# Lysosomal acid phosphatase is internalized via clathrin-coated pits

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**The presence of lysosomal acid phosphatase (LAP) in coated pits at the plasma membrane was investigated by immunocytochemistry in thymidine kinase negative mouse L-cells (Ltk$^-$) and baby hamster kidney (BHK) cells overexpressing human LAP (Ltk-LAP and BHK-LAP cells). Double immunogold labeling showed that at various stages of invaginating coated pits LAP colocalized with clathrin and plasma membrane adaptors (HA-2 adaptors). Quantitation of the immunogold label showed similar density of wild-type LAP in coated over non-coated areas of the plasma membrane, whereas an internalization-deficient, truncated mutant of LAP which lacks the cytoplasmic tail was less efficiently included into coated pits. Internalization of anti-LAP antibodies into endosomal vesicles was accompanied by rapid dissociation of the coat proteins as shown by an immunofluorescence assay. The role of clathrin-coated vesicles in internalization of LAP was further corroborated by microinjecting monoclonal antibodies against clathrin or HA-2 adaptors into BHK-LAP cells. Internalization of LAP as detected by an immunofluorescence assay was transiently blocked by microinjected antibodies against clathrin or HA-2 adaptors, whereas unrelated antibodies did not affect internalization. These data suggest that LAP is included into clathrin-coated pits of the plasma membrane for rapid internalization.**

*Abbreviations.* BHK cells Baby hamster kidney cells. — EGF Epidermal growth factor. — ER Endoplasmic reticulum. — FITC Fluorescein isothiocyanate. — HA Hydroxyapatite. — IGF Insulin-like growth factor. — LAP Lysosomal acid phosphatase. — LDL Low density lipoprotein. — Ltk$^-$ Thymidine kinase negative mouse L-cells. — MEM Eagle's minimum essential medium. — PBS Phosphate-buffered saline.

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## Introduction

Lysosomal acid phosphatase (LAP) is synthesized as an integral membrane protein with a single membrane-spanning domain and a short cytosolic tail of 19 amino acids [35]. Its transport to the lysosome is independent of the mannose 6-phosphate transport signal and involves passage of endoplasmic reticulum (ER), Golgi and plasma membrane followed by internalization. From a pool that rapidly recycles between endosomes and the plasma membrane, LAP is transported to lysosomes, where it is proteolytically processed to the mature soluble form [4, 17, 18]. A truncated form of LAP without cytosolic tail, or a mutant in which the tyrosine residue of the cytosolic tail has been replaced by phenylalanine, are internalized with low efficiency and consequently accumulate at the plasma membrane. Transport of these mutants to lysosomes takes places only slowly [29].

The tyrosine-containing signal for efficient internalization of LAP resembles the signal for endocytosis of receptors via clathrin-coated pits. For instance, a tyrosine residue in the cytosolic tail as signal for efficient endocytosis has been found in low density lipoprotein (LDL) receptor [11], mannose 6-phosphate/insulin-like growth factor II (M6P-IGFII) receptor [23], polyIg receptor [5] and transferrin receptor [1, 20, 25]. Influenza virus hemagglutinin, a protein that is normally excluded from coated pits at the plasma membrane, was converted to a mutant form that is rapidly endocytosed and recycles between endosomes and plasma membrane, by replacement of a cysteine residue in the cytosolic tail with a tyrosine [22].

The tyrosine-containing signal of endocytic receptors is thought to be directly involved in the assembly of clathrin-coated pits at the plasma membrane by binding to an oligomeric complex of cytosolic proteins called plasma membrane adaptors or HA-2 adaptors. These proteins are proposed to interconnect clathrin molecules of the coat to the cytosolic tail of membrane receptors (reviewed in [28]). The cytosolic tail of the M6P-IGFII receptor has been shown to

 

bind to HA-2 adaptors, and binding was abolished by mutation of the two tyrosines in the cytosolic tail [16]. Furthermore, binding of HA-2 adaptors to the cytosolic tail of the LDL receptor was competed by a peptide corresponding to the mutated, tyrosine-containing cytosolic tail of influenza virus hemagglutinin, whereas a peptide corresponding to wild-type hemagglutinin did not compete [27].

Requirement of a tyrosine residue in the cytosolic tail of LAP for its efficient internalization [29] suggested that LAP is internalized via clathrin-coated pits. When we investigated the presence of LAP in coated pits of the plasma membrane by double-labeling immunocytochemistry, we found that LAP was only partly colocalized with HA-2 adaptors in clathrin-coated pits at the plasma membrane. We did not observe an enrichment of immunogold-labeled LAP in coated pits as was expected in view of the tyrosine-containing internalization signal. We therefore examined the role of clathrin and HA-2 adaptors for internalization of LAP by a functional assay that was based on microinjection of monoclonal antibodies against clathrin and α-adaptin.

## Materials and methods

### *Antibodies and cell lines*

Monoclonal antibodies against clathrin heavy chain (X19 and X22) and the α-adaptin were as described [8, 10]. To localize clathrin in immunoelectron microscopy, a polyclonal antibody against the light chain was used which was kindly provided by Dr. E. Ungewickell, Max-Planck-Institut für Biochemie, Martinsried/Germany. Monoclonal antibodies against the leukocyte cell surface antigen Leu10 [9] and the lysosomal enzyme arylsulfatase A (Sommerlade and Hille, unpublished) were used for control injections. All injected antibodies were mouse IgG1. Polyclonal antibodies against LAP were purified from rabbit serum [15] by protein A-Sepharose chromatography according to the manufacturers advice (Pharmacia, Freiburg/Germany). $F_{ab}$ fragments were prepared by digestion with papain (Sigma, Deisenhofen/Germany) as described [24] except that the reducing agent was 5 mM cysteine. $F_c$- and $F_{ab}$ fragments were separated by protein A-Sepharose chromatography. Fluorescein isothiocyanate (FITC)-conjugated anti-mouse Ig was from Janssen (Rüsselsheim/Germany), and Texas Red-conjugated anti-rabbit Ig was from Southern Biotechnology (Birmingham/UK).

Baby hamster kidney (BHK) cells which overexpress human LAP (BHK-LAP cells) were as described [35]. Thymidine kinase negative mouse L-cells which overexpress wild-type LAP (Ltk-LAP cells) or a tail-less mutant of LAP (Ltk-LAP-trc cells) were prepared as described [6] using the vectors published by Peters et al. [29]. According to tartrate-inhibitable enzyme activity, expression of human LAP was 70-fold (BHK-LAP cells), 45-fold (Ltk-LAP cells) or 120-fold (Ltk-LAP-trc cells) above the level of endogenous LAP in the parental cell line.

