Individual Phosphoinositide 3-Kinase C2α Domain Activities Independently Regulate Clathrin Function*

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Phosphoinositide 3-kinase C2α (PI3K-C2α) is a member of the class II PI3 kinases, defined by the presence of a second C2 domain at their C termini. The cellular functions of the class II enzymes are incompletely understood, though they have been implicated in receptor activation pathways initiated by epidermal growth factor, insulin, and chemokines. PI3K-C2α was recently found to be localized to clathrin-coated membranes in the trans-Golgi network and at endocytic sites on the plasma membrane. Further, a specific binding site was identified for clathrin on the N terminus of PI3K-C2α, whose occupancy resulted in lipid kinase activation. Expression of PI3K-C2α in cells dramatically affected clathrin distribution and function in cells, leading to accumulation of intracellular clathrin-coated structures, which are visualized here at the ultrastructural level, and inhibition of clathrin-mediated transport from both the plasma membrane and the trans-Golgi network. In this study we have demonstrated that the isolated clathrin binding domain of PI3K-C2α can drive clathrin lattice assembly and that both the lipid kinase activity of the protein can independently modulate clathrin distribution and function when expressed in cells. Together, these results suggest that PI3K-C2α employs both protein-protein interaction and localized production of 3-phosphoinositides to affect clathrin dynamics at sites of membrane budding and targeting.

It is well established that both cargo and informational movement across the plasma membrane, as well as certain aspects of intercellular signaling, involve vesicular transport initiated by endocytosis from clathrin-coated pits. The machinery of endocytosis has been extensively studied, and its complexity would seem to reflect the challenge of providing a highly efficient yet controllable interface between the environment and the cell (1). In addition to the structural protein clathrin, numerous other receptor binding adaptors are present, as are proteins involved in membrane curvature, detachment, coat dissociation, and recycling; these include AP-2 and other adaptors, epsin, dynamin, synaptojanin, and others (reviewed in Refs. 2–5). How the functions of these factors are controlled in space and time during the lifetime of the coated pit and the initial phase of the endocytic process remains a major challenge to our understanding. It has recently become apparent that membrane lipids, and specifically phosphoinositides, play major roles in the coordination of these processes.

There is considerable evidence for an important regulatory role of phosphoinositide lipids in membrane traffic between multiple intracellular compartments. In the endocytic pathway, phosphoinositides were initially demonstrated to bind with high affinity to the plasma membrane adaptor AP-2 and to modulate its properties (6, 7). Subsequent studies have confirmed these observations and demonstrated the interaction of phosphoinositides with other proteins involved in clathrin-mediated trafficking (reviewed in Ref. 8). The actin cytoskeleton has also been strongly implicated in the function of the endocytic machinery (9–11), and phosphoinositide regulators (12, 13) tightly control its assembly and disassembly. Thus there is considerable in vitro evidence for roles of both PtdIns(4,5)P2 and 3-phosphorylated inositides in membrane trafficking, and the identity of many of the targets to which they bind has now been established. To dissect the spatiotemporal aspects of these interactions in the modulation of endocytic and cytoskeletal function, it is critical to identify and study the enzymes responsible for inositol metabolism at specific sites of action.

We have recently shown that a specific phosphoinositide 3-kinase, phosphoinositide 3-kinase C2α (PI3K-C2α)1, is a component of the clathrin-mediated transport machinery at the plasma membrane and in the trans-Golgi network (TGN) (14). PI3K-C2α is a member of the class II PI3 kinases and is distinguished from other PI3 kinases by its additional C-terminal C2 domain (15, 16). Three distinct mammalian members of the class II PI3 kinase family have been characterized (17), PI3K-C2α, PI3K-C2β, and PI3K-C2γ. Although all share the distinguishing C-terminal C2 domain as well as the characteristic 3-kinase motifs, the enzymes differ predominantly in their N-terminal regions. The cellular functions of class II enzymes are not well understood, though there is evidence that PI3K-C2β, and to a lesser extent PI3K-C2α, can be stimulated by activation of several receptor systems including epidermal growth factor (18), insulin (19), chemokines (20), and integrins (21); the former is also implicated in cell adhesion and movement (22). By analogy with the Class I PI 3-kinase family, it has been suggested that activation of class II enzymes by signaling receptors may mediate specific aspects of downstream receptor activity. In vivo evidence for this possibility has recently been reported in studies of the single Class II PI 3-kinase in Drosophila melanogaster, PI3K-68D, which demonstrated its involvement in patterning processes involving the epidermal growth factor and Notch receptor pathways (23). Interestingly, the N-terminal region of PI3K-68D more closely resembles that of mammalian PI3K-C2β, consistent with the receptor-mediated signaling role for this family member implied by the studies in mammalian cells noted above.

