Endocytic Adaptor Molecules Reveal an Endosomal Population of Clathrin by Total Internal Reflection Fluorescence Microscopy*§

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Received for publication, November 20, 2003, and in revised form, January 8, 2004
Published, JBC Papers in Press, January 12, 2004, DOI 10.1074/jbc.M312717200

Most eukaryotes utilize a single pool of clathrin to assemble clathrin-coated transport vesicles at different intracellular locations. Coat assembly is a cyclical process. Soluble clathrin triskelia are recruited to the membrane surface by compartment-specific adaptor and/or accessory proteins. Adjacent triskelia then pack together to assemble a polyhedral lattice that progressively invaginates, budding off the membrane surface encasing a nascent transport vesicle that is quickly uncoated. Using total internal reflection fluorescence microscopy to follow clathrin dynamics close to the cell surface, we find that the majority of labeled clathrin structures are relatively static, moving vertically in and out of the evanescent field but with little lateral motion. A small minority shows rapid lateral and directed movement over micrometer distances. Adaptor proteins, including the α subunit of AP-2, ARH, and Dab2 are also relatively static and exhibit virtually no lateral movement. A fluorescently labeled AP-2 β2 subunit, incorporated into both AP-2 and AP-1 adaptor complexes, exhibits both types of behavior. This suggests that the highly motile clathrin puncta may be distinct from plasma membrane-associated clathrin structures. When endocytosed cargo molecules, such as transferrin or low density lipoprotein, are followed into cells, they exhibit even more lateral motion than clathrin, and gradually concentrate in the perinuclear region, consistent with classical endosomal trafficking. Importantly, clathrin partially colocalizes with internalized transferrin, but diverges as the structures move longitudinally. Thus, highly motile clathrin structures are apparently distinct from the plasma membrane, accompany transcytosis, and contain AP-1, revealing an endosomal population of clathrin structures.

In eukaryotic cells, clathrin coats assemble to transport select cargo molecules from the trans-Golgi network (TGN)1 and the plasma membrane (1–4), although clathrin has also been detected in association with various endosomal compartments (5–8). A clathrin coat forms upon membranes as a polygonal lattice, which progressively curves to incorporate membrane, cargo, and extracellular fluid into a vesicular transport intermediate (1–4). Whereas the trafficking itineraries of many cargo molecules sorted into clathrin-coated vesicles are known, the kinetics of clathrin coat assembly, and the mechanics of adaptor protein interaction are not well understood. Transected, fluorescently-tagged clathrin, combined with live cell imaging, shows that clathrin-coated structures are extremely dynamic (9). Total internal reflection fluorescence microscopy (TIR-FM) has extended the time-resolved study of clathrin structures by allowing the selective imaging of molecular events very close to the plasma membrane. This permits analysis of the temporal colocalization of clathrin with other proteins (10–12). In these studies, four distinct behaviors of clathrin-coated structures have been reported: stasis, disappearance, reappearance, and lateral motility (9–12).

The evanescent field utilized in TIR-FM has a decay or penetration depth of 70–110 nm, exciting only those fluorophores close to the basal plasma membrane (13). Thus, clathrin-coated structures that disappear from the TIR-FM field could be internalized vesicles moving deeper into the cell, the loss of concentrated and hence detectable clathrin because of vesicle uncoating or, finally, because of the physical deformation of the plasma membrane out of the TIR-FM field (10–12, 14). These dynamics are coupled, in part, to the activity of dynamin, actin, and tubulin (9–11). The selective excitation of TIR-FM also allows imaging of adaptor proteins, which link transmembrane receptors to the clathrin coat. Endocytic adaptor proteins, including AP-2, Disabled-2 (Dab2), and the autosomal recessive hypercholesterolemia (ARH) protein, bind to the plasma membrane via phosphatidylinositol 4,5-bisphosphate, recruit and polymerize clathrin on the membrane, and synchronously bind cargo molecule receptors such as transferrin or low density lipoprotein (LDL) receptors (15–19). With the demonstration that AP-2 is not absolutely required for clathrin-mediated endocytosis, understanding the operation of these alternate adaptors is now a priority (20–22). There is an emerging role for these proteins as part of the endocytic cargo selection machinery, analogous to the role GGA proteins play at the TGN (17, 22, 23).

Here, we describe two other phenomena associated with temporal changes in clathrin-coated structures: coalescence and separation. Examination of the endocytic adaptor proteins total internal reflection fluorescence microscopy; Dab2, Disabled-2; ARH, autosomal recessive hypercholesterolemia; AP, adaptor protein; LDL, low density lipoprotein; EGF, epidermal growth factor; GFP, green fluorescent protein; Tfn488, transferrin conjugated to Alexa 488; EGF488, EGF conjugated to Alexa 488; LCa, light chain a.

In eukaryotic cells, clathrin coats assemble to transport select cargo molecules from the trans-Golgi network (TGN)1 and

* This work was supported in part by National Institutes of Health Grant R01 DK53249 and the Senior Vice Chancellor for the Health Sciences, University of Pittsburgh in support of the Competitive Medical Research Fund. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ The on-line version of this article (available at http://www.jbc.org) contains movies of live cell TIR-FM corresponding to the experiments shown in Figs. 1 (GFP-LCa), 3 (AP-2 α subunit-YFP), 6 (AP-2 β2 subunit-YFP and DabRed-LCa), and 7 (Tfn488).

† Supported by the Renal and Epithelial Biology Training Grant 5T32 DK061296-02.