### *Immunogold labeling*

To immunolabel LAP and clathrin, nearly confluent 6-cm dishes of Ltk-LAP cells were fixed for 2 h at room temperature with 1% acrolein and 0.1% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4 and stored in phosphate buffered saline (PBS). To localize LAP and HA-2 adaptors, cells were fixed for 2 h at room temperature with 2% paraformaldehyde in PBS, and stored in PBS. Fixation with glutaraldehyde or acrolein completely abolished labeling for HA-2 adaptors. Cells were scraped off the dish and embedded in gelatin as described previously [33], without fixation of the gelatin [30]. Ultrathin cryosections of the prefixed cells were prepared, immunolabeled with protein A-gold, poststained with uranyl acetate and embedded in methylcellulose as described [33]. Double labeling was performed by sequential protein A-gold labeling, whereby a 1% glutaraldehyde intermediate fixation was used to prevent loss of the first antibody and cross-labeling artefacts [34]. The monoclonal antibody AP6 against α-adaptin was visualized with an intermediate rabbit anti-mouse IgG antibody, which was subsequently labeled by protein A-gold.

To determine the density of immunogold-labeled LAP at the plasma membrane, the grid in the microscope was moved according to a fixed pattern encountering cells in a random manner. Each time when a plasma membrane reached a mark at the electron microscope screen, the image was projected on a TV screen connected to a Jeol 1200 EX electron microscope. Screening and counting were performed at a final magnification of 20650× at the TV screen. As an arbitrary unit for measuring membrane length, we counted the amount of intersections of the plasma membrane with a line raster (width 2 cm) superimposed on the TV screen. Gold particles up to a distance of 30 nm from the membrane were taken into account. The density of the gold particles was expressed as number of gold particles per intersection [36]. To establish the average amount of label in coated pits, plasma membranes were screened and whenever a coated pit was encountered, the amount of gold particles present in the coated pit and the amount of intersections through the membrane were counted at the TV screen. To avoid differences in labeling intensity of coated membranes and non-coated parts of the plasma membrane due to potential differences in the efficiency of penetration of antibodies, only coated pits that were in clear continuity with the plasma membrane were considered. For the final calculation of the number of gold particles per intersection, also intersections through coated pits devoid of gold particles were included. All measurements were performed at 2 different grids. The final sample number (35) was determined by progressive mean analysis.

### *Microinjection*

Cells were grown on polylysine-coated coverslips for 18 to 24 h. During microinjection, the pH of the culture medium was stabilized by addition of 10 mM HEPES. Antibodies (X 19:24.6 mg/ml, X22: 10.7 mg/ml, AP6: 17.5 mg/ml, 20B1: 20 mg/ml, αLeu10: 20.2 mg/ml) were dialyzed against 10 mM Tris-HCl, 150 mM KCl, pH 7.4, and centrifuged for 30 min at 100000g immediately before use. Capillaries (Clark, Pangbourne, Reading/UK) were prepared with a Sachs Flaming puller PC-84 (Sutter Instruments, San Raphael, CA/USA), and microinjection was performed with the microinjector 5242 (Eppendorf, Hamburg/Germany). Previous studies suggest that molar equivalence or excess of monoclonal antibodies over the antigen was required to affect endocytosis [12]. Assuming an average content of clathrin and adaptors in transfected BHK cells, a 3- to 30-fold molar excess of antibody can be introduced by injecting 20 to 200 fl of a solution containing 20 mg/ml of antibody [10, 12].

After microinjection, cells were incubated for 1 to 4 h at 37 °C, 5% $CO_2$ before internalization assays were performed. Where indicated, FITC-bovine serum albumin (1 mg/ml) was used as coinjection marker.

### *Internalization of antibodies and immunofluorescence staining*

Internalization of anti-LAP antibodies was performed as described [29] with the following modifications. Cells were cooled on ice, washed two times with ice-cold PBS, incubated for 5 min with

0.2% gelatin in PBS followed by a 60-min incubation with anti-LAP antiserum diluted 1:150 in PBS, 0.2% gelatin. Cells were washed twice with ice-cold PBS and once with bicarbonate-free Eagle's minimum essential medium (MEM) containing 10 mM HEPES, pH 7.4 (referred to as MEM-HEPES). Cells were either kept on ice (control) or incubated for 2 to 20 min at 37 °C in MEM-HEPES (internalization). Internalization was stopped by washing cells twice with ice-cold PBS followed by addition of ice-cold 3% paraformaldehyde. After 40 min at room temperature, the formaldehyde was quenched by a 10-min incubation with 50 mM $NH_4Cl$ in PBS. Unless indicated otherwise, cells were permeabilized for 2×5 min with 0.5% Triton X-100 in PBS. Immunofluorescence staining was performed as described [35].

For staining of cell surface LAP on prefixed cells, cells were fixed and incubated with $NH_4Cl$ as above. Cells were washed three times with PBS and incubated for 5 min with PBS, 0.2% gelatin. Anti-LAP antiserum (diluted as above) was bound for 60 min at room temperature followed by three washes with PBS, 0.2% gelatin. Cells were then permeabilized and processed for immunofluorescence as above.

Mouse anti-clathrin (X22) and mouse anti-α-adaptin (AP6) were used at 10 to 20 μg/ml, and fluorochrome-labeled antibodies were diluted 1:100 to 1:200. All antibodies were bound for 1 h at room temperature. Coverslips were routinely embedded with Mowiol (Calbiochem, Frankfurt a. M./Germany) in PBS, pH 7.4, containing 2.5 mg/ml 3,4,5-trihydroxybenzoic acid propyl ester to stabilize fluorescence of Texas Red. Samples shown in Figure 3 were embedded with Mowiol containing 100 mg/ml 1,4-diazabicyclo[2.2.2]octane to stabilize fluorescence of FITC. Immunofluorescence was visualized either by an inverted fluorescence microscope (IM35; Zeiss, Oberkochen/Germany) or a confocal laser scanning microscope (Zeiss). Excitation of fluorescence was done with an argon laser (488 nm) for FITC, or a helium-neon laser (543 nm) for Texas Red. Barrier filters for selective detection of fluorochromes were BP 515-565 for FITC and LP 590 for Texas Red. Photographs were taken with Kodak TMAX film.

