**In Vitro Binding of Plasma Membrane-coated Vesicle Adaptors to the Cytoplasmic Domain of Lysosomal Acid Phosphatase***

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Sorting of the newly synthesized membrane-bound precursor of lysosomal acid phosphatase (LAP) involves internalization from the plasma membrane via clathrin-coated pits. Using an in vitro system, we present direct evidence for high affinity interaction of the cytoplasmic domain of LAP with the amino-terminal trunk portion of plasma membrane-coated vesicle adaptors. Coated vesicle adaptors of the trans-Golgi network displayed poor binding to LAP, but high affinity binding to the cytoplasmic tail of the 46-kDa mannose 6-phosphate receptor, which is included in clathrin-coated pits of the trans-Golgi network. Binding of plasma membrane adaptors to the tail peptide of LAP required an internalization signal that contains either tyrosine or phenylalanine.

Transport of newly synthesized lysosomal enzymes to lysosomes requires their segregation from resident proteins of the endoplasmic reticulum, the Golgi complex, and the secretory pathway. This study is focused on lysosomal acid phosphatase (LAP), which is synthesized as an integral membrane protein with a single membrane-spanning domain and a short cytoplasmic domain composed of 18 amino acids (Waheed et al., 1988). After exit from the trans-Golgi network (TGN), LAP passes the plasma membrane, from where it is internalized via clathrin-coated pits (Braun et al., 1989; Peters et al., 1990; Hille et al., 1992), and transported to lysosomes by an as yet unknown mechanism. Within lysosomes, the cytoplasmic and membrane-spanning domains of LAP are proteolytically cleaved, and the resulting soluble form of LAP cannot recycle from lysosomes (Gottschalk et al., 1989).

Sorting of newly synthesized LAP is independent of mannose 6-phosphate receptors, which mediate transport of soluble lysosomal enzymes (Kornfeld and Mellman, 1989). In contrast to transport of LAP, mannose 6-phosphate-dependant transport of newly synthesized lysosomal enzymes does not involve passage of the plasma membrane. Instead, mannose 6-phosphate receptors and their ligands are segregated from the secretory pathway in the TGN by inclusion into clathrin-coated vesicles. Whether newly synthesized LAP can be segregated from the secretory pathway at the level of the TGN is not known. The kinetics of transport suggest, albeit does not prove, that LAP reaches the plasma membrane with the bulk flow of the secretory pathway (Braun et al., 1989). Mannose 6-phosphate receptors are also found at the plasma membrane and perform clathrin-mediated endocytosis of exogenous ligands.

Sorting of membrane proteins and receptors into clathrin-coated transport vesicles is thought to require recognition of their cytoplasmic domains by complexes of cytosolic proteins, called adaptors. Binding of adaptors to the cytoplasmic domain then mediates assembly of the clathrin coat which is required for formation of transport vesicles (Pearse and Robinson, 1990). To date, two types of adaptors have been described, HA-1 and HA-2 adaptors, which are restricted to coated vesicles of the TGN and the plasma membrane, respectively (Ahle et al., 1988; Geuze et al., 1991; Pearse and Robinson, 1990). Adaptors are heterotetrameric complexes of two 100-kDa subunits and two smaller subunits of about 50 kDa and 20 kDa. The 100-kDa α- and β-subunit of HA-2 adaptors (α- and β-adaptins) are homologous to the γ- and β'-subunits (γ- and β'-adaptns), respectively, of HA-1 adaptors. The β-adaptin has been shown to bind both clathrin (Ahle and Ungewickell, 1989) and the cytoplasmic domain of the asialoglycoprotein receptor (Beitzel and Spiess, 1991).

Binding of HA-2 adaptors to the cytoplasmic domain of the receptors requires an intact internalization signal (Pearse, 1988; Glickman et al., 1989). This signal for clathrin-mediated internalization contains a tyrosine residue, which is exposed in a β-turn conformation, but does not display a strict amino acid consensus sequence (Kristakis et al., 1990; Bansal and Gierasch, 1991). In some, but not all, receptors, the tyrosine residue can be replaced by phenylalanine. The signal for internalization of LAP resembles the internalization signal of endocytic receptors. It also contains a tyrosine residue that is exposed in a β-turn conformation within the cytoplasmic domain (Peters et al., 1990; Eberle et al., 1991). In the case of LAP, the tyrosine residue cannot be substituted by the aromatic residue phenylalanine or by alanine (Peters et al. 1990; Lehmann et al., 1992). Mutant forms of LAP that contain within their cytoplasmic domain a phenylalanine or alanine residue instead of the essential tyrosine, accumulate at the plasma membrane and are internalized slowly. These findings, and our recent observation that internalization of LAP can be inhibited by microinjection of antibodies against clathrin or α-adaptin (Hille et al., 1992), suggested that LAP, like endocytic receptors, is sorted into clathrin-coated pits of the plasma membrane. So far, however, direct evidence that the cytoplasmic domain of LAP interacts with HA-2 adaptors was not available.

