Agonists Cause Nuclear Translocation of Phosphatidylinositol 3-Kinase γ

A Gβγ-DEPENDENT PATHWAY THAT REQUIRES THE p110γ AMINO TERMINUS*

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In hematopoietic cells, the signals initiated by activation of the phosphoinositide 3-kinase (PI3K) family have been implicated in cell proliferation and survival, membrane and cytoskeletal reorganization, chemotaxis, and the neutrophil respiratory burst. Of the four isoforms of human PI3K that phosphorylate phosphatidylinositol 4,5-bisphosphate, only p110γ (or PI3Kγ) is associated with the regulatory subunit, p101, and is stimulated by G protein βγ heterodimers. We performed immunolocalization of transfected p110γ in HepG2 cells and found that, under resting conditions, p110γ was present in a diffuse cytoplasmic pattern, but translocated to the cell nucleus after serum stimulation. Serum-stimulated p110γ translocation was inhibited by pertussis toxin and could also be induced by overexpression of Gβγ in the absence of serum. In addition, we found that deletion of the amino-terminal 33 residues of p110γ had no effect on association with p101 or on its agonist-regulated translocation, but truncation of the amino-terminal 82 residues yielded a p110γ variant that did not associate with p101 and was constitutively localized in the nucleus. This finding implies that the intracellular localization of p110γ is regulated by p101 as well as Gβγ. The effect of PI3Kγ in the nucleus is an area of active investigation.

Phosphoinositide 3-kinases are a group of enzymes that phosphorylate the D-3 position of the inositol ring of phosphatidylinositol to produce phosphatidylinositol 3-phosphate (PI1-3-P), PI-3,4-P2, and PI-3,4,5-P3. After stimulation by growth factor- or G protein coupled-receptors, there is a transient increase in PI-3,4-P2 and PI-3,4,5-P3 (1). The relatively larger pool of PI-3-P remains stable. Much of what is known about the role of PI3K in blood cells derives from the overexpression of PI3K mutants and the use of pharmacologic inhibitors such as wortmannin and LY294002. Studies have suggested that PI3K is involved in several aspects of signaling in hematopoietic cells: 1) cell proliferation, 2) chemotaxis, 3) histamine release from basophils, 3) phagocytosis by monocytes, 4) platelet aggregation, and 5) the respiratory burst of neutrophils (2).

The four isoforms of human PI3K that phosphorylate Pl, PI-4-P, and PI-4,5-P2 are classified by their catalytic subunits: p110α, p110β, p110γ, and p110δ. The most studied human PI3K is a heterodimer composed of p110α, p110β, or p110δ coupled to an adapter protein, p85. These p85-associated PI3K isoforms are tightly linked to signaling mediated by growth factor receptors. After stimulation by extracellular growth factors, the cytoplasmic tail of the growth factor receptor autophosphorylates, enabling it to associate with numerous signaling proteins, including p85, via its two SH2 domains. This recruits p110α to the cellular membrane, which appears to be sufficient to activate its lipid kinase activity (3). The binding of activated Ras to p110α also appears capable of activating p85-p110, but whether this enhances its membrane association is currently unknown (4). Intracellular localization studies have shown that p85-p110 is present in the cytoplasm with a small component at the extracellular membrane (3); yet two studies using PC12 or human embryonic kidney 293 cells have suggested that p85-associated PI3K can translocate to the nucleus after neuronal growth factor stimulation (5) or in H2O2 exposure (6). However, these observations remain controversial.

Several years ago, it was shown that hematopoietic cells possess a PI3K that can be directly stimulated by Gβγ heterodimers (26). Several groups have demonstrated that this G protein-activated PI3K is a heterodimer composed of a catalytic subunit, p110γ, and an adapter protein, p101 (7, 8). In addition to blood cells, Northern blot analysis demonstrated that p110γ mRNA is also abundant in skeletal and cardiac muscle, liver, and pancreas (9). This PI3K plays a role in the activation of mitogen-activated protein kinase by G protein-coupled receptors and Btk (10, 11). In reconstitution assays, the p101-p110γ complex was inhibited by the pleckstrin homology domain-containing protein pleckstrin, whereas the p85-p110δ complexes were unaffected (12).

