Clathrin Terminal Domain-Ligand Interactions Regulate Sorting of Mannose 6-Phosphate Receptors Mediated by AP-1 and GGA Adaptors

Wiebke Stahlschmidt, Mark J. Robertson, Phillip J. Robinson, Adam McCluskey, and Volker Haucke

From the Leibniz-Institut für Molekulare Pharmakologie, 13125 Berlin-Buch, Germany, the Centre for Chemical Biology, Chemistry, The University of Newcastle, Callaghan, New South Wales 2308, Australia, and the Cell Signalling Unit, Children’s Medical Research Institute, The University of Sydney, Sydney, New South Wales 2145, Australia

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Abstract

Clathrin plays important roles in intracellular membrane traffic including endocytosis of plasma membrane proteins and receptors and protein sorting between the trans-Golgi network (TGN) and endosomes. Whether clathrin serves additional roles in receptor recycling, degradative sorting, or constitutive secretion has remained somewhat controversial. Here we have used acute pharmacological perturbation of clathrin terminal domain (TD) function to dissect the role of clathrin in intracellular membrane traffic. We report that internalization of major histocompatibility complex I (MHCI) is inhibited in cells depleted of clathrin or its major clathrin adaptor complex 2 (AP-2), a phenotype mimicked by application of Pitstop® inhibitors of clathrin TD function. Hence, MHCI endocytosis occurs via a clathrin/AP-2-dependent pathway. Acute perturbation of clathrin also impairs the dynamics of intracellular clathrin/adaptor complex 1 (AP-1)- or GGA (Golgi-localized, γ-ear-containing, Arf-binding protein)-coated structures at the TGN/endosomal interface, resulting in the peripheral dispersion of mannose 6-phosphate receptors. By contrast, secretory traffic of vesicular stomatitis virus G protein, recycling of internalized transferrin from endosomes, or degradation of EGF receptor proceeds unperturbed in cells with impaired clathrin TD function. These data indicate that clathrin is required for the function of AP-1- and GGA-coated carriers at the TGN but may be dispensable for outward traffic en route to the plasma membrane.

Background: Clathrin is a coat protein involved in intracellular membrane traffic.

Results: Acute inhibition of clathrin-ligand association by Pitstop® compounds perturbs intracellular receptor sorting mediated by AP-1 and GGA adaptors.

Conclusion: Clathrin is required for intracellular receptor sorting by AP-1 and GGA adaptor proteins.

Significance: Pitstop® compounds are tools for the functional dissection of intracellular trafficking pathways.

Clathrin is comprised of three heavy (HC) and three light chains (LC) and is the main component of clathrin coats that cover the cytoplasmic face of cellular membranes (1). At the plasma membrane, clathrin-mediated endocytosis (CME) regulates the cell surface levels and internalization of important plasma membrane proteins including receptors, transporters, ion channels, and synaptic vesicle proteins in brain (1–5). CME is also hijacked by pathogens such as HIV or bacteria (i.e., Listeria) to gain access to the cell interior. Assembly of endocytic clathrin-coated pits (CCPs) is initiated by the recruitment of early endocytic adaptors such as AP-2 and FCHO1/2 to plasma membrane sites enriched in phosphatidylinositol 4,5-bisphosphate. Together with other endocytic proteins, these factors couple clathrin recruitment and assembly to the selection of transmembrane cargo and the remodeling of the plasma membrane to eventually generate endocytic vesicles destined for endosomal fusion (3–5). In addition to CME, eukaryotic cells also express several clathrin-independent endocytic pathways (6). For example, Shigata toxin uses an actin-regulated route involving glycosphingolipids for internalization (7).

At the trans-Golgi network (TGN)-endosomal interface, clathrin is implicated in sorting of cargo such as mannose 6-phosphate receptors (MPRs) (8), a pathway required for the lysosomal delivery of soluble acid hydrolases (9). CCP formation at the TGN involves the essential (10) heterotetrameric adaptor complex AP-1 (comprising the γ, β1, μ1, and σ1 subunits), as well as the monomeric GGA (Golgi-localized, γ-ear-containing, Arf-binding proteins) adaptors (11, 12), in addition to a variety of accessory proteins including γ-synergpin, Eps15, Gadkin, and amphiphysin (13) among others (14). A major cellular function of AP-1- (15) and GGA-containing clathrin-coated carriers (16) is the bidirectional traffic of cargo such as MPRs between endosomes and the TGN. GFP-MPRs exit from the TGN via clathrin/AP-1-containing tubules (17) that appear to be distinct from those involved in constitutive secretion of...
vesicular stomatitis virus G protein (VSVG) (18, 19). Acute or sustained loss of AP-1 depletes MPRs from clathrin-coated vesicles and impairs MPR retrieval to the TGN (10, 20). Loss of AP-1 from TGN/endosomal membranes in addition results in co-depletion of GGA2 from clathrin-coated structures (21), suggesting that AP-1 and GGA2 are functionally linked (22).

Clathrin coats have also been observed on endosomal compartments (23, 24), although their precise function at these sites is less clear. Loss of function of the clathrin-associated Arf6-GTPase-activating protein ACAP1 impairs recycling of several cargos including transferrin (Tf) receptor and integrins (25, 26). Furthermore, so-called gyrating clathrin (G-clathrin) has been suggested to operate in an Arf6-dependent rapid recycling route (27). By contrast, dominant interference with clathrin function by inducible overexpression of the clathrin hub domain or ligand-induced cross-linking of clathrin LCs had only minor effects on transferrin recycling (28, 29). Likewise, it remains unclear to what degree flat clathrin coats on endosomes are required for Hrs-mediated degradative sorting of epidermal growth factor (23). Thus, whether and how clathrin or its association with ligands is required for endosomal sorting remains an open question.