In the present study, we have investigated the interaction of the cytoplasmic domain of human LAP with clathrin-coated vesicle adaptors in vitro. An immobilized peptide that corresponds to the cytoplasmic domain of human LAP effi-
ciently bound HA-2 adaptors, whereas HA-1 adaptors bound poorly. The specificity of binding was confirmed by using modified peptides that contained phenylalanine or alanine instead of the single tyrosine residue and by competition with free peptides. A peptide corresponding to the cytoplasmic domain of M_{r} = 46,000 mannose 6-phosphate receptor (MPR 46) efficiently bound both HA-1 and HA-2 adaptors.

**EXPERIMENTAL PROCEDURES**

**Materials**—Monoclonal antibodies against γ-adaptin (mAb 100/2) or γ-adaptor (mAb 100/3) (Ahle et al., 1988) were kindly provided by E. Ungewickell (Martinried, FRG), and the monoclonal antibody against α-adaptin (AC1-M11) (Robinson, 1987) by M. Robinson (Cambridge, UK). Biotinylated anti-mouse IgG was from Dianova (Hamburg, FRG), and peroxidase-conjugated streptavidin was from Zymed Laboratories (San Francisco). Peptides were synthesized and purified as described (Nadimpalli et al., 1991).

**Preparation of Adaptors from Bovine Brain-coated Vesicles—**Bovine brain was obtained from a local slaughterhouse and processed for 10 min at 37°C in buffer A. After addition of phenylmethylsulfonyl fluoride, 0.005% phenylmethylsulfonyl fluoride, 0.02% NaN₃, pH 7.8 for 30 min at 37°C, precipitated clathrin was collected by centrifugation at 100,000 × g for 30 min and applied to a 2.6 × 75 cm Sepharose CL-4B column (Pharmacia, Freiburg, FRG), which had been equilibrated in buffer A as described by Keen et al. (1979). Fractions containing adaptor complexes (referred to as mixed adaptors and peptides) were pooled and concentrated to 0.75 mg/ml by ultrafiltration under nitrogen atmosphere (PM 10 membrane, AMICON, Witten, FRG) and stored at 4°C for up to 2 weeks.

**Hydroxyapatite chromatography for separation of HA-1 and HA-2 adaptors was performed as described by Manfredi and Bazari (1987).**

**Limited Trypsin Proteolysis of Adaptor Complexes—**Adaptors were treated with 1-1-tosylamide-2-phenylethylchloromethyl ketone-treated trypsin (Sigma, Deisenhofen, FRG) at a ratio of 1:10 (w/w) for 10 min at 37°C in buffer A. After addition of phenylmethylsulfonyl fluoride (2.5 mM final concentration), the digest was cleared by centrifugation before loading to the affinity column.

**Affinity Chromatography—**15-20 mg of peptide in 0.5 ml of 0.2 M HEPES (pH 7.5) were coupled to 0.75 ml of Affi-Gel 10 (Bio-Rad, München, FRG) for 20 h at 4°C with rotation according to the manufacturer's instructions. The coupling efficiencies were from 80 to 90% for all the peptides, resulting in a density of 8-10 μmol/ml for acid phosphatase-derived peptides and about 1.5 μmol/ml for the tail peptide, followed by a 30-min centrifugation at 100,000 × g before loading to the affinity column.

**Adaptors were dialyzed overnight against buffer C (0.1 M MES/NaOH, 0.15 M NaCl, 1 mM EDTA, 0.5 mM MgCl₂, 2 mM CHAPS, 0.2 mM dithiothreitol, 0.005% phenylmethylsulfonyl fluoride, 0.02% NaN₃, pH 7.0) and centrifuged at 100,000 × g for 30 min. About 80% of the protein was recovered in the supernatant. Typically, 10-60 μg of adaptors in 250 μl of buffer C were applied to each affinity column, which had been equilibrated with buffer C containing 0.5 mg/ml bovine serum albumin. After 15 min of incubation, the columns were washed with 10 volumes of buffer C and then eluted with 5 volumes of buffer B, pH 7.4. Columns were washed extensively with buffer B, stored in buffer C, and used for at least 6 months without loss of binding activity.