## Results

### *Immunocytochemical localization of LAP in coated pits at the plasma membrane*

To investigate whether LAP is included into coated pits at the plasma membrane, cryosections of Ltk cells which overexpress human LAP (Ltk-LAP cells) were immunolabeled for LAP and analyzed by electron microscopy. Immunogold-labeled LAP was found at the plasma membrane, in a variety of vesicles of the endocytic pathway, and in lysosomes. Figure 1 shows the presence of LAP in coated areas of the plasma membrane and in different stages of invaginating coated vesicles. The electron-dense coat of these structures was shown to contain clathrin by labeling with a polyclonal antibody directed against clathrin light chain (Fig. 1, *left*). HA-2 adaptors were also found in the coated membranes together with LAP, as shown by labeling with a monoclonal antibody against α-adaptin, the 100 kDa α-subunit of the HA-2 adaptor complex (Fig. 1, *right*).

To compare the abundance of LAP at the plasma membrane and in coated pits of the plasma membrane, cryosections of Ltk-LAP cells were stained for LAP, and the number of gold particles was counted in at random sampled membranes and divided by the number of intersections in a superimposed line raster. Twenty-four percent of the coated membranes, which were identified by their electron-dense coat, were found to contain label for LAP. The average density of LAP was similar in coated and non-coated areas of the plasma membrane of Ltk-LAP cells (Tab. I).

**Tab. I.** Density of immunolabeled LAP in coated and non-coated areas of the plasma membrane.

| Cell line | Coated pits labeled for LAP | Number of gold particles per intersection | | Enrichment of LAP in coated pits |
|---|---|---|---|---|
| | | CP | PM | CP/PM |
| | (%) | (arbitrary units) | | |
| Ltk-LAP | 24 | 0.21 | 0.24 | 0.88 |
| Ltk-LAP-trc | 56 | 0.67 | 1.36 | 0.49 |

Ltk-LAP cells were fixed by successive 1-h incubations with 1% and 2% paraformaldehyde. Cryosections were labeled with anti-LAP Ig and protein A-gold. Gold particles were counted at the plasma membrane (PM) and in coated pits (CP) which were in clear continuity with the plasma membrane and that were identified by their electron-dense coat. The length of membranes was determined using intersections with a superimposed grid as arbitrary units (for details see Materials and methods).

To investigate whether the distribution of LAP at the plasma membrane depends on a signal contained within its cytoplasmic tail, we investigated Ltk cells overexpressing a truncated mutant of LAP that lacks the cytoplasmic tail (Ltk-LAP-trc cells). Due to a high level of expression and the low rate of endocytosis [29], the tail-less mutant accumulated at the cell surface resulting in a 5.7-fold higher density at the plasma membrane, when compared with Ltk cells overexpressing wild-type LAP (Tab. I). The average density of the tail-less mutant in coated areas of the plasma membrane was two-fold lower than in non-coated areas. Thus, despite the high density at the plasma membrane, the tail-less mutant seems to be partly excluded from coated pits.

The immunocytochemical colocalization of LAP with clathrin and HA-2 adaptors is compatible with internalization of LAP via clathrin-coated pits. Partial exclusion of the tail-less mutant from coated pits supports the view that the cytoplasmic tail of LAP contains a signal that favors inclusion into coated pits. However, since immunogold-labeled LAP was not enriched in coated areas of the plasma membrane, internalization by an alternative, clathrin-independent pathway could not be ruled out. We therefore sought to obtain additional evidence for clathrin-mediated endocytosis by studying the effect of monoclonal antibodies against clathrin and HA-2 adaptors on internalization of LAP.

### *Immunofluorescence assay for internalization of LAP*

To study the effect of microinjected antibodies on internalization of LAP, we optimized a recently published immunofluorescence assay, which allows to monitor internalization of LAP at the level of individual cells [29]. Antibodies against LAP were bound at 4 °C to the surface of BHK cells overexpressing LAP (BHK-LAP cells), cells were recultured at 37 °C for internalization, and anti-LAP Ig was detected by immunofluorescence (Fig. 2). To discriminate cell surface and endosomal staining, non-permeabilized