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1 The abbreviations used are: TGN, trans-Golgi network; TIR-FM,
AP-2, Dab2, and ARH, along with the cargo molecules transferrin, LDL, and epidermal growth factor (EGF) shows that the previous appearance and disappearance of labeled structures observed using TIR-FM is common to plasma membrane-localized proteins, while proteins and cargo that are associated with endosomes exhibit significant lateral motion within the TIR-FM field. Dual-color TIR-FM defines the relationship between structures, and establishes whether endosomal clathrin population sensitive to nocodazole treatment. The depth of the evanescent field is also determined empirically, and endosomes are found within this plane. Thus, the highly mobile clathrin structures observed by TIR-FM appear to be endosomal in nature.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—To label clathrin with the enhanced green fluorescent protein (GFP) we used a plasmid containing a human neuronal-specific clathrin light chain a (LCa) isoform ligated into the BamHI and XbaI sites of pEGFP-C3 (GFP-LCa). By inserting the brain LCa via the internal BamHI site, the four amino-terminal residues (MAEL) of the 248-amino acid light chain sequence are removed and replaced with residues (PRAR) encoded by the vector polyn linker joining the carboxyl terminus of GFP to the amino-terminal end of LCa. The plasmid encoding dReRed-LCa was kindly provided by Tom Kirchhausen, the plasmid encoding the AP-2 α2-subunit fused via the carboxyl terminus to YFP (α2-YFP) was a kind gift from Jim Keen, whereas the AP-2 β2 subunit fused via the carboxyl terminus to YFP (β2-YFP) was a kind gift from Alexander Sorkin. The ARH-GFP construct was prepared by ligating full-length human ARH, generated by PCR using the primers 5’-TAT-AGCTAAGCGAGCCGACATGGACGCGCTCAAGTCGG-3’ and 5’-GCCG-CAGATCTGCGAAGCTGAAGAGGTCATCCTG-3’, into the Nhel and BglII sites of pEGFP-N1 (Clontech). The Dab2-GFP construct has been cloned into the NheI and XbaI sites of pEGFP-N1 (Clontech). The Dab2-GFP construct is prevented by automated dideoxynucleotide sequencing.

**Cell Culture—**CV-1 (CCL-70, ATCC) and A431 (CRL-1555, ATCC) cells were cultured in Dulbecco’s modified Eagle’s medium (Cellgro) supplemented with 10% fetal calf serum (HyClone) and 2% L-glutamine. All plasmids and constructs were verified by automated dideoxynucleotide sequencing.

**Cell Culture—**CV-1 (CCL-70, ATCC) and A431 (CRL-1555, ATCC) cells were cultured in Dulbecco’s modified Eagle’s medium (Cellgro) supplemented with 10% fetal calf serum (HyClone) and 2% L-glutamine (Invitrogen) at 37 °C in a humidified atmosphere with 10% CO2. Normal human fibroblasts (GM 01386, Human Genetic Cell Repository at the Coriell Institute) were cultured in Eagle’s minimal essential medium (BioWhittaker) supplemented with 10% fetal calf serum and 2% L-glutamine. All cells were plated onto glass-bottom 35-mm tissue culture dishes (Mat-tek) at ~40% confluence 1 day prior to transfection. Cells grown on glass-bottom dishes were transfected with 500 ng of DNA 1 day prior to imaging using LipofectAMINE 2000 (Invitrogen). Cells were imaged either in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and 2% L-glutamine or phenol-free Dulbecco’s modified Eagle’s medium buffered with 25 mM Hepes (pH 7.2) (starvation medium), neither of which affected the dynamics or the imaging of the cells, although 0.45 μM sucrose had a higher refractive index than normal medium, so the evanescent wave penetrated deeper. Cells were imaged on a Nikon 2000E inverted microscope (Melville, NY) with a 1.45 NA oil immersion objective capable of epifluorescence and TIR-FM illumination through the objective. GFP, YFP, Alexa 488, and DIO were excited with a 488 nm line of an argon laser, whereas DsRed was excited with a 567 nm line of a krypton laser (laser bench provided by Prairie Technologies, Madison, WI). All laser lines were selectively blocked within the laser bench and illumination (both intensity and blanking) was controlled by acousto-optical tunable filters. To image both green and red fluorophores simultaneously, an Optical Insights image splitter was used. Images were collected using a water-cooled Orca II ER (Hamamatsu, Tokyo, Japan). Data sets were acquired using SimplePCI (C-imaging systems, Cranberry, PA) software. In all experiments images were collected at ~1 frame/s. The only post-processing performed was to ensure appropriate registration (using fiduciary points) of two color images, and image scaling such that labeled structures are clearly visible. No other filters or enhancements were applied to the data sets. For sucrose treatment, the culture medium in the incubation chamber was replaced after acquiring several images of a single cell by TIR-FM with 0.45 M sucrose in starvation medium. The cell was located and imaging resumed ~30 s after addition of the sucrose solution. Cells were then imaged for up to 1 h after treatment, during which they remained the same in appearance. To depolymerize the microtubule cytoskeleton, cells were incubated with 10 μM nocodazole (Sigma) for 30 min at 37 °C in starvation medium prior to imaging, and then imaged in the continued presence of the nocodazole.

**Ligand Internalization—**For the transferrin or EGF uptake experiments, cells were serum starved by incubation in starvation medium for 1 h at 37 °C prior to placement on the microscope stage. Once cells were ready for imaging, transferrin conjugated to Alexa 488 (Thy488) (Molecular Probes), or EGF conjugated to Alexa 488 (EGF488) (Molecular Probes) was added to a final concentration of 50 μg/ml or 10 ng/ml, respectively, and the cells were imaged immediately. For LDL uptake experiments, cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% lipoprotein-deficient fetal calf serum and 2% L-glutamine, or 1 h prior to imaging, up-regulated using LDL receptors. Cells were given 20 μg/ml Dio-LDL (Biomedical Technologies) and imaged immediately. For transferrin pulse experiments, cells transfected with DsRed-LCa were serum starved as above, incubated with 50 μg/ml Thy488 for 30 min, washed, and imaged in starvation medium.