In addition to localizing PI3K-C2α to endocytic and TGN clathrin-coated membranes (14), we found that PI3K-C2α is enzymatically activated by clathrin through binding to a discrete clathrin binding domain

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3 The abbreviations used are: PI3K-C2α, phosphoinositide 3-kinase C2α; TGN, trans-Golgi network; CBD, clathrin binding domain; WT, wild type; GST, glutathione S-transferase; MES, 4-morpholinonethanesulfonic acid.
(CBD) present in the unique N-terminal region of the protein (24). Exogenous expression of PI3K-C2α in cells dramatically affected both clathrin distribution and function. Although plasma membrane clathrin-coated pits could still be observed, PI3K-C2α expression induced a striking accumulation of intracellular clathrin staining consistent with the presence of numerous cytoplasmic clathrin-coated structures. Such structures, distinct from those in the TGN, are virtually undetectable in control cells (24, 25). Inhibition of clathrin-mediated transport from both the plasma membrane and TGN was also observed under these conditions. It should be noted that the proliferation of uniform, punctate intracellular clathrin signal under these conditions was quite distinct from the aggregates of clathrin in granules that have been observed upon expression of dominant-negative forms of other clathrin-binding proteins such as auxilin or AP180 (26).

Given the ubiquitous roles played by many different phosphoinositides in membrane trafficking, clathrin binding and activation of PI3K-C2α suggests a possible mechanism for the localized and timed formation of 3-phosphoinositides at sites of membrane budding during endocytosis or Golgi transport. This notion is supported by the observation that both PI3K-C2α overexpression (24) and the putatively reciprocal knockout of synaptojanin phosphatase activity (27) result in the accumulation of unusual intracellular clathrin-coated structures, together suggesting defects in the coat recycling pathway. Elucidation of PI3K-C2α function under these conditions can be expected to provide insight into the mechanisms by which phosphoinositides regulate these aspects of membrane trafficking. To provide a basis for understanding PI3K-C2α function in cells, we report here an analysis of the roles of the CBD and of the lipid kinase activity in modulating PI3K-C2α effects on clathrin behavior in cells. Interestingly, we found that both the enzymatic and the clathrin binding activities of PI3K-C2α play distinct and apparently independent roles in regulating clathrin distribution and function.

MATERIALS AND METHODS

Constructs—Glu-tagged full-length and (Δ1–142) human PI3K-C2α in pMPT-SM have been described (24). The regions of PI3K-C2α cDNA corresponding to constructs noted in Fig. 1A were cloned into SmallEcoRI sites of pMPT-SM (Sigma) with a Glu tag at the 5’-end (14). Similarly, pMPT-SM-KD PI3K-C2α and pMPT-SM-KD-(143–1686) (ΔCBD/KD) were generated in which Lys-1138, Asp-1157, and Asp-1250 were mutated to Ala. For generation of stable cell lines, cDNAs encoding WT- and KD-PI3K-C2α were cloned into SacII-EcoRI sites of pTRE (Clontech).

