Differential Recognition of Tyrosine-based Basolateral Signals by AP-1B Subunit μ1B in Polarized Epithelial Cells

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To investigate the importance of tyrosine recognition by the AP-1B clathrin adaptor subunit μ1B for basolateral sorting of integral membrane proteins in polarized epithelial cells, we have produced and characterized a mutant form of μ1B. The mutant (M-μ1B) contains alanine substitutions of each of the four conserved residues, which in the AP-2 adaptor subunit are critical for interacting with tyrosine-based endocytosis signals. We show M-μ1B is defective for tyrosine binding in vitro, but is nevertheless incorporated into AP-1 complexes in transfected cells. Using LLC-PK1 cells expressing either wild type or M-μ1B, we find that there is inefficient basolateral expression of membrane proteins whose basolateral targeting signals share critical tyrosines with signals for endocytosis. In contrast, membrane proteins whose basolateral targeting signals are distinct from their endocytosis signals (transferrin and low-density lipoprotein receptors) accumulate at the basolateral domain normally, although in a manner that is strictly dependent on μ1B or M-μ1B expression. Our results suggest that μ1B interacts with different classes of basolateral targeting signals in distinct ways.

INTRODUCTION

The plasma membrane of epithelial cells is physically separated by the tight junction into two distinct domains: the apical and the basolateral membranes. These two membrane domains have distinct lipid and protein compositions, which is thought to be important for the polarity and function of epithelial cells (Mellman, 1996; Aroeti et al., 1998; Mostov et al., 2000). To maintain the “polar” distribution of newly synthesized membrane proteins, as well as those endocytosed from the cell surface, proteins must be transported to the proper plasma membrane domain from the trans-Golgi network (TGN) or from the endosomal compartments, respectively.

Polarized targeting of basolateral plasma membrane proteins is largely dependent on distinct sorting signals present in their cytoplasmic domains (Mellman, 1996; Aroeti et al., 1998; Mostov et al., 2000). Some of these basolateral sorting signals show a sequence similarity with tyrosine-based or dileucine-based endocytosis signals, which are well known as clathrin-coated pit targeting signals (Matter and Mellman, 1994). Because these coated pit targeting signals directly interact with adaptor protein (AP) complexes of clathrin coats (Ohno et al., 1995; Boll et al., 1996; Dell’Angelica et al., 1997; Rapoport et al., 1998; Rodionov and Bakke, 1998; Hofmann et al., 1999), it had been hypothesized early on that an AP or AP-like complex may play a similar role in basolateral sorting in epithelial cells (Hunziker et al., 1991).

AP complexes comprise a family of heterotetrameric protein complexes (AP-1 through AP-4) consisting of two large (α, γ, δ or ε, and β), one medium (μ), and one small (σ)
subunit (Hirst and Robinson, 1998; Bonifacino and Dell’Angelica, 1999). Recently, we cloned a novel medium subunit, μIB, which is expressed only in epithelial cells (Ohno et al., 1999). μIB can assemble in combinatorial manner with three subunits of AP-1A (γ, β1, and α1) to generate an AP-1B complex (Folsch et al., 1999). Importantly, AP-1B plays an essential role in basolateral targeting of a variety of membrane proteins such as the transferrin receptor (TfR) and the low-density lipoprotein receptor (LDLR) (Folsch et al., 1999, 2001). AP-1A cannot substitute for AP-1B in basolateral sorting, consistent with the fact that only AP-1B, and not AP-1A, complexes interact physically with basolateral targeting signals (Folsch et al., 2001). Because the only apparent difference between these complexes is identity of their μ subunits, it is reasonable to suspect that the μIB subunit itself is responsible for recognizing basolateral targeting signals.

Indeed, it is well known that all μ subunits at least in vitro interact directly with sorting signals that contain critical tyrosine residues, where those signals conform to the consensus sequence YXXØ (where Y is tyrosine; X is any amino acid; and Ø is a bulky, hydrophobic residue) (Ohno et al., 1995, 1999; Boll et al., 1996; Dell’Angelica et al., 1997; Aguilar et al., 2001). However, μ subunits interact with distinct subsets of tyrosine-based signals with different affinities, a feature that is likely to reflect their ability to select different cargo proteins during transport (Ohno et al., 1996, 1998). An interesting feature of basolateral targeting signals is that they tend to be highly heterogeneous, with many not conforming to the YXXØ motif. Even in these instances, however, transport to the basolateral surface is completely dependent on AP-1B (Folsch et al., 1999). Conceivably, these different classes of signals interact with μIB in distinct ways.