An inherent problem in answering these issues is the multiple interdependent roles of clathrin in CME, TGN/endosomal membrane traffic, and mitosis that complicate a precise molecular analysis of its function by genetic means or by RNA interference. A possible alternative strategy is the use of pharmacological or chemical perturbations of clathrin function that allow the design of stage-specific assays, i.e., to experimentally separate endocytosis from downstream endosomal sorting. The downside of such approaches is potential off target side effects caused by drug application that would cloud interpretation of the results as recently demonstrated for dynamin inhibitors (30). Hence, care must be taken to ascertain the specificity of the chemical perturbations.

Recently, we identified Pitstop®-1 and Pitstop®-2 as small molecule inhibitors of clathrin function in CME (31). CME inhibition is based on the ability of Pitstop compounds to interfere with the function of the clathrin TD, a seven-bladed β-propeller, as a recruitment hub for accessory proteins harboring clathrin box motifs (31) including AP and GGA adaptors, Eps15, epsins, amphiphysin, SNXs, or CALM (1, 3, 5, 11, 32, 33). Although Pitstop-2 has been shown to inhibit CME of transferrin and HIV uptake via arrest of CCP dynamics (31), its activity inhibition is based on the ability of Pitstop compounds to intervene in pEGFP-C1 vector GGA2, and GGA3 cDNA was inserted into pEGFP-C2 (Clontech, respectively). VSVG-SP-GFP was described before (36). Bovine eCOP-eGFP (37) and GPI-GFP (38) were from Felix T. Wieland (University of Heidelberg).

For knockdown experiments, siRNAs were purchased from MWG. The sequences used were for clathrin heavy chain (AUCCAAUUGGAAGACCAAU and AAGCAGUGAGCGUUUGAGA) and for AP-2 μ2 subunit (GGUGAACCUUUCGCGGUATT). Pitstop® is a registered trademark of Children’s Medical Research Institute, Newcastle Innovation Ltd., and Freie Universität Berlin. These compounds are available from Abcam (Cambridge, UK).

**Experimental Procedures**

**Antibodies, Cell Lines, Plasmids, siRNAs, and Inhibitors** — For immune fluorescence experiments, antibodies were used against clathrin heavy chain (Abcam, ab21679), AP-1 (γ1-adaptin, Sigma, A4200), MHC class I (HLA-ABC clone w6/32, eBioscience, 14-9983-82), LC3 (MBL, M152-3), and p62 LCK ligand (BD Transduction, 610832). For Western blot experiments, antibodies were used against clathrin heavy chain (TD1, homebrew), AP-1 (γ1-adaptin, BD Transduction, 610836), AP-2 (α-adaptin AC1M11, Dianova, MA3-061), AP-3 (μ3-adaptin p47, BD Transduction, 610901), EGF receptor (D38B1, Cell Signaling, 4267S), and β-actin (ac-15, Sigma, A-5441).

For live cell imaging, BSC-1 cells stably expressing the AP-1 σ1 subunit fused to eGFP (kind gift from T. Kirchhausen (34)) and HeLa cells stably expressing GFP-Cl-MPR (kind gift from B. Hoflack (17)) were used. For overexpression in HeLa cells, clathrin light chain sequence was cloned into CoralHuTM pKaede-MN1 vector (MBL (35)). GGA1 cDNA was cloned into the pEGFP-C1 vector GGA2, and GGA3 cDNA was inserted into pEGFP-C2 (Clontech, respectively). VSVG-SP-GFP was described before (36). Bovine eCOP-eGFP (37) and GPl-GFP (38) were from Felix T. Wieland (University of Heidelberg).

**Transfection of Cells** — For depletion of endogenous protein, two rounds of siRNA knockdown were performed using Oligofectamine (Invitrogen) as a transfection reagent according to the manufacturer’s manual. The day after the second transfection, cells were split on Matrigel-coated coverslips or into 6-well plates for lysate preparation and used in experiments the following day.

Transient overexpression of fluorescently tagged proteins was done with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s manual. Cells split on Matrigel-coated coverslips were used 1 day after transfection.

**MHC Class I Internalization** — For MHC class I internalization experiments, HeLa cells were seeded on Matrigel-coated glass coverslips. 24 h after seeding, the cells were incubated with antibody against MHC class I (clone w6/32, eBioscience) in DMEM supplemented with 10 mM HEPES, pH 7.4, and 0.1% FCS for 15 min on ice. After brief washing in PBS + 10 mM MgCl₂, cells were allowed to internalize the surface bound antibody in the presence of 30 μM compound (clathrin-active compounds: Pitstop-2 and Pitstop-2-100) and their respective clathrin-inactive compounds: Pitstop-2 negative control compound (Pitnot-2) and Pitstop-2-100 negative control compound (Pitnot-2-100) or 0.1% DMSO in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 0.1% FCS and 10 mM.
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HEPES, pH 7.4, for 30 min at 37 °C. After washing three times in ice-cold PBS + 10 mM MgCl₂, the cells were fixed in 4% paraformaldehyde, 4% sucrose in PBS, pH 7.4, for 15 min at room temperature.

