The N-terminal 34 residues of the 55 kDa regulatory subunits of phosphoinositide 3-kinase interact with tubulin

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There are five regulatory subunit isoforms of phosphoinositide 3-kinase (PI 3-kinase), which are classified into three groups: proteins of 85 kDa (p85α and p85β), 55 kDa (p55α and p55γ) and 50 kDa (p50α). Structural differences between the three groups reside in the N-terminus. To elucidate the unique functional role of the 55 kDa regulatory subunits, GST (glutathione S-transferase) fusion proteins containing a unique N-terminal portion consisting of a 34-amino-acid sequence of p55α or p55γ (GST–p55α/γN$^{1-34}$) were used as affinity matrices to screen rat brain cell extracts for proteins to which this portion binds specifically. A protein that bound was identified as β-tubulin by protein sequencing. In addition, not only the β isoform of tubulin, but also the α and γ isoforms, were detected in the protein absorbed from cell lysates with GST–p55γN$^{1-34}$ and GST–p55αN$^{1-34}$ by immunoblotting. Indeed, the only regulatory subunit present in the purified microtubule assembly from rat brain was the 55 kDa isoform; neither 85 kDa nor 50 kDa subunits were detected. These results indicate endogenous binding of 55 kDa regulatory subunits of PI 3-kinase to tubulin in the brain. Finally, we measured tubulin-associated PI 3-kinase activity in CHO/IR cells overexpressing each of the five regulatory subunit isoforms. Only in cells expressing p55α or p55γ was there a significant elevation of tubulin-associated PI 3-kinase activity in response to insulin. These results suggest that the p55α and p55γ regulatory subunits have important roles in regulating PI 3-kinase activity, particularly for microtubules at the cell periphery.

Key words: insulin, microtubules, p55, PI 3-kinase.

INTRODUCTION

The interaction of growth factors and hormones with their cell-surface receptors activates a range of intracellular signalling cascades that result in the regulation of a variety of important metabolic events within the cell [1–3]. Phosphoinositide 3-kinase (PI 3-kinase) has been identified through its ability to associate with cellular protein kinases, such as growth factor receptors and oncogene products [4,5]. This lipid kinase phosphorylates phosphatidylinositol at the *D*-3 position of the inositol ring in response to stimulation with a variety of growth factors and hormones [6]. Although the biological role of these phospholipids remains unclear, activation of PI 3-kinase has been implicated in the regulation of various cellular activities, including proliferation, differentiation, membrane ruffling, prevention of apoptosis and insulin-stimulated glucose transport [7–13].

PI 3-kinase is composed of a catalytic 110 kDa protein (p110) associated with a regulatory subunit. To date, five mammalian regulatory subunit isoforms have been identified, including two 85 kDa proteins (p85α and p85β), two 55 kDa proteins (p55α and p55γ) and one 50 kDa protein (p50α) [14–18]. These isoforms share two SH2 (Src homology 2) domains and an inter-SH2 domain, but contain different N-terminal sequences. The most well known isoforms, p85α and p85β, contain SH3 and Bcr homology domains in their N-termini. The more recently cloned 55 kDa isoforms (p55α and p55γ) contain a unique 34-amino-acid sequence in their N-termini. These 34 amino acids exhibit 50 % identity and 62 % similarity between p55α and p55γ. The 50 kDa isoform, an alternative splice variant of the p85α gene, contains only a unique six-amino-acid sequence in its N-terminal portion, which is apparently too short to interact with other molecules. These sequence data suggest that the 55 kDa and 85 kDa isoforms may have more functions than the 50 kDa isoform through interactions with other proteins via their N-terminal portions. The N-terminal portion of p85α, the SH3 domain, was shown to interact with dynamin, resulting in increased GTPase activity [19]. In contrast, the function of the N-terminal portion of the 55 kDa proteins remains unknown. The conservation of this 34-amino-acid N-terminal domain between p55α and p55γ suggests that this portion possesses a specific function. Thus we attempted to identify a protein that associates with this N-terminal domain. Herein we report a functional property that is distinct to the 55 kDa regulatory subunits p55α and p55γ, which is attributable to the difference in their N-terminal sequence when compared with those of the other PI 3-kinase regulatory subunits. This difference may be important for determining the distinct subcellular distributions of the PI 3-kinase protein.

EXPERIMENTAL

Glutathione S-transferase (GST) fusion proteins

Sequences corresponding to the N-terminal 34 amino acids of p55γ (p55γN$^{1-34}$), the two SH2 domains plus the inter-SH2 sequence of p55γ (p55γSH2; amino acids 53–455), the N-terminal 34 amino acids of p55α (p55αN$^{1-34}$), the SH3 domains of p85α

Abbreviations used: PI 3-kinase, phosphoinositide 3-kinase; SH2 domain; Src homology 2 domain; GST, glutathione S-transferase; PDGF, platelet-derived growth factor; HA, haemagglutinin.

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and p85β (p85αSH3, amino acids 3–87; p85βSH3, amino acids 3–85) and the two SH2 domains plus the inter-SH2 sequence of p50/55/85α and p85β (p85αSH2, amino acids 326–725; p85βSH2, amino acids 318-719) were amplified by PCR and cloned into the pGEX-3T expression vector (Pharmacia). pGEX-3 constructs were transformed into *Escherichia coli* (JM109). The expression of GST fusion proteins was induced by the addition of 1 mM isopropyl β-D-thiogalactoside, and the protein was purified on glutathione–Sepharose 4B beads according to the manufacturer's instructions (Pharmacia).

