Antibody to AP1B Adaptor Blocks Biosynthetic and Recycling Routes of Basolateral Proteins at Recycling Endosomes[□]

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The epithelial-specific adaptor AP1B sorts basolateral plasma membrane (PM) proteins in both biosynthetic and recycling routes, but the site where it carries out this function remains incompletely defined. Here, we have investigated this topic in Fischer rat thyroid (FRT) epithelial cells using an antibody against the medium subunit μ 1B. This antibody was suitable for immunofluorescence and blocked the function of AP1B in these cells. The antibody blocked the basolateral recycling of two basolateral PM markers, Transferrin receptor (TfR) and LDL receptor (LDLR), in a perinuclear compartment with marker and functional characteristics of recycling endosomes (RE). Live imaging experiments demonstrated that in the presence of the antibody two newly synthesized GFP-tagged basolateral proteins (vesicular stomatitis virus G [VSVG] protein and TfR) exited the *trans*-Golgi network (TGN) normally but became blocked at the RE within 3–5 min. By contrast, the antibody did not block trafficking of green fluorescent protein (GFP)-LDLR from the TGN to the PM but stopped its recycling after internalization into RE in ~45 min. Our experiments conclusively demonstrate that 1) AP1B functions exclusively at RE; 2) TGN-to-RE transport is very fast and selective and is mediated by adaptors different from AP1B; and 3) the TGN and AP1B-containing RE cooperate in biosynthetic basolateral sorting.

INTRODUCTION

Epithelial cells display an asymmetric distribution of plasma membrane (PM) proteins into apical and basolateral PM domains (Yeaman *et al.*, 1999; Mostov *et al.*, 2003; Rodriguez-Boulan *et al.*, 2005). Early studies demonstrated that this polarity was achieved by sorting of newly synthesized proteins at the level of the *trans*-Golgi network (TGN; Rindler *et al.*, 1984; Fuller *et al.*, 1985; Griffiths and Simons, 1986) and of endocytosed proteins at the level of recycling endosomes (RE; Matter and Mellman, 1994; Mostov and Cardone, 1995; Odorizzi and Trowbridge, 1997). Trafficking in both biosynthetic and recycling routes is controlled by apical sorting signals (e.g., N- and O-glycans, lipid anchors, and protein

domains with affinity for lipid rafts), by microtubule motor determinants (Rodriguez-Boulan and Gonzalez, 1999; Schuck and Simons, 2004; Rodriguez-Boulan *et al.*, 2005) and by basolateral sorting signals (e.g., tyrosine, dileucine, and monoleucine motifs, some similar to endocytic determinants, and noncanonical motifs not yet matching any consensus sequence; Matter *et al.*, 1994; Rodriguez-Boulan *et al.*, 2005). Tyrosine-based signals are recognized by a family of organelle-specific tetrameric AP (adaptor protein) adaptors via their μ subunit, whereas dileucine motifs may be recognized via large (γ and δ) and small (σ 1, σ 3) subunits of AP adaptors acting together (Bonifacino and Lippincott-Schwartz, 2003; Janvier *et al.*, 2003).

The early paradigm of two separate sorting sites for proteins in biosynthetic or recycling pathways has, however, progressively shifted over the past decade to a different paradigm in which both TGN and RE share a sorting role in the biosynthetic route. This new paradigm emerged from the observations by several laboratories showing that newly synthesized PM proteins leaving the Golgi apparatus may traverse the endosomal compartment before arrival at the cell surface (Futter *et al.*, 1995; Leitinger *et al.*, 1995; Brachet *et al.*, 1999; Orzech *et al.*, 2000; Ang *et al.*, 2003; Lock and Stow, 2005). These early observations, however, did not elucidate the magnitude of the transendosomal route nor the individual contribution of TGN or RE to the biosynthetic sorting of individual proteins. The molecular sorting ma-

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Abbreviations used: AP, adaptor protein (complex); ARE, apical recycling endosomes; FRT, Fisher rat thyroid; LDLR, low-density lipoprotein receptor; RE, recycling endosomes; TfR, transferrin receptor; VSVG, vesicular stomatitis virus glycoprotein G.

chinery operating in each compartment is also poorly understood.

Some insight into these important questions was provided by the discovery of the basolateral sorting adaptors AP1B (Ohno *et al.*, 1999) and AP4 (Simmen *et al.*, 2002). The role of AP4 in basolateral sorting remains poorly understood. By contrast, considerable advances have been made in our understanding of AP1B function (Folsch, 2005; Ellis *et al.*, 2006). AP1B is a variety of AP1 that displays an epithelial-specific subunit, μ 1B, instead of the more ubiquitous μ 1A subunit found in AP1A (Ohno *et al.*, 1999). Reconstitution of μ 1B into LLC-PK1 epithelial cells, which lack this protein, showed that AP1B is required for the correct sorting of a group of basolateral proteins (Folsch *et al.*, 1999; Gan *et al.*, 2002; Sugimoto *et al.*, 2002; Folsch *et al.*, 2003). Early electron microscopy (EM) studies suggested that AP1B sorts basolateral proteins in the TGN (Folsch *et al.*, 2001). However, more detailed functional and immunofluorescence experiments showed that AP1B sorts transferrin receptor (TfR) and LDL receptor (LDLR) postendocytically in LLC-PK1 cells, probably at the level of RE (Gan *et al.*, 2002; Folsch *et al.*, 2003).

More recently, however, μ 1B knockdown experiments in Madin-Darby canine kidney (MDCK) cells demonstrated for the first time that AP1B sorts two basolateral proteins, VSVG and TfR, in the biosynthetic route (Gravotta *et al.*, 2007). Interestingly, for TfR this was only true in recently polarized (1–2 d confluent) MDCK cells, as in 4–5 d confluent monolayers TfR followed an AP1B-independent biosynthetic route, suggesting that as monolayers develop their polarity, they establish a direct route from TGN to PM (Gravotta *et al.*, 2007). Cell fractionation data demonstrated enrichment of endogenous AP1B in endosomal fractions in both 1- and 4.5-d polarized monolayers, suggesting that basolateral sorting in the biosynthetic route might be carried out in RE. However, the experiments did not quantitatively demonstrate the transport of basolateral proteins between TGN and RE or directly show that the sorting role of AP1B took place at RE. Similarly, another report (Ang *et al.*, 2004) demonstrated that ablation of RE with horseradish peroxidase (HRP)-peroxidase blocked transport of VSVG in the biosynthetic route, but did not demonstrate that under those conditions a significant mass of VSVG had left the TGN and quantitatively moved to RE, and more precisely, to RE containing AP1B.

Finally, a recent study (Fields *et al.*, 2007) reported the interaction of various basolateral proteins with different AP adaptors by a yeast two-hybrid approach, but the data obtained provide no information on specific routes in which these adaptors perform their function or on the role of RE in biosynthetic transport. Thus, the role of RE in biosynthetic transport and the sorting role of AP1B in RE is currently supported only by circumstantial evidence.

