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The tyrosine-based lysosomal targeting signal in lamp-1 mediates sorting into Golgi-derived clathrin-coated vesicles

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Diversion of membrane proteins from the trans-Golgi network (TGN) or the plasma membrane into the endosomal system occurs via clathrin-coated vesicles (CCVs). These sorting events may require the interaction of cytosolic domain signals with clathrin adaptor proteins (APs) at the TGN (AP-1) or the plasma membrane (AP-2). While tyrosine- and di-leucine-based signals in several proteins mediate endocytosis via cell surface CCVs, segregation into Golgi-derived CCVs has so far only been documented for the mannose 6-phosphate receptors, where it is thought to require a casein kinase II phosphorylation site adjacent to a di-leucine motif. Although recently tyrosine-based signals have also been shown to interact with the μ chain of AP-1 *in vitro*, it is not clear if these signals also bind intact AP-1 adaptors, nor if they can mediate sorting of proteins into AP-1 CCVs. Here we show that the cytosolic domain of the lysosomal membrane glycoprotein lamp-1 binds AP-1 and AP-2. Furthermore, lamp-1 is present in AP-1-positive vesicles and tubules in the trans-region on the Golgi complex. AP-1 binding as well as localization to AP-1 CCVs require the presence of the functional tyrosine-based lysosomal targeting signal of lamp-1. These results indicate that lamp-1 can exit the TGN in CCVs and that tyrosine signals can mediate these sorting events.

Keywords: adaptor complexes/clathrin assembly proteins/clathrin-coated vesicles/endocytosis/mannose 6-phosphate receptor

Introduction

Segregation of membrane proteins into clathrin-coated vesicles (CCVs) is thought to involve the interaction of cytosolic determinants with clathrin assembly proteins at the level of the trans-Golgi network (TGN) (AP-1) (Pearse and Robinson, 1990; Geuze *et al.*, 1992) or the plasma membrane (AP-2) (Robinson, 1994). Sorting into the endosomal system at the level of the TGN has to be tightly regulated to avoid resident plasma membrane proteins being delivered to lysosomes and prematurely degraded. Integral membrane proteins that enter this pathway are the mannose 6-phosphate receptors (MPRs). Sorting of the MPRs into Golgi-derived CCVs (Campbell and Rome, 1983; Geuze *et al.*, 1985; Lemanski *et al.*, 1987) has been thought to involve the interaction of a di-leucine-based cytosolic determinant with AP-1 (Glickman *et al.*, 1989; Johnson and Kornfeld, 1992a,b; Chen *et al.*, 1993; Sosa *et al.*, 1993), although more recent evidence indicates that the adjacent casein kinase II phosphorylation site may be sufficient for AP-1 binding (Mauxion *et al.*, 1996). Di-leucine-based determinants have also been implicated in directing CD3 γ/δ chains and limp-II from the TGN to endosomes and lysosomes (Letourneur and Klausner, 1992; Sandoval *et al.*, 1994) and the invariant chain also contains di-leucine-type signals required for localization to the endosomal system (Bremnes *et al.*, 1994). With the exception of the MPR, however, an involvement of Golgi CCVs remains to be established for all these proteins. In contrast, although some tyrosine-based signals have been shown to interact with the μ subunit of AP-1 *in vitro* (Ohno *et al.*, 1995), there is no evidence to date that these signals also bind intact AP-1, nor that they can mediate sorting of proteins into Golgi CCVs *in vivo* (Chang *et al.*, 1993; Sosa *et al.*, 1993).

While most membrane proteins avoid lysosomes, the major constituents of the lysosomal membrane reach this organelle due to tyrosine- (lamp-1, lamp-2, limp-I and lysosomal acid phosphatase; LAP) or di-leucine (limp-II)-based lysosomal targeting signals (Hunziker and Geuze, 1996). Endosomal and lysosomal delivery of lysosomal membrane glycoproteins could occur directly from the TGN or indirectly via the plasma membrane and endocytosis. The pathway via the cell surface and endocytosis has been documented (Lippincott-Schwartz and Fambrough, 1987; Furuno *et al.*, 1989a,b; Mane *et al.*, 1989; Williams and Fukuda, 1990; Nabi *et al.*, 1991; Mathews *et al.*, 1992) and is best established for LAP (Braun *et al.*, 1989). Mutation of the tyrosine residue in the targeting signal of LAP has been shown to prevent endocytosis and, consequently, appearance of LAP in lysosomes (Peters *et al.*, 1990; Lehmann *et al.*, 1992). Furthermore, immobilized peptides encoding the cytosolic tail of LAP were found to bind AP-2 but not AP-1 (Sosa *et al.*, 1993), strongly arguing for the indirect route. Although direct transport from the TGN to endosomes and lysosomes has been postulated for some lysosomal membrane glycoproteins (D'Souza and August, 1986; Green *et al.*, 1987; Hunziker *et al.*, 1991; Harter and Mellman, 1992; Höning and Hunziker, 1995), there is no direct or conclusive evidence for such a pathway and its existence for this class of proteins has remained controversial (Kornfeld and Mellman, 1989; Fukuda, 1991; Peters and Von Figura, 1994; Sandoval and Bakke, 1994). If the direct transport of lysosomal membrane glycoproteins exists, it could, by analogy to the MPRs, occur via Golgi-derived CCVs and may therefore be restricted to

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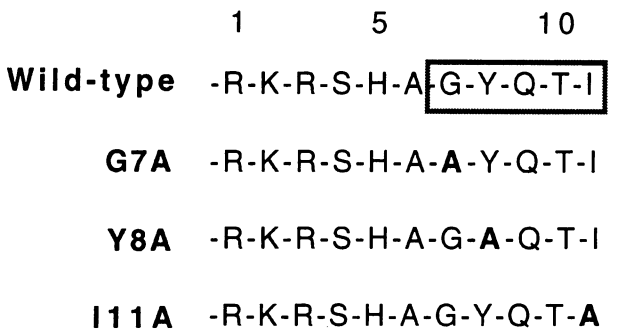

Fig. 1. Peptides containing wild-type and mutant lamp-1 cytosolic tails. The amino acid sequence of the peptides containing the complete cytosolic tail of the wild-type lamp-1 or the G7A, Y8A, I11A mutants defective in correct lysosomal sorting (Höning and Hunziker, 1995) is shown in the one letter code; alanine substitutions are shown in bold, the lysosomal targeting motif is boxed.

proteins encoding di-leucine-based signals (Sandoval and Bakke, 1994). Alternatively, a direct route could differ from that taken by MPRs and, for example, involve transport via a clathrin-independent pathway. Furthermore, it is unclear whether all lysosomal membrane glycoproteins would follow a direct, an indirect, or both routes, or whether the pathway taken would depend on a particular lysosomal membrane glycoprotein.