Thus far, the only μ chain whose structure has been at least partially solved is the μ2 subunit of the AP-2 adaptor complex (Owen and Evans, 1998). By analyzing the position of a peptide containing YXXØ-type signal bound to μ2, several residues in μ2 were identified that seemed to be responsible for signal binding. Because these residues are also conserved in the sequence of μIB, we asked whether they were also important for the binding of basolateral signals. Indeed, they were but only in the case of signals that depended on critical tyrosine residues that were also required for endocytosis.

MATERIALS AND METHODS

Antibodies

A rabbit polyclonal antibody specific for a μIB C-terminal peptide was described previously (Folsch et al., 1999). A rabbit antiserum recognizing β4 was raised against a glutathione S-transferase-β4 fragment (corresponding amino acid residues 492–506 of human β4). An anti-human asialoglycoprotein receptor (AGPR) subunit H1 antisera was a gift from Dr. Martin Spiess (University of Basel, Switzerland). The following antibodies were obtained from the American Type Culture Collection (Manassas, VA): 7C7B6, a monoclonal antibody (mAb) recognizing Tac, the interleukin-2 receptor β subunit; L5.1, a mAb specific for the human TfR; and a mAb specific for the human LDLR, C7. A mouse anti-γ-adaptin mAb, 100/3, was purchased from Sigma-Aldrich (St. Louis, MO). The following were purchased from Molecular Probes (Eugene, OR): Alexa Fluor 488-conjugated anti-mouse and anti-rabbit IgG antibodies; Alexa Fluor 546-conjugated anti-mouse and anti-rabbit IgG antibodies; and an Alexa Fluor 488 phalloidin. Anti-mouse and anti-rabbit IgG, 125I-labeled whole antibody, were purchased from Amershams Biosciences (Piscataway, NJ).

Plasmids

Gal4ad-μ1B, Gal4ad-ETYWF, and Gal4ad-RSLYRL were described previously (Ohno et al., 1999). A mutant human μIB cDNA (M-μIB), in which four amino acids (Phe172, Asp174, Trp408, and Arg410) were replaced with alanine, was produced by polymerase chain reaction-based site-directed mutagenesis, and subcloned into pcDNA3 (Invitrogen, Carlsbad, CA) for transfection, or into pACT2 (CLONTECH, Palo Alto, CA) for two-hybrid analyses. The expression vector for the human AGPR subunit H1 and its tyrosine-to-alanine mutant, AGPR-H1(SA), was a gift from Dr. Martin Spiess. Tac-lysosomal-associated membrane protein-1 (Lamp-1) was made by polymerase chain reaction-based recombination and subcloned into pcDNA3 as described previously (Humphrey et al., 1993) and has the sequence of the luminal and transmembrane domains of Tac and the cytoplasmic domain of Lamp-1. In Tac-Lamp1YA, tyrosine in the cytoplasmic domain of Lamp-1 was substituted with alanine. cDNA encoding the human TfR (a gift from Dr. Juan S. Bonifacino, National Institutes of Health, Bethesda, MD) was subcloned into pcDNA3. Expression constructs for LDLR were described previously (Matter et al., 1992).

Yeast Two-Hybrid Analysis

The yeast strain H7C (CLONTECH) was maintained on YEPD (rich) medium. Transformation and two-hybrid analyses were performed as described in the instructions for the MATCHMAKER two-hybrid system (CLONTECH). In brief, GAL4-binding domain (bd) and GAL4-activation domain (ad) constructs were cotransformed into H7C. Half of the transformants were cultured on dropout media lacking leucine and tryptophan (indicated as -His) as a control of transformation, and half were plated on media lacking leucine, tryptophan, and histidine (denoted as –His). Transformants growing on the –His plate were judged positive for protein–protein interactions.

