Phosphoinositid 3-Kinase C2α (PI3K-C2α) is a type II PI-3-kinase that has been implicated in several important membrane transport and signaling processes. We previously found that overexpression of PI3K-C2α inhibits clathrin-mediated membrane trafficking and induces proliferation of novel clathrin-coated structures within the cytoplasm. Using fluorescently tagged fusions of PI3K-C2α, coated structures within the cytoplasm. Using fluorescently tagged fusions of PI3K-C2α and clathrin, we explored the behavior of these structures in intact cells. Both proteins are present in the structures, and using rapid image acquisition and fluorescence photoactivation probes, we find that they exhibit localized, rapid mobility (5–20 μm/s). The movement is microtubule-based as revealed by use of inhibitors, and PI3K-C2α accumulates on microtubules rapidly and reversibly following cytoplasmic acidification, which also blocks movement. Dynactin mediates the movement of these clathrin-PI3K-C2α structures, since disruption of dynactin function by overexpression of its p50 subunit also inhibits movement. Finally, immunoprecipitation experiments reveal an interaction between endogenous PI3K-C2α and dynactin subunits. Together, these results reveal a molecular linkage between PI3K-C2α and the microtubule motor machinery, with implications for membrane trafficking in intact cells.

Clathrin-coated membranes are ubiquitous in all eukaryotic cells. They play key roles in physiological processes, such as receptor-mediated endocytosis and other forms of vesicular transport (reviewed in Refs. 1 and 2), and derangements of the clathrin membrane transport system have been implicated in many pathological states (3–5). Clathrin-coated structures (CCSs) are normally present in several recognizable forms within cells: as coated pits on the plasma membrane (PM) and coated buds on the trans-Golgi network (TGN); as coated vesicles that have detached from these membranes and are thought to have a transient existence; and as coated buds on endosomal and other internal membranes. An appreciation of the dynamic behavior of CCS was initially inferred from biochemical and cell biological experiments (reviewed in Ref. 6) and was directly observable with the advent of fluorescently tagged coat proteins microinjected into cells or transiently expressed as a GFP-tagged fusion protein (7–9). Several lines of evidence identify coated pits as relatively stationary spots on the plasma membrane of cells that periodically disappear and reappear in a “blinking” process, construed to reflect internalization into short lived coated vesicles that undergo rapid uncoating.

It is becoming increasingly apparent that the cytoskeleton interfaces with vesicular transport at many levels. Involvement of actin in endocytosis in yeast has been well established (10–12) and is strongly implicated in the initial stages of clathrin-mediated endocytosis in mammalian cells (13, 14). The available evidence indicates that microtubules (MTs) also function at several stages in vesicular transport. For example, the long range movement of AP-1- and GGA1-coated structures can be abolished by disruption of MT (15). Structures that are likely to be clathrin-coated endosomes (9) and Rab5- and Rab7-decorated early endosomes (16–18) have also been demonstrated to undergo microtubule-dependent transport.

Phosphoinositides play a regulatory role in many of these processes, since they bind to proteins that regulate both coated pit assembly and cargo binding, and the cytoskeletal motor machinery (reviewed in Refs. 19–21). We have been interested in a novel PI3K implicated in clathrin-dependent functions. Besides being a downstream effector of growth factor receptors like other PI3Ks (22, 23), phosphoinositide 3-kinase C2α (PI3K-C2α) has recently been found to be essential for ATP-dependent priming of neurosecretory granule exocytosis (24). Our previous work has shown that PI3K-C2α is highly enriched in clathrin-coated pits and vesicles (25), and this localization may reflect both its clathrin binding and catalytic activities (26). Further, overexpression of PI3K-C2α results in inhibition of clathrin-mediated endocytosis and membrane trafficking (27) and is also accompanied by the appearance of numerous CCSs occurring throughout the cytoplasm of the cell. This is in striking contrast to the localization of clathrin in virtually all eukaryotic cells, in which the coat structure is localized on the PM and TGN, and to a considerably lesser extent on peripheral, endosomal membranes (8, 28, 29).

Here we use live cell imaging techniques to demonstrate that these unique structures contain both clathrin and PI3K-C2α.
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Further, they exhibit rapid, localized motility, which may reflect their presence as coated buds or segments on membrane tubules. The movement is attributable to interaction with the MT motor machinery, probably mediated by interaction of PI3K-C2α with dynactin. Our results indicate that the dynamics and function of CCSs in cells can be modulated by their interaction with PI3K-C2α.

