Insulin Activates the $\alpha$ Isoform of Class II Phosphoinositide 3-Kinase*

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The novel class II phosphoinositide (PI) 3-kinases are characterized by the presence of a C-terminal C2 domain, but little is known about their regulation. We find insulin causes a rapid 2–3-fold increase in the activity of PI3-kinase C2a (PI3K-C2a) in CHO-IR cells, 3T3-L1 adipocytes, and fully differentiated L5L6 myotubes. No insulin-induced activation of PI3K-C2a was observed in cell types known to have low responsiveness to insulin including HEK 293 cells, 3T3-L1 preadipocytes, and undifferentiated L5L6 myoblasts. The mechanism of activation of PI3K-C2a by insulin differs from that of class Ia PI 3-kinases in that insulin stimulation did not cause PI3K-C2a to associate with IRS-1 or insulin receptor. PI3K-C2a existed as a doublet, and insulin stimulation caused a redistribution from the lower molecular weight band to the higher molecular weight band, suggesting phosphorylation-induced bandshift. Consistent with this, in vitro phosphatase treatment reduced the intensity of the upper band back to that seen in unstimulated cells. This suggests that insulin-induced phosphorylation could play a role in regulation of the activity of PI3K-C2a. The finding that insulin activates PI3K-C2a in cell types known to possess a wide range of responses to insulin suggests that PI3K-C2a is a novel component of insulin-stimulated signaling cascades.

A great deal of evidence is now available to indicate that phosphorylation of the D-3 position of the inositol ring of phosphoinositides is a critical step in many intracellular signaling pathways activated by insulin and growth factors (1, 2). A family of phosphoinositide (PI) 3-kinases have been identified that are capable of phosphorylating the D-3 position of the inositol head group (3, 4). Of these, the class Ia PI 3-kinases have been most intensely studied, and enzymes in this class all possess a catalytic subunit tightly associated with an adapter subunit containing two SH2 domains. The binding of these SH2 domains to specific phosphotyrosine containing peptide motifs facilitates recruitment of class Ia PI 3-kinases into receptor tyrosine kinase-induced signaling complexes and thus provides the most clearly identified mechanism by which growth factors can regulate PI 3-kinase activity. However, the ability of growth factors to regulate PI 3-kinase activity via other classes of PI 3-kinase has not been extensively studied.

The class II PI 3-kinases represent a novel group of PI 3-kinases characterized by the presence of a C2 domain at the C terminus. These were originally cloned from Drosophila (5, 6), but three mammalian isoforms of class II PI 3-kinase have also been cloned. PI3K-C2a (6–8) and PI3K-C2b (9, 10) are widely expressed, whereas expression of a third isoform, PI3K-C2γ, is restricted to liver (11, 12). The in vitro substrate specificity of all the class II PI 3-kinases is similar in that they prefer phosphatidylinositol as a substrate but they also phosphorylate PI 4-phosphate under certain conditions. However, the class II PI 3-kinases do not phosphorylate PI 4,5-bisphosphate. The class II PI 3-kinase isoforms can be distinguished pharmacologically because PI3K-C2a is relatively resistant to the PI 3-kinase inhibitors wortmannin and LY294002 (7, 8), whereas PI3K-C2β is sensitive to these inhibitors (10).

The role of class II PI 3-kinases in the cell and how they might be involved in signaling cascades is poorly understood, and it is clearly of interest to determine whether these enzymes can be activated or recruited to signaling complexes by different agonists. In this regard there are reports that the activity of class II PI 3-kinases can be increased by stimuli using heterotrimeric G-protein-linked signaling pathways. The chemokine MCP-1 induces a transient activation of PI3K-C2a via a Goi-linked mechanism in monocytes (13), whereas platelet aggregation induces a transient activation of PI3K-C2b (14).