Purification of proteins that associate with the N-terminal 34 amino acids of p55

Samples of 10 μg of the three immobilized GST fusion proteins (GST alone, GST–p85αSH3 and GST–p55γN$^{1-34}$) were incubated with 5 ml of a 1% Triton X-100 extract of rat brain (1 mg/ml) for 1 h at 4 °C. After thorough washing of the beads, proteins were released by boiling in Laemmli buffer, subjected to SDS/PAGE, transferred on to a PVDF membrane (Bio-Rad), and visualized by Ponceau S staining. The region of the membrane containing protein bound to GST–p55γN$^{1-34}$ was cut out and its amino acid sequence was determined using a Shimazu protein sequencer PSQ-1 system.

Cell culture and preparation of cell lysates

CHO cells overexpressing the human insulin receptor (CHO/IR cells) were maintained in Ham's F-12 medium containing 10% (v/v) fetal calf serum. The cells from one 100-mm plate (10^7 cells) were treated with insulin, washed twice with ice-cold PBS, and lysed in 5 ml of ice-cold lysis buffer A (1% Nonidet P-40, 10% glycerol, 2 mM vanadate, 10 mM NaF, 10 mM sodium pyrophosphate, 50 mM Hepes, pH 7.5, 137 mM NaCl, 1mM $CaCl_2$, 1 mM $MgCl_2$, 2 mM EDTA, 2 mg/ml aprotinin and 34 mg/ml PMSF). The lysate was cleared by centrifugation at 14000 ***g*** for 10 min. The resulting supernatants were used for experiments.

Microtubule assembly and purification

Purification of the fraction containing tubulin was performed using a previously described method [20]. Briefly, 2 g of rat brain tissue was homogenized with a Teflon/glass homogenizer in 10 ml of ice-cold PEM buffer (0.1 M Pipes/NaOH, 1 mM EGTA and 1 mM $MgSO_4$, pH 6.6). The homogenate was centrifuged at 30000 ***g*** for 15 min at 4 °C. The supernatant was then centrifuged at 180000 ***g*** for 90 min at 4 °C. Taxol was added to a concentration of 20 μM and GTP to 1 mM in the resulting supernatant. The cytosolic extract was warmed to 37 °C for 10 min and transferred to a centrifuge tube. A prewarmed sucrose underlayer solution [PEM buffer containing 10% (w/v) sucrose, 20 μM taxol and 1 mM GTP] was introduced at the bottom of the tube. The sample was finally centrifuged at 30000 ***g*** (37 °C). The whole cytosolic extract and the microtubule pellet were each suspended in Laemmli buffer and then subjected to SDS/PAGE. The regulatory subunits in these fractions were detected by immunoblotting using antisera purchased from UBI (αp85PAN-UBI; a mixture of antisera raised against the entire p85α sequence and the N-teminal SH2 region of p85α).

Binding of GST fusion proteins and immobilized synthetic peptides to the α, β and γ isoforms of tubulin

CHO cell lysates (1 ml) were incubated with 50 μl of GST beads containing 10 μg of the GST fusion proteins for 30 min at 4 °C. After thorough washing in ice-cold TNE buffer (10 mM Tris/HCl, pH 7.8, 0.15 M NaCl, 1% Nonidet P-40, 1 mM EDTA and 34 mg/ml PMSF), the precipitated proteins were subjected to SDS/PAGE and then transferred to nitrocellulose. Immunoblotting was performed using an ECL® kit (Amersham) with monoclonal antibodies obtained from Sigma against the α, β or γ isoforms of tubulin.

In addition, we investigated the binding *in vitro* of various synthetic peptides to each tubulin isoform. The synthetic peptides corresponded to the following sequences: p55γN$^{2-34}$, YNTVWSMDRDDADWREVMMPYSTELIFYIEMDP (amino acids 2–34 of p55γ); p55γN$^{11-28}$, DDADWREVMMPYSTELIF (amino acids 11–28 of p55γ); p55αN$^{2-34}$, YTTVWTMEDLDLECAKTDINCGTDLMFYIEMDP (amino acids 2–34 of p55α); p55αN$^{2-27}$, YTTVWTMEDLDLECAKTDINCGTDLM (amino acids 2–27 of p55α); p85αSH3, HLGDILTVNKGSLVALGFSDGQEARPEDIGWLNGYN (amino acids 25–60 of p85α); p85βSH3, YPFRRERPEDLELLPGDLLVVSRVALQALGVA (amino acids 13–44 of p85β); c-Akt, ERRPHFPQFSYSASGTA (amino acids 485–501 of c-Akt); 14-3-3, MDDREDLVYQAKLAEQAERYDEMVESMKKVAGMDV (amino acids 1–35 of 14-3-3 protein); p50αN$^{1-10}$, MHNLQTLPPK (amino acids 1–10 of p50α). These peptides were covalently coupled to Affi-gel 10 beads (Bio-Rad) as described previously [21], and 50 μl samples of the beads were incubated with 1 ml of CHO cell lysate for 30 min at 4 °C. After thorough washing in ice-cold TNE buffer, the precipitated proteins were separated by SDS/PAGE and subjected to immunoblotting using antibodies against each tubulin isoform. Furthermore, we examined whether addition of various concentrations of the synthetic peptide p55αN$^{2-34}$ can inhibit the binding of GST fusion proteins to tubulin, in order to investigate whether the N-terminal region and the region consisting of two SH2 domains plus the inter-SH2 domain bind to the same or different portions of tubulin.

