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Inhibition of the receptor-binding function of clathrin adaptor protein AP-2 by dominant-negative mutant μ 2 subunit and its effects on endocytosis

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Although interactions between the μ 2 subunit of the clathrin adaptor protein complex AP-2 and tyrosine-based internalization motifs have been implicated in the selective recruitment of cargo molecules into coated pits, the functional significance of this interaction for endocytosis of many types of membrane proteins remains unclear. To analyze the function of μ 2–receptor interactions, we constructed an epitope-tagged μ 2 that incorporates into AP-2 and is targeted to coated pits. Mutational analysis revealed that Asp176 and Trp421 of μ 2 are involved in the interaction with internalization motifs of TGN38 and epidermal growth factor (EGF) receptor. Inducible overexpression of mutant μ 2, in which these two residues were changed to alanines, resulted in metabolic replacement of endogenous μ 2 in AP-2 complexes and complete abrogation of AP-2 interaction with the tyrosine-based internalization motifs. As a consequence, endocytosis of the transferrin receptor was severely impaired. In contrast, internalization of the EGF receptor was not affected. These results demonstrate the potential usefulness of the dominant-interfering approach for functional analysis of the adaptor protein family, and indicate that clathrin-mediated endocytosis may proceed in both a μ 2-dependent and -independent manner.

Keywords: clathrin adaptor protein AP-2/EGF receptor/endocytosis/transferrin receptor

Introduction

Clathrin-mediated vesicle formation is an essential step in membrane recycling along the endocytic and secretory pathways. In mammalian cells, the plasma membrane clathrin-coated pits are responsible for the uptake of many macromolecules and viruses into the cell and for endocytosis of integral membrane proteins (reviewed in Schmid, 1997). Clathrin vesicles in the *trans*-Golgi network (TGN) serve to deliver newly synthesized proteins to endosomes and lysosomes via a receptor-mediated mechanism (reviewed in Traub and Kornfeld, 1997).

Clathrin coats are also found in endosomes and immature secretory granules, although the functional role of clathrin in these organelles is not defined (Tooze and Tooze, 1986; Stoorvogel *et al.*, 1996). The main structural components of plasma membrane-coated pits and vesicles are the clathrin triskelions and adaptor protein AP-2 complexes (Brodsky, 1988; Kirchhausen, 1993; Schmid, 1997). AP-2 is a heterotetramer consisting of two large (100–115 kDa) α - and β -subunits or adaptins, and one medium μ 2 (50 kDa) and one small (17 kDa) σ 2-subunit (Robinson, 1997). In clathrin-coated vesicles, AP-2 is located between the lipid bilayer and clathrin lattice, and presumably is anchoring clathrin to membrane. The precise mechanisms of specific targeting and docking of AP-2 to the plasma membrane are not understood. The α -adaptin appears to contain the major membrane-binding interface, although other subunits of AP-2 are also implicated in membrane binding (Robinson, 1993; Page and Robinson, 1995; Gaidarov *et al.*, 1996). The hinge domain of the β -subunit binds the clathrin heavy chain, providing a mechanism for membrane anchoring of the clathrin triskeleons via AP-2 and formation of the polyhedral lattices (Gallusser and Kirchhausen, 1993).

Apart from their structural role in coat assembly, APs are also implicated in the selective recognition and recruitment of cargo proteins into coated pits. Effective targeting to coated pits requires that receptors contain specific internalization signals, also known as endocytic codes, which function as address tags recognized by the endocytic machinery. These signals include tyrosine-based motifs with consensus sequence either NPXY or YXX Θ (where X stands for any amino acid and Θ for a bulky hydrophobic residue), di-leucine motifs and acidic clusters (reviewed in Kirchhausen *et al.*, 1997). AP-2 has been shown to bind polypeptides containing di-leucine (Glickman *et al.*, 1989), NPXY (Pearse, 1988) and YXX Θ motifs *in vitro* (Sosa *et al.*, 1993) as well as the YXX Θ motif in co-immunoprecipitation experiments (Nesterov *et al.*, 1995a; Sorkin *et al.*, 1996; Fire *et al.*, 1997; Vincent *et al.*, 1997; Zhang and Allison, 1997). However, whether all these interactions occur *in vivo* and are functionally important is not always established. For instance, a recent report suggests that the NPXY motif binds directly to the terminal domain of the clathrin heavy chain rather than to AP-2 (Kibbey *et al.*, 1998). The exact mechanism of di-leucine motif function is even less clear. Using *in vitro* binding techniques, Bremnes *et al.* (1998) reported that di-leucine motifs interact with the μ 2 subunit of AP-2, whereas others show that the interaction occurs through the β -adaptin (Rapoport *et al.*, 1998). The di-leucine motif is also reported to interact with AP-2 indirectly, through the viral protein Nef (Hua and Cullen, 1997; Piguet *et al.*, 1998).

The direct interaction of YXX Θ internalization signals with μ 2 has been demonstrated by several types of protein–

protein interaction assays (Ohno *et al.*, 1995; Rapoport *et al.*, 1997). Nonetheless, binding of receptors to AP-2 or the presence of the YXX Θ motif does not necessarily correlate with the internalization capacity of the receptors. For instance, epidermal growth factor (EGF) receptor strongly binds AP-2 via the sequence YRAL (Sorkin *et al.*, 1996). However, mutations in this motif, which abolish EGF receptor interaction with AP-2, do not significantly affect internalization of the receptor (Nesterov *et al.*, 1995b; Sorkin *et al.*, 1996). In contrast, transferrin receptors whose internalization is dependent on the presence of the YTRF motif (Collawn *et al.*, 1990) displayed very weak, if any, detectable interaction with AP-2 (Nesterov *et al.*, 1995a; Ohno *et al.*, 1995). Moreover, EGF and transferrin receptors do not compete for the saturable elements of the endocytic machinery (Wiley, 1988; Warren *et al.*, 1997). Therefore, despite the presence of similar internalization signals, these two receptors may be endocytosed by different mechanisms.

Thus, although AP-2 adaptors are implicated in several types of cargo recruitment mechanisms, the molecular details and the functional significance of their interaction with receptor cytoplasmic tails remain poorly understood. Here we describe dominant-interfering mutants which allow the direct assessment of the role of AP-2–receptor interactions in receptor-mediated endocytosis. The amino acid residues of μ 2 essential for binding to the YXX Θ internalization signal were mapped, and a mutant μ 2 incapable of binding to this endocytic code was engineered. When overexpressed in HeLa cells, the mutant μ 2 assembled into AP-2 complexes at the expense of the endogenous μ 2 and blocked the interaction of AP-2 with internalization signals. The analyses of endocytic trafficking in these cells revealed different effects of mutant μ 2 on endocytosis of transferrin and EGF receptors.

Results

μ 2 with an internal epitope tag assembles into AP-2 complexes and is targeted to coated pits

Structure–function analysis of μ 2 interactions required the biochemical and morphological detection of heterologously expressed μ 2 protein. Therefore, it was essential to engineer an epitope-tagged version of μ 2 that retained the ability to incorporate into AP-2 complex and be targeted to clathrin-coated pits. In initial experiments, an influenza virus hemagglutinin-1 (HA) epitope was placed at the N- or C-terminus of μ 2 and these proteins were transiently expressed in HEK293 and COS-1 cells. Expression experiments yielded essentially similar results in both cell lines, although the immunofluorescence analysis of coated pit proteins was facilitated in COS-1 cells flattened on the glass coverslips. The ability of epitope-tagged μ 2 to co-immunoprecipitate with the α - or β -adaptins was considered as evidence of its assembly into AP-2 complexes. Figure 1A demonstrates that μ 2 containing an HA tag on either terminus failed to co-immunoprecipitate with β -adaptin. Placement of Myc or FLAG epitopes at the C-terminus also prevented μ 2 incorporation into AP-2 complexes (data not shown).