For competition assays, the adaptors in buffer C were incubated overnight at 4°C with up to 15,000-fold molar excess of the soluble peptide, followed by a 30-min centrifugation at 100,000 × g before loading to the affinity column.

**Electrophoresis and Western Blotting—**For SDS-PAGE (Laemmli, 1970), proteins were precipitated by trichloroacetic acid (10% final concentration). Detection of proteins by silver staining was done according to Ansorge (1985).

Proteins were transferred from SDS-polyacrylamide gels to nitrocellulose membranes (0.2-μm pore size, Schleicher and Schuell, Dassel, FRG) as described (Burnette, 1981). Nonspecific binding sites were blocked overnight at 4°C with buffer D (5% delipidated milk powder, 0.05% Tween 20 in phosphate-buffered saline). All the following steps were carried out at room temperature. Membranes were incubated with anti-adaptin antibody for 3 h, washed 3 times for 15 min with buffer D, and incubated with biotinylated anti-mouse IgG (diluted 1:5000 in buffer D) for 2 h. After washing 5 times with buffer E (0.05% SDS, 0.9% NaCl, 0.5% Triton X-100) and once with buffer F (0.05% Tween, 0.15 M NaCl, 10 mM sodium phosphate, pH 7.4), the membranes were incubated with peroxidase-conjugated streptavidin (diluted 1:5000 in buffer F) and stored in buffers E and F as before. Detection by the enhanced chemiluminescence method (ECL) was carried out according to the manufacturer's advice (Amersham Buchcher, Braunschweig, FRG) using Kodak XAR-5 film. Signals were quantitated by densitometric scanning of the films (Ultrascan XL, LKB-PHARMACIA, Freiburg, FRG). A linear increase of the chemiluminescence signal was observed with up to 20 μg of loaded protein for HA-1 and up to 45 μg of loaded protein for HA-2 (see Fig. 3C).

**Protein Determination—**Protein was quantitated either according to Lowry et al. (1951) or, when adaptors were in buffer B, according to Peterson (1977).

**RESULTS**

**The Cytoplasmic Domain of Lysosomal Acid Phosphatase Selectively Binds HA-2 Adaptors—**For in vitro binding of coated vesicle adaptors, synthetic peptides that correspond to the cytoplasmic domain of human lysosomal acid phosphatase were immobilized on Affi-Gel 10 at a density of about 8–10 μmol per ml of gel. The coupling procedure (covalent linkage of the amino terminus to the activated agarose matrix) resulted in immobilization of monomeric peptides with freely exposed carboxyl termini (see Fig. 1). Thus, the orientation of the immobilized peptides mimics that of the cytoplasmic domain as it is exposed in cellular membranes. Covalently bound proteins were extracted from bovine brain-coated vesicles by high ionic strength, and HA-1 and HA-2 adaptors were separated from clathrin by gel filtration chromatography. A protein fraction, referred to as mixed adaptor preparation, which contains predominantly HA-1 and HA-2 adaptors together with a small amount of assembly protein AP180 and low molecular weight proteins, but essentially no clathrin (see Fig. 7, lane a), was loaded to the peptide columns. Bound material was eluted with 1 M Tris-HCl, pH 7.4, conditions which elute adaptors from coated membranes. Flow-through and bound fractions were analyzed by SDS-PAGE and Western blotting, using monoclonal antibodies against γ- and α-adaptin of HA-1 and HA-2 adaptors, respectively. As shown in Fig. 2, HA-2 adaptors were selectively bound to the cytoplasmic domain of human LAP (LAP-wt), whereas HA-1 adaptors were hardly detectable in the bound fraction. Under identical conditions, the immobilized peptide LAP-AS, which contains alanine in place of the single tyrosine residue of the cytoplasmic domain of LAP bound HA-2 adaptors 5.5 times less efficiently than the LAP-wt peptide, as determined by densitometric analysis of the chemiluminescence signal (Table I). This result is in agreement with the internalization-deficient phenotype of mutated LAP with tyrosine replaced by alanine (Lehmann et al., 1992).