To bind surface epitopes of noninternalized MHC class I, cells were incubated overnight at 4 °C with an unlabeled goat anti-mouse antibody (1:10) in goat serum dilution buffer (10% goat serum, 15 mM sodium phosphate buffer, pH 7.4, 100 mM NaCl) without detergent. The next day, the cells were permeabilized with goat serum dilution buffer complemented with 1 mg/ml saponin for 20 min at room temperature before staining of internalized MHC class I antibody with an Alexa 488-labeled fluorescent goat anti-mouse secondary antibody.

Transferrin Recycling—HeLa cells were split on coverslips in 12-well plates. The next day, 70% confluent cells were starved for 1 h in serum-free DMEM. Then a transferrin uptake was performed using 20 μg/ml transferrinAlexa 488 (Invitrogen) for 30 min at 37 °C. After washing the cells twice in PBS + 10 mM MgCl₂ on ice, an acidic wash was done on ice in 0.1 M sodium acetate, pH 5.3, with 0.2 M NaCl for 3 min, and then cells were washed twice more in PBS + 10 mM MgCl₂. The 0-min time point was fixed for 15 min in 4% paraformaldehyde. To the remaining cells, prewarmed DMEM containing 1 mg/ml unlabeled transferrin and 30 μM Pitstop-2, 30 μM control Pitnot-2 or corresponding amounts of DMSO, respectively, were added, and they were shifted to 37 °C immediately. After 7.5 or 15 min the cells were transferred on ice, washed twice with PBS + MgCl₂, and then subsequently fixed.

VSVG Secretion—HeLa cells were split on coverslips in 12-well plates. The next day, the 80–90% confluent cells were transfected with VSVG-SP-GFP using Lipofectamine 2000 (Invitrogen) for 30 min at 37 °C. After washing the cells twice in PBS + 10 mM MgCl₂, and they were shifted to 37 °C immediately. After 7.5 or 15 min the cells were transferred on ice, washed twice with PBS + MgCl₂, and then subsequently fixed.

VSVG Recycling—HeLa cells were split on coverslips in 12-well plates. The next day, the 80–90% confluent cells were transfected with VSVG-SP-GFP using Lipofectamine 2000 (Invitrogen). After 4 h of incubation at 37 °C, the medium was replaced, and cells were shifted to 39 °C for additional 6 h, to trap VSVG-SP-GFP in the ER during expression. After 10 h of total expression time, the cells were incubated with 10 μg/ml cycloheximide and shifted to 19 °C to allow VSVG-SP-GFP trafficking to the TGN. One hour later, 30 μM Pitstop-2, 30 μM Pitnot-2, or the according amount of DMSO (0.1%) in DMEM containing 0.1% FCS and 10 mM HEPES, pH 7.4, were added to the cells and preincubated for additional 10 min at 19 °C. Then the time point “0 min chase” was washed with PBS + 10 mM MgCl₂, on ice and fixed for 15 min in 4% paraformaldehyde. The remaining cells were transferred to 32 °C, chased for 60 min, washed, and fixed as described above.

Immunofluorescence and Confocal Microscopy Analyses—For immunostaining HaCaT cells were seeded onto Matrigel-coated glass coverslips 24 h before the experiment. Cells were fixed in 4% paraformaldehyde, 4% sucrose in PBS, pH 7.4, for 15 min at room temperature or in ice-cold methanol for 7 min at −20 °C. Cells were permeabilized in goat serum dilution buffer (10% goat serum, 15 mM sodium phosphate buffer, pH 7.4, 100 mM NaCl) supplement with 0.3% Triton X-100 or 1 mg/ml saponin and processed for immunostaining in 30 μl of primary antibody solution (1 h at room temperature). Following extensive washes, samples were stained with secondary antibody (1:200, 1 h at room temperature).

Transferrin recycling and VSVG secretion were imaged using an inverted epifluorescence microscope (Zeiss). Sum intensities were analyzed using Slidebook software. VSVG-SP-GFP distribution in the cell was analyzed using ImageJ.

For all other experiments, cells were imaged by spinning disc confocal microscopy (PerkinElmer Life Sciences). For live cell microscopy, cells were split onto Matrigel-coated coverslips. Time series for 2 min at 0.5 Hz were acquired at 37 °C 5–30 min after the addition of 0.1% DMSO or 30 μM compound (Pitstop-2, Pitstop-2-100, Pitnot-2, or Pitnot-2-100) in imaging buffer (Hanks’ balanced salt solution complemented with 10 mM HEPES, pH 7.4, and 0.1% FCS). Analysis of time lapse series and generation of kymographs was done using Volocity software (PerkinElmer Life Sciences).

Fluorescence recovery after photobleaching (FRAP) assays were performed with a spinning disc confocal microscope (PerkinElmer Life Sciences). For live cell imaging, HeLa cells expressing GPI-GFP were washed once in PBS and then incubated in imaging buffer supplemented with 30 μM Pitstop-2, 30 μM Pitstop-2-100, 30 μM Pitnot-2, 30 μM Pitnot-2-100, or 0.1% DMSO. After 5–30 min of compound incubation, FRAP experiments were performed at 37 °C. Cells were imaged for 5 s before bleaching at a frame rate of 1 frame/s, and afterward bleached in a round area of 125-μm² diameter. Immediately after bleaching the cells were imaged for another 60 s at a frame rate of 2.5 images/s. Fluorescence recovery was analyzed in the bleached area by comparing fluorescence intensities before and after bleaching after correction of photobleaching relative to a control area that was not actively bleached.

