The two known mannose 6-phosphate receptors (MPR46 and MPR300) both mediate the transport of Man-6-P-containing lysosomal proteins to lysosomes. However, the MPRs cannot be detected in lysosomes, instead they recycle between the plasma membrane and endosomes and between endosomes and the trans-Golgi network. Both, endocytosis from the plasma membrane and budding of transport vesicles from the trans-Golgi network involves the interaction of the receptor with the clathrin-coated vesicles-associated protein complexes AP1 and AP2. We have analyzed this interaction between the Golgi-restricted AP1 complex and the plasma membrane-restricted AP2 complex with the MPR46 tail in vitro by using a biosensor.

AP1 and AP2 both bind to and dissociate from the MPR46 tail with similar kinetics. Using synthetic peptides corresponding to different MPR receptor tail regions in inhibition and binding studies, a common high affinity binding site for AP1 and AP2 and two separate binding sites for AP1 and AP2, respectively, were identified.

Clathrin-coated vesicles (CCVs) are transport intermediates of vesicular traffic from the trans-Golgi network (TGN) and from the plasma membrane to endosomes. CCVs from the plasma membrane and from the TGN have clathrin as their major structural protein component in common, but can be distinguished by their different sets of assembly proteins (APs) (reviewed in Ref. 1). The AP1 complex has been shown to be restricted under normal conditions to the TGN, whereas the AP2 complex can only be detected in coated pits and CCVs derived from the plasma membrane. However it is possible to induce relocation of adaptors to endosomal structures.

Although it is still not known how the AP complexes are recruited to their respective target membrane and released thereof, the complexes itself are well characterized biochemically and genetically (4–6). Both AP complexes are heterotetrameric, the AP1 complex composed of two 100-kDa subunits (γ and β) together with a medium subunit of 47 kDa (μ1) and a small subunit of 19 kDa (δ1) (4, 7). The AP2 complex is of similar size and composition with two large subunits (α and β) in association with a 50-kDa (μ2) and a 17-kDa subunit (δ2) (7).

The two β subunits, the medium chains, and the small subunits all show a relatively high homology between their counterparts. The greatest diversity is found between the α and the γ subunits. In vitro reconstitution assays and the use of proteolytically truncated AP complexes have revealed that the two β subunits mediate the interaction with clathrin (8). Experiments using cells that express AP complexes where different regions of the α subunit are replaced by the respective γ sequence have shown that these subunits play the major role in the correct targeting of the AP complexes (9).

Several in vitro studies using immobilized receptor tail peptides have also demonstrated that AP1 and AP2 can bind to the cytoplasmic tail of receptors. The interaction between AP1 and a receptor tail has so far been reported for MPR46 (10), MPR300 (11), and for the lysosomal membrane protein lamp-1 (12). In this context it was shown that AP2 interacts with the tail of the LDL receptor (13), the ASGP receptor (14), lamp-1 (12), lysosomal acid phosphatase, and the MPR46 tail (10).

For the interactions between adaptor complexes with different receptor tails, the necessity of a specific sequence motif involving a tyrosine (10–12, 14, 15) or a di-leucine (16) was demonstrated. Theoretical considerations and experimental data made it likely that the α and γ subunits mediate this interaction (1). Beltzer and Spiess (14) observed an interaction between the asialoglycoprotein receptor tail and the β subunit of AP2. Recent studies using the yeast two-hybrid system have shown an interaction between tyrosine based sorting signals and the μ-chains of AP1 and AP2 (17–19).

Two major constituents of TGN-derived CCVs are the MPRs (reviewed in Ref. 20), two proteins which both are involved in sorting of lysosomal enzymes carrying Man-6-P recognition marker. Recent studies using Golgi membranes from MPR-deficient cells transfected with MPR46 and tail mutants of MPR46 suggested that recruitment of AP1 relies mainly on the presence of MPR. This interaction was dependent on ARF-1, which is thought to promote the membrane association of AP1 (21, 22). The authors also presented evidence that a specific sequence near the carboxyl terminus of the MPR46 tail comprising a casein kinase II phosphorylation site is the AP1 binding site. However, it is still not known, whether the MPR tail is a direct target of AP1 at the TGN membrane or whether other yet undefined factors mediate the AP1-MPR tail interaction.