EXPERIMENTAL PROCEDURES

Constructs—Glu epitope-tagged full-length human PI3K-C2α in pMTSM has been described (27). GFP/YFP and a photoactivatable form of GFP (PA-GFP) (30), the latter a generous gift from Drs. George Patterson and Jennifer Lippincott-Schwartz (National Institutes of Health), were cloned into the Sal and Smal sites of pMTSM-PI3K-C2α. pMTSM-Glu-p50 was generated by cloning full-length p50 (excised from GFP-p50-N1, a generous gift of Dr. Richard Vallee, Columbia University, New York) into the Sal and EcoRI sites of pMTSM vector, and Glu was inserted into the Sal and Smal sites. pRSET-B mCherry was from Dr. Roger Y. Tsien (University of California San Diego). PA-GFP-Clathrin-C1 and mCherry-Clathrin-C1 were constructed by the same strategy as GFP-Clathrin-C1 (7).

Antibodies and Reagents—Affinity-purified rabbit polyclonal anti-human PI3K-C2α was generated by injection of His-tagged PI3K-C2α–2–365. Monoclonal antibodies against p150 and p50 were purchased from BD Biosciences. A monoclonal antibody against the Glu epitope was from Research Diagnostics. Monoclonal anti-tubulin antibody DM1A and polyclonal anti-p50 antibody were generous gifts from Dr. Mark Black (Temple University, Philadelphia, PA). A monoclonal antibody anti-clathrin (X22) was from the American Type Culture Collection, and polyclonal anti-clathrin antibody R5 has been described (25). Nocodazole, taxol, latrunculin B, and cytochalasin D were purchased from Sigma. All other chemicals were reagent grade or better.

Immunoprecipitation—Cells with or without ectopically expressed PI3K-C2α were lysed in 100 mM MES (pH 6.8), 0.1% Triton, 1 mM EGTA, 0.5 mM MgCl₂, 0.02% NaN₃, containing a proteinase inhibitor mixture (27). Lysates were centrifuged at 12,000 × g for 10 min, and supernatants were challenged with polyclonal anti-p50 or anti-PI3K-C2α antibody and blotted with anti-p150, anti-p50, and anti-PI3K-C2α antibodies.

Fluorescence Microscopy—Immunostaining of transiently transfected COS1 cells was performed as described previously (7, 27) except that for p50 staining cells were permeabilized in 0.05% saponin, 100 mM PIPES (pH 6.8), 1 mM MgCl₂, 5 mM EGTA for 30 s before fixation. For live cell imaging, transfected COS1 cells were seeded into glass bottom culture dishes (MatTek). After spreading, the complete Dulbecco’s modified Eagle’s medium was replaced with HEPES-supplemented F-12 medium, and images were acquired at the indicated time intervals. For pharmacological treatments, cells were first imaged before treatment and then incubated in complete medium containing 10 μg/ml nocodazole, 1 μM taxol, 1.2 μM latrunculin B, or 30 μM cytochalasin D for the indicated periods. The cells were then transferred into HEPES-supplemented F-12 medium containing the corresponding chemical reagents, and images were acquired as indicated. Cold treatment was performed by incubation on ice for 2–3 h, followed by cold 3.7% formaldehyde fixation. For acidification treatment, cells were incubated in acetic acid-adjusted Ringer buffer (pH 5) for 2 min before imaging or fixation; no effects were seen at pH 7. Cellular ATP was depleted by the addition of 0.3% NaN₃ in culture medium lacking glucose and serum for 10–15 min, and images were acquired in the same medium. Samples were imaged using X63 Planapo/1.4 numerical aperture objectives on either a Zeiss 510 Meta laser confocal system or a Zeiss Axiovert S100 TV wide field microscope system equipped with a Photometrics Cascade camera controlled by Metamorph software (Universal Imaging, Inc.). Simultaneous imaging of YFP and mCherry signals was accomplished using a Dual-view system (Optical Insights, Inc.). Photoactivation experiments were performed on a Zeiss 510 Meta laser confocal system. Regions of interest of ~7–14 μm² were photoactivated using a diode laser (405 nm, 25 milliwatts) at a 52% setting, and images were recorded by excitation with an argon laser (488 nm, 30 milliwatts) at a 5% setting. GraphPad Prism was used for statistical analysis of normalized pixel intensities.