In the present study, we analyzed the initial steps in the pathway of lamp-1 from the TGN to endosomes and lysosomes. We provide direct biochemical and morphological evidence that lamp-1 is able to exit the TGN in CCVs. The lamp-1 tail bound AP-1 and was present in AP-1-positive vesicles and tubules in the TGN region. AP-1 binding and localization of lamp-1 to AP-1-positive compartments required the tyrosine-based cytosolic signal. Thus, proteins that like lamp-1 contain tyrosine determinants can be sorted into AP-1 CCVs.

Results

Lamp-1 tail peptides containing a functional lysosomal sorting signal bind AP-1 and AP-2

Inclusion of proteins into CCVs involves the interaction of cytosolic sorting signals with either AP-1 or AP-2 (Pearse and Robinson, 1990; Robinson, 1994). To determine whether lamp-1 may be able to exit the TGN in CCVs, we analyzed if a peptide containing the complete 11 residue cytosolic tail of lamp-1 (Figure 1) was able to bind AP-1. Following the incubation of purified APs with the immobilized peptides, unbound material was removed by washing and bound APs were eluted. Adaptors present in the fractions of the wash and elution steps were analyzed on slot or Western blots using antibodies specific for the γ or α subunits of AP-1 or AP-2 respectively.

As shown in Figure 2A and B, up to 75% of the AP-1 added to the binding assay was retained by the lamp-1 tail peptide (wild-type), while <8% bound to an unrelated control peptide. Correct lysosomal transport of lamp-1 depends on the presence of an intact tyrosine-dependent GYXXI cytosolic sorting determinant (Figure 1) and substitution of amino acids G7, Y8 or I11 by alanines leads to the delivery of a significant fraction of the protein to the cell surface (Hunziker *et al.*, 1991; Harter and Mellman, 1992; Höning and Hunziker, 1995). Therefore, we next analyzed whether peptides encoding G7A, Y8A or I11A substitutions were still able to interact with AP-1. Although binding of AP-1 to the G7A tail peptide was reduced by ~50% as compared with the wild-type peptide, a significant fraction (35%) of the added AP-1 was still retained (Figure 2A and B), indicating that G7A was able to interact with AP-1, albeit with a reduced efficiency or affinity. In contrast, binding of AP-1 to peptides encoding the I11A or Y8A substitution was reduced by ~80% and only 16% (I11A) or 15% (Y8A) of the AP-1 present in the binding assay was retained. The importance of Y8 and I11 for AP-1 binding therefore closely correlated with the known critical role of these residues in correct lysosomal targeting of lamp-1 *in vivo* (Hunziker *et al.*, 1991; Harter and Mellman, 1992; Höning and Hunziker, 1995).

Although the specificity of the binding of AP-1 to the lamp-1 tail peptide was apparent from the effects of the single amino acid substitutions (Y8A and I11A) in the peptide, the specificity and the requirement of the functional tyrosine signal were confirmed by inhibition experiments. If APs were pre-incubated with an excess of soluble wild-type lamp-1 peptide, binding of AP-1 to the immobilized wild-type tail was inhibited by >70% (Figure 3A). Although a large excess of soluble peptide was needed to obtain maximal inhibition (1 mg peptide/40 μg APs; data not shown), this may reflect differences in the affinities of APs for soluble versus immobilized peptides, since peptides coupled via their N-terminus to a solid support may more accurately reflect the exposure of the small lamp-1 tail on the cytosolic face of biological membranes. Consistent with the ability of the G7A peptide to bind AP-1 less efficiently than the wild-type tail (Figure 2A and B), the soluble G7A peptide displayed a reduced but still significant inhibitory effect (50%). In contrast, pre-incubation of APs with soluble Y8A and I11A peptides inhibited AP-1 binding to the immobilized wild-type tail by <10%. Since a second lysosomal membrane glycoprotein, LAP, has been suggested to reach lysosomes via the cell surface and endocytosis (Braun *et al.*, 1989; Peters *et al.*, 1990; Lehmann *et al.*, 1992), we also tested the ability of a peptide containing the complete LAP tail to compete for binding of AP-1 to immobilized lamp-1 tails. As shown in Figure 3A, the LAP peptide did not affect binding of AP-1 to the lamp-1 tail, consistent with the failure of the cytosolic domain of LAP to interact with AP-1 (Sosa *et al.*, 1993). Despite the inability of the LAP peptide to inhibit binding of AP-1, it did interfere with the association of AP-2 with the lamp-1 tail (Figure 3B, see below).

In addition to the G7A, Y8A or I11A substitution mutants, wild-type lamp-1 can also reach the plasma membrane under certain circumstances, such as in cells overexpressing the protein (Harter and Mellman, 1992; Höning and Hunziker, 1995). Whereas wild-type lamp-1 and the G7A mutant are rapidly internalized from the cell surface, the Y8A and I11A mutants are not endocytosed (Hunziker *et al.*, 1991; Harter and Mellman, 1992; Höning and Hunziker, 1995). Therefore, we next used antibodies specific for the α-adaptin subunit of the AP-2 complex to analyze the ability of peptides containing the wild-type and mutant lamp-1 tails to interact with plasma membrane adaptors. As shown in Figure 2C and D, 50–60% of