For CLC-Kaede photoconversion experiments, cells transiently overexpressing CLC-Kaede were imaged 5–30 min after the addition of 30 μM Pitstop-2, 30 μM Pitnot-1, or DMSO (0.1%) in imaging buffer by spinning disc confocal microscopy. After 10 s of imaging, the TGN region of a cell was photoconverted from Kaede 488 to Kaede 568 using the 405-nm laser line. Immediately afterward, a time lapse series was acquired for additional 2 min with a frame rate of 2 s/frame. Quantifications of fluorescence intensities and Pearson’s correlations were done using Volocity software (Improvision).

Preparation of Cell Lysates and Immunoblotting—To analyze knockdown efficiency or protein degradation, whole cell lysates were prepared from HaCaT cells in a 6-well plate. The cells were transfected to ice, washed three times with cold PBS + 10 mM MgCl₂, and scraped in 500 μl of PBS. After centrifugation for 3 min at 4 °C and 1000 × g, the PBS was replaced by 50 μl of cell lysis buffer (20 mM HEPES, pH 7.4, 100 mM KCl, 2 mM MgCl₂, 1% Triton X-100, 1 mM PMSF, and 0.3% (v/v) protease inhibitor mixture). After 30 min of incubation on ice, the cells were centrifuged 10 min at 17,000 × g, 4 °C to remove cell debris. The protein concentrations were determined, sample buffer (62.5 mM Tris, pH 6.8, 10% glycerol, 5% 2-mercaptoethanol, 3% SDS, and 0.05% bromphenol blue final concentration) was added, and the lysates were boiled for 5 min.

To prepare cytosol membrane fractionations, HeLa cells depleted of endogenous clathrin heavy chain were split onto 6-cm dishes and incubated with 30 μM Pitstop-2 or 0.1% DMSO in DMEM supplemented with 0.1% FCS and 10 mM HEPES-NaOH (pH 7.4) for 20 min at 37 °C. Then they were washed twice on ice with cold PBS + 10 mM MgCl₂. After complete removal of the washing buffer, cells were harvested in 100 μl of
lysate buffer without detergent (20 mM HEPES-NaOH, pH 7.4, 100 mM KCl, 2 mM MgCl₂, 0.3% protease inhibitor mixture (Sigma), and 1 mM PMSF) using a cell scraper. Cells were lysed by repetitive freeze-thaw cycles (three times). Cell lysates were drawn through a 27-gauge needle 20 times to homogenize them. Subsequent centrifugation for 3 min at 4 °C with 1000 × g and 4 °C was performed to remove nuclei and large debris. The supernatant was collected, 20 μl were saved for analysis as total cell lysates in Western blot, whereas the rest was transferred to TLA tubes and spun at 4 °C with 180,000 × g. The supernatant, defined as the cytosolic fraction, was collected, protein concentration was determined, and sample buffer was added. The membrane pellet was resuspended in a volume of sample buffer that equals the volume of the cytosolic fraction. Samples from three independent experiments were used in SDS-PAGE and Western blot to compare the degree of membrane binding of different proteins. Quantifications were done using the ImageJ gel analysis tool.

**RESULTS**

**MHC Class I Endocytosis Depends on Clathrin and AP-2**—To dissect the role of clathrin and its ligand-binding TD in intracellular membrane traffic, we used a chemical biology approach based on recently identified small molecule inhibitors of clathrin function (Pitstop® compounds (31)) (Fig. 1A) combined with siRNA-mediated knockdown as a control for specificity. Pitstop® compounds bind to the clathrin TD, thereby competing with TD access of endogenous accessory protein ligands. Because Donaldson and co-workers (39) reported Pitstop-2-mediated inhibition of MHCI endocytosis, a process claimed to be clathrin-independent (40), with a dose dependence identical to that of the CME cargo transferrin, we started out by reinvigorating the role of clathrin and its major endocytic adaptor AP-2 in MHCI uptake. Antibodies against surface MHCI were allowed to internalize at physiological temperature for 30 min and visualized by confocal microscopy using previously established protocols and cell types (39). Application of Pitstop-2 potently inhibited MHCI endocytosis (Fig. 1, D and F) with an IC₅₀ of ~10–15 μM, similar to that reported for CME of transferrin (31). Two control compounds (Pitnot-2 and Pitnot-2-100) structurally related to Pitstop-2 (Fig. 1A) but unable to associate with the clathrin TD had no effect on either CME of transferrin or on MHCI uptake (Fig. 1D). To challenge these data, we sought to identify novel clathrin inhibitors by employing a medicinal chemistry approach based on the synthesis of a small focused library of Pitstop-2 analogs. This resulted in the identification of Pitstop®-2-100, a compound that inhibits clathrin TD association with amphiphysin 1 with an IC₅₀ of ~7.5 μM, similar to that of Pitstop-2 (Fig. 1, A and B, and Ref. 31). Application of Pitstop-2-100 also potently inhibited MHCII endocytosis (Fig. 1, D and F), akin to its effect on CME of transferrin (Fig. 1C). The effect of the Pitstop compounds on MHCII endocytosis could reflect off target side effects. Alternatively, MHCII internalization may indeed depend on clathrin and/or AP-2, in agreement with several recent studies reporting a crucial role for clathrin/AP-2 and associated epsins in MHCII endocytosis (41–43). To distinguish between these possibilities, we treated HeLa cells with well characterized clathrin heavy chain (CHC)- or AP-2-specific siRNAs resulting in depletion of clathrin or AP-2 to less than 10% of the levels expressed in control cells (Fig. 1I) and a potent inhibition of transferrin CME (by ~80–90%; data not shown). Strikingly, clathrin- or AP-2-depleted cells showed no overt alterations in MHCII surface expression (Fig. 1H) but failed to internalize MHCII from the plasma membrane (Fig. 1, E and G), similar to effects exhibited by application of Pitstop-2 or Pitstop-2-100. Combined application of CHC or AP-2-specific siRNAs and Pitstop-2 resulted in a slight further reduction of MHCII endocytosis to near undetectable levels (Fig. 1, E and G), presumably caused by remaining amounts of clathrin and AP-2 (we estimate 5–8%) in siRNA-treated cells.

