Mouse p170 Is a Novel Phosphatidylinositol 3-Kinase Containing a C2 Domain*

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Phosphatidylinositol (PI) 3-kinases catalyze the formation of 3'-phosphoinositides, which appear to promote cellular responses to growth factors and such membrane trafficking events as insulin-stimulated translocation of intracellular glucose transporters. We report here the cloning of a novel PI 3-kinase, p170, from cDNA of insulin-sensitive mouse 3T3-L1 adipocytes. Mouse p170 utilizes PI and to a limited extent PI 4-P as substrates, in contrast to the PI-specific yeast VPS34 homolog PtdIns 3-kinase and the p110 PI 3-kinases, which phosphorylate PI, PI 4-P, and PI 4,5-P2. Mouse p170 is also distinct from PtdIns 3-kinase or the p110 PI 3-kinases in exhibiting a 10-fold lower sensitivity to wortmannin. Unique structural elements of p170 include C-terminal sequences strikingly similar to the phosphoinositide-binding C2 domain of protein kinase C isoforms, synaptotagmins, and other proteins. These features of mouse p170 are shared with a recently cloned Drosophila PI 3-kinase, DmPI3K_68D. Together, these proteins define a new class of PI 3-kinase likely influenced by cellular regulators distinct from those acting upon p110- or VPS34-like PI 3-kinases.

Multiple species of 3'-phosphorylated inositol lipids produced in reactions catalyzed by phosphatidylinositol (PI)3-kinases are thought to be involved in cellular signaling and membrane trafficking pathways. A relatively large, constitutive pool of PI 3-phosphate is present in resting cells, while very low levels of PI 3,4-bisphosphate and PI 3,4,5-trisphosphate are rapidly increased in response to a number of external cellular stimuli (reviewed in Refs. 1 and 2). The pool of PI 3-phosphate may be largely due to PI 3-kinases such as Ptdlns 3-kinase (3), a mammalian homolog of the yeast VPS 34 protein (4), which can utilize only PI as substrate. In contrast, a second category of PI 3-kinases, isoforms of the p110 PI 3-kinase, are capable of phosphorylating PI 4-phosphate and PI 4,5-bisphosphate at the 3' position (5-8). These enzymes apparently contribute to the regulated pools of PI 3,4-P2 and PI 3,4,5-P3, stimulated by receptor or non-receptor tyrosine kinase activation (in the case of the isoforms p110 and p110(j1)) or G-protein activation (in the case of p110(y)). The existence of multiple PI 3-kinase isoforms suggests the influence of multiple signaling pathways on these enzymes and, possibly, divergent functions of the individual 3'-phosphoinositides.

A role for polyphosphoinositides as second messengers has been suggested by their activation of protein kinases such as certain protein kinase C species (9, 10). Products of p110-type PI 3-kinases also appear to be necessary for activation of p70 S6 kinase (11, 12) and protein kinase B/c-Akt (13, 14). An additional role for polyphosphoinositide products of p110 PI 3-kinases in regulated membrane trafficking is suggested by the requirement for yeast VPS34 PI 3-kinase activity in proper targeting of soluble hydrolases to the vacuole (15). Strong evidence implicates a direct role for PI 3-kinase in ligand-induced lysosomal targeting of the platelet-derived growth factor receptor (16, 17), as well as insulin-mediated translocation of intracellular GLUT4 glucose transporters to the cell surface (Ref. 18; reviewed in Ref. 19). The importance of PI 3-kinases in these and other pathways has been inferred from association and/or activation of the enzyme in receptor or downstream signaling complexes, but also from sensitivity to PI 3-kinase inhibitors such as wortmannin (reviewed in Ref. 20). Insulin-stimulated glucose uptake and GLUT4 translocation, for example, are inhibited by low nanomolar concentrations of wortmannin in intact fat cells (21). All of the PI 3-kinase enzymes cloned to date from mammalian cells are highly sensitive to this fungal metabolite (IC50 in vitro of 2-5 nM; Refs. 3, 8, and 22). The yeast VPS34 protein, however, is relatively insensitive to wortmannin (23), and evidence for a wortmannin-insensitive PI 3-kinase activity in mammalian cells (24) suggests that this compound is not a universally potent inhibitor of PI 3-kinases.

