Binding of a Diphosphotyrosine-containing Peptide That Mimics Activated Platelet-derived Growth Factor Receptor β Induces Oligomerization of Phosphatidylinositol 3-Kinase*

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Phosphatidylinositol 3-kinase (PI3K) is a heterodimeric enzyme comprising a p110 catalytic subunit and a p85 regulatory subunit. We have recently shown that the isolated p85 subunit exists as a dimer; therefore, we examined whether the heterodimeric enzyme was capable of further self-association. Size-exclusion chromatography demonstrated that PI3K was a 1:1 complex of p85 and p110 under native conditions. However, binding of a diphosphotyrosine-containing peptide that mimics an activated platelet-derived growth factor receptor β induced an increase in the apparent molecular mass of PI3K. This increase was due to dimerization of PI3K and was dependent on PI3K concentration but not diphosphopeptide concentration. Dimer formation was also observed directly using fluorescence resonance energy transfer. Diphosphopeptide-induced activation of PI3K (Carpenter, C. L., Auger, K. R., Chanudhuri, M., Yoakim, M., Schaffhausen, B., Shoeolson, S., and Cantley, L. C. (1993) J. Biol. Chem. 268, 9478–9483; Rordorf-Nikolic, T., Van Horn, D. J., Chen, D., White, M. F., and Backer, J. M. (1995) J. Biol. Chem. 270, 3662–3666) was not a direct result of dimerization and occurred only when phosphatidylinositol, and not phosphatidylinositol-4,5-diphosphate, was the phosphorylation substrate. Binding of the tandem SH2 domains of the p85 regulatory subunit to activated receptor tyrosine kinases therefore induces dimerization of PI3K, which may be an early step in inositol lipid-mediated signal transduction.

Propagation of extracellular signals to the nucleus is mediated by a wide range of second messengers, which employ a number of common signaling mechanisms, such as dimerization or oligomerization, phosphorylation of tyrosine, serine, or threonine residues, the binding of SH21 or phosphotyrosine binding domains to phosphotyrosine-containing sequences, or binding of SH3 domains to proline-rich sequence motifs.

Phosphatidylinositol 3-kinase (PI3K) is a dual-specificity kinase that has been implicated in a wide range of cellular events, including proliferation and cell migration (1). In response to a number of cell stimuli, this two-subunit enzyme phosphorylates the 3′ position of inositol lipids to produce the second messengers phosphatidylinositol 3,4-diphosphate (PI-3,4-P₂) and phosphatidylinositol 3,4,5-triphosphate (PI-3,4,5-P₃), which have been shown to bind to and activate plekstrin homology domain-containing proteins such as protein kinase B (AKT) and PDK1 (2). PI3K is also a serine/threonine kinase that can autophosphorylate either subunit (3, 4) and has been reported to phosphorylate the intracellular docking protein, IRS-1 (5).

A number of PI3K isoforms are known, which can be classified on the basis of their subunit composition and sequence homology. The class IA PI3Ks are heterodimers of a p110 catalytic subunit, of which there are three isoforms, p110α, p110β, and p110δ (6), and a p85 adapter or regulatory subunit, of which there are five isoforms, p85α, p85β, p55γ, p55α, and p50α (7). The 85-kDa isoforms, p85α and p85β (8), contain several homology domains, including two SH2 domains, a BCR homology domain and SH3 domain, whereas the lower molecular mass isoforms, p55γ, p55α, and p50α, lack the BH and SH3 domains. The catalytic subunit, p110, also contains several homology domains, including the kinase domain, a PIK domain whose sequence is conserved in PI3-kinases and PI4-kinases, as well as putative N-terminal p85-binding and Ras binding domains (6).