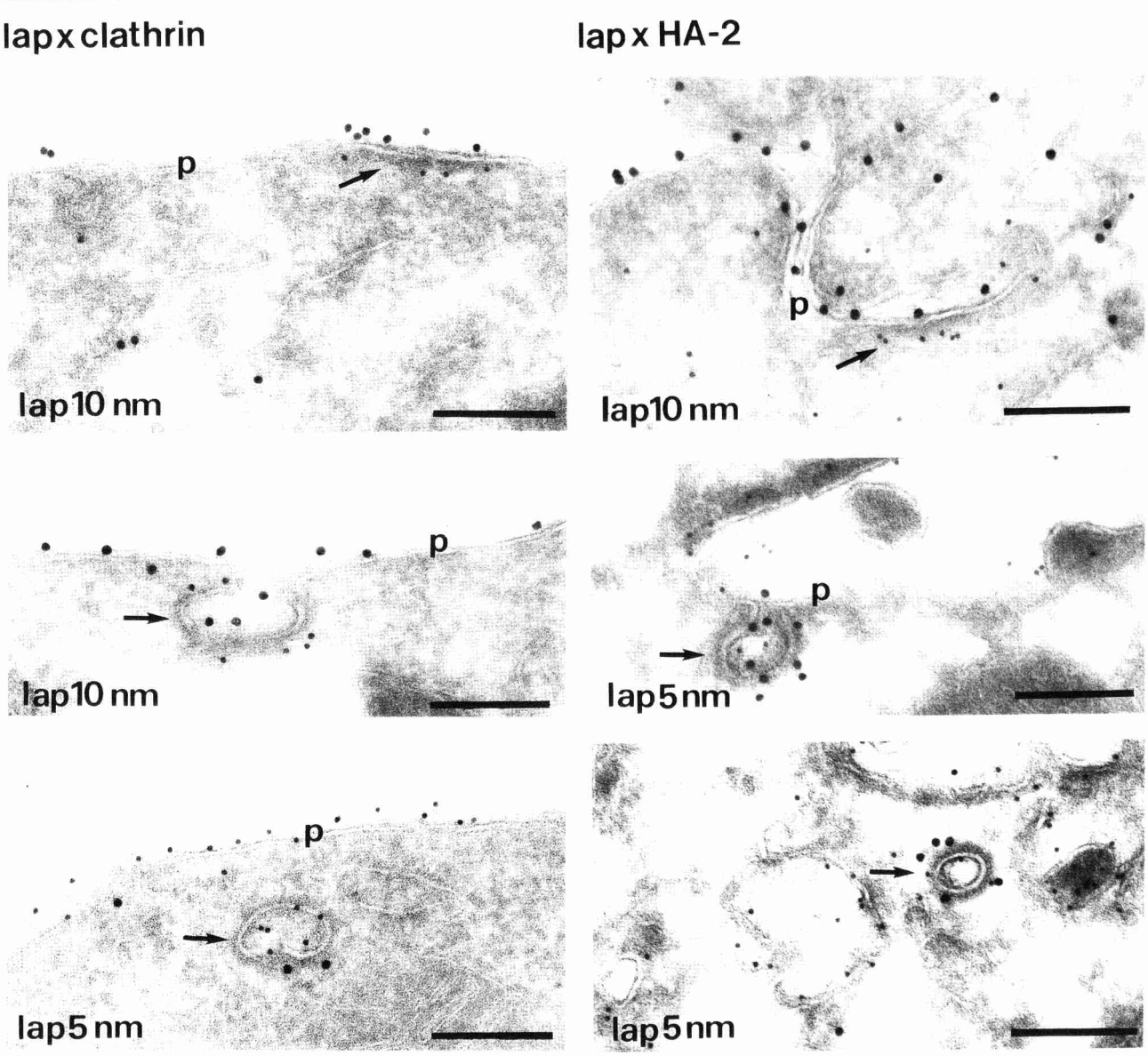


**Fig. 1.** Colocalization of LAP with clathrin and α-adaptin (HA-2) in coated pits of the plasma membrane and coated vesicles examined by immunogold labeling. Cells were fixed with acrolein and glutaraldehyde for staining of clathrin (*left*), and with formaldehyde for staining of α-adaptin (*right*). For double labeling of cryosections, gold particles of 5 and 10 nm were used. The size of the colloidal gold representing LAP is indicated in the left corner of each panel. *Arrows* indicate the association of LAP with clathrin or α-adaptin at different stages of coated vesicle formation. — p Plasma membrane. — Bar 200 nm.

cells (Figs. 2b, d, f) were compared with cells that had been permeabilized by detergent (Figs. 2a, c, e). After binding of anti-LAP Ig at 4 °C, a diffuse immunolabeling was observed, together with fine dots scattered all over the cell surface (Figs. 2a, b). As expected, the pattern at the cell surface was similar for permeabilized and non-permeabilized cells. When cells were recultured at 37 °C for 2 or 5 min, anti-LAP Ig was included into large vesicular structures, which were detectable only after permeabilization of cells (Figs. 2c, e) and therefore are assumed to be intracellular and represent endosomes. Left-over surface staining was visible only with non-permeabilized cells (Figs. 2d, f), suggesting that in permeabilized cells the amount of surface-bound anti-LAP is reduced during the immunofluorescence protocol. Thus, the immunofluorescence assay most likely overestimates the rate of internalization due to selective loss of surface-bound anti-LAP Ig. Nevertheless, it provides a clearcut discrimination between surface-bound and internalized antibodies on the basis of their immunofluorescence patterns.

The dotted pattern of surface-bound anti-LAP Ig obtained by immunofluorescence in BHK-LAP cells (Figs. 2a,

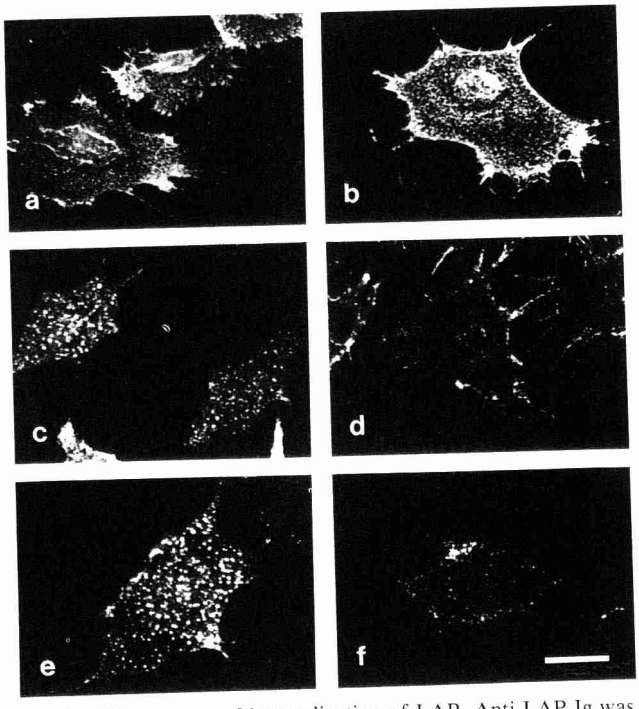


**Fig. 2.** Time course of internalization of LAP. Anti-LAP Ig was bound to the cell surface of BHK-LAP cells at 4 °C (**a, b**) followed by internalization of the antibodies at 37 °C for 2 min (**c, d**) or 5 min (**e, f**). Cells were fixed and anti-LAP Ig was detected by staining with Texas Red-conjugated anti-rabbit Ig. **a, c, e.** Permeabilized cells showing total anti-LAP Ig. — **b, d, f.** Non-permeabilized cells showing surface-bound anti-LAP Ig. — Bar 20 μm.

b) and also in Ltk-LAP cells (not shown) was in contrast to the uniform surface distribution shown above by immunogold labeling of prefixed cryosections (Fig. 1). Since a dotted pattern in immunofluorescence was also observed when cells had been fixed before binding of anti-LAP Ig (not shown), it is not likely to represent a mere artifact of antibody-induced clustering. To find out whether the dotted immunofluorescence pattern of LAP at the cell surface indicates coated pit localization, we combined the internalization assay with immunofluorescence staining for clathrin or α-adaptin. Anti-LAP antibodies that had been bound to the cell surface either at 4 °C or after fixation of cells, partly colocalized with clathrin (not shown) or α-adaptin (Figs. 3a, c). A reliable analysis of the colocalization with clathrin was hampered due to the strong perinuclear staining of clathrin, which represents coated vesicles of the trans Golgi network. The monoclonal antibody against α-adaptin exclusively stained small dot-like structures, which by analysis with confocal laser scanning microscopy, were shown to be located at or near to the plasma membrane (Figs. 3c, d). At the dorsal side of the cell, which is exposed to the culture medium and is accessible to anti-LAP Ig, 77% of α-adaptin-containing coated pits were positive for LAP, when anti-LAP antibodies were bound to the surface of fixed cells. When anti-LAP antibodies were bound to the surface of cells at 4 °C before fixation, this value increased to 89%. When internalization of anti-LAP antibodies was allowed for 5 min at 37 °C, endosomal vesicles labeled for LAP did not contain detectable amounts of anti-α-adaptin (Figs. 3b, d), indicating rapid dissociation of the clathrin coat from endocytic vesicles.

