, further dissociation of adaptors was very low. The association constant K_a , the dissociation constant K_d and the calculation of the equilibrium constant $K_D = k_d/k_a$ were determined by using the BIA evaluation software version 1.2, assuming a first order kinetic $A + B = AB$. The model used calculates the association rate constant k_a and the steady state response level R_{eq} by fitting data to the equation:

$$R = R_{eq}(1 - e^{-(K_a C_n + K_d)(t-t_0)})$$

where t is the time in s; R_{eq} , the steady state response level; and C , the molar concentration of adaptors in the injection solution. The steric interference factor N , which describes the valency of the interaction between the adaptors and the MPR46 tail, was set to 1. The dissociation rate constant k_d was determined by fitting data to the equation:

$$R = R_0 e^{-K_d(t-t_0)}$$

where R_0 is the response level at the beginning time t_0 of the dissociation phase. This model, which has recently been applied to describe adaptor tail interaction (Heilker *et al.*, 1996), is described in more detail elsewhere (Karlsson *et al.*, 1991; O'Shannessy *et al.*, 1993). It should be noted that the models described above allow the determination of rate constants without reaching equilibrium during the experimental cycle. The relative rate constants given illustrate the affinity differences between different tail peptides and are independent of adaptor concentration.

Electrophoresis and Western blotting

The cytosolic fractions derived from gel filtration were subjected to SDS-PAGE analysis (Laemmli, 1970) and subsequently transferred onto nitrocellulose membranes. The membranes were then probed for AP-2 or AP-3 using the specific first antibodies (see above) followed by horseradish peroxidase-labelled second antibodies, and detected by using the ECL system (Amersham).

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