A number of regulatory mechanisms for PI3K have been described, but no single event has been demonstrated to result in full activation of this enzyme. It is likely that multiple events are required to transduce a signal via this lipid/protein kinase. Binding of the tandem SH2 domains in the regulatory subunit to specific phosphotyrosine-containing motifs present on a range of molecules, such as receptor tyrosine kinases (RTKs) and cytoplasmic signaling proteins, including IRS-1, has been

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1 The abbreviations used are: SH, src homology; BH, BCR homology; FCS, fluorescence correlation spectroscopy; FRET, fluorescence resonance energy transfer; HP-SEC, high performance size-exclusion chromatography; IR, insulin receptor; IRS, insulin receptor substrate; MALDI, matrix-assisted laser desorption and ionization; FDOGβ-R, platelet-derived growth factor receptor β; PI, phosphatidylinositol; PI3K, phosphatidylinositol 3-kinase; pY, phosphotyrosine; RTK, receptor tyrosine kinase; 2-ME, 2-mercaptoethanol; Bicine, N,N-bis(2-hydroxyethyl)glycine.
reported to increase the intrinsic activity of PI3K (9–11) and trigger a conformational change in the regulatory subunit (12). Binding of a range of other intracellular ligands, such as SH3 domains and small GTPases, has also been reported to activate PI3K (13–15). Alternatively, binding of these ligands may result in activation simply by translocating PI3K to the plasma membrane, where its lipid substrates are located (16). For example, activation of PI3K by binding of Ha-Ras\(^\text{V12} \) has been most convincingly demonstrated when Ras was immobilized in phosphatidylinositol-containing lipid vesicles (17), suggesting that the role of Ras may be to recruit PI3K to its membrane substrate, in a mechanism analogous to that of c-Raf activation by Ras (18).

Another common theme in activation of intracellular signaling proteins is dimerization or oligomerization, which can be induced by ligand binding and, in the case of RTKs, lead to auto- or trans-phosphorylation of the RTK and up-regulation of catalytic activity. Several intracellular protein kinases, such as c-Raf, have also been shown to be activated by dimerization (19, 20). We have recently demonstrated that the 85-kDa subunits of PI3K exist as dimers in the absence of the catalytic subunit, whereas the low molecular mass isoforms of the regulatory subunit are monomeric.\(^2\) In contrast, PI3K activity in unstimulated cells has been reported to have an apparent molecular mass of approximately 200 kDa (21, 22), suggesting the whole complex is dimeric or oligomeric. Two common themes in activation of intracellular signal transduction proteins is dimerization or oligomerization, which can be induced by ligand binding and, in the case of RTKs, lead to auto- or trans-phosphorylation of the RTK and up-regulation of catalytic activity. Several intracellular protein kinases, such as c-Raf, have also been shown to be activated by dimerization (19, 20). We have recently demonstrated that the 85-kDa subunit of PI3K exist as dimers in the absence of the catalytic subunit, whereas the low molecular mass isoforms of the regulatory subunit are monomeric.\(^2\) In contrast, PI3K activity in unstimulated cells has been reported to have an apparent molecular mass of approximately 200 kDa (21, 22), suggesting the whole complex is dimeric or oligomeric.

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| EXPERIMENTAL PROCEDURES |
|---------------------------|

**Materials**—Peptides were synthesized by Synthesyn Analytic and were of the following sequences: Tyr-740/Tyr-751, GGpYMDMDSKDESVDYVPML; Tyr-740, GGpYMDMDSK; Tyr-751, GGpYMDMD. Phosphatidylinositol-4-phosphate and phosphatidylinositol-4,5-biphosphate were from Calbiochem.

**Experimental Procedures**

**PI3 Kinase Assays**—PI3-kinase assays were carried out essentially as described previously (27). Lipid kinase assays contained 2 mM MgCl\(_2\), 1 mM ATP, 20 μM of [γ\(^32\)P]ATP, and 200 μM of either phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PI-4-P), or phosphatidylinositol 4,5-biphosphate (PI-4,5-P\(_2\)).

**Protein Kinase Assays**—Protein kinase assays were carried out in TBS containing 10 mM MnCl\(_2\), 1 mM ATP, and 20 μM of [γ\(^32\)P]ATP. Phosphorylated proteins were separated from free [γ\(^32\)P]ATP, by SDS-polyacrylamide gel electrophoresis, exposed to a phosphor screen (Molecular Dynamics), and analyzed using ImageQuant software (Molecular Dynamics).