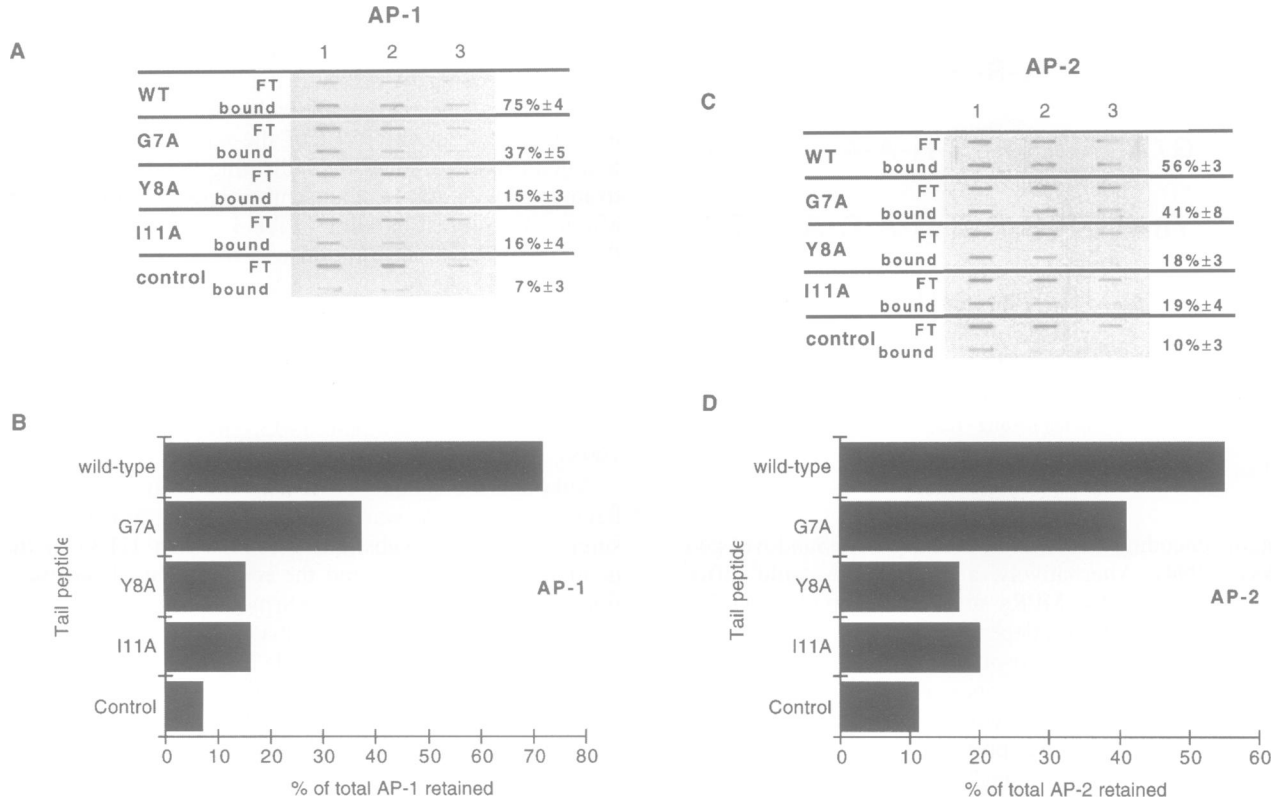

Fig. 2. Binding of APs to lamp-1 tail peptides. Immobilized peptides encoding the wild-type (wt) or mutant lamp-1 tails or an unrelated control peptide were incubated with equal amounts of purified APs in binding buffer (see Materials and methods). After washing the columns with 3×2 ml binding buffer (flow-through, FT, lanes 1–3), bound APs were eluted with 3×2 ml elution buffer (bound, lanes 1–3). The three 2 ml fractions from the wash and elution steps were each transferred to nitrocellulose (lanes 1–3) using a slot blot device and detected using monoclonal antibodies specific for the γ subunit of AP-1 (**A**) or the α subunit of AP-2 (**C**). A typical experiment is shown in (A) and (C). Most of the non-retained APs were removed in the first 2 ml of the wash (lane 1), the bulk of the bound APs were recovered in the first two fractions of the elution (lanes 1 and 2). For quantitation, autoradiographs from four independent experiments were analyzed by densitometry and the fraction of AP-1 (**B**) or AP-2 (**D**) that bound to the different peptides was expressed as a percentage of the total APs added to the binding assay.

the AP-2 present in the binding assay was retained by immobilized peptides containing either the wild-type or the G7A tail peptide. Binding to the Y8A and I11A peptides, in contrast, was reduced by 60–70% and <20% of the added AP-2 was retained. The AP-2 binding results therefore closely correlated with the known importance of Y8 and I11 in lamp-1 internalization (Williams and Fukuda, 1990; Hunziker *et al.*, 1991; Harter and Mellman, 1992; Guarnieri *et al.*, 1993; Höning and Hunziker, 1995).

Pre-incubation of APs with excess soluble wild-type or G7A peptide led to a significant if partial inhibition of AP-2 binding to the immobilized wild-type lamp-1 tail (35 and 25% inhibition respectively; Figure 3B). In contrast, soluble peptides encoding the Y8A and I11A substitutions failed to compete for AP-2 binding. Despite the lack of an inhibitory effect of the soluble LAP tail peptide on AP-1 binding (Figure 3A), this peptide interfered with AP-2 binding to a similar extent (35% inhibition) as the soluble wild-type lamp-1 peptide (Figure 3B).

Analyses by surface plasmon resonance (SPR) spectroscopy, a technique able to detect protein–protein interactions (Jonsson, 1991), were carried out to confirm by an independent method the signal-dependent binding of AP-1 and AP-2 to the lamp-1 tail. Peptides that had been coupled to the sensor surface via their N-terminal amino group at comparable densities were exposed to purified AP-1 and AP-2 adaptors and the binding and dissociation of APs were recorded in real time. As shown in Figure 4, binding to the wild-type tail was detected for AP-1 and AP-2, but both adaptor types were no longer absorbed to peptides containing the Y8A and I11A substitutions.

In conclusion, these experiments show that the lamp-1 tail can bind intact AP-1 and AP-2 *in vitro* and that the association of APs is dependent on the functional GYXXZ lysosomal sorting signal in the cytosolic domain of lamp-1.

Lamp-1 localizes to AP-1 clathrin-coated membranes in the trans-Golgi region

Although the observed binding of AP-1 to the lamp-1 tail is consistent with the sorting of lamp-1 into CCVs at the TGN, AP-1 binding does not necessarily prove that lamp-1 is also sorted into AP-1 CCVs *in vivo*. Therefore, we next used immunogold electron microscopy to directly visualize whether lamp-1 or the Y8A mutant were present in AP-1-positive vesicles in the trans-region of the Golgi. For this purpose we used stably transfected MDCK cells (Höning and Hunziker, 1995) which, by immunoprecipitation of metabolically labeled protein and Western blot analysis (Figure 5), expressed similar levels of the wild-type or mutant lamp-1. Lamp-1 expression levels in the transfected cells were furthermore comparable with those of the endogenous protein in NRK cells.

In cells expressing the wild-type protein, lamp-1 labeling

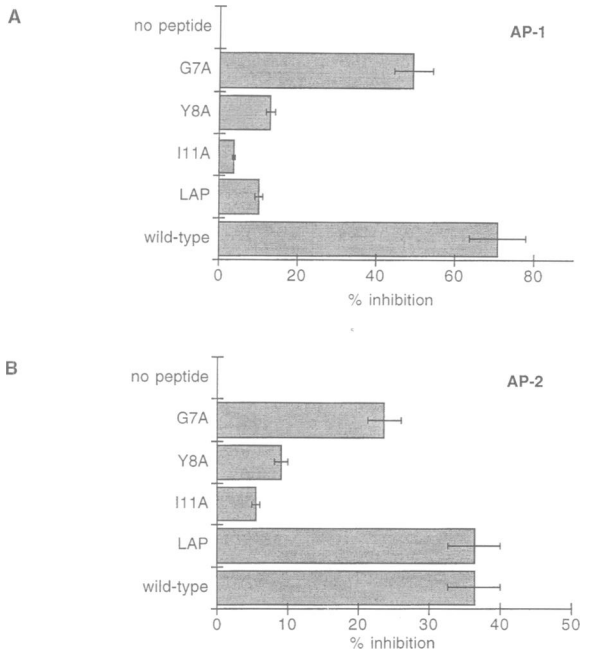

Fig. 3. Inhibition of AP-1 (**A**) and AP-2 (**B**) binding to immobilized wild-type tails by soluble lamp-1 and LAP peptides. APs (40 μg/ml) were pre-incubated with the indicated soluble peptides (1 mg/ml) prior to incubation with the immobilized lamp-1 tail peptide. Binding of AP-1 or AP-2 to the immobilized lamp-1 peptide was determined as in Figure 2. Inhibition was calculated as the difference between binding in the absence (0% inhibition) or the presence of the peptides indicated and the averaged data from three independent experiments are shown.

