Phosphoinositide 3-Kinase Binds Constitutively to α/β-Tubulin and Binds to γ-Tubulin in Response to Insulin*

(Received for publication, May 3, 1995, and in revised form, July 13, 1995)

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Recently we reported the localization of phosphoinositide 3-kinase (PI 3-kinase) by immunofluorescence to microtubule bundles and the centrosome (Kapeller, R., Chakrabarti, R., Cantley, L. F., and Corvera, S. (1993) Mol. Cell. Biol. 13, 6052–6063). In complementary experiments we used the recombinant p85 subunit of PI 3-kinase to identify proteins that associate with phosphoinositide 3-kinase and found that phosphoinositide 3-kinase associates with α/β-tubulin. The association occurs in vivo but was not significantly affected by growth factor stimulation. We localized the region of p85 that interacts with α/β-tubulin to the inter-SH2 domain. These results support the immunofluorescence data and show that p85 directly associates with α/β-tubulin. We then determined whether phosphoinositide 3-kinase associates with γ-tubulin. We found a dramatic growth factor-dependent association of phosphoinositide 3-kinase with γ-tubulin. Phosphoinositide 3-kinase associates with γ-tubulin in response to insulin and, to a lesser extent, in response to platelet-derived growth factor.

Neither epidermal growth factor nor nerve growth factor treatment of cells results in association of phosphoinositide 3-kinase and γ-tubulin. Phosphoinositide 3-kinase is also immunoprecipitated with antibodies to pericentrin in response to insulin, indicating that phosphoinositide 3-kinase is recruited to the centrosome. Neither phosphoinositide 3-kinase activity, nor intact microtubules are necessary for the association. Treatment of cells with 0.5 M NaCl dissociates γ-tubulin from the centrosome and disrupts the association of phosphoinositide 3-kinase with pericentrin, but not γ-tubulin. Recombinant p85 binds to γ-tubulin from both insulin-stimulated and quiescent cells. These results suggest that the association of phosphoinositide 3-kinase with γ-tubulin is direct. These data suggest that phosphoinositide 3-kinase may be involved in regulating microtubule responses to insulin and platelet-derived growth factor.

Phosphoinositide 3-kinase (PI 3-kinase) is a heterodimer of a 110-kDa catalytic subunit and an 85-kDa regulatory subunit and has both phosphoinositide and protein kinase activities (2, 3). In response to a number of growth factors PI 3-kinase is activated resulting in the production of PtdIns-3,4,5-P3 and PtdIns-3,4-P2 (4). The downstream targets of PI 3-kinase are still not well understood. Members of the novel group of protein kinases C are activated by PtdIns-3,4,5-P3 and these proteins are likely targets of PI 3-kinase signaling (5). Studies using both the PI 3-kinase inhibitor, wortmannin and platelet-derived growth factor (PDGF) receptor mutants, have shown that PI 3-kinase is necessary for ruffling in response to PDGF, insulin, and insulin-like growth factor-1 (6–8). Mutants of the PDGF receptor that do not bind PI 3-kinase are also defective in cell motility in an assay of cell migration toward a PDGF gradient (9). PI 3-kinase clearly has effects on the actin cytoskeleton.

Recently we have found evidence suggesting an interaction of PI 3-kinase and microtubules (1). Immunofluorescence studies using an antibody to the 85-kDa subunit of PI 3-kinase have shown that PI 3-kinase is concentrated in the perinuclear region, especially around the centriole. Stimulation of cells with PDGF increases the staining of p85 in the perinuclear region. PI 3-kinase also localizes to some microtubule bundles. The stimulation of PI 3-kinase localization to the perinuclear region suggests that PI 3-kinase, in a complex with growth factor receptors, moves along microtubules to reach the perinuclear area (1, 10). Microtubule-dependent vesicular and organelle transport has also been described in response to serum (11, 12). The PI 3-kinase localized to microtubules could be either a component of vesicles moving along microtubules or directly associated with microtubules. There is no explanation for localization of p85 to the centrosome.