**Peptide Binding to PI3K**—The procedure for measuring SH2 domain interactions with phosphopeptides using the BIAcore biosensor (Biacore AB) has been described previously (28). Briefly, a biotinylated Tyr-751 phosphopeptide was captured on immobilized avidin, and then PI3K was injected in running buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 4 mM dithiothreitol, 0.005% Tween 20). The response at equilibrium was recorded and compared with that obtained upon preincubation of the PI3K solution with increasing amounts of free phosphopeptide (Tyr-740, Tyr-751, or Tyr-740/Tyr-751). In order to estimate the IC\(_50\) values, the results were plotted as resonance units (RU) versus competitor concentration, and the curve obtained fitted with the equation \( R = R_{\text{max}} \frac{C}{C + K_C} \), where \( R \) is the response; \( R_{\text{max}} \) is the response obtained in the absence of competitor; and \( P \) is the Hill coefficient. The equilibrium dissociation constant (\( K_D \)) for the interaction of PI3K with the immobilized Tyr-751 was estimated by injecting increasing concentrations of PI3K, plotting the response at equilibrium versus the free concentration (C), and fitting the equation with \( R = R_{\text{max}} \frac{C}{C + K_D} \). In order to estimate the \( K_D \) value for the interaction with Tyr-740/Tyr-751, the formula \( K_D = K_D/IC_{50} \) was used.

**Fluorescent Labeling of PI3K**—PI3K was further purified and exchanged into Buffer C (20 mM Bicine, pH 8.2, 150 μM NaCl, 5 mM 2-ME) using NP-30 as described above. 5 μM PI3K in Buffer C was incubated with a 10--50-fold molar excess of the sulfoiodoaniline fluorescent dyes, Cy3 or Cy5, for 30–60 min at room temperature. Unreacted dye was inactivated by the addition of 100 mM Tris, pH 8.0, and labeled PI3K was separated from free dye and exchanged into TBS using pre-packed Sephadex G-25 columns (PD10, Amersham Pharmacia Biotech); then labeled PI3K was concentrated using Centricon-10 microconcentrators (Amicon). The number of Cy dye molecules per PI3K molecule was determined by measuring the ratio of the optical densities at 280 and 550 nm (Cy3) or 649 nm (Cy5) using a scanning spectrophotometer. The calculated extinction coefficient for PI3K-EF tag was 251,970 M\(^{-1}\) cm\(^{-1}\) whereas those specified by the manufacturer for Cy3 and Cy5 were 150,000 M\(^{-1}\) cm\(^{-1}\) and 250,000 M\(^{-1}\) cm\(^{-1}\), respectively.

**FRET Measurements**—Time-based emission and steady-state emission spectra were collected using a PPT QM1 Quantum Master T fluorimeter, equipped with a Power Arc 75 Xe arc lamp. The donor fluorophore (Cy5) was excited at 550 nm, and emitted fluorescence was recorded at 649 nm (Cy5) using a scanning spectrophotometer. The calculated extinction coefficient for PI3K-EF tag was 251,970 M\(^{-1}\) cm\(^{-1}\) whereas those specified by the manufacturer for Cy3 and Cy5 were 150,000 M\(^{-1}\) cm\(^{-1}\) and 250,000 M\(^{-1}\) cm\(^{-1}\), respectively.

| Fluorescence Correlation Spectroscopy (FCS) Measurements | The principles of FCS have been reviewed elsewhere (29). Experiments were... |

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performed with a Confocor instrument (Zeiss-Evotec), using a He/Ne laser and an excitation wavelength of 543 nm. Cy3-PI3K (1 nM) was mixed with unlabeled PI3K to a final concentration of 1 μM in buffer A. Measurements were obtained before and after addition of Tyr-740/Tyr-751 to a final concentration of 10 μM. FCS Access software was used to analyze the plots of the autocorrelation function G(t) versus time.