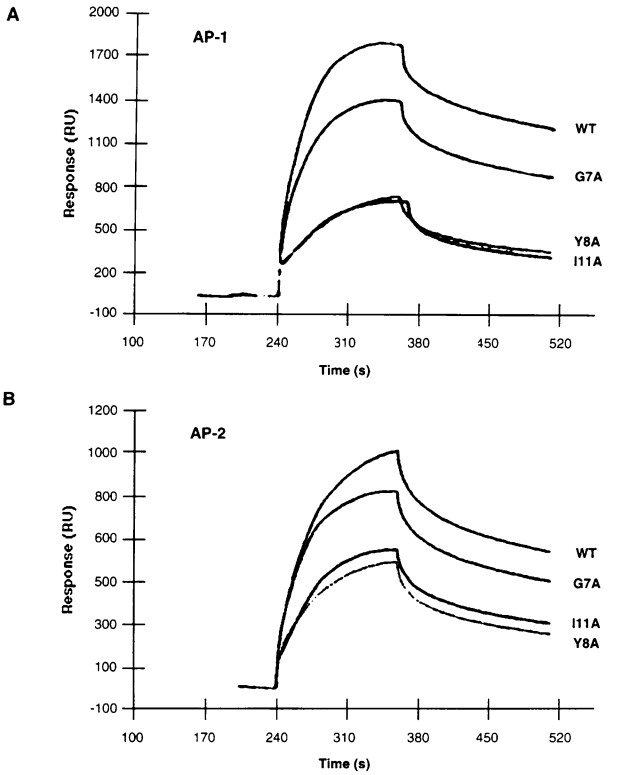

Fig. 4. Binding of AP-1 (**A**) and AP-2 (**B**) complexes to immobilized lamp-1 peptides analyzed by SPR in real time. Peptides containing wild-type (WT) or the G7A, Y8A and I11A mutant lamp-1 tails were coupled to sensor chips at similar densities (0.6–0.8 ng/mm^2) and exposed to purified adaptor complexes (200 nM) for 2 min.

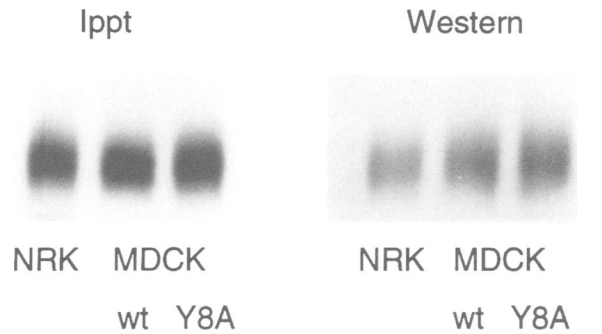

Fig. 5. Expression of endogenous lamp-1 in NRK cells and wild-type (wt) or mutant (Y8A) rat lamp-1 in transfected MDCK cells. Immunoprecipitation: lamp-1 was isolated by immunoprecipitation from equal numbers of cells labeled with [^{35}S]methionine and analyzed by SDS–PAGE and autoradiography. Western blot: equal amounts of total protein (20 μg) were fractionated by SDS–PAGE, transferred to nitrocellulose and probed with a lamp-1 antiserum.

was found in lysosomes and multivesicular endosomes, but was mostly absent from the plasma membrane (Figure 6A). In the trans-region of the Golgi complex, lamp-1 labeling was also detected in coated vesicles and tubules that stained for AP-1 (Figures 6A and 7A and B). In contrast, the Y8A mutant was expressed abundantly on the plasma membrane (Figure 6B) and did not show extensive co-localization with AP-1 (Figure 7C and D). The bulk of the lamp-1-containing AP-1-positive membranes were closely associated with the trans-region of Golgi stacks (Figures 6 and 7).

Quantitation of the immunogold label for lamp-1 confirmed the predominant intracellular localization of the wild-type protein at steady-state, with <5% of the protein being on the plasma membrane (Figure 8A). The intracellular distribution of lamp-1 in transfected MDCK cells thus closely resembled that of the endogenous protein in rat hepatoma cells, also determined by immunogold labeling (Geuze *et al.*, 1988). In contrast, 74% of the the labeling was concentrated on the cell surface in cells expressing the Y8A mutant. At equilibrium, 3% of wild-type lamp-1 was found on TGN membranes that also stained for γ-adaptin, but <0.1% of the Y8A mutant was present in AP-1-positive structures. In cells expressing either the wild-type or the Y8A mutant protein, a similar fraction (~77%) of the intracellular labeling for lamp-1 was associated with endosomes and lysosomes.

The apparent importance of the tyrosine residue in localizing lamp-1 to AP-1 CCVs was confirmed by quantitation of the fraction of γ-adaptin-positive membranes that also carried wild-type or Y8A mutant lamp-1 (Figure 8B). In cells expressing wild-type lamp-1, 30% of the γ-adaptin-positive vesicles labeled for lamp-1. In contrast, <10% of the membranes carrying γ-adaptin stained for lamp-1 in cells transfected with the Y8A mutant. These results confirm the lower fraction of total Y8A detected in AP-1-positive compartments (Figure 8A) and show that inactivation of the lysosomal targeting signal reduces the extent of localization of lamp-1 to γ-adaptin-positive membranes by ~70%. Thus, these results closely correlate with the observed cell surface delivery of the Y8A mutant *in vivo* (Höning and Hunziker, 1995) and the inability of the Y8A tail peptide to bind AP-1 *in vitro* (see above).