γ-Tubulin is a recently discovered form of tubulin which is localized primarily to the centrosome in mammalian cells and is proposed to be the link between the microtubule organizing center and the microtubule polymers (13, 14). It is able to nucleate microtubule formation and is also able to bind to the centrosome. This central role in microtubule formation makes γ-tubulin an attractive candidate for regulation of microtubule formation.

Although there is quite suggestive evidence that microtubule-dependent processes occur in response to growth factors (10, 11) and pp60c-src and insulin-dependent phosphorylation of α- and β-tubulin have been described (15, 16), it is not clear that significant changes in microtubules themselves occur in response to growth factors. It might be expected that cells responding to growth factors would alter microtubules to facilitate any number of responses from shape change to transport.
These sorts of changes in microtubules are technically difficult to study in response to growth factors and little data are available describing alterations in microtubule dynamics or structure in response to growth factors.

In an effort to better understand the function of PI 3-kinase, we analyzed proteins that associated with recombinant p85. Surprisingly, we found that p85 associates directly with αβ-tubulin. These data provide a mechanism for the previous observation that a fraction of p85 localizes with microtubules by immunofluorescence. Since p85 bound to αβ-tubulin we also examined whether PI 3-kinase associates with γ-tubulin. We found that PI 3-kinase associates with γ-tubulin and that there is a dramatic increase in the association in response to insulin and PDGF. The growth factor-dependent association of PI 3-kinase with γ-tubulin suggests that microtubules themselves could be regulated in response to growth factors and that PI 3-kinase might play a role in this regulation.

**EXPERIMENTAL PROCEDURES**

Reagents—Tissue culture media, bovine crystalline insulin, and genetin (G418) were obtained from Life Technologies, Inc. (Grand Island, NY). Nocodazole, PtdIns-4-P, PtdIns-4,5-P2, glutathione-Sepharose (GSH) beads, and protein A-Sepharose beads were purchased from Sigma. PtdIns (Avanti, Birmingham, AL), Acrylagel, and Bisc-acylagel (National Diagnostics, Manville, NJ) and the ECL kit (Amer sham) were obtained from commercial sources. EGF, PDGF, and NGF were purchased from Upstate Biotechnology. p85 antibodies used in this study were raised in this laboratory against a GST fusion protein containing either the N-terminal SH2 domain (GST-NSH2) or the N-terminal portion (GST-NT) of rat p85a. Polyclonal and monoclonal antibodies generated against the C-terminal SH2 domain of p85a were purchased from Transduction Laboratories (Lexington, KY). The α- and β-tubulin monoclonal antibodies were obtained from Sigma. The γ-tubulin antibody was a gift of Tim Stearns (Stanford University). The antibody to pericentrin was a gift of Stephen Doxsey (University of Massachusetts). The antibody to tubulin was a gift of Vlado T. Lisac (University of California, San Diego). Glutathione S-Transferase Fusion Proteins—The GST fusion proteins containing full-length p85a (GST-85b) with cell lysates and analyzed the associated proteins. We identified a protein by Coomassie staining that ran at 55 kDa. To test whether this protein was tubulin, immobilized GST or GST-85b was incubated with tubulin from quiescent or insulin stimulated CHO-IR cells. The GST and GST-85b precipitates were processed as described under "Experimental Procedures" and analyzed for the presence of α-tubulin by Western blotting. As shown in Fig. 1A, no α-tubulin was precipitated by GST-coated beads (lanes 3 and 4), whereas GST-85b immunoprecipitated α-tubulin (lanes 5 and 6). Since tubulin can be tyrosine phosphorylated we determined whether a phosphopeptide based on the middle T sequence that binds the SH2 domains of p85 (EEY[PO2]IMPME or EEY[MPME]) would block the association. The peptide has a kD in the nanomolar range for the SH2 domains of PI 3-kinase (22). At a concentration of 10 μM no inhibition of the association of GST-85b and α-tubulin was found (lanes 7 and 8). Insulin did not affect the amount of α-tubulin precipitated by GST-85-coated beads.