**Image Processing and Data Presentation—**Visualization of subtle motion is difficult in monochromatic image sequences. However, using color superposition of these monochrome stacks, it is possible to see quite small changes in object position. Thus, by sequencing temporal imaging sets in the red, green, and blue color channels of a color image, non-motile structures will appear as white structures, whereas motile structures will show a green-red-blue trail. For image motion over time, an XY time image can be generated such that the position is visualized in the x-axis, and time visualized in the y-axis, which is similar to a classical kymograph, and used extensively here. On the kymographs, a solid, vertical line represents a non-moving vesicle, whereas breaks in an individual line represent the appearance/disappearance of the object from the region, and slanted lines represent lateral particle motion. Even breaks across the entire projection represent a temporary adjustment of focus. Two-color TIR-FM images were processed similarly, except they were not time colored.

**Online Supplemental Material—**Movies of live cell TIR-FM corresponding to experiments shown in Fig. 1 (GFP-LCa), Fig. 3 (AP-2 α2-subunit-YFP), Fig. 6 (AP-2 β2 subunit-YFP and DsRed-LCa), and Fig. 7 (Thy488). Additional movies can be found at www.chi.pitt.edu/movies/keyel/keyel.html.

**RESULTS**

**Novel Spatial Clathrin Phenomena**—When cells are transiently transfected with the clathrin LCa fused to the carboxyl terminus of GFP (GFP-LCa), the fluorescent clathrin appears as bright puncta that colocalize extensively with endogenous clathrin (data not shown) (9, 26). By epifluorescence, the cytosolic pool of unpolymerized fluorescent clathrin is visible as a diffuse haze, along with bright TGN and surface puncta (Fig. 1B). When the same fibroblasts are imaged using TIR-FM optics, the signal is restricted to fluorescent structures close to the plasma membrane surface, hence the improved signal-to-noise ratio of the image (Fig. 1C). In agreement with previous studies examining clathrin dynamics (9–12, 27), we observe fluorescent clathrin structures with four different behaviors in living cells (Fig. 1, D–I): appearing (Fig. 1, open arrows), disappearing (white arrows), moving laterally (arrowheads), and relatively stationary. Importantly, the high signal-to-noise ratio in TIR-FM allows images to be collected using low laser power, restricting the evanescent field and minimizing photo-bleaching. The lack of photobleaching is evident from the persistence of the fluorescence in non-motile structures immediately adjacent to abruptly disappearing spots (Fig. 1F).

Two different types of lateral motion are apparent in our data sets. The first is the uncoordinated gyration of a coated structure about a ~1-μm region, which we term local motion (Fig. 1, D–F, open arrowheads). The second type of lateral motion is the dynamic (0.5–0.67 μm/s), directed transit of a structure down a roughly linear path (1.5–9 μm travel distance), designated long distance motion (Fig. 1G, white arrowheads). Others consider this long distance motion to be planar clathrin-coated lattice or invaginated coated pits moving in a microtubule-dependent fashion while still associated with the
Fig. 1. Different clathrin dynamics in living cells. Normal human fibroblasts were transfected with GFP-LCa 18 h prior to imaging. Two expressing cells, visualized by DIC (A), epifluorescence (B), and TIR-FM (C), show that most of the cytosolic and TGN-associated GFP-LCa is eliminated and the signal-to-noise ratio is improved in the TIR-FM field. To demonstrate appearance (open arrows), disappearance (white arrows), splitting (asterisks), coalescence, long distance (white arrowheads), and local (open arrowheads) motion, kymographs (xt projection) from selected regions of TIR-FM data set are shown (D–I). The accompanying still images show traditional representations from specific time points in the kymographs, indicated by the lines. The vertical green line in D is included to show the degree of local motion. The scale bar is 10 μm for A, and 20 s for D–I.
plasma membrane, although localization to endosomes was not excluded (10, 27). However, Fig. 1G shows a large (~0.6 μm diameter) clathrin-coated structure moving laterally (white arrowheads, and seen by the green-red-blue transition in the image sequence stills). Along its entire trajectory, this structure travels by several non-motile clathrin-positive structures without evidence of interaction; there is no change in speed, surface area, or collisional activity (see Supplemental Materials movie 3). Because these objects are larger than the diffraction limit of the optical system being used, and invaginated coated pits are ~100 nm in depth (14), this suggests that the evanescent wave allows visualization of subcellular structures beyond that indicated by the absolute value of the decay depth (9–11).