Here, we utilized a function-blocking antibody against μ 1B in Fischer rat epithelial (FRT) cells, a model epithelial cell line with similar sorting characteristics as MDCK cells (Zurzolo *et al.*, 1993), and quantitative live imaging to study these important questions in detail. Because the experiments were carried out in recently confluent FRT cells, to facilitate live imaging, our data can be compared with the results obtained by Gravotta *et al.* (2007) in recently (1–2 d) confluent MDCK cells. Our experiments demonstrate that two basolateral proteins, VSVG and TfR, move quantitatively with very fast kinetics from TGN to RE ($t_{1/2} \sim 3$ –5 min) and are blocked by AP1B antibody at the level of RE in the biosynthetic route. By contrast, LDLR moves from TGN to RE with slow kinetics (~ 45 min), reflecting that it traffics first from PM to the basolateral surface, before being

stopped by the AP1B antibody at the level of RE. Our data demonstrate directly and quantitatively, for the first time, that some newly synthesized basolateral proteins move obligatorily through AP1B-containing RE and that AP1B performs its basolateral sorting role in RE.

MATERIALS AND METHODS

Plasmids

A carboxyterminal spacer-GFP-tagged version of VSVGts045 (vesicular stomatitis virus G [VSVG] protein-green fluorescent protein [GFP]) was constructed by cloning the tsO45-VSVG cDNA into NheI/XhoI sites of pEGFP-N1 plasmid, followed in frame by the spacer 5'-ATT-CIT-TCA-GGG-GGA-TCA-GGG-GGA-TCA-GGG-GGA-TCA-GGG-ATA-GGG-GAA-GGG-3' inserted in EcoRI/BamHI sites. p-CB6-LDLR-GFP and p-CB6-LDLR-YA18-GFP plasmids were provided by K. Matter (University College London, United Kingdom). Plasmids for dominant-negative proteins: Eps15 (GFP-Eps15-H29; previously called GFP-EA95/295; Benmerah *et al.*, 1999) provided by A. Benmerah (Institut Cochin, U567 INSERM/UMR8104 CNRS, Paris France), protein kinase D2 (pME-Py-GST-PKD2-KD; Yeaman *et al.*, 2004) provided by V. Malhotra (University of California, San Diego). The VSVG3-GFP plasmid (Toomre *et al.*, 1999) encoding an apical version of tsO4-VSVG-GFP (Ang *et al.*, 2004; Yeaman *et al.*, 2004) was provided by K. Simons (Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany). The vector for Rab11-GFP was a gift of M. Zerial (Max Planck Institute of Molecular Cell Biology and Genetics).

Antibodies

μ 1B- and μ 1A/B-specific antibodies were produced by immunizing rabbits with synthetic peptides conjugated with mollusk *Concholepas concholepas* hemocyanin (Blue Carrier, Biosonda Biotechnology, Santiago, Chile). The peptides correspond to N-terminal 44–56 residues (K-GALAPLLSHGQVH) and C-terminal 366–378 residues (CK-EKEEVEGRPPIGV) of human μ 1B (Folsch *et al.*, 1999) and 261–271 residues (CK-VLFNTGRGKS) of μ 1A. These antibodies were affinity-purified. Immunoglobulin G (IgG) from preimmune sera (PI-IgG) was purified with Econo-Pac Serum IgG Purification Kit (Bio-Rad, Richmond, CA). Secondary antibodies: Alexa488/555-tagged goat-anti-mouse and goat-anti-rabbit (Molecular Probes, Eugene, OR). Monoclonal antibodies were as follows: anti-TGN38 and anti-EEA-1 (Affinity BioReagents, Golden, CO), anti- γ -adaptin clone 100/3 (Sigma, St. Louis, MO), anti- γ -adaptin (BD Transduction Laboratories, Lexington, KY), anti-TfR (Zymed, South San Francisco, CA) and anti-ZO-1 (Chemicon, Temecula, CA). Polyclonal antibodies were as follows: anti-Rab11 antibody (Affinity BioReagents), HRP-conjugated secondary anti-rabbit (Chemicon), and anti-mouse antibodies (Rockland, Gilbertville, PA).

Glutathione S-Transferase-Fusion Protein Purification

Recombinant human μ 1A or μ 1B glutathione S-transferase (GST)-fusion protein were produced in transformed *Escherichia coli* cells induced with 0.2 mM isopropyl-1-thio- β -D-galactopyranoside (Invitrogen, Carlsbad, CA) for 3 h. GST-fusion proteins were purified by affinity chromatography on glutathione-Sepharose as described by the manufacturer.

Cell Culture and Microinjection

LLC-PK1 and LLC-PK1- μ 1B cells (Gan *et al.*, 2002) were maintained in DMEM (Invitrogen-BRL) supplemented with 5% FBS (Invitrogen), L-glutamine and nonessential amino acids (GeminiBio-Products, Woodland, CA). FRT cells were maintained in F-12 Coon's modification (Sigma) supplemented with 10% fetal bovine serum (FBS). The cells were plated at subconfluent levels on glass coverslips and microinjected 1 d after reaching confluence. The expression plasmids (25 μ g·ml⁻¹) and antibodies (40 μ g·ml⁻¹), were prepared in HKCl microinjection buffer (Kreitzer *et al.*, 2000; 10 mM HEPES, 140 mM potassium chloride, pH 7.4). The mix was centrifuged at 14,000 rpm in an Eppendorf-refrigerated centrifuge (Fremont, CA) for 15 min at 4°C. Microinjections were made in the nucleus (plasmids) and cytosol (antibodies) using back-loaded glass capillaries and an Eppendorf Transjector 5246 system mounted on a Zeiss Axiovert S100 inverted microscope (Thornwood, NY), keeping the cells in bicarbonate-free DMEM supplemented with 5% FBS, 20 mM HEPES. After incubating the cells for 1 h at 37°C, the medium was changed to serum-free medium supplemented with 100 μ g·ml⁻¹ cycloheximide for the rest of the experiment. Control experiments for μ 1Bn antibody were made by preincubating the antibody for 30 min at 37°C in HKM buffer (25 mM HEPES-KOH, pH 7.6; 50 mM KCl; 5 mM MgCl₂) with either the N-terminal (μ 1Bnp) or C-terminal (μ 1Bcp) peptides.