Binding of the HA-2 adaptor complex to the LAP-wt peptide was observed at pH values between 6.6 and 7.4. The interaction was resistant to high ionic strength (0.5 M NaCl) and mild, anionic detergent (2 mM CHAPS, 10 mM n-octyl glucoside, or 0.1% saponin) (not shown). Purified clathrin did not bind to the cytoplasmic tail of LAP (not shown). These results suggest specific binding of HA-2 adaptors to the LAP-wt peptide.

To determine the capacity of the LAP-wt column for binding of HA-2, columns were loaded with 5.5–33 μg of the mixed adaptor preparation per μmol of immobilized peptide. Flow-through and bound fractions were pooled and analyzed by SDS-PAGE and Western blotting. When low amounts of protein were loaded (up to 8.5 μg of the mixed adaptor preparation per μmol of tail peptide), quantitative binding of
Fig. 1. Peptides used for binding of adaptors. LAP-wt, peptide corresponding to the tail peptide of wild-type LAP.

Fig. 2. The tail peptide of LAP specifically binds HA-2 adaptors. A mixed adaptor preparation (40 μg) was bound to 1.8 μmol of immobilized peptide LAP-wt (left panel) or peptide LAP-A8 (right panel) and eluted with 1 M Tris-HCl, pH 7.4. Flow-through (FT) and bound (B) fractions were analyzed by SDS-PAGE (7.5% polyacrylamide) and Western blotting. HA-1 and HA-2 adaptors were detected by anti-γ-adaptin or anti-α-adaptin antibodies, respectively, followed by chemiluminescence detection.

HA-2 adaptors was observed within 15 min of incubation, suggesting that the adaptor preparation contained HA-2 adaptors exclusively in a binding-competent form (Fig. 3A, top panel). When increasing amounts of protein were loaded, part of the HA-2 adaptors were observed in the flow-through fraction. Quantitation of the chemiluminescence signal by densitometry revealed that binding of HA-2 adaptors reached saturation at about 30 μg of mixed adaptors per μmol of LAP-wt peptide (Fig. 3A, bottom panel). The linear response of the chemiluminescence signal to increasing amounts of adaptor proteins shown in Fig. 3C illustrates that this was not due to saturation of the immunodetection method.

In view of the low binding of HA-1 adaptors to the LAP-wt peptide, it was necessary to investigate whether the coated vesicle extract contained HA-1 adaptors in a binding-competent form. An immobilized peptide which corresponds to the amino acids of the cytoplasmic domain of human MPR 46 (see Pohlmann et al., 1987) bound both HA-1 and HA-2 adaptors as expected for a receptor which is sorted into clathrin-coated vesicles at the TGN and plasma membrane (Fig. 3B). When increasing amounts of adaptors were loaded to the MPR 46 tail peptide, the binding of HA-1 and HA-2 adaptors increased almost in parallel and reached saturation at about 100 μg per μmol of immobilized peptide. These data suggest that both types of adaptors display similar affinity to the tail peptide of MPR 46 and both are in a binding-competent form.

We next asked whether the weak binding of HA-1 adaptors to the LAP-wt peptide was a result of competition with HA-2 adaptors present in the mixed adaptor preparation. HA-1 and HA-2 adaptors were therefore separated by hydroxyapatite chromatography. Analysis by silver staining and Western blotting showed that HA-1 and HA-2 adaptors had been efficiently separated, even though they were contaminated with assembly protein AP180 (not shown). In contrast to HA-2 adaptors, purified HA-1 adaptors did not bind to the LAP-wt peptide, even at a concentration (18 μg/μmol) which resulted in saturation of the column with HA-2 (Fig. 4). Specificity of HA-2 adaptor binding to the cytoplasmic tail of LAP was further investigated using columns with modified tail peptides and by competition with an excess of free peptide. The peptide LAP-F8, which contains phenylalanine in place of the essential tyrosine, bound HA-2 adaptors with similar efficiency as LAP-wt, whereas alanine could not substitute for tyrosine in the in vitro binding assay (Fig. 5 and Table I).