The significance of PI 3-kinase in insulin action suggested an examination of the particular enzymes involved. To this end we sought to identify PI 3-kinase isoforms that are present in insulin-responsive cells. We report here that mouse 3T3-L1 adipocytes contain, in addition to p110 isoforms and a VPS34 homolog, a novel PI 3-kinase, which we call p170. This protein is biochemically distinct from the p110 or VPS34 family in both substrate specificity and sensitivity to wortmannin. The unique structure of p170 includes a C-terminal region with striking sequence similarity to the phospholipid/inositol polyphosphate-binding C2 domain previously identified in many membrane-associated proteins. These observations suggest that p170 defines a novel category of PI 3-kinases potentially the target of distinct activators and capable of producing 3'-phosphoinositides essential to specific cellular functions.

EXPERIMENTAL PROCEDURES

Identification and Cloning of p170—Oligonucleotide primers A (5'-GCCTCTAGACAC(G/A)CTTGT(C/T)CG/CAAGCAAGCA(A/G)GA-3'), B (5'-CGGA-AGCTT(G/A)TGC/CG(A/G)TCC/CC/CCC/CC(T/C)-3'), and C (5'-CCGAGCTT- TAR/GA/AC(A/G)CTGGC-3') were designed based on the peptide sequences GDDLQD, DRHNND, and FHIDFG conserved in known lipid kinases. The last primer was used to prime reverse transcription of 3T3-L1 adipocyte RNA using avian myeloblastosis virus reverse transcriptase (Promega) according to the manufacturer's protocol. Complementary DNA from this reaction was amplified by polymerase chain rea..
plified with primers A and B and the products subcloned and sequenced. A clone encoding the novel p170 sequence was in turn used as a hybridization probe for screening a 3TF-F442A adipocyte cDNA library (a gift of Dr. Bruce Spiegelman, Dana-Farber Cancer Institute). Overlapping clones totalling 5067 base pairs were isolated and sequenced. All sequences reported were determined on both strands using an Applied Biosystems 373 sequencer. Rapid amplification of cDNA ends (RACE) was performed according to Frohman (25) using a primer complementary to nucleotides 380–398 of the p170 sequence.

Expression, Preparations, and Assay of PI 3-Kinase Enzymes—For expression in SF9 cells, p170 and bovine p110 (a gift of Dr. Michael Waterfield, Ludwig Institute for Cancer Research) were cloned into the baculovirus expression vector pAcG3x (Pharmingen). BamHI sites were created by polymerase chain reaction at the initiation codon of p170 and p110 cDNAs placed the remaining of the coding sequence in frame with the glutathione S-transferase (GST) coding sequence of the vector. Resulting BamHI-EcoRI fragments of each cDNA were ligated to the same site of pAcG3x and the resulting plasmid cotransfected with linearized wild-type viral DNA into SF9 cells as directed by the manufacturer.