An epitope tag was then inserted within the μ 2 polypeptide chain. Analysis of AP-2 domain structure by limited proteolysis (Aguilar *et al.*, 1997) reveals the presence of

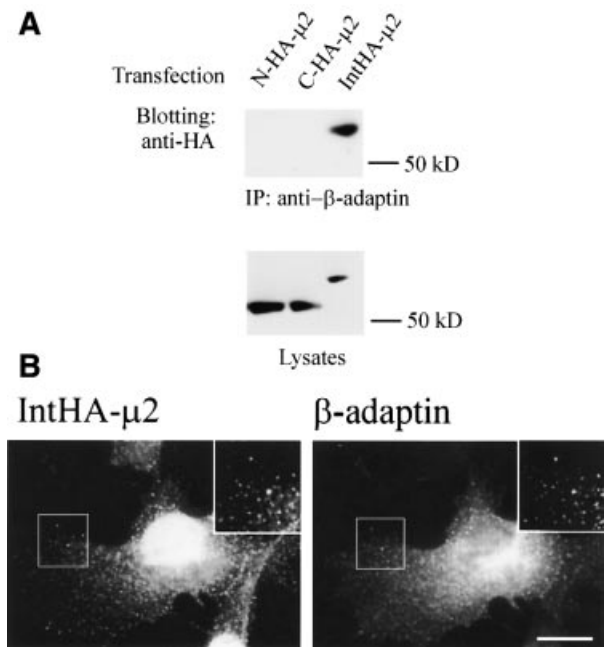


Fig. 1. Assembly of μ 2 containing an internal HA tag into AP-2 complexes. (A) COS-1 cells transfected with μ 2 constructs containing an HA tag at the N-terminus (N-HA- μ 2), C-terminus (C-HA- μ 2) or between residues 236 and 237 (IntHA- μ 2) were lysed, and APs were immunoprecipitated using Ab32 to β -adaptins. Lysates and immunoprecipitates (IP) were analyzed by Western blotting with anti-HA antibody 16B12. Note that IntHA- μ 2 migrates aberrantly slow on SDS-PAGE. (B) COS-1 cells expressing IntHA- μ 2 were processed for double-label immunofluorescence staining using mouse monoclonal anti-HA and rabbit Ab32 to β -adaptins. Rabbit and mouse primary antibodies were detected with the corresponding secondary IgGs labeled with Texas red and FITC, respectively. The serial optical sections were acquired through the Texas red (red) and FITC (green) channels, and deconvoluted as described in Materials and methods. The images represent individual optical sections (0.2 μ m thick). Inserts represent the peripheral regions of the cells, indicated by white boxes, at higher magnification and with enhanced contrast. Bar = 10 μ m.

two trypsin-sensitive sites in μ 2 which are situated in the regions of low sequence similarity between μ subunits of different APs (amino acids 146–163 and 220–250 of rat μ 2). The protease sensitivity of these regions suggests that they are exposed on the surface of the AP-2 core and may not be critical for the assembly of the AP-2 complex. Insertion of an HA tag between residues 236 and 237 of μ 2 produced a protein (IntHA- μ 2) that could be co-immunoprecipitated with β -adaptins (Figure 1A) or α -adaptins (data not shown).

Double-label immunofluorescence staining of COS-1 cells transiently transfected with IntHA- μ 2 revealed co-localization of IntHA- μ 2 and β -adaptin in plasma membrane coated pits (Figure 1B). This co-localization was clearly seen in cells expressing moderate levels of the protein, but not easily visualized in cells overexpressing IntHA- μ 2 due to the substantial aggregation of this protein. In contrast, μ 2 containing an N- or C-terminal HA tag was found in cytosol and in the form of large aggregates and was never detected in coated pits, regardless of expression levels (data not shown). The capacity of IntHA- μ 2 to co-immunoprecipitate with other subunits of AP-2 and to be targeted to coated pits made this construct suitable for experiments designed to generate dominant-negative AP-2 complexes.

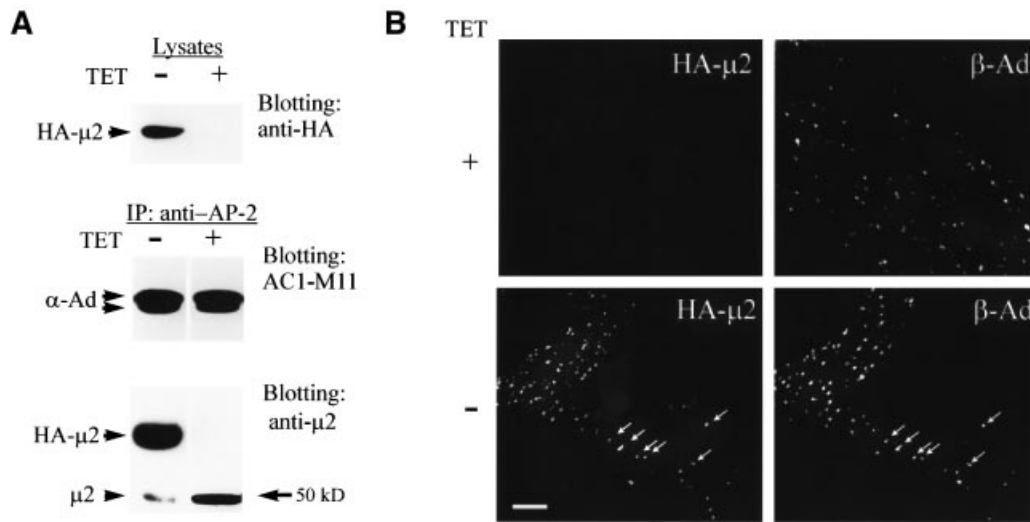


Fig. 2. Expression of epitope-tagged $\mu 2$ in HeLa cells using the tetracycline-controlled system. **(A)** HeLa cells expressing HA- $\mu 2$ under the tetracycline-regulated promoter were grown for 4 days in the presence (+TET) or absence of tetracycline (-TET), lysed and AP-2 was precipitated using antibody AP.6. Aliquots of cell lysates were resolved by SDS-PAGE, and expression of HA- $\mu 2$ was determined by Western blotting with anti-HA. The immunoprecipitates were analyzed for the presence of α -adaptins (αA and αC) and $\mu 2$ -proteins (endogenous and HA-tagged) by blotting with AC1-M11 antibody and anti- $\mu 2$ serum, respectively. **(B)** HeLa cells expressing HA- $\mu 2$ were grown in the presence or absence of tetracycline. Cells were permeabilized with 0.1% Triton X-100, fixed and processed for double-label immunofluorescence staining using Ab32 and anti-HA as described in Materials and methods. Serial optical sections were acquired through the Texas Red (red) and FITC (green) channels, and deconvoluted. The images represent individual optical sections (0.2 μm thick). The arrows indicate examples of co-localization of HA and β -adaplin staining. Bar = 5 μm .

Epitope-tagged $\mu 2$ replaces endogenous $\mu 2$ in the AP-2 complex

To interfere efficiently with the receptor-binding function of AP-2, heterologous $\mu 2$ should be expressed in cells at a level high enough to substitute for its endogenous counterpart in newly synthesized AP-2 complexes under conditions that avoid the toxic effects of overexpression. Inducible expression meets these requirements and, in contrast to transient expression, allows quantitative analysis of the cell population homogeneously expressing a protein of interest. IntHA- $\mu 2$ (further referred to as HA- $\mu 2$) was expressed in HeLa cells using a tetracycline-regulated expression system (Gossen and Bujard, 1992). Removal of tetracycline results in expression of HA- $\mu 2$ detected by anti-HA antibodies, whereas no expression was detected in the presence of tetracycline (Figure 2A, top panel). Immunoprecipitation with the α -adaplin antibody AP.6 followed by Western blotting with α -adaplin antibody AC1-M11 revealed that the number of AP-2 complexes was not changed, regardless of the presence of tetracycline (Figure 2A, middle panel). HA- $\mu 2$, which can be distinguished from endogenous $\mu 2$ by a slower electrophoretic mobility, co-immunoprecipitates with α -adaptins as shown by blotting with the antibody to $\mu 2$, confirming the assembly of HA-tagged $\mu 2$ into the AP-2 complex (Figure 2A, bottom panel).