To investigate whether tubulin and PI 3-kinase associate in intact cells, p85 and α-tubulin immunoprecipitates from insulin-treated and control CHO-IR cells were examined for the presence of p85 and α-tubulin by Western blotting. As shown in Fig. 1B, α-tubulin was detected in p85 immunoprecipitates from both insulin-stimulated and control CHO-IR cells (lanes 3 and 4). Treatment of CHO-IR cells with insulin, however, did not change the amount of α-tubulin that immunoprecipitated with p85. The α-tubulin antibody also immunoprecipitated p85 (Fig. 1B, lanes 5 and 6). Neither did insulin treatment affect the presence of p85 observed in α-tubulin immunoprecipitates (Fig. 1, lanes 5 and 6). An irrelevant antibody did not immunoprecipitate either tubulin or p85 from CHO-IR cell lysates (Fig. 1, lanes 7 and 8), indicating that association of tubulin with PI 3-kinase is specific. This results indicate that tubulin and PI 3-kinase interact in vivo and that this association is not changed by insulin. Based on Western blotting, we estimate that 5–10% of cellular p85 associates with αβ-tubulin. Similar
results were obtained when PI 3-kinase activity, rather than Western blotting, was measured on the immunoprecipitates (data not shown).

To determine whether the interaction between p85 and α-tubulin was direct, we incubated GST-85-coated beads with tubulin purified from rat brain. Coomassie Blue staining showed that tubulin was purified to homogeneity (not shown). We also incubated GST- or GST-85-coated beads with untreated CHO-IR cell lysates as a control. The precipitates were analyzed for the presence of α-tubulin by Western blotting. GST-85 precipitated α-tubulin from the purified preparation, indicating that the association between p85 and tubulin is direct (Fig. 1C).

Characterization of the Domains of p85 That Associate with α/β- and γ-Tubulin

To determine which domain(s) of p85 mediates the association with α/β-tubulin, we incubated GST fusion proteins containing different domains of p85 with CHO-IR cell lysates and the precipitates were analyzed for the presence of α- and β-tubulin by Western blotting (Fig. 2A). As shown in Fig. 2B, α-tubulin was precipitated by GST-85 and the GST-N+C (which contains both SH2 domains and the inter-SH2 region). There was also a small amount of binding to the GST-CSH2 protein. This construct contains a portion of the inter-SH2 domain. Since tyrosine-phosphorylated peptides do not block the association of p85 and tubulin (Fig. 1), we do not think that the phosphopeptide binding pocket of the SH2 domain mediates binding. We have made an inter-SH2 domain construct, lacking the SH2 domains, but it is quite hydrophobic.
and is insoluble. In addition, when the GST constructs were incubated with purified tubulin we found binding to GST-85 and GST-N+C, but we did not see binding to the GST-CSH2 construct (Fig. 2C).

With each of these constructs there is a small increase in the amount of tubulin that associates with the p85 constructs after insulin stimulation. Since we have not been able to reproducibly find a difference in the association of PI 3-kinase and α/β-tubulin following insulin stimulation, as judged by activity measurements on α/β-tubulin immunoprecipitates, we are uncertain of the physiologic relevance of this finding. Since tubulin exits as a heterodimer of α and β subunits we wanted to confirm that the β subunit was also present. The membrane was stripped and reprobed with β-tubulin antibody (Fig. 2B, panels E and F). β-Tubulin was also precipitated from CHO-IR cells with GST-85, GST-N+C, and to some extent by the GST-CSH2, but not by other GST fusion proteins containing other domains of p85. The inter-SH2 domain of p85 is predicted to contain a coiled-coil (23) and tubulin also has a coiled-coil structure. It is possible that interaction of these two coiled-coil domains mediates the binding of p85 to tubulin.

PI 3-Kinase Associates with γ-Tubulin in Response to Insulin—Since we found that PI 3-kinase associates with α/β-tubulin we also wanted to determine whether PI 3-kinase associates with γ-tubulin. We first determined whether PI 3-kinase and γ-tubulin are associated by Western blotting of immunoprecipitates from CHO-IR. We found that in response to insulin we could detect p85 in γ-tubulin immunoprecipitates (Fig. 3A). We were unable to detect γ-tubulin in p85 immunoprecipitates. Only a fraction of p85 associates with γ-tubulin in response to insulin, so our inability to detect γ-tubulin may be due to lack of sensitivity. We estimate, based on Western blotting that about 1% of cellular p85 associates with γ-tubulin in response to insulin. It is also possible that the antibody does not recognize the fraction of p85 bound to γ-tubulin.

