# The α-isoform of class II phosphoinositide 3-kinase is more effectively activated by insulin receptors than IGF receptors, and activation requires receptor NPEY motifs

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**Abstract Little is known about the physiological role and mechanism of activation of class II phosphoinositide 3-kinases (PI3Ks), although it has been shown that the PI3K-C2α isoform is activated by insulin. Using chimaeric receptor constructs which can be activated independently of endogenous receptors in transfected cells, we found that PI3K-C2α activity was stimulated to a greater extent by insulin receptors than IGF receptors in 3T3-L1 adipocytes. Activation of PI3K-C2α required an intact NPEY motif in the receptor juxtamembrane domain. We conclude that PI3K-C2α is a candidate for participation in insulin-specific intracellular signalling.**


*Key words:* Insulin; Insulin-like growth factor; Receptor; Phosphoinositide 3-kinase; C2α; 3T3-L1 adipocyte

## 1. Introduction

A great deal of evidence has accumulated to indicate that phosphoinositide 3-kinase (PI3K) activity is a necessary component in the signalling pathways emanating from receptor tyrosine kinases [1]. There are four distinct classes of enzymes with PI3K activity, two of which are known to be activated by receptor tyrosine kinases [2]. The three isoforms of the catalytic subunits of class-Ia PI3K associate with adapter subunits containing two SH2 domains and are activated when these interact with phosphotyrosines in specific sequence motifs. Three class-II PI3K isoforms have been identified, which lack adapter subunits and SH2 domains but are characterised by the presence of a C-terminal C2 domain. We have recently shown that the α isoform of class-II PI3K (PI3K-C2α) can be activated by insulin in cells with high numbers of insulin receptors [3]. However, even high concentrations of insulin produced little stimulation of PI3K-C2α in 3T3-L1 fibroblasts. This is somewhat surprising as these cells have significant numbers of IGF-I receptors which should be activated by high concentrations of insulin. In general insulin and IGF-I induce very similar metabolic responses in cells [4], although we have recently demonstrated that their receptors activate certain pathways to different extents [5]. Therefore our finding that PI3K-C2α is not activated by high doses of insulin in 3T3-L1 fibroblast suggests that the insulin receptor (IR) may be more effective than the type I IGF-I receptor (IGFR) in activating this form of PI3K.

The mechanism by which insulin activates class-II PI3Ks is not known. PI3K-C2α is not itself tyrosine phosphorylated but it does associate with a 160 kDa protein that becomes tyrosine phosphorylated after insulin stimulation in HEK293 cells [3]. One clue to the mechanism of activation may be provided by a sequence homology identified between the carboxy-terminal region of PI3K-C2α and the phosphotyrosine binding (PTB) domain of IRS 1 (M.D. Waterfield, personal communication) suggesting direct association with receptors might be involved in class-II PI3K activation. The tyrosine 960 on the IR and the corresponding tyrosine 950 on the IGFR, within NPEY sequence motifs, have been identified as binding sites for the PTB domains in SHC and IRS-1 [6–9].

We have recently developed chimaeric receptors, in which the extracellular portion of the TrkC receptor is linked to the intracellular portion of either the IR or IGFR, as tools to study mechanism and specificity in insulin/IGF-I signalling [5,10]. Addition of the TrkC ligand (NT-3) allows specific activation of either the IR or IGFR chimaeras without activation of endogenous receptors in transfected cells. Using this system we obtained evidence that insulin and IGF-I may activate distinct pools of class-Ia PI3K [5]. In the present study, we have used the chimaeric receptor system for a direct comparison of the efficacy of insulin and IGF-I receptors in activating class-II PI3K. We have also modified these chimaeric constructs by mutating tyrosine to phenylalanine at position 960 in the IR and 950 in the IGFR to determine whether these residues are required for stimulation of class-II PI3K.

## 2. Materials and methods

### 2.1. Chimaeras and cell lines

Chimaeras containing the extracellular and transmembrane portions of the neurotrophin receptor TrkC together with the intracellular portion of the human IR or IGFR (TIR and TIGR respectively) were constructed as previously described [10]. Point mutations (TIR Tyr960→Phe, TIR960; TIGR Tyr950→Phe, TIGR950) were created by Quick Change site directed mutagenesis kit (Promega), and the sequence verified using a Sequenase version 2.0 DNA sequencing kit (Amersham). The chimaeras were expressed under the regulation of the EF-1α promoter (a kind gift from Dr R.E. Lewis, Eppley Institute for Research in Cancer, University of Nebraska) in the vector