Cell Culture and Transfection

LLC-PK1 porcine kidney cells were cultured in Dulbecco’s modified Eagle’s medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma-Aldrich) (regular medium). LLC-PK1 cells stably transfected with human μIB (LLC-PK1:μIB) (Folsch et al., 1999) were grown in regular medium supplemented with 1.8 mg/ml genetin (Invitrogen). To obtain LLC-PK1 cells stably expressing M-μ1B, cells were transfected using the calcium phosphate precipitation, and the positive clones were selected and maintained in regular medium supplemented with 1.8 mg/ml genetin.

Immunoprecipitation, Gel Filtration, and Immunoblotting

LLC-PK1 transfectants were split in six-well plates 1 d before the experiment. The cells (on ice) were washed twice with ice-cold phosphate-buffered saline (PBS), and then buffer A (1% Triton X-100 [wt/vol], 0.3 M NaCl, 50 mM Tris-HCl [pH 7.4], 0.1% bovine serum albumin [wt/vol], and protease inhibitors [240 μg/ml pBASF, 2 μg/ml aprotinin, 157 μg/ml benzamidine, 10 μg/ml leupeptin, 10 μg/ml chymostatin, and 10 μg/ml pepstatin A]) was added. The cells were recovered using a cell scraper and passed four times through a 21-gauge needle. Lysis was judged complete after a 30-min incubation on ice. The lysates were clarified by centrifugation for 15 min at 13,000 rpm in an Eppendorf centrifuge at 4°C. The resulting supernatants were used for immunoprecipitation with the 100/3 anti-γ-adaptin antibody prebound to protein G-Sepharose.
Enhanced chemiluminescence system (Amersham Biosciences). For gel filtration analysis, 400 µl/well of buffer A, once with buffer A without Triton X-100, was subjected to SDS-PAGE, transferred onto Hybond-ECL membranes (Amersham Biosciences), immunoblotted with the anti-µ1B antibody or the anti-γ-adaptin antibody, and detected using the enhanced chemiluminescence system (Amersham Biosciences).

For immunofluorescence, 400 µl/well of buffer A without bovine serum albumin was used for lysis, and 200 µl of lysis supernatant was subsequently applied to a Superose 6 gel filtration column equilibrated with buffer B (0.5 mM EDTA, 1% Triton X-100, 0.3 M NaCl, 50 mM Tris-HCl [pH 7.4]). Fractions (0.5 ml) were collected and precipitated by adding trichloroacetic acid to a final concentration of 10% (wt/vol). Samples were resolved on SDS-PAGE and subjected to Western blot analysis by using anti-µ1B, anti-γ-adaptin, anti-α-adaptin, anti-α,3, and anti-β4 antibodies.

**Immunofluorescence**

LLC-PK1 cells stably expressing µ1B or M-µ1B were plated on polycarbonate membrane filters at a density of 5.6 × 10^4 cells/6.5-mm filter (Transwell units, 0.4-µm pore size; Corning-Costar, Corning, NY) and cultured for 4 d with daily changes of medium. Cells were transfected with the indicated expression plasmids by using GenePORTER2 (Gene Therapy Systems, San Diego, CA). After 2 d of incubation, the cells were washed twice with PBS, and the indicated antibodies were added to both the apical and the basolateral sides. After an incubation of 30 min at 4°C, cultures were washed twice with PBS and fixed in 3% paraformaldehyde/PBS for 15 min at room temperature. Subsequently, the filters were washed twice with PBS and incubated with the secondary antibodies Alexa Fluor 488 anti-mouse IgG for the apical side and Alexa Fluor 546 anti-mouse IgG for the basolateral side, respectively, for 1 h. When parental LLC-PK1 cells were stained, cells were plated at a density of 1.7 × 10^5 cells/12-mm filter (0.4-µm pore size), washed twice in PBS, incubated with the primary antibodies for 30 min at 4°C, washed twice in PBS, fixed in 3% paraformaldehyde/PBS, and incubated with Alexa Fluor 546 anti-mouse IgG for 1 h. This is because the parental cells usually fail to make a continuous monolayer. The filters were then cut off and washed four times with PBS over a period of 30 min.