In conclusion, AP-1 not only binds to the lamp-1 tail

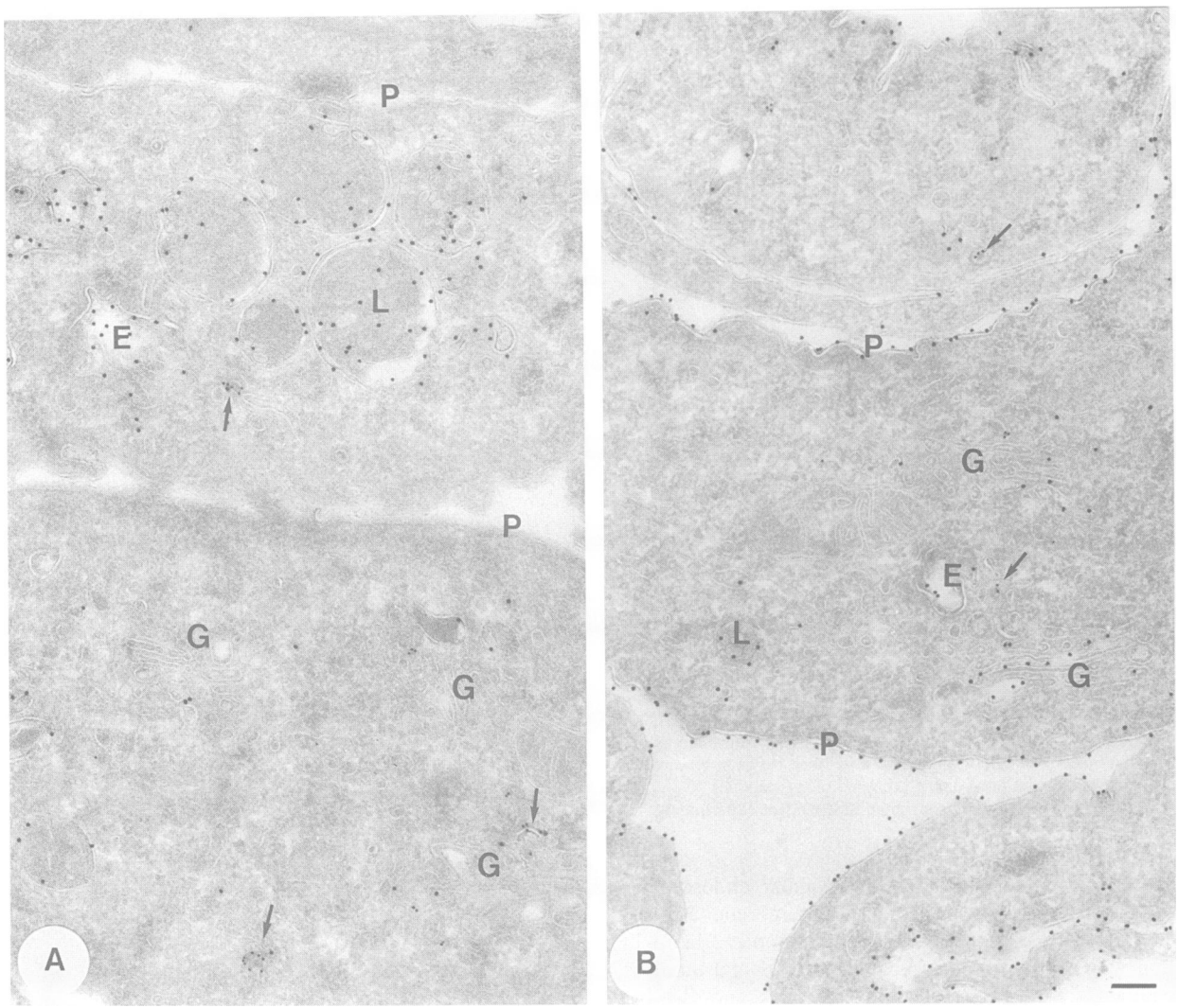

Fig. 6. Survey immunoelectron micrographs of ultrathin cryosections of MDCK cells transfected with wild-type (A) or Y8A mutant (B) rat lamp-1. Sections were double immunolabeled with anti-lamp-1 (15 nm gold) and anti-γ-adaptin (10 nm gold). (**A**) Parts of three cell profiles, the lower of which shows a Golgi region with three Golgi stacks (G) and two AP-1-coated CCVs positive for lamp-1 (arrows). One such CCV is also present in the middle cell (arrow) together with endosomal (E) and lysosomal (L) vacuoles showing abundant lamp-1 labeling. No lamp-1 is present on the plasma membrane (P). (**B**) Comparable view of cells expressing the Y8A mutant lamp-1 in Golgi stacks (G), abundantly on the plasma membrane (P) and to a lesser extent in endosomal (E) and lysosomal (L) vacuoles. Bar 0.2 μm.

in vitro, but the tyrosine-based lysosomal targeting signal is also able to mediate inclusion of lamp-1 into AP-1 CCVs.

Discussion

Several studies have so far provided evidence that delivery of lamp-1 into the endosomal system may occur directly from the TGN and not require transit through the plasma membrane (D'Souza and August, 1986; Green *et al.*, 1987; Hunziker *et al.*, 1991; Harter and Mellman, 1992; Höning and Hunziker, 1995). In the present manuscript we provide two lines of evidence for a role of TGN-derived CCVs in the biosynthetic route of lamp-1. First, peptides encoding the cytosolic tail of lamp-1 were able to bind to Golgi and plasma membrane adaptor complexes. Second, lamp-1 was detected in AP-1-positive membranes in the TGN region. Both adaptor binding and localization to AP-1 membranes required the presence of a functional GYXXI lysosomal targeting signal.

Binding of adaptors to lamp-1 tails

Recent experiments using the yeast two-hybrid system and *in vitro* binding assays have shown that the μ subunits of AP-1 and AP-2 can interact with the tyrosine signals in several proteins, including lamp-1 (Ohno *et al.*, 1995). Our results based on classical binding and SPR spectroscopy experiments are consistent with these findings and extend them by indicating that intact AP-1 complexes can also bind to the lamp-1 tail. In both experimental approaches, the interaction of AP-1 and AP-2 with the lamp-1 tail required an intact GYXXI sorting signal. Binding experiments using immobilized peptides may be subject to several pitfalls, including the possibility that they may not behave like an affinity matrix but as a simple ion exchanger. Against this possibility argues the fact that adaptor binding was not only affected by substitution of the polar tyrosine residue by a hydrophobic alanine, but also by the conserved substitution of hydrophobic residues in the case of the G7A and I11A peptides. Furthermore,