With this study we wanted to define the MPR tail-adaptor interaction kinetically, and we aimed to identify the MPR46 tail sequences that bind AP1 and AP2. For this purpose we analyzed the interaction of purified adaptors with the MPR46 tail immobilized to the surface of a biosensor. Using peptides corresponding to different sequences of the MPR46 tail as

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1) (4, 7). The AP2 complex is of heterotetrameric, the AP1 complex composed of two 100-kDa subunits (γ and β) together with a medium subunit of 47 kDa (μ1) and a small subunit of 19 kDa (δ1) (4, 7). The AP2 complex is of similar size and composition with two large subunits (α and β) in association with a 50-kDa (μ2) and a 17-kDa subunit (δ2) (7).

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2. H. Honing and W. Hunziker, unpublished data.
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Fig. 1. Sequence of the MPR46 tail. The amino acid sequence of the wild-type MPR46 tail is shown in the one-letter code, above the sequence boxes. Shaded boxes indicate peptides that interact with adaptors.

Inhibitors of adaptor-MPR46 tail interaction or as ligands for adaptors, we could show that the MPR46 tail contains at least three high affinity binding sites for the adaptor complexes, one of which interacts with both AP1 and AP2.

MATERIALS AND METHODS
Preparation of AP1 and AP2—Clathrin-coated vesicles were prepared from porcine or bovine brain according to Keen et al. (23) and Honing et al. (24). Coat proteins were extracted from CCVs with 1 mM Tris-HCl, pH 7.8, containing 2 mM EDTA and 0.2 mM dithiothreitol. After centrifugation at 100,000 × g for 30 min, the material was applied to a Superose-6 column (2.5 × 75 cm) connected to a fast protein liquid chromatography system (Pharmacia Biotech Inc.) at a flow rate of 0.5 ml/min. The column was equilibrated in 0.5 mM Tris-HCl, pH 7.5, or 0.2 mM dithiothreitol. Fractions (1.5 ml) containing APs were identified by SDS-polyacrylamide gel electrophoresis, pooled, and concentrated. For the separation of AP2 from AP1 the mixed adaptor pool derived after gel filtration was applied to hydroxyapatite chromatography exactly as described by Manfredi and Bazari (25). The purity of AP1 and AP2 was analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting using monospecific antibodies to α- or γ-adaptin.

AP1 and AP2 were concentrated to 0.2 mg/ml, dialyzed, and stored in 50 mM Tris, pH 7.0. For interaction analysis, the adaptors were diluted or dialyzed into 20 mM HEPES-NaOH, pH 7.0, 150 mM NaCl, 10 mM KCl, 2 mM MgCl₂, 0.2 mM dithiothreitol (buffer A). Prior to all experiments described, APs were centrifuged for 15 min at 13,000 × g.

Peptide Synthesis—A full-length MPR46 tail peptide and all small peptides were synthesized using amino acids protected with N-(9-fluorenyl)methoxycarbonyl (Fmoc) and activated with benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate and an 9050 peptide-derivatized surfaces, no binding was observed (not shown). Thus AP containing fractions show a specific binding to the immobilized MPR46 tail.

Interaction Analysis by SPR—The adaptor-MPR tail interaction was analyzed in real-time by SPR (26) detection using a BIACore AB. The HPLC pure MPR tail peptide was coupled to a CM sensor chip, which has a streptavidin-derivatized dextran matrix. To study the interaction between the adaptors and the MPR46 tail, was set to 1. The dissociation rate constant k₅ was determined by fitting data to the following equation,

\[ R = R_0 e^{-kt} \]  

where \( R_0 \) is the response level at the beginning time \( t \) of the dissociation phase. This model, which has recently been applied to describe adaptortail interaction (15), is described in more detail elsewhere (28, 29). For the calculation of adaptor concentration, a molecular mass of 280,000 Da was taken for both complexes.

RESULTS
The immobilized MPR46 Tail Specifically Interacts with Clathrin AP Complexes—To analyze the kinetics of the adaptor-MPR tail interaction and to define the MPR tail sequences that bind AP1 or AP2, we used the SPR technology. For this purpose we coupled the full-length MPR46 tail peptide (Fig. 1) to the activated dextran surface of a biosensor via its primary amino group. Biosensor surfaces that were derivatized with an unrelated control peptide or that were kept free of peptide served as controls. Around 500 pmol of the MPR46 tail and the control peptide were covalently coupled, which resulted in a base-line shift of 1500 resonance units (for MPR46). As shown in Fig. 2, only protein fractions that contain adaptor complexes of CCVs (Tris extract, mixed AP pool) gave a typical sensorgram showing association and dissociation phase. In contrast, clathrin, a low molecular weight (adaptor-free) protein fraction derived from coated vesicles, as well as BSA, only changed the baseline due to a change in the refractive index of the buffer. When AP containing fractions were passed over non- or control peptide-derivatized surfaces, no binding was observed (not shown). Thus AP containing fractions show a specific binding to the immobilized MPR46 tail.