For staining with Alexa Fluor 488 phalloidin, cells were cultured for 6 d with daily changes of medium, fixed in 3% paraformaldehyde, permeabilized with 0.1% Triton X-100, washed two times with PBS, and incubated for 30 min. Samples were analyzed using an LSM 510 laser scanning confocal microscope (Carl Zeiss, Thornwood, NY).

**Binding of Radiiodinated Antibodies**

Parental LLC-PK1 cells or cells stably expressing µ1B or M-µ1B were plated (at a density of 1.7 × 10^5 cells/12-mm filter) and transfected as described for immunofluorescence experiments. After 2 d of incubation, the cells were washed twice with ice-cold PBS, and the indicated antibodies were added from either the apical or basolateral side. After an incubation of 30 min at 4°C, cultures were washed twice with PBS and fixed in 3% paraformaldehyde/PBS for 15 min at room temperature. Subsequently, the filters were washed twice with PBS and incubated with secondary antibodies (anti-mouse or anti-rabbit IgG, 125I-labeled whole antibody), added to both sides of the filter membrane, for 1 h at room temperature. Finally, the filters were washed twice with PBS, cut off, and cell-associated radioactivity was measured with a gamma counter. Non-specific binding was determined by measuring binding to cultures incubated with the secondary antibodies alone, which were subtracted from the cell-associated radioactivity determined as described above. All given values represent the mean of three independent experiments performed in duplicate. Mean values of the three experiments for the sum of the apically and basolaterally associated radioactivity in parental, µ1B, and M-µ1B–expressing LLC-PK1 cells were 481, 443, and 524 cpm for AGFPR-H1 transfection; 296, 264, and 222 cpm for Tac-Lamp1 transfection; 133, 147, and 125 cpm for LDLR; and 143, 118, and 113 cpm for TIR transfection, respectively.

**RESULTS**

**Production of a Mutant µ1B Deficient at Binding Tyrosine-based Motifs**

To investigate the importance of µ1B-recognition of tyrosine-based sorting signals in basolateral sorting, we generated a mutant form of µ1B in which those residues possibly involved in tyrosine recognition were altered. The residues selected for mutagenesis were those identified from the µ2 crystal structure as being involved in binding YXXØ signals, four of which were precisely conserved in the µ1B sequence (Owen and Evans, 1998; Boniface and Dall’Angelica, 1999). Phe^{172}, Asp^{174}, Trp^{408}, and Arg^{410} were each replaced with alanines to produce the M-µ1B mutant. Initially, we examined the ability of this protein to bind tyrosine motifs in a yeast two-hybrid assay. We picked two YXXØ sequences from combinatorial library clones according to the previous study (Ohno et al., 1999); YWFL as a negative control and YRRL as a positive control, respectively, for µ1B binding. As expected, M-µ1B failed to interact with a test YXXØ motif, YRRL, which interacted with µ1B (Figure 1). Thus, altering the four conserved residues required for tyrosine interactions in µ2 greatly reduced the ability of µ1B to interact with YXXØ motifs.
stably expressing M-plexes. For this purpose, we established LLC-PK1 cell lines membranes, immunoblotted with the anti-
ants were subjected to SDS-PAGE, transferred onto Hybond-ECL (LLC-PK1::
We next asked whether the mutations introduced into
Mutant 1B is incorporated into AP-1B complexes.
We next tested another tyrosine-based basolateral sorting
We also determined the distribution of AGPR-H1(5A), in
We have demonstrated that 1B is required for the basolat-
erald sorting of membrane proteins containing basolateral
targeting signals, such as TfR and LDLR (Folsch et al., 1999,
Because 1B was incorporated into AP-1B complexes (Figure 2), we asked whether it could support the
targeting, such as Lamp-1. Lamp-1 is a lysosomal mem-
and subjected to Western blot analysis by using anti-AP
subunits as described in MATERIALS AND METHODS.
Mutant 1B (1B) Is Incorporated into AP-1B Complexes
We next asked whether the mutations introduced into
M-1B affected the incorporation of M-1B into AP-1B com-
opments. For this purpose, we established LLC-PK1 cell lines
ably expressing M-1B and determined whether an anti-gamma adaptin antibody could coprecipitate M-1B from these
cells. As previously shown, wild-type 1B coprecipitates with gamma adaptin, a large-chain adaptin of AP-1 (Figure 2A)
(Folsch et al., 1999, 2001). In the present study, M-1B was
detected in anti-gamma adaptin precipitates from lysates of three
stable cell lines expressing M-1B (Figure 2). This suggests that
M-1B is incorporated into AP-1B complexes.
We also verified that M-1B was specifically assembled
into AP-1 but not into the other AP complexes (i.e., AP-2,
AP-3, or AP-4). Cytosol from LLC-PK1 cells stably expressing
1B was fractionated by gel filtration chromatography on a Superose 6 column, and fractions were collected
and subjected to Western blot analysis by using anti-AP
subunit antibodies. As shown in Figure 2B, and consistent
with previous observations for 1B stably expressed in LLC-
PK1 (Folsch et al., 1999), M-1B was eluted in two peaks.
One peak coeluted with AP-1, as indicated by the presence of gamma adaptin in the same fractions. The second peak likely
represented unassembled monomeric M-1B, as previously
reported for 1B (Folsch et al., 1999). Figure 2B also showed
that the subunits of other AP complexes tested had different
elution profiles from M-1B. Our elution profiles are consistent with studies demonstrating that each AP complex exhibits
somewhat different apparent molecular weights (Dell’Angelica et al., 1997, 1999). In combination, these data suggest that
M-1B assembles into an AP-1 complex, with some not incor-
porating and existing as a monomer, in our LLC-PK1 cells.
Also, M-1B does not seem to incorporate into the other AP
complexes, a finding in agreement with previous studies of
1B. Comparison of the expression levels by immunoblotting
of the serial dilution of the lysates showed a similar amount of
1B expression for LLC-PK1::1B and LLC-PK1::M-1B.1 cells
(our unpublished data). The results presented in this study
were obtained using LLC-PK1::M-1B.1, but similar results
were observed using LLC-PK1::M-1B.2 cells (our unpub-
lished data).