The literature suggests that the regulation of p110α/β/δ catalytic subunits is controlled by their intracellular localization, which, in turn, is controlled by their p85-binding partners. In addition, several reports now provide evidence that p85-associated PI3K can translocate to the cell nucleus (5, 6). However, no published reports specifically address the intracellular localization of p101-p110γ. Therefore, in this study, we determined, first, the intracellular localization of p110γ and, second, whether the localization is influenced by binding to p101. Our results show that p110γ, upon serum stimulation, translocates to the cell nucleus. This serum-induced nuclear translocation is pertussis toxin-sensitive and can be mimicked by overexpression of Gβγ heterodimers, but does not appear to be cell cycle-regulated. However, the nuclear localization is regulated by p101 since the Δ1–82 truncation variant of p110γ, which cannot associate with p101, is constitutively localized in the nucleus.

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1 The abbreviations used are: PI, phosphatidylinositol; PI3K, phosphatidylinositol 3-kinase; HA, hemagglutinin; GFP, green fluorescent protein; PDE, porcine aortic endothelial.
MAMMALIAN EXPRESSION VECTORS—The cDNA clone of human p110γ was described previously (13). The p110γ variants were generated by polymerase chain reaction mutagenesis using the techniques of Ho et al. (14) and Landt et al. (15). The deletion variants, p110γ (Δ1–34) and p110γ (Δ1–82), also contained the 5′-untranslated region from β-globin fused upstream of the initiator methionine. All of these cDNAs were cloned into pcCMV3 and contain a carboxyl-terminal additional 9-amino acid hemagglutinin (HA) epitope tag (YPYDVPDYA) recognized by the monoclonal antibody 12CA5. The GFP-p110γ fusion-expressing plasmid contained the sequence for GFP in place of the stop codon and was generated by the technique of polymerase chain reaction splice overlap extension and cloned into pcDNA3.1 (Invitrogen, Carlsbad, CA). The sequences of all clones were fully confirmed. The plasmids cloned the expression of an EE epitope (EEDKLISETEDL)-tagged bovine p110γ were a generous gift from Dr. Len Stephens (Babraham Institute, Cambridge, United Kingdom). Plasmids that direct the synthesis of Gβ1 and Gγ2α were a generous gift from Dr. Janet Robishaw (Geisinger Institute, Danville, PA).

Co-immunoprecipitation Studies—COS-7SH cells were transfected by the calcium phosphate technique as described previously (16) using plasmids that direct the synthesis of HA-p110γ variants, with and without EE-p101 or Gβγ. Forty-eight hours later, the cells were washed with phosphate-buffered saline and then lysed on ice in 1 ml of 1% Triton X-100, 0.14 mM NaCl, 1 mM MgCl2, 1 mM EGTA, 20 mM HEPES (pH 7.4), 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 0.1% aprotinin. After clarification at 13,000 rpm for 30 min, the supernatants were incubated with Sepharose G coupled to an anti-EE antibody (BAbCO, Berkeley, CA) overnight. The beads and associated proteins were pelleted at 2000 × g for 30 s, washed extensively in the lysis buffer, and boiled in Laemmli loading buffer. The anti-EE immunoprecipitates were then fractionated by 7.5% SDS-polyacrylamide gel electrophoresis and immunoblotted with the anti-HA antibody HA.11 (BAbCO) to detect the co-immunoprecipitation of HA-p110γ variants along with EE-p101.

Indirect Immunofluorescence—HepG2 cells were transiently transfected with the calcium phosphate technique and stained as described previously (17). Platelet-derived growth factor-transformed porcine aortic endothelial (PAE) cells were electroporated as described previously (18). Staining of cells with ethidium monoazide (Molecular Probes, Inc., Eugene, OR) was performed following the manufacturer’s protocol.