These studies have not addressed the question of whether class II PI 3-kinases are stimulated following activation of growth factor receptor tyrosine kinases. We have therefore investigated the regulation of PI3K-C2a and PI3K-C2b by insulin in a range of cell types. These studies show that although PI3K-C2a was not activated by insulin in HEK293 cells, 3T3-L1 preadipocytes, or L5L6 myoblasts, there was a 2–3-fold increase in activity of PI3K-C2a in fully differentiated L5L6 myotubes, differentiated 3T3-L1 adipocytes, and Chinese hamster ovary cells overexpressing insulin receptors (CHO-IR) cells. PI3K-C2b was not activated by insulin in HEK 293 cells and only poorly activated in transfected CHO-IR cells. PI3K-C2a existed as a doublet in all cells tested. In the cells in which insulin activates PI3K-C2a, insulin also caused a redistribution from the lower molecular weight band to the higher molecular weight band. This suggests that insulin was stimulating phosphorylation of PI3K-C2a. Consistent with this, in vitro phosphatase treatment reduced the intensity of the upper band back to that seen in unstimulated cells. The ratio of the upper to lower bands was altered in the HEK 293 cells in which PI3K-C2α was not stimulated. These findings suggest that insulin-induced phosphorylation could play a role in regulation of the activity of PI3K-C2a. The finding that PI3K-C2a is activated by insulin in cell culture models of physiologically relevant insulin target tissues raises the possibility that in addition to class Ia PI 3-kinases, class II PI 3-kinases also play a role in insulin signaling pathways.

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§ The abbreviation used is: PI, phosphoinositide.
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**Fig. 1.** Short term stimulation with insulin activates PI 3-kinase C2α while having little effect on PI 3-kinase C2β activity. Cells were serum-starved overnight and then stimulated with 100 nM insulin for 5 min. Cells were lysed and immunoprecipitated using PI3K-C2α-specific antisera (solid bars) or PI3K-C2β-specific antisera (hatched bars). PI 3-kinase assays were performed on these immunoprecipitates as described under “Experimental Procedures.” Results are the means of at least three experiments and expressed as fold stimulation over basal (± S.E.).

**EXPERIMENTAL PROCEDURES**

All chemical reagents were from Sigma unless stated. Radiochemicals were obtained from New England Biolabs. PI 3-kinase assay reagents and antibodies to IRS-1 and p85 were as described previously (15), PY99 antiphosphotyrosine antibody was from Santa Cruz Biotechnologies. 3T3-L1 cells were cultured and differentiated as described previously (15). CHO-IR cells were obtained from Prof. K. Siddle (University of Cambridge), L5L6 murine muscle cells were kindly provided by Dr. F. Giorgino (University of Bari). PI3K-C2α and antibodies were raised to a glutathione S-transferase fusion protein corresponding to the first 331 amino acids of the human PI3K-C2β sequence (10). PI3K-C2α antibodies were raised to glutathione S-transferase fusion protein corresponding to amino acids 2–337 of the human PI3K-C2α sequence. The PI3K-C2α expression construct was in PBK-CMV, and the PI3K-C2β expression construct was cloned into pCDNA3.1. Both contained a Glu tag at the N terminus. The Myc-tagged IRS-1 was supplied by Dr. J. Whitehead (Cambridge University). HEK 293 cells were transiently transfected using the calcium phosphate method, and CHO-IR cells were transiently transfected using Superfect (Qiagen).

For protein analysis and PI 3-kinase assays, cells were serum-starved for 16 h prior to insulin stimulation and then treated with 100 nM insulin for 5 min (unless otherwise stated) at 37 °C. Cells were washed once in phosphate-buffered saline and lysed in a buffer containing 10 mM Tris-HCl (pH 7.4), 5 mM EDTA (pH 8.0), 50 mM NaCl, 100 mM NaF, and 1% (v/v) Triton X-100 supplemented with 2 mg/ml aprotinin, 1 mM pepstatin, 1 mg/ml leupeptin, 2 mM phenylmethylsulfonyl fluoride, and 10 mM sodium orthovanadate. Where indicated immunoprecipitates were incubated with 200 units of λ-protein phosphatase at 30 °C for 30 min. Equal amounts of cell lysate were resolved by SDS-polyacrylamide gel electrophoresis on a 6% gel with an 80:1 ratio of bis-acrylamide:acrylamide (National Diagnostics) and transferred to polyvinylidene difluoride for Western blotting. Immunoprecipitations were performed from the Triton-soluble fraction using the indicated antibodies, and PI 3-kinase assays were performed on the immunoprecipitates with phosphatidylinositol (Lipid Products, Redhill, Surrey, UK) as a substrate using methods previously described (15). TLC plates and radiolabeled Western blots were analyzed using Fuji FLA-2000 phosphorimagere, whereas Western blots visualized with ECL were analyzed us a Fuji LAS1000 chemiluminesence imager.