These experiments also demonstrate the ability of epitope-tagged $\mu 2$ to substitute for endogenous $\mu 2$ in AP-2 complexes. Since the half-life of AP-2 is ~ 24 h (Sorkin *et al.*, 1995), the complete turnover of the AP-2 pool was expected to take several days. After 4 days of expression, HA- $\mu 2$ represented at least 80–90% of the total amount of $\mu 2$ protein assembled into AP-2 (Figure 2A). Interestingly, analysis of cellular lysates revealed that overexpression of HA- $\mu 2$ caused almost complete disappearance of endogenous $\mu 2$ (data not shown). This observation suggests

the existence of mechanisms that eliminate excessive $\mu 2$ which is not assembled into AP-2, and may serve at a post-translational level to control the stoichiometry between subunits of the AP-2 complex.

The localization of HA- $\mu 2$ in HeLa cells was examined by double-label immunofluorescence staining after cell permeabilization with Triton X-100. Figure 2B shows the appearance of punctate staining with anti-HA in the absence of tetracycline and the co-localization of HA- $\mu 2$ staining with β -adaptins, indicating that AP-2 containing HA- $\mu 2$ is targeted correctly to plasma membrane and allows formation of coated pits.

Point mutations D176A and W421A disrupt the interaction of $\mu 2$ with internalization signals without interfering with incorporation into AP-2

To identify residues in $\mu 2$ that are essential for the interaction with YXX Φ internalization signals, several $\mu 2$ mutants were generated. The wild-type or mutant $\mu 2$ constructs were either translated *in vitro* in the presence of [35 S]methionine (Figure 3A) or transiently expressed in COS-1 or HEK293 cells (Figure 3B). The $\mu 2$ proteins were tested for their ability to interact with GST fused either with triple repeats of the internalization signal SDYQRL from the transmembrane protein TGN38 (GST-TGN38) or with the residues 908–1186 of the C-terminus of the EGF receptor (GST-EGFR). To control for non-specific interactions, GST alone or a GST-EGFR mutant, whose AP-binding sequence Y 974 RAL (Sorkin *et al.*, 1996) was mutated to ARAL (GST-EGFR974A), were used. Both GST-TGN38 and GST-EGFR were equally potent in binding *in vitro* translated $\mu 2$. GST-EGFR also efficiently precipitated cellular AP-2 complexes containing transiently expressed HA- $\mu 2$. In contrast, we have not been able to detect the specific binding of cellular AP-2 to GST-TGN38.

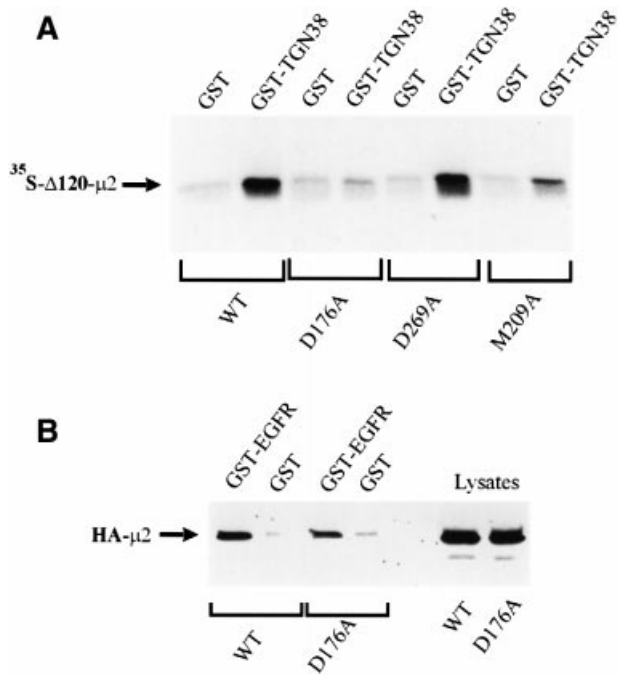


Fig. 3. Effects of point mutations in $\mu 2$ on interactions with TGN38 and EGF receptor. **(A)** Fragments of $\mu 2$ ($\Delta 120\mu 2$, residues 121–435) were translated in the presence of [^{35}S]methionine using the TNT system. Where indicated, residues D176, D269 or M209 of $\mu 2$ were changed to alanine. *In vitro* translated $\mu 2$ fragments were incubated with glutathione–agarose beads containing either GST–TGN38 or GST alone. Precipitates were analyzed by electrophoresis followed by fluorography. **(B)** COS-1 cells transfected with the wild-type or D176A mutant HA- $\mu 2$ were lysed, and the lysates were incubated with glutathione–agarose beads containing GST–EGFR or GST alone. The agarose precipitates and aliquots of lysates were analyzed by Western blotting with anti-HA.

It has been reported that mutation of $\mu 2$ residues D176, M209 or F265 to alanine inhibits the interaction of $\mu 2$ with the TGN38 internalization signal in yeast two-hybrid assays (Aguilar *et al.*, 1997). Similar mutations were made in $\mu 2$ lacking the first 120 N-terminal residues and the mutants were expressed in reticulocyte lysates. The N-terminal truncated $\mu 2$ was used because deletion of the 120 residues of the N-terminus of $\mu 2$ has been shown to increase the affinity of $\mu 2$ binding to TGN38 in yeast two-hybrid and *in vitro* pull-down assays (Ohno *et al.*, 1995, 1996). In GST pull-down experiments with *in vitro* translated $\mu 2$, only the D176A mutation completely abolished the $\mu 2$ interaction with TGN38, whereas the M209A mutation resulted in a partial inhibition (Figure 3A, upper panel). Similar results were obtained in pull-down experiments with GST–EGFR (data not shown). Surprisingly, binding of $\mu 2$ transiently expressed in COS-1 cells to GST–EGFR was only moderately affected by D176A mutation (Figure 3B).

To generate mutations in $\mu 2$ that would inhibit interaction with both TGN38 and EGF receptor internalization signals, several $\mu 2$ mutants with C-terminal truncations were prepared. GST pull-down experiments revealed that whereas $\mu 2$ truncated at residue 428 (C'428) partially retained the ability to bind GST–EGFR and GST–TGN38, $\mu 2$ truncated at residues 420 (C'420) and 409 (C'409) (Figure 4A and B) did not bind internalization signals. Therefore, the sequence between residues 420 and 428 is

important for HA- $\mu 2$ interaction with YXX Θ motifs. However, truncated $\mu 2$ mutants were not useful because these mutants were not incorporated into AP-2 (Figure 5A). The observation that modifications of the C-terminus of $\mu 2$ by small deletions or extensions with epitope tags (Figures 1 and 5) abrogate $\mu 2$ co-precipitation with adaptins implicates this region in AP-2 assembly.

The region 420–428 contains the sequence WVRYI that is conserved in $\mu 2$ from yeast to mammals and in the $\mu 1$ subunit of AP-1. This region, and particularly amino acid residue W421, could be part of a hydrophobic pocket involved in the interaction with either the first tyrosine or the last hydrophobic residue of the internalization signal. Consistent with this hypothesis, the substitution of W421 by alanine (W421A) completely abolished the interaction of HA- $\mu 2$ with both GST–TGN38 (Figure 4B) and GST–EGFR (Figure 4C). The interaction of wild-type $\mu 2$ with GST–EGFR required Tyr974 of the EGF receptor (Figure 4C). Thus, mutagenesis and GST pull-down assays revealed that at least two regions of the $\mu 2$ molecule containing residues D176 and W421 are engaged in $\mu 2$ interaction with YXX Θ motifs. That D176A and W421 are directly involved in the interaction with internalization signal peptides has now been demonstrated by the crystal structure data published during the preparation of this manuscript (Owen and Evans, 1998).