RESULTS

Purification of the Recombinant p110α-EE Tag-p85α Complex—The regulatory subunit of PI3K, p85α, expresses at up to 5-fold higher levels than p110α-EE tag in Sf9 cells. The inclusion of a C-terminal epitope tag in the p110α amino acid sequence allowed the purification of an enzyme complex with a 1:1 ratio of regulatory:catalytic subunits, as excess p85α did not bind to the anti-EE tag affinity matrix. The C-terminal tag did not appear to significantly alter PI3K, as the recombinant p110α-EE tag-p85α complex had the stability, specific activity, and substrate specificity expected for wild type PI3K (data not shown). In contrast, N-terminal tags have been shown to activate PI3K by enhancing the stability of the p110 subunit (30).

The 1:1 ratio of subunits was confirmed using Coomassie Blue-stained SDS-polyacrylamide gel electrophoresis, and MALDI mass spectrometry was used to confirm the molecular masses of each subunit. MALDI mass spectrometric analysis of tryptic peptides from PI3K failed to identify any post-translational modifications in these recombinant PI3K preparations. Although recombinant PI3K was greater than 95% pure after competitive elution from the affinity matrix using the EE tag peptide (data not shown), its solubility and stability were enhanced by a further purification step using anion-exchange chromatography.

Determination of the Oligomeric Status of Recombinant p110α-EE Tag-p85α by HP-SEC—As previously reported (21, 22, 31), recombinant PI3K made up of a complex of EE-tagged p110α (predicted molecular mass 125.3 kDa) and p85α (predicted molecular mass 83.5 kDa) had a retention time on HP-SEC that corresponded with a molecular mass of approximately 200 kDa (Fig. 1A). The peak of PI3K lipid kinase activity correlated with the protein peak as determined by absorbance at 220 or 280 nm (Fig. 1A). During an investigation of the effect of binding of a number of ligands for PI3K to this p110α-EE tag-p85α complex, it was observed that binding of a diphosphotyrosine-containing peptide to the amino acid sequence of the p85α-binding site on the PDGFB-R (Tyr-740/Tyr-751) altered the retention time of the p110α-EE tag-p85α complex, increasing its apparent molecular mass. Again, the PI3K activity profile correlated with the protein peak (Fig. 1A). In contrast, binding of peptides with the same amino acid sequence but only one phosphotyrosine residue (corresponding to either Tyr-740 or Tyr-751 of the PDGFB-R) did not result in the same increase in apparent molecular mass (Fig. 1B).

There are several possible causes of an apparent increase in molecular mass by HP-SEC, including protein oligomerization, changes in hydrophobicity, or conformational changes. Hydrophobic proteins can interact nonspecifically with the column matrix, retarding their elution and therefore decreasing their apparent molecular mass. In contrast, Tyr-740/Tyr-751 binding caused an increase in the apparent molecular mass of PI3K. Ionic interactions of hydrophilic or charged proteins with the column matrix are negligible at ionic strengths above 0.1 M (32).

An increase in apparent molecular mass could also be caused by a conformational change in PI3K that increased its hydrodynamic volume. The hydrodynamic volume of a protein is defined as the diameter of the spherical volume created by that protein as it rapidly tumbles in solution. Asymmetrical proteins will elute with an abnormally high apparent molecular mass compared with the globular proteins used to calibrate the column. If a change in conformation was induced by ligand binding, the equilibrium position of the reaction (K2; see Equation 1),

\[
P_{13K} + pep_{m} \rightleftharpoons P_{13K}^{*} - pep_{m} \text{ (Equation 1)}
\]

(where P13K* is the alternate conformation of P13K, k2 is the rate constant, pep is the diphosphotyrosine-containing peptide Tyr-740/Tyr-751, and the solid arrows represent the equilibrium reaction that would be detectable by HP-SEC) would be independent of the concentration of either of the two reactants. In the presence of a constant 10-fold molar excess of Tyr-740/Tyr-751, the apparent molecular mass of PI3K was dependent on PI3K concentration. When the concentration of PI3K applied to the column was less than 0.1 μM, the apparent molecular mass was approximately 200 kDa, equivalent to that of P13K in the absence of Tyr-740/Tyr-751. As the concentration of PI3K was increased, the apparent molecular mass correspondingly increased, asymptoting toward 400 kDa (Fig. 2A).