These data demonstrate that MHCII endocytosis strictly depends on clathrin/AP-2 and epsin (43), in agreement with earlier data from a number of laboratories (41–43) and with its inhibition by Pitstop compounds but not by clathrin-inactive analogs. The specific effect of Pitstop compounds on CME is further underscored by the observation that plasma membrane dynamics of GPI-eGFP in FRAP experiments are nearly unaffected by application of Pitstop-2 or Pitstop-2-100 (Fig. 2, B and C). A minor effect of Pitstop-2 in this assay (Fig. 2C) may reflect the accumulation of stalled CCPs that may somewhat restrict diffusion of plasma membrane proteins unable to enter CCPs. Conversely, effects of Pitstop-2 on AP-2 dynamics at the plasma membrane (31) are mimicked in clathrin knockout cells (Fig. 2A). Hence, a previous claim that Pitstop-2 displays off target action on clathrin-independent endocytosis (39) is not supported by these data.

**Clathrin TD-Ligand Interactions Regulate TGN/Endosomal Clathrin Dynamics**—Previously, it was shown that Pitstop-2 does not alter clathrin recruitment and distribution in cells but...
FIGURE 1. MHCI endocytosis depends on clathrin and AP-2. A, chemical structures of Pitstop-2, Pitstop-2-100, Pitnot-2, and Pitnot-2-100. B, IC₅₀ values of Pitstop-2- or Pitstop-2-100-mediated interference with clathrin TD complex formation determined by ELISA. C, internalization of transferrin-Alexa 488 into HeLa cells is inhibited by Pitstop-2 or Pitstop-2-100 (30 μM). The amount of transferrin-Alexa 488 internalized into DMSO-treated cells was set to 100%. D, MHCI internalization into HeLa cells is inhibited by Pitstop-2 or the related compound Pitstop-2-100 but not by two different non-clathrin-binding control compounds. Scale bar, 10 μm. E, representative images of MHCI internalization into CHC- or AP-2(k.d.)-depleted HeLa cells. Cells were treated with either DMSO or Pitstop-2. Scale bar, 10 μm. F, quantification of data shown in D (n = 3 independent experiments). The amount of MHCI internalized into DMSO-treated cells was set to 100%. G, quantification of MHCI internalization into clathrin (CHC k.d.)- or AP-2(μ)-depleted (μ2 k.d.) cells treated with DMSO or Pitstop-2 as shown in E. The amount of MHCI internalized into DMSO-treated control cells was set to 100% (n = 3 independent experiments; *, p < 0.05; **, p < 0.005; ***, p < 0.0005). H, representative images of MHCI surface levels of CHC- or AP-2(μ)-depleted HeLa cells. Cells were treated with either DMSO or Pitstop-2. Scale bar, 10 μm. I, quantification of clathrin (CHC) or AP-2(α) levels in cells treated with siRNA against AP-2(μ) or CHC or mock transfected (n = 3 independent experiments).

FIGURE 2. Pitstop-2-mediated inhibition of CCP dynamics has no major effects on general plasma membrane mobility. A, time lapse series of cells stably expressing AP-2(α2)-eGFP were collected after clathrin HC depletion or mock treatment using live cell TIRF microscopy. Cells were imaged for 2 min at 37 °C in presence of 30 μM Pitstop-2 or DMSO (0.1%). AP-2(α2)-eGFP distribution is unaffected by clathrin HC knockdown (CHC k.d.) or application of Pitstop-2, respectively. B, FRAP analysis of HeLa expressing GPI-eGFP and either left untreated or treated with 0.1% DMSO or with 30 μM of the indicated compounds (Pitstop-2, Pitstop-2-100, Pitnot-2, or Pitnot-2-100; see Fig. 1A). 60 s after bleaching, Pitstop-2-treated cells show similar recovery to DMSO-treated controls. Scale bar, 10 μm. C, quantitative analysis of fluorescence recovery as shown in B. Graphs represent the relative fluorescence recovery normalized to the fluorescent intensity before bleaching (n = 3 independent experiments; *, p < 0.05).
as a result of impaired association with its accessory protein ligands reduces clathrin dynamics at plasma membrane CCPs (44). A similar, partially overlapping accessory protein network regulates clathrin function in TGN/endosomal membrane traffic (14), although this has never been addressed directly. To test the hypothesis that clathrin TD-ligand interactions regulate TGN/endosomal clathrin function, we analyzed clathrin dynamics in HeLa cells expressing clathrin LC fused to the photoswitchable fluorescent protein Kaede. Exposure to UV light causes photoswitching of Kaede from green to red fluorescence (35). We used this property to study the dynamic behavior of clathrin at the TGN. In DMSO-treated control cells, local phototransformation of clathrin LC-Kaede at the TGN caused a strong gain of red and a corresponding loss of green fluorescent clathrin LC puncta in the converted area. Green fluorescent clathrin puncta recovered within 3 min because of exchange with clathrin molecules from the surrounding nonphotoconverted area (Fig. 3, A and C). Much less efficient recovery was observed in cells treated with Pitstop-2, suggesting that interference with clathrin TD function impairs clathrin dynamics at the TGN (Fig. 3, B and C). This conclusion was further supported by the reduced intermixing of green and red fluorescent clathrin populations in Pitstop-2-treated cells (Fig. 3D). Thus, clathrin TD-ligand interactions regulate clathrin dynamics at the TGN/endosomal interface, similar to what has been reported for plasma membrane CCPs.