To inhibit maximally the effects the receptor-binding function of $\mu 2$, both D176 and W421 residues of $\mu 2$ were changed to alanines. The double mutant was expressed in HeLa cells using a tetracycline-regulated system, and interaction of the mutant $\mu 2$ protein with GST–EGFR was examined. As shown in Figure 4D, the D176A/W421A HA- $\mu 2$ was severely impaired in its ability to bind GST–EGFR.

In contrast to truncations, the point mutations D176A and W421A did not affect the ability of $\mu 2$ to co-immunoprecipitate with α -adaptins (Figure 5A). Figure 5B demonstrates that the double mutant D176A/W421A expressed in HeLa cells was also immunoprecipitated with α -adaptins. As demonstrated for wild-type HA- $\mu 2$ (Figure 2) when the expression of D176A/W421A $\mu 2$ was induced by tetracycline withdrawal, the mutant displaced endogenous $\mu 2$ from the cellular AP-2 complexes (Figure 5B).

Inducible overexpression of the D176A/W421A $\mu 2$ mutant inhibits interaction of AP-2 with the EGF receptor

To investigate how the displacement of endogenous $\mu 2$ by the mutant $\mu 2$ affects interaction between the whole AP-2 complex and YXX Θ signals, lysates from HeLa cells expressing either wild-type or D176A/W421A $\mu 2$ were incubated with GST–EGFR. The amount of bound AP-2 was determined by the presence of α -adaptin in GST precipitates. It was found that AP-2 obtained from cells expressing wild-type HA- $\mu 2$ bound very efficiently to GST–EGFR and did not bind to either the GST–EGFR974A mutant or GST alone (Figure 6A). In contrast, when expression of HA- $\mu 2$ -D176A/W421A was induced by the removal of tetracycline, binding of AP-2 to GST–EGFR was abolished completely (Figure 6B).

To confirm that the inhibition was specific to the plasma membrane adaptor AP-2, the effect of mutant $\mu 2$ overexpression on the interaction between GST–EGFR

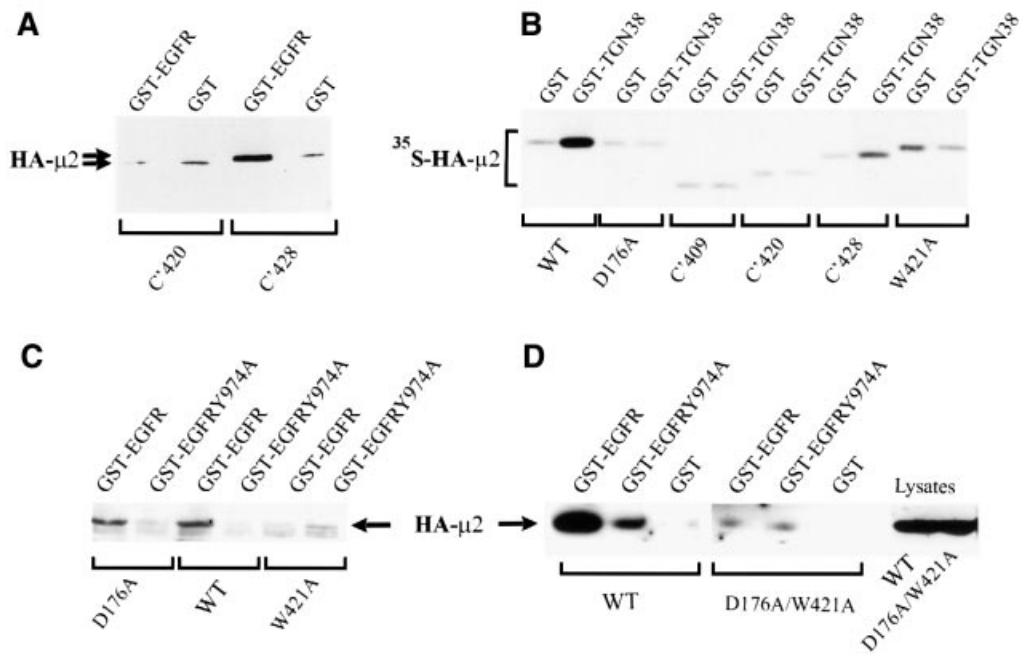


Fig. 4. Effects of mutations in the C-terminus of $\mu 2$ on the interaction with TGN38 and EGF receptor. (A) COS-1 cells transfected with HA- $\mu 2$ truncated at residues 420 (C'420) or 428 (C'428) were lysed, and the lysates were incubated with glutathione-agarose beads containing GST-EGFR or GST alone. The agarose precipitates were analyzed by Western blotting with anti-HA. The level of expression of two mutants was identical. (B) Wild-type (WT), truncation mutants (C'409, C'420 and C'428) and point mutants D176A and W421A of HA- $\mu 2$ were ^{35}S -labeled by *in vitro* translation in reticulocyte lysates, and the lysates were incubated with glutathione-agarose beads containing GST-TGN38 or GST alone. The agarose precipitates were resolved by SDS-PAGE and analyzed by radioautography. The W421A mutant consistently displayed high non-specific binding to glutathione-agarose beads. (C) HEK293 cells transfected with wild-type HA- $\mu 2$, D176A or W421 mutants of HA- $\mu 2$ were lysed, and incubated with glutathione-agarose beads containing GST-EGFR or GST-EGFR Y974A. The agarose precipitates were analyzed by Western blotting with anti-HA. The level of expression of all three constructs was identical. (D) HeLa cells grown in the absence of tetracycline to express wild-type HA- $\mu 2$ or HA- $\mu 2$ D176A/W421A mutant were lysed, and the lysates were incubated with glutathione-agarose beads containing GST-EGFR, GST-EGFR Y974A or GST alone. The agarose precipitates and the aliquots of cell lysates (5% of the amount used for pull-down experiments) were analyzed by Western blotting with anti-HA.

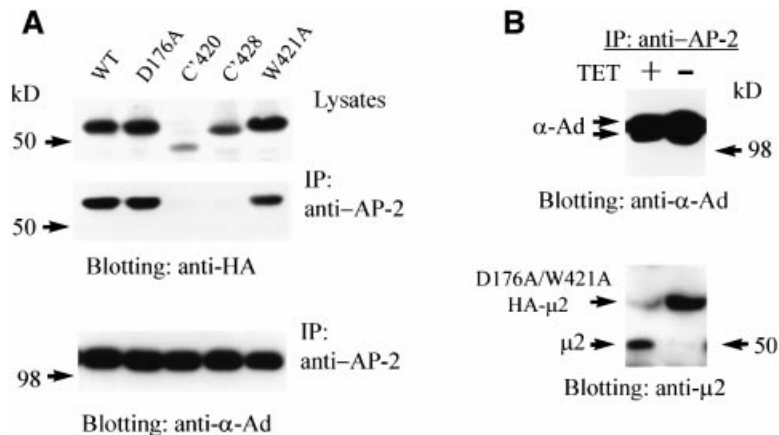


Fig. 5. Assembly of $\mu 2$ mutants into AP-2. (A) HEK293 cells were transfected with wild-type HA- $\mu 2$ (WT), truncation mutants of HA- $\mu 2$ with the last residue being 420 (C'420) or 428 (C'428), HA- $\mu 2$ D176A (D176A) or HA- $\mu 2$ W421A constructs. AP-2 was immunoprecipitated with AP.6 antibody, and HA- $\mu 2$ proteins and α -adaptins were detected in immunoprecipitates using anti-HA and AC1-M11 antibodies, respectively. The aliquots of cell lysates were analyzed by Western blotting with anti-HA. (B) HeLa cells expressing (-TET) or not expressing (+TET) HA- $\mu 2$ D176A/W421A were lysed and AP-2 was precipitated using AP.6 antibody. The α -adaptins and $\mu 2$ proteins (endogenous and HA-tagged) were detected by Western blotting with AC1-M11 antibody and anti- $\mu 2$ serum, respectively.

and the *trans*-Golgi adaptor protein AP-1 was tested. Binding of the EGF receptor to AP-1 *in vitro* and *in vivo* has been documented (Sorkina *et al.*, 1999). Figure 6B demonstrates that the EGF receptor interaction with AP-1, detected by the presence of γ -adaptin in GST-EGFR precipitates, was not affected by the overexpression of

D176A/W421A mutant $\mu 2$. These results provide strong evidence that the $\mu 2$ subunit of AP-2 is solely responsible for the interaction between coated pit adaptors and internalization signals, and validate the use of $\mu 2$ mutant-expressing cells as a tool for the analysis of the functional importance of this interaction *in vivo*.