Recognition of Tyrosine by 1B Is Required for
YXXO-Motif-dependent Basolateral Sorting In Vivo
We have demonstrated that 1B is required for the basolat-
erald sorting of membrane proteins containing basolateral
targeting signals, such as TfR and LDLR (Folsch et al., 1999,
2001). Because M-1B was incorporated into AP-1B complexes (Figure 2), we asked whether it could support the
proper targeting of basolateral membrane proteins, as does
1B. We first examined the steady-state localization on the
plasma membrane of AGPR-H1 transiently expressed in
filter-grown LLC-PK1 cells stably expressing 1B or M-1B.
AGPR-H1 is a basolateral membrane protein that cycles
between the plasma membrane and endosomes in hepato-
cytes and transfected Madin-Darby canine kidney (MDCK)
cells. A tyrosine-based sorting motif YQDL is essential for
both efficient internalization and polarized expression of
AGPR-H1 (Fuhrer et al., 1991; Geffen et al., 1993).

An analysis of the transfected AGPR-H1 localization by
using immunofluorescence confocal microscopy is pre-
sented in Figure 3. As expected, AGPR-H1 was localized
predominantly on the basolateral plasma membrane in
LLC-PK1::1B cells (Figure 3B). However, it was detected on
the apical and basolateral plasma membranes in
LLC-PK1::M-1B cells, much as it was when expressed in the
1B-negative parental LLC-PK1 cells (Figure 3, A and B). We
also determined the distribution of AGPR-H1(5A), in
which the tyrosine in the YQDL motif was substituted with
alanine (Geffen et al., 1993). Herein, AGPR-H1(5A) distrib-
uted on both apical and basolateral plasma membranes even
in LLC-PK1::1B cells (Figure 3B). These results were con-
firmed by a quantitative antibody binding assay (see below).