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*Abbreviations:* IGF, insulin-like growth factor; IR, insulin receptor; IGFR, type I IGF receptor; TIR, TrkC-insulin receptor chimaera; TIGR, TrkC-IGF receptor chimaera; IRS, insulin receptor substrate; PI3K, phosphoinositide 3-kinase; PTB, phosphotyrosine binding; NT-3, neurotrophin-3; PBS, phosphate-buffered saline

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pcDNA3 (Invitrogen). 3T3-L1 fibroblasts (from ATCC) were maintained at no higher than 80% confluence in DMEM containing 10% NCS, 4.5 g/l glucose, 2 mM glutamine and antibiotics (DMEM/NCS) and for differentiation they were grown as previously described [5]. Differentiated cells were used after 10–12 days and only when at least 90% of the cells showed adipocyte phenotype by accumulation of lipid droplets. Clones of 3T3-L1 fibroblasts expressing TIR and TIGR were as previously described and clones expressing the TIR960 and TIGR950 mutants were isolated at the same time.

*2.2. Antibodies*

Rabbit antibodies directed against the carboxy-terminal domains of the IR or IGFR were as previously described [5]. Rabbit antibody to PI3K-C2α was also as described [3]. Mouse monoclonal anti-phosphotyrosine antibody 4G10 was from NEB. HRP-linked secondary antibodies were from Dako and $^{125}$I-labelled secondary antibodies were prepared by in-house iodination of antibodies from Sigma.

*2.3. Receptor autophosphorylation*

Serum starved adipocytes were stimulated for 5 min and solubilised in lysis buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 30 mM NaF, 1% Triton X-100, 1 mM $Na_3VO_4$, 10 mM $Na_2P_2O_7$, 0.1 mM AEBSF, 2.5 mM benzamidine, 1 μg/ml antipain, 1 μg/ml leupeptin, 1 μg/ml pepstatin A) and the lysate clarified by centrifugation at 13 500×*g* for 15 min at 4°C. Crude lysates were resolved by SDS-PAGE before electroblotting to PVDF membranes (Millipore), and receptor autophosphorylation levels detected by incubation with anti-phosphotyrosine antibody in TBST buffer (150 mM NaCl, 50 mM Tris, 0.1% Tween 20), after blocking in 1% BSA, followed by $^{125}$I-labelled secondary antibodies (approximately 0.2 μCi per blot) and quantified on a Fujix BAS2000 Phosphorimager.

*2.4. Analysis of association between receptors and PI3K-C2α*

Lysates prepared as described for receptor autophosphorylation were subjected to immunoprecipitation using either anti-IR (1:100), anti-IGFR (1:100) or anti-PI3K-C2α (1:100) antibodies plus protein A-agarose (2 mg/sample). Immunoprecipitates were washed once in lysis buffer and twice in PBS in the cold, resolved by SDS-PAGE, and Western blotted onto PVDF membranes which were then probed with anti-PI3K-C2α or anti-phosphotyrosine antibodies as previously described [3]. The blots were developed by incubation with HRP-linked secondary antibodies and detection by enhanced chemiluminescence (ECL) and exposure against Hyperfilm MP autoradiography film (Amersham International).

*2.5. PI3K-C2α activity*

Cells were stimulated for 5 min, lysed and immunoprecipitated with anti-PI3K-C2α antibody. The immunoprecipitates were washed and assayed for lipid kinase activity as previously described [3], and the radiolabelled phosphatidylinositol 3-phosphate produced was quantified on a Fujix BAS2000 Phosphorimager.

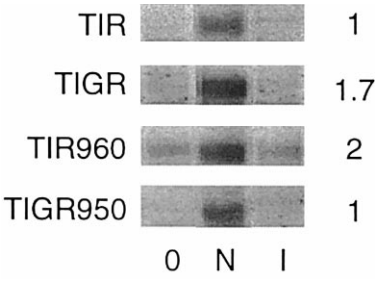


Fig. 1. Characterisation of chimaeras. Clonal lines of 3T3-L1 adipocytes as indicated were left unstimulated (0) or stimulated with 4nM NT-3 (N) or 100 nM insulin (I) for 5 min, lysed and resolved by SDS-PAGE before Western blotting with anti-phosphotyrosine antibody. The region of the blots containing the chimaeric receptors (approx. 160 kDa) is shown. Numbers indicate average amount of auto-phosphorylated receptor relative to TIR from three independent experiments.