Both AP1 and AP2 Bind to the MPR46 Tail with Similar Kinetics—To study the adaptor interaction in more detail, we effect of short MPR46 tail peptides, isolated AP1 or AP2 was incubated with a 1000-fold molar excess of peptide for 30 min prior to an interaction analysis, which was performed exactly as described above.

Determination of Kinetic Parameters—The kinetic parameters of adaptor binding and dissociation to the immobilized MPR tail was determined 15–20 s after switching from buffer flow to adaptor solution (association) or after switching back to running buffer (dissociation) to avoid distortions due to injection and mixing.

The association constant \( k₄ \), the dissociation constant \( k₅ \), and the steady state response level \( R_{ss} \) by fitting data to the following equation,

\[ R = R_{ss} - R_{eq} \]  

where \( R_{ss} \) is the response level at the beginning time \( t₂ \) of the dissociation phase. This model, which has recently been applied to describe adaptor-tail interaction (15), is described in more detail elsewhere (28, 29). For the calculation of adaptor concentration, a molecular mass of 280,000 Da was taken for both complexes.
next purified and separated AP1 and AP2 (Fig. 3, inset). Both AP1 and AP2 bound to the sensor chip derivatized with the MPR46 tail (Fig. 3, bar A). To control for the specificity of binding, subsequent to the binding of AP1 or AP2, the sensor chip was perfused with monoclonal antibodies to AP1 or AP2 (Fig. 3, bar B). The sensorgrams demonstrate that after binding of AP1 only the antibody to AP1 but not that to AP2 was bound. The reverse was observed after binding of AP2. These data demonstrate that the adaptors were bound to the MPR46 tail and that the preparations of AP1 and AP2 were essentially free of each other.

When AP1 or AP2 were passed over the MPR46 derivatized sensor chip at concentrations ranging from 2.5 to 250 nM, both AP complexes bound with similar kinetics. For AP1 a $K_D$ of 12 nM $\pm$ 0.15 was calculated and 16 nM $\pm$ 0.16 for AP2 (Fig. 4).

At the highest concentration of adaptors the stoichiometry between immobilized tails and bound adaptors was about 1:15. Theoretically a ratio of 1:1 may be reached. Several factors may contribute to a lower stoichiometry of AP binding. These include misfolding of tail peptides during immobilization, inaccessibility of tail peptides to adaptors in the three-dimensional matrix (100 nm thickness) to which they are immobilized and steric hindrance by AP complexes bound to neighboring cytoplasmic tails.

**Short MPR Tail Peptides Specifically Inhibit the Receptor-Adaptor Interaction**—With the experiments described above it was possible to demonstrate the high affinity binding of AP1 and AP2 to the MPR46 tail. However, the sequences of the MPR46 tail required for binding of AP1 and AP2 are not known. To identify these sequences, we synthesized a set of peptides corresponding to different regions in the MPR46 tail (see Fig. 1). Assuming that one (or more) of the peptides have an adaptor binding capacity they should be able to inhibit the binding of the adaptors to the MPR46 tail when applied together with the adaptors to the Biosensor surface. We incubated AP1 and AP2 with a 1000-fold molar excess of peptide for 30 min at 4°C prior to an SPR analysis. The sensorgrams were recorded in comparison with those obtained with adaptors not preincubated with peptides or preincubated with BSA or a nonrelated control peptide. As shown in Fig. 5A several peptides colinear with the MPR46 tail sequence inhibited the binding of AP1 and AP2 to the MPR tail. A peptide (residues 2–16) comprising the proximal quarter of the receptor tail inhibited binding of AP2, but not of AP1. The opposite was found for peptide 27–43. This peptide inhibited binding of AP1, while that of AP2 was nearly unaffected. A third peptide (residues 49–67) inhibited binding of both AP1 and AP2. The inhibition was sequence-specific, as addition of BSA or an MPR46-unrelated 21-mer peptide to the adaptor solution at the same molar concentration as the inhibitory peptides (Fig. 5A) did not interfere with adaptor binding. Moreover two other MPR46 tail peptides (peptides 17–31 and 39–53) inhibited neither AP1 nor AP2 binding. Thus three different peptides inhibit the binding of the AP complexes, residues 27–43 and 49–67 inhibiting binding to AP1, residues 2–16 and 49–67 that to AP2.