Indirect Immunofluorescence

Indirect immunofluorescence was performed as described (Soza *et al.*, 2004). Briefly, cells grown on coverslips were washed in phosphate-buffered saline (PBS) supplemented with 0.1 mM CaCl₂ and 1 mM MgCl₂ (PBS-CM) and

fixed in freshly prepared PBS-CM 4% paraformaldehyde (Merck, Rahway, NJ) for 30 min. Cells were permeabilized in PBS-CM with 0.1% Triton X-100 for 10 min and blocked with PBS-CM 0.2% gelatin (Sigma type Bloom G2500, PBS-CM-G) for 10 min at room temperature. Incubation with specific primary antibodies against μ 1B (1:200), TGN38 (1:250), TfR (1:500), Early Endosomal Antigen-1 (EEA-1) (1:50), γ -adaplin (1:100), GST (1:500), and Rab-11 (1:50) was done during 30 min at 37°C in PBS-CM-G buffer. Cells were subsequently labeled with appropriate fluorescent-conjugated secondary antibodies Alexa 488/555-tagged goat-anti-mouse and goat-anti-rabbit (1:2500). Experiments with μ 1Bn antibody blocked with either μ 1Bnp or μ 1Bcp peptides included 1% DMSO in the incubation buffer.

Permanent Silencing of μ 1B in FRT Cells

Stable knockdown clones were generated by transfecting FRT cells with a retroviral vector carrying a μ 1B-targeting sequence. A 60-mer sense (5'-GATCCCCGAACGAGGTCTTCATTGATTCAAGAGAATCAATGAAGACCTCGTTCTTTTC-3') and antisense (5'-TCGAGAAAAAGAACGAGGTCTTCATTGATTCTCTTGAAATCAATGAAGACCTCGTTCCGGG-3'). Oligonucleotide encoding the 19-m34 μ 1B-targeted sequence with a BglIII/XhoI

restriction sites were annealed and cloned into a pSuper.retro.puro vector (OligoEngine, Seattle, WA) following the manufacturer's protocol. Cells were selected for puromycin resistance and characterized by Western blotting.

TGN Block and Time-Lapse Fluorescence Microscopy

Microinjected cells expressing GFP-tagged proteins for 1 h at 37°C were cooled at 4°C and then placed in a 20°C incubator for 2 h in the presence of 100 μ g/ml⁻¹ cycloheximide. Cooling was necessary to achieve a better block of the proteins at the TGN with minimal spill into the μ 1B compartment. After the 20°C block, the cells were cooled again on ice before reestablishing the transport at 37°C or 32°C for VSVG-GFP. This was done in the absence of FBS to improve the detection of the proteins at the cell surface. The cells were either processed for immunofluorescence or mounted on a POC-R thermal-controlled chamber (Zeiss) for time-lapse imaging *in vivo*. Images were collected at 10-min intervals.

Image Acquisition and Analysis

Digital fluorescence images were acquired on a Zeiss Axiophot microscope with a Plan-APOCHROMAT 63 $\times$ /1.4 oil immersion objective and the 14-bit

Figure 1. A polyclonal μ 1B antibody (μ 1Bn-Ab) suitable for immunofluorescence. (A) Immunogenic peptides used to produce antibodies to N- (μ 1Bn) and C-terminal (μ 1Bc) regions of μ 1B and to the indicated region of μ 1A. (B) Immunoblots with affinity-purified antibodies to the peptides described in A were performed on partially isolated recombinant μ 1B-GST and μ 1A-GST produced in *E. coli*. (C) LLC-PK1 cells microinjected with expression vector encoding human μ 1B (arrowheads) display intense fluorescence upon staining with μ 1Bn-Ab, whereas surrounding control cells are negative. Bar, 10 μ m. (D) FRT cells permanently knocked down for μ 1B (μ 1B-KD) with RNAi. Note the perinuclear localization of μ 1B in control cells, by indirect immunofluorescence with μ 1Bn-Ab, and the decreased staining of μ 1B-KD cells. Measurement of integrated fluorescence with MetaMorph software demonstrated ~50% decrease in μ 1B expression in μ 1B-KD cells (bar graph, mean $\pm$ SD; n = 60 cells). Bar, 10 μ m. (E) Immunoblot with μ 1Bc and μ 1A antibodies show ~50% decrease of μ 1B expression in μ 1B-KD cells (bar graph, mean $\pm$ SD; three independent experiments). (F) Indirect immunofluorescence of FRT cells stained with μ 1Bn-Ab. Control cells show perinuclear staining that disappears in the presence of μ 1Bn peptide (μ 1Bnp) but not μ 1Bc peptide (μ 1Bcp). Bar, 10 μ m. (G) Comparison of human, rat, and dog μ 1Bn region. Note that the canine sequence differs from the human sequence only in the conservative change of an arginine for a lysine (letters in bold), a change that is also present in rat μ 1Bn.

Zeiss Axiocam camera, transferred to a computer workstation running Axiovision imaging software (Zeiss), processed, and analyzed with MetaMorph imaging software (Universal Imaging, West Chester, PA). For quantification, all images from a single experiment were acquired under identical settings (14 bits; 1300×1030 pixels, and the same exposure times, avoiding signal saturation). Their integrated fluorescence intensities (equivalent to the sum of all grayscale values for every pixel in the region) were analyzed after 2D deconvolution and threshold adjustment to select the perinuclear region. The percentage of colocalization, measured as integrated pixel intensity in the regions of overlap of LDLR-GFP, VSVG-GFP α and TfR-GFP with either μ 1B or TGN38 (detected by immunofluorescence) was calculated for each individual cell ($n = 20$ – 30). Laser scanning microscopy was used to assess the polarity of FRT cells under the same conditions of the microinjection experiments. Images were acquired on a LSM 5 Zeiss microscope using 488/543 laser and a $63\times/1.4$ oil immersion objective.

RESULTS

A Polyclonal Antibody That Recognizes μ 1B by Immunofluorescence

A caveat of all μ 1B localization studies to date is that they were carried out in transfected cells overexpressing epitope-tagged μ 1B (Folsch *et al.*, 1999, 2001, 2003; Gan *et al.*, 2002). None of the anti- μ 1B subunit antibodies generated to date (Folsch *et al.*, 1999; Gravotta *et al.*, 2007) have turned out to be suitable for immunofluorescence. We raised rabbit antibodies against described N- (μ 1Bn) and C-terminal (μ 1Bc) antigenic peptides of human μ 1B (Folsch *et al.*, 1999) and a peptide of μ 1A (Gravotta *et al.*, 2007; Figure 1A). Both μ 1B-specific antibodies (μ 1Bn-Ab and μ 1Bc-Ab) distinguished in immunoblots human μ 1B from the 80% identical μ 1A (Ohno *et al.*, 1999) produced in *E. coli* (Figure 1B). Surprisingly, μ 1Bn-Ab turned out to be suitable for immunofluorescence. This antibody decorated human μ 1B expressed by microinjection of its cDNA in LLC-PK1 cells, whereas nonmicroinjected cells showed no staining (Figure 1C). μ 1Bn-Ab decorated as well endogenous μ 1B expressed by polarized FRT cells (Figure 1, D and F), but not endogenous μ 1B expressed by MDCK cells (data not shown). In FRT cells, μ 1Bn-Ab decorated mainly a perinuclear compartment (Figure 1D). An FRT cell line in which μ 1B was permanently knocked down by RNAi (μ 1B-KD) exhibited decreased levels of μ 1B, as detected by immunofluorescence with μ 1Bn-Ab and by

immunoblot with μ 1Bc-Ab (Figure 1, D and E). The immunofluorescence staining was specifically competed by the μ 1Bn peptide but not by the μ 1Bc peptide (Figure 1F). These observations indicate that the μ 1Bn-Ab specifically recognizes endogenous μ 1B in FRT cells. It seems unlikely that the lack of reaction with canine μ 1B reflects a difference in the sequence between canine and rat/human μ 1B peptide used as immunogen (Figure 1G). Rather, it must reflect a different organization of the AP1B adaptor in different cells (see *Discussion*).