RESULTS

PI3K-C2α Induces Cytoplasmic Clathrin-coated Structures with Rapid Movement—Our previous work has shown that overexpression of PI3K-C2α inhibits clathrin-mediated endocytosis and membrane trafficking (27). It is also accompanied by the appearance of numerous intracellular CCSs. To learn more about the nature of these novel structures, we investigated their behavior in live COS1 cells transiently expressing both PI3K-C2α and GFP-clathrin. Upon direct observation through the microscope eyepiece, we unexpectedly found that these structures were moving rapidly within the cytoplasm of cells. This is readily apparent in single maximum projection images (Fig. 1A) formed by combining stacks of 15 images captured at 36-ms intervals from cells expressing GFP-clathrin alone (control) or cells expressing both GFP-clathrin and PI3K-C2α. Whereas the CCSs in the control cells appear virtually stationary on this approximately 0.5-s total time scale, as observed previously (7, 8, 31), the blurriness in the PI3K-C2α projection suggests considerable movement. Another appreciation of this difference is evident on examination and overlay of individual frames (Fig. 1B); CCS spots in the control over this ~360-ms time span show virtually no movement, and overlay of the frames reveals almost perfect superimposition. In contrast, the position of the GFP-clathrin signal changes dramatically between frames in the PI3K-C2α-expressing cells, as revealed by the lack of spot coincidence in the overlay image. Finally, a complete animation sequence of these images in real time provides dramatic evidence of the remarkable rate and nature of this movement (available as supplemental Video 1, right panel).

It was possible to discern several kinds of movement in the animation sequences of cells expressing PI3K-C2α and GFP-clathrin. Occasionally, we could see GFP-CCSs decorating ring-like structures of 1–2 μm diameter, presumably vesicles or vacuoles, and these CCSs consistently ran along the perimeter during a several-s observation time. Interestingly, very few of...
the dynamic CCSs in the PI3K-C2α-expressing cells appeared to be moving vectorially over substantial distances. This is evident both in the general absence of long tracks in the maximum projection image in Fig. 1A (in which two short atypical translational movements are indicated by the arrows) and on viewing the video sequence (supplemental Video 1, right panel). Rather, within several frames (i.e. comprising less than 0.4 s), the overwhelming majority of CCSs seem to completely disappear from their origins, and new structures appear in the field.

We reasoned that the highly motile behavior of the intracellular CCS might depend directly on the presence of PI3K-C2α in the coat structure. To test this hypothesis, we prepared GFP/YFP-PI3K-C2α fusion protein constructs and confirmed that, like nonfluorescent PI3K-C2α, they also caused the appearance of intracellular CCSs and inhibition of transferrin uptake upon overexpression in cells (Fig. S1). We then expressed both mCherry-clathrin and YFP-PI3K-C2α in COS1 cells and monitored the behavior of the proteins using simultaneous imaging of both signals. YFP-PI3K-C2α was almost entirely associated with mCherry-clathrin spots (Fig. S2), and both proteins were observed to move together in the cytoplasm with the rapid dynamics described above (supplemental Video 2). This result is consistent with our previous observation that ≥90% of exogenous PI3K-C2α spots colocalized with intracellular clathrin in fixed cells (27). Since these structures contain both clathrin and PI3K-C2α, we refer to them here as PI3K-C2α-CCSs. The remarkable mobility of PI3K-C2α-CCSs, as compared with CCSs in control cells, also suggests that PI3K-C2α is capable of imparting motility to CCSs.

Upon examining the movement of some GFP-PI3K-C2α-CCS spots, it was occasionally possible to follow their positions for short periods and when they could be tracked in three or more successive frames to estimate velocities (see examples in Fig. 1, C and D, and supplemental Video 3). The apparent speed of CCSs measured in this way varied greatly, with many moving at 5–10 μm/s but some exhibiting much higher apparent velocities, occasionally exceeding 20 μm/s (Fig. 1C). These enlarged images also suggested that the GFP-clathrin signal might comprise a coated tubular region as well as a discrete bud, although movement could also affect the appearance of these structures. The proliferation of intracellular CCSs with rapid dynamics was also observed upon expression of PI3K-C2α in human 293 or HeLa cells, as well as in Chinese hamster ovary cells (data not shown). Together, these observations indicate that PI3K-C2α expression induces the appearance of novel CCSs that move extremely rapidly but linearly only over very short distances if at all.

