Interaction of Wild Type and Dominant-Negative $p55^{PIK}$ Regulatory Subunit of Phosphatidylinositol 3-Kinase with Insulin-Like Growth Factor-1 Signaling Proteins

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In a first series of experiments done in the yeast two-hybrid system, we investigated the nature of protein-protein interaction between the regulatory subunit of phosphatidylinositol 3-kinase (PI 3-kinase), $p55^{PIK}$, and several of its potential signaling partners. The region between the Src homology 2 (SH2) domains of $p55^{PIK}$ bound to the NH_2 terminus region of p110α, as previously shown for p85α. Moreover, we found that the insulin-like growth factor-1 receptor (IGF-IR) bound to $p55^{PIK}$; the interaction occurred at the receptor tyrosine 1316 and involved both $p55^{PIK}$ SH2 domains. Interaction between $p55^{PIK}$ and IGF-IR was seen not only in the yeast two-hybrid system, but also using *in vitro* binding and coimmunoprecipitation of lysates from IGF-1 stimulated 293 cells overexpressing $p55^{PIK}$. Further, IGF-I stimulation of these cells led to tyrosine phosphorylation of $p55^{PIK}$. In 293 cells association of $p55^{PIK}$ with insulin receptor substrate-1 and with IGF-IR was dependent on PI 3-kinase, since it was increased by wortmannin, an inhibitor of PI 3-kinase. Further, by deleting amino acids 203–217 of $p55^{PIK}$ inter-SH2 domain, we engineered a $p55^{PIK}$ mutant unable to bind to the p110α catalytic subunit of PI 3-kinase. This mutant had a dominant-negative action on insulin-stimulated glucose transport, since insulin's effect on Glut 4 myc translocation was inhibited in adipocytes expressing mutant $p55^{PIK}$. Importantly, this dominant-negative mutant was more efficient than wild type $p55^{PIK}$ in associating to IGF-IR and insulin receptor substrate-1 in 293 cells. Taken together, our results show that $p55^{PIK}$ interacts with key elements in the IGF-I signaling pathway, and that these interactions are negatively modulated by PI 3-kinase itself, providing circuitry for regulatory feedback control. (Molecular Endocrinology 11: 1911–1923, 1997)

INTRODUCTION

Phosphatidylinositol 3-kinase (PI 3-kinase) is a heterodimeric enzyme consisting of an 85-kDa regulatory subunit (p85) and a 110-kDa catalytic subunit (p110) with dual specificity, *i.e*. lipid kinase and serine kinase activity (1, 2). Stimulation of the p110 lipid kinase activity leads to production of 3-phosphoinositides. While the precise role of these lipid products is still unclear, it is believed that they could act as second messengers that deliver specific signals in the cell. Recently, Cantley *et al.* reported that phosphatidylinositol-(3,4,5)-triphoshate is able to bind to Src homology 2 (SH2) domains of p85 PI 3-kinase and of $pp60^{c\text{-}src}$, suggesting a role in the control of SH2 domain binding to phosphoproteins (3). In neuron-like PC12 cells, PI 3-kinase is necessary for neurite outgrowth and prevention of apoptosis upon nerve growth factor (NGF) stimulation (4). Moreover, PI 3-kinase appears to be involved in insulin-induced actin rearrangement, $pp70^{S6k}$ activation, and translocation of glucose transporters Glut4 (5–9).

p85 carries in its C-terminal part two SH2 domains separated by an inter-SH2 (IS) domain. These three domains are highly conserved between the different p85 isoforms, suggesting that they are likely to play an important role. Indeed, the IS domain is the binding

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site for the N terminus of p110, and SH2 domains are high-affinity binding sites for the phosphorylated motifs, pYXXM, occurring in tyrosine kinase receptors and receptor substrates. This suggests that binding of p85 SH2 domains to phosphotyrosine-containing sequences leads to conformational changes in the protein. These structural modifications would be transmitted to p110 through its association with the IS domain of p85, resulting in p110 activation (10, 11).