The concentration dependence of this phenomenon suggests it is unlikely to be due to a conformational change.

The predicted molecular mass of a P13K dimer is 418 kDa, which correlates with the observed maximal apparent molecular mass at P13K concentrations greater than 1 μM (Fig. 2A), suggesting that the increase in apparent molecular mass upon binding of Tyr-740/Tyr-751 is due to dimerization. Discrete monomer and dimer populations were not observed, but two populations would not be expected if the monomer-dimer equilibrium was in fast exchange compared with the retention time on the column. In the case of a fast equilibrium, one would...
FIG. 2. Determination of the mechanism of PI3K dimerization. A, apparent molecular mass of p110α-EE tag/p85α at a range of concentrations in the absence (○) or presence (●) of a constant 10-fold molar excess of Tyr-751/Tyr-740. Molecular masses were determined by HP-SEC and were calculated from the retention time (min) of the maximum peak height of p110α-EE tag/p85α. The retention time was converted to apparent molecular mass by comparison to a plot of log molecular mass (kDa) versus retention time (min) of the maximum height of the peak for a range of standard proteins (Bio-Rad) chromatographed using the same buffer conditions as in Fig. 1. B, inhibition of PI3K (100 nM) binding to immobilized Tyr-751 by increasing concentrations of Tyr-740 (△), Tyr-751 (○), and Tyr-740/Tyr-751 (●). C, apparent molecular mass of 2.4 μM p110α-EE tag/p85α at a range of concentrations of Tyr-751/Tyr-740. Molecular masses were determined as above.

Dimerization of Phosphatidylinositol 3-Kinase

expect to see a single population corresponding to a weighted average of amount of PI3K in monomeric or dimeric states, as was observed (Fig. 2A).

Because p85α has two SH2 domains and the Tyr-740/Tyr-751 has two phosphotyrosine residues, there are two ways in which dimerization of PI3K could occur. First, both SH2 domains in p85α could bind both phosphotyrosine residues in a single Tyr-740/Tyr-751 peptide, inducing a change that increases the affinity of PI3K for another peptide-bound PI3K molecule. Alternatively, a single Tyr-740/Tyr-751 peptide could bind SH2 domains from different PI3K molecules, generating a “cross-linked” dimer. If the latter model were correct, the affinity of dimerization would be the same as the affinity of Tyr-740/Tyr-751 for SH2 domains. The affinity for dimerization, as estimated by the concentration of PI3K required to produce a half-maximal increase in apparent molecular mass, is in the 10⁻⁷ M range (Fig. 2A). Determination of the IC₅₀ for competition of Tyr-740/Tyr-751 with immobilized Tyr-751 for binding to PI3K allowed an estimation of the affinity of the Tyr-740/Tyr-751 for the p85α SH2 domains. The IC₅₀ values for Tyr-740, Tyr-751, and Tyr-740/Tyr-751 were 75.6, 13.4, and 2.8 nM, respectively. Based on these values and a K₆ of 25.9 nM for the direct interaction of PI3K with the immobilized Tyr-751 (data not shown), the K₆ for the interaction of the p85α SH2 domains with Tyr-740/Tyr-751 was estimated at 5.4 nM, which is approximately 100-fold greater than the apparent affinity of dimerization (Fig. 2B). In addition, the affinity of Tyr-740/Tyr-751 for PI3K was greater than that of either Tyr-740 or Tyr-751 (Fig. 2B), suggesting that both phosphotyrosine residues in the Tyr-740/Tyr-751 bind to a single PI3K molecule.