Because structures with sizes on the order of the depth of the evanescent wave can apparently pass each other within the TIR-FM field, we determined the depth of the excitatory evanescent wave empirically. The intensity of the evanescent wave decreases exponentially with distance,

\[ I_z = I_0 e^{-d/\lambda} \]  

(Eq. 1)

where \( I_z \) is the intensity at depth \( z \), \( I_0 \) is the initial intensity, and \( d \) is the characteristic decay depth, defined as,

\[ d = \frac{\lambda}{(4 \pi n_2)(\sin^2 \theta_2 - \sin^2 \theta_1)^{1/2}} \]  

(Eq. 2)

where \( \lambda \) is the wavelength of the incident light in a vacuum, \( \theta_2 \) is the angle of the incident light, \( \theta_1 \) is the critical angle, defined as \( \sin^{-1} (n_2/n_1) \), and \( n_1 \) and \( n_2 \) are the refractive indices of the substrate and liquid medium, respectively (13). The refractive indices of the glass coverslip and aqueous culture medium (Fig. 6B shows Tfn488 in the medium under ~85% of the cell in the TIR-FM field) are 1.518 and 1.33, respectively, making the calculated critical angle 61.2°. Thus, using 69° incident light and a 488-nm excitation wavelength, \( d \) is calculated to be 79.4 nm. Empirically, in our system, we measured the critical angle (now 63.6°), then the calculated decay depth becomes 97.5 nm. These values are all within the 70–110 nm range for \( d \) quoted in the literature. However, \( d \) is only the depth at which the intensity of the evanescent wave is 37% of the initial intensity. To empirically determine the experimental depth at which fluorophores are visible, 1-μm fluorescent beads were imaged in the TIR-FM field. A measured diameter of 786 nm indicates a visible depth of 190 nm (using the Pythagorean theorem), which is over twice the calculated decay depth. Precise determination of the depth of the TIR-FM field is difficult because of the physical limits of the optical system and the diffraction limit of light. We find that, typically, the nucleus of CV-1 cells is ~150 nm from the ventral plasma membrane surface and in some data sets, the nucleus is occasionally observed within the TIR-FM field as an oval, poorly stained region (see Figs. 5 and 6). Therefore, we conclude that in our system, the evanescent wave penetrates deep enough into the cell to excite two vertically aligned clathrin-coated vesicles, and that these structures can be distinct from the plasma membrane.

We also report two other clathrin dynamics seen using TIR-FM: the coalescence and separation/splitting of clathrin-containing structures (Fig. 1, E–G). The structures that coalesce range in size from diffraction-limited spots to structures 0.8–1.0 μm in diameter. These structures commonly exhibit local motion prior to coalescence. The most common sequence seen is a static structure around which another moves, followed by merging of the two (Fig. 1H). The reverse dynamic is also seen, where clathrin structures actively separate from others prior to moving laterally or disappearing from the TIR-FM field (Fig. 1, E–G, asterisks).

The clathrin structures observed by TIR-FM range in diameter from diffusion-limited objects up to 0.6–1 μm. Importantly, the different sized structures distribute unevenly through the cell. Diffraction-limited structures are most abundant in the periphery of the cell (7% large structures), whereas larger objects are prevalent in the perinuclear region (75% large structures; Fig. 1C). As the TGN lies deep within the cell, this perinuclear clathrin is probably not TGN associated. This
is reinforced by epifluorescent images showing that TGN-associated clathrin is localized to a Golgi surface near the top of the perinuclear region (Fig. 1B). The size and perinuclear clustering of the larger clathrin structures is unusual for coated pits or coated vesicles at the cell surface.

Sucrose Halt All Clathrin Activity—As clathrin movement out of the TIR-FM field is assumed to be because of endocytosis, we tested the validity of this assumption by placing cells transfected with GFP-LCa into 0.45 x sucrose dissolved in culture medium. This treatment is known to block endocytosis by driving the polymerization of clathrin into microcages incapable of participating in vesicle formation (28). Under these conditions, all motion, from appearance and disappearance to local and long distance motion, stops within 30 s following treatment (Fig. 2). Additionally, there is an increase in the number of clathrin-positive structures to 120% of pre-sucrose levels, consistent with the formation of microcages that deposit on the membrane. These structures also rapidly bleach (t½ ~ 100 s, using laser power that shows minimal bleaching in untreated cells), strongly suggesting that there is no molecular exchange with cytosolic clathrin, as occurs under normal conditions (29).

Endocytic Adapter Proteins Do Not Display Long Distance Motion—Although the dynamics of clathrin in relation to the actin cytoskeleton, dynamin, and, most recently, AP-2 have been examined by TIR-FM (9–11, 27), the spatio-temporal behaviors of alternate endocytic adapter proteins have not been reported. We expressed and imaged YFP fusions of the αc and β2 subunits of the AP-2 endocytic adaptor (Fig. 3), as well as the alternate endocytic adaptors ARH, Hip1R, and Dab2, fused to GFP, for comparison (Fig. 4). Our TIR-FM observations are consistent with previous reports on the distribution of the endogenous proteins that, in each case, localizes to punctate structures on the membrane (16–18, 24, 30–32). However, in living cells, the motion of these adaptors is quite different from that of clathrin. The AP-2 αc subunit, ARH, and Hip1R all localize to small, diffraction-limited punctate structures that appear and disappear in common with clathrin puncta (Fig. 3, A–C, and Fig. 4, A–D and F, arrows), but less than 1% of the structures show any long distance motion. Dab2 also localizes to appearing/disappearing/static structures, but many of these are considerably larger, up to 1 μm in diameter (Fig. 4E). These tagged proteins do appear and disappear in the same spot, supporting the idea that there are endocytic “hot spots” on the plasma membrane (14).

Standard immunofluorescence microscopy shows that Hip1R localizes to surface clathrin-coated puncta at steady state (24), as do AP-2 and Dab2 (16, 18, 27, 33). Rapid freeze/deep etch EM shows that the Hip1R puncta correspond to both planar clathrin structures and coated pits (24). If either of these structures display lateral motion, the fluorescent Hip1R should also exhibit lateral motion. We find that only ~3% of Hip1R-positive structures exhibit local motion, compared with 30% for clathrin-positive structures, and they have minimal long distance motion (<0.4% structures) (Fig. 4, C and F). Similarly, AP-2 and Dab2, both of which colocalize extensively with clathrin at steady state, have fewer puncta exhibiting local motion than GFP-LCa, <1 and 9%, respectively (Figs. 3, A–C, and 4). ARH does show some lateral motion (16%), although the protein localizes less well with clathrin-containing structures (Fig. 4, A and B) (17). Thus, we conclude that the clathrin-positive structures exhibiting long-range motion are not associated with plasma membrane-associated coat components.