Reduced Mobility of AP-1- and GGA-coated Carriers upon Acute Interference with Clathrin TD Function—Clathrin and AP-2 recruitment to plasma membrane CCPs has been shown to proceed unperturbed in the presence of Pitstop-2. To analyze whether interference with clathrin function affects membrane recruitment of AP-1 or AP-3 adaptors to the TGN or endo-

**FIGURE 3. Acute inhibition of clathrin TD function impairs exchange of clathrin pools at the TGN/endosomal interface.** A and B, UV excitation of clathrin LC-Kaede causes photoconversion from green to red in the illuminated TGN area. 180 s of postphotoconversion mixing of the red and green clathrin LC populations is observed in DMSO-treated control cells (A, arrowheads), but not in cells treated with 30 μM Pitstop-2 (B). Scale bar, 10 μm. C, quantification of data displayed in A and B (n = 3 independent experiments; t = 0 min set to zero). Recovery of green fluorescence in the TGN is impaired in Pitstop-2-treated cells. D, reduced co-localization of the red and green populations of clathrin LC-Kaede in cells treated with Pitstop-2 compared with cells incubated with DMSO or control compound. Depicted are Pearson’s correlation coefficients at different time points postphotoconversion subtracted by the value at 0 min (n = 3 independent experiments; *, p < 0.05; **, p < 0.005).
somal membranes, we fractionated HeLa depleted of endogenous clathrin or treated with Pitstop-2 (Fig. 4A). As expected, Pitstop-2 had no effect on the association of clathrin or AP-2 with membranes, confirming earlier results (31). Similar to AP-2, Pitstop-2 also had no effect on the partitioning of AP-1 and AP-3 adaptors between cytosol and membrane fractions, suggesting that clathrin is not required to recruit or stabilize AP complexes at membranes. The clathrin independence of AP-1, AP-2, and AP-3 recruitment was further confirmed in clathrin knockdown cells (Fig. 4A).

To investigate the effect of Pitstop-2 on AP-1 dynamics, living BSC-1 cells stably expressing eGFP-tagged AP-1σ (eGFP-AP-1σ) (34) were imaged by time-resolved spinning disc confocal microscopy for 2 min with a frame rate of 0.5 Hz. In control cells, AP-1-coated carriers exhibited a highly dynamic behavior with characteristics similar to those of AP-2 at the plasma membrane in agreement with published data (45). The addition of Pitstop-2 had no apparent effect on AP-1 association with membranes but within minutes led to stalling of AP-1 dynamics as observed from kymographs (Fig. 4B), whereas inactive control compounds had no effect (not shown). Quantitative analysis of the mean lifetimes of eGFP-AP-1σ positive structures revealed a significant increase from 46 to 84 s in presence of Pitstop-2 (Fig. 4C). Moreover, the mean square displacement of individual eGFP-AP-1σ puncta was reduced by more than half from ~124 nm × s⁻¹ to 54 nm × s⁻¹ in Pitstop-2- versus DMSO-treated control cells (Fig. 4D).

In addition to AP-1 the TGN/endosomal pools of clathrin are associated with GGA proteins, monomeric adaptors that regulate sorting of MPRs between the TGN and endosomes, among other cargo (reviewed in Ref. 46). Live cell spinning disc confocal imaging revealed that acute perturbation of clathrin TD function by Pitstop-2 interfered with the dynamics of GGA1-eGFP, GGA2-eGFP, and GGA3-eGFP positive structures (Fig.
similar to the effects seen for AP-1. In contrast, Pit-stop-2 had no effect on the dynamics of COPI-coated carriers (e.g., COP-I-eGFP (37)) at the Golgi (Fig. 5D), consistent with its on target activity toward clathrin. These results suggest that clathrin TD function regulates the dynamics of AP-1 and GGA carriers at the TGN/endosomal interface.