Assay of Pl 3-Kinase Activity—COS1 cells transfected with WT- or KD-PI3K-C2α were analyzed as described previously (24). Briefly, cells were lysed in 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 15 mM glycerol, 1% Nonidet P-40, 2 mM EDTA. Clarified lysates (75,000 rpm, 20 min, 4 °C, TLA 100 rotor) were then immunoprecipitated with a monoclonal antibody against the Glu epitope (Research Diagnostics, Inc.), and equivalent amounts (assayed by anti-Glu immunoblotting) of immunoprecipitate were assayed for PI 3-kinase activity. Sonicated and equivalent amounts (assayed by anti-Glu immunoblotting) of immunoprecipitated protein sample for 10 min in 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.5 mM EGTA. The kinase reaction was initiated upon addition of 4 mM MgCl₂ and 40 μM [γ-32P]ATP (2 μCi). After incubation at 30 °C for 30 min, lipids were extracted with acidified chloroform/methanol, analyzed by thin-layer chromatography using silica-gel-60 plates and chloroform/methanol/4 mM ammonium hydroxide (9:7:2, v/v/v), and quantified using a PhosphorImager (Amersham Biosciences).

Electron Microscopy—MEF Tet-Off cells (Clontech), stably co-transfected with pTK-Hyg and pTRE-PI3K-C2α or pTRE-KD, were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 μg/ml G418, 150 μg/ml hygromycin, and 1 μg/ml doxycycline. One week after removing doxycycline, PI3K-C2α and KD expressing MEF Tet-Off cells (confirmed by immunoblotting and immunofluorescence) and parental cells were rinsed in 0.1 M sodium cacodylate (pH 7.4) and then fixed in this solution supplemented with 2.5% glutaraldehyde and 2% paraformaldehyde. Samples were processed for conventional thin-section electron microscopy as described previously (28). Quantification of coated membrane profiles was obtained by evaluating 8–11 fields comprising 2.25 square microns (roughly 1.5 × 1.5 μm) at high magnification (∼50,000) for each cell line.

GST-TD (Clathrin Terminal Domain) Binding Assay—pGEX-2T and pGEX-2T-TD-(1–579) were expressed in Escherichia coli BL21 and purified as described previously (29). COS1 cells expressing Glu-tagged PI3K-C2α residues 2–873 were lysed 48 h after transfection in 25 mM Na-HEPES, (pH 7.3), 0.1% Triton X-100, 50 mM KCl, supplemented with protease inhibitors and centrifuged at 80,000 rpm for 15 min in a TLA100 rotor. Ten μg of glutathione-S-transferase (GST) or GST-TD-bound glutathione-agarose beads were incubated with clarified lysate, and binding was detected by immunoblotting with anti-Glu antibody.

Miscellaneous Procedures—For immunofluorescence studies and transferrin uptake experiments, COS1 cells transiently transfected with vector, full-length wild type, or mutant PI3K-C2α constructs were processed as described previously (24). Quantitation of Alexa-594 transferrin uptake (20 μg/ml for 10 min) and of clathrin-coated structure number was performed by analyzing pairs of PI3K-C2α-expressing and non-expressing cells in the same field, following background correction. Ten separate fields from multiple experiments were evaluated for each construct. For transferrin uptake, average pixel intensity in the perinuclear recycling compartment was quantitated, while the number of anti-clathrin antibody (R5 antibody, Ref. 25)-labeled spots within a 30.25 μm² (50 × 50 pixel) box toward the periphery of the cell were counted for determination of clathrin coat structures. Image processing was performed using MetaMorph software (Molecular Devices, Inc.). For immunoprecipitation studies, cells were lysed 48 h after transfection in 100 mM MES-Na (pH 6.8), 0.1% Triton X-100, 1 mM EGTA, 0.5 mM MgCl₂, 0.02 Na3VO4, supplemented with protease inhibitors. Clarified lysates (12,000 rpm, 10 min) were centrifuged at 80,000 rpm for 15 min in a TLA100 rotor. Pellets were resuspended in lysis buffer and challenged with anti-Glu antibody. The immunoprecipitates were blotted with anti-Glu or anti-clathrin antibody TD1 (ATCC, Manassas, VA). Clathrin coat assembly was performed as described (30). Briefly, 100 μg of clathrin alone or mixed with equimolar amounts of either AP-2 or purified PI3K-C2α (2–134) was dialyzed overnight against 0.1 M MES-Na, pH 6.5, 1 mM EGTA, 0.5 mM MgCl₂, 0.02% NaN₃. Assembled clathrin structures were sedimented by ultracentrifugation (80,000 rpm, 10 min, TLA100 rotor), and equal proportions of pellets were analyzed by SDS-PAGE. Assembled clathrin structures induced by PI3K-C2α-(2–134) were further visualized by negative staining electron microscopy as described previously (30).