If the dimer was cross-linked, the equilibrium position of the reaction (Kₑ; see Equation 2)  

$$2 \text{PI3K} + 1 \text{peptide} \rightleftharpoons K_1 \text{pep-PI3K}_2$$

and therefore the apparent molecular mass would be dependent on both the PI3K and Tyr-740/Tyr-751 concentrations. However, when increasing concentrations of Tyr-740/Tyr-751 were added to 2.4 μM PI3K, the apparent molecular mass remained constant (approximately 350 kDa) (Fig. 2C), suggesting that the Tyr-740/Tyr-751 does not participate directly in the observed equilibrium. The model that best fits the observed phenomenon is shown in Equation 3.

$$2 \text{PI3K} + 2 \text{peptide} \rightleftharpoons K_1 \text{pep-PI3K}_2 \rightleftharpoons K_2 \text{pep-PI3K}_3 \rightleftharpoons K_3 \text{pep-PI3K}_4$$

(Eq. 3)

In this model, it is necessary to invoke a conformational or other change as an intermediate step in the reaction scheme to explain why dimerization does not occur in the absence of Tyr-740/Tyr-751. One Tyr-740/Tyr-751 binds both SH2 domains in a single PI3K molecule (Kₑ), inducing a conformational or other change in the PI3K molecule (K₁), which exposes a binding site on PI3K that forms the dimer interface, resulting in the monomer-dimer equilibrium detected by HP-SEC (Kₑ).

Detection of Dimerization of PI3K by FRET and FCS—Fluorescence resonance energy transfer (FRET) is observed when the emission of fluorescence from one fluorophore, the donor, is of a suitable wavelength to excite a second fluorophore, the acceptor. The efficiency of FRET is dependent on the inverse sixth power of the distance between the donor and acceptor molecules; therefore, emission from the acceptor fluorophore is only observed when the two fluorophores are in close proximity (10–100 Å). Observance of FRET upon dimerization is not dependent on hydrodynamic volume, unlike HP-SEC, and therefore is a direct measurement of protein-protein interactions.

PI3K was covalently labeled with either a donor fluorophore, the sulfoindocyanine dye Cy3 (33), or an acceptor fluorophore, Cy5 (33). Between two and five dye molecules were incorporated per PI3K heterodimer. To ensure that labeling of PI3K did not disrupt its structure, Cy5-PI3K and Cy5-P3IK were assayed for their specific activity which was found to be inversely proportional to the molar ratios of dye:protein (data not shown). Therefore, Cy5-PI3K and Cy5-P3IK with labeling ratios of between 2:1 and 3:1 were used for the following FRET experiments.

Analysis of FRET by fluorimetry demonstrated that incubation of Cy3-PI3K with Cy5-PI3K in the presence of Tyr-740/Tyr-751 resulted in a transfer of fluorescence energy from the donor to the acceptor fluorophore, resulting in the measured decrease in donor emission (Fig. 3A). In contrast the addition of Tyr-751 or buffer alone did not result in an energy transfer...
from the donor to the acceptor (Fig. 3A), suggesting that there was a Tyr-740/Tyr-751-induced association of the two labeled PI3K populations.

Further evidence of the dimerization of PI3K was provided using FCS. FCS measures the translational diffusion time of fluorescent molecules in solution, which is proportional to the fourth power of the hydrodynamic volume and therefore increases when they bind to other proteins. The diffusion time of Cy3-PI3K in solution increased following the addition of Tyr-740/Tyr-751 (Fig. 3B), suggesting that there was an induced self-association of Cy3-PI3K.

Effect of Dimerization on PI3K Activity—Binding of both mono- and di-phosphotyrosine-containing peptides has been shown to stimulate PI3K activity approximately 3-fold when PI was the phosphorylation substrate (9, 11). However, half-maximal activation was achieved at lower concentrations of the diphosphopeptide compared with the monophosphopeptides (10). In order to determine whether activation is a direct consequence of Tyr-740/Tyr-751-induced dimerization, we analyzed the dependence of PI3K PI kinase activity on enzyme concentration. Enzyme activity is generally proportional to enzyme concentration, but if activation was a result of dimerization, a plot of enzyme concentration versus activity would have an upward curve (34). The plot of PI3K activity versus concentration for the p110α-EE tag-p85α complex was linear (Fig. 4A), suggesting that dimerization is not the mechanism behind Tyr-740/Tyr-751-induced activation.