Distinct Dynamic Behavior of the AP-2 αc and β2 Subunits—In contrast to the AP-2 αc subunit, ARH, Dab2, and Hip1R, the AP-2 β2 subunit exhibits both long distance and local motion (5 and 32%, respectively), in common with clathrin (Fig. 3, D and E). To better understand this difference in the dynamics of the αc and β2 subunits, we correlated the relative motion of clathrin and AP-2 by simultaneous expression of DsRed-LCa and AP-2 αc-YFP or β2-YFP fusions in CV-1 cells. When expressed together, clathrin does not show perfect colocalization with the αc subunit-YFP fusion, a specific marker for AP-2 (Fig. 5A). In fact, αc–YFP is only seen in static and disappearing/appearing structures and not in long distance moving structures (Fig. 5B), in agreement with the single-color experiments. Therefore, the division of clathrin structures into laterally mobile or stationary/appearing/disappearing is a classification that appears to discriminate between AP-2 adaptor-positive clathrin, and AP-2 adaptor-negative clathrin.

However, long distance motion is clearly apparent when the β2-YFP protein fusion is expressed alone. When CV-1 cells cotransfected with β2-YFP and DsRed-LCa are examined, cells that express low levels of β2-YFP (Fig. 6, A–C) show colocalization of β2-YFP with ~10% of long distantly moving clathrin structures (~8% of total clathrin). About 20% of the DsRed-LCa and β2-YFP structures in these cells exhibit disappearing/appearing behavior per minute. By contrast, in cells expressing high levels of β2-YFP (Fig. 6, D and E), all of the long range mobile clathrin structures are now positive for the β2-YFP signal and colocalization between all β2-YFP and DsRed-LCa structures increases from ~75 to 85%. As the primary sequence of the AP-2 β2 subunit shows 85% homology to the β1 subunit of the AP-1 adaptor complex it can act as a functional replacement for the β1 subunit in AP-1 complexes. This is shown experimentally (34, 35) and the promiscuity is also observed in vivo, where native AP-1 complexes purified from brain contain the β2 subunit (36). Thus, upon overexpression of the β2-YFP construct, the puncta observed probably represent both AP-1 and AP-2-positive structures. AP-1 localizes to the TGN, which generally cannot be observed by TIR-FM (37, 38), but is also found on endosomes (39–41). Because the AP-2 αc subunit exhibits virtually no long distance and limited local motion, one explanation for the highly mobile clathrin population could be that it contains AP-1 adaptors, which are not present on the plasma membrane. Importantly, the extent of AP-1 labeling will also increase as the expression level of the β2-YFP subunit rises. Therefore, we propose that the clathrin observable by TIR-FM is accounted for by both AP-1 and AP-2 positive structures, the former correlating with the long distance motile structures, and the latter correlating with the static, appearing and disappearing structures.

In general, our data agree with a previous study showing that laterally mobile clathrin structures are devoid of AP-2 (27). They differ in two important respects, however. First, the correlation between the β2 subunit-clathrin overlap and the β2 subunit expression level shown here suggests that the long distance mobile population does contain an associated adaptor complex, AP-1. Second, within the static population of fluorescent αc and β2 subunits, the appearance and disappearance behavior typical of budding clathrin structures is clearly seen. For example, in a selected image sequence from αc-YFP expressing CV-1 cells (Fig. 3D, circles), two labeled structures disappear abruptly from the field. Similarly, in dual-color images of DsRed-LCa and β2-YFP, synchronous exit of double-labeled yellow structures is seen (Fig. 6C, circles). We believe that this demonstrates that AP-2 and clathrin leave the plasma membrane together in the form of a polymerized coat, in contrast to the view that AP-2 positioned on the cell surface is entirely static and not seen in structures during internalization (27).
Fig. 3. **Dynamics of the large subunits of the AP-2 adaptor.** CV-1 cells were transfected as described in the legend to Fig. 1 with either AP-2 \( \alpha_c \)-YFP (A–C) or \( \beta_2 \)-YFP (D and E) and imaged by TIR-FM. The boxed regions in the TIR-FM images (A and D) are shown by the kymographs (B, C, and E). Dynamics are indicated with the same symbols used in Fig. 1. The black asterisks denote focal shifts in the data set. Although the AP-2 \( \alpha \) subunit shows appearance and disappearance (arrows and circles in still images), it exhibits little to no lateral motion. The \( \beta_2 \) subunit, however, displays nearly identical dynamics to clathrin. The scale bar represents 10 \( \mu \)m (A and D) or 20 s (B, C, and E).
Cargo Molecules followed into the Cell by TIR-FM—There is compelling evidence for the formation of clathrin-coated buds and vesicles upon transferrin-positive early and recycling endosomal elements (5, 8). In whole mount EM images, these structures are often in close proximity to the plasma membrane (5, 8), probably within the constraints of the evanescent field in TIR-FM. Indeed, the uptake and intracellular trafficking of Tfn488 can be clearly observed by TIR-FM in living cells (Fig. 7, A–D) (42). The media appears intensely fluorescent because of the presence of unbound Tfn488. The unlabeled regions in the field correspond to focal adhesions (Fig. 7A, white arrows) where the cell is in tight contact with the coverslip and thus excludes the fluorescent probe. Elsewhere, the cell surface is separated from the glass substrate such that media diffuses freely underneath, accounting for the diffuse background fluorescence observed in these experiments. During Tfn488 uptake, diffraction-limited peripheral structures appear throughout the cell, which then move rapidly (0.5 μm/s) toward the perinuclear region, where they separate and fuse with each other, forming structures up to 1 μm in diameter over time (Fig. 7B). At the periphery of the cell, the motile properties of the transferrin-positive structures mimic that of the static clathrin and AP-2 populations. Between this region and the larger juxtanuclear recycling endosomes, an intermediate zone is characterized by rapid, bidirectional long distance trajectories (Fig. 7B). In the continued presence of Tfn488, the larger vesicles are apparent after 15 min of uptake, characteristic of recycling endosomes (Fig. 7, A and B). Tfn488 exhibits more long distance motion than GFP-LCa (15 versus 7%) with a longer average path length (5 versus 2.5 μm). When CV-1 cells are pulsed with fluorescently labeled LDL particles (DiO-LDL), similar motion is observed with the LDL moving to ~1-μm diameter perinuclear structures, although, in contrast, no recycling is seen (Fig. 7, E and F). This shows clearly that portions of the endocytic pathway can be observed by TIR-FM.