RESULTS AND DISCUSSION

PI3K-C2α is a multidomain protein (Fig. 1) containing two protein modules directly related to the catalytic activity of the enzyme (HR1 and HR2), PX and C2 domains at its C terminus, and a distinct clathrin binding domain (CBD) in the N-terminal part of the protein (24). Expression of PI3K-C2α in cells leads to gross changes in clathrin distribution and function, manifested as proliferation of intracellular clathrin...
rin-coated structures and inhibition of clathrin function in endocytosis (Fig. 2A) and TGN sorting (24). To investigate the role of the kinase activity in these processes, we generated a lipid kinase-defective mutant. In this mutant, residue Asp-1250 in the catalytic loop region (corresponding to Asp-166 in the protein kinase A catalytic subunit), conserved between all classes of PI 3-kinases as well as other lipid and protein kinases, was changed to alanine. In addition, two residues (Lys-1138 and Asp-1157) involved in ATP binding, and corresponding to protein kinase A catalytic subunit residues Lys-72 and Asp-89, were replaced with alanine. The resulting epitope-tagged kinase-dead mutant (KD-PI3K-C2/H9251) and wild type (WT) proteins were immunoprecipitated from lysates of expressing cells and tested for kinase activity. At similar levels of overall expression, no catalytic activity toward phosphatidylinositol was detected in immunoprecipitates containing the KD protein in contrast to the wild type protein (Fig. 1B), confirming that kinase activity had indeed been ablated by the mutations.

When epitope-tagged KD-PI3K-C2/H9251 protein was transiently expressed in COS1 cells and subjected to immunofluorescence, it exhibited a punctate pattern throughout the cytoplasm with some concentration in the perinuclear region characteristic of the TGN, a pattern indistinguishable from that of the WT protein (Fig. 2A and Ref. 24). As with the wild type protein, localization in the nucleus (31) was only very occasionally observed. These results suggest that, as expected, the protein was correctly folded. Further, the results indicate that the kinase activity of the protein is not required for its correct targeting.

Next we wished to evaluate the effect of transiently expressing KD-PI3K-C2/H9251 on the proliferation of novel intracellular clathrin-coated structures and inhibition of clathrin-mediated endocytosis that characterize expression of the wild type protein. Surprisingly, expression of the mutant protein also resulted in proliferation of intracellular clathrin-coated structures, virtually indistinguishable from the effect of wild type enzyme (Fig. 2A). Similarly, cells expressing KD-PI3K-C2/H9251 were also defective in endocytosis of transferrin (Fig. 2B). Quantitation of multiple fields confirmed these effects (Fig. 3). The effects on clathrin distribution and on transferrin internalization were observed at similar levels of wild type and mutant enzyme overexpression. These data indicate that determinants other than the lipid kinase activity of PI3K-C2/H9251 are sufficient to elicit the effects on clathrin distribution and function.

Stable Cell Lines Reveal Ultrastructural Changes upon PI3K-C2α Expression—To more fully characterize the effects of WT- and KD-PI3K-C2α expression upon clathrin morphology, we established stable inducible cell lines expressing each of these proteins in Tet-regulatable MEF cells. As with the transiently expressing cells (Fig. 2A),
light microscopy revealed that both the WT and the KD-PI3K-C2α proteins are localized to punctate spots throughout the cytoplasm and are concentrated in the perinuclear region; further they show an increase in intracellular clathrin and a modest inhibition of transferrin uptake (data not shown). Ultrastructural analysis of the stable cell lines revealed important changes in coated membrane profiles. In parental MEF cells, characteristic bristle-coated pits were observed on the plasma membrane and occasionally as discrete profiles nearby (Fig. 4, upper left). In the WT-PI3K-C2α-expressing cells, no significant change was observed in the frequency of plasma membrane-coated pits (data not shown). However, a striking increase in the number of intracellular coated profiles was observed (Fig. 4, right panels). Most frequently these had the appearance of coated buds on the ends of tubules. Quantitation of the number of these coated buds suggests that their appearance is ∼10-fold more frequent in the WT-PI3K-C2α-expressing cells (2.4 coated structures/μm²; 9 fields analyzed) than in the parental line (0.23 coated structures/μm²; 8 fields analyzed). Evaluation of the KD-PI3K-C2α cell line (Fig. 4, lower left panel) also revealed a pronounced increase in intracellular coated membrane profiles (1.8 coated structures/μm²; 11 fields analyzed).