Recently, two novel regulatory subunits of PI 3-kinase have been cloned: 1) p55α, which corresponds to an alternatively spliced form of p85α (12, 13), and 2) p55^{PIK} (14). These newly identified proteins have, in common with p85, a highly conserved region comprising the two SH2 domains separated by an IS domain and differ from it in their NH_2 terminus. Indeed, the Bcr, the SH3 domains, and one of the proline-rich motifs found in p85 isoforms are absent in p55α and p55^{PIK} and are replaced by a unique sequence of 34 amino acids with a pronounced homology between the two p55 proteins. With regard to the tissue distribution of the two newly disclosed PI 3-kinase-regulatory subunits, they are found in most tissues. However, p55α is more abundant in brain and muscle (13), whereas p55^{PIK} expression is the highest in brain and testis (14).

Insulin-Like Growth Factor-1 (IGF-I) binding to its transmembrane receptor (IGF-IR) leads to receptor autophosphorylation and phosphorylation of intracellular substrates including insulin receptor substrate-1 (IRS-1) (15). It is generally believed that, upon IGF-I stimulation, the major pathway leading to activation of p110 PI 3-kinase is triggered by binding of p85 SH2 domains to tyrosine-phosphorylated IRS-1 (16, 17). However, PI 3-kinase is also able to bind directly to the IGF-IR (18–21). Indeed, the carboxy-terminal tyrosine 1316 of IGF-IR, contained in the YAHM sequence, is a potential binding site for p85 PI 3-kinase, but the role of this association is still unclear.

The newly identified regulatory subunits of PI 3-kinase, p55^{PIK}, and IGF-I receptors are both highly expressed in brain, especially in the early stages of development (14, 22). Therefore, we speculated that p55^{PIK} could act as a downstream effector in the IGF-IR-signaling pathway. In the present study, we wished to characterize in the yeast two-hybrid system the interaction of p55^{PIK} with a series of potentially associated proteins. We also investigated whether p55^{PIK} was associated with these molecules in intact mammalian cells and whether PI 3-kinase modulated these associations. Finally, we engineered a mutant of p55^{PIK} unable to bind to p110 and examined its effect on insulin-stimulated glucose transport and association with IGF-IR-signaling proteins in mammalian cells.

RESULTS

Interaction of p55^{PIK} and its subdomains with different proteins was first investigated using the yeast two-hybrid system. To do so, we engineered several hybrid proteins with either the bacterial repressor LexA, which contains a DNA-binding domain (LexA), or the activation domain of the transcription factor GAL4 (GAD). Both types of hybrids were coexpressed in the yeast strain L40. When these domains fused to LexA and GAL4 are able to interact, a functional transcription factor is reconstituted that recognizes the LexA upstream-activating sequences contained in the reporter genes LacZ and HIS3. The specificity of our system was evaluated by testing all our hybrids with unrelated proteins (LexA-lamin and GAD-RAF). In these instances, no reporter gene activity was detected (data not shown). Taking advantage of the hemagglutinin epitope, we also verified that p55^{PIK} and its different subdomains in fusion with GAD were expressed in yeast (not shown).

Mapping of the p110α and p55^{PIK} Domains Involved in Interaction between Both Proteins

To study the interaction of p110α with p55^{PIK} in the yeast two-hybrid system, the entire protein and several constructs of p110α in fusion with LexA were cotransformed with p55^{PIK} fused to GAL4 activation domain (GAD-p55^{PIK}). Figure 1 shows the β-galactosidase activity obtained for each cotransformation, which reflects the transcriptional level of the reporter gene LexA-LacZ. p110α strongly interacted with p55^{PIK}, since their coexpression led to a high level of β-galactosidase activity ($\sim$5000 U). Increased deletions in the p110α C-terminal domain from amino acids 1068 to 870 (BP), to 576 (BX), and to 127 (R1) reduced interaction with p55^{PIK}, since the β-galacto-