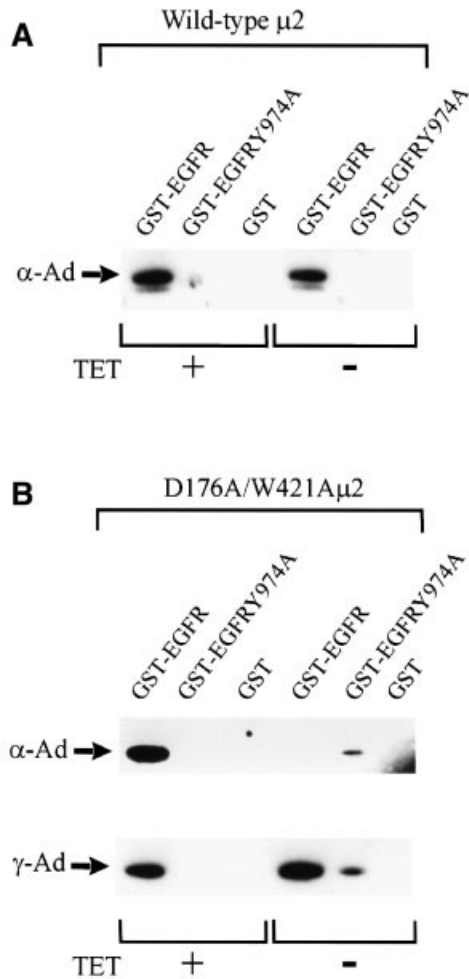


Fig. 6. D176A/W421A μ 2 mutant inhibits interaction of AP-2 with the EGF receptor C-terminus. GST-EGFR, GST-EGFR Y974A or GST alone were bound to glutathione-agarose beads, and incubated with lysates from HeLa cells expressing (–TET) or not expressing (+TET) either wild-type HA- μ 2 (A) or D176A/W421A HA- μ 2 (B). The agarose precipitates were resolved through SDS-PAGE and the amounts of bound AP-2 and AP-1 were determined by Western blotting with antibodies AC1-M11 to α -adaptins or 100/3 to γ -adaptin.

Inhibition of the interaction between AP-2 and tyrosine-based internalization signals produces contrasting effects on endocytosis of different receptors

To investigate how inhibition of the AP-2 receptor-binding function affects receptor-mediated endocytosis, we analyzed the effect of dominant-negative μ 2 on the internalization kinetics of EGF and transferrin receptors. Both receptors are naturally expressed in HeLa cells and contain tyrosine-based internalization signals with the AP-binding consensus sequences (Collawn *et al.*, 1990; Chang *et al.*, 1993a). Three cell lines, inducibly expressing wild-type, single mutant D176A μ 2 and double mutant D176A/W421A μ 2, were examined for the rates of internalization of labeled transferrin and EGF. As shown in Figure 7, [125 I]transferrin was internalized rapidly in all cell lines grown in the medium containing tetracycline. However, induction of the expression of D176/W421 μ 2 produced a 4-fold decrease in the rate of internalization of transferrin, whereas this rate was unaffected by the expression of wild-type HA- μ 2. Moreover, a continuous inhibition of

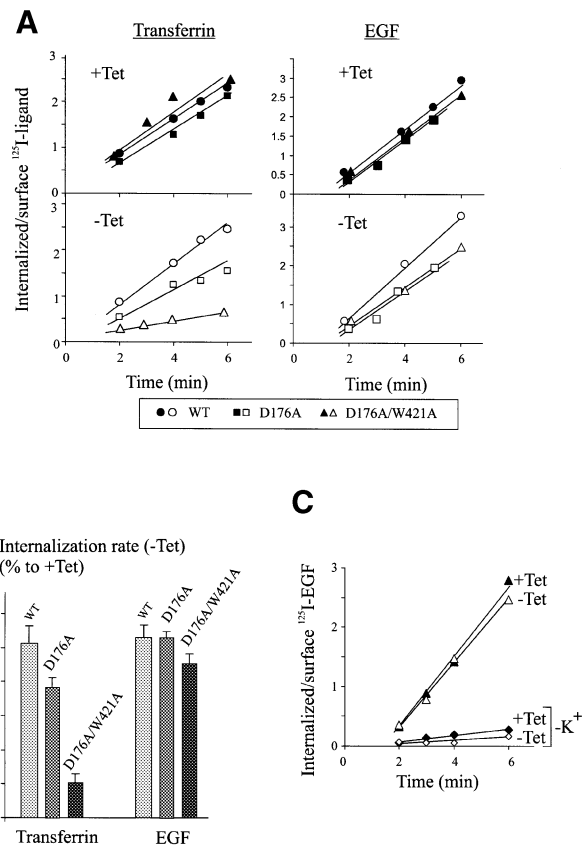


Fig. 7. Internalization of [125 I]EGF and [125 I]transferrin in HeLa cells. HeLa cells expressing wild-type HA- μ 2 (WT, circles), HA-D176A μ 2 (D176A, squares) or HA-D176A/W421A μ 2 (D176A/W421A, triangles) mutants were grown in the presence (+Tet) or absence of tetracycline (–Tet). Cells were incubated with 1 μ g/ml [125 I]transferrin or 1 ng/ml [125 I]EGF for 2–6 min and the amount of surface-bound and internalized radioactivity was determined as described in Materials and methods. The rate of internalization is expressed as the ratio of internalized and surface [125 I]ligand for each time point. (A) Time course of transferrin or EGF internalization. (B) Internalization rates averaged from several experiments in cells grown without tetracycline. Four single cell clones expressing the D176A/W421A mutant were examined. The rates are expressed as a percentage of these rates in cells grown with tetracycline where expression of HA- μ 2 proteins is inhibited. (C) Time course of [125 I]EGF internalization in control and K^+ -depleted (– K^+) cells. The results are representative of several experiments with two clones of cells expressing the D176A/W421A μ 2 mutant.

transferrin receptor endocytosis as a result of the expression of the D176A/W421A mutant resulted in a 4-fold increase in the surface pool of these receptors (data not shown). The single mutant D176A μ 2 imposed an intermediate effect on transferrin endocytosis (Figure 7). These data indicate that the internalization of the transferrin receptor requires interaction of the receptor with the μ 2 subunit of AP-2.

In parallel experiments, the internalization rates of [125 I]EGF were measured. As with the transferrin receptor, the internalization parameters of EGF were similar in different cell clones in the presence of tetracycline (Figure 7). However, in contrast to transferrin endocytosis, internalization of EGF was not significantly affected by overexpression of D176/W421 μ 2 (Figure 7). Furthermore, the extent and the rate of EGF-induced down-regulation of EGF receptors was independent of the expression of the wild-type or mutant μ 2 (data not shown).

To test whether the internalization of EGF receptors in cells expressing mutant $\mu 2$ is clathrin dependent, the effect of K^+ depletion on [^{125}I]EGF uptake was examined. K^+ depletion of the cells is known to block the assembly of coated pits and clathrin-mediated endocytosis (Larkin *et al.*, 1983). As shown in Figure 7C, K^+ depletion abolished EGF internalization in cells grown in the presence or absence of tetracycline. These data suggest that endocytosis of the EGF receptor is mediated by clathrin-coated vesicles, but does not require the interaction of the receptor with $\mu 2$.