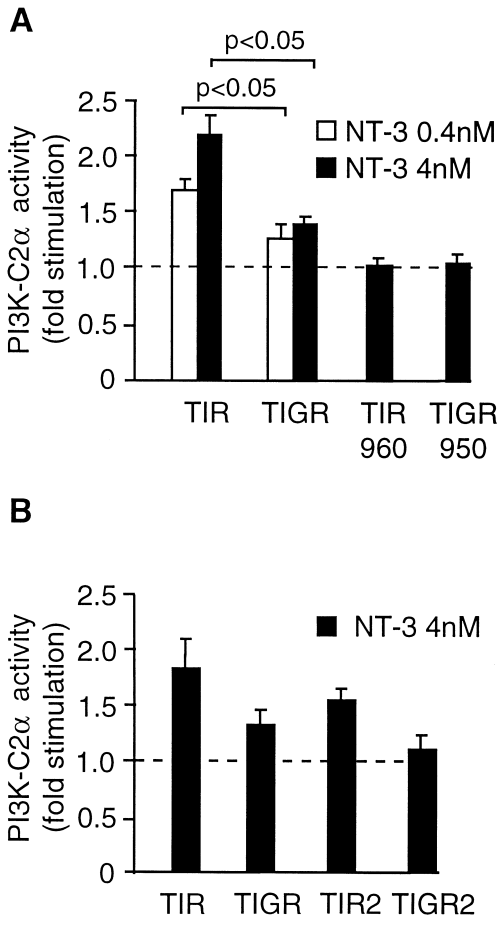


Fig. 2. PI3K-C2α activity. Experiments were conducted with clonal lines of 3T3-L1 adipocytes (A) or fibroblasts (B). Cells were left unstimulated, or stimulated with 0.4 nM NT-3 (white bars) or 4 nM NT-3 (black bars) or with insulin (A: 100 nM; B: 1 μM) for 5 min, before lysis and determination of lipid kinase activity in anti-PI3K-C2α immunoprecipitates. Results are presented as fold stimulation over basal and are means ± S.E.M. from 3–5 independent experiments performed in duplicate. Statistically significant differences between activities in TIR and TIGR cells are indicated. Fold stimulations with insulin were (A): TIR 1.82 ± 0.12; TIGR 1.69 ± 0.09; TIR960 1.64 ± 0.15; TIGR950 1.58 ± 0.05; and (B): TIR 1.58 ± 0.40; TIGR 1.35 ± 0.23; TIR960 1.43 ± 0.31; TIGR950 1.54 ± 0.19.

## 3. Results

*3.1. Expression of chimaeric receptors*

Clones of stably transfected 3T3-L1 fibroblasts were selected which expressed chimaeric receptors consisting of the extracellular portion of TrkC fused to TIR, TIGR, or the same receptors mutated within the juxtamembrane region at IR Tyr960→Phe (TIR960) or the corresponding IGFR Tyr950→Phe (TIGR950). Autophosphorylation of these tyrosine residues has been shown to be crucial for recruitment of receptor substrates, and mutation of these sites compromises signalling via IRS- and SHC-dependent pathways and downstream metabolic responses.

Cells were stimulated to differentiate into adipocytes, and the levels of chimaeras compared by performing anti-phosphotyrosine blotting to assess receptor autophosphorylation in NT-3-stimulated cells. The various clones expressed similar levels of chimaeras, within a factor of 2 (Fig. 1), and the

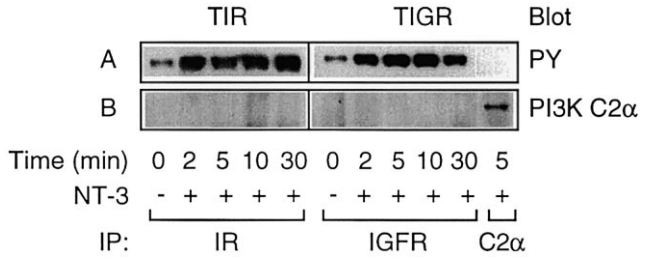


Fig. 3. Lack of association between PI3K-C2α and the IR or IGFR. Clonal lines of 3T3-L1 adipocytes were left unstimulated, or stimulated with 4nM NT-3 for 0–30 min as indicated. Cells were then lysed, immunoprecipitated (IP) with IR-, IGFR- or PI3K-C2α-specific antibody, resolved by SDS-PAGE, blotted onto PVDF membranes and the blots probed with phosphotyrosine (PY) or PI3K-C2α-specific antibody. The regions of the blots containing the chimaeric receptors (approx 160 kDa) (A) or PI3K-C2α (approx. 180 kDa) (B) are shown.

results of these blotting experiments were confirmed by binding studies with radio-iodinated NT-3 (data not shown).