Endogenous AP1B Associates in a BFA-sensitive Manner with RE, Which in Contrast with TGN Are Not Affected by Protein Kinase D

Published work indicates that μ 1B colocalizes better with endosomal than with TGN markers, whereas the opposite is true for μ 1A (Gan *et al.*, 2002; Folsch *et al.*, 2003). In agreement with these observations, the perinuclear compartment of FRT cells that contained endogenous μ 1B was enriched in the recycling endosomal marker TfR and was depleted of the TGN marker TGN38 (Figure 2A, fluorescence panels). Quantitative image analysis demonstrated $72.3 \pm 1.9\%$ colocalization between μ 1B and TfR and $23.7 \pm 0.9\%$ colocalization between μ 1B and TGN38 (Figure 2A, graph). Consistently, γ -adaptin, a subunit shared by both AP1B and AP1A adaptors, showed an intermediate extent of colocalization with μ 1B (35%), consistent with the fact that a substantial amount of this protein localizes to TGN as a component of AP1A (Figure 2A, graph). EEA1, a marker of sorting endosomes (the earliest of the early endosomes) colocalized very poorly with μ 1B (Figure 2A). Rab11, which in polarized MDCK cells localizes to apical recycling endosomes (ARE) rather than to Tf-rich common RE (Brown *et al.*, 2000), did not colocalize either with TfR in FRT cells (Figure 2A). Although our rabbit μ 1B antibody precluded colocalization studies with the polyclonal antibody to Rab11, we observed that Rab11-GFP expressed by microinjection did not colocalize with μ 1B (not shown), thus confirming that the μ 1B compartment is distinct from ARE and providing additional evidence for its identification as the equivalent of

Figure 2. Characterization of the μ 1B compartment stained by μ 1Bn-Ab: correspondence with recycling endosomes and not with TGN, and BFA sensitivity. (A) Left fluorescence panels, FRT cells were fixed and processed for double indirect immunofluorescence with μ 1Bn-Ab or anti-Rab-11 antibody (red) and monoclonal antibodies against γ -adaptin, TGN38, TfR, and EE1A (green). Top fluorescence panels, merged images with the region enclosed by a square magnified in the bottom panels. Each fluorescence image was processed by 2D deconvolution. Bar, 10 μ m. Right bar graph, integrated fluorescence intensity colocalization values calculated for individual cells ($n = 40$), as indicated in *Materials and Methods*. (B) BFA abrogates the association of AP1B with perinuclear compartment. Treatment of FRT cells with 5 μ g/ml BFA for 30 min results in disappearance of μ 1B fluorescence and reduction of γ -adaptin staining, suggesting that the association of AP1B with RE is dependent on ARF, similar to AP1A in the TGN. Bar, 10 μ m.

Figure 3. The μ 1B compartment is functionally distinct from the TGN in its protein kinase D (PKD)-dependency. FRT cells were injected intranuclearly with an expression vector encoding a kinase-dead form of PKD linked to GST (PKD2-KD), incubated for 1 h at 37°C and then for an additional hour in the presence of cycloheximide (100 μ g/ml). The cells were then processed for double immunofluorescence with a goat polyclonal antibody against GST (green) and μ 1Bn-Ab or monoclonal antibodies against TfR or TGN38 (red). Note that expression of PKD2-KD promotes tubulation of the TGN38 compartment, but no tubulation of the μ 1B or TfR compartments. Quantitative assessment of the images shows PKD2-KD colocalizing mainly with TGN38 (top graph; $n = 20$ cells) and producing tubules that contain TGN38 but not μ 1B or TfR (bottom graph; $n = 60$ cells). Arrowheads point to tubules displaying TGN38 and PKD2-KD.

common RE in MDCK cells (Brown *et al.*, 2000). The association of AP1A with TGN requires ARF GTPase activity (Stamnes and Rothman, 1993; Traub *et al.*, 1993). In FRT cells, the perinuclear staining of both μ 1B and γ -adaptin vanished upon BFA treatment, indicating that recruitment of AP1B to RE also depends on an ARF-like GTPase activity (Figure 2B).

Additional experiments supported a non-TGN localization of AP1B. Recently, Yeaman *et al.* (2004) reported that protein kinase D (PKD) is involved in the generation of basolateral transport vesicles from the TGN; expression of dominant-negative PKD-2 in MDCK cells leads to TGN tubulation and apical missorting of model basolateral markers (Yeaman *et al.*, 2004). Expression of the PKD-2-KD protein by microinjection of its cDNA into FRT cells and subsequent incubation for 1 h resulted in the formation of tubules rich in TGN38 without affecting the μ 1B/TfR compartment (Figure 3). PKD-2-KD colocalized with TGN-derived tubules rich in TGN38 but not with μ 1B or TfR. These results further strengthen the notion that μ 1B resides in a TfR-rich compartment distinct from the TGN, both spatially and functionally.

μ 1Bn Antibody Blocks Basolateral Recycling of TfR and LDLR in FRT Cells

A serendipitous finding was that the μ 1Bn-Ab interfered with the traffic of AP1B-dependent cargo. To our knowledge, no function-blocking antibodies have been described for AP complexes. Control experiments with the N-terminal peptide and with LLC-PK1 cells lacking μ 1B demonstrated that the antibody blocked the function of μ 1B.

We have reported that AP1B participates in postendocytic basolateral recycling of TfR and LDLR in LLC-PK1 cells (Gan *et al.*, 2002); hence, we first used the μ 1Bn-Ab to interfere with the recycling of these receptors in FRT cells. To facilitate surface detection of TfR returning from RE, we coinjected the antibody together with an expression plasmid encoding a dominant negative mutant of Eps15 protein, Eps15-EH29-GFP, that inhibits the endocytosis of TfR (Benmerah *et al.*, 1998). Expression of Eps15-EH29-GFP increased the percentage of cells displaying detectable TfR staining at the cell surface, from <10% to nearly 80% (Figure 4), as expected for PM accumulation of receptors returning from RE. Under these conditions, comicroinjection of the μ 1Bn-Ab, but not of the corresponding PI-IgG fraction, inhibited the appearance of TfR at the cell surface. The specificity of this effect was further demonstrated by its abrogation with

the μ 1B N-terminal peptide (μ 1Bnp) but not by the μ 1Bc peptide (μ 1Bcp; Figure 4). The simplest interpretation of these data are that μ 1Bn-Ab arrested recycling of TfR in RE by blocking the function of AP1B at RE.