We next tested another tyrosine-based basolateral sorting
signal, YQTI, from Lamp-1. Lamp-1 is a lysosomal mem-
brane protein, and the YQTI sequence in its cytoplasmic tail

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has been reported to be required for direct lysosomal sorting, endocytosis as well as basolateral targeting (Hunziker et al., 1991; Harter and Mellman, 1992; Honing and Hunziker, 1995). We used a chimeric protein Tac-Lamp1, in which the luminal and transmembrane domains of Tac, the α subunit of interleukin-2 receptor, is appended with the Lamp-1 cytoplasmic tail containing the YQTI motif. Similar results were obtained as described above for AGPR-H1. As shown in Figure 4B, Tac-Lamp1 was primarily localized on the basolateral plasma membrane when expressed in LLC-PK1:μ1B cells. In contrast, it was expressed both apically and basolaterally in LLC-PK1:M-μ1B cells as well as in parental LLC-PK1 cells (Figure 4, A and B). Tac-Lamp1,YA, bearing a tyrosine-to-alanine substitution in YQTI motif, was similarly expressed on both apical and basolateral plasma membranes in LLC-PK1:μ1B cells, as expected (Figure 4B).

Finally, we determined the steady-state distribution of AGPR-H1 and Tac-Lamp1 quantitatively (Figure 7, A and B). As expected from the qualitative immunofluorescence results, both AGPR-H1 and Tac-Lamp1 were predominantly (80–90%) expressed at the basolateral surface of LLC-PK1:μ1B cells but were randomly expressed on both the apical and basolateral plasma membranes in parental LLC-PK1 as well as in LLC-PK1:M-μ1B cells.

Taken together, these results suggest that the tyrosine-based basolateral sorting signals of AGPR-H1 and Lamp-1 require interactions with the presumptive tyrosine-binding pocket of μ1B for proper basolateral targeting in vivo.

Tyrosine Binding by μ1B Is Not Required for Basolateral Targeting of TfR and LDLR

We next studied the steady-state plasma membrane distribution of TfR transiently expressed in parental LLC-PK1 cells and LLC-PK1 cells containing μ1B or M-μ1B (Figure 5). As shown previously (Folsch et al., 1999), the localization of TfR at the basolateral surface of LLC-PK1 cells was dependent on μ1B expression. Interestingly, and in contrast to results obtained for AGPR-H1 and Lamp-1, TfR was also found at the basolateral surface of cells expressing M-μ1B. Although the basolateral targeting signal of TfR has not been precisely defined, it is clear that the signal is distinct from the tyrosine-containing motif (YTRF) that is required for TfR endocytosis in clathrin-coated pits (Dargemont et al., 1993; Odorizzi and Trowbridge, 1997).

We next determined whether basolateral localization of LDLR was dependent on the tyrosine-recognition ability of μ1B. The LDLR cytoplasmic domain contains two basolateral targeting signals, both of which depend on critical tyrosines but only one of these (tyrosine 18) is also required for
endocytosis. The second signal (involving tyrosine-35) is the dominant of the two and interacts with μ1B (Folsch et al., 2001). As shown in Figure 6, and like TfR, LDLR was targeted to the basolateral plasma membrane of LLC-PK1 cells expressing either μ1B or M-μ1B, although it is predominantly expressed on the apical plasma membrane in parental LLC-PK1 cells.

The polarized distribution of both TfR and LDLR was then determined by a quantitative antibody binding assay (Figure 7, C and D). As found previously in parental LLC-PK1 cells (Folsch et al., 1999), TfR was randomly distributed on the apical and basolateral plasma membranes, whereas LDLR was predominantly (75%) expressed at the apical plasma membrane; the latter finding is consistent with the notion that LDLR possesses a recessive apical determinant (Matter et al., 1992; Matter and Mellman, 1994). As expected from the immunofluorescence data (Figures 5 and 6), both TfR (~90%) and LDLR (80%) were predominantly expressed on the basolateral plasma membrane in LLC-PK1::M-μ1B cells as well as LLC-PK1::μ1B cells.

Taken together, these results suggest that the basolateral targeting signals of TfR and LDLR do not require the tyrosine-motif binding ability of μ1B for their proper targeting to the basolateral plasma membrane. This feature is consistent with the fact that, unlike AGPR-H1 and Lamp-1, neither depends exclusively on a tyrosine-containing endocytosis-type signal for polarity.