PI3K-C2α-CCSs Show Restricted Mobility—The nonvectorial movement of the PI3K-C2α-CCSs suggested that the overall mobility of the particles, though extremely rapid, might actually be limited to a local region. To learn more about their behavior, we utilized a photoactivatable variant of GFP (PA-GFP) (30). Expressing this reporter alone, we found that after photoactivating the PA-GFP signal first with laser light to activate it, we observed highly dynamic spots moving over short distances but did not see long tracks or buds extending from them (Fig. 2A). This was true for both cell types that we examined. When we expressed both YFP-PI3K-C2α and PA-GFP, we observed that the typical distribution of dynamic spots showed up in the PA-GFP images of PI3K-C2α-expressing cells (Fig. 2B). YFP-PI3K-C2α showed up in the overlays of all the images, indicating that PI3K-C2α and the PA-GFP signal were distributed in the same way (Fig. 2C). However, there were no spots formed, indicating that PA-GFP was not being recruited to any new sites, such as clathrin patches.
activation of a small region, PA-GFP fluorescence intensity dropped rapidly (see a representative example in Fig. 2 and supplemental Video 4) with an apparent half-time of about 1 s ($n = 11$ cells), although this is likely to be an overestimate, since it is on the time scale of photoactivation and signal acquisition. This probably reflects the rate of diffusion of the $\sim 27$ kDa protein in situ, since GFP has been shown to have minimal interaction with cellular constituents (32), and the diffusion properties of PA-GFP in cells are experimentally indistinguishable from those of GFP.$^4$

The PA-GFP-PI3K-C2$\alpha$ fusion protein was then expressed. When subjected to whole cell photoactivation, its overall fluorescence pattern was indistinguishable from that obtained upon expression and conventional imaging of GFP-PI3K-C2$\alpha$, confirming the similar behavior of the two proteins (data not shown). Following photoactivation of a small area of cells expressing PA-GFP-PI3K-C2$\alpha$, a biphasic decay curve was observed with a rapid component and much slower phase (representative examples in Fig. 2 and supplemental Video 5). An experimentally indistinguishable curve was observed when cells co-expressing nonfluorescent PI3K-C2$\alpha$ and PA-GFP-clathrin were subjected to photoactivation (Fig. 2 and supplemental Video 6), indicating that under these conditions the two proteins not only colocalize (Fig. S2) but also move together at this level of resolution.

Analysis of 15 data sets from PA-GFP-PI3K-C2$\alpha$-expressing cells revealed a minor fast decay component of about 1.7 s. This may reflect a soluble pool of PA-GFP-PI3K-C2$\alpha$ that contributes a diffuse background signal. The 8-fold greater mass of PA-GFP-PI3K-C2$\alpha$ compared with PA-GFP would be expected to exhibit a 2-fold slower diffusion rate.

The major PA-GFP-PI3K-C2$\alpha$ fluorescence decay component exhibited a much greater half-time of $\sim 14.3 \pm 1.3$ s ($n = 15$ cells), suggesting several possible interpretations. The $\sim 20$-fold decrease in signal decay rate (compared with PA-GFP) could indicate that PA-GFP-PI3K-C2$\alpha$ is incorporated into free particles of more than $10^8$ Da. This would correspond to extremely large coat structures, containing more than 100 clathrin and PI3K-C2$\alpha$ molecules. Alternatively, it could reflect restriction of PI3K-C2$\alpha$-CCS movement by interaction with cellular components (e.g. the cytoskeleton). It is also possible that the PI3K-C2$\alpha$-CCSs are present as coated segments on membrane tubules, as implied by recent ultrastructural images (26). This tethering might then be expected to restrict long range movements. Although further ultrastructural work will be required to fully resolve these or other possibilities, we chose here to explore the potential involvement of motor machinery in the movement of the PI3K-C2$\alpha$-CCS in intact cells.