Additional experiments with a second fast recycling basolateral receptor, GFP-LDLR, provided additional support for this hypothesis. We microinjected recently confluent FRT cells with a cDNA encoding GFP-tagged LDLR (LDLR-GFP), incubated them successively for 1 h at 37°C and 2 h at 20°C to accumulate the protein in the TGN and then transferred the cells to 37°C to allow for synchronized release of the protein from the TGN, according to an established protocol (Kreitzer *et al.*, 2000). To make more evident the blocking effect of the μ 1B antibody, we adjusted the conditions of the experiment (expression time and serum concentration) to maximize the PM localization and minimize the endosomal localization of LDLR-GFP. Incubating the cells in serum-free medium was sufficient to cause such an effect (Supplementary Figure S1), suggesting that LDLR spends less time in the endocytic pathway in the absence of ligand. Under these conditions, control FRT cells comicroinjected with PI-IgG displayed LDLR-GFP mainly at the basolateral PM (Figure 5A, top three panels). By contrast, when μ 1Bn-Ab was comicroinjected with the cDNA, LDLR-GFP moved normally to the PM and was internalized, but accumulated in a perinuclear compartment (Figure 5A, middle three panels). This accumulation was much slower with an endocytosis-deficient mutant of LDLR (LDLR_{Y18A}-GFP; Figure 5A, bottom three panels), further supporting the view that the antibody blocks LDLR trafficking postendocytically. Immunofluorescence experiments showed that the compartment that accumulated LDLR-GFP was enriched in both endogenous μ 1B and TfR, but not in TGN38 (Figure 5, B and C). These results and the TfR experiments previously described (Figure 4) demonstrate that μ 1Bn-Ab blocks the recycling of LDLR and TfR to the basolateral surface, promoting their accumulation in RE closely apposed to the TGN. To our knowledge, this is the first report of an antibody against a subunit of an AP adaptor that has trafficking-blocking properties.

μ 1Bn Antibody Blocks Biosynthetic Basolateral Transport of TfR and VSVG Protein, But Not LDLR

The function-blocking capability of the μ 1B antibody provided us with a unique functional assay to investigate in detail whether acute block of AP1B would block trafficking

Figure 4. The μ 1Bn-Ab blocks basolateral recycling of TfR. (A) TfR-increment at the cell surface after endocytic inhibition. FRT cells were microinjected with dominant-negative Epsin15 (GFP-Eps15-EH29) to prevent endocytosis in the indicated conditions, fixed 3 h later, and processed for indirect immunofluorescence with TfR antibody (red). In control samples (without coinjected antibody), most FRT cells expressing GFP-Epsin15-EH29 displayed TfR (red) at their borders (arrows), in addition to a perinuclear compartment. By contrast, cells coinjected with μ 1Bn-Ab at $t = 0$ displayed no surface TfR. This effect was abrogated by preincubating the μ 1Bn antibody with μ 1Bn peptide (μ 1Bnp) but not with μ 1Bc peptide. Bar, 10 μ m. (B) Quantification of the results in A. The percentage of cells displaying detectable levels of TfR at the cell surface increase from 6% in not microinjected cells to nearly 80% in cells expressing the Eps15-EH29-GFP for 3 h. Microinjection of the μ 1B antibody but not the preimmune immunoglobulin G (PI-IgG) fraction (not shown in A) reduced to <25% the cells displaying cell surface TfR. The μ 1Bn peptide, but not the μ 1Bc peptide, inhibited the effect of μ 1Bn-Ab, demonstrating its specificity. Graph shows mean $\pm$ SEM ($n = 60$). These results indicate that μ 1Bn antibody inhibited the return of TfR from endosomes to the cell surface.

of basolateral proteins in the biosynthetic route. We first studied the effect of μ 1Bn-Ab on the exit kinetics of three different GFP-tagged basolateral markers, LDLR, VSVG protein, and TfR, from the TGN of polarized FRT cells (1–2 d after confluency; see *Materials and Methods*). A live imaging protocol that quantifies GFP fluorescence in the Golgi area at different times after reversal of the 20°C block (Musch *et al.*, 2001) showed striking results (Figure 6A). The μ 1Bn-Ab did not alter the kinetics of LDLR-GFP release from the Golgi area, in agreement with the results described above (Figure 5). Instead, it drastically inhibited the release of VSVG pro-

tein and TfR from the perinuclear region (Figure 6A). Images with higher exposure times, which saturate the perinuclear staining but are necessary to visualize the weaker surface GFP fluorescence, demonstrated that VSVG-GFP and TfR-GFP reached the basolateral membrane in cells microinjected with control PI-IgG but were blocked intracellularly in the presence of μ 1Bn-Ab (Figure 6B).

It should be pointed out that all of the experiments described above were performed on FRT cells grown on coverslips and confluent for ~ 2 d. Under our experimental conditions, FRT cells exhibited a relatively flat phenotype that facilitated our microinjection and quantitative live imaging assays; however, they were sufficiently polarized to localize TfR, VSVG, and LDLR predominantly to the basolateral membrane and the apical variant of tsO45-VSVG-GFP (VSVG3-GFP; Keller *et al.*, 2001), to the apical surface, relative to the tight junction marker protein ZO-1 (Supplementary Figure S2).

Newly Synthesized VSVG Protein and TfR Reach RE Directly from the TGN, Whereas LDLR Reaches RE Postendocytically

The μ 1B-Ab blocking effects allowed us to investigate a second major question regarding the biology of AP1B, i.e., does the adaptor work in TGN or in RE intersecting the biosynthetic pathway? The experiments described above did not elucidate the nature of the perinuclear compartment, where the AP1B antibody stopped the biosynthetic route of basolateral proteins. To identify this compartment, we accumulated GFP-tagged LDLR, TfR, and VSVG proteins in the TGN using microinjection and the 20°C block as in Figure 5 and quantified with MetaMorph software the extent of colocalization of GFP with TGN38 and μ 1B before and 10 min after releasing the temperature block (Figure 7). As expected, more than 75% of all three proteins colocalized with TGN38 before release of the 20°C block and exited the TGN quickly upon release of the block (Figure 7, A–C). Remarkably, different cargo proteins showed strikingly different rates of transport from TGN to the AP1B-positive compartment. LDLR-GFP did not colocalize significantly with μ 1B 10 min after release of the 20°C block (Figure 7A), whereas the bulk ($\sim 80\%$) of TfR-GFP and VSVG-GFP moved to the AP1B compartment within that time (Figure 7, B and C), and these proteins were blocked from exiting this compartment by μ 1Bn-Ab. Time-course analysis (Figure 7D) demonstrated that TfR and VSVG achieved 75% colocalization with μ 1B in just 5 min and reached a plateau at $\sim 80\%$ colocalization. By contrast, LDLR only started to colocalize with μ 1B after a long lag period of 60 min, likely reflecting its passage through the basolateral PM, internalization, and endosomal trafficking before accumulation at the AP1B-positive compartment (Figure 7D, see also Figure 5). These experiments clearly demonstrate that the major site of function of AP1B in the biosynthetic route is not the TGN, but closely apposed RE (see model in Figure 9).