To analyze internalization of LAP by immunocytochemistry, antibodies must be bound to the cell surface at 4 °C. Under these conditions, divalent antibodies may induce clustering of cell surface antigens. It was therefore necessary to control whether internalization of divalent anti-LAP-Ig is representative for the rate of internalization of LAP that is not tagged by antibodies. As will be described in detail elsewhere (L. Lehmann, W. Eberle, S. Krull, V. Prill, B. Schmidt, C. Sander, K. v. Figura, C. Peters, submitted), the rate of internalization of anti LAP-Ig was quantitated by an anti-LAP-Ig/[$^{125}$I]protein A internalization assay and compared to the rate of internalization of LAP that was biotinylated at the cell surface. In the anti-LAP-Ig/[$^{125}$I]protein A internalization assay, on average 55% of cell surface LAP was internalized within 5 min. In the biotinylation assay, which avoids tagging of LAP by antibodies, 70% of cell surface LAP was internalized within 5 min. These data suggest that internalization of anti-LAP-Ig occurs with a similar rate as internalization of untagged LAP. The immunofluorescence assay therefore allows to compare the relative rates of internalization in cells microinjected with various antibodies.

### *Inhibition of clathrin-mediated endocytosis by microinjection of antibodies against clathrin or HA-2 adaptors*

To provide additional evidence for clathrin-mediated internalization of LAP by a functional assay, we injected monoclonal antibodies against clathrin heavy chain and α-adaptin, which inhibit clathrin-mediated endocytosis [10,12].

A monoclonal antibody, X19, which recognizes clathrin heavy chain and prevents assembly of clathrin baskets in vitro [3], was microinjected into BHK-LAP cells. The injected antibodies were allowed to bind to the intracellular antigen for 1 h before anti-LAP Ig was bound to the cell surface at 4 °C. Bound anti-LAP Ig was detected by immunofluorescence using Texas Red-conjugated anti-rabbit Ig (Figs. 4a–c, *left*) and microinjected cells were identified by FITC-conjugated anti-mouse Ig (*right panels*). The pattern for anti-LAP Ig bound to the cell surface of injected cells apparently did not differ from the pattern of anti-LAP Ig bound to the cell surface of non-injected cells (Fig. 4a). After reculturing the cells at 37 °C for 2 min, the pattern of anti-LAP Ig bound to most of the microinjected cells still resembled cell surface staining, whereas in non-injected cells the anti-LAP Ig had been internalized into endosomes (Fig. 4b). After 5 min at 37 °C, microinjected cells were found to contain endosome-like structures positive for anti-LAP Ig (Fig. 4c). At this time point, the pattern of anti-LAP Ig was similar for microinjected and control cells except for residual surface staining of microinjected cells. The latter was evident especially with non-permeabilized cells (not shown). To demonstrate that the endosome-like structures of microinjected cells represent indeed internal-

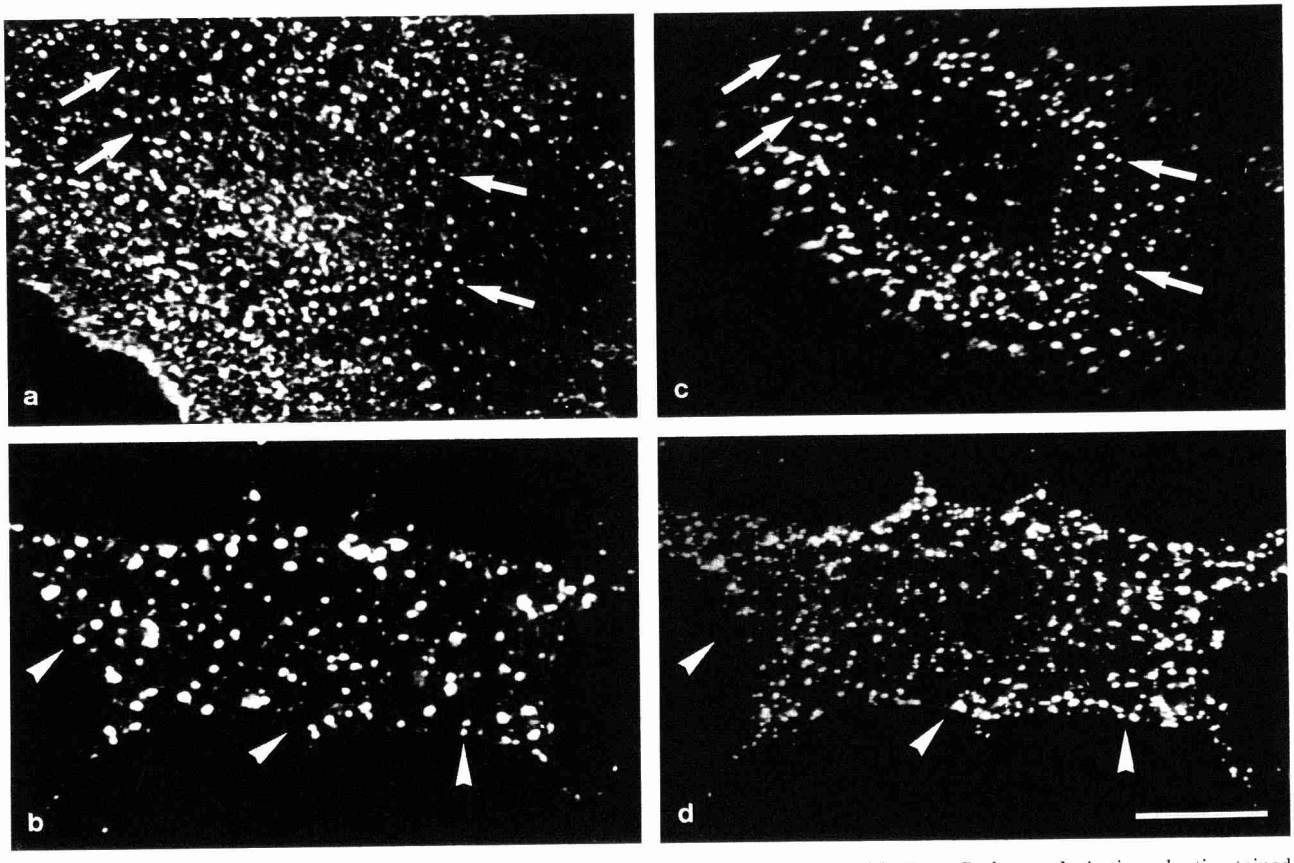


**Fig. 3.** Double immunofluorescence labeling of surface-bound or internalized LAP antibodies and α-adaptin (HA-2). Anti-LAP Ig from rabbit was bound to BHK-LAP cells at 4 °C and cells were either fixed immediately (**a, c**) or warmed to 37 °C for 5 min and then fixed (**b, d**). After permeabilization of the cells, α-adaptin was stained with the mouse monoclonal antibody AP6. — **a, b.** Anti-LAP stained with Texas Red. — **c, d.** Anti-α-adaptin stained with FITC. Shown is an overlay of confocal optical sections (0.8 μm steps) through the dorsal part of the cell, which is accessible to anti-LAP Ig. *Arrows* in (**a**) and (**c**) indicate structures stained for LAP and α-adaptin; *arrowheads* in (**b**) and (**d**) indicate identical positions that are labeled only for LAP or α-adaptin. — Bar 10 μm.