Fast Motility of CCSs Induced by PI3K-C2$\alpha$ Is MT-dependent—The characteristic rapid movement of PI3K-C2$\alpha$-CCS was greatly inhibited by metabolic poisoning (e.g. 0.3% sodium azide, 10 min). However, incubation of cells with either cytochalasin D (30 $\mu$M, 40 min) or latrunculin B (1 $\mu$M, 30 min), which both ablated microfilament structure in cells, was without noticeable effect, indicating that actin microfilaments were not critical to mobility (data not shown). In contrast, the MT-targeted drugs nocodazole (data not shown) and taxol significantly affected the movements of PI3K-C2$\alpha$-CCS. This is reflected by the sharpening of the maximum projection image of time stacks for GFP-clathrin observed in the same cell before and after treatment for 60 min with taxol (Fig. 3). Similarly, examination of real time GFP-clathrin image sequences also shows a significant inhibition of movement after taxol treatment (compare supplemental Videos 7 and 8). This is consistent with published results indicating taxol inhibition of MT-based vesicle transport, a consequence of MT accumulation and bundling (33–36).

Further evidence for the interaction of PI3K-C2$\alpha$ and clathrin with microtubules came from two other lines of experiments. In the course of utilizing an acid wash step to dissociate cell surface receptor-ligand complexes, we serendipitously observed a radical change in the distribution of GFP-PI3K-C2$\alpha$ in intact cells. Remarkably, incubation of cells at pH 5 for 1–2 min, which has been shown to cause cytoplasmic pH to drop to $n$.

$^4$E. N. Pugh, Jr., personal communication.
pH ~6 (37), caused the characteristic punctate pattern of PI3K-C2α to now be supplemented by a striking filamentous localization (Fig. 4, compare A and B). Immunostaining for tubulin confirmed that these linear structures were indeed MTs and that essentially all were decorated with PI3K-C2α (Fig. 4B). The punctate component of the PI3K-C2α signal, which for the most part continued to colocalize with clathrin, was also often found on MTs, although there was some cell variability in this distribution. Importantly, acidification virtually abolished the rapid movement of PI3K-C2α-CCS (supplemental Video 9). These effects were readily reversible, since replacement of the acidification buffer with neutral medium restored the characteristic rapid movement of PI3K-C2α-CCS within 2–5 min (data not shown).

We found further evidence for an interaction of PI3K-C2α and clathrin with MTs upon chilling cells. Although cold treatment largely depolymerized MTs in most COS1 cells and yielded a punctate tubulin staining pattern, some cells retained noticeable filament staining, as often seen in cells of fibroblastic origin (38). In these cells, GFP-PI3K-C2α underwent a significant redistribution to a largely filamentous pattern, periodically decorated by punctate signal (Fig. 4C). The punctate signal corresponded precisely with clathrin, as revealed by immunostaining in these cells (Fig. 4C), whereas the filamentous pattern comprised decoration of apparently cold-stable MT along their length with GFP-PI3K-C2α.

Together, these experiments reveal a clear interaction of PI3K-C2α with MT and suggest that this interaction is required for the rapid motility of the PI3K-C2α-CCS. Inhibition of this movement is probably a consequence of arrest at a pH- and temperature-sensitive step of a functional complex involving PI3K-C2α and MTs.

**Dynactin Subunits Associate with PI3K-C2α and Contribute to Motility**—It is well known that dynactin is a critical factor in MT-dependent movements (39). To confirm the dependence of PI3K-C2α-CCS movement on MT and to explore the possible role of dynactin in its rapid motility, we employed several approaches. First, we expressed the p50 subunit of dynactin (also termed dynamitin) and examined its distribution in comparison with endogenous PI3K-C2α. Expression of Glu (epitope-tagged)-p50 yielded a largely punctate pattern, which substantially overlapped with that of endogenous PI3K-C2α (Fig. 4D).

Next, we found that a stable interaction of PI3K-C2α with dynactin components could be demonstrated by immunoprecipitation. When endogenous dynactin p50 was immunoprecipitated from detergent extracts of COS1 cells expressing epitope-tagged PI3K-C2α (Fig. 5A), the immunoprecipitates contained PI3K-C2α as well as the dynactin p50 and p150 subunits as expected (40, 41). Association of dynactin with PI3K-C2α was also evident in the absence of ectopic expression. When endogenous PI3K-C2α was immunoprecipitated from either COS1 or HeLa cells, the endogenous dynactin p50 and p150 subunits were also detected in the immunoprecipitates (Fig. 5B), demonstrating the physiological relevance of this association.