To confirm the Western blot findings we also immunoprecipitated γ-tubulin from CHO-IR cells and assayed the immunoprecipitates for PI 3-kinase activity and determined the time course of the association. In quiescent cells we found minimal PI 3-kinase activity associated with γ-tubulin, but stimulation with 100 nM insulin resulted in the appearance of PI 3-kinase in the immunoprecipitates (Fig. 3B). The associated activity peaked between 5 and 15 min after insulin stimulation and decayed over an hour. We found maximal association of PI 3-kinase with γ-tubulin at an insulin concentration of 10 nM, indicating that the effect is mediated at physiologic insulin concentrations (not shown). The increase in PI 3-kinase activity in γ-tubulin immunoprecipitates could be explained either by a stimulation of activity of PI 3-kinase constitutively associated with γ-tubulin or recruitment of PI 3-kinase in response to insulin. The Western blots demonstrate that PI 3-kinase is recruited to γ-tubulin in response to insulin (Fig. 3A).

A comparison of the amount of activity that immunoprecipitates with α- or β-tubulin antibodies to γ-tubulin antibodies showed that much less activity was associated with the α- or β-tubulin immunoprecipitates. Since more p85 protein is present in α- or β-tubulin immunoprecipitates, this indicates that the specific activity of PI 3-kinase associated with γ-tubulin is higher.

As a control for the effect of the 0.5 M NaCl and to determine whether PI 3-kinase associated with γ-tubulin at the centrosome we determined the presence of PI 3-kinase activity in immunoprecipitates using an antibody to pericentrin, another centrosomal protein (25). We found that PI 3-kinase activity was present in the pericentrin immunoprecipitates from insulin-stimulated, but not quiescent cells (Fig. 4B). This confirmed that PI 3-kinase is recruited to the centrosome in response to insulin. These results do not exclude the possibility that PI 3-kinase also associates with γ-tubulin that is not at the centrosome. Furthermore, pericentrin immunoprecipitates from...
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**Fig. 4.** Association of PI 3-kinase with γ-tubulin and pericentrin. CHO-IR cells were treated or not with insulin as indicated. Cells were then lysed in the presence of 0.2 M or 0.5 M NaCl. The NaCl was then diluted to 200 mM and immunoprecipitates were done using either the γ-tubulin or pericentrin antibodies. The immunoprecipitates were washed and assayed for PI 3-kinase activity as described in the legend to Fig. 3. PIP, phosphatidylinositol phosphate; PIP₃, phosphatidylinositol-3,4,5-trisphosphate.

Insulin-stimulated cells lysed in 0.5 M NaCl lack PI 3-kinase activity. Western blots of similar immunoprecipitates show no difference in the amount of pericentrin present (data not shown). These data suggest that PI 3-kinase is in a tight complex with γ-tubulin and pericentrin and remains associated with γ-tubulin even after salt disruption of γ-tubulin binding to the centrosome.

GST-85 Associates with γ-Tubulin—Purified or recombinant γ-tubulin is not available so we were unable to investigate whether purified or recombinant PI 3-kinase would associate directly with γ-tubulin. We did find, however, that the recombinant p85 subunit of PI 3-kinase associated with γ-tubulin from cell lysates. GST and GST-85 fusion proteins expressed in bacteria and purified with GSH beads were incubated with insulin-treated or control CHO-IR lysates and the precipitates probed with an γ-tubulin antibody. As shown in Fig. 5, GST-85 (lane 3), but not GST (lane 2) precipitated γ-tubulin. Insulin treatment did not change the amount of γ-tubulin precipitated by GST-85. The γ-tubulin that associates with GST-85 runs at a slightly lower molecular weight than the γ-tubulin in the cell lysate. This could be due to proteolysis or due to association with a modified form of γ-tubulin. We have found no evidence of tyrosine phosphorylation of γ-tubulin by phosphorytrosine blotting but in vivo[^32P]P₀₅ labeling indicates that γ-tubulin is phosphorylated (not shown).