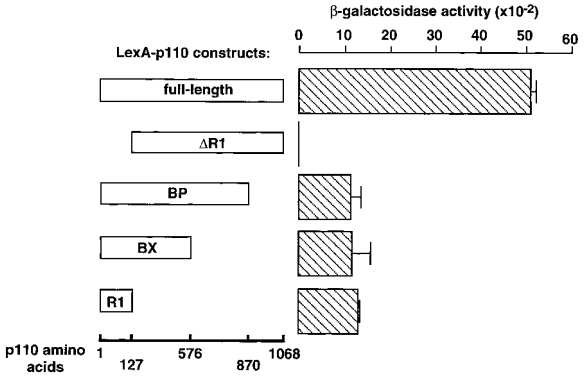

Fig. 1. Mapping of p110α Subdomains Interacting with p55^{PIK}

The yeast reporter strain L40 was cotransformed with cDNAs encoding GAD-p55^{PIK} and the indicated LexA-p110α constructs, which were obtained as described in *Materials and Methods*. Double transformants were grown in liquid culture, and transactivation of the reporter gene LacZ was assayed by measuring β-galactosidase activity and using the substrate chlorophenol red-β-D-galactopyranoside. β-Galactosidase units were determined according to Miller (42). Values represent the average $\pm$ SE of three independent transformants. One representative experiment of three is shown.

Table 1. Construction of Fusion Proteins between GAL4 Activation Domain and Domains of p55PIK

p55PIK	Amino Acid Sequence	Primers 5′	Primers 3′
Full-length	1–454	A	I
NH2	1–51	A	B
nSH2	52–160	C	D
IS	154–348	E	F
cSH2	348–447	G	H
nSH2 IS	52–348	C	F
IS cSH2	154–447	E	H
nSH2 IS cSH2	52–447	C	H

Complementary DNAs coding for full-length p55PIK and its subdomains were amplified by PCR using appropriate primers, and then inserted in-frame into the polylinker of pACTII yeast expression vector. The different subdomains are schematized, and their corresponding amino acids sequence is listed. The following primers (5′ to 3′) were used for PCR: A, catgccatggcgatggaccgcgatgacgc; B, ccggaattcaatgaaactgtccttcattcc; C, catgccatggcgtctcttcaagatgcag; D, ccggaattcctgatcctgttggaatctgg; E, catgccatggcgtccagattccaacagg; F, ccggaattcctcatcataatgcggcagg; G, catgccatggcggagaaaacctggtttgtg; H, ccggaattctgcatggacagggtaggc; I, ccggaattcttatctgcagagcgtaggc.

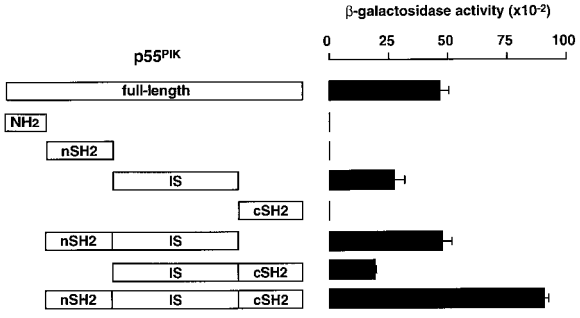

Fig. 2. Mapping of p55PIK Subdomains Interacting with p110α

The LexA-p110α fusion protein was coexpressed with GAD-p55PIK and a series of GAD-p55PIK deletion mutants that were obtained as described in Table 1. After selection of the cotransformants on selective plates and growth in a liquid selection medium, β-galactosidase activity was measured as described in *Materials and Methods*. One experiment of three is shown and data represent the average ± SE of three isolated colonies.

sidase activity was decreased by about 5-fold for the three constructs. Moreover, when GAD-p55PIK was coexpressed with p110α deleted from amino acids 1–127 (LexA-p110ΔR1), no β-galactosidase activity was measured. These results demonstrate that the N-terminal domain of p110α is absolutely required for interaction with p55PIK but that additional sequences in the C terminus are also involved.