ized LAP rather than LAP that had been clustered at the cell surface, immunofluorescence was performed with and without permeabilization. Regardless whether the cells had been injected or not, endosome-like structures were observed only after permeabilization of the cells indicating true internalization as shown for the 20 min time point in Figure 5. Microinjection of a second monoclonal antibody which is also directed against clathrin heavy chain, X22, demonstrated essentially the same inhibition of endocytosis (not shown).

To rule out nonspecific damage of cells by the microinjection itself, we injected control antibodies directed against the leukocyte surface antigen Leu10 or the lysosomal enzyme arylsulfatase A, which were of the same IgG subclass as the anti-clathrin antibodies (IgG1). Neither of these antibodies altered the pattern of internalized anti-LAP Ig as shown for the 2 min internalization time point after injection of anti-arylsulfatase A Ig (Fig. 4e).

The monoclonal antibody against α-adaptin, AP6, when microinjected into BHK-LAP cells also delayed the internalization of LAP from the cell surface, similar to the results shown above for microinjection of anti-clathrin X19. In cells injected with AP6 anti-α-adaptin, the internalization assay revealed cell surface staining of LAP for up to 2 min after warming cells to 37 °C (Fig. 4d). After 5 min at 37 °C, cells injected with anti-α-adaptin had internalized anti-LAP antibodies into endosome-like structures, but in comparison to control cells confocal laser scanning microscopy revealed that these endosomes were located more towards the periphery of the cells (not shown). These results suggest that microinjection of antibodies directed against clathrin heavy chain or α-adaptin specifically delay, but do not abolish, the internalization of LAP from the cell surface.

For a semiquantitative evaluation of the results, the pattern of anti-LAP Ig in individual microinjected cells was inspected in the fluorescence microscope to discriminate cells that had internalized anti-LAP Ig from those that showed cell surface staining. Cells were divided into two classes defined by the presence or absence of clusters of anti-LAP Ig that indicate internalization as shown above (compare Fig. 2). Only cells that showed a clearcut cell sur-

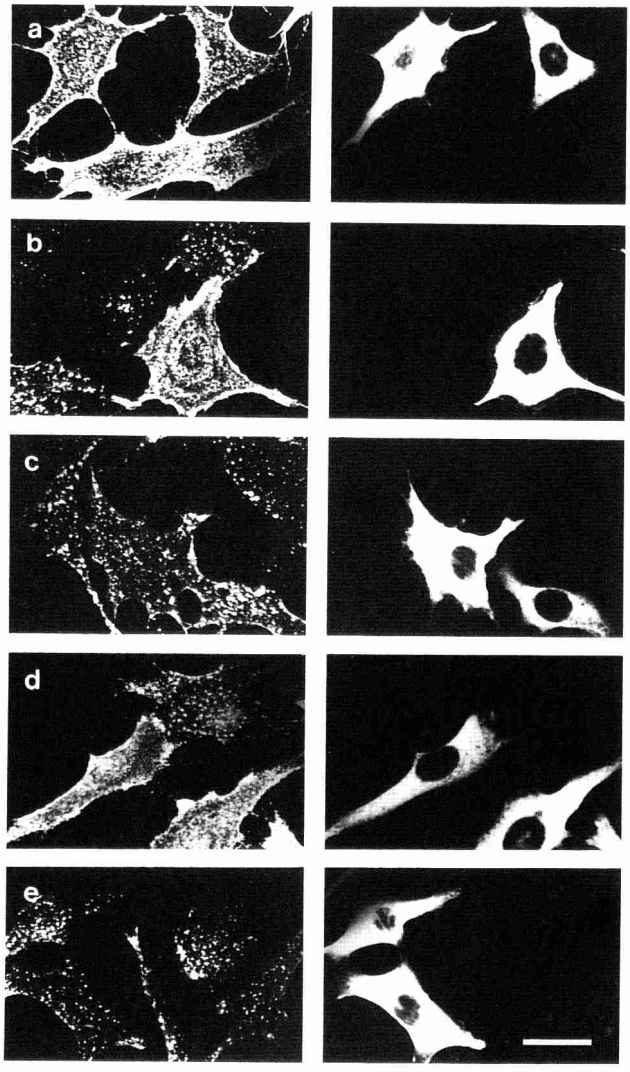


**Fig. 4.** Microinjection of antibodies against clathrin or α-adaptin causes a delay in internalization of LAP. Antibodies against clathrin (X19, **a–c**), α-adaptin (AP6, **d**) or control antibodies (20B1, **e**) were injected into BHK-LAP cells followed by a 1-h incubation at 37 °C. After binding of anti-LAP Ig to the cell surface at 4 °C, cells were either fixed immediately (**a**), or internalization was allowed for 2 min (**b, d, e**) or 5 min (**c**) at 37 °C. After fixation and permeabilization, Texas Red-conjugated anti-rabbit Ig was used to detect LAP (*left*), and FITC-conjugated anti-mouse Ig was used to detect the injected mouse antibody (*right*). An overlay of confocal optical sections (1 μm steps) through the whole depth of the cells is shown. — Bar 20 μm.

face staining (compare Figs. 2a, b) were counted as internalization negative. Cells that showed a mixture of clustered anti-LAP staining and residual cell surface staining were defined as internalization positive. The time course of the appearance of internalization-positive cells was followed in cells that had been injected 1 h before the internalization assay (Fig. 6a). When cells were processed for immunofluorescence immediately after binding of anti-LAP