The association of GST-85 with γ-tubulin from quiescent cells suggests that no modification of γ-tubulin is necessary for association with PI 3-kinase in response to insulin, but that PI 3-kinase is modified in some manner that allows it to interact with γ-tubulin. It is likely that a domain of p85 is revealed in response to insulin that is able to interact with γ-tubulin. The presence of p110 associated with p85 in native PI 3-kinase may change the structure of p85 such that a covalent modification or association with another protein is required to unmask the domain. We attempted to identify the domain of p85 that binds to γ-tubulin, as we did for α/β-tubulin. We were not able to precipitate γ-tubulin with any of the subdomains of p85 that were used in the experiment shown in Fig. 2 (not shown). There are several possible explanations. More than one domain may be necessary to bind γ-tubulin or our constructs may have split the domain necessary for interaction with γ-tubulin. These results indicate that γ-tubulin associates with a different domain of p85 than α/β-tubulin.

Neither PI 3-Kinase Activity nor Microtubules Are Necessary for Recruitment of PI 3-Kinase to γ-Tubulin—Since the association of PI 3-kinase with γ-tubulin seems to require an insulin-independent alteration of PI 3-kinase, we determined whether the enzymatic activities of PI 3-kinase are necessary for the association. PI 3-kinase has both phosphoinositide and protein kinase activity and either activity might be necessary for association with γ-tubulin. We used LY294002, a reversible inhibitor of PI 3-kinase, to determine whether the association of PI 3-kinase with γ-tubulin was blocked. Since LY294002 is a reversible inhibitor, PI 3-kinase activity could be assayed in γ-tubulin immunoprecipitates following treatment (26). We treated CHO-IR cells for 20 min with 25 µM LY294002 (a condition that blocks PtdIns-3,4,5-P₃ production in response to insulin in these cells)² and then stimulated with insulin for 5 min. γ-Tubulin immunoprecipitates were then assayed for PI 3-kinase activity. LY294002 had no effect on recruitment of PI 3-kinase to γ-tubulin (Fig. 6A). These results indicate that the enzymatic activities of PI 3-kinase are not necessary to mediate recruitment.

Since the centrosome is the origin of microtubules we thought that PI 3-kinase might move along microtubules to reach the centrosome and γ-tubulin. This could occur as insulin receptors undergo endocytosis and carry PI 3-kinase with them. We used nocodazole to disrupt microtubules to determine whether intact microtubules were necessary for the recruitment of PI 3-kinase to γ-tubulin. We found that there was no effect of nocodazole on the association of PI 3-kinase with γ-tubulin (Fig. 6B). We also determined by immunofluorescence staining of microtubules that a 20-min treatment with nocodazole caused significant microtubule depolymerization. This indicates that intact microtubules are not necessary for PI 3-kinase to associate with γ-tubulin and the centrosome.

Insulin Is the Best Stimulus to Recruit PI 3-Kinase to γ-Tubulin—Since the association of PI 3-kinase and γ-tubulin was stimulated by insulin we expected that other growth factors would also stimulate the association. We determined whether EGF, PDGF, NGF, and insulin in other cell lines result in the recruitment of PI 3-kinase to γ-tubulin as determined by PI 3-kinase activity in γ-tubulin immunoprecipitates (Fig. 7). We compared EGF and insulin as stimuli in A431 cells and found little response to EGF, but a marked response to insulin. We compared PDGF and insulin in NIH 3T3 cells transfected with the insulin receptor and found a response to PDGF, but a more marked response to insulin. The responses to both factors was smaller in the NIH 3T3 cells than in other cell lines. We also compared the response to insulin, EGF, and NGF in PC-12 cells. In response to insulin PI 3-kinase associated with γ-tubulin, but this effect was not seen in response to EGF or NGF. EGF and NGF are known to activate PI 3-kinase in these cells (27, 28). Insulin is clearly the best stimulus for the association of PI 3-kinase and γ-tubulin, but there is also cell type-dependent variation in the magnitude of the response. The data suggest that a response unique to insulin and PDGF is necessary for the association of PI 3-kinase with γ-tubulin. The unique aspect of insulin signaling is that the downstream signaling

² L. Rameh and L. Cantley, unpublished observations.
proteins interact with IRS-1. We examined γ-tubulin immunoprecipitates from insulin stimulated cells for the presence of IRS-1 by Western blotting for IRS-1 and with antiphosphotyrosine antibodies. We did not detect IRS-1 in γ-tubulin immunoprecipitates (data not shown).