**RESULTS AND DISCUSSION**

The current study aimed to determine whether class II PI 3-kinases are activated following insulin stimulation. Initial studies found that all the cell types used in the current study express PI3K-C2α (data not shown). Insulin causes a 2–3-fold increase in the PI 3-kinase activity present in PI3K-C2α immunoprecipitates from CHO-IR cells, L5L6 myotubes, and 3T3-L1 adipocytes but not in immunoprecipitates from 3T3-L1 preadipocytes, undifferentiated L5L6 myoblasts, or HEK 293 cells (Fig. 1). In the case of 3T3-L1 cells, the levels of PI3K-C2α protein and PI 3-kinase activity in PI3K-C2α immunoprecipitants from fibroblasts into adipocytes were very similar, increasing only 2-fold during differentiation. Therefore the lack of insulin stimulation of PI3K-C2α in 3T3-L1 fibroblasts is not due to a deficiency of PI3K-C2α. To show that the observed activation of PI3K-C2α was not an artifact of the antibody used, Glu-tagged PI3K-C2α was transiently expressed in CHO-IR cells, and the expressed protein was specifically immunoprecipitated via the epitope tag. The transiently transfected PI3K-C2α was stimulated by insulin to a similar extent to that of the endogenous enzyme (data not shown). Of the cells studied only the human-derived HEK 293 cells express detectable levels of PI3K-C2β (data not shown). However, as for PI3K-C2α, insulin did not cause an increase in the activity of the endogenous PI3K-C2β in HEK293 cells (Fig. 1). Therefore PI3K-C2β was transiently expressed in CHO-IR cells to study the effects of insulin on this isoform and to compare it with the effects on PI3K-C2α. In CHO-IR cells insulin consistently induced only a very small (1.22-fold ± 0.12) increase in the activity of the PI 3-kinase (Fig. 1). One explanation for this is that PI3K-C2β may be genuinely less able to be activated by insulin, although an alternative explanation could be that insulin is activating only a small part of the total pool of PI3K-C2β. It is also possible that because PI3K-C2β is not normally expressed in the CHO cells, the cellular machinery necessary for its activation may be absent. Therefore these data do not preclude the possibility that insulin or other growth factors may activate PI3K-C2β to a greater extent in appropriate cell types. However, as insulin clearly activated the cellular pool of PI3K-C2α, we chose to further study this isoform.

The insulin-induced increase in PI 3-kinase activity associated with the PI3K-C2α immunoprecipitates was rapid reaching nearly maximum activation within 1 min (Fig. 2A). We also...
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Serum-starved CHO-IR cells were stimulated with 100 nM insulin for 5 min, and cells were then lysed and immunoprecipitated with PI3K-C2a antibody. The PI 3-phosphate product was shown. A, PI 3-kinase activity was assayed in the immunoprecipitates in the presence of varying concentrations of wortmannin as indicated, and radiolabeled lipid product was separated by TLC and visualized using a phosphorimager. The PI 3-phosphate product is shown. B, a quantitation of the PI3-kinase activity in immunoprecipitates from the insulin-stimulated CHO-IR cells above. C, CHO-IR cells transfected with PI3K-C2a and Western blotted with PY99 antibody. Where indicated samples were incubated with protein phosphatase as described under “Experimental Procedures.” C, HEK 293 cells were immunoprecipitated with PI3K-C2a and blotted with PI3K-C2a antibody. Bands corresponding to PI3K-C2a are shown. D, CHO-IR cells transfected with PI3K-C2a, immunoprecipitated with PI3K-C2a antibody, and blotted with PI3K-C2a antibody. Bands corresponding to PI3K-C2a are shown. Ins, insulin.