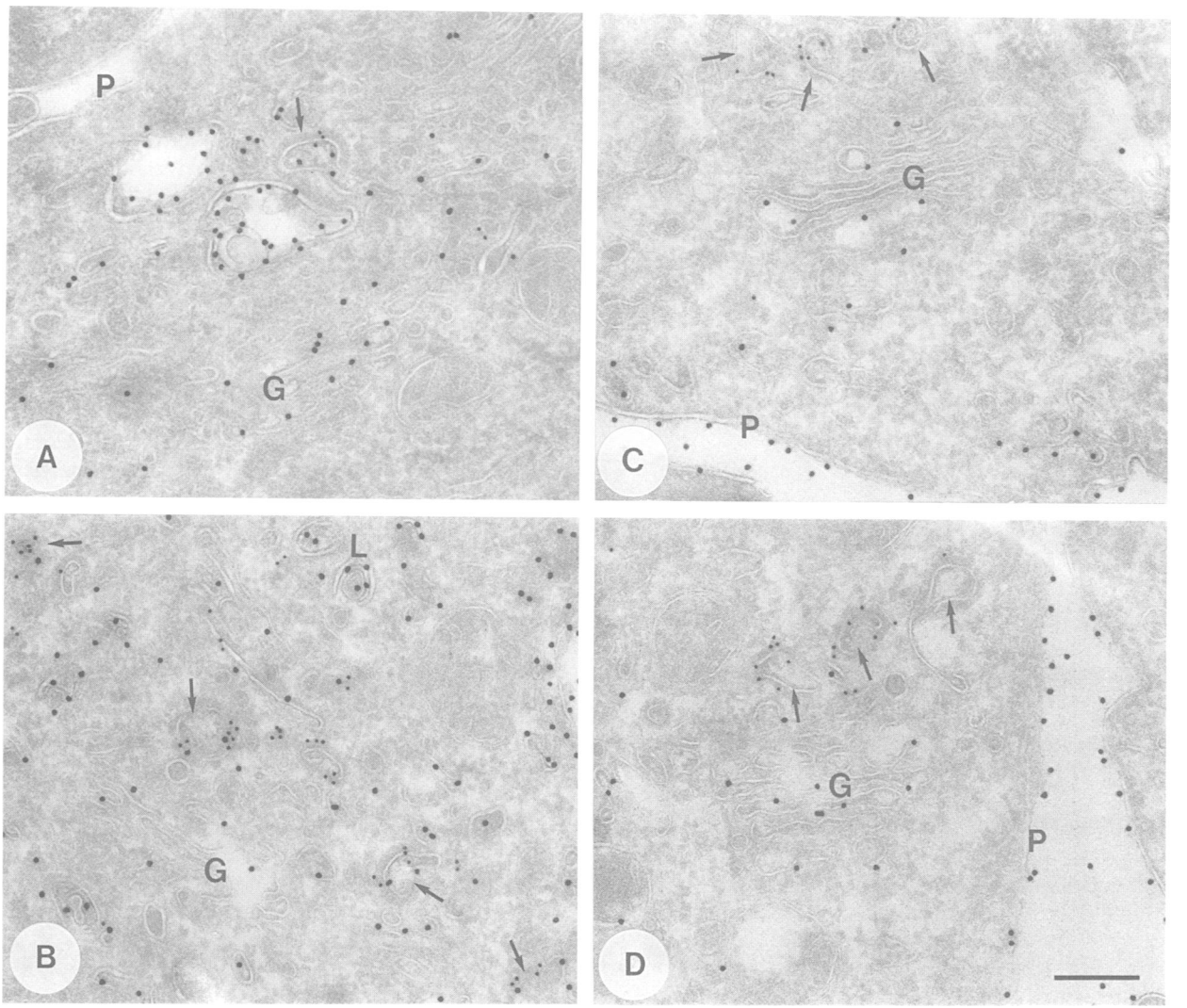

Fig. 7. Higher magnification views of sections of MDCK cells expressing wild-type (**A** and **B**) and Y8A mutant (**C** and **D**) lamp-1. The sections were labeled as in Figure 6 and show Golgi areas with CCVs (arrows) labeled for both γ-adaptin and lamp-1 in (A) and (B), but for γ-adaptin or Y8A only in (C) and (D). G, Golgi stacks; L, lysosomes; P, plasma membrane. Bar 0.2 μm.

binding of adaptors to the immobilized wild-type lamp-1 peptide could be inhibited by pre-incubating adaptors with excess soluble wild-type but not with Y8A or I11A peptide. Although the extent of inhibition by the different peptides closely correlated with their adaptor binding properties, inhibition required a large excess of soluble peptide. This may reflect differences in the affinity of APs for soluble versus immobilized peptides. Consistent with the selective interaction of a LAP peptide with AP-2 but not AP-1 (Sosa *et al.*, 1993), the LAP tail did not inhibit binding of AP-1 to lamp-1 tails, even though it did interfere with binding of AP-2. The apparent equilibrium dissociation constants of adaptors for the wild-type peptide estimated from the SPR spectroscopy data were in the range 10^{-7}–10^{-8} M and are similar to those obtained for the the interaction of AP-1 with the MPR in permeabilized cells (Le Borgne *et al.*, 1996; Mauxion *et al.*, 1996) or of AP-1 and AP-2 with influenza hemagglutinin tail peptides carrying a tyrosine or di-leucine motif (Heilker *et al.*, 1996). Thus, despite the caveats discussed above, our results are consistent with the idea that, at least *in vitro*, the lamp-1 tail can bind AP-1 and AP-2. Adaptor binding by the wild-type and mutant peptides closely correlated with the presence of an intact GYXXI lysosomal targeting signal, with the observed effect that mutations in this signal have on lamp-1 trafficking (Höning and Hunziker, 1995) and with the degree of co-localization of lamp-1 or Y8A with AP-1, indicating that adaptor binding to the lamp-1 tail is relevant for lamp-1 routing.

Co-localization of lamp-1 and AP-1

Although our binding results indicate that AP-1 is able to interact with the lamp-1 tail *in vitro*, this may not be the case *in vivo* or the interaction with AP-1 may not lead to the inclusion of lamp-1 into CCVs at the level of the TGN. Therefore, it was important to show that lamp-1 indeed is present in AP-1 CCVs. As observed for AP-1 binding, localization of lamp-1 to AP-1-positive vesicles required an intact GYXXI signal and was significantly reduced for the Y8A mutant. The localization of lamp-1 to AP-1-positive structures indicates that the protein can exit the Golgi in the same CCVs involved in delivery of MPRs from the TGN to endosomes. MPR-deficient fibroblasts still display 25–35% of the original AP-1

A

Lamp-1 (lgp120)	Plasma membrane	Endosomes Lysosomes	TGN Golgi	AP-1 membranes	ER Cytosol
wild-type	5%	73%	8%	3%	10%
Y8A	74%	20%	3%	<0.1%	3%

B

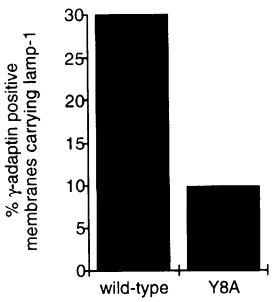

Fig. 8. (**A**) Quantitation of immunogold for wild-type and Y8A lamp-1. Gold particles in random cell profiles were counted and allocated to the structures listed. Background over nuclei and mitochondria was 2%. (**B**) Fraction of AP-1-positive vesicles that also labeled for lamp-1. The fraction of AP-1-positive membranes carrying one or more lamp-1 gold particles was determined in transfected MDCK cells expressing wild-type or Y8A mutant lamp-1.

binding sites (Le Borgne *et al.*, 1996), consistent with the notion that proteins other than the MPRs also leave the TGN in CCVs. Although we cannot draw any conclusions as to what fraction of lamp-1 exits the TGN in CCVs, several considerations suggest that the quantitation of our immunogold labeling experiments underestimates the number of lamp-1 molecules in transit in AP-1-positive vesicles. First, in contrast to the MPRs, lamp-1 does not cycle between endosomes and the TGN (Rohrer *et al.*, 1995) and only a few newly synthesized lamp-1 molecules may be leaving the TGN at any time. Second, CCVs may rapidly uncoat, yielding lamp-1-positive, AP-1-negative profiles. Third, the limited number of molecules that can be packed into a given CCV may lead to the formation of a large fraction of CCVs that exclusively carry MPRs but are devoid of lamp-1 or that carry lamp-1 at amounts that are below the detection level. Although we cannot exclude that some lamp-1 molecules may also reach endosomes and lysosomes via an alternative, AP-1-independent pathway, the failure to detect passage of significant amounts of lamp-1 through the cell surface *in vivo* (Höning and Hunziker, 1995) and the observation that 30% of the γ-adaptin-positive membranes carry lamp-1 indicates that the protein is at least able to exit the TGN in CCVs. Furthermore, lamp-1 and the CD-MPR could be detected in common AP-1-coated vesicles (Hunziker and Geuze, 1996), suggesting that lamp-1 and the MPRs may indeed exit the TGN in common transport vesicles.