Discussion

We applied a dominant-interfering approach to investigate the functional significance of the interaction between the tyrosine-based internalization YXX Θ signal and the plasma membrane adaptor protein AP-2. Since *in vitro* interactions between YXX Θ motifs and AP-2 were shown to be mediated by $\mu 2$, we generated a mutant of $\mu 2$ that does not bind internalization sequences, but is capable of substituting for the endogenous $\mu 2$ in AP-2 complexes. The expression of such a mutant was expected to inhibit the interaction of receptor cytoplasmic tails with AP-2, and thus could be used to assess the importance of this interaction for endocytosis of different receptors.

Generation of suitable $\mu 2$ mutants first required construction of an epitope-tagged version of $\mu 2$ that assembles into AP-2 and does not interfere with the intracellular targeting of AP-2. Placing a small HA sequence at the N-terminus of $\mu 2$ inhibited incorporation into AP-2 complexes. This result is consistent with the direct interaction of the N-terminus of $\mu 2$ with β -adaptin demonstrated in two-hybrid assays (Aguilar *et al.*, 1997). Unexpectedly, $\mu 2$ proteins containing small epitope tags at the C-terminus or missing only the last seven amino acid residues were unable to associate with adaptins, indicating that the C-terminus of $\mu 2$ is also important for the AP-2 complex assembly. Since this region of $\mu 2$ did not appear to be important for binding to β -adaptins, it may be involved in the interaction with other AP-2 subunits or required for the proper folding of the $\mu 2$ protein. Insertion of the HA epitope into an internal region of low sequence similarity among adaptor medium chains did not prevent $\mu 2$ assembly into AP-2 or its targeting to coated pits. An antibody to the epitope tag recognized AP-incorporated $\mu 2$ under non-denatured conditions in immunoprecipitation and immunofluorescence experiments. Together with data obtained by limited proteolysis (Aguilar *et al.*, 1997), these results suggest that the region encompassing residues 236 and 237 is exposed on the surface of the AP-2 core.

Several residues important for the binding of $\mu 2$ to the YQRL internalization signal in TGN38 have been identified previously in yeast two-hybrid assays (Aguilar *et al.*, 1997). In GST pull-down experiments, however, only mutation D176A significantly affected $\mu 2$ interaction. The reason for this discrepancy is presently unclear, and may be related to the differences in folding of $\mu 2$ proteins expressed in yeast, translated *in vitro* or expressed in mammalian cells. Interestingly, whereas the D176A mutation strongly inhibited $\mu 2$ binding to the YQRL motif of TGN38, the interaction of this mutant with the YRAL sequence of the EGF receptor was only partially affected.

Further mapping of the receptor-binding sequences revealed that mutation of the conserved W421 yielded $\mu 2$ protein defective in binding to both the EGF receptor and TGN38. To suppress completely the interaction of $\mu 2$ with several types of YXX Θ consensus internalization signals, the double mutant D176A/W421A was used in expression studies.

During the preparation of this manuscript, the crystal structure of the part of the $\mu 2$ protein (residues 158–435) complexed with tyrosine-containing peptides was published (Owen and Evans, 1998). Interestingly, both function-suppressive mutations that we identified involve residues that are located within the tyrosine peptide-binding pocket and directly participate in the interaction with this peptide. The crystal structure demonstrates multiple interactions within the binding pocket, possibly explaining why the mutation D176A had only partial effects on the EGF receptor interaction with $\mu 2$. Apparently, in the absence of hydrogen bonds between the hydroxyl group of Y974 of the EGF receptor and D176 of $\mu 2$, the hydrophobic interactions of Y974 with W421 and F174 of $\mu 2$ as well as that of L977 of the EGF receptor with several aliphatic residues within the binding pocket of $\mu 2$ are sufficient to support the complex. The structure also revealed that the site of insertion of the HA tag lies within the unfolded loop between the $\beta 3$ - and $\beta 4$ -strands of $\mu 2$, which explains why this modification did not affect $\mu 2$ functions.

Thus, independently of the structural data, mutational analysis identified residues D176 and W421 as a part of the internalization signal-binding interface of $\mu 2$ and, in addition, demonstrated that this binding pocket is the binding site in the holo $\mu 2$ protein. Another YXX Θ -binding interface was proposed to exist within the N-terminal domain of $\mu 2$ (residues 102–125) (Bremnes *et al.*, 1998) that is situated outside of the structurally characterized part of the protein. However, as the D176A/W421A $\mu 2$ mutations completely abrogated the binding abilities of full-length $\mu 2$, region 102–125 does not appear to be essential for the interaction with YXX Θ motifs.

Several approaches to achieve a maximal dominant-negative effect of mutant $\mu 2$ were tested. Transient expression resulted in accumulation of a large fraction of $\mu 2$ protein in cytosolic aggregates and did not allow quantitative measurements of endocytosis. The selection of constitutively expressing stable cell lines yielded clones containing low levels of exogenous protein. The tetracycline-controlled expression system proved to be the most suitable: it generated high expression levels sufficient to substitute metabolically for endogenous $\mu 2$ in the majority of cellular AP-2 complexes in all cells in the population, making possible quantitative measurements of endocytic kinetics. Importantly, the generation of dominant-negative AP-2 incapable of binding to YXX Θ motifs did not increase the total amount of AP-2 in the cell. Thus, in contrast to dominant-negative approaches that involve simple overexpression of a protein or a fragment of a protein, this strategy allowed investigation of functions for a particular type of adaptor protein without interfering with other APs. AP-2 complexes containing mutant $\mu 2$ were properly targeted to the plasma membrane, and in general behaved indistinguishably from control AP-2 (data not shown), indicating that the interaction of the cyto-

plasmic tails with AP-2 via $\mu 2$ does not play a significant role in AP-2 targeting and docking to the membrane.

To investigate how inhibition of the signal recognition function of AP-2 affects receptor-mediated endocytosis, we measured the endocytic kinetics of EGF and transferrin receptors in cells expressing a dominant-interfering mutant of $\mu 2$. Both receptors are internalized via clathrin-coated pits in HeLa cells, and contain YRAL and YTRF sequences, respectively, which are implicated in internalization (Collawn *et al.*, 1990; Chang *et al.*, 1993b; Sorkin *et al.*, 1996). Mutational inactivation of the YTRF motif of the transferrin receptor abolished endocytosis (Collawn *et al.*, 1990). Despite the apparent low affinity interaction between the transferrin receptor and AP-2 (Nesterov *et al.*, 1995a; Ohno *et al.*, 1995) that, to our knowledge, has not been confirmed by co-immunoprecipitation, the experiments in cells expressing mutant $\mu 2$ directly demonstrate that the interaction of the transferrin receptor with the $\mu 2$ subunit of AP-2 is essential for the receptor endocytosis.

Studies of EGF receptor endocytosis have suggested several mechanisms. EGF receptors can be co-immunoprecipitated with AP-2, bind AP-2 *in vitro* and interact with the $\mu 2$ subunit in yeast two-hybrid assays (Sorkin and Carpenter, 1993; Boll *et al.*, 1995; Nesterov *et al.*, 1995a; Sorkina *et al.*, 1999). The strong EGF receptor interaction with AP-2 is mediated by the Y⁹⁷⁴RAL sequence of the receptor (Sorkin *et al.*, 1996). However, mutations in this sequence had no effect on EGF receptor endocytosis in cells expressing physiological levels of receptors (Nesterov *et al.*, 1995b; Sorkin *et al.*, 1996). Based on these observations, it was concluded that the high affinity interaction of the EGF receptor with AP-2 was not important for endocytosis. However, the possibility that EGF receptors associate with AP-2 via other weak binding sites or through the formation of heterodimers with other members of the Erb receptor family (Gilboa *et al.*, 1995; Lenferink *et al.*, 1998) could not be excluded. The present experiments with a dominant-negative mutant of $\mu 2$ demonstrate that the interaction of $\mu 2$ with EGF receptors is not essential for receptor internalization, and support the notion that alternative mechanisms control the endocytosis of this receptor (Lamaze *et al.*, 1993). These mechanisms may involve newly characterized clathrin-binding molecules such as β -arrestins (Ferguson *et al.*, 1996) participating in the recruitment of other classes of receptors into coated pits or the terminal domains of clathrin heavy chain (Kibbey *et al.*, 1998). A number of proteins containing SH2 or PTB domains that are capable of binding to the activated EGF receptor may also play a role in the targeting of the EGF receptors to coated pits.