Overexpressed p50 has been demonstrated to be an inhibitor of dynactin function, presumably by sequestering other dynactin components and thus disrupting the functional complex (40). Accordingly, we asked whether overexpression of p50 could affect the rapid motility of the PI3K-C2α-CCS. Indeed, in cells expressing both PI3K-C2α and p50 (the latter confirmed by subsequent immunofluorescence), we found that most of the GFP-clathrin spots now appeared to be moving considerably slower than in control (supplemental Video 10). This was most clearly demonstrated by identifying the minimal intensity value at each pixel location throughout the time stack, which highlights spots that exhibit little or no mobility (Fig. 5C). This analysis revealed that in a 20 × 14-μm sampling region of a p50-expressing cell, there were 92 clear spots. Ninety-five percent of these GFP-CCSs persisted throughout the 10 frames of the time stack (spanning 0.5 s). Furthermore, because of their comparatively slower movements, many of these spots could be reliably tracked during the time stack, moving within an area of diameter ~0.5–1 μm and with an average speed of about 3 μm/s. In contrast, analysis of GFP-clathrin in similarly sized regions of control cells lacking exogenous p50 expression revealed only 25 discrete spots. Further, the vast majority of these motile spots could not be reliably tracked for more than three frames (as noted earlier) (Fig. 1). This functional evidence in live cells for the sensitivity of fast PI3K-C2α-CCS movement to disruption of the dynactin complex complements the biochemical data in support of an interaction of PI3K-C2α with dynactin.

**DISCUSSION**

Previous immunofluorescence studies have shown that endogenous PI3K-C2α is detectable in a subset of clathrin-coated pits at the PM as well as being localized on intracellular vesicular structures in the TGN and throughout the cytoplasm (25, 27). We found that upon modest overexpression, exogenous PI3K-C2α decorated PM clathrin-coated pits but also induced the formation of novel, intracellular CCSs that appeared in great number (27). Given the likelihood that these intracellular PI3K-C2α-CCSs reflect a physiologically important component of the clathrin membrane traffic system in cells, although exaggerated upon overexpression, we extended
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![Image](https://example.com/image.png)

**FIGURE 4. PI3K-C2α associates with microtubules and dynactin.** Compared with control (A), PI3K-C2α (green in merge) diffusely decorates MTs after acidification (B) and chilling (C) as well as localizing with clathrin in discrete spots on the MTs. For details, see “Results.” The boxed insets are enlarged 2-fold. Bar, 10 μm. D, exogenous dynactin p50 shows substantial colocalization with endogenous PI3K-C2α. Bar, 10 μm.

Our studies to evaluate their behavior in live cells. Our findings indicate that PI3K-C2α can confer remarkable mobility on clathrin-coated membranes in intact cells. They also reveal a structural and functional association of PI3K-C2α with dynactin subunits that provides a molecular basis for the interaction of PI3K-C2α with the MT motor machinery, with implications for membrane trafficking.

The PI3K-C2α-CCS exhibited localized and fast motility. In fact, despite using very short exposure times in the 10–30-ms range, it was difficult to reliably track individual PI3K-C2α-CCS for more than three frames before they vanished or their fates became uncertain. This phenomenon is not consistent with long range vectorial movement; given the apparent speed and field of view, a significant fraction of transiting GFP-labeled particles (>15%) should be evident, and this is not observed. Spot transience could conceivably reflect extremely rapid coat dissociation, but the kinetics of appearance and disappearance of coated pits, which generally occur over several s (7, 31), also makes this possibility unlikely. Further arguing against this possibility is the observation that in cells overexpressing dynamin p50, the PI3K-C2α-CCS spots are observable but show slower speeds and longer persistence in the field of view. p50 overexpression would not be expected to affect coat dissociation but does inhibit coupling to the MT motor machinery through inhibition of dynactin function (42). Finally, the slow rate of attenuation of the PA-GFP-PI3K-C2α signal after photoactivation suggests that PI3K-C2α-CCS are tethered or that their long range movement is restricted in some way.