Next we mapped the region of p55PIK responsible for its interaction with p110α. LexA-p110α was coexpressed with the different subdomains of p55PIK fused to GAD (Table 1). Figure 2 illustrates the β-galactosidase activity obtained for each cotransformant. No activity was detected when p110α was expressed with the isolated p55PIK N terminus, or amino-terminal SH2 (nSH2), or carboxy-terminal SH2 (cSH2) domains. In contrast, the full-length protein as well as the constructs containing the IS domain (IS alone, nSH2/IS, IS/cSH2, and IS flanked with both n- and c-SH2 domains, nSH2/IS/cSH2) gave a β-galactosidase activity varying from 2000–9000 U. Note that increased β-galactosidase activity seen with GAD-nSH2/IS/cSH2, compared with other constructs, was due to higher expression level of this hybrid protein (not shown). Our data thus show that the different hybrid proteins containing p55PIK IS domain are able to interact with p110α. Since IS is the only domain able to bind by itself to p110α, this region appears to be necessary and sufficient to make possible interaction between p110α and p55PIK. We also cotransformed the N-terminal domain of p110α (p110RI) and the IS domain of p55PIK and found that both isolated domains are sufficient to create an interaction (not shown).

We conclude that p55PIK binds to the amino-terminal domain of p110α, comprising amino acids 1–127. For p55PIK, this interaction occurs through its IS domain, corresponding to amino acid sequence 154–348.

In Vitro Association of p55PIK to the IGF-IR

Next we investigated whether p55PIK interacts with the IGF-IR. To address this issue, we produced ^{35}S-labeled p55PIK by *in vitro* translation. We also prepared partially purified IGF-IR fixed on wheat germ agglutinin (WGA) beads, which were autophosphorylated or not with unlabeled ATP. ^{35}S-labeled p55PIK was then incubated with IGF-IR for 3 h at 4 C, and pellets were extensively washed. Finally, the samples were submitted to SDS-PAGE and autoradiographed (Fig. 3). ^{35}S-

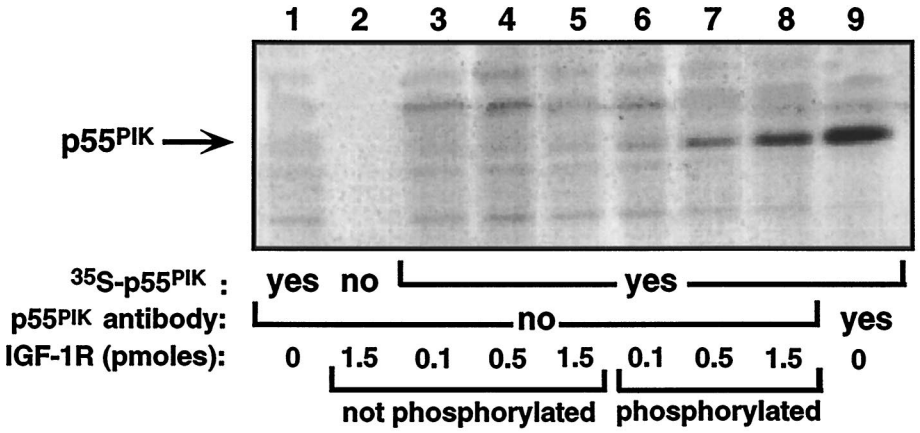

Fig. 3. *In vitro* Association of IGF-IR with p55^{PIK}

Different quantities of WGA-purified IGF-IR were adsorbed on WGA-coupled agarose beads and autophosphorylated or not with unlabeled ATP. ^{35}S-labeled p55^{PIK} protein was prepared by performing an *in vitro* transcription/translation and added to either pellets containing phosphorylated or unphosphorylated IGF-IR or protein A-Sepharose beads preincubated with antibodies to p55^{PIK}. After 3 h at 4 C, samples were extensively washed and subjected to SDS-PAGE under reducing conditions. A representative autoradiogram of three independent experiments is shown.