Although AP-1 has been considered to be a specific marker for the TGN and Golgi-derived CCVs, recent evidence indicates that in certain cell types a small fraction may also be present on early endosomes (Le Borgne *et al.*, 1996). While AP-1 in the TGN is found on membranes and associated buds, the sparse labeling on endosomes appears to be restricted to buds, which may reflect partially uncoated TGN vesicles that have fused with endosomes (Le Borgne *et al.*, 1996). The bulk of the lamp-1-positive AP-1-coated vesicles and tubules were closely associated with Golgi stacks and the labeling for AP-1 was evenly distributed (Figures 6 and 7). This, together with the failure to detect lamp-1 in early endosomes (Green *et al.*, 1987; Griffiths *et al.*, 1988), indicates that most of the lamp-1 is present in TGN-derived CCVs. It is unlikely that AP-1 CCVs are involved in the transfer of lamp-1 from endosomes to lysosomes. Such a role would also not be consistent with the observation that proteins that interact with AP-1 can be delivered from endosomes to different cellular locations, such as the TGN (MPRs), lysosomes (lamp-1) or the cell surface (FcRII-B2, Höning and Hunziker, unpublished data; mutant influenza hemagglutinin, Heilker *et al.*, 1996) and that LAP is delivered to lysosomes even though it does not bind AP-1 (Sosa *et al.*, 1993). Thus, the most likely site at which lamp-1 is segregated into AP-1 CCVs is the TGN.

Intracellular trafficking of lysosomal membrane proteins

Under certain circumstances, variable amounts of lamp-1 may be routed to endosomes and lysosomes via the cell surface and endocytosis (Lippincott-Schwartz and Fambrough, 1987; Furuno *et al.*, 1989a,b; Mane *et al.*, 1989; Williams and Fukuda, 1990; Mathews *et al.*, 1992). Expression of high levels of lamp-1, for example, is known to lead to the appearance of the overexpressed transfected and the endogenous protein on the plasma membrane, possibly reflecting saturation of the Golgi sorting machinery (Harter and Mellman, 1992; Höning and Hunziker, 1995). Thus, whether lamp-1 is delivered to the cell surface or not could depend on the cellular levels of cargo molecules, such as MPRs or lysosomal membrane proteins, to be packed into CCVs or of AP-1 or other sorting components present in particular cell types or certain physiological and/or experimental conditions. The presence of variable amounts of lamp-1 on the plasma membrane could, however, also reflect differences in the ability of lamp-1 to reach the cell surface from endosomes or lysosomes (Rohrer *et al.*, 1996).

The observation that lamp-1 can exit the TGN in CCVs cannot *a priori* be generalized for other lysosomal membrane glycoproteins, even though lamps-1–3 share a similar GYXXZ signal. Since the C-terminal Ile at position Z in lamp-1 is critical for direct lysosomal sorting (Höning and Hunziker, 1995) and AP-1 binding, its substitution by a Phe, Val or Leu in lamp-2 (Hatem *et al.*, 1995) or a Met in limp-3 (Metzelaar *et al.*, 1991) could modulate the efficiency or affinity of AP-1 binding. Also LAP carries a sorting signal (GYXXV) (Lehmann *et al.*, 1992) similar to that of lamp-1 but does not bind AP-1 (Sosa *et al.*, 1993). This may reflect the presence of a Val at position Z or the location of the LAP signal within the cytosolic tail. In contrast to other lysosomal membrane glycoproteins, limp-II encodes a leucine–isoleucine sorting signal similar to that found in the MPR (Ogata and Fukuda, 1994; Sandoval *et al.*, 1994). Although limp-II appears to reach lysosomes directly from the TGN (Vega *et al.*, 1991), the involvement of Golgi-derived CCVs remains

to be established. Indeed, introduction of a tyrosine or a di-leucine in the tail of influenza hemagglutinin confers AP-1 and AP-2 binding activity (Heilker *et al.*, 1996) and also the endogenous di-leucine signal in the tail of FcRII-B2 (Hunziker and Fumey, 1994) binds both adaptors (Höning and Hunziker, unpublished data), indicating that di-leucine motifs may also be able to mediate sorting into Golgi CCVs. Thus, whether a protein is segregated into AP-1 CCVs or not does not seem to depend on a particular type of signal (i.e. di-leucine versus tyrosine), but rather on structural features of the two determinants, either at the level of their primary sequence, their position within the tail or their secondary structure.

The residues at positions −1 and +3 relative to the Y in the GYXXZ motif seem to play an important role for recognition in the TGN, since conservative substitutions at these locations (G7A and I11A) interfere with AP-1 binding and *in vivo* sorting (Hunziker *et al.*, 1991; Harter and Mellman, 1992; Höning and Hunziker, 1995). Since G7A still displays significant AP-1 binding activity, an important fraction of the G7A protein may be routed directly, even though detectable levels reach endosomes and lysosomes via the cell surface (Hunziker *et al.*, 1991; Harter and Mellman, 1992; Höning and Hunziker, 1995). Thus, the efficiency or fidelity of sorting of a given protein in the Golgi could depend on the particular residues present at positions −1 and +3 in the signal. Although many endocytic receptors carry tyrosine-dependent internalization signals that resemble the GYXXI motif, these determinants are generally located within the tail (Sandoval and Bakke, 1994; Hunziker and Geuze, 1996). Whether exposure at the end of the cytosolic domain, a minimal distance from the membrane or a particular secondary structure controls the recognition of a given signal in the TGN will have to be analyzed. An intriguing possibility is that some endocytic receptors encoding tyrosine signals may reach the plasma membrane indirectly (Futter *et al.*, 1995; Leitinger *et al.*, 1995), possibly via Golgi-derived CCVs and endosomes.