**Monolayer Formation of LLC-PK1 Cells Is Supported in the Presence of M-μ1B as Well as μ1B**

LLC-PK1 cells, unlike MDCK cells, do not always produce perfect monolayers typical of epithelial cells in culture, but occasionally pile up instead (Folsch et al., 1999). Expression of μ1B in LLC-PK1 cells corrects this phenotype resulting in monolayer-type growth (Folsch et al., 1999). Herein, we took advantage of this morphological difference in LLC-PK1 cells in the presence or absence of μ1B to measure the function of M-μ1B in monolayer formation. When LLC-PK1 cells expressing M-μ1B were grown on filter membranes, they grew in monolayers similar to cells expressing μ1B (Figure 8). This finding suggests that the molecule(s) required for growth of LLC-PK1 cells in a monolayer depend on the presence of μ1B or M-μ1B for function, but do not seem to require the tyrosine-binding ability by μ1B.

**DISCUSSION**

Although it is clear that expression of μ1B plays a critical role in ensuring the polarized targeting of a wide array of basolateral plasma proteins in epithelial cells, little is understood about how this one AP-1B subunit interacts with the diverse set of basolateral sorting signals it seems to decode. Some basolateral signals depend on tyrosine residues that are also critical for AP-2-dependent clathrin-mediated endocytosis (e.g., Lamp-1 and AGPR-H1), some depend on tyrosines that are not required for endocytosis (LDLR), and some do not involve tyrosine residues at all (TfR). We characterized the importance of tyrosine recognition by replacing in four residues conserved among μ family members thought to be important for tyrosine binding (Owen and Evans, 1998). A similar strategy has successfully been applied to study the importance of tyrosine recognition by μ2 in endocytosis (Nesterov et al., 1999). Although the mutant μ1B (M-μ1B) was incorporated into functional AP-1B complexes, it lost the ability to decode tyrosine-dependent basolateral signals, or at least those that share tyrosines important for endocytosis such as AGPR-H1 and Lamp-1.
In contrast, basolateral expression of TfR and LDLR was not obviously affected by the removal of residues required for tyrosine binding \( \mu \)1B. It has been reported that the basolateral sorting of TfR is mainly determined by the GDNS sequence downstream of the YTRF endocytosis/coated pit localization signal (Dargemont et al., 1993; Odorizzi and Trowbridge, 1997). Although the precise features of the TfR basolateral targeting signal have yet to be characterized, it is clear that the tyrosine required for endocytosis is not involved. Thus, it was interesting to learn that four residues in \( \mu \)1B that are required for the coordination of tyrosine-containing determinants were not required for the basolateral targeting of TfR.

LDLR was an even more interesting case. This receptor's cytoplasmic tail contains two independent basolateral targeting determinants, both of which are tyrosine-dependent for activity (Matter et al., 1992, 1994). The membrane proximal signal overlaps with, but is distinct from, the NPVY endocytosis signal. The distal signal's critical tyrosine, on the other hand, does not direct endocytosis. Basolateral expression of LDLR was not affected by the \( \mu \)1B mutations, suggesting that at least one of the LDLR basolateral signals does not bind to the tyrosine-binding pocket of \( \mu \)1B. This may not be surprising, because the sequence surrounding the tyrosine of either signal does not conform to the canonical YXY\( \Omega \) sequence that is recognized by \( \mu \) chains, including \( \mu \)1B (Ohno et al., 1995, 1999; Boll et al., 1996; Dell'Angelica et al., 1997; Aguilar et al., 2001). Moreover, recent work has demonstrated that it is the distal basolateral targeting signal in LDLR that serves primarily to control basolateral targeting of this receptor (Koivisto et al., 2001).

Based on the present study, together with previous reports (Roush et al., 1998; Folsch et al., 1999), basolateral sorting signals so far identified can be divided into at least the following three classes. First, there are signals such as the dileucine-based determinant found in the IgG receptor FcRRII-B2 (Hunziker and Fumey, 1994; Matter et al., 1994), which can mediate basolateral targeting in the absence of \( \mu \)1B. Second, YXX\( \Omega \)-type basolateral signals such as those in AGPR-H1 (Fuhrer et al., 1991; Geffen et al., 1993) and Lamp-1 (Hunziker et al., 1991; Honing and Hunziker, 1995), which require the interaction of a critical tyrosine residue with \( \mu \)1B for their sorting function. This same tyrosine is also required for rapid endocytosis of these membrane proteins via the AP-2 adapter complex. Finally, signals such as those in TfR and LDLR, which clearly require the presence of \( \mu \)1B (and by extension the AP-1B complex), but not the ability of \( \mu \)1B to bind tyrosine via \( \mu \)1B residues required for interacting with tyrosines involved in endocytosis.