Microinjection—Microinjection was performed using an Eppendorf 5171 Micromanipulator with an Eppendorf 5246 Transrector. Plasmid DNA was diluted in 2X injection buffer (100 mM HEPES [pH 7.2], 200 mM KCl, and 10 mM NaPO4) to a final concentration of 25 ng/μl. Transrector settings were injection pressure = 60 hectopascals, compensatory pressure = 20 hectopascals, and time of injection = 0.1 s. Cells were incubated for 6 h following injection and were then fixed in 10% formaldehyde buffered Formalin for 30 min prior to image analysis. Two methods were used for image collection and analysis. Conventional fluorescence microscopy was performed using a Nikon Microphot-SAP fluorescence microscope and camera. We also used the resources of the University of Pennsylvania Cancer Center Confoal Microscopy Core Facility. Confoal images were acquired from a TCS 4D upright microscope and processed on an IBM OS9 workstation using Scanware software. All light microscopic figures were shot at ×40 magnification.

RESULTS AND DISCUSSION

p110γ Translocates to the Cell Nucleus of HepG2 Cells after Serum Stimulation—To begin to understand the role of PI3Kγ in vivo, we performed indirect immunofluorescence of transfected p110γ in human HepG2 hepatoma cells. Liver cells naturally express PI3Kγ; therefore, we reasoned that HepG2 cells should contain any accessory proteins needed for proper p101-p110γ signaling. Since all available antibodies were unable to detect endogenous p110γ by immunofluorescence, we expressed the HA epitope-tagged p110γ that was recognized by the anti-HA monoclonal antibody 12CA5. Twenty-four hours after transfection, the cells were placed in medium without serum for 16 h. The cells were then washed, fixed, and stained with an anti-HA antibody.

As shown in Fig. 1 (A and B), when HepG2 cells were transfected with epitope-tagged p110γ and analyzed under serum-deprived conditions, the cells appeared large and flat. Under these resting conditions, indirect immunofluorescence showed that p110γ was present in a diffuse cytoplasmic pattern. In contrast, after stimulation of the cells with serum, immunolocalization of p110γ revealed that it was no longer detected in the cytoplasm, but was found almost exclusively in the nucleus (Fig. 1, C and D). Confocal microscopy verified this finding and revealed that the transported PI3Kγ was diffusely present throughout the nucleus, but most concentrated at the nuclear membrane. Moreover, PI3Kγ staining did not coincide with simultaneous staining of the Golgi apparatus with the antibody G2404, a monoclonal antibody directed against the Golgi 58-kDa protein (data not shown). As has been reported previously, overexpression of PI3Kγ did induce morphologic changes, including shrinking of the cytoplasm and ruffling of the cell membrane (5). Although cells overexpressing this protein had dramatic changes in their appearance, they were still viable. This was demonstrated by the exclusion of the DNA-staining agent ethidium monoazide in the absence of cell permeabilization. In addition, the cells did not show any evidence of apoptosis by the terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling immunofluorescence assay. Therefore, in response to serum stimulation, overexpressed p110γ induces dramatic morphologic changes and translocates to the nucleus.

Overexpression of p101, which is endogenously present in HepG2 cells, had no influence on p110γ nuclear translocation and, under either resting or stimulated conditions, was present in both the cytoplasm and nucleus (data not shown). We performed time course experiments to determine how rapidly after cell stimulation p110γ migrates to the nucleus. In these experiments, cells were transfected with plasmids encoding PI3Kγ. Twenty-four hours later, the cells were serum-deprived for an additional 16 h. At this point, all of the PI3Kγ protein was cytoplasmic. The medium was then changed to Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, and the time course of nuclear translocation was measured using the addition of serum as the defined time 0. These experiments showed that the translocation of p110γ began as rapidly as 2 h after serum exposure and was almost complete by 7 h, but
do not endogenously contain PI3Kγ also contain the necessary accessory proteins for PI3Kγ nuclear translocation. As shown in Fig. 3A, when p101 and p110γ were transfected into stably platelet-derived growth factor-expressing PAE cells, staining for p110γ demonstrated that it was found in both the cytoplasm and nucleus. In these platelet-derived growth factor-overexpressing cells, this pattern of distribution was independent of serum (data not shown). As shown in Fig. 3 (B and C), the intracellular localization was also independent of cell cycle as demonstrated by thymidine block (arresting cells at the G1/S boundary) or by thymidine block and release (arresting cells in S phase). In contrast, transfection of p101 and p110γ into COS-7SH cells demonstrated an exclusively cytoplasmic distribution for p110γ (data not shown). Together, this suggests that, like p101 and p110γ, the accessory proteins required for p110γ nuclear translocation are not ubiquitously expressed.