The tyrosine signal in lamp-1 does not only mediate Golgi sorting, but also endocytosis and, in polarized cells, basolateral targeting (Hunziker *et al.*, 1991; Höning and Hunziker, 1995). Furthermore, the spacing of the tyrosine signal relative to the membrane is critical for the delivery of lamp-1 from endosomes to lysosomes (Rohrer *et al.*, 1996). Consistent with the ability of lamp-1 to be endocytosed, the lamp-1 tail also bound plasma membrane APs and AP-2 binding closely correlated with the presence of a functional endocytosis signal. A number of tyrosine signals as well as di-leucine determinants can also specify basolateral targeting (Rodriguez-Boulan and Zurzolo, 1993). Since at least some basolateral signals can bind AP-1, a possible role for this or a related adaptor complex in basolateral sorting will have to be explored. It will also be important to elucidate how adaptors discriminate between signals to be recognized at different cellular locations and what the significance of the observed redundancy in sorting determinants is.

Materials and methods

Isolation and binding of adaptor complexes to immobilized lamp-1 tail peptides

APs were isolated from pig brain as described (Keen and Beck, 1989; Höning *et al.*, 1994) and stored in binding buffer (100 mM MES, 150 mM NaCl, 1 mM EGTA, 0.005% phenylmethylsulfonyl fluoride, 0.5 mM $MgCl_2$, 2 mM CHAPS, 0.2 mM dithiothreitol, 0.02% NaN_3, pH 7.0). AP-1 and AP-2 complexes were separated by hydroxyapatite chromatography as described (Ungewickell *et al.*, 1994) and their purity was assessed by SDS–PAGE and immunoblot analysis (see below).

Aliquots of 20 mg of synthetic peptide containing the wild-type or mutant lamp-1 tails or the unrelated peptide QRAGDTGSSRPQEGEAVA were coupled to 2 ml Affigel 10 (Bio-Rad) according to the manufacturer's instructions, resulting in a 60–70% coupling efficiency as judged by spectrophotometry. Immobilized peptides (500 μg) were incubated with equal amounts (10–100 μg) of purified APs in binding buffer for 60 min at 20°C. Columns were washed with 2×3 ml binding buffer (flow-through) and bound APs were eluted in 2×3 ml elution buffer (1 M Tris, pH 8.0, 0.02% NaN_3, 0.2 mM EDTA, 0.2% β-mercaptoethanol) essentially as described (Sosa *et al.*, 1993). More than 90% of the applied APs were recovered in the flow-through and bound fractions. Adaptors present in the different fractions were directly transferred to nitrocellulose using a Schleicher & Schuell SRC072 Minifold-II Micro-Sample filtration unit. Alternatively, the fractions were TCA precipitated, fractionated by SDS–PAGE (7.5%) and transferred to nitrocellulose (not shown). The nitrocellulose was incubated for 15 min in 1% formaldehyde in phosphate-buffered saline (PBS), washed in PBS and incubated for 10 min in PBS, 50 mM NH_4Cl. After blocking in 5% dry milk, 0.05% Tween 20 in PBS for 12 h, AP-1 or AP-2 were detected using monoclonal antibodies specific for γ-adaptin (clone 100/3) or α-adaptin (clone 100/2) respectively (Ahle *et al.*, 1988). Following incubation with a peroxidase-conjugated goat anti-mouse secondary antibody (Sigma Chemical Co.), visualization was carried out by chemiluminescence (ECL; Amersham Co.). For quantitation, autoradiograms from five independent experiments were analyzed by densitometry and AP-1 or AP-2 bound to the different peptides was normalized to the amount of peptide immobilized to account for the small differences in coupling efficiency of the different peptides. The numbers show the fraction of total APs in percent added to the binding assay that was retained by the different peptides. For inhibition experiments, the adaptors were incubated overnight at 4°C with the soluble peptides (0.01–5 mg). The adaptor/peptide mixture was applied to the immobilized tail peptides after spinning for 30 min at 100 000 *g*.

Surface plasmon resonance (SPR) spectroscopy

The adaptor–tail interactions were analyzed in real time by detecting SPR spectroscopy with a BIAcore 2000 instrument (Pharmacia Biosensor AB). All experiments were performed using CM5 sensor chips (research grade) at 25°C with a flow rate of 20 μl/min. The tail peptides were covalently coupled via their N-terminal amino group to the activated sensor chip surface according to the manufacturer's instructions (BIAcore methods manual) at similar peptide densities of 0.6–0.8 ng/mm^2 (400–500 RU). Purified adaptors were exposed to the immobilized peptides at 50–250 nM in running buffer (20 mM HEPES, pH 7.3, 2 mM $MgCl_2$, 10 mM KCl, 0.2 mM dithiothreitol) for 2 min and the chip was then flushed with buffer for 2 min. After each binding cycle, bound adaptors were removed in a regeneration step by injecting 20 μl 0.01 N NaOH, 0.1% SDS. Kinetic parameters and equilibrium dissociation constants were determined from sensograms recorded at different adaptor concentrations and were calculated using the BIAcore kinetic evaluation software, assuming pseudo-first order kinetics. To exclude distortions due to injection and mixing, segments of the sensograms 30 s after switching from buffer to adaptor solution (association rate) and 25 s after switching back to protein-free buffer (dissociation rate) were used for the calculations. Unexpectedly, but as observed in other similar studies (Ohno *et al.*, 1995; Heilker *et al.*, 1996), the dissociation rates for the different peptides were very similar.

Immunoprecipitation and Western blot analysis

Immunoprecipitation of lamp-1 or Y8A from metabolically labeled cells was previously outlined (Hunziker *et al.*, 1991). Western blot analysis of 20 μg total protein was carried out as described (Geuze *et al.*, 1988), except that a peroxidase-conjugated goat anti-rabbit antibody and chemiluminescence were used to visualize the lamp-1 protein. Cells used for immunoprecipitation and Western blot analysis were cultured under identical conditions as those used for immunoelectron microscopy.

Immunoelectron microscopy

Stably transfected MDCK cells were treated with 1 mM butyrate for 12 h prior to fixation to moderately increase expression levels (Höning and Hunziker, 1995). The cells were fixed in 1% glutaraldehyde, 0.1 M sodium phosphate buffer, pH 7.4, for 2 h at room temperature, washed

in buffer, embedded in 10% gelatin in buffer, solidified on ice and incubated in 2.3 M sucrose in phosphate buffer for 2 h at 4°C. Ultrathin cryosections were single or double immunolabeled with protein A–gold probes as described (Slot *et al.*, 1991). The sections were collected, contrasted and embedded in methyl cellulose (Liou and Slot, 1995). For quantitation, gold particles were counted at 10 000× instrumental magnification and allocated to the structures listed. Background over nuclei and mitochondria was 2%. In 20 random cell profiles of transfected MDCK cells expressing wild-type or Y8A mutant lamp-1, 5975 and 4124 gold particles respectively were counted. To determine the fraction of AP-1-labeled vesicles that also labeled for wild-type or Y8A mutant lamp-1, a total of 186 and 104 γ-adaptin-labeled vesicles were counted in a different set of 32 random cell profiles and the fraction of vesicles carrying one or more lamp-1 gold particles was determined.

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