Labeled p55^{PIK} incubated with beads alone reflected the experimental background, which corresponds to nonspecific binding (lane 1). In lane 9, ^{35}S-labeled p55^{PIK} was immunoprecipitated with an antibody to p55^{PIK} as a positive control. Compared with lane 1, we observed a specific band that we identified as p55^{PIK} given its appropriate electrophoretic mobility. Regardless of the quantity of receptors, when [^{35}S]p55^{PIK} was incubated with unphosphorylated IGF-IR, no labeled species was retained (lanes 3–5). In contrast, even with the lowest quantity of phosphorylated IGF-IR (0.1 pmol), we could detect a signal corresponding to p55^{PIK} (lane 6). This indicates that a fraction of labeled p55^{PIK} bound to the phosphorylated IGF-IR. Moreover, p55^{PIK} associated to the receptor in a dose-dependent manner, since we could visualize more associated p55^{PIK} when the amount of phosphorylated IGF-IR was increased (lanes 6–8). Our results demonstrate that p55^{PIK} bound to the IGF-IR *in vitro* and that this process requires receptor autophosphorylation.

Interaction of p55^{PIK} with the IGF-IR in the Yeast Two-Hybrid System and Role of the Receptor Kinase and Autophosphorylation Sites in This Interaction

Cotransformants expressing GAD-p55^{PIK} and LexA-IGF-IRβ (wild type and mutant forms of the receptor β-subunit) were tested for their ability to give blue colonies on a X-gal filter assay (Table 2). We previously showed that the IGF-IRβ hybrid expressed in L40 corresponds to a constitutively active receptor (23). When p55^{PIK} was coexpressed with wild type IGF-IR, β-galactosidase activity was strong, indicating binding of p55^{PIK} to IGF-IR. This interaction was dependent on the receptor tyrosine kinase since yeast failed to give X-gal activity with kinase-deficient IGF-IR, in which lysine 1003 has been replaced by threonine (LexA-IGF-IRβ K1003T). These results are in agreement with those found for *in vitro* binding experiments described above (Fig. 3).

Table 2. Role of the IGF-IR Kinase and of its Autophosphorylation Sites in Association with p55^{PIK}

Expression of GAD-p55^{PIK} with LexA-IGF-1Rβ Constructs	β-Galactosidase Activity
Wild type	Strong
K1003T	Absent
Y1316F	Weak
Y950F	Strong
Y950F/Y1316F	Weak
Y1131F	Strong
Y1135F	Strong
Y1136F	Strong
Y1131F/Y1135F	Absent
Y1135F/Y1136F	Weak
Y1131F/Y1135F/Y1136F	Absent

The yeast reporter strain L40 was cotransformed with pACTII plasmid encoding the GAD-p55^{PIK} fusion protein and pBTM116 plasmid encoding wild type and the indicated mutant LexA-IGF-1Rβ hybrid proteins. Double transformants were isolated on selective plates. Transcription of the reporter gene LacZ was monitored by evaluating the β-galactosidase activity, using the substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside in a filter assay. "Strong" and "weak" reflect the relative intensity of the β-galactosidase activity. "Absent" indicates that no enzymatic activity was detectable. Similar results were obtained for activation of the reporter gene HIS3, which was estimated by observing, for each cotransformant, the His$^+$ phenotype on His$^-$ plates.