We have found that PI 3-kinase, probably through the SH2 domain of p85, binds to α/β-tubulin. This association is direct, as indicated by detection of the complex formed from recombinant p85 and purified tubulin. Although we saw a small effect of insulin on the association of GST-85 constructs with α/β-tubulin, the physiologic relevance of this difference is not clear. Immunofluorescence studies using antibodies to p85 suggested that p85 localizes near microtubule bundles (1). Our data suggest that PI 3-kinase truly is localized to microtubules, through a direct association with the α/β heterodimer.

It was previously proposed that PI 3-kinase moves with the PDGF receptor in stimulated cells along microtubules to localize near the microtubule organization center (1). The direct association of PI 3-kinase with tubulin suggests a distinct function of PI 3-kinase bound to microtubules. There is little effect of growth factors on the association and if PI 3-kinase were moving with a vesicle one would expect a motor, not PI 3-kinase, to directly contact tubulin. For these reasons it does not seem likely that the PI 3-kinase associated with tubulin is part of a vesicle moving along the microtubule. We do not know the function of PI 3-kinase associated with microtubules. It could play a role in microtubule formation and stability or perhaps in regulating the budding or fusion of vesicles localized on microtubules.

The association of PI 3-kinase with α/β-tubulin led us to examine whether PI 3-kinase might also bind to γ-tubulin. We found that insulin and PDGF stimulate the association of PI 3-kinase with γ-tubulin. EGF and NGF do not stimulate the association. PI 3-kinase is recruited to γ-tubulin at the centrosome, but we cannot exclude the possibility that PI 3-kinase is also recruited to γ-tubulin at sites other than the centrosome. Recruitment of PI 3-kinase to γ-tubulin does not seem to require either the enzymatic activity of PI 3-kinase nor intact microtubules. We cannot be certain that γ-tubulin associates directly with PI 3-kinase since a purified form of γ-tubulin is not available. Since GST-85 associates with γ-tubulin from quiescent as well as insulin-stimulated cells, the effect of insulin that allows association of PI 3-kinase and γ-tubulin is probably mediated by a modification of PI 3-kinase and not γ-tubulin.

The immunofluorescence study of p85 identified two areas of perinuclear localization (1). A more diffuse localization whose intensity increases in response to PDGF and is blocked by nocodazole is consistent with a late endosomal compartment (29). Additionally p85 was found to localize to the centrosome (1). The localization was not affected by nocodazole. The association of PI 3-kinase with γ-tubulin explains the localization of p85 to the centrosome. It is not clear how p85 is recruited to γ-tubulin. It is not likely that PI 3-kinase is transported with endosomes containing the receptor, since nocodazole blocks the redistribution of p85 in response to PDGF (1), but does not block recruitment of PI 3-kinase to γ-tubulin.

Insulin is the primary stimulus that leads to the association of γ-tubulin and PI 3-kinase. Insulin and other growth factors share many common downstream signals. One unique aspect of insulin signaling is the phosphorylation of IRS-1. We thought that IRS-1 might mediate the association with γ-tubulin and explain why insulin seems to be the primary stimulus leading to association. We found no evidence for the presence of IRS-1 in γ-tubulin immunoprecipitates. Also, since IRS-1 is not phosphorylated in response to PDGF it could not explain the PDGF response. We are uncertain of the upstream signal that allows PI 3-kinase to associate with γ-tubulin in response to insulin.

The obvious question raised by these data is what does PI 3-kinase do at the centrosome in response to insulin? These
data suggest that there are growth factor-dependent effects on microtubules. The centrosome is known to orient in the direction of cell movement in cells undergoing chemotaxis and it is possible that PI 3-kinase plays a role in this orientation (30).

Acknowledgments—We thank Tim Stearns and Stephen Doxsey for providing antibodies and Luyba Varticovski for sharing data prior to publication.

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