Acute Interference with Clathrin TD Function Impairs MPR Retrieval to the TGN—MPR sorting of newly synthesized lysosomal enzymes to the endolysosomal system is mediated by carriers coated with clathrin, AP-1, and/or GGAs (15–17). A hallmark of AP-1 dysfunction is the peripheral dispersion of MPRs caused by defective retrograde sorting to the TGN (10). Because Pit-stop-2-mediated interference with clathrin TD function stalls AP-1 and GGA dynamics, it seemed likely that it would also cause MPR missorting. To investigate this, we analyzed HeLa cells stably expressing the transmembrane and cytoplasmic domain of the cation-independent (CI) MPR fused to eGFP (GFP-CI-MPR) (17). In control cells, GFP-CI-MPR was concentrated in tubular elements at the TGN that were frequently seen to detach and move toward the cell periphery, sometimes breaking into smaller tubular fragments. Earlier work had shown that these tubules contain AP-1 (17) and that AP-1 loss causes the peripheral dispersion of MPR-containing structures (10). To assess whether the clathrin TD is functionally required for the maintenance of MPRs at the TGN, we treated cells with Pit-stop-2 and monitored GFP-CI-MPR dynamics over a time course of 90 min. Within 30 min of Pit-stop-2 application, GFP-CI-MPR-containing structures became progressively fenestrated, and by 60–90 min, the former TGN pool of GFP-CI-MPR had become dispersed into multiple smaller fragments (Fig. 6, A–C). Quantitative analysis showed that Pit-stop-2-mediated interference with clathrin TD function within 60–90 min led to a 2-fold increase in the number of GFP-CI-MPR-containing fragments and a corresponding decrease in object size (4.2 ± 0.9 μm² in control versus 1.7 ± 0.3 μm² in presence of Pit-stop-2-treated cells).

To rule out that these changes in GFP-CI-MPR distribution were caused by a general effect on Golgi function, we investigated secretory traffic of VSVG from the Golgi complex to the cell surface. To this aim, we capitalized on a temperature-sensitive VSVG-SP-eGFP fusion protein that is trapped in the Golgi complex during incubation at 19 °C. A shift to 32 °C allows exit of VSVG-SP-eGFP and secretion to the plasma membrane (18). Quantitative analysis of the ratio between VSVG-SP-eGFP localized to the TGN and to the plasma membrane after 60 min of chase at 32 °C showed no difference between cells incubated with DMSO, Pit-stop-2, or an inactive control compound (Pitnot-2) (Fig. 7). These findings confirm that constitutive secretion of VSVG from the TGN is clathrin/
AP-1-independent and, thus, unaffected by Pitstop-2, in agreement with earlier data (18, 19).

Recycling of Transferrin Is Independent of Clathrin TD Function—Clathrin coats in addition to the plasma membrane and the TGN have also been observed on endosomes (23, 24, 47), where they are involved in polarized sorting to the basolateral surface in epithelial cells, among possible other functions (48). A highly dynamic form of endosomal clathrin (termed G-clathrin) has been postulated to regulate rapid recycling (27). Recycling transferrin, on the other hand, has been localized on tubular endosomes lacking apparent clathrin coats but covered with the dynamin-related EHD1/3 proteins (49–51). Because transferrin endocytosis strictly depends on clathrin function, the role of clathrin in endosomal recycling of transferrin cannot be addressed genetically or by siRNA knockdown.

To address this point, we loaded HeLa cells with Alexa 488-labeled transferrin (Tf\textsuperscript{488}) and then assayed Tf\textsuperscript{488} recycling following addition of DMSO, Pitstop-2, or a non-clathrin-binding inactive control compound (Pitnot-2). Tf\textsuperscript{488} recycling from perinuclear endosomes proceeded unperturbed over the time course of 15 min (Fig. 8). These data thus suggest that clathrin TD function is dispensable for recycling of transferrin from perinuclear endosomes to the cell surface.

As a further control, we also analyzed the acute effects of Pitstop-2 on the degradation of internalized ligand-bound EGFRs, a clathrin-independent process involving ESCRT-mediated sorting into multivesicular bodies and lysosomal proteolysis but not clathrin (although clathrin in the long run may be required to sort lysosomal enzymes via MPRs; see Fig. 6). Cells were stimulated with EGF 30 min prior to the addition of Pitstop-2, inactive control compound or DMSO. During the subsequent chase period, EGFRs were degraded with near identical half-times irrespective of the addition of Pitstop-2 (Fig. 9, A and B). Similarly, degradation of fluorescently labeled internalized EGF\textsuperscript{Alexa647} proceeded unperturbed in the presence of Pitstop-2 (Fig. 9, C and D). Thus, acute inhibition of clathrin TD function has no apparent effect on the degradation of internalized EGFRs or its ligand. Taken together, our data reveal a selective role for clathrin TD-ligand interactions in regulating AP-1 and GGA dynamics and MPR sorting, whereas secretory traffic of VSVG, transferrin recycling, and EGF/EGFR degradation are unaffected.