To narrow down the sequences required for the inhibition of adaptor binding, shorter peptides of 9–10 residues were tested (Fig. 5C). The peptide 10–19 inhibited binding of AP2 but not of AP1 similar to peptide 2–16 (Fig. 5C). A 50% inhibition of AP2 binding was observed at 41 $\mu$M peptide 2–16 and 100 $\mu$M peptide 10–19 (Fig. 6B), indicating that residues 10–19 are sufficient for inhibition. The sequence of peptide 27–43, which inhibited binding of AP1, was covered by three overlapping 10-mer peptides. Of the three peptides, 29–38, 34–43, and 39–48, only peptide 34–43 inhibited AP1 binding and none AP2 binding. The peptides 27–43 and 34–43 both inhibited AP1 binding by 50% at 40 $\mu$M concentration (Fig. 6A) showing that the inhibition of peptide 27–43 can be attributed to its COOH-terminal 10 residues.

The sequence of the COOH-terminal peptide 49–67 was covered by the three overlapping peptides: 49–58, 54–62, and 58–67. Each of the three shorter peptides inhibited binding of AP1 and AP2 as observed for the longer peptide 49–67 (Fig. 5C). The inhibitory activity of peptide 54–62, however, was weaker (50% inhibition of AP1 and AP2 binding at 180 and 450 $\mu$M, respectively) than that of the other peptides. The two peptides, 49–58 and 58–67, overlapping by one residue, exhibited an inhibitory activity comparable with that of peptide 49–67 with 34–100 $\mu$M required for 50% inhibition of AP1 binding and 28–64 $\mu$M peptide for that of AP2 binding (see Fig. 6, A and B). This suggests that adaptors and the C terminus of the MPR46 tail contact over an extended sequence.
Taken together the results obtained by the inhibition assays have shown that peptides corresponding to at least three distinct regions of the MPR46 tail inhibit the binding of the adaptor complexes to the MPR tail in a dose-dependent manner. One explanation for these observations is that the MPR tail has more than one binding site for AP1 and AP2, respectively. It is also conceivable that the peptides somehow indirectly disrupt the binding and are not colinear with the sequences mediating AP binding. The former assumption would greatly be strengthened, if the peptides identified in the inhibition experiments could be shown to mediate binding of AP1 and/or AP2.

**FIG. 4.** Kinetics of the adaptor-MPR46 tail interaction. AP1 or AP2 were analyzed for their binding to the immobilized MPR46 tail at the indicated concentrations. The kinetic values for on-rate $k_a$ (M$^{-1}$s$^{-1}$) and off-rate $k_d$ (s$^{-1}$) were determined in a 30-s time interval (indicated by the shaded bars), the equilibrium constant $K_D$ (M) was calculated as described under “Material and Methods.”

**FIG. 5.** Inhibition of the adaptor-tail interaction by short MPR46 tail peptides. Synthetic peptides (100 μM) corresponding to the indicated MPR46 tail sequence were incubated with 100 nM AP1 (dashed columns) or 100 nM AP2 (filled columns) prior to SPR analysis using the immobilized full-length MPR46 tail. The resonance units after an association/dissociation cycle of 4 min (see Fig. 4) were taken as 100% binding. The values represent the data obtained from two to three independent experiments. A, effect of a 22-mer peptide (100 μM) unrelated to MPR46 and of BSA (100 μM). B, 15–18-mer peptides covering the entire length of the MPR46 tail. C, 9–10-mer peptides overlapping with the inhibitory peptides 2–16, 27–43, and 49–67.

**FIG. 6.** Analysis of the concentration dependence of inhibitory peptides. Adaptors (100 nM) were incubated in the presence of a 10–3000-fold molar excess with the indicated peptides and then analyzed for their binding to the immobilized MPR46 tail. The resonance units obtained after a 4-min association/dissociation cycle are plotted as percentage of controls without peptide. The values correspond to the results obtained from three independent experiments. The peptide concentration required for 50% inhibition of binding was calculated after linear regression of the data.