GST-p170 and GST-p110 were prepared from SF9 cells at 3–4 days following infection. Cells were collected by centrifugation, washed once in phosphate-buffered saline, and then lysed in 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, 10% glycerol, 1% Nonidet P-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 5 μg/ml leupeptin, 5 μg/ml aprotenin. The lysate was clarified by centrifugation (16,000 × g, 15 min) and the supernatant incubated with glutathione-agarose beads (approximately 100 μl of beads/1 mg of lysate) for 1-2 h. Beads were washed twice in phosphate-buffered saline with 1% Nonidet P-40; twice in 100 mM Tris-HCl (pH 7.5), 500 mM LiCl; once in 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA; and once in 20 mM Tris-HCl (pH 7.5), 100 mM NaCl; 3.5 mM MgCl2, 0.5 mM EGTA (assay buffer). For PI 3-kinase assays, 10 μl of beads prepared as above with 50–100 μg of infected cell lysates were suspended in 40 μl of assay buffer. After a 10-min incubation with lipid (0.2 mg/ml each phosphatidylinositol and phosphatidylinositol-4-phosphate), Avanti Polar Lipids the reaction was carried out for an additional 10 min in the presence of 10 μM ATP containing 12.5 μCi of [γ32P]ATP. Samples prepared for HPLC analysis used 10 μg of brain lipid extract (Folch fraction I, Sigma) in the presence of 10 μM ATP containing 75 μCi of [γ32P]ATP. Lipid products were extracted and separated by thin layer chromatography as described previously (26) or deacylated and the glycerophosphoinositidase headgroups analyzed by HPLC as described (27). Standards for HPLC analysis included [3H]PI, [3H]PI 4,5-P2 as well as products of PI 3-kinase reactions carried out on p85 immunoprecipitates.

RESULTS AND DISCUSSION

Using a strategy based on PCR amplification of conserved lipid kinase sequences, we identified four species of PI 3-kinase expressed in 3T3-L1 adipocytes. Two of these corresponded to two of the known isoforms of the p110 catalytic subunit of PI 3-kinase (p110α and p110β), while the third was found to be more closely related to the yeast VPS34 gene product (4) and likely represents the mouse homolog of that protein and the similar PtdIns 3-kinase recently cloned from human cDNA (3). The fourth sequence, although highly similar to the catalytic region sequences of known PI 3-kinases, was clearly distinct and was characterized further. Screening of 600,000 plaques of an adipocyte cDNA library with a probe derived from this PCR product identified seven overlapping clones. The two that covered the maximum extent of the 5′ and 3′ end were fully sequenced and were found to encompass a total of 5076 base pairs. To verify that these clones included the full coding region of this species, RACE-PCR clones were obtained and sequenced. Four independent clones were sequenced. The longest of these extended the cDNA sequence by only 6 nucleotides, while each of the others extended within 5 nucleotides of the longest. We conclude that the sequence reported represents the full extent of its 5′ end.

The sequence of this cDNA includes an open reading frame of 4530 nucleotides, which is predicted to encode a protein of about 1500 amino acids. All four of the predicted PI 3-kinases are shown. Sequences with significant homology to other PI 3-kinases (designated as region I and II, Fig. 1) originally obtained as a PCR product. The catalytic region of p170 is more similar to those of the p110 PI 3-kinases than to the p85 subunit of PI 3-kinase (Fig. 2A), sharing, for example, the additional charged residues in the putative substate pocket (Fig. 2B, bracketed) predicted to accommodate 4- and 5-phosphorylated phosphoinositide substrates (3). In addition, the p170 sequence displays significant similarity to the region just N-terminal to the probable catalytic domain, which is also conserved among a number of known PI 3-kinases (region I, Figs. 1 and 2). Region I displays the most similarity to the analogous region of the various p110 isoforms.

Despite this similarity in regions I and II, p170 is distinguished from other PI 3-kinases in having additional sequences on the C-terminal side of the catalytic domain not found in other known mammalian PI 3-kinases. This region of p170 contains a striking similarity (Fig. 2C) to a domain denoted C2 originally identified in a number of protein kinase C isoforms (28). It is more recently noted to mediate the binding of such proteins as synaptotagmins, raphilin 3A, p120 Ras-GAP, and others (29–32). This C2 domain has been identified as a phosphatidylinositol/polysphosphate binding motif, which in some cases is dependent on Ca2+ for lipid binding activity. This feature of p170 is shared with a recently identified novel PI 3-kinase in Drosophila called DmP13K_68D (22), with which it also shares high levels of similarity in regions I and II (Fig. 2). Both mouse and Drosophila proteins, however, lack the conserved aspartate residues involved in Ca2+ coordination by a calcium-independent fashion similar to the non-calcium-regulated C2B domain of synaptotagmin I (22).