The Function-blocking Effect of μ 1Bn-Ab Requires μ 1B Expression and Basolateral Sorting Signal

Additional control experiments with wild-type LLC-PK1 cells (which lack μ 1B) and LLC-PK1 permanently transfected with μ 1B (Gan *et al.*, 2002) showed that μ 1Bn-Ab elicited the perinuclear accumulation of all three GFP-tagged reporters only in cells expressing μ 1B (Figure 8A). Furthermore, the apical variant of tsO45-VSVG-GFP (VSVG3-GFP; Keller *et al.*, 2001), bearing a basolateral sorting motif masked by GFP (Ang *et al.*, 2003, 2004; Folsch *et al.*, 2003; Yeaman *et al.*, 2004), was not perinuclearly arrested by μ 1Bn-Ab (Figure 8B).

Figure 5. Microinjected $\mu 1\text{Bn-Ab}$ blocks basolateral recycling of LDLR from a perinuclear compartment that contains both $\mu 1\text{B}$ and Tfr. (A) $\mu 1\text{Bn-Ab}$ provokes postendocytic perinuclear accumulation of LDLR. FRT cells were comicroinjected with expression vectors for either wild-type LDLR-GFP or the endocytosis-defective mutant LDLR_{Y18A}-GFP (Gan *et al.*, 2002) and either preimmune IgG fraction (PI-IgG) or $\mu 1\text{B}$ antibody, as indicated. The cells were then incubated at 37°C for 1 h and then at 20°C for 2 h to allow accumulation of the fluorescent proteins in the TGN, as previously described (Kreitzer *et al.*, 2000, 2003; left column of panels). After transfer to 37°C for 1 h, both LDLR and LDLR_{Y18A}-GFP reached the basolateral PM (middle column of panels). The presence of $\mu 1\text{B}$ antibody, but not PI-IgG, during the next 2 h caused accumulation of LDLR-GFP but not of LDLR_{Y18A}-GFP in a perinuclear compartment (right column of panels). Bar, 10 μm . (B) Perinuclearly accumulated LDLR colocalizes with $\mu 1\text{B}$ and Tfr. Using a similar experimental protocol, LDLR-GFP colocalized with TGN38 but not with $\mu 1\text{B}$ or Tfr in cells fixed during the TGN block, whereas 3 h after release of the TGN block it accumulated in a compartment enriched in $\mu 1\text{B}$ and Tfr. Bars, 5 μm . (C) Quantitative data of the colocalization results in B, shown as mean $\pm$ SEM ($n = 30$).

Together with the results described before (Figure 5), these experiments demonstrate that the $\mu 1\text{Bn-Ab}$ inhibits basolateral transport in cargo- and compartment-specific manners: it blocks biosynthetic delivery of VSVG protein and Tfr and the postendocytic recycling of LDLR and Tfr to the basolateral membrane.

DISCUSSION

A polyclonal antibody raised against an N-terminal immunogenic peptide of $\mu 1\text{B}$ allowed us to define the subcellular localization of endogenous AP1B by immunofluorescence and its site of function by acute function-blocking experiments. The use of this antibody in recycling assays and in Golgi exit assays allowed us to conclusively demonstrate that 1) AP1B functions not in the TGN but in physically and kinetically very close RE; 2) RE are an obligatory step in the biosynthetic route of some but not all basolateral proteins; 3) PM proteins with tyrosine-dependent motifs require adaptors different from AP1B at the TGN to be transported to either AP1B-containing RE or directly to the PM; and 4) cargo proteins move with very fast kinetics ($t_{1/2}$, ~3–5 min) from the TGN to AP1B-containing RE. These results provide solid experimental support for the emerging paradigm that

RE and TGN have a cooperative sorting role in the biosynthetic route.

The ability of the antibody to react with $\mu 1\text{B}$ in live or fixed FRT cells was unexpected because crystallography and modeling data (Heldwein *et al.*, 2004) indicate that the N-terminal epitope of $\mu 1\text{B}$ used as the antigen is likely buried within the fully assembled AP1 complex. Furthermore, the antibody did not react with MDCK cells even though the N-terminal sequence of canine $\mu 1\text{B}$ is highly homologous to both rat and human $\mu 1\text{B}$. However, the following controls confirmed that the antibody reacted specifically with rat and human $\mu 1\text{B}$: 1) the peptide antigen abrogated both the immunostaining and the transport-blocking effects of the antibody; 2) $\mu 1\text{B}$ immunostaining was observed upon transfection of $\mu 1\text{B}$ into LLCPK1 cells (which normally lack this protein) and was decreased after partial RNAi-mediated knockdown of $\mu 1\text{B}$ expression in FRT cells; and 3) the antibody displayed traffic-blocking effects in LLCPK1 cells that were only observed upon $\mu 1\text{B}$ transfection. Our experiments suggest that AP1B may be present not only as a fully assembled adaptor, but also in incompletely assembled configurations that are essential for its biological function and that can vary in different cells. This latter property could explain the higher reactivity of the antibody in FRT cells than in MDCK

Figure 6. The μ 1Bn-Ab blocks the biosynthetic routes of VSVG and TfR but not LDLR. (A) Exit from the perinuclear region. FRT cells were microinjected intranuclearly with cDNAs encoding the indicated GFP protein vectors together with PI-IgG (control) or μ 1Bn-Ab. After incubation at 37°C for 1 h followed by 20°C for 2 h (TGN block), the cells were mounted in a live-cell recording chamber, and the temperature was shifted to 37°C to allow exit from the TGN. The left panels show representative perinuclear fluorescence patterns that were quantified as integrated fluorescence, and they are graphically represented in the panels on the right; each data point in the graph is the mean $\pm$ SEM ($n = 10$ cells). The μ 1Bn-Ab inhibited the exit of VSVG-GFP and TfR-GFP from the perinuclear region (Golgi area) but did not affect the exit of LDLR-GFP. Bar, 10 μ m. (B) Cargo detection at the basolateral cell surface. The cells were comicroinjected with the indicated vectors together with either a PI-IgG fraction or the μ 1Bn-Ab, but the pictures were taken using longer exposure times that saturated the perinuclear images, in order to detect the proteins at the cell surface. As shown for the LDLR in Figure 4, both TfR and VSVG were detected at the basolateral cell surface after releasing the TGN block for 1 h; this was not observed in cells coinjected with the μ 1Bn-Ab. Bar, 10 μ m.