What is the functional role of EGF receptor interaction with AP-2? It is possible that this interaction has a role in the internalization of EGF receptors in cells overexpressing these receptors. The highest extent of EGF receptor association with AP-2 was detected in A-431 cells which display an extraordinary high density of EGF receptors (Sorkin and Carpenter, 1993). Moreover, mutations of the Y974-containing motif of the receptor resulted in impaired endocytosis in NIH 3T3 cells expressing high levels of receptors (Sorkin *et al.*, 1996).

The results presented in this study thus demonstrate that endocytosis may proceed through both $\mu 2$ -dependent and -independent mechanisms. The data also illustrate

the usefulness of a dominant-interfering approach to investigate functions of various internalization signals and, potentially, other adaptor protein complexes.

Materials and methods

Reagents

Pfu polymerase, *Taq* precision polymerase and the QuickChange site-directed mutagenesis kit were from Stratagene Cloning Systems (La Jolla, CA). The *in vitro* coupled transcription and translation system (TNT) was from Promega Corporation (Madison, WI). The mammalian expression vector pcDNA3 was from Invitrogen Corp. (Carlsbad, CA) and the tetracycline-controlled expression vector pUHG 10-3 was a gift of H.Bujard (ZMBH, Heidelberg, Germany). The bacterial expression vector pGEX-4T3 was purchased from Amersham Pharmacia Biotech (Piscataway, NJ), B-PER reagent from Pierce (Rockford, IL), tetracycline and puromycin from Calbiochem-Novabiochem Corporation (La Jolla, CA), and sodium butyrate and glutathione-agarose beads from Sigma (St Louis, MO).

Iron-saturated human transferrin was purchased from Sigma and iodinated using Iodo-Beads (Pierce, Inc.). Mouse receptor-grade EGF was obtained from Collaborative Research Inc. and iodinated using a modified chloramine-T method as described previously (Carpenter and Cohen, 1976). The specific activity of [¹²⁵I]transferrin and [¹²⁵I]EGF was 3.0 × 10⁵ c.p.m./ μ g and 1.5–1.9 × 10⁵ c.p.m./ng, respectively.

Antibodies

Monoclonal antibody to the α -subunit of AP-2 (AP.6) (Chin *et al.*, 1989) was obtained from ATCC, whereas the monoclonal antibody 100/3 specific to γ -adaptin was from Sigma. Monoclonal AC1-M11 antibody to α -adaptin and polyclonal antiserum specific to the $\mu 2$ subunit were provided by M.Robinson (Cambridge University, UK). AC1-M11 antibody was also purchased from Affinity Bioreagents Inc. (Golden, CO). Rabbit polyclonal antibody Ab32 specific to β -adaptins was described previously (Sorkin *et al.*, 1996). Monoclonal antibody 16B12 to the HA epitope tag was purchased from BABCO (Richmond, CA).

Plasmid constructs

cDNA encoding rat $\mu 2$ (Thurieu *et al.*, 1988) provided by T.Kirchhausen (Harvard University, Boston, MA) was amplified using *Pfu* polymerase and subcloned either into the constitutive expression vector pcDNA3 or into the tetracycline-controlled expression vector pUHG 10-3 (Gossen and Bujard, 1992) provided by H.Bujard (ZMBH, Heidelberg, Germany). In order to generate $\mu 2$ containing a C-terminal HA-1 epitope tag, the sequence encoding YPYDVPDYA was introduced into the 3' amplification oligonucleotide. $\mu 2$ containing an internal HA epitope was generated by a two-step amplification procedure, which introduced the sequence YPYDVPDYALE between residues 236 and 237. To place an HA tag at the N-terminus, $\mu 2$ cDNA encoding residues 2–435 was subcloned in-frame to a modified pcDNA3 plasmid generating an N-terminal extension MEYPYDVPDYAEFCRYPCHWRPLE. N-terminal truncation mutant N-120 and C-terminal truncation mutants C'409, C'420 and C'428 were generated by amplification of $\mu 2$ cDNA using *Pfu* polymerase. All point mutations in $\mu 2$ constructs were generated using the QuickChange site-directed mutagenesis kit according to the manufacturer's protocol.

A fragment of EGF receptor corresponding to the C-terminus of the receptor (amino acids 908–1186) was amplified using *Taq* precision polymerase and subcloned into the bacterial expression vector pGEX-4T3 to yield GST-EGFR. A tyrosine to alanine mutation of the residue equivalent to Tyr974 in the holo-EGF receptor was introduced into GST-EGFR using the QuickChange method to yield GST-EGFR974A. Synthetic oligonucleotides encoding a triple repeat of the internalization signal of TGN38 (SDYQRSLSDYQLLSYQRLNLKL) were annealed and cloned in-frame in the GST expression vector pGX-KG to yield GST-TGN38.

All constructs were verified by dideoxynucleotide sequencing. The sequences of oligonucleotides used for cloning are available upon request.

Expression of GST fusion proteins

GST-TGN38 was made in *Escherichia coli* BL21 strain. Expression of proteins was induced by 0.2 mM isopropyl- β -D-thiogalactopyranoside (IPTG) for 5 h, cells were collected by centrifugation, washed in ice-cold 100 mM NaCl, supplemented with 20 mM Tris-HCl pH 8.0 and frozen. The cell pellet was resuspended in B-PER reagent containing

protease inhibitors (5 ml of B-PER per 1 l of bacterial culture), sonicated and cleared by centrifugation. Aliquots of bacterial lysate were stored at -80°C .

GST-EGFR and GST-EGFR974A were made in *E. coli* B834 (DE3) pLysS, which were grown in LB (supplemented with ampicillin and chloramphenicol) to $A_{260} \sim 0.6$. The expression was induced with 0.1 mM IPTG for 2–3 h at 37°C . The cells were harvested and washed once in 50 mM Tris, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF) pH 8.0 and frozen at -80°C . The cell pellets were sonicated in 50 mM Tris, 4 mM EDTA, 2 mM EGTA, 1 mM dithiothreitol (DTT), 25% (w/v) sucrose pH 8.0 supplemented with 1% Triton X-100, 1% protease cocktail (Sigma), 10 mg/ml aprotinin, 10 mg/ml leupeptin and 10 mg/ml iodoacetamide. After 20 min centrifugation at 100 000 g, the supernatants were bound to glutathione-Sepharose 4B beads (Pharmacia) at the ratio of 700 ml per 50 ml of starting culture for 3 h at 4°C . The beads were washed three times in ice-cold Ca^{2+} , Mg^{2+} -free phosphate-buffered saline (CMF-PBS), resuspended in an equal volume of fresh CMF-PBS to give a 50% (v/v) slurry, and stored at 4°C .

Mammalian cell culture and transfections

HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), antibiotics and glutamine. COS-1 cells were grown in DMEM containing 10% newborn calf serum, antibiotics and glutamine. HeLa cells expressing tet-off transactivator (Damke *et al.*, 1995) were provided by S. Schmid (Scripps Institute, La Jolla, CA). HeLa cells were grown in DMEM containing 10% FBS, antibiotics and glutamine, and supplemented with G418.

For transient expression, cells grown to 50–80% confluency were transfected with pcDNA3 plasmids using a calcium phosphate transfection kit (5'–3', Boulder, CO), Lipofectamine (Gibco-BRL) or Effectene (Qiagen). Transient transfections were used for all experiments with COS-1 and HEK293 cells. Transfected cells were split 1 day after transfection to dishes and glass coverslips and used for experiments on the second or third day. Cells were grown to ~90 or 50% confluency for co-immunoprecipitation or immunofluorescence experiments, respectively.