Overexpression of β-tubulin and regulatory subunits in Sf-9 insect cells

cDNAs encoding the full-length amino acid sequences of p85α, p85β, p55γ, p55α and p50α plus the HA (haemagglutinin)-tag amino acid sequence (YPYDVPDYA) at the C-terminus, and the full-length sequence of β-tubulin, were each subcloned into pBacPAK9, and baculoviruses were prepared according to the manufacturer's instructions (Clontech). The cDNA coding for β-tubulin was donated by Dr S. Lewis (New York University Medical Center, New York, NY, U.S.A.). The Sf-9 cells, infected with baculoviruses expressing control lacZ, β-tubulin alone or both β-tubulin and one of the five regulatory subunit isoforms, were cultured for 48 h and then lysed in ice-cold lysis buffer A. The lysates were cleared by centrifugation at 14000 ***g*** for 10 min. Immunoprecipitation was performed using the anti-HA-tag antibody, covalently coupled to Protein A–Sepharose beads. After thorough washing of the beads in TNE buffer, the precipitated proteins were subjected to SDS/PAGE and immunoblotting was performed using an antibody against β-tubulin.

Transient expression of regulatory subunits of PI 3-kinase with an adenovirus expression system, and PI 3-kinase assay

The cDNAs encoding the full-length amino acid sequences of p85α, p85β, p55γ, p55α and p50α, as well as the HA-tagged common C-terminal amino acid sequence (YPYDVPDYA), were ligated into the *Swa*I sites of pAdex1wt. Recombinant adenoviruses were obtained as previously described [22]. CHO/IR cells were infected with these viruses for 1 h, then grown for 48 h.

Prior to insulin treatment, the medium was replaced with Ham's F-12 medium containing 0.2% (w/v) BSA, and the cells were incubated for 18 h. Then 1 μM insulin was added to the medium and the cells were incubated for 10 min at 37 °C. After insulin treatment, the medium was removed and the cells were collected into ice-cold lysis buffer A. The expression of each protein was confirmed by immunoblotting with anti-HA antibody. Cell lysates corresponding to each 35-mm plate (10^6 cells) were immunoprecipitated using antibodies against the β or γ isoforms of tubulin, and were assayed for PI 3-kinase activity as described previously [10]. Radioactivity was measured using a Molecular Imager instrument.

RESULTS

Purification of a protein that binds to the N-terminal domain of p55α and p55γ

To identify the protein that bound to the N-terminal 34 amino acids of p55γ, the three immobilized GST fusion proteins (GST alone, GST–p85αSH3 and GST–p55γN$^{1-34}$) were incubated in the presence or absence of cell extracts of rat brain. Figure 1(A) shows the result of Coomassie Blue staining of the gel to which multiple proteins released from GST fusion proteins were applied. While GST alone did not bind any particular protein, GST–p85αSH3 and GST–p55γN$^{1-34}$ bound specifically to a 100 kDa (Figure 1A, lane 4) and a 55 kDa (lane 6) protein respectively. The 100 kDa protein in lane 4 was considered to be dynamin, since Gout et al. [19] reported previously that the αSH3 domain of p85α binds to dynamin with a molecular size of approx. 100 kDa on SDS/PAGE. To identify the 55 kDa protein detected in lane 6, it was transferred on to a PVDF membrane and subjected to reverse-phase HPLC. The N-terminal protein sequence of the 55 kDa protein is presented in Figure 1(B). A total of 21 amino acids from the N-terminus were identified definitively and revealed to be identical with the equivalent sequence of rat β-tubulin. These data suggest that the unique N-terminal domain of p55γ binds to β-tubulin *in vitro*.

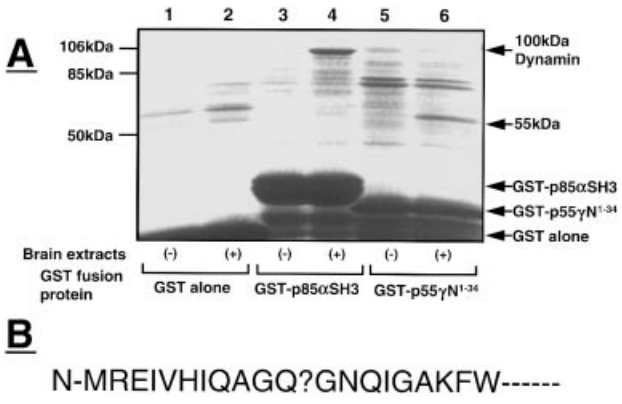

Figure 1 Identification of a protein that binds to the N-terminal domain of p55 subunits

(**A**) Proteins from rat brain extracts bind to recombinant GST proteins. Portions of 10 μg of three GST fusion proteins (GST alone, lanes 1 and 2; GST–p85αSH3, lanes 3 and 4; GST–p55γN$^{1-34}$, lanes 5 and 6) were incubated in the presence (lanes 2, 4 and 6) or absence (lanes 1, 3 and 5) of 5 ml of a 1% Triton X-100 extract of rat brain (1 mg/ml) for 1 h at 4 °C. After thorough washing, proteins were released by boiling in Laemmli buffer, and subjected to SDS/PAGE. The gel was visualized by Coomassie Blue staining. (**B**) N-terminal protein sequence of 55 kDa protein. The 55 kDa protein that bound specifically to GST–p55γN$^{1-34}$ was purified and applied to a protein sequencer. A total of 21 residues from the N-terminus were determined, and revealed to be identical to the equivalent sequence of rat β-tubulin.