**DISCUSSION**

Our data question the conclusion of a previous study that the Pitstop-2 compound has an off target action in clathrin-independent endocytosis (39). Using the same cell type and conditions as reported in that study (and, thus, ruling out technical or cell type-specific differences), we show based on both RNAi against clathrin and AP-2(\(\mu\)) and two different Pitstop\(\texttrademark\) compounds as well as inactive analogs that MHCI endocytosis is indeed mediated by clathrin and AP-2 (Fig. 1). We note that in
none of the previously published works by Donaldson and co-workers, including Ref. 39, is a direct quantitative comparison of control versus clathrin- or AP-2-depleted cells with respect to their ability to internalize MHC class I presented. Instead, it was claimed based on overexpression of a supposedly GTP-locked dominant-negative Arf6 mutant that MHC class I follows an Arf6-specific clathrin-independent endocytosis pathway (6, 40, 52). Recent data from several other laboratories have called this conclusion into question because MHC class I endocytosis is unaffected by knockdown of Arf6 (41) but depends on the clathrin/AP-2 binding endocytic protein epsin 1 (42, 43), consistent with our data reported here. Because Arf6 regulates rapid recycling, it is possible that the previously reported effects of overexpression of dominant-negative mutant Arf6 on MHC class I uptake (6, 40, 52) may have resulted from general perturbations of the endosomal system rather than reflecting clathrin-independent MHC class I internalization. The specificity of Pitstop compounds for CME is further underscored by the lack of effect on general plasma membrane mobility (Fig. 2; in contrast to Ref. 39) and by recent studies on the entry of various animal viruses that either use CME or clathrin-independent routes (53). Collectively, these results highlight the importance of using multiple approaches, including compounds and their inactive analogs to increase probability that on target actions are being observed.

The results reported here also reveal a crucial role for clathrin and its TD in AP-1- and GGA-mediated sorting of MPRs between the TGN and endosomes. Live imaging of the dynamics of AP-1- or GGA-coated carriers shows that application of Pitstop-2 causes a profound arrest in AP-1 (Fig. 4) and GGA (Fig. 5) dynamics, a conclusion further supported by photoconversion experiments involving clathrin LC-Kaede (Fig. 3). These changes are accompanied by the progressive dispersion of MPRs to peripheral endosomal puncta (Fig. 6), a phenotype mimicking AP-1 deficiency in cells and in vivo (10). The specificity of these phenotypes is underscored by a number of observations. First, despite its profound block of AP-1 and GGA dynamics, Pitstop-2 had no effect on the mobility or function of COPI-coated carriers formed at the Golgi complex (Fig. 5D) or on the general mobility of plasma membrane proteins (Fig. 2). Second, stalled CCP dynamics at the TGN were readily reversible after washout of the drug. Third, pathways known to be clathrin-independent, such as the traffic of constitutive cargo such as VSVG from the Golgi to the plasma membrane (Fig. 7) or Shiga toxin internalization (31) proceed unperturbed in the presence of Pitstop-2.

Our data therefore confirm and extend previous work on the role of clathrin-associated adaptors such as AP-1 and GGAs in TGN/endosomal sorting of MPRs and, thus, in the delivery of lysosomal hydrolases (9). Specifically, the results described here indicate that clathrin TD association with its ligands not only regulates CCP dynamics at the cell surface but also at the TGN/endosomal boundary. Putative TD ligands (also termed clathrin-associated sorting proteins, CLASPs) at the TGN comprise the β- and γ-subunits of AP-1, GGAs, epsin R/Clint, amphiphysin, and auxilin 2, as well as members of the sorting nexin family of BAR domain proteins (i.e., SNX4 and SNX18) that associate with clathrin (54). Future work will need to address precisely which of these interactions underlie the stalled dynamics of AP-1- and GGA-containing clathrin carriers observed in Pitstop-2-treated cells. Based on previous work on CME (31), we predict that clathrin TD-ligand interactions may regulate CCP

![Figure 9](image-url)

**FIGURE 9.** EGF receptor degradation proceeds unperturbed in the presence of Pitstop-2. A, 30 μM Pitstop-2, 30 μM Pitnot-2, or 0.1% DMSO were added to HeLa cells 30 min after stimulation with 500 ng/ml unlabeled EGF and chased for the indicated times. Pitstop-2 treatment had no effect on EGF receptor degradation. B, quantification of data shown in A using ImageJ (n = 3 independent experiments). C, HeLa cells loaded for 20 min with Alexa647-labeled EGF (EGF647) were treated with DMSO or 30 μM Pitstop-2 and allowed to degrade EGF647 for the indicated times. Scale bar, 10 μm. D, quantification of the data shown in C (n = 2 independent experiments). The data were normalized to the amount of EGF647 present in DMSO-treated cells at 0 min of chase.
formation and turnover at multiple stages within their life cycle at the TGN.

Finally, our work has revealed that clathrin TD-ligand interactions are apparently dispensable for the recycling of internalized transferrin from perinuclear endosomes (Fig. 8) or for the ligand-induced degradation of EGF receptors (Fig. 9), a pathway involving the ESCRT machinery. These results correlate well with observations by live imaging and by electron microscopy that have shown the presence of recycling transferrin in endosomal EHD-coated tubules emanating from perinuclear sites devoid of discernible clathrin coats (49–51). However, because of experimental constraints (our assay is based on the prior accumulation of transferrin in perinuclear endosomes), we cannot rule out the possibility that clathrin and its partners play a role in rapid recycling from early sorting endosomes, a pathway shown to depend on the small GTPases Rab35 and Arf6 (55), the actin cytoskeleton, and possibly clathrin (27).

Overall, our data suggest that Pitstop® compounds apart from their use as CME inhibitors are important tools for the functional dissection of intracellular trafficking pathways involving lysosomes and lysosome-related organelles including melanosomes or lytic granules in cytotoxic T cells (56). As with all small molecule inhibitors, it is important to carefully monitor activities as well as potential side effects caused by the chemical scaffold of the drug itself (i.e., by assaying clathrin inactive control compounds in parallel). Future studies directed at the generation of more potent clathrin inhibitors on new chemical scaffolds will hopefully accelerate these efforts.

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