Occasionally, several independent Tfn488-labeled structures are seen sequentially trafficking several micrometers along a single track within a short time period (Fig. 7C, white arrowheads). This long distance motion of Tfn488, and of GFP-LCa, is arrested by treating cells with 10 μM nocodazole for 30 min prior to imaging (data not shown), demonstrating that the labeled structures move in a motor-dependent fashion along microtubule tracks, as previously reported (10, 43). Furthermore, following nocodazole treatment, the larger 0.6–1-μm structures do not accumulate in the perinuclear region. No-
The AP-2 α subunit does not colocalize with clathrin structures undergoing long distance motion. CV-1 cells were transfected as described with DsRed-LCa and AP-2 α=YFP and imaged by TIR-FM. There are two distinct populations of DsRed-LCa-labeled structures (middle panels, red in merged image) visible in these cells (A), a laterally motile population devoid of AP-2 and a relatively immobile, AP-2-associated population. The kymograph (B) demonstrates that AP-2 (left panels, green in merged image) is present in appearing and disappearing structures, but the adaptor is absent from structures undergoing lateral motion. Dynamics are indicated with the same symbols used in Fig. 1. The scale bar represents 10 μm (A) or 20 s (B).
codazole treatment does not alter the appearance/disappearance behavior of either in the TIR-FM field, however, confirming that endocytosis from the plasma membrane is unaffected by microtubule depolymerization. In related experiments, we find that when cells pulsed with Tfn488 are treated with 0.45 M sucrose, most motion halts within 30 s of sucrose treatment, similar to GFP-LCa (2% post-sucrose motion versus 15% presucrose) (data not shown). The only motion remaining after 30 s of sucrose treatment is the continued long distance travel of a few Tfn488 puncta that are moving much slower (0.1 μm/s) than usual. These results suggest that the effect of hyperosmotic stress goes beyond the arrest of clathin-mediated endocytosis. In fact, incubating cultured cells in hyperosmotic sucrose fragments the Golgi/TGN (33, 44) and osmotic stress can exert profound effects on ER-Golgi trafficking and COPI coat dynamics within 5 min (44).

**Relationship between Dynamic Clathrin and Internalized Transferrin**—Although long distance motion is observed with

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**Fig. 6. The AP-2 β2 subunit colocalizes with clathrin structures undergoing long distance motion.** CV-1 cells were transfected as described with DsRed-LCa and AP-2 β2-YFP and imaged by TIR-FM. In a cell expressing relatively low levels of the β2-YFP fusion (A), only some of the laterally moving DsRed-LCa structures are also β2-YFP positive, as shown in the kymograph (B). The AP-2 β2 subunit does colocalize with DsRed-LCa in appearing and disappearing structures as shown by the kymograph, and in the series of still images (C, circles). In a cell expressing greater levels of β2-YFP (D), however, the laterally motile clathrin structures also contain the β2 subunit, as shown in the kymograph (E). In each set, the left panel is the AP-2 β2-YFP (green in merged images), the middle panel is DsRed-LCa (shown in red in merged images), and the right panel is the merged image. Dynamics are indicated with the same symbols used in Fig. 1. The scale bar represents 10 μm (A and D) or 20 s (B and E).
both GFP-LCa and Tfn488, the motile Tfn488-positive structures are more numerous and travel further (Fig. 7 compared with Figs. 4 and 5). To examine the spatio-temporal relationship between clathrin and Tfn488 structures, we pulsed Tfn488 into DsRed-LCa-transfected cells for 30 min and then removed the transferrin before imaging. Perinuclear accumulation of Tfn488 is found within compact recycling endosomes by epi-fluorescence (Fig. 8A), although, as expected, it is not readily seen in the TIR-FM image (Fig. 8B). Yet TIR-FM does show that both Tfn488- and DsRed-LCa-positive structures are concentrated in the perinuclear region, with significant (36%) colocalization. When TIR-FM is performed in the continued presence of Tfn488, the colocalization is increased to 56%. Interestingly, long distance clathrin motion appears to be aligned, but not exactly coincident with transferrin-positive elements. Upon initiation of long distance motion, the two fluorescent labels are often close, but spatially resolved. For example, while moving rapidly along a similar overall trajectory, a double-labeled structure separates into a DsRed-LCa structure trailing a Tfn488 structure (Fig. 8C, white arrow-head). These observations may be related to the complex and temporally malleable morphology of transferrin-bearing endosomes as they progress from early to recycling structures. Nevertheless, the data do indicate that at least a portion of the motile transferrin-positive structures have closely apposed clathrin.  