The shortened tail peptide LAP-Δ7, which contains the es-

Table 1
Quantitation of adaptor binding to tail peptides
Adaptors were subjected to affinity chromatography on tail peptides as described in Fig. 5, and chemiluminescence signals were quantitated by densitometry. The number of experiments (n) is given in parentheses. Mean of duplicate determinations or standard deviation (for n ≥ 3) are given. ND, not done.

| Peptide | Adaptors loaded | HA-1 adaptors bound | HA-2 adaptors bound |
|---------|-----------------|---------------------|---------------------|
| LAP-wt  | 8.3             | ND                  | 98 ± 2 (n = 5)      |
|         | 11.0            | 18 ± 12 (n = 3)     | 89 ± 7 (n = 4)      |
| LAP-F8  | 11.0            | ND                  | 97 ± 5 (n = 3)      |
| LAP-A8  | 8.3             | ND                  | 25 (n = 1)          |
|         | 11.0            | 8 ± 3 (n = 2)       | 16 ± 5 (n = 3)      |
| LAP-Δ7  | 8.3             | ND                  | 98 ± 3 (n = 2)      |
|         | 11.0            | ND                  | 78 (n = 1)          |
| MPR 46  | 65.0            | 84 ± 8 (n = 3)      | 96 ± 5 (n = 6)      |
Binding of HA-2 Adaptors to the Tail of LAP

FIG. 3. Binding of adaptors to tail peptides is saturable (A and B). Up to 60 µg of a mixed adaptor preparation were subjected to affinity chromatography on peptides LAP-wt (1.8 µmol, closed symbols), LAP-A8 (1.8 µmol, open symbols) (A), or MPR 46 tail (0.3 µmol) (B). Equal proportions of the pooled flow-through (1) and bound (2) fractions were analyzed by SDS-PAGE and Western blotting. 20% and 75% of the sample were used for detection of HA-1 and HA-2 adaptors, respectively. For selected samples, the section of the film containing the chemiluminescence signal of the 100-kDa adaptins is shown (upper panels). For all samples, signals were quantitated by densitometry (lower panels). Loaded material is given as micrograms of mixed adaptors per µmol of immobilized peptide. C, standard curve for quantitation of adaptors. After separation by SDS-PAGE, HA-1 or HA-2 adaptors were detected by Western blotting. Chemiluminescence signals were quantitated by densitometry. ▲, HA-1 adaptors; ○, ○, HA-2 adaptors. Single values of a representative experiment are given.

sential tyrosine and represents the minimal length required for internalization of LAP in vivo (Lehmann et al., 1992), also bound HA-2 adaptors selectively. For competition experiments, adaptors were preincubated overnight with excess soluble peptide before they were loaded to the LAP-wt column. The peptide LAP-wt decreased binding of HA-2 adaptors to the LAP-wt column in a concentration-dependent fashion (Fig. 6A). 1–2 mg of soluble peptide (0.5–1 µmol) reduced binding to 50% of the control without soluble peptide. Thus, about an equimolar amount of soluble peptide over immobilized peptide (1.8 µmol) or 15,000-fold molar excess of the soluble peptide over HA-2 adaptor complex was required for competition. When modified peptides were used for competition, LAP-A8 inhibited with similar efficiency as the LAP-wt peptide, whereas peptide LAP-A8 did not affect binding, and the shortened peptide LAP-Δ7 competed for binding to the LAP-wt column with very low efficiency (Fig. 6B).

The Cytoplasmic Domain of LAP Binds to the Trunk of the HA-2 Adaptors—Analysis of the structure of adaptor proteins had revealed that the complex consists of a compact trunk portion, which is formed by the amino-terminal part of the 100-kDa adaptins together with the 20- and 50-kDa small subunits, and two heads formed by the carboxyl-terminal part of each 100-kDa adaptin, which is separated from the trunk by a hinge region (Kirchhausen et al., 1989; Schröder and Ungewickell, 1991). Trunks and heads can be separated by limited trypic cleavage in the hinge region of the 100-kDa adaptins. In our hands, an incubation of adaptors with trypsin at a ratio of 19:1 (w:w) for 10 min at 37 °C was sufficient to cleave more than 95% of the 100-kDa adaptins. As shown by SDS-PAGE and silver staining, the 100-kDa adaptins (filled arrowhead in Fig. 7, lanes a and b) were cleaved to polypeptides with an apparent molecular mass of about 70 kDa which contribute to the trunks (open arrowheads) and 45–50 kDa which contribute to the heads (arrows). The heads appeared as a ladder of four polypeptides with heterogenous molecular
Binding of HA-2 Adaptors to the Tail of LAP

**Fig. 4.** Purified HA-1 adaptors do not bind to LAP tail peptide. Enriched preparations of HA-2 or HA-1 adaptors, which had been separated by hydroxyapatite chromatography (100 µg of protein each), were subjected to affinity chromatography on peptide LAP-wt (5.5 µmol). Flow-through (FT) and bound (B) fractions were analyzed by SDS-PAGE (7.5% polyacrylamide) and silver staining. The 100-kDa and 47-50-kDa subunits of the adaptor complex and the 180-kDa assembly protein AP180 are indicated. BSA, bovine serum albumin.