The IGF-IR autophosphorylation sites corresponding to juxtamembrane tyrosine 950 and C terminus tyrosine 1316 have been implicated in binding cytosolic proteins. Indeed, tyrosine 950 binds to the phos-

photyrosine binding (PTB) domain of IRS-1, IRS-2, and Shc (23, 24), whereas tyrosine 1316 is the binding site for p85α of PI 3-kinase (19) and for phosphotyrosine phosphatase SHP-2 (25). Compared with wild type receptor, the IGF-IR mutant Y950F displayed the same level of β-galactosidase activity, indicating that interaction with p55PIK was not affected by the tyrosine 950 mutation. Interestingly, interaction was dramatically decreased with the Y1316F and with the Y950F/Y1316F mutants. Next, we examined the involvement of the three major IGF-IR autophophosphorylation sites, tyrosines 1131, 1135, and 1136, located in the kinase domain. As shown in Table 2, the β-galactosidase activity, seen with yeast expressing p55PIK and the IGF-IR singly mutated on tyrosine 1131, 1135, and 1136, was similar to that obtained with wild type receptor. This suggests that, taken individually, none of these three autophosphorylation sites is involved in p55PIK binding. However, IGF-IR mutated on two tyrosines (LexA-IGF-IRβ Y1131F/Y1135F or Y1135F/Y1136F) or on three tyrosines (LexA-IGF-IRβ Y1131F/Y1135F/Y1136F) could interact poorly, if at all, with p55PIK. These sites have been shown to be essential for kinase activity, since phosphorylation of the three sites is necessary to obtain a fully active receptor (26–28). Mutation of only one site is unlikely to be sufficient to impair receptor kinase activity, so that interaction with p55PIK is unchanged. In contrast, when more than one site is mutated, receptor tyrosine kinase activity is severely decreased (two sites) or abolished (three sites). This leads to a loss in p55PIK binding, which is very likely to be due to a loss in phosphorylation of receptor tyrosine 1316.

Taken together, our data suggest that 1) IGF-IR tyrosine kinase activity is required for receptor binding to p55PIK; 2) IGF-IR C-terminal tyrosine 1316 is the major p55PIK binding site to the receptor, and 3) the receptor kinase domain autophosphorylation sites are probably not directly implicated as part of a binding surface, but are necessary for the modulation of IGF-IR autophosphorylation.

Mapping of the p55PIK Domain Responsible for Its Interaction with the IGF-IR β-Subunit

The p55PIK constructs depicted in Table 1 were tested for their ability to interact with wild type IGF-IR. The β-galactosidase activity measured with cotransformants expressing LexA-IGF-IRβ and GAD-p55PIK constructs is shown in Fig. 4. In contrast to the full-length protein, the p55PIK NH_2-terminus and IS domains failed to interact with IGF-IR. Moreover, isolated nSH2 and cSH2 domains and constructs containing at least one of the two SH2 domains in combination with the IS domain (nSH2/IS, IS/cSH2 and nSH2/IS/cSH2), were able to associate with the receptor. As was previously observed in Fig. 2, GAD-nSH2/IS/cSH2 gave a stronger β-galactosidase activity, compared with those obtained with other fusion proteins. This can be explained by a stronger expression in yeast of GAD-nSH2/IS/cSH2, compared with other p55PIK fusion proteins. These results demonstrate that both nSH2 and cSH2 domains of p55PIK are binding sites for the IGF-IR β-subunit. Additional experiments with IGF-IR mutated on tyrosine 1316 coexpressed with different subdomains of p55PIK allowed us to confirm that this tyrosine was the target for both SH2 domains, since we were unable to detect β-galactosidase activity in yeast coexpressing the Y1316F IGF-IR and isolated nSH2 or cSH2 domains (data not shown). However, a residual interaction could be observed with full-length and nSH2/IS/cSH2 constructs, suggesting that p55PIK could associate to another site on the IGF-IR, albeit with very low efficiency compared with binding to tyrosine 1316.