**Studies of the Interaction between p110γ and p101**—There is some controversy in the literature regarding the necessity of the p101 subunit for p110γ function (7, 9, 10). We next sought to determine whether G protein-coupled or growth factor-receptor-mediated signaling pathways were responsible for the serum-induced nuclear translocation of p110γ. To examine this issue, we preincubated cells with pertussis toxin to inhibit the release of Gβγ heterodimers from Gαi-coupled receptors. Cells were then examined in the presence or absence of serum stimulation. In the absence of serum, pertussis toxin did not affect the diffuse cytoplasmic distribution of p110γ in serum-starved cells (data not shown). However, pertussis-toxin inhibited the serum-induced p110γ nuclear translocation (Fig. 1, E and F). This suggests that the serum-induced nuclear translocation of PI3Kγ is dependent on a Gβγ-mediated signaling pathway. To test this hypothesis, we attempted to mimic serum-mediated nuclear translocation by overexpression of Gβγ heterodimers. The overexpression of Gβγ heterodimers resulted in the nuclear translocation of p110γ in the absence of serum stimulation (data not shown). As expected, pertussis toxin failed to alter the nuclear localization of p110γ induced by overexpression of Gβγ heterodimers (Fig. 1, G and H). Together, these observations suggest that serum contains a factor that initiates the nuclear translocation of p110γ by causing release of Gβγ heterodimers downstream from a Gαi-coupled receptor.

To confirm this Gβγ-mediated translocation of p110γ, a plasmid directing the expression of a chimeric protein composed of GFP fused to the carboxy terminus of p110γ was microinjected into HepG2 cells. Cells were analyzed 6 h after microinjection of the plasmid under serum-starved conditions. Similar to our observations with indirect immunofluorescence, under resting conditions, the GFP-p110γ fusion protein was found in a diffuse cytoplasmic pattern (Fig. 2, A and C). In contrast, when plasmids that direct the expression of Gβγ heterodimers were simultaneously microinjected with the GFP-p110γ plasmid, the GFP fusion protein was exclusively localized in the nucleus (Fig. 2, B and D). Thus, in these cells, which endogenously express PI3Kγ, there is a Gβγ-mediated transport of this lipid kinase from the cytoplasm to the nucleus.

We questioned whether cell lines derived from tissues that do not endogenously contain PI3Kγ also contain the necessary accessory proteins for PI3Kγ nuclear translocation. As shown in Fig. 3A, when p101 and p110γ were transfected into stably platelet-derived growth factor-expressing PAE cells, staining for p110γ demonstrated that it was found in both the cytoplasm and nucleus. In these platelet-derived growth factor-overexpressing cells, this pattern of distribution was independent of serum (data not shown). As shown in Fig. 3 (B and C), the intracellular localization was also independent of cell cycle as demonstrated by thymidine block (arresting cells at the G1/S boundary) or by thymidine block and release (arresting cells in S phase). In contrast, transfection of p101 and p110γ into COS-7SH cells demonstrated an exclusively cytoplasmic distribution for p110γ (data not shown). Together, this suggests that, like p101 and p110γ, the accessory proteins required for p110γ nuclear translocation are not ubiquitously expressed.

**Studies of the Interaction between p110γ and p101**—There is some controversy in the literature regarding the necessity of the p101 subunit for p110γ function (7, 9, 10). We next sought to determine whether cell lines derived from tissues that do not endogenously contain PI3Kγ also contain the necessary accessory proteins for PI3Kγ nuclear translocation. As shown in Fig. 3A, when p101 and p110γ were transfected into stably platelet-derived growth factor-expressing PAE cells, staining for p110γ demonstrated that it was found in both the cytoplasm and nucleus. In these platelet-derived growth factor-overexpressing cells, this pattern of distribution was independent of serum (data not shown). As shown in Fig. 3 (B and C), the intracellular localization was also independent of cell cycle as demonstrated by thymidine block (arresting cells at the G1/S boundary) or by thymidine block and release (arresting cells in S phase). In contrast, transfection of p101 and p110γ into COS-7SH cells demonstrated an exclusively cytoplasmic distribution for p110γ (data not shown). Together, this suggests that, like p101 and p110γ, the accessory proteins required for p110γ nuclear translocation are not ubiquitously expressed.
Specifically, we have shown that factors present in serum cause responses to serum can be inhibited by pertussis toxin and can associate with p101, translocated from the cytoplasm to the nucleus at heterodimers. A mutant protein (data not shown).