Isoforms of tubulin that bind to the N-terminal domain and to the inter-SH2 domain of p55α and p55γ

The *in vitro* interactions between the various tubulin isoforms and the inter-SH2 region or SH2 domain of p85α were reported previously [23]. Our results mentioned above and the previous report [23] indicated that the 55 kDa regulatory subunits contain two tubulin-binding domains, one in the N-terminus and the other in the inter-SH2 and SH2 region. To compare the binding capability of the N-terminal domain for each tubulin isoform with that of the inter-SH2 and SH2 region, several GST fusion proteins were prepared and incubated with extracts of quiescent CHO cells. To investigate the interaction of these GST fusion proteins with each of the three isoforms of tubulin, the proteins associated with the GST fusion proteins were subjected to immunoblotting using antibodies specific to each isoform (Figure 2, upper panels). The amounts of the various GST fusion proteins used for precipitation were very similar (results not shown), and the ratios of the amount of tubulin bound in relation to the amount of the immobilized GST fusion protein were calculated (Figure 2, lower panels). The results of Western blotting of 5 μg of rat brain extract, as a positive control (lane 9), are also presented. Based on a previous observation [20] that 5 μg of brain cytosolic protein contains about 0.5 μg of β-tubulin, we estimated the relative binding affinities between GST fusion proteins and tubulin. GST alone, GST–p85αSH3 and GST–p85βSH3 did not interact with any of the tubulin isoforms (Figure 2, lanes 1–3). GST fusion proteins containing the N-terminal 34 amino acids of p55α or p55γ (GST–p55αN$^{1-34}$ and GST–p55γN$^{1-34}$ respectively) associated efficiently with the α and β isoforms of tubulin, while those containing two SH2 domains and the inter-SH2 domain of the regulatory subunits (GST–$\alpha/\beta/\gamma$SH2) bound smaller amounts of the α and β isoforms (Figures 2A and 2B, lanes 4–8). In contrast, with the γ isoform of tubulin, GST–p55αN$^{1-34}$ and GST–p55γN$^{1-34}$ exhibited a binding capacity similar to that of GST–$\alpha/\beta/\gamma$SH2 proteins (Figure 2C, lanes 4–8). These results suggest that p55α and p55γ have the two tubulin-binding sites, and also that the N-terminal 34-amino-acid domain shows greater binding affinity *in vitro* for the α and β isoforms of tubulin than does the region consisting of two SH2 domains and an inter-SH2 domain.

We also investigated the *in vitro* binding between the GST fusion proteins GST–p85α and GST–p55γ (i.e. containing the entire regulatory subunit proteins) and tubulin (Figures 2D and 2E, lanes 1–4). In the case of the β isoform of tubulin in particularly, significant binding to GST–p55γ was observed. This suggests that the whole p55γ molecule exhibits high-binding affinity to tubulin.

Synthetic peptides based on the N-terminal 34 amino acids of p55α and p55γ bind to tubulin

To investigate which region of the N-terminal 34 amino acids of p55α and p55γ is essential for the association with tubulin, we prepared four peptides as described in the Experimental section, and examined which peptides were capable of binding to tubulin. None of the irrelevant peptides bound to any isoform of tubulin (Figures 3B–3D, lanes 4–7 and 10), whereas the peptides p55αN$^{2-34}$ and p55γN$^{2-34}$ were shown to bind to the α, β and γ isoforms (Figures 3B–3D, lanes 8 and 9), a result similar to that observed in the experiments using GST fusion proteins (Figure 2). Figure 3(A) shows a comparison of the two N-terminal regions of 34 amino acids for p55α and p55γ. This domain has

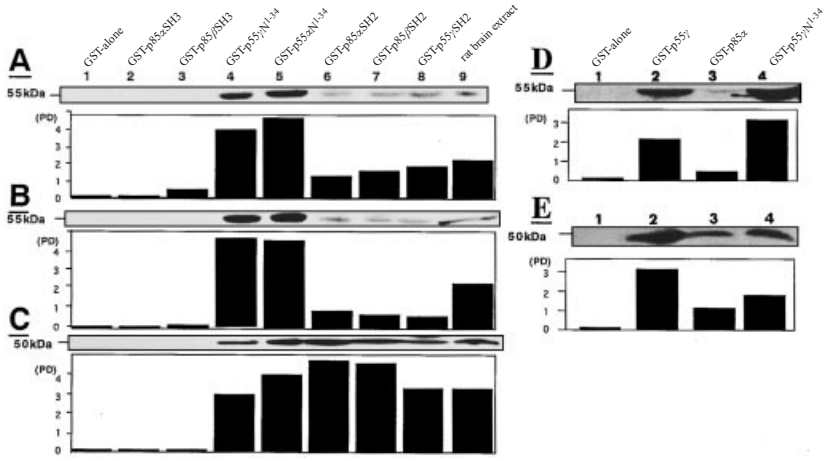

Figure 2 Interactions between recombinant GST fusion proteins and $\alpha/\beta/\gamma$-tubulin

Portions of 10 μg of GST fusion proteins containing different domains of the regulatory subunit of PI 3-kinase (50 μl of GST beads) were incubated for 30 min at 4 °C with 1 ml of CHO/IR cell lysates. The beads were washed, and the precipitated proteins were subjected to SDS/PAGE and transferred to nitrocellulose. The upper panels show the immunoblots, which were obtained using antibodies against α-tubulin (**A**), β-tubulin (**B**, **D**) and γ-tubulin (**C**, **E**). The lower panels show the ratios of the amount of bound tubulin to the amount of immobilized GST fusion protein. (**A**)–(**C**) Lanes: 1, GST alone; 2, GST–p85αSH3; 3, GST–p85βSH3; 4, GST–p55γN$^{1-34}$; 5, GST–p55αN$^{1-34}$; 6, GST–p85αSH2; 7, GST–p85βSH2; 8, GST–p55γSH2; 9, 5 μl of rat brain extract (1 mg/ml). (**D**), (**E**) Lanes: 1, GST alone; 2, GST–p55γ; 3, GST–p85α; 4, GST–p55γN$^{1-34}$. PD, pixel density.