find that the activation was observed at doses as low as 1 nM insulin with near full activation at 10 nM insulin in CHO-IR cells (Fig. 2B). This indicates that the activation of PI3K-C2a is likely to occur in physiological contexts. We next used the inhibitor wortmannin to determine whether contamination by class Ia PI 3-kinases or PI3K-C2a is contributing to the insulin stimulation of PI 3-kinase activity in PI3K-C2a immunoprecipitates. The class Ia PI 3-kinases and PI3K-C2a are at least 1 order or magnitude more sensitive to wortmannin than the PI3K-C2a (7, 8, 10). Our studies show that there is no contamination by class Ia PI 3-kinases or PI3K-C2b in PI3K-C2a immunoprecipitates because the PI 3-kinase activity found in PI3K-C2a immunoprecipitates from basal or insulin-stimulated cells was resistant to wortmannin (IC50 > 100 nM), whereas the PI 3-kinase activity present in antiphosphotyrosine or anti p85 immunoprecipitates had the characteristics of class Ia PI 3-kinase activity because it was sensitive to wortmannin (IC50 = ~10 nM) (Fig. 3). Overall these results provide strong evidence that insulin actually increases the catalytic activity of the PI3K-C2a in CHO-IR cells, L5L6 myotubes, and 3T3-L1 adipocytes.

The finding that growth factors activate class II PI 3-kinases immediately raises the question of how this occurs. Binding of Ca2+ or phospholipid to the C2 domains can play a role in regulating protein function (16), making this a potential candidate to explain growth factor regulation of class II PI 3-kinases. Indeed there is evidence that this domain is involved in regulating the activity of class II PI 3-kinases because deletion of this domain in PI3K-C2b increases catalytic activity (10). Further, a recent report has indicated that PI3K-C2b is activated following platelet aggregation by a mechanism that is blocked by addition of calpain inhibitors, suggesting a calcium regulated pathway (14). However, it appears unlikely that a similar mechanism operates in the case of insulin activation because we only see a very weak activation of PI3K-C2b by insulin. Further we find calpain inhibitors do not block the ability of insulin to activate PI3K-C2a (data not shown), and previous studies demonstrate that insulin does not acutely increase cytosolic free calcium levels (17–19), making it unlikely that a calcium-dependent mechanism is involved. It has also been reported that the chemokine MCP-1 activates PI3K-C2a via a Gαi-linked signaling pathway (13). However, it is unlikely that the insulin-induced activation of PI3K-C2a acts via this mechanism because there is no evidence that insulin acts via heterotrimeric G-proteins. Therefore, the mechanism by which insulin activates PI3K-C2a is unclear.