Figure 7. VSVG and TfR but not LDLR are blocked by μ 1B-Ab in RE after release from the 20°C block. (A–C) Cargo colocalization with either TGN38 or μ 1B. FRT cells were microinjected with the indicated expression vectors together with μ 1Bn-Ab and then incubated for 2 h at 20°C. After the TGN block, all three GFP-proteins colocalized well with TGN38 (~75%, as assessed by integrated fluorescence, right panels). Ten minutes after release of the TGN block, LDLR colocalized poorly with both TGN38 and μ 1B (A), whereas ~80% of both TfR (B) and VSVG (C) colocalized with μ 1B. Bar, 5 μ m. Other data identify this compartment as RE. Data shown in right panels represent mean $\pm$ SEM ($n = 30$ cells). (D) Kinetics of transport from TGN to RE is cargo dependent. Colocalization of VSVG, TfR, and LDLR with μ 1B was assessed after releasing the TGN block for the indicated time periods in cells comicroinjected

with μ 1Bn-Ab. The graph shows that 75% of VSVG and TfR reached RE within 5 min and continued to accumulate, reaching and keeping a plateau during the next 3 h of the experiment. Instead, LDLR-GFP began to be detected at RE only after a lag period of 1 h and had not yet reached maximal accumulation by 3 h. Each data point represents mean $\pm$ SEM ($n = 30$ cells).

cells. Indeed, published evidence suggests the presence of α - σ and β - μ dimers in cells (Deneka *et al.*, 2003) and indicates that μ 2, the medium subunit of AP2 (Liu *et al.*, 1994)

and μ 1B (Folsch *et al.*, 1999; Sugimoto *et al.*, 2002) may exist unincorporated into tetrameric AP complexes. Other experiments suggest that conformational changes leading to ex-

Figure 8. The blocking effect of μ 1B-Ab requires expression of μ 1B and a functional basolateral sorting signal. (A) μ 1Bn-Ab provokes perinuclear accumulation of basolateral markers only in cells that express μ 1B. LLC-PK1 cells, either wild type or permanently transfected with μ 1B, were comicroinjected with expression vectors encoding LDL-GFP, TfR-GFP, or VSVG-GFP and the μ 1B antibody. After a 20°C block for 2 h, the cells were incubated at 37°C (LDLR and TfR) or at 32°C (VSVG) for the indicated time periods to reestablish the traffic out of the TGN. Only cells expressing μ 1B reproduce the effects of μ 1B antibody previously seen in FRT cells, showing perinuclear accumulation of cargo. Bar, 10 μ m. (B) Exit from the perinuclear region of VSVG3-GFP is not inhibited by μ 1Bn-Ab. FRT cells were comicroinjected with μ 1B-Ab and the vector VSVG3-GFP encoding an apical version of tsO45 VSVG, which has shielded its basolateral sorting signal. After incubating the cells for 1 h at 37°C and then for 2 h at 20°C to block transport at the TGN, exit from the TGN was allowed by shifting the temperature to 32°C for 1 h. The fluorescence intensity in individual cells ($n = 10$) in two independent experiments and is graphically represented as the mean $\pm$ SD. Bar, 10 μ m. μ 1B-Ab did not provoke perinuclear arrest of VSVG3-GFP.

posure of the μ 2 subunit in AP2 (Haucke and De Camilli, 1999; Collins *et al.*, 2002) and μ 1A in AP1A (Ghosh and Kornfeld, 2003) might be triggered during the functional cycle of AP adaptors. It is also possible that the attachment of the adaptor to its binding site (in this manuscript shown to involve ARF activity) may promote an open configuration different from that observed by crystallography. Unfortunately, our μ 1B antibody was unsuitable for immunoprecipitation, which prevented us from carrying out additional experiments to discern among these possibilities.

Experiments using this antibody localized AP1B to a perinuclear compartment that, although physically very close to the TGN, lacked typical TGN markers. This compartment was enriched in TfR and did not contain EEA1, a marker of early sorting endosomes (Wilson *et al.*, 2000), or Rab11, a marker of AREs in MDCK cells (Casanova *et al.*, 1999; Wang *et al.*, 2001; Figure 2A). All of these characteristics are typical of a compartment denominated common RE, involved in the recycling of TfR and LDLR to the basolateral membrane and in the transcytosis of Polymeric IgA Receptor to the apical membrane via ARE (Futter *et al.*, 1998; Brown *et al.*, 2000; Wang *et al.*, 2000). Additional experiments showed that the compartment decorated by μ 1Bn-Ab, unlike the TGN, was insensitive to tubulation induced by a kinase-dead form of PKD (Figure 3), a protein recently implicated in the regulation of the trafficking of basolateral proteins (Yeaman *et al.*, 2004). Taken together, these experiments provide strong support to our original suggestion that AP1B localizes predominantly to RE (Gan *et al.*, 2002).

Surprisingly, the μ 1Bn antibody blocked the recycling of TfR and LDLR to the basolateral membrane, promoting the accumulation of these fast-recycling receptors in perinuclear RE (Figures 4 and 5). These results suggest that RE is a site of function of μ 1B, congruently with previous evidence (Gan *et al.*, 2002), but cannot discard an additional sorting role of AP1B at the TGN. The possibility that AP1B might function at the TGN, originally proposed on the basis of EM immunogold data (Folsch *et al.*, 1999, 2001), gained further traction

based on our recent observations in siRNA knockdown MDCK cells, which demonstrated μ 1B-dependent basolateral sorting both during biosynthetic and recycling traffic (Gravotta *et al.*, 2007). Although AP1B was mainly detected in an endosomal fraction, it cannot be disregarded that a small, highly dynamic pool of this adaptor actually functions at the TGN. We took advantage of the trafficking-arresting properties of μ 1Bn-Ab to provide a definitive answer to this question. The μ 1Bn-Ab blocked biosynthetic trafficking of VSVG and TfR not in the TGN but in a closely apposed μ 1B-enriched RE that they reached within 5 min after leaving the TGN (Figure 7). Importantly, LDLR only started to accumulate in RE ~45 min after leaving the TGN, reflecting the time required for this protein to be exocytosed, internalized, and transported to perinuclear RE (Figure 7). These data conclusively demonstrate that the sorting role of AP1B in the biosynthetic route takes place not at both TGN and RE, but exclusively in RE located physically and kinetically very close to the TGN. Our results are also the first to show that AP1B-containing RE are an obligatory step in the biosynthetic route of some but not all basolateral proteins and that the TGN discriminates among distinct tyrosine-dependent basolateral motifs.