The above considerations indicate that mouse p170 represents the first known mammalian form of a new class of PI 3-kinase molecules, which also includes DmP13K_68D. In the N-terminal half of p170, however, there is little similarity to DmP13K_68D, or to other PI 3-kinases. Of special note, there is no sequence resembling the potential SH3-binding proline-rich motif identified in DmP13K_68D (22). Neither is there similarity to the N-terminal sequence of the p110 or p110β isoforms responsible for their association with the p85 regulatory sub-
Previously identified PI 3-kinases can be divided into two groups based on substrate specificity. Three known isoforms of the p110 catalytic subunit are known to utilize PI as well as PI 4-phosphate and PI 4,5-bisphosphate as substrates. VPS34, however, utilizes only PI. In order to test this property of p170, the complete coding region of p170 was fused to GST-encoding sequences of a baculovirus expression vector, and the enzyme was expressed in Sf9 cells as a GST fusion protein. PI3-kinase activity has been shown to be stimulated in the pathways emanating from these stimuli has been further suggested by the parallel sensitivity of these enzymes and the resulting biological responses to the fungal product wortmannin. This compound inhibits all p110 isoforms and the mammalian VPS34-like PtdIns3-kinase at low nanomolar concentrations (3). However, wortmannin is not a potent inhibitor of the yeast VPS34 (22). We tested the sensitivity of p170 to increasing amounts of wortmannin in assay reactions containing glutathione-agarose-bound GST-p170 using PI as a substrate (Fig. 4). Under these conditions p170 activity was inhibited only by relatively high concentrations of wortmannin (50% inhibition by approximately 40–50 nM wortmannin). Even at 100 nM wortmannin, a concentration widely used for in vitro or in vivo studies of PI 3-kinase activity, about 20% of the activity of p170 remained. By contrast, in parallel reactions the activity of GST-p110 PI 3-kinase was readily inhibited by low nanomolar concentrations of the same phosphatidylinositol.
Furthermore, even a small amount of PI 3,4-P₂, if directed to corresponding to ³²P-PI were cut out and radioactivity measured by scintillation counting. The graph shows the mean ± standard error of four determinations of activity relative to control reactions without wortmannin. This result differs from that obtained with DmPI3K_68D, with previous reports with cellular or recombinant p110(3,24).

It is likely not accurately reflected in vitro (discussed in Ref. 36). Furthermore, even a small amount of PI 3,4-P₂, if directed to critical cellular locations, could have physiological significance. Like other second messengers, the signal from a small amount of this phosphoinositide could be amplified in a cascade of subsequent signaling events. In any case our results argue for caution in the interpretation of results obtained by the use of inhibitors such as wortmannin. At low concentrations of this drug, p110 and VPS34-like PI 3-kinases may be inhibited, but others such as p170 may still be partially or fully functional. A recent report of a PI 4-kinase with wortmannin sensitivity similar to p170 PI 3-kinase (37) suggests that multiple enzymatic activities may be affected at such increased concentrations of wortmannin. Alternative methods will be required to dissect rigorously the distinct contributions to cell function made by the multiple PI 3-kinase species now known to be present in mammalian cells. The identification of p170 and isolation of its cDNA should facilitate experimental approaches to this problem.

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**FIG. 4. Wortmannin sensitivity of mouse p170 PI 3-kinase.** PI 3-kinase assays were carried out with glutathione-agarose-bound p170 or p110 in the presence of PI, [γ³²P]ATP, and the indicated concentrations of wortmannin. A, upper panel, a representative experiment showing a thin layer chromatogram of labeled lipid products of GST-p170 without wortmannin or in the presence of 2–100 nM wortmannin. The position of ³²P-PI is indicated; lower panel, thin layer chromatogram of products of GST-p110 without or with wortmannin. B, spots corresponding to ³²P-PI were cut out and radioactivity measured by scintillation counting. The graph shows the mean ± standard error of four determinations of activity relative to control reactions without wortmannin for GST-p170 (closed circles) of GST-p110 (closed triangles).