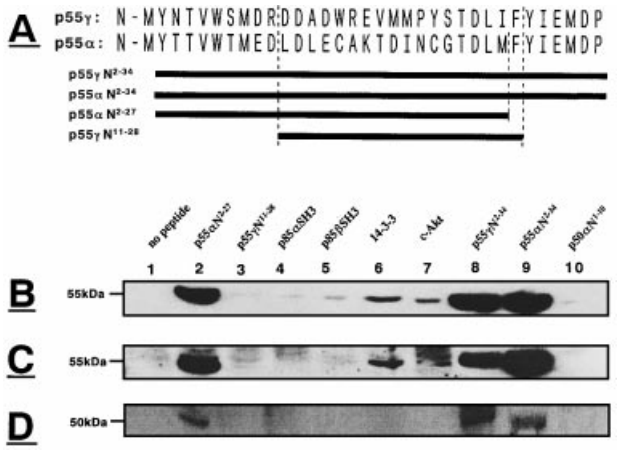

Figure 3 Association between the $\alpha/\beta/\gamma$ isoforms of tubulin and synthetic peptides based on the N-terminal region of p55

(**A**) Alignment of the N-terminal 34 amino acids of the p55γ and p55α regulatory subunits of PI 3-kinase, and sequences of the synthetic peptides used in this study. The two regions that share more than 50% identity are amino acids 1–9 and 24–34. The sequences of the four synthetic peptides p55γN$^{2-34}$, p55αN$^{2-34}$, p55γN$^{11-28}$ and p55αN$^{2-27}$ are indicated. (**B**)–(**D**) Interactions between synthetic peptides and $\alpha/\beta/\gamma$-tubulin. Synthetic peptides were covalently coupled to Affi-gel 10 beads, and 50 μl of the beads were incubated in 1 ml of CHO cell lysate for 30 min at 4 °C. After thorough washing in ice-cold TNE buffer, the precipitated proteins were analysed by SDS/PAGE and immunoblotting, which was performed using antibodies against α-tubulin (**B**), β-tubulin (**C**) and γ-tubulin (**D**). Lanes: 1, beads only; 2, p55αN$^{2-27}$; 3, p55γN$^{11-28}$; 4, p85αSH3; 5, p85βSH3; 6, 14-3-3 protein; 7, c-Akt; 8, p55γN$^{2-34}$; 9, p55αN$^{2-34}$; 10, p50αN$^{1-10}$.

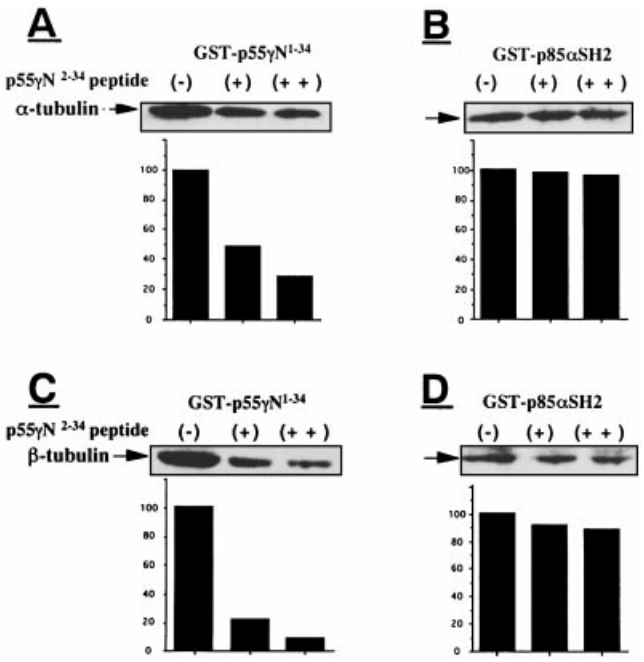

Figure 4 Effects of p55γN$^{2-34}$ peptide on the association of GST–p55γN$^{1-34}$ and GST–p85αSH2 with α/β-tubulin

Portions of 10 μg of immobilized GST–p55γN$^{1-34}$ (**A** and **C**) or GST–p85αSH2 (**B** and **D**) fusion proteins were incubated with 1 ml of CHO cell lysate for 30 min at 4 °C in the absence (−) or presence of 40 μg/ml (+) or 200 μg/ml (+ +) p55γN$^{2-34}$ peptide. After extensive washing, the precipitated proteins were subjected to SDS/PAGE and transferred to nitrocellulose. Immunoblotting was performed using antibodies against α-tubulin (**A** and **B**) or β-tubulin (**C** and **D**).

two regions that are highly conserved between p55α and p55γ, one at the N-terminal end (MYN/TTVWS/TMD/E; amino acids 1–9 of p55α/γ) and the other at the C-terminal end (TDLI/MFYIEMDP; amino acids 24–34). To determine which region of the N-terminal 34-amino-acid domain is critical for binding to tubulin, two short peptides were examined. p55αN$^{2-27}$, a synthetic peptide which lacks the C-terminal seven amino acids, was shown to retain tubulin binding ability (Figures