CONCLUSION

Although PI3Kγ has been well described as a mediator of signaling events at the plasma membrane, this work suggests that PI3Kγ may also play a role at the nuclear membrane. Specifically, we have shown that factors present in serum cause p110γ to move to the nucleus in transfected HepG2 cells. This response to serum can be inhibited by pertussis toxin and can be mimicked by overexpression of Gγ proteins. A mutant form of p110γ that is unable to bind to p101 is constitutively localized in the nucleus.

These observations raise a number of issues, including the mechanism by which PI3Kγ is transported to the nucleus, the impact of PI3Kγ on nuclear signaling, and whether these findings also apply to growth factor-activated p110α/β. The mechanism by which PI3Kγ is localized to the nucleus at this point is unclear. This process certainly can be regulated by the release of Gγ heterodimers. Our studies suggest that p101 appears to regulate this translocation since a variant of p110γ that does not associate with p101 is constitutively found in the nucleus. This is similar to the mechanism by which mitogen-activated protein kinase shuttles between the nucleus and the cytoplasm by alternatively interacting with cytoplasmic and nuclear retention (or anchoring) proteins (20). This model would imply sequences for nuclear import and retention, the existence and perhaps sequences for nuclear export and cytoplasmic retention within p110γ. Examination of the p110γ sequence reveals two potential nuclear import signals: R17RRRR and K806KKP. Since the p110γ(A1–34) variant is capable of nuclear translocation, it implies that R17RRRR is not critical. Whether K806KKP is required for nuclear transport is currently unknown, as is the role of second messenger formation.

The effect of PI3K on mitosis and survival is a topic of recent interest. Evidence derived from the use of inhibitors (or over-expressed effectors) of PI3K implies that a tight regulation of PI3K is critical for both cell growth and cell death. Most likely, this effect of PI3K is the result of an increase in lipid second messengers. Although cytoplasmic p101-p110γ will phosphorylate PI, PI-4-P, and PI-4,5-P2, only cytoplasmic PI-3,4-P2 and PI-3,4,5-P3 appear to change significantly after G protein-coupled receptor stimulation. Nuclear membranes, which probably contain the substrate for nuclear lipid kinases, contain abundant quantities of PI, PI-4-P, and PI-4,5-P2. But at this point, the substrate for PI3Kγ in the nucleus remains to be determined. It has long been appreciated that individual phospholipid concentrations vary with the cell cycle. For example, Dobos et al. (21) demonstrated that PI-3-P is elevated during the G2/M phase of the cell cycle. Recently, it has also been suggested that the cell cycle may actually be influenced by phospholipid content (22–24). Consistent with this hypothesis, fibroblasts deprived of choline and synchronized in G1 phase by serum starvation do not efficiently enter S phase after serum stimulation (25). This implies that phospholipid synthesis is required for S phase entry. It is possible that nuclear PI3Kγ influences the cell cycle by transiently elevating the PI-3-P concentration. Recent evidence suggests that p110γ may also have protein kinase activity (19). This raises the alternative possibility that the substrate for nuclear PI3Kγ is a protein, instead of a lipid.

Although most reports in the literature show a cytoplasmic (or plasma membrane) intracellular distribution for p85, two reports have suggested that it may relocate to the nucleus after neuronal growth factor or H2O2 stimulation (5, 6). Consistent with this observation, preliminary studies in our laboratory have demonstrated that hemagglutinin epitope-tagged p110α expressed in HepG2 cells will also translocate to the nuclear membrane after serum stimulation.2 This implies that phospholipid synthesis is required for S phase entry. It is possible that nuclear PI3Kγ influences the cell cycle by transiently elevating the PI-3-P concentration. Recent evidence suggests that p110γ may also have protein kinase activity (19). This raises the alternative possibility that the substrate for nuclear PI3Kγ is a protein, instead of a lipid.

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