Regardless of enzyme concentration, Tyr-740/Tyr-751 did not induce activation of PI3K when PI-4,5-P2 was used as the lipid kinase substrate or when the regulatory subunits were used as the substrates for protein serine/threonine kinase activity (Fig. 4, B and C). Clearly, activation of PI3K is not induced by dimerization, and indeed it is questionable whether the Tyr-740/Tyr-751-induced increase in phosphorylation of a non-physiological substrate, PI, would have a biological consequence.

DISCUSSION

One of the first events in RTK-mediated signal transduction is growth factor-induced receptor dimerization or oligomerization (35). Receptor dimerization triggers the intrinsic kinase activity, resulting in trans- and auto-phosphorylation of tyrosine residues and creation of specific binding sites for SH2 domain-containing proteins. SH2 domains bind phosphotyrosine-containing sequence motifs with a high degree of specificity (36, 37), and the consensus sequence motifs that bind a number of SH2 domains have been defined, including that of the p85 subunit of PI3K (37). Both SH2 domains in p85α bind a linear epitope with the sequence pYXXM (where X is any amino acid), and this sequence motif is present in a number of RTKs and intracellular docking proteins. Some signaling proteins, such as IRS-1 and Erb B3 (9, 38), have more than one sequence pYXXM motif, and the PDGFβ-R has two pYXXM motifs with seven amino acid residues between them (39).

The binding of a peptide containing both pYXXM motifs from the PDGFβ-R (Tyr-751/Tyr-740) (11) and Fig. 4A) or from IRS-1 (10) has been shown to activate the intrinsic lipid kinase activity of PI3K when PI was the phosphorylation substrate, whereas peptides containing only one p85-binding site bind

![Figure 3](http://www.jbc.org/)

**Fig. 3. Detection of PI3K dimerization by FRET and FCS.** A, following preincubation of Cy3-PI3K (0.2 μM) and Cy5-PI3K (0.2 μM), time-based recording of donor fluorescence was carried out, prior to and immediately following the addition of buffer or Tyr-740/Tyr-751 (---) or Tyr-751 (- - -) to the reaction (at the indicated times). Cy3-PI3K was excited at 550 nm and donor emission recorded at 554 nm. B, comparison of the correlation curves for Cy3-PI3K (10 nM) with 7.5 μM unlabeled p110α-EE tag/p85α obtained in the presence (---) or absence (- - -) of 200 ng/ml Tyr-740/Tyr-751. Measurements for fluorescence correlation spectroscopy were recorded as described elsewhere (29). Note the shift to slower diffusion times upon peptide addition.
with lower affinity and activate the enzyme to a lesser extent. A mechanism for PI3K activation has been postulated in which both SH2 domains in p85 are simultaneously occupied by both phosphorysine residues in a diphosphopeptide, which causes a conformational change within the inter-SH2 region of the p85 subunit and activates p110 (10). Binding of Tyr-740/Tyr-751 has been shown to induce a conformational change in the p85α regulatory subunit (12), which may be the same conformational change that is involved in the self-association of the PI3K heterodimer (Figs. 1–3). Binding of the two phosphorysine residues in the diphosphopeptide to SH2 domains in different PI3K molecules did not seem to be the mechanism by which dimerization occurs, as the formation of higher molecular mass complexes of PI3K was independent of diphosphopeptide concentration (Fig. 2C), suggesting that the diphosphopeptide does not participate directly in the dimerization event.