**Multiple Adaptor Binding Sites**—In the above described experiments peptides defining three distinct sequences were most effective in inhibiting the binding of AP1 and/or AP2 to the full-length MPR46 tail. To analyze the ability of these peptides to bind adaptors, we coupled the peptides 2–16 (inhibiting binding of AP2), 27–43 (inhibiting binding of AP1), and 49–67 (inhibiting binding of AP1 and AP2) to the sensor chip.
under standard conditions, binding was analyzed at 800 nM 2–16 and of AP2 to peptide 27–43 that would escape detection length tail. To exclude a low affinity binding of AP1 to peptide 19888

AP2 binding not only to itself (Fig. 8 67LL/AA prior recording the adaptor binding to the immobi-

length tail. AP1 or AP2 was incubated in the presence of a can also be inhibited by soluble peptide as found for the full-

leucine pair. AP1 and AP2 bound to the mutant peptide 49– 67LL/AA, a peptide in which two alanines replaced the di-

ly by MPR46 (31, 32). We therefore recorded AP binding to 49– 64/65 known to be critical for the sorting of lysosomal enzymes

boxyl-terminal peptide 49–67 and its mutant counterpart 49– 67LL/AA (Fig. 8 7) and inhibition of adaptor binding (Fig. 8) between the car-

somes (33, 34). The receptor itself is not found in lysosomes but recycles between endosomes and the plasma membrane or endosomes and the TGN (35). Some of the sorting signals that mediate intracellular sorting of MPR46 have been characterized during the last 10 years mainly by using cells expressing tail mutants of the receptor. The carboxyl-terminal di-leucine motif is essential for the enzyme sorting function of the receptor (36) and it has also been characterized as an internalization signal mediating endocytosis of MPR46 from the plasma membrane (32).

Two other sequence determinants involving Tyr-45 and Phe-13 have also been shown to act as endocytosis signals (31, 32). In this study we directly tried to identify sequence determinants of MPR46 that are able to interact with AP1 and/or AP2 in vitro. For this purpose short MPR46 tail peptides (10–19 amino acids in length) and a 67-amino acid full-length tail peptide were immobilized on a sensor chip surface. Subsequently we analyzed the adaptor interaction by SPR. This enabled us to record the interaction between the MPR46 tail and adaptors in real-time and thus estimate the affinity of the interaction.

If the full-length MPR46 tail peptide was immobilized on the sensor chip surface, we recorded a specific interaction with purified adaptors. No interaction was detected with other protein fractions, including clathrin, a low molecular weight protein fraction, and BSA (see Fig. 2). After separating AP2 from

### Table I

| Peptide | AP1 | AP2 |
|---------|-----|-----|
| 2–16    | 23  | 55  |
| 10–19   | ND  | 23  |
| 27–43   | 58  | 62  |
| 34–43   | 14  | 14  |
| 49–67   | 122 | 108 |
| 49–58   | 15  | 17  |
| 54–62   | 15  | 17  |
| 58–67   | 15  | 17  |
| 49–67LL/AA | 15 | 17 |

a $K_D$ was determined as shown in Fig. 3.
b Dash indicates that AP1 and/or AP2 did not bind to the peptide.
c ND, not determined.

d and one sequence determinant (residues 49–67) that bound both AP1 and AP2.

### DISCUSSION

High Affinity Binding of AP1 and AP2 to the MPR46 Tail—

One of the major functions of MPR46 is the sorting of newly synthesized lysosomal enzymes from the TGN to lysosomes (33, 34). The receptor itself is not found in lysosomes but recycles between endosomes and the plasma membrane or endosomes and the TGN (35). Some of the sorting signals that mediate intracellular sorting of MPR46 have been characterized during the last 10 years mainly by using cells expressing tail mutants of the receptor. The carboxyl-terminal di-leucine motif is essential for the enzyme sorting function of the receptor (36) and it has also been characterized as an internalization signal mediating endocytosis of MPR46 from the plasma membrane (32). Two other sequence determinants involving Tyr-45 and Phe-13 have also been shown to act as endocytosis signals (31, 32).