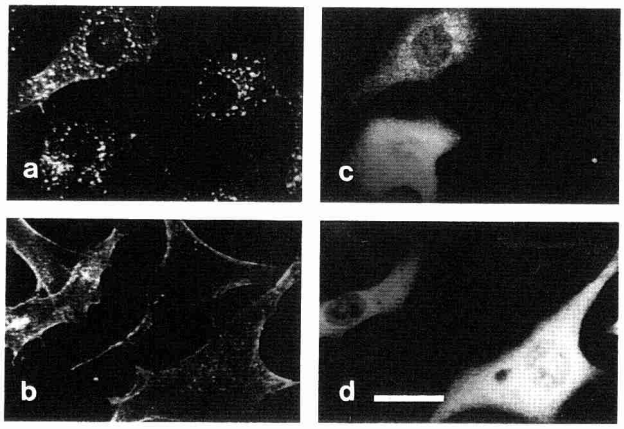


**Fig. 5.** Clustering of anti-LAP antibodies in microinjected cells reflects internalization into intracellular membranes. Microinjection of anti-clathrin IgG (X19) and FITC-BSA (**c, d**) as coinjection marker, and a 20-min internalization of anti-LAP Ig (**a, b**) were performed with BHK-LAP cells as in Figure 4. Immunofluorescence was performed with (**a, c**) and without (**b, d**) permeabilization of cells. An overlay of confocal optical sections (1 μm steps) through the whole depth of the cells is shown. — Bar 20 μm.

Ig to the cell surface at 4 °C, 72 to 85% of all cells showed the typical cell surface pattern. Fifteen to 28% of the cells showed clusters of anti-LAP Ig, which represents the background of aggregated LAP at the cell surface, but which cannot be discriminated from endosomes in the immunofluorescence of permeabilized cells. After 2 min reculturing at 37 °C, more than 95% of non-injected control cells or cells injected with control antibodies contained anti-LAP Ig in clusters that represent endosomes. After microinjection of antibodies recognizing clathrin heavy chain or α-adaptin, the percentage of cells with clustered anti-LAP Ig at the 2-min internalization was only 30 to 57%. When internalization was allowed for 5 min, the percentage of cells containing clustered anti-LAP Ig was similar for microinjected and control cells (95–98% of all cells counted). It should be noted, however, that the pattern of anti-LAP Ig in microinjected cells at this time point shows a mixture of cell surface and endosomal staining, indicating that endocytosis was not complete after 5 min in these cells (see above). The extent to which transport was blocked differed among individual microinjected cells. A similar heterogeneity in the response to microinjected antibodies against coat proteins has been observed earlier [10] and may be explained partly by variations in the amount of injected antibody. Alternatively, the susceptibility of individual cells to microinjected antibodies may vary, for example due to cell cycle effects.

In another set of experiments, microinjected antibodies were allowed to bind to their intracellular antigen for up to 3 h, and internalization was allowed for 2 min at 37 °C. When X19 anti-clathrin or AP6 anti-α-adaptin were injected, the maximal effect on internalization of anti-LAP Ig was reached at about 2 h after microinjection (Fig. 6b). The percentage of cells containing clustered anti-LAP Ig

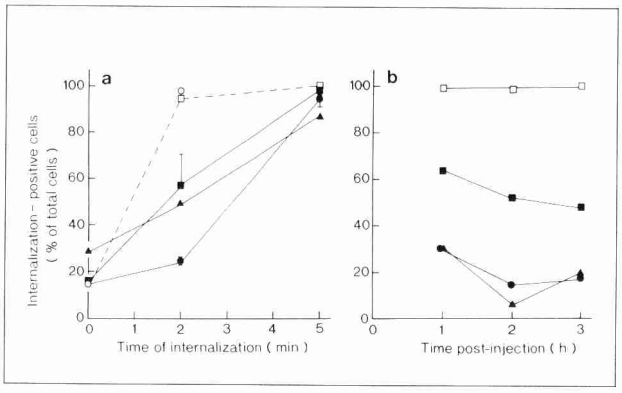


**Fig. 6.** Semiquantitative analysis of the inhibitory effect of injected antibodies on internalization of LAP. Microinjection of antibodies, binding and internalization of anti-LAP Ig, and immunofluorescence staining were performed as in Figure 4. The pattern of anti-LAP Ig was inspected by conventional fluorescence microscopy. Between 52 and 248 cells (average 127) were evaluated for each time point. Cells were divided into two classes defined by the presence or absence of clustered Ig that indicates onset of internalization. The percentage of internalization-positive cells is shown. —**a**. After microinjection, cells were incubated for 1 h at 37 °C followed by internalization of surface-bound anti-LAP Ig for up to 5 min at 37 °C. —**b**. After microinjection cells were incubated at 37 °C for 1 to 3 h followed by a 2-min internalization of surface-bound anti-LAP Ig. —●: X19 anti-clathrin; ■: X22 anti-clathrin; ▲: AP6 anti-α-adaptin; □: 20B1 anti-arylsulfatase A; ○: non-injected cells. Where indicated, bars give the deviation of duplicate determinations.

was reduced to 6 to 19%, corresponding to the background of clustering observed after binding of anti-LAP Ig at 4 °C. This result indicates that the inhibitory effect on anti-LAP internalization was complete after about 2 h. After microinjection of X22 anti-clathrin, the percentage of cells containing clustered LAP showed a less pronounced decrease from 64% to 48% within the 3 h post-microinjection period. When cells were injected with the control antibody that is specific for lysosomal arylsulfatase A, no inhibition of endocytosis was observed throughout the 3 h post-microinjection period. Besides the inhibitory effect on internalization, microinjected cells did not reveal apparent morphological changes for at least 4 h after microinjection.

The results of the microinjection experiments support the conclusion that rapid endocytosis of LAP occurs via clathrin-coated vesicles. The transient inhibition of internalization by antibodies against α-adaptin suggests that HA-2 adaptors are an essential component for assembly of these vesicles.

## Discussion

The role of clathrin-coated vesicles for receptor-mediated endocytosis has been studied in great detail. All receptors that are known to be internalized via clathrin-coated vesicles are transmembrane proteins and contain, in their cytosolic tail, a tyrosine residue, which is critical for efficient internalization (for review, see [28]). Only recently, an alternative pathway of endocytosis has been described for tetrahydrofolate receptor, which does not span the membrane, but is anchored in the membrane by glycophospholipid, and which is excluded from coated pits [31]. Signals that trigger inclusion of membrane proteins into endocytic vesicles of this clathrin-independent pathway have not been identified yet. So far, little is known about the mechanisms for internalization of membrane proteins other than receptors, e.g., lysosomal membrane proteins that reach the lysosome after passage of the plasma membrane. Internalization of LAP, which rapidly recycles between plasma membrane and endosomes, requires a tyrosine residue in its cytosolic tail [4, 29]. Analogous to the endocytosis signal of receptors, this tyrosine is preceded by turn-inducing amino acids and may be exposed in a tight turn [14]. These observations suggested that the tyrosine-containing turn might represent a signal for binding of HA-2 adaptors to the cytosolic tail of LAP, but so far, direct evidence that clathrin and HA-2 adaptors are involved in internalization of LAP was not available. Similar to LAP, the members of the lysosomal membrane glycoprotein family require a tyrosine in their cytosolic tail for efficient endocytosis [19, 38].