Binding of a diphosphopeptide to the SH2 domains of PI3K therefore seems to affect another region of the protein, resulting in self-association of the enzyme (Figs. 1 and 3). In contrast, binding of singly phosphorylated peptides did not induce dimerization of PI3K (Fig. 1B), even though Tyr-751 binds both SH2 domains of p85 with similar affinities (28) and can presumably occupy both SH2 domains simultaneously. Occupation of SH2 domains is therefore not sufficient to induce dimerization, and a subsequent event must be required which, given the distance constraints imposed by the seven residues between the two pYXXM motifs in this peptide, may involve the SH2 domains moving closer together. This would suggest that the conformational change occurs within the inter-SH2 region, which may act as a flexible linker between the two SH2 domains. The inter-SH2 region is the binding site for the p110 subunit (40), which may explain how an SH2 domain-mediated event could be transmitted to the p110 subunit. The variable length of the sequence between phosphorysine residues in proteins with more than one pYXXM motif suggests that not all binding events involving both SH2 domains would lead to dimerization. The insulin receptor (IR) has only 6 residues between tandem pYXXM motifs, whereas IRS-1 has 20 residues. PI3K is activated by a peptide derived from the IRS-1 sequence (10) but not the IR sequence (41), supporting the idea that it is the precise distance constraint imposed on the two SH2 domains that is important for inducing a conformational change in the inter-SH2 region. Different arrangements pYXXM motifs in three-dimensional space may therefore mediate signal specificity in PI3K signaling pathways.

The conformational change in the inter-SH2 region that is transmitted to the p110 subunit results in the unmasking of a binding site for another diphosphopeptide-bound PI3K. This binding site must be obscured when the SH2 domains are not peptide-bound or when they are bound to singly phosphorylated peptides. It has previously been observed that immunoprecipitation of PI3K using a polyclonal antibody against p85α was less efficient in the presence of Tyr-740/Tyr-751 (11), suggesting that some surfaces on PI3K are less accessible when the SH2 domains are bound to diphosphopeptide. We have previously shown that p85α forms a homodimer and that the dimerization interface resides in the N-terminal half of the protein, which contains the SH3 and BH domains and two proline-rich motifs.2 Combinations of these N-terminal domains have also been shown to be dimers (42, 43). The SH3 and BH domains are therefore free to form a dimeric interface in p85α alone but not in the p110α-EE tag-p85α complex. This implies that p110 may interact with the SH3 and BH domains, preventing their interaction with each other, and that this interaction is released when the SH2 domains bind diphosphopeptide. This may also explain how the binding of SH3 or BH domain ligands to PI3K could regulate the catalytic activity (13, 14).

Dimerization of receptor tyrosine kinases (RTKs) (44, 45), transmembrane phosphatases (46), and cytoplasmic protein kinases (19, 20) is often an early step in signal transduction. Another emerging theme in receptor-mediated signaling is the formation of multiprotein complexes, some of which bind to dimeric receptors, and may also contain dimeric components. Formation of these complexes can result in the activation of one or more components of the complex, in recruitment to the juxtamembrane region of the cell, or in the creation of novel protein-binding surfaces. New binding sites can be generated by heterotypic or homotypic protein-protein interactions, which may allow recruitment of other components of the complex. Dimerization of PI3K does not directly activate the intrinsic kinase activity in vitro; therefore, the role of dimer formation may be to generate novel binding surfaces for recruitment of other cellular signaling proteins that may be the factors that moderate PI3K activity in vivo. The differences in the three-dimensional arrangements of pYXXM motifs in signaling proteins and their ability or inability to induce PI3K dimerization increases the repertoire of PI3K-containing multiprotein complexes and may be a mechanism that directs specificity in PI3K-mediated signal transduction downstream of different cell stimuli.
Dimerization of Phosphatidylinositol 3-Kinase

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Phosphatidylinositol 3-Kinase Induces Oligomerization of Platelet-derived Growth Factor Receptor β Induced by Binding of a Diphosphotyrosine-containing Peptide That Mimics Activated Phosphatidylinositol 3-Kinase

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J. Biol. Chem. 1998, 273:33379-33385.
doi: 10.1074/jbc.273.50.33379

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