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MPR46 Contains Multiple Clathrin Adaptor Binding Sites

AP1 by hydroxypatite chromatography, we could show that both complexes interact with the MPR46 tail with similar \( K_D \) values of about 13 nM (AP1) and 17 nM (AP2) (Fig. 4). This strong interaction is in good agreement with the \( K_D \) of 25 nM, which was obtained for the binding of AP2 to internal membranes (22). In this study, cells overexpressing wild-type or mutant MPR46 were permeabilized with streptolysin-O, endogenous APs were removed and subsequently recruitment of exogenous AP1 from bovine brain cytosol was monitored via enzyme-linked immunosorbent assay. A similar approach was used to determine the binding of AP2 to membrane fragments of MDBK cells (38). In the latter study, high affinity binding of AP2 (30 nM) to membranes was found to be dependent on the MPR46 tail, which was subsequently recorded in the presence of a 3000-fold molar excess of soluble peptide 2–16, 27–43, 49–67, or 49–67LL/AA. Adaptor binding was calculated as described in the legend to Fig. 5 and expressed as percentage of controls analyzed in the absence of soluble peptides (see Fig. 7).

Fig. 8. Inhibition of adaptor binding to MPR46 tail peptides. The MPR46 tail peptides 2–16 (A), 27–43 (B), 49–67 (C) and 49–67 (D) were coupled to the sensor chip surface. Binding of AP1 (dashed bars) and AP2 (filled bars), 100 nM each, was subsequently recorded in the presence of a 3000-fold molar excess of soluble peptide 2–16, 27–43, 49–67, or 49–67LL/AA. Adaptor binding was calculated as described in the legend to Fig. 5 and expressed as percentage of controls analyzed in the absence of soluble peptides (see Fig. 7).

AP1 by the binding of monomeric adaptor complexes.

Several studies have recently used the biosensor technology for monitoring adaptor-tail interaction (12, 15, 17, 39). In several of these studies the affinity constants were not calculated. When binding of AP2 to the EGF receptor was calculated, the dissociation rate was too low to allow determination of \( K_D \) (39). Hemagglutinin tail derived peptides carrying amino acid substitutions that created functional tyrosine or di-leucine based sorting signals were shown to interact with both AP1 and AP2 with \( K_D \) values ranging from 350 to 900 nM (15). In the study of Höning et al. (12), binding of AP1 and AP2 with the cytoplasmic tail of lamp1 was characterized. The \( K_D \) values calculated for this interaction varied between 30 and 50 nM for AP1 and AP2. When comparing the different \( K_D \) values for the interaction of adaptors with different cytoplasmic tails, it should be noted that posttranslational modifications and oligomerization of membrane proteins may greatly affect their binding to adaptors.

MPR Tail Sequences That Bind AP1 with High Affinity—To identify sequences that mediate binding of AP1 we first used short tail peptides to inhibit adaptor binding to the MPR46 tail (Figs. 5 and 6). Next we examined peptides that exhibited an inhibitory activity for their ability to bind adaptors (Figs. 7 and 8). In summary we were able to identify two independent sequence determinants (amino acids 27–43 and 49–67) that bound AP1 with nearly identical affinity as the full-length tail. By using short peptides of 9–10 residues in length, the sequences critical for inhibition could be narrowed down to amino acids 34–43 for the proximal determinant, while the carboxy-terminal AP1 binding determinant appears to involve an extended sequence covering residues 49–67. The experiments using a mutant form of peptide 49–67, in which the leucine residues 64 and 65 were replaced by two alanines (peptide 49–67LL/AA), clearly show that the di-leucine motif is not a prerequisite of adaptor binding.

Residues 56–60, which represent a casein kinase II phosphorylation site, are known to be critical for AP1 recruitment to Golgi membranes of MPR-deficient fibroblasts transfected with wild-type or mutant MPR46 (22). The functional importance of the casein kinase II phosphorylation site is under dispute. While Mauxion et al. (22) suggested that it acts as a dominant TGN-sorting determinant for packaging of MPR46 in Golgi-derived vesicles, the substitution of Ser-57 by alanine or aspartate did not affect the sorting of lysosomal enzymes in mouse cells (36, 40). A functional casein kinase II phosphorylation site, however, was required for transport of MPR46 to the plasma membrane (40), suggesting that phosphorylation controls a sorting step within the endosomal system. Our experiments showing high affinity binding of AP1 to residues 49–67 have been performed without phosphorylation of the peptide. Moreover, Chen et al. (41) have recently described that phosphorylation of the equivalent carboxyl-terminal caseine kinase-II sequence of MPR300 is not essential for receptor function. Thus phosphorylation of serine 57 is not a prerequisite for high affinity binding of AP1, although it may regulate the affinity of binding.