Work from other groups has shown that CCSs on or near the PM and in the TGN can move with an average velocity of ∼1 μm/s (9, 15). These rates are comparable with most of the movement that we and others have seen in control cells expressing only GFP-clathrin. However, they are at least 10-fold slower than the apparent movements of PI3K-C2α-CCS reported here (often ≥5–20 μm/s), whether monitored by GFP-clathrin or by GFP-PI3K-C2α. MT motors are capable of producing these velocities, both in vitro and in vivo. Mitochondria carried on MTs can move as fast as 15 μm/s (43), and movement up to 12 μm/s for multiple kinesins or dyneins (44) and over 400 μm/s for axonemal dynein-mediated motility (45) have been reported. Based on our results, we speculate that coat structures containing multiple PI3K-C2α molecules may transiently recruit multiple MT-based motor molecules, resulting in rapid speeds over short distances as noted above. These PI3K-C2α-CCSs may be present as coated segments on the ends or along membrane tubules, and their intermittent coupling to MTs and movement in three dimensions would yield the motion observed. We have obtained ultrastructural evidence suggesting the presence of coated buds on membrane tubules in PI3K-C2α-expressing cells (26), but more definitive ultrastructural studies will be required to fully resolve this possibility.

Several lines of evidence reported here indicate that PI3K-C2α and PI3K-C2α-CCS are associated with MT in intact cells and that their rapid mobility depends on MT-based motors, probably through interaction with dynactin. Dynactin is a multisubunit protein complex that is required for most dynein- and some kinesin-dependent movement (39, 46). There are several lines of evidence implicating dynactin in endocytic trafficking. Perturbation of dynactin function in vivo affects endomembrane dynamics and trafficking (42). A functional dynein-dynactin interaction is also required for proper microtubule organization and for the transport and localization of endomembrane compartments (47). It is important to note that the association of PI3K-C2α with dynactin reported here (Fig. 5) is more intimate than simply presence on the same transport vesicle, since binding was revealed in a detergent extract.
that through interactions with both clathrin and dynactin, treatment or GFP-tau expression have been reported, as have ratios (53) and susceptible to modulation by either nocodazole imaged by TIRF (9) or in broken and resealed cytoplast preparation of a small fraction of CCS moving on MT near the PM as pension but not in adherent cell lines (52). In addition, association of microtubule disruption in hemopoietic cells growing in suction vesicles (51). Functionally, transferrin entry was inhibited by as a tightly bound component of highly purified clathrin-coated PI3K-C2α of COS1 cells.

of COS1 and HeLa cells.

FIGURE 5. Dynactin p50 and p150 subunits co-immunoprecipitate with PI3K-C2α. A, anti-p50 antibody co-immunoprecipitates both exogenous PI3K-C2α (detected by anti-Glu antibody) and p150 from detergent extracts of COS1 cells. B, both endogenous dynactin p50 and p150 subunits are present in immunoprecipitates of endogenous PI3K-C2α from detergent extracts of COS1 and HeLa cells. Input lanes comprise 7.5% of each reaction, and antibody was omitted from control samples.

It is well established that MTs play major roles in the cellular localization of some intracellular organelles (37) and transport of other vesicular structures. These include movement of CCS between the TGN and endosomal compartments that have been shown to be mediated, at least in part, by MT (15). In addition to PI3K-C2α, several other proteins of the clathrin-mediated membrane trafficking pathway have been shown to be directly or indirectly associated with MT. These include endophilin A3 (48), epsin (49), and AP-1 (50), which may link appropriate CCSs to MTs. Indeed, tubulin has been identified as a tightly bound component of highly purified clathrin-coated vesicles (51). Functionally, transferrin entry was inhibited by microtubule disruption in hemopoietic cells growing in suspension but not in adherent cell lines (52). In addition, association of a small fraction of CCS moving on MT near the PM as imaged by TIRF (9) or in broken and resealed cytoplasm preparations (53) and susceptible to modulation by either nocodazole treatment or GFP-tau expression have been reported, as have subsets of motile, MT-bound early endosomes (18).

In light of these observations, it seems reasonable to propose that through interactions with both clathrin and dynactin, PI3K-C2α can provide a structural link between CCS and MTs. Roles for clathrin-MT interactions in mitosis have also been demonstrated (54, 55), raising the possibility of PI3K-C2α involvement in this process as well. Finally, PI3K-C2α has also recently been implicated in control of neurosecretory vesicle release (24), a process in which MT play key roles in concert with the actin machinery. As the role of actin in endocytosis in yeast is well established (10–12) and becoming more evident in mammalian cells (13, 14, 56), a role for PI3K-C2α in modula-

tion of the interplay between phosphoinositides, actin, and MT networks will also have to be considered.

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