Long Distance Motion and Enlarged Endosomal Structures in Cuboidal Cells—CV-1 cells and fibroblasts are extremely flat cells. Thin section EM analysis of CV-1 cells reveals a distance of ~150 nm from membrane to membrane at the extreme periphery, with a similar distance between the nucleus and the basal plasma membrane (data not shown). One plausible reason for observing endosomes in these cells by TIR-FM is that these structures cannot be accommodated elsewhere in these cultured cells. Alternatively, a fraction of endosomes may generally traffic within ~200 nm of the plasma membrane. To distinguish between these possibilities, we examined GFP-LCa dynamics and cargo uptake in the cuboidal, epithelial-like A431 carcinoma cell line. GFP-LCa dynamics include the complete range of motion observed in CV-1 cells (Fig. 9, A and C).
Fig. 7. Internalization of LDL and transferrin observed by TIR-FM. CV-1 cells were serum-starved for 1 h (transferrin) or grown in lipoprotein-deficient serum overnight (LDL) prior to placement on the microscope. Tfn488 (A–D) or DiO-LDL (E and F) was added and the cells were imaged about 5–15 min later in the continued presence of the fluorescent cargo. The regions boxed in the TIR-FM images (A and E) are shown by the kymographs (B, D, and F). The kymograph in C comes from a different Tfn488 uptake dataset. The various dynamics are marked with the same symbols used in Fig. 1. Both DiO-LDL and Tfn488 display greater long distance motion than the AP-2 β2 subunit or clathrin, as shown in the kymographs. The scale bar represents 10 μm (A and B) or 20 s (C and D).
Again, the 0.6–1-μm structures are present, although their abundance is similar to the peripheral abundance of these structures in fibroblasts (8% in A431 cells compared with 7% in fibroblasts). This supports the idea that there is a population of endosomes generally within 200 nm of the plasma membrane, although this population is increased because of cytoplasmic space constraints in CV-1 cells and fibroblasts.

A431 cells express millions rather than thousands of EGF receptors, so we examined the uptake of fluorescent EGF (EGF488) in addition to Tfn488 dynamics. Both EGF488 and Tfn488 initially label only the periphery because A431 cells are tightly associated with the basal glass surface and apparently do not freely allow diffusion of these molecules below the cell. As time progresses, Tfn488 and EGF488 are internalized and penetrate deeper into the cell, where they either disappear or merge into larger structures (Fig. 9, B and D, and data not shown). As in CV-1 cells, the larger structures form as time progresses. The peripheral fluorescent EGF488 is very dynamic because the plasma membrane undergoes extensive actin-dependent ruffling upon EGF stimulation (45). Together, these experiments show...

**Fig. 8.** Simultaneous visualization of clathrin and Tfn488 containing structures. CV-1 cells transfected with DsRed-LCa (middle panels) and pulsed with Tfn488 (left panels) were imaged by TIR-FM. Epifluorescent images (A) clearly show prominent accumulation of transferrin in juxtanuclear recycling endosomes and clathrin in the TGN region. The TIR-FM images (B) reveal a different population of structures also concentrated in the perinuclear region. The regions shown in the kymographs (C) are boxed in the TIR-FM image. Dynamics are indicated with the same symbols used in Fig. 1. Notice in C that the structure showing long distance motion is yellow prior to motion and then separates into green and red structures moving along the same tract. The scale bar represents 10 μm (A and B) or 20 s (C).
that the population of surface proximal endosomes is not wholly because of cellular geometry constraints but rather a feature of several morphologically distinct cultured cell types.

**DISCUSSION**

The diversity of intracellular clathrin-coated structures now known to exist makes it difficult to definitively assign fluorescent clathrin signals to one particular subcellular site without additional compartmental information. In other studies utilizing TIR-FM, the conclusion that the observed fluorescent structures are confined to the plasma membrane is based upon idealized optical properties of the illumination system, with a typical decay depth of 70–110 nm (10–12, 27). However, at this depth, the evanescent field still maintains 37% of the maximal intensity. At three decay depths from the interface, the signal diminishes to 5% of maximal intensity, so the excitatory field approaches extinction at 200–300 nm into the cell. Our empirical measurements concur and indicate that under our TIR-FM imaging conditions, molecules 150–200 nm from the interface are readily detected. We believe that this expands the number and type of structures that must be considered when interpreting the TIR-FM data sets. In fact, intracellular actin rockets propelling endocytic vesicles (46), fluorescently tagged secretory vesicles (37, 47, 48), labeled endosomes (42), and lysosomes (49) have all previously been observed with TIR-FM by others.

Our data showing that AP-2 and several alternate adaptors generally do not move in a rapid, directed fashion parallel to the plane of the membrane agree well with an independent, recently published TIR-FM study (27). The absence of adaptor subunits from both the disappearing and motile clathrin populations prompted the suggestion that these coat components dissociate before the internalization phase begins (27). Because experiments combining TIR-FM with wide field imaging show that clathrin-positive puncta persist in the epifluorescence images after departing from the TIR-FM field (11), one possibility is that AP-2 is disassembled/released before the outermost clathrin lattice, which accompanies the transport vesicle (27). We find this hypothesis unlikely for a number of reasons. First, there is good evidence for enrichment of adaptor proteins like AP-2, AP180/CALM, and HIP1/Hip1R in preparations of biochemically purified clathrin-coated vesicles. Second, clathrin trimers alone have no affinity for biological membranes and require AP-2, alternate adaptors, or accessory proteins like amphipysin to be targeted to the cell surface (3, 16, 50, 51). Third, although it is now known that AP-2 is not absolutely necessary for the endocytic uptake of EGF or LDL (20, 22),