Fig. 5. Binding of HA-2 adaptors to the cytoplasmic domain of LAP requires an aromatic amino acid. A mixture of HA-1 and HA-2 adaptors (15 µg of protein) was subjected to affinity chromatography on wild-type or modified LAP tail peptides (1.8 µmol) (for abbreviations, see Fig. 1), or MPR 46 tail peptide (0.3 µmol). Proteins from the flow-through (1) or bound (2) fractions were pooled and analyzed by Western blotting. The part of the film containing the signal for 100-kDa adaptins is shown.

**Fig. 6.** Binding of HA-2 adaptors to the LAP tail peptide is competed by excess of soluble peptide. A mixed adaptor preparation was preincubated with an excess of soluble peptide, and affinity chromatography on peptide LAP-wt (1.8 µmol) was performed. HA-2 adaptors in the combined flow-through (1) or bound (2) fractions were detected by Western blotting and quantitated by densitometry. A, 20 µg of adaptors were incubated with peptide LAP-wt as indicated; B, 15 µg of adaptors were incubated with 2 mg of wild-type or modified LAP tail peptides.

**In Vitro Assay for Binding of Adaptors to Tail Peptides**—In the present study we introduce an improved protocol for in vitro binding of coated vesicle adaptors to tail peptides, which is based on the affinity chromatography published by Pearse and co-workers (Pearse, 1988; Glickman et al., 1989). These authors bound radioiodinated coated vesicle adaptors to tail peptides of low density lipoprotein receptor and 300-kDa goods.

**DISCUSSION**

LAP has been shown to enter coated pits of the plasma membrane (Hille et al., 1992) and requires a tyrosine residue in its cytoplasmic domain for rapid endocytosis (Peters et al., 1990), providing indirect evidence that the cytoplasmic domain of LAP may bind plasma membrane adaptors (HA-2 adaptors). Here we present direct evidence for high affinity binding of plasma membrane adaptors to the cytoplasmic domain of LAP using an in vitro system. In contrast, Golgi adaptors bound with very low efficiency, if at all.

**In Vitro Assay for Binding of Adaptors to Tail Peptides**—In the present study we introduce an improved protocol for in
mammose 6-phosphate receptor. While these studies were in progress, Beltzer and Spiess (1991) reported a different assay system for binding of asialoglycoprotein receptor to the β-chain of HA-2 adaptors, which were blotted on nitrocellulose. In our assay, which is based on immunodetection of adaptors to the tail peptides, whereas assembly protein AP180, which in binding efficiency of HA-1 and HA-2 adaptors observed in our assay most likely represent physiological differences in the context of the membrane anchor or lipid bilayer. Moreover, binding of adaptors may be favored by clustering or oligomerization of tail peptides, as hypothesized by Beltzer and Spiess (1991) for asialoglycoprotein receptor. In fact, LAP has been shown to form dimers (Gieselmann et al., 1984), which might result in an ordered structure of the cytoplasmic domain that is achieved only partly by the synthetic tail peptide.

Specific Binding of Plasma Membrane Adaptors to the Tail Peptide of LAP—Modified peptides were used to study the signals required for binding of plasma membrane adaptors to the cytoplasmic tail of LAP. A modified tail peptide with the essential tyrosine replaced by alanine (LAP-A8) bound to plasma membrane adaptors with low efficiency, which is in agreement with the 20-fold lower internalization rate of the Tyr → Ala mutant in overexpressing cells (Lehmann et al., 1992). The shortened peptide (LAP-Δ7) represents the minimal length required for efficient endocytosis of LAP in overexpressing cells (Lehmann et al., 1992). When immobilized on Affi-Gel 10, LAP-Δ7 bound HA-2 adaptors with similar efficiency as LAP-wt peptide.