In the case of class Ia PI 3-kinases, insulin stimulation causes phosphorylation of tyrosines in YMXM motifs in IRS proteins that results in SH2 domain-mediated recruitment of the PI 3-kinase. This interaction both increases the activity of the PI 3-kinase (20, 21) and targets the PI 3-kinase to specific locations in the cell (15, 22–24). It is clear that PI3K-C2a does not form a tight complex with the insulin receptor because in PI3K-C2a immunoprecipitates from insulin-stimulated cells no tyrosine phosphorylated bands are observed at 95 kDa, which would correspond to the insulin receptor (Fig. 4A). However, these co-immunoprecipitation experiments do suggest that the PI3K-C2a associates with two tyrosine phosphorylated proteins, one of 207 kDa and the other of 160 kDa (Fig. 4A). The intensity of the 207-kDa phosphotyrosine band is similar in both basal and insulin-stimulated cells (Fig. 4A), suggesting that regulating the tyrosine phosphorylation level of this protein by the insulin receptor is not the mechanism by which insulin controls the activity of PI3K-C2a. The identity of the 207-kDa phosphoprotein is currently unknown, but the fact that the *Drosophila* homologue of PI 3-kinase C2 also associates with a tyrosine phosphorylated protein of a similar size (6) indicates this may represent a protein important in the regulation of members of the class II PI 3-kinases. The 160-kDa tyrosine phosphorylated protein is only associated with PI3K-C2a following insulin stimulation, suggesting that it could play a role in regulating the activity of this enzyme. At first appearance the size of the band suggested it might represent IRS-1, although several lines of evidence suggest that this is not the case. Firstly, although we clearly see an increase in wortmannin-sensitive PI 3-kinase activity in antiphosphotyrosine immunoprecipitations after insulin stimulation (Fig. 3A), no significant amount of wortmannin-resistant PI 3-kinase activity is observed in anti-phosphotyrosine immunoprecipitates from either basal or insulin-stimulated cells. Because the activity in
P13K-C2α is clearly resistant to wortmannin (Fig. 3), this suggests that P13K-C2α is not recruited to the same complexes as class Iα PI 3-kinase. Secondly, Western blotting revealed that no IRS-1 or IRS-2 was present in the P13K-C2α immunoprecipitates from stimulated CHO-IR cells (data not shown), and transient transfection of CHO-IR cells with a Myc-tagged IRS-1 construct and subsequent immunoprecipitation of IRS-1 via the Myc tag also failed to reveal any association of P13K-C2α with IRS-1 (data not shown). The above clearly indicates that the mechanism of activation of P13K-C2α is distinct from that of the class Iα PI 3-kinases. We cannot exclude the possibility that the 207- and 160-kDa tyrosine phosphorylated proteins may play a role in regulating the activity of P13K-C2α, but further progress on this issue will await the identification of these proteins.

Immunoblots of P13K-C2α immunoprecipitates blotted back with P13K-C2α antibody consistently revealed two bands of 193 and 195 kDa, respectively, in all cell types tested (Fig. 4B). One possibility is that these could represent a bandshift due to the presence of two differently phosphorylated forms of the P13K-C2α. This is supported by the finding that in CHO-IR cells insulin causes the intensity of the lower band to decrease and the intensity of the upper band to increase (Fig. 4B). To quantitate this, the results of seven observations from four independent experiments were compared by calculating the intensity of each band as a percentage of the combined intensity of the upper and lower bands. This showed that in the basal state 27 ± 4% of P13K-C2α immunoreactivity was in the upper band, and following insulin stimulation this increased to 46 ± 3%. This represents a 1.7-fold increase in the proportion of P13K-C2α in the higher molecular weight form. The phosphatase treatment of the immunoprecipitates from insulin-stimulated cells returned the ratio of the bands back to that seen in the basal state (Fig. 4B). Identical results were seen in 3T3-L1 adipocytes (not shown). In HEK293 cells P13K-C2α activity did not increase with insulin stimulation (Fig. 1). In these cells a significant proportion of the P13K-C2α was present in the upper band in unstimulated cells, and there was no appreciable change in the ratio of the upper and lower bands following insulin stimulation (Fig. 4C). Therefore the changes in the proportion of P13K-C2α in the upper band in response to insulin correlates well with changes in the activity of P13K-C2α, with a band shift being observed in cells in which P13K-C2α was stimulated but not in those in which it was not. Conversely, Western blots of P13K-C2β from unstimulated cells show only a single band, and this was not altered by insulin stimulation of CHO-IR cells transiently transfected with P13K-C2β (Fig. 4D).

In summary these results clearly indicate that insulin causes a rapid activation of P13K-C2α, which is consistent with a role in downstream signaling. Further, the mechanism of activation of P13K-C2α differs from that of class Iα PI 3-kinases and may involve insulin-induced phosphorylation of the P13K-C2α. Given the important role of D-3 phosphoinositides in insulin signaling, these findings suggest that P13K-C2α is a novel downstream effector in insulin signaling cascade, although the end point effects that this is involved in regulating remain unknown.

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