Although it is clear that basolateral proteins such as LDLR and TfR interact directly and selectively with the AP-1B adaptor complex, the actual interacting subunit has not been identified. A priori, \( \mu \)1B is the most likely candidate. It is clear that its homolog \( \mu \)2 directly binds the internalization motifs in endocytic receptors. Moreover, the single substitution of \( \mu \)1B for \( \mu \)1A in the AP-1B complex switches the affinity of the complex from those proteins involved in TGN/endosome transport in all cells to proteins that are transported to the basolateral surface of epithelial cells. Only 47 (of \( \sim 270 \)) amino acids differ between the carboxyl-terminal domains of \( \mu \)1A and \( \mu \)1B. The \( \mu \)1 carboxyl-terminal domain is thought to protrude from the trunk of the AP-1 complex and to be important for interactions with sorting signals (Owen and Evans, 1998; Bonifacino and Dell'Angelica, 1999). These carboxyl-terminal \( \mu \)1B residues may, therefore, participate in
providing the binding surface for the signals from Tfr and LDLR. Alternatively, these signals may bind to a region of \( \mu \)1B that overlaps where the YXXO-type signal binds, but bind in a different register, or perhaps interacting with different residues in this region. Some flexibility in the mode of interaction of internalization signals with \( \mu \)2 has recently been observed (Owen et al., 2001).

Another explanation, although we think it less likely, is that the signals could interact with AP-1B subunits other than \( \mu \)1B. The presence of AP-1A cannot support the basolateral sorting of Tfr or LDLR (Roush et al., 1998; Folsch et al., 1999). Because AP-1A and AP-1B are believed to share the subunits other than \( \mu \)1A and \( \mu \)1B (Folsch et al., 1999), it is difficult to imagine that these common subunits cause the difference in sorting phenotype. Nevertheless, it might be possible that the difference between \( \mu \)1A and \( \mu \)1B could cause the conformational change(s) of the other subunits to generate the binding surface for the basolateral sorting signals from Tfr and LDLR. Thirty-six residues differ between \( \mu \)1A and \( \mu \)1B in their amino-terminal domains, the region thought to be involved in mediating interactions with other adaptor subunits; conceivably, alterations in such interactions may lead to alterations in substrate specificity. Final understanding of how \( \mu \)1B can accommodate such seemingly different signals for such a common, fundamental function as polarized targeting in epithelia will require direct structural information on the \( \mu \)1B and adaptors in general.

Finally, it should be pointed out that the precise site of action of AP-1B in polarized sorting remains to be determined. Other kidney epithelial cells, such as MDCK cells, have been shown to sort apical from basolateral proteins upon their emergence from the Golgi complex, before their first appearance at the plasma membrane. Hepatocytes, which are \( \mu \)1B negative, sort by an indirect route whereby both apical and basolateral proteins are transported from the Golgi to the basolateral surface from which they are internalized and then sorted from each other in endosomes. Because in MDCK cells the signals that mediate biosynthetic and endocytic basolateral sorting are similar (Matter et al., 1994; Odorizzi and Trowbridge, 1997), it is conceivable that \( \mu \)1B acts on both pathways. Indeed, there is ample evidence that AP-1 adaptors can be found at the TGN and in endosomes (Futter et al., 1998; Folsch et al., 2001). It is also possible that expression of \( \mu \)1B confers upon the TGN the ability to mediate apical vs. basolateral sorting. Thus, it is possible that LLC-PK1 cells sort indirectly (like hepatocytes), whereas \( \mu \)1B-expressing LLC-PK1 cells sort directly (like MDCK cells). The fact that a tyrosine mutant of AGPR-H1 was found apically argues against indirect sorting in \( \mu \)1B-expressing LLC-PK1 cells. For such a mutant to reach the apical surface by the indirect route, transcytosis from the basolateral domain would be required. Yet, transcytosis might be rendered less efficient because the same tyrosine residue required for basolateral targeting is also required for rapid endocytosis.

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