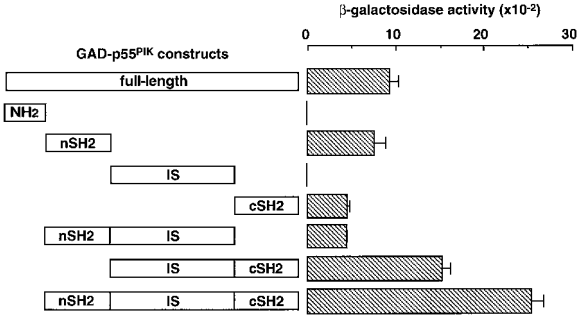

Fig. 4. Mapping of p55PIK Subdomains Interacting with IGF-IR

Hybrid constructs between GAD and p55PIK or its subdomains were obtained as described in Table 1. These fusion proteins were coexpressed with LexA-IGF-IRβ in the yeast reporter strain L40. Activity of the reporter gene LacZ was assayed by measuring β-galactosidase activity as described in *Materials and Methods*. Values calculated according to Miller (42) are the mean ± SE of one representative experiment done in triplicate, *i.e.* with three independent cotransformants.

Association of p55PIK with IGF-IR in Intact Mammalian Cells

We examined whether in intact mammalian cells p55PIK was able to associate with IGF-IR-signaling molecules. Therefore, 239 EBNA cells expressing p55PIK were stimulated with IGF-I, and cell extracts were subjected to immunoprecipitation with antibodies to p55PIK. Immunoblots with antiphosphotyrosine antibodies revealed three proteins with a molecular mass of approximately 170, 95, and 50 kDa (Fig. 5). No proteins were detected when cell extracts were subjected to immunoprecipitation with nonimmune serum, or when the experiment was performed with nontransfected cells, suggesting that the phosphoproteins seen in transfected cells after IGF-I stimulation were due to the presence of p55PIK. Using blotting with specific antibodies, we identified the molecular species of 170, 95, and 50 kDa as being IRS-1, IGF-IR β-subunit, and p55PIK, respectively (data not shown).

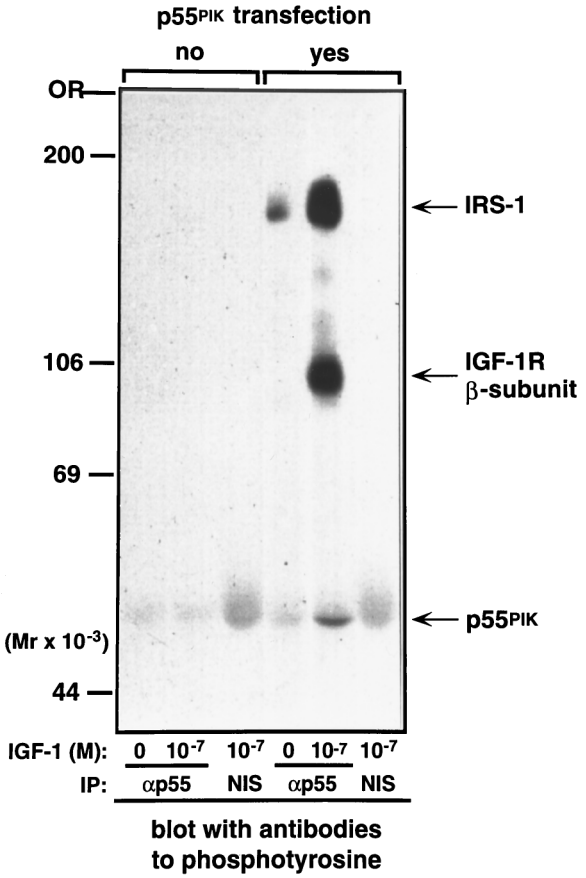

Fig. 5. Association of Tyrosine-Phosphorylated Proteins with $p55^{PIK}$ in Mammalian Cells

$p55^{PIK}$-expressing or nonexpressing 293 EBNA cells were treated or not with 10^{-7} M IGF-I for 5 min. After cell lysis, extracts were submitted to immunoprecipitation with an antibody to $p55^{PIK}$ or with nonimmune serum. Then the samples were subjected to SDS-PAGE, followed by transfer to an Immobilon membrane. Proteins were revealed with antibodies to phosphotyrosine and [^{125}I]protein A. A representative autoradiogram of three independent experiments is shown.