These results confirm the immunofluorescence observations of a dramatic proliferation of intracellular clathrin-coated structures reported here (Fig. 2) and previously (24). Further, they indicate that the lipid kinase activity of the exogenous PI3K-C2α molecule is not required to elicit the effect. In most cases the ultrastructural images suggest the presence of coats on small, circular membrane profiles of ∼70–90-nm diameter. Whether these structures are in fact discrete coated vesicles, coated buds on the end of tubular segments, or other coated membrane structures will require more elaborate ultrastructural studies such as serial section analysis. Observation of fluorescently tagged protein molecules in live cells may provide an alternative perspective, and preliminary results in our laboratory are consistent with the presence of clathrin-coated buds exhibiting highly dynamic but restricted local mobility.4

Functional Domains in PI3K-C2α—The finding that the lipid kinase activity of PI3K-C2α was not required to elicit either the marked changes in clathrin distribution or inhibition of transferrin uptake prompted us to further analyze which domain(s) of the protein contributed to these phenomena. Accordingly, several constructs comprising different domains of PI3K-C2α were prepared and transiently expressed in cells. First, we expressed the N-terminal half of PI3K-C2α (amino acids 2–873), truncated before the catalytic regions (Fig. 1A, NT). This N-terminal fragment expressed well and exhibited the punctate pattern of cytoplasmic fluorescence with perinuclear concentration (Fig. 5A) characteristic of both the endogenous and WT protein (14, 24). Furthermore, it was able to potently induce formation of intracellular clathrin-coated structures and, like the WT protein, it colocalized with these structures (Fig. 5A, insets). Finally, again like the full-length WT protein, it was able to inhibit endocytosis of transferrin at expression levels comparable with those of full-length WT or KD proteins (Fig. 5D).

These results (quantitated in Fig. 3) extend the finding that neither functional kinase activity nor the structural presence of the C-terminal half of the protein is required to elicit the proliferation of intracellular clathrin structures and concomitant inhibition of endocytosis. As the N-terminal half of PI3K-C2α contains the clathrin binding domain that we have identified previously at the extreme N terminus of the protein (24), we then focused our attention on this region. First, expression of the N-terminal half of the protein lacking the CBD (residues 143–873) neither colocalized with clathrin nor had any demonstrable effect on its distribution (Fig. 5B), suggesting that the activity of this half of the protein can indeed be ascribed to the CBD. In confirmation, we found that expression of this fragment alone (residues 2–143) was indeed able to induce the formation of intracellular clathrin-coated structures, though only at higher levels of expression (Fig. 5C). This may reflect less effective folding or stability of this short fragment upon expression or that there are additional functional determinants located toward the C terminus of the protein. In any case, the results demonstrate that the ability of the catalytically inactive PI3K-C2α to elicit

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changes in clathrin distribution is attributable at least in part to the CBD of the enzyme.

The PI-3KC2α CBD Possesses Clathrin Assembly Activity—Given the ability of PI3K-C2α fragments that contain the CBD to induce the proliferation of intracellular clathrin-coated structures, we hypothesized that the CBD might itself possess clathrin assembly activity that could account for the formation of these structures through clathrin polymerization. Our previous work demonstrated that this region of the protein could indeed bind to intact clathrin triskelia in preincubated COS1 cells (24). The clathrin binding motif we noted in PI3K-C2α has been shown to interact specifically with the terminal domain of the clathrin heavy chain (29, 32). As expected from these characteristics, we found that clathrin terminal domain–containing epitope-tagged PI3K-C2α (residues 1–579) fused to GST was able to efficiently pull out the PI3K-C2α fragment of amino acids 2–873 from expressing COS1 cells (Fig. 6A).