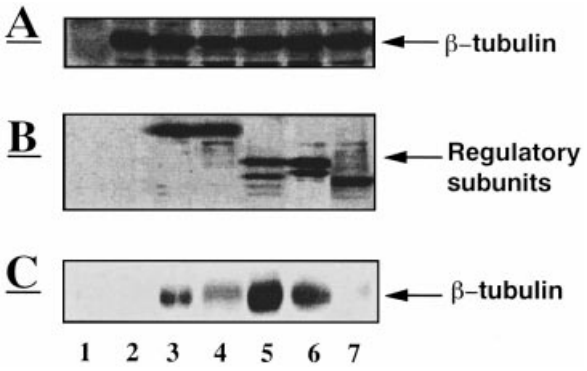

Figure 5 Interactions between recombinant β-tubulin and regulatory subunits in Sf-9 insect cells

The Sf-9 cells were infected with baculovirus expressing β-tubulin and a baculovirus expressing lacZ, p85α, p85β, p55γ, p55α or p50α. Cells were then cultured for 48 h and lysed in ice-cold lysis buffer A. The cell lysates corresponding to each 35-mm plate (10^6 cells) were subjected to SDS/PAGE, and immunoblotting was performed using the antibody against β-tubulin (**A**). Immunoprecipitation was performed using anti-HA-tag antibody, covalently coupled to Protein A–Sepharose beads. After thorough washing, the samples were subjected to SDS/PAGE, and immunoblotting was performed using anti-HA-tag antibody (**B**) or antibody against β-tubulin (**C**). Lysates were derived from control Sf-9 cells (lane 1), or from Sf-9 cells expressing β-tubulin plus lacZ (lane 2), p85α (lane 3), p85β (lane 4), p55γ (lane 5), p55α (lane 6) or p50α (lane 7).

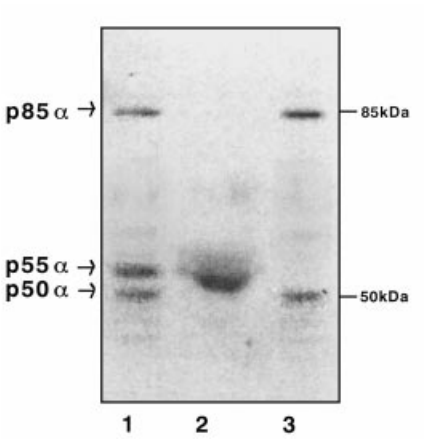

Figure 6 Co-precipitation of endogenous regulatory subunits with brain microtubule assembly

Brain tissue was homogenized with a Teflon/glass homogenizer, and the post-microtubule supernatant and microtubule pellet were prepared as described in the Experimental section. These fractions were subjected to SDS/PAGE, and immunoblotting was performed using an antibody against the whole p85α molecule. Lane 1, brain cell lysate; lane 2, microtubule pellet; lane 3, post-microtubule supernatant.

3B–3D, lane 2). In contrast, another synthetic peptide, p55γN$^{11–28}$, which lacks not only the C-terminal six amino acids but also the N-terminal 10 amino acids, lacked the ability to bind tubulin (Figures 3B–3D, lane 3). These results suggest that the N-terminal 10 amino acids of p55 are essential for tubulin binding.

Inhibition by synthetic peptides of binding of GST fusion proteins to tubulin

To investigate whether the N-terminal portion of p55 and the portion consisting of two SH2 domains and an inter-SH2 domain bind to the same region of tubulin, we examined whether the synthetic peptide p55γN$^{2–34}$ inhibited the binding of each GST–p55α/γN$^{1–34}$ protein or GST–SH2 protein to tubulin. The association between α/β-tubulin and GST–p55γN$^{1–34}$ proteins was inhibited on addition of the p55γN$^{2–34}$ peptide, in a concentration-dependent manner (Figures 4A and 4C), since GST–p55γN$^{1–34}$ and p55γN$^{2–34}$ contain the same tubulin-binding sequence. In contrast, the p55γN$^{2–34}$ peptide had no effect on the binding between α/β-tubulin and GST–p85αSH2 fusion proteins (Figures 4B and 4D). These results suggest that the N-terminal domain and that consisting of two SH2 domains and an inter-SH2 domain bind to different regions of the α and β isoforms of tubulin *in vitro*. Similar results were obtained for binding to γ-tubulin (results not shown).

Association of each of the five regulatory subunits with β-tubulin *in vivo* in Sf-9 cells

To investigate the binding of each of the five regulatory subunits to tubulin *in vivo*, we first utilized a baculovirus expression system. Baculoviruses expressing each of the five regulatory subunits and β-tubulin were prepared. Without the co-expression of regulatory subunits, expressed tubulin was not detected in the precipitates obtained with the antibody against the HA-tag (Figure 5, lane 2). On co-expression of any of the regulatory subunit isoforms, a significant amount of β-tubulin was immunoprecipitated by the HA-tag antibody (which recognizes the C-terminal end of the expressed regulatory subunits); however, a larger amount of β-tubulin was observed in cells expressing p55α or p55γ (Figure 5, lanes 5 and 6) than in those expressing p85α, p85β or p50α (Figure 5, lanes 3, 4 and 7). These results suggest that any regulatory subunit of PI 3-kinase can bind a small amount of β-tubulin via the SH2 and inter-SH2 domains, but also that p55α and p55γ bind a larger amount of β-tubulin via their unique N-terminal portion.

p55, but not p85 or p50, regulatory subunits are present in the purified microtubule assembly from rat brain

To further demonstrate the association of endogenous p55 regulatory subunits with microtubules, we prepared a precipitated purified microtubule assembly. The immunoblot of rat brain cytosolic extracts with αp85PAN-UBI (which contains antisera raised against the entire p85α sequence and the N-teminal SH2 region of p85α), revealed bands of 85 kDa, 55 kDa and 50 kDa, corresponding to p85α, p55α and p50α respectively (Figure 6, lane 1), as previously described [17]. Interestingly, p55α was detected only in the microtubule pellet (lane 2), i.e. there was none in the post-microtubule supernatant (lane 3). In contrast, p85α and p50α were detected only in the post-microtubule supernatant (lane 3). These results strongly suggest endogenous association of the p55α regulatory subunit with microtubules in the brain.