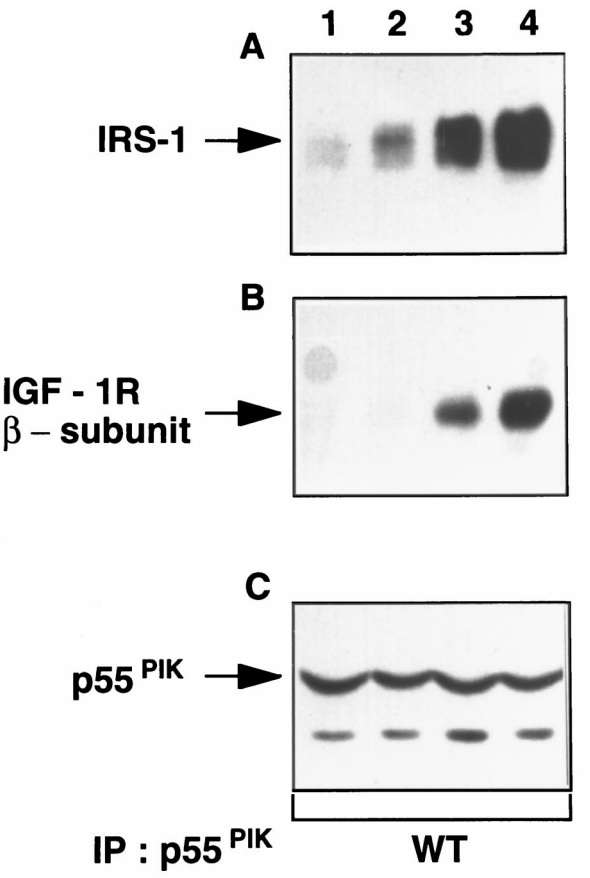

Fig. 6. Effect of PI 3-Kinase Inhibition on Association of $p55^{PIK}$ with IGF-IR- Signaling Proteins in Mammalian Cells

293 EBNA cells were transfected with 8 μg pcDNA3-$p55^{PIK}$. After 24 h, the cells were pretreated or not with 100 nM wortmannin and stimulated for 5 min with 10^{-7} M IGF-I (1: buffer, 2: wortmannin, 3: IGF-I, 4: wortmannin and IGF-I). Cell lysates were immunoprecipitated with antibodies to $p55^{PIK}$. Proteins in the immune complexes were separated by SDS-PAGE and transferred to an Immobilon membrane, which was blotted with antibodies to phosphotyrosine (panels A and B) or $p55^{PIK}$ (panel C). The autoradiograms are representative of three independent experiments.

Therefore, it would appear that in 293 cells expressing $p55^{PIK}$, IGF-I induces tyrosine phosphorylation of $p55^{PIK}$ and stimulates immunoprecipitation of a ternary complex between IGF-IRs, IRS-1, and $p55^{PIK}$ or binary complexes between $p55^{PIK}$ and IRS-1 and between $p55^{PIK}$ and IGF-IR.

Inhibition of PI 3-Kinase Associated with $p55^{PIK}$ Increases its Coimmunoprecipitation with IRS-1 and IGF-IR

We looked at the wortmannin effect on association of $p55^{PIK}$ with IRS-1 and with IGF-IR in intact cells. The same experiment as the one described in Fig. 5 was performed, except that cells were pretreated with wortmannin before being exposed to IGF-I (Fig. 6). In cells expressing comparable $p55^