**Fig. 9.** Epithelial-like cells also demonstrate long distance motion. A431 cells were transfected with GFP-LCa as described (A and C) or serum starved for 1 h followed by addition of EGF488 (B, D, and E) and imaged by TIR-FM. The boxed regions in the TIR-FM images (A and B) are shown by the kymographs (C–E). In these cuboidal cells, clathrin and endocytic ligands display a similar range of motion to the flatter fibroblast or CV-1 cells. Dynamics are indicated with the same symbols used in Fig. 1. The scale bar represents 5 µm (A and B) or 20 s (C–E).
alternate clathrin- and cargo-binding endocytic adaptors like epsin, Dab2, and ARH must promote clathrin coat assembly in the absence of AP-2 (52). Yet we do not find that Dab2, ARH, or the endocytic protein Hip1R display long range lateral mobility, apparently eliminating these proteins as potential linkers for membrane-associated clathrin. Fourth, the available data indicates that the kinesin family microtubule motor KIF13A binds physically to AP-1 (53) and there is no evidence currently for binary associations between clathrin and motor proteins that we are aware of. In our TIR-FM data sets, we see abrupt disappearance of both AP-2 subunits and clathrin indicating to us that AP-2 and clathrin bud from the plasma membrane assembled together in a polyhedral clathrin coat.

We also find that the minor, highly motile clathrin population contains the β2 subunit of AP-2, but the augmented coincidence of this subunit with DsRed-clathrin at higher expression levels suggests that the β2 subunit is being incorporated into the AP-1 heterotetramer. Together with our results showing endosomal movement in the TIR-FM field and the 150–200 nm depth of the excitatory field, our current interpretation of the data is that the fluorescent clathrin population associated with long-range motion represents AP-1-positive structures of endosomal origin. Is there any evidence to support a role for AP-1 and clathrin in endosomal sorting? In polarized Madin-Darby canine kidney cells, AP-1, identified by the unique γ subunit, is found associated with a population of pleiomorphic clathrin-coated endocytic tubules that are responsible for proper sorting of the transferrin receptor to the basolateral clathrin-coated endocytic tubules that are responsible for membrane-associated clathrin.

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Based on our observations, and those of others (9, 11, 26), the appearance and disappearance of clathrin-positive puncta is almost certainly because of the physical formation and release of coated vesicles from the plasma membrane, because both clathrin and adaptors show a parallel behavior. What is surprising is that adaptors appear less motile than might be expected. The majority of the clathrin and AP-2-positive structures at the periphery of the cell appear relatively static and long-lived. The local gyrational motion of these coat components probably reflects the extent of free diffusion of the coated pits along the membrane. Indeed, it has been previously shown that local motion increases in the presence of latrunculin B, an actin depolymerizing agent (9). Many structures persist for several minutes without showing dramatic alterations in either fluorescence intensity or position. These observations are consistent with the published wide field (9) and confocal (26) data, and with live cell fluorescence recovery after photobleaching experiments with both GFP-tagged Lcα and the AP-2 α subunit (29, 33). In these studies, a large fraction of peripheral fluorescent clathrin or AP-2 remains in the same relative position over a 2-min period. The dynamic properties of clathrin Lcα-GFP transfected into primary hippocampal neurons after in vitro culture for 20–40 days (26, 57) are also remarkably similar to our observations in non-neuronal cells. The relatively static structures found in the older cultured neurons prompted the designation of a “stable endocytic zone” (26) that is clearly endocytically active despite the apparently limited clathrin dynamics (26, 57). In this system, clathrin Lcα-GFP overexpressed in younger neurons, cultured for fewer than 10 days, is considerably more dynamic (26). This significant finding rules out that the observed static behavior is a consequence of the transfection procedure and suggests instead that the dynamic properties of surface clathrin lattices may depend on cell type, culture conditions, and physiology.

The dynamic properties of fluorescent AP-2 and clathrin over time are also similar to the behavior of an assembled, non-clathrin coat, the COPI coatomer coat. In e-COP-GFP image sequences covering about 60 s, many COPI-coated structures near the Golgi region of the cell remain roughly static with relatively little fluorescent intensity fluctuation (56). However, we do find that about 20% of the clathrin structures appear and disappear per minute, suggesting that all of the plasma membrane clathrin could turn over in ~5 min, roughly consistent with the time required for receptor internalization. We have considered the possibility that the GFP tag interferes with normal functioning of the fusion proteins, altering the kinetics observed. However, the various tagged proteins we use have the COP at different positions and the relatively static behavior of the AP-2 α subunit, Dab2, ARH, and Hip1R suggests a common dynamic. The fact that the β2-YFP moves into the highly mobile fraction in higher overexpressers also argues for two distinct populations of clathrin. Studies on the internalization of fluorescent influenza virus, which is internalized via clathrin-mediated endocytosis, reveal three stages during viral entry and transport (25). The first stage, which corresponds to endocytic uptake and local actin-dependent movement, takes the longest, about 6 of the ~8 min required for viral fusion after initial binding to the cell surface (25). The second stage is rapid, microtubule-dependent transport of the virus to the perinuclear region preceding the third stage fusion event (25). Thus, the overall kinetics and intracellular trajectory of virus internalization mirrors the behavior we observe for clathrin.

Acknowledgments—We are extremely grateful to our colleagues for readily providing important plasmids used in this work. We also thank Matthew Hawryluk and Sanjay Mishra for critical reading of the manuscript and Romesh Draviam for technical assistance with TIR-FM.

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