The mechanisms utilized by AP1B to recognize different basolateral sorting signals have not yet been fully elucidated. AP1B's μ 1B subunit shares with AP2's μ 2 subunit a pocket lined with a cluster of conserved amino acid residues that recognize Yxx Φ motifs (Owen and Evans, 1998; Bonifacino and Dell'Angelica, 1999). This pocket is required for the sorting of basolateral proteins with typical Yxx Φ motifs, e.g., VSV G protein, but not for basolateral sorting of AP1B-dependent TfR and LDLR, suggesting that AP1B utilizes nonpocket regions to interact with these proteins' atypical basolateral signals (Sugimoto *et al.*, 2002). In TfR, the basolateral sorting signal is a GDNS sequence downstream of the endocytic motif YTRF, which lacks basolateral signaling ability (Dargemont *et al.*, 1993; Odorizzi and Trowbridge, 1997). LDLR displays a "weak" membrane-proximal

basolateral signal constituted by its endocytic motif NPVY and a more distal "strong" basolateral signal constituted by an atypical tyrosine motif, which depends on Tyr 35, the adjacent Gly34, and a downstream acidic cluster (EDD; Matter *et al.*, 1992, 1993; Koivisto *et al.*, 2001). It is conceivable that AP1B may interact with these signals via subunits other than μ 1B, as shown for the interaction between AP1 or AP3 with (DE)XXXL(LI) motifs (Janvier *et al.*, 2003).

The observation that the μ 1Bn antibody blocked transport out of RE, rather than promoting diversion of the basolateral proteins into an apical route, as typically observed by many groups when basolateral sorting signals are mutated, suggests that the antibody trapped basolateral proteins into a complex that prevented any type of transport. This possibility is supported by the finding that the transport of the three basolateral markers in LLC-PK1 cells, which lack μ 1B expression, as well as the transport of VSVG3-GFP variant with its basolateral sorting signal inactivated (Keller *et al.*, 2001), was not blocked by the μ 1Bn-Ab (Figure 8). Only upon expression of exogenous μ 1B did LLC-PK1 cells become sensitive to the blocking effects of μ 1Bn-Ab. Thus, our antibody seems to arrest basolateral proteins into a complex that prevented transport out of RE in a μ 1B- and basolateral motif-dependent manner. Other experiments also showed that BFA promoted dissociation of μ 1B from RE (Figure 2B), thus indicating for the first time that BFA-sensitive ARF GTPase activity is crucially involved in AP1B recruitment to RE membranes. This result provides a mechanistic explanation for previous published data showing that BFA promotes missorting of recycling basolateral proteins (Matter *et al.*, 1993; Futter *et al.*, 1998; Wang *et al.*, 2001).

Early reports using cell fractionation detected TfR (Futter *et al.*, 1995) and other basolateral proteins (Leitinger *et al.*, 1995; Orzech *et al.*, 2000) in endosomes while en route from the TGN to the PM. The resolution of these experiments was insufficient to determine the magnitude of the TGN-endosome-PM trafficking or to identify the compartments involved. A more recent study (Ang *et al.*, 2004), used video-microscopy and biochemical assays in MDCK cells to reveal transient associations of VSVG-GFP protein with transferrin-positive RE minutes after release from the TGN block. These authors, utilizing an endosomal ablation procedure based on HRP-peroxidase, suggested that ~85% of newly synthesized VSVG traveled through the endosomal compartment before reaching the cell surface. However, these experiments did not identify the site of the transport block as either RE containing Rab11 or AP1B, or other endocytic compartments (early endosomes), nor excluded the possibility that the traffic arrest might have taken place at the TGN itself rather than at RE, because of indirect effects of the ablation procedure on TGN function (e.g., blocking of a recycling route essential for TGN function). Our results clearly go beyond this and other previous studies by demonstrating that two newly synthesized basolateral proteins, TfR and VSVG, must obligatorily transit AP1B-containing RE and that the bulk of these cargo proteins reach the AP1B-RE with very fast kinetics after exiting the TGN.

Our results also provide new mechanistic insights about the sorting machinery of the TGN and RE. Rab11-containing endosomes have been found to intersect the biosynthetic pathway of E-cadherin in HeLa cells, and the evidence suggested that Rab11 is required for dileucine-mediated basolateral sorting in MDCK cells (Lock and Stow, 2005). However, as mentioned, Rab11 associates with ARE but not with common RE involved in basolateral recycling of TfR or LDLR (Brown *et al.*, 2000; Wang *et al.*, 2000). In agreement with these reports, we did not observe an overlap between

Figure 9. AP1B-mediated biosynthetic and recycling sorting pathways involving RE. Newly synthesized VSVG and TfR intersect the perinuclear μ 1B-containing compartment upon exiting the TGN en route to the basolateral surface, whereas LDLR is directly sorted from the TGN to the basolateral surface. The LDLR and TfR are then endocytosed and recycle basolaterally from this μ 1B compartment. Transport from the TGN to the μ 1B compartment is mediated by sorting signals and might hypothetically involve AP3. Direct basolateral sorting from the TGN might involve AP4.

Rab11 and μ 1B-containing endosomes in FRT cells (Figure 2A) and E-cadherin was only marginally affected in μ 1B-knockdown MDCK cells (Gravotta *et al.*, 2007). Therefore, different basolateral proteins might traverse different sets of RE in their biosynthetic routes. In addition, the evidence also indicates that basolateral proteins use different sorting elements and transport pathways to exit the TGN. We have shown in acute trafficking-blocking experiments with membrane-permeant peptides that the TGN discriminates between basolateral cargo bearing tyrosine-based or nonconventional sorting motifs (Soza *et al.*, 2004). Our present results imply that the TGN also distinguishes basolateral proteins that possess distinct classes of tyrosine-based motifs (VSVG and LDLR) and sorts them either to AP1B-containing RE (VSVG) or to the PM bypassing this compartment (LDLR). Thus, our results are the most clear in predicting that different adaptors, distinct from AP1B, mediate sorting from TGN into routes to either RE or the PM. On the basis of previous reports (Nishimura *et al.*, 2002; Simmen *et al.*, 2002), we suggest that these adaptors might be AP3 and AP4 (Figure 9).

Our previous experiments in MDCK cells knocked down for μ 1B expression showed that in recently polarized cells (1–2 d), TfR was missorted only in the biosynthetic route, whereas at later times (4.5 d) it was missorted only in the recycling route (Gravotta *et al.*, 2007). These experiments suggested that TfR might switch to a direct route from TGN to PM in more established MDCK monolayers; however, given the slow installation of the RNAi inhibition of AP1B expression, we were not able to eliminate other possibilities, e.g., the activation of a different basolateral adaptor at RE in more mature MDCK monolayers. Because the experiments reported here were carried out only in recently polarized FRT cells (2 d of confluency), we cannot discard the possi-

bility that these cells, like MDCK cells, might develop an AP1B-independent biosynthetic route for TfR at later confluency times.

In summary, our results provide definitive evidence for the functional sorting role of AP1B in RE, and for the hypothesis, so far supported only by circumstantial data, that RE carry out an important sorting role in both the biosynthetic and recycling basolateral routes. Future work must complete the mapping of the basolateral routes and the adaptors involved in transport between TGN and RE or the PM and determine the role of RE in sorting events previously thought to occur in the TGN.

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