Tubulin-associated PI 3-kinase activity mediated by the five regulatory subunits

To investigate what role the N-terminal portion of p55α and p55γ, which was demonstrated to be responsible for the binding to tubulin, plays in the regulation of PI 3-kinase activity associated with tubulin, each of the five regulatory subunit isoforms was expressed in CHO/IR cells using an adenovirus transfection system. The expression levels of the five regulatory subunits were very similar, as described previously [17]. In cells overexpressing control lacZ, p85α, p85β or p50α, no significant

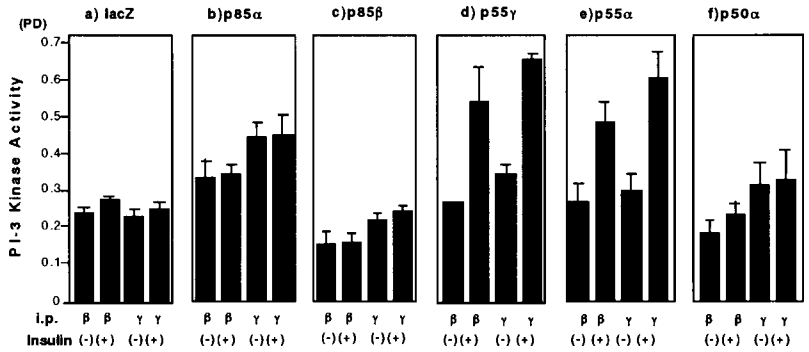

Figure 7 Tubulin-associated PI 3-kinase activation in response to insulin in CHO-IR cells overexpressing regulatory subunits

CHO/IR cells were infected with adenoviruses expressing (**a**) lacZ, (**b**) p85α, (**c**) p85β, (**d**) p55γ, (**e**) p55α or (**f**) p50α for 1 h, then grown for 48 h. Prior to insulin treatment, the medium was replaced with Ham's F-12 medium containing 0.2% (w/v) BSA, and cells were incubated for 18 h. Then the cells were incubated for 10 min at 37 °C in the presence or absence of 1 μM insulin. After insulin treatment, the medium was removed and the cells were collected in ice-cold lysis buffer A. The cell lysates corresponding to each 35-mm plate (10^6 cells) were immunoprecipitated (i.p.) using an antibody against either β- or γ-tubulin, and assayed for PI 3-kinase activity. PD, pixel density.

alteration of PI 3-kinase activity associated with the β or γ isoforms of tubulin was observed in response to insulin stimulation; however, in those overexpressing p55α or p55γ there was an apparent elevation of tubulin-associated PI 3-kinase activity (Figure 7). These results suggest that the p55 regulatory subunit proteins play a particularly important role in elevating PI 3-kinase activity associated with tubulin in response to insulin stimulation.

DISCUSSION

The primary role of the regulatory subunits of PI 3-kinase is to recognize an upstream signal of tyrosine phosphorylation and to then transfer this signal to the p110 catalytic subunits, which generate phospholipid products as a downstream signal [24]. To date, three groups of regulatory subunits have been identified in terms of their structure: proteins of 85 kDa, 55 kDa and 50 kDa [14–18]. It would be of great interest to elucidate the different roles of each of these regulatory subunits in the various signal transduction pathways. The well studied isoforms p85α and p85β have SH3 and Bcr homology domains in the N-terminal portion of the molecule, and their roles have been reported [19]. In contrast, p55α and p55γ contain a short N-terminal portion consisting of a 34-amino-acid sequence [14–16]. Since the shortest isoform, p50α, has only six amino acids in its N-terminus [17,18], it is natural to speculate that the 34-amino-acid portions in the N-termini of p55α and p55γ have particular functions. Thus we attempted to identify the protein that binds to this portion, and found that this N-terminal region binds to tubulin. The reason why only β-tubulin (but not α- or γ-tubulin) was identified from a protein which binds to GST–p55N$^{1-34}$ is unclear. We speculate that the binding affinity of α-tubulin for p55N$^{1-34}$ is much less than that of β-tubulin and that the expression level of γ-tubulin is much lower than that of β-tubulin in the brain, which could explain why β-tubulin was the predominant isoform precipitated by GST–p55N$^{1-34}$.

Tubulin exists as a heterodimer consisting of two tightly linked α and β isoforms, and both subunits polymerize to form microtubules that provide a cytoskeletal network. In contrast, γ-tubulin is a specialized minor form of tubulin and is one of the centrosome-specific proteins, and is also thought to interact with the heterodimer of α/β-tubulin to help nucleate microtubules [25]. Thus the differences in tubulin binding ability could be a mechanism involved in the distinct subcellular distribution of the PI 3-kinase catalytic subunit, p110, which binds to the different regulatory subunit isoforms.