### 3.2. Activation of PI3K-C2α

The capacity of IR and IGFR to mediate stimulation of class-II PI3K was assessed by measuring lipid kinase activity in anti-PI3K-C2α immunoprecipitates from chimaera-transfected adipocytes which had been stimulated with NT-3 or insulin (Fig. 2A). These studies focussed on the C2α isoform as we were unable to detect the C2β isoform in 3T3-L1 adipocytes [3] and expression of the C2γ isoform is restricted to liver [11]. In all cells (TIR, TIGR, TIR960, TIGR 950) PI3K activity in the anti-PI3K-C2α immune complex assay was stimulated 1.5–1.8-fold by insulin, acting through endogenous IR. In TIR cells, NT-3 at submaximally and maximally effective concentrations (0.4 and 4 nM) induced increases in PI3K-C2α activity which were comparable to or greater than those with insulin (2.2- and 1.7-fold respectively). However, in TIGR cells NT-3 was less effective than insulin in stimulating PI3K-C2α activity (1.3- and 1.4-fold at 0.4 and 4 nM NT-3), and compared to TIR cells this difference in effectiveness of NT-3 was statistically significant.

A very similar pattern of responses was observed when PI3K-C2α activity was studied in 3T3-L1 fibroblasts (Fig. 2B). In this case two independently derived pairs of matched TIR and TIGR clones were available, and in both pairs the stimulation of PI3K-C2α activity via TIR was greater than via TIGR. The level of PI3K-C2α activity in fibroblasts, as measured in immunoprecipitates and expressed per mg of total protein, was approximately half of that in adipocytes (data not shown) and the fold stimulations by insulin and NT-3 were also somewhat lower in fibroblasts than adipocytes.

No effect of NT-3 on PI3K-C2α activity was observed in adipocytes expressing chimaeras with juxtamembrane mutations (TIR960 and TIR950) (Fig. 2A). This lack of stimulation suggested either that the juxtamembrane tyrosine residues might be involved directly in PI3K-C2α recruitment, or indirectly in activation of PI3K-C2α by established IRS-dependent signalling pathways.

### 3.3. Association between PI3K-C2α and receptors

To test whether the PI3K-C2α associates directly with receptors, we stimulated chimaera-expressing adipocytes with NT-3 and analysed anti-receptor immunoprecipitates by anti-PI3K-C2α immunoblotting. PI3K-C2α was readily detected in anti-PI3K-C2α immunoprecipitates, but not in either anti-IR or anti-IGFR immunoprecipitates (Fig. 3). Conversely, phospho-receptor was readily detected by anti-phosphotyrosine blotting of anti-receptor immunoprecipitates, but no phospho-receptor was detected in anti-PI3K-C2α immunoprecipitates (Fig. 3).

## 4. Discussion

The use of chimaeric receptors has allowed us to extend our previous findings [3] and to demonstrate clearly that the IR is able to activate PI3K-C2α. This approach further allowed us to establish that activation of the IR causes a much greater increase in PI3K-C2α activity than does activation of the IGFR. Although the cellular roles of class-II PI3Ks are as yet unclear, this finding highlights yet another difference between IR and IGFR in their capacity to activate PI3Ks, our previous work having established that the receptors also differ in their capacity to stimulate class Ia (IRS-1-associated) PI3K activity [5]. These differences in the extent of PI3K stimulation are likely to result in divergence in signalling outcome and in metabolic effects such as the stimulation of glucose transport [1].

The present data also provide insights into the mechanism by which insulin regulates class-II PI3K activity. Mutation of the NPXY motifs on the IR and IGFR completely abrogated activation of the PI3K-C2α suggesting that association with a PTB domain-containing protein is required [12]. IRS proteins are candidates to play such a role, although there is no obvious mechanism by which class II PI3Ks and IRS proteins could interact directly. Indeed we have previously shown that PI3K-C2α does not associate with IRS-1 in insulin-stimulated cells, and that no tyrosine phosphorylated proteins of a size corresponding to IRS-1 or IRS-2 are observed in anti-PI3K-C2α immunoprecipitates [3]. Another possibility is that activation of PI3K-C2α requires signalling pathways initiated by IRS proteins, perhaps involving class Ia PI3K and downstream protein kinase cascades [1]. A final and intriguing possibility is the fact that PI3K-C2α itself possesses a putative PTB domain (M.D. Waterfield, personal communication) which might mediate direct association with the receptor. Although we were unable to detect PI3K-C2α in anti-IR immunoprecipitates, this does not rule out the occurrence of such an interaction within intact cells, as it has also proved difficult to demonstrate direct association between IRS proteins and the IR even though there is a large amount of indirect evidence that interaction of the IRS-1 PTB domain with the insulin receptor is essential for insulin signalling [13]. It is notable that the residues flanking NPEY motifs, which are responsible for fine specificity of interactions with PTB domains of IRS and SHC proteins [14,15], are conserved between the IR and IGFR. Therefore, whatever the nature of the PTB-receptor interaction underlying the activation of PI3K-C2α, it seems likely that additional mechanisms must be invoked to account for the relative specificity of IR compared to IGFR in mediating activation of PI3K-C2α.

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