Next we incubated purified clathrin triskelia with bacterially expressed polyhistidine-tagged PI3K-C2α-(2–134) under conditions in which clathrin assembly requires a separate factor to polymerize into coat structures (30). Clathrin assembly was analyzed by ultracentrifugation followed by SDS-PAGE analysis. The N-terminal PI3K-C2α fragment supported efficient polymerization of clathrin into a sedimentable state, comparable in extent to AP-2–mediated assembly used as a control (Fig. 6B). The macromolecular products of the assembly reaction were visualized by negative staining electron microscopy, revealing the formation of complete clathrin lattices in the presence of the PI3K-C2α N-terminal fragment (Fig. 6C). These data demonstrate that PI3K-C2α interacts with the clathrin domain and can induce clathrin assembly. It seems likely that this activity is responsible for the formation of assembled clathrin structures observed upon expression of PI3K-C2α constructs.

PI3K-C2α Kinase Activity and the CBD Operate Independently—Our results to this point indicate that the CBD is sufficient to induce the proliferation of intracellular clathrin structures and that the lipid kinase activity of PI3K-C2α is not required. To test whether the catalytic activity of PI3K-C2α can independently play a role in the modulation of clathrin distribution in cells it was necessary to remove the clathrin binding domain from PI3K-C2α. Accordingly epitope-tagged deletion mutants of PI3K-C2α lacking N-terminal residues 1–142 in catalytically active (ΔCBD) or inactive (ΔCBD/KD) proteins (Fig. 1) were constructed and expressed in COS1 cells.

Cells transiently expressing the indicated constructs were lysed, the epitope-tagged proteins were immunoprecipitated from resuspended high-speed pellets, and the presence of associated clathrin was then determined by immunoblotting (Fig. 7A). As expected, the presence of the CBD conferred the ability to bind clathrin, as the latter was present in immunoprecipitates of both full-length WT- and KD-PI3K-C2α. Moreover, immunoprecipitation of the CBD itself also revealed the presence of clathrin, providing evidence of a stable association. These
FIGURE 7. Both the PI 3-kinase activity and the CBD of PI3K-C2α contribute independently to binding and perturbation of clathrin function in intact cells. A, indicated forms of wild type, truncated, or mutant PI3K-C2α were expressed and immunoprecipitated from clarified lysates of transfected COS1 cells as described under “Materials and Methods.” Bound clathrin present in the immunoprecipitates was detected by blotting. Binding of clathrin was dependent on the presence of either the clathrin binding domain or the catalytic activity of PI3K-C2α. B, the kinase activity of PI3K-C2α is sufficient, even in the absence of the CBD, to induce the formation of intracellular clathrin-coated structures. Clathrin distribution was visualized in cells expressing either catalytically active (upper panels) or inactive (lower panels) PI3K-C2α mutants lacking the N-terminal CBD (residues 1–142). Images shown are representative of many fields examined. Bar, 10 μm.

data support our immunofluorescence (Figs. 3 and 5) and ultrastructural (Fig. 4) results showing that enzymatically inactive PI3K-C2α as well as the CBD alone are each able to mediate the formation of intracellular clathrin-coated vesicular structures and support the hypothesis that this activity is a direct consequence of the presence of the clathrin binding and assembly activity of the CBD (Fig. 6).

To evaluate the contribution of the kinase activity to the effect of PI3K-C2α on clathrin, cells expressing truncated PI3K-C2α constructs lacking the CBD (ΔCBD) were then examined. Interestingly, clathrin was efficiently recovered in immunoprecipitates of cells expressing the ΔCBD at levels comparable with the full-length protein (when corrected for expression level). However, it was greatly diminished or absent in immunoprecipitates from cells expressing the kinase-dead version of PI3K-C2α/ H9004. The enzymatically inactive PI3K-C2α/ H9004 and PI3K-C2α/ H9262 lack the N-terminal CBD at levels comparable with the full-length protein (when corrected for expression level). However, it was greatly diminished or absent in immunoprecipitates from cells expressing the kinase-dead version of wild type, truncated, or mutant PI3K-C2α.

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