While Mauxion et al. (22) could not find evidence for an AP1 binding site within the amino-terminal 51 residues of the MPR46 tail, we identified a second sequence, amino acids 27–43, which displays high affinity binding of AP1. It is of interest that this sequence includes the motif that has recently been proposed to function as a lysosomal avoidance signal (amino acids 34–39; Ref. 42) for which the reversibly palmitoylated cysteine residue C34 is critical (43). Again the importance of

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3 S. Höning, J. Griffith, H. Geuze, and W. Hunziker, personal communication.
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this modification for regulation of the affinity to AP1 remains to be determined. The present data show that the nonmodified sequence 27–43 mediates interaction with AP1 at and off rates similar to those of the full-length MPR46 tail.

**MPR46 Tail Sequences That Mediate AP2 Binding**—Expression of tail mutants of MPR46 and determining their internalization rate has allowed definition of three distinct sequences critical for rapid internalization (31, 32). Two of these sequences containing Tyr-45 and the di-leucine pair 64/65, respectively, fall into the classes of the canonical tyrosine- and di-leucine-based internalization signals that have been identified in the cytoplasmic tail of a growing number of plasma membrane receptors. The third internalization sequence is represented by amino acids 7–13 with a critical phenylalanine in position 13. This sequence lacks similarity to any of the known internalization motifs. The three internalization sequences are partially redundant. Loss of either of the three has only a minor effect on internalization, while loss of two of them, irrespective in which combination, reduces internalization by more than 50%. Furthermore, the two sequences harboring amino acids 7–13 and the di-leucine pair 64/65 were shown to function as autonomous internalization sequences (31). Interestingly, the two MPR46 tail sequences that were identified in this study to mediate high affinity binding of AP2 (peptides 2–16 and 49–67) comprise residues 7–13 and 64/65, respectively. For the carboxyl-terminal peptide 49–67, we were able to show that Leu 64/65 are not critical for binding of AP2 to the tail peptide.

It should be noted that none of the peptides comprising Tyr-45 (peptides 39–53, 39–48, and 44–53) inhibited AP2 binding to the MPR46 tail. This may indicate that none of the peptides adopts the conformation of the corresponding sequence in the MPR46 tail. Since experimental evidence for an autonomous function of a Tyr-45-containing sequence as an internalization sequence is missing, the alternative possibility has to be considered that Tyr-45 is not part of an AP2 binding site, but affecting AP2 binding indirectly.

**MPR46 Contains Multiple Adaptor Binding Sites**—Our results have shown that the MPR46 tail contains multiple high affinity binding sites for AP1 and AP2, respectively. In addition some of the binding sites show high affinity binding to only one type of adaptor. The residual affinity to the other receptor peptides, if existing at all, is at least 500-fold lower. Whether in vivo all signals are utilized, whether they are utilized simultaneously or alternatively, and to which extent they are regulated by posttranslational modifications remains to be determined.

Presence of several distinct binding sites for one type of adaptor complex in one cytoplasmic tail suggests that also adaptor complexes have several distinct binding sites for sequences in cytoplasmic tails. We observed that peptides inhibited not only binding of adaptors to the same but also to other peptides. For example peptide 2–16 inhibited binding of AP2 to itself and to peptide 49–67. This could indicate that peptide 2–16 and peptide 49–67 compete for the same binding site in AP2. However peptide 49–67 did not inhibit binding of AP2 to peptide 2–16 as would be predicted by such a model. This renders an alternative explanation more likely. Binding of peptide 2–16 to its binding site in the adaptor complex could induce a conformational change that lowers the affinity at the site to which peptide 49–67 binds to, while an analogous effect would not be produced by binding of peptide 49–67. In addition the inhibition of AP1 binding to peptide 27–43 by peptide 49–67 can be explained analogous.

Multiple interactions between cytoplasmic tails and adaptor complexes would greatly facilitate in membranes the formation of subdomains enriched in transmembrane cargo proteins designed for incorporation in clathrin coated vesicles through cross-linking via adaptors. Future work has to characterize the binding between tail peptides and adaptor complexes or their mutants by techniques such as photocross-linking, x-ray crystallography, or multidimensional NMR, to provide structural information about their interaction.

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