Direct evidence for binding of HA-2 adaptors to tyrosine-containing internalization signals is available for a limited number of receptor peptides [2, 16, 27]. Peptides containing tyrosine as internalization signal most likely share structural similarities [21], but do not reveal a strict consensus sequence for binding of HA-2 adaptors. Therefore, the role of tyrosine residues for binding of HA-2 adaptors to other tyrosine-containing receptors of membrane proteins need to be established. Moreover, recent reports that in polarized cells the critical tyrosine of the internalization signal exerts an additional function in basolateral sorting of some, but not all receptors [7, 19], raise the question whether tyrosine-containing signals may be heterogenous in their binding properties.

Using two independent experimental approaches, we now show internalization of LAP via clathrin-coated pits. First, by immunocytochemistry, we investigated colocalization of LAP with clathrin and α-adaptin at the plasma membrane. Second, antibodies against clathrin or α-adaptin were tested for their effect on rapid internalization of LAP.

### *Coated pit localization of LAP*

At the level of electron microscopy, LAP was found in various stages of electron-dense coated vesicles invaginating from the plasma membrane. In view of recent reports on non-clathrin-coated vesicles that are involved in intracellular trafficking of membranes [13, 26, 32], double immunogold labeling was performed for unequivocal identification of clathrin-coated pits. Our results show that LAP colocalized both with clathrin and HA-2 adaptors in coated pits and coated vesicles. According to immunoelectron microscopy, LAP was apparently not enriched in coated pits compared to non-coated parts of the plasma membrane, which is in contrast to most of the cell surface receptors investigated so far. On the other hand, a tail-less mutant of LAP

was found to occur less frequently in coated pits of the plasma membrane, suggesting that inclusion of LAP into coated pits is not a random event. That the tail-less mutant was not completely excluded from coated pits may be due to its high level of cell surface expression.

In contrast to the results obtained by immunoelectron microscopy, immunofluorescence double staining revealed a dot-like pattern of LAP which partly colocalized with clathrin and HA-2 adaptors. This result can be explained in either of two ways. Colocalization of LAP with clathrin and HA-2 adaptors could reflect enrichment of LAP in coated pits and the different pattern obtained by immunoelectron microscopy would result from an underestimation of the amount of LAP in coated pits due to steric hindrance or less efficient penetration of the immunogold label. Alternatively, interaction of LAP with other components of coated pits may result in more efficient fixation compared to diffusely distributed LAP, the latter being selectively lost during the washing steps of the immunofluorescence protocol. The second explanation may be supported by the observation that, using radioiodinated protein A to quantitate anti-LAP Ig under conditions of the immunofluorescence protocol, surface-bound LAP was more readily washed out than intracellular LAP. However, this method does not allow to discriminate LAP that is localized in coated pits or diffusely distributed in the plasma membrane.

In view of the rapid internalization of LAP from the plasma membrane of BHK-LAP cells [4], it was surprising to find immunogold labeling for LAP with almost the same density in coated pits and non-coated areas of the plasma membrane in Ltk-LAP cells. In contrast, most of the cell surface receptors have been described to accumulate in coated pits of the plasma membrane either spontaneously or after binding their ligands. This indicates that BHK-LAP cells need to rapidly incorporate a high percentage of their plasma membrane in order to achieve efficient clearance of LAP from the cell surface. In fact, as much as 5% of the plasma membrane was found to be coated with clathrin by electron microscopy (not shown). On the other hand, the similar density of immunogold labeling for LAP in coated and non-coated areas of the plasma membrane may reflect saturation of clathrin-dependent endocytosis due to the overexpression of LAP in the transfected cell line. That the endocytic system may indeed be saturable was concluded from studies on internalization of epidermal growth factor (EGF) receptor [37]. Clustering of EGF receptor in coated pits was shown to be independent of its ligand in human fibroblasts. In contrast, EGF receptor was found to be diffusely distributed in the plasma membrane of A431 cells, and clustering into coated pits at the plasma membrane of these cells was observed only in the presence of ligand. Wiley explained the different behavior of EGF receptors in the two cell lines by the high level of expression of EGF receptor in A431 cells, which may lead to saturation of a component required for clustering of receptors into coated pits [37]. So far, it was not possible to investigate the distribution of LAP at the surface of non-transfected cells because of their low level of endogenous LAP.

*Inhibition of clathrin-mediated internalization of LAP*

Due to the ambigous results concerning enrichment of LAP in clathrin-coated areas of the plasma membrane, we used an independent approach to corroborate the involvement of clathrin and HA-2 adaptors in internalization of LAP. Antibodies that are known to inhibit clathrin-mediated endocytosis were microinjected to investigate their inhibitory effect on internalization of LAP. Internalization of LAP was specifically blocked upon microinjection of these antibodies against clathrin or α-adaptin, but not upon microinjection of unrelated antibodies. The block was transient resulting in delayed internalization of LAP. One feasible explanation for this result is that the injected antibodies decrease the rate of formation of clathrin-coated pits or the rate of budding of clathrin-coated vesicles, either by steric hindrance or by disturbing the conformation of the coat proteins. Alternatively, clathrin-mediated endocytosis might be efficiently blocked by the injected antibodies; the internalization of LAP in injected cells would then reflect a slower, clathrin-independent pathway. The available methods do not allow discrimination of the two possibilities.

Taken together, we have presented the first direct evidence that clathrin and HA-2 adaptors are involved in internalization of a lysosomal membrane protein from the plasma membrane. Our results raise the question whether inclusion of LAP into clathrin-coated pits of the plasma membrane is regulated by direct interaction of its cytosolic tail with HA-2 adaptors, which mediates the assembly of the clathrin coat. Alternatively, LAP might associate with other protein components of clathrin-coated pits via as yet unknown interactions, the coat being assembled by binding of HA-2 adaptors to receptors that are concentrated in the same area of the membrane. As will be shown elsewhere, direct interaction of LAP with HA-2 adaptors has been obtained in vitro. An immobilized peptide that corresponds to the cytosolic tail of LAP was shown to bind HA-2 adaptors from a clathrin vesicle extract of bovine brain (Sosa et al., unpublished). Studying the molecular interactions of LAP and adaptor proteins will lead to a more detailed understanding of intracellular transport of lysosomal membrane proteins.

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