Surprisingly, a modified peptide with tyrosine replaced by the aromatic amino acid phenylalanine bound plasma membrane adaptors with similar efficiency as LAP-wt, whereas the Tyr → Phe mutant is internalized 3.5 times more slowly than wild-type LAP in overexpressing cells. Two observations may resolve this apparent contradiction. First, two-dimensional NMR analysis of the synthetic peptides used in this study has shown that substitution of the tyrosine residue by phenylalanine reduces the tendency of the peptide to form a β-turn by 25% only, whereas substitution by alanine results in 50% reduced tendency to form a β-turn (Lehmann et al., 1992). The peptide LAP-F8 is therefore expected to possess a higher affinity for binding of adaptors than the peptide LAP-A8. Reduction of the tendency to form a β-turn by 25% obviously is tolerable for binding of adaptors in vitro to LAP-F8. In contrast, peptide LAP-A8, which has a 50% reduced tendency to form a β-turn, showed only residual binding of HA-2 adaptors, which was difficult to discriminate from unspecific binding. In situ, however, small differences in the affinity for binding adaptors may be critical, since the cytoplasmic domain of LAP must compete with a large number of receptor molecules that are destined to enter clathrin-coated pits with high affinity. Another explanation for the different effect of the Tyr → Phe substitution in vitro and in overexpressing cells may be the presence of adaptors for the in vitro assay. In two different types of overexpressing cells (baby hamster kidney cells, BHK, and Madin-Darby canine kidney cells, MDCK), internalization of LAP tolerates different mutations in the cytoplasmic domain (Prill et al., 1993). This result points to species or cell type-specific differences in the affinity for adaptors to tail peptides. Binding of bovine plasma membrane adaptors to the Tyr → Phe mutant suggests that adaptors from bovine brain have a higher affinity for a phenylalanine-containing peptide than adaptors from BHK or MDCK cells. Phenylalanine can indeed substitute for tyrosine in the internalization signal, as has been shown for the transferrin receptor, low density lipoprotein receptor, and MPR 300, suggesting that an aromatic residue rather than a specific tyrosine constitutes part of the signal for binding of
plasma membrane adaptors (McGraw and Maxfield, 1990; Davis et al., 1987; Canfield et al., 1991).

The binding site of HA-2 adaptors for the tail peptide of LAP was shown to be located in the trunk portion of the molecule, which is known to contain also the binding site for clathrin (Schröder and Ungewickell, 1991). The head portion did not bind to the tail peptide of LAP. This result is in agreement with a recent report of Beltzer and Spiess (1991) about the interaction of asialoglycoprotein receptor with the trunk of β-adaptin. Thus, there is increasing evidence against the view that the head portion mightaccommodate binding of a large number of receptor tails, since its amino acid sequence displays higher variability than that of the trunk portion (Kirchhausen et al., 1989; Ponnambalam et al., 1990).

Low Efficiency Binding of Golgi Adaptors to the Tail Peptide of LAP—In contrast to the highly efficient binding of HA-2 adaptors to LAP-wt peptide, HA-1 adaptors bound poorly to the same peptide, if at all. In 2 out of 12 experiments, 30–35% of HA-1 adaptors bound to the LAP-wt peptide, whereas, usually, binding was less than 10% of loaded material. Elevated binding of HA-1 adaptors to LAP-wt peptide was always correlated with quantitative binding of HA-2 adaptors, suggesting that these adaptor preparations were highly active for binding to tail peptides. Binding of HA-1 adaptors to LAP-wt peptide seemed to be slightly higher than binding of HA-1 adaptors to LAP-F8 or LAP-A5, but, due to the weak signal, it was difficult to decide whether the difference was significant.

In contrast, the cytoplasmic domain of MPR 46 bound both plasma membrane and Golgi adaptors with similar efficiency. These data are in agreement with the sorting function of MPR 46, which delivers newly synthesized lysosomal enzymes to the endosomal-prelysosomal compartment, and immunocytochemical studies showing that MPR 46 is present in clathrin-coated vesicles at the TGN and the plasma membrane. (Klumperman et al., 1993). In contrast, the fast transport of newly synthesized LAP to the cell surface had suggested that LAP is transported directly from the TGN to the plasma membrane by the constitutive pathway (Braun et al., 1989). The low efficiency binding of Golgi adaptors to the tail peptide of LAP supports the view that newly synthesized LAP will be sorted into coated vesicles of the TGN much less efficiently than mannose 6-phosphate receptors. Since mannose 6-phosphate receptors will compete for binding of Golgi adaptors in situ, it is reasonable to assume that most, if not all, of the newly synthesized LAP will leave the TGN via the constitutive pathway.

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