HeLa cell lines expressing $\mu 2$ constructs under the tetracycline-off-controlled promoter were generated as outlined in Damke *et al.* (1995). The cells were grown in DMEM supplemented with 10% fetal calf serum, 400 $\mu\text{g}/\text{ml}$ G418, 100 U/ml penicillin, 100 U/ml streptomycin and 0.25 $\mu\text{g}/\text{ml}$ amphotericin B. Cells (5×10^5) were transfected using the calcium phosphate method with 17 μg of expression vector pUHG10-3 containing various $\mu 2$ constructs mixed with 1.5 μg of plasmid pBSpac, containing the puromycin resistance selection marker. Individual colonies selected in the presence of 200 ng/ml puromycin and 2 $\mu\text{g}/\text{ml}$ tetracycline were isolated and analyzed for the expression of epitope-tagged $\mu 2$ constructs by Western blotting using antibody 16B12.

For the experiments, cells were plated in the growth medium that did not contain selection markers with or without tetracycline. At 24 h after plating, medium was replaced with the fresh medium with or without tetracycline supplemented with 2 mM sodium butyrate to ensure the high rates of HA- $\mu 2$ expression necessary to replace endogenous $\mu 2$ from AP-2 complexes. Experiments were performed 3–4 days after plating.

GST pull-down experiments

COS-1, HEK293 or HeLa cells expressing $\mu 2$ proteins were washed with CMF-PBS and lysed in Triton/glycerol/HEPES buffer (TGH): 50 mM HEPES pH 7.4, 1% Triton X-100, 10% glycerol, 100 mM NaCl, 1 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 10 mg/ml leupeptin, 10 mg/ml aprotinin, 10 mM benzamide for 10 min at 4°C . The lysates were then cleared by centrifugation for 45 min at 125 000 g in a Beckman TLA 45 rotor.

^{35}S -Labeled $\mu 2$ proteins were made by *in vitro* transcription and translation from a T7 promoter using the Promega TNT kit, according to the supplier's protocol. Prior to pull-down experiments, aliquots of TNT reactions (10% of the total reaction mixture) were electrophoresed and the amounts of labeled $\mu 2$ proteins were determined by phosphorimaging. Typically, these amounts varied by not more than 15%. Between 43 and 47 μl of TNT reactions corresponding to equivalent amounts of the labeled proteins were diluted with 1 ml of TGH containing 25 mg/ml bovine serum albumin (BSA) and cleared by centrifugation for 45 min at 125 000 g.

For GST-TGN38 binding experiments, aliquots (0.5 ml) of *E. coli* lysates were incubated with 10 μl of glutathione-agarose beads for 2–3 h at 4°C . The beads were then washed once with 1 ml of TGH containing 25 mg/ml BSA, and incubated with 0.2–0.5 ml of TGH-diluted TNT

reactions for 2–5 h at 4°C , washed three times with TGH and heated in 30 μl of SDS sample buffer. The precipitates from the TNT reactions were separated by SDS-PAGE, gels were dried and exposed to X-ray films and analyzed by phosphorimaging. The pull-down experiments with GST-EGFR and *in vitro* translated $\mu 2$ were carried out similarly. However, because of the low yield of GST-EGFR protein, the ratio of specific and non-specific signals in these experiments was lower than that in experiments with GST-TGN38.

In binding experiments with HA- $\mu 2$ expressed *in vivo*, beads loaded with GST, GST-EGFR and GST-EGFR974A were washed once in CMF-PBS and incubated with mammalian cell lysates for 3 h at 4°C . The beads were washed three times with 600 ml of 150 mM KCl, 20 mM HEPES, 2 mM MgCl_2 pH 7.2, then heated in sample buffer for 5 min at 80°C . The precipitates from cell lysates were then resolved by SDS-PAGE, and the proteins were transferred to nitrocellulose membranes and probed by Western blotting with various antibodies to AP-2 subunits or to the HA epitope tag followed by species-specific secondary antibodies or protein A (Zymed, Inc.) conjugated with horseradish peroxidase. The enhanced chemiluminescence kits were from Pierce and Amersham.

Immunoprecipitation of APs

TGH lysates were prepared from $\mu 2$ -expressing cells, and cleared as described above. APs were immunoprecipitated with AP.6 or Ab32 antibodies specific to α - or β -adaptins, respectively. The precipitates were washed with TGH, denatured by heating in sample buffer, and resolved on SDS-PAGE followed by transfer to the membrane and Western blotting with various antibodies to AP subunits and anti-HA.

Immunofluorescence staining

COS-1 cells transiently expressing $\mu 2$ proteins were grown on coverslips. The cells were washed with CMF-PBS and fixed with freshly prepared 4% paraformaldehyde (Electron Microscopy Sciences) for 12 min at room temperature, and mildly permeabilized using a 3 min incubation in CMF-PBS containing 0.1% Triton X-100 and 1% BSA at room temperature. HeLa cells expressing $\mu 2$ proteins under the control of the tetracycline-dependent promoter were permeabilized mildly with 0.1% Triton X-100 for 3 min at 4°C before fixation. This pre-treatment eliminated a significant background staining of HeLa cells with anti-HA antibodies but does not affect staining of clathrin-coated pits with various antibodies to clathrin and AP-2. We found that monoclonal 16B12 antibody is the only anti-HA antibody that recognizes the internal HA tag. Therefore, for double-labeling, this monoclonal antibody was paired with a polyclonal antibody to β -adaptin, Ab32.

Fixed cells were incubated in CMF-PBS containing 1% BSA at room temperature for 1 h with rabbit Ab32 and mouse anti-HA antibodies, washed intensively and then incubated with the secondary donkey anti-mouse and anti-rabbit IgG labeled with Texas red and fluorescein isothiocyanate (FITC), respectively (Jackson Tec.). Both primary and secondary antibody solutions were pre-cleared by centrifugation at 100 000 g for 10 min. After staining, the coverslips were mounted in Fluoromount-G (Fisher) containing 1 mg/ml *p*-phenylenediamine. To obtain high-resolution three-dimensional images of cells, we used an imaging workstation consisting of the thermoelectrically cooled charged-coupled device (CCD) Micromax camera with Sony Interline area array (Princeton Instruments) and a Nikon Diaphot microscope equipped with z-step motor and dual filter wheel controlled by QED Imaging or Intelligent Imaging Innovation (Denver, CO) software. Typically, 15–40 serial two-dimensional images were recorded at 100–200 nm intervals. A Z-stack of images obtained was deconvoluted using a modification of the constrained iteration method. Final analysis of all images was performed using AdobePhotoshop 4.03.

Internalization of [^{125}I]EGF and [^{125}I]transferrin

To monitor [^{125}I]EGF or [^{125}I]transferrin internalization, HeLa cells were grown in 12-well dishes in the presence or absence of tetracycline. The cells were incubated in binding medium for 1 h prior to experiments. To measure the internalization rates, cells were incubated with [^{125}I]EGF (1 ng/ml) or [^{125}I]transferrin (1 $\mu\text{g}/\text{ml}$) in binding medium at 37°C for 1–6 min. A low concentration of [^{125}I]EGF was used to avoid saturation of the internalization machinery. After the indicated times, the medium was aspirated, and the monolayers were washed rapidly three times with DMEM to remove unbound ligand. The cells were then incubated for 5 min with 0.2 M acetic acid (pH 2.8) containing 0.5 M NaCl at 4°C . The acid wash was combined with another short rinse in the same buffer, and used to determine the amount of surface-bound [^{125}I]ligand. The cells were lysed in 1 M NaOH to determine the intracellular (internalized)

radioactivity. The ratio of internalized: surface radioactivity was plotted against time. The linear regression coefficient of the dependence of this ratio on time represents the specific rate constant for internalization. Non-specific binding was measured for each time point in the presence of 100-fold molar excess of unlabeled EGF, and was not more than 3–5% of the total counts. The K⁺ depletion experiments were performed exactly as described (Sorkin *et al.*, 1996).

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