A number of previous studies have examined the chemicals and molecules that bind directly to tubulin [26–30]. Kapeller et al. [23,26] showed that p85α binds to tubulin via its inter-SH2 domain, and that p85α is located near microtubule bundles and moves to the perinuclear region when cells are stimulated by platelet-derived growth factor (PDGF). Their work suggested that PDGF-receptor–PI 3-kinase complexes internalize and transit in association with the microtubule cytoskeleton. More recently, Itoh et al. [27] demonstrated that the SH2 domain of p85α regulatory subunits binds to β-tubulin via a region different from the tyrosine-phosphorylated protein-recognition site. Although there seems to be some disagreement between these two reports regarding the domain of p85α that binds tubulin, the GST fusion protein containing two SH2 domains and an inter-SH2 domain (GST–p85αSH2) used in the present study showed *in vitro* association with tubulin. We have shown that GST–p55α/γN$^{1-34}$ proteins precipitated much more α/β tubulin than did the GST–SH2 proteins, suggesting that the N-terminal 34-amino-acid domain binds tubulin more tightly than the SH2 and inter-SH2 regions. In addition, based on the observation that p55γN$^{2-34}$ peptide did not inhibit the binding of GST–p85αSH2 fusion protein to β-tubulin, the site of β-tubulin that binds to the N-terminal domain of p55α and p55γ is considered to be different from that which binds to the portion consisting of two SH2 domains and an inter-SH2 domain.

We also demonstrated that p55α and p55γ bound much more β-tubulin *in vivo* in Sf-9 cells than did p50α, p85α or p85β. In the experiments using Sf-9 cells, the expression levels were very high, such that an association of p85α and p50α with tubulin might have been observed despite the low affinity of binding between tubulin and these subunits. We speculate that the p55 subunits associate with tubulin efficiently, while the p85 and p50 regulatory subunits also bind to microtubules, but with much lower affinity.

To demonstrate an association of endogenous p55 regulatory subunits with microtubules, we first attempted to show the co-localization of p55α with tubulin by employing immunostaining. However, since the epitope of the specific anti-p55α antibody is the unique N-terminal portion of p55α, which is involved in the association with tubulin, it appeared that p55α bound to tubulin was not detectable by immunostaining with anti-p55α antibody

(results not shown). Thus we prepared the precipitated purified microtubule assembly, in an effort to demonstrate that it contains the p55 regulatory subunit. We found that only p55α (i.e. not p85α or p50α) was detectable in the purified microtubule assembly precipitated from rat brain. These results suggest that the unique N-terminal domain of the 55 kDa regulatory subunit is indeed important for the association with tubulin *in vivo*.

To investigate the effects of the various N-terminal structures of the regulatory subunits of PI 3-kinase, we transiently expressed each regulatory subunit isoform in CHO/IR cells using an adenovirus expression system, and investigated the regulation of PI 3-kinase activity associated with tubulin in response to insulin stimulation. In CHO/IR cells overexpressing the p85α regulatory subunit, we did not observe an elevation of PI 3-kinase activity in response to insulin in the precipitates associated with β- or γ-tubulin. A previous study [23] showed that PI 3-kinase activity is recruited to γ-tubulin in response to insulin in CHO/IR cells. We cannot explain this apparent contradiction, although the results do raise the possibility that the different antibodies against γ-tubulin used in the two studies may recognize different portions of tubulin and thus account for the difference. In our study, an elevation in tubulin-associated PI 3-kinase activity in response to insulin was observed specifically in cells expressing p55α or p55γ. This series of results suggests an important role for the unique N-terminal portion of p55α and p55γ in the regulation of PI 3-kinase activity associated with tubulin. Therefore p55α and p55γ are likely to play a distinct role, not only in carrying PI 3-kinase activity to the centrosome in the perinuclear area, but also in regulating PI 3-kinase activity associated with the microtubule cytoskeleton in the cell periphery.

Activation of PI 3-kinase has been reported to play an essential role in the regulation of numerous cellular activities, including proliferation [7,8], differentiation [9], membrane ruffling [10], prevention of apoptosis [11], and insulin-stimulated glucose transport [12,13]. Although the molecules downstream of the lipid product produced by PI 3-kinase have been revealed (i.e. c-Akt, Rac, several isoforms of protein kinase C and p70 S6 kinase [31–35]), why this lipid kinase induces so many different functions remains unclear. We speculate that the site where PI 3-kinase activation occurs in cells is extremely important for induction of its individual functions. One report [36] has suggested the importance of the localization of PI 3-kinase activation, showing that the translocation of PI 3-kinase activity to the intracellular vesicle fraction is necessary for translocation of the glucose transporter to the cell surface, which appears to be mediated by association with insulin receptor substrate-1 and possibly -2. That report clarified why insulin, but not epidermal growth factor or PDGF, can induce GLUT4 translocation efficiently, despite the fact that insulin, epidermal growth factor and PDGF activate PI 3-kinase activity to a similar extent. Thus not only the total PI 3-kinase activity of cells but also the localization of the PI 3-kinase protein need to be investigated in order to fully understand the mechanisms by which PI 3-kinase induces cellular activities. Although we have no data concerning the function of tubulin-associated PI 3-kinase, it is conceivable that it may have effects on microtubule formation and/or stability, or may be involved in regulating the budding or fusion of vesicles localized on microtubules. Alternatively, γ-tubulin-associated PI 3-kinase may have a specific function in the centrosome. If this is the case, the p55α and p55γ regulatory subunits may have crucial roles. The importance of the subcellular localization of lipid kinases, including PI 3-kinase, is just beginning to be understood, and further studies are needed to address this issue.

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Received 26 July 1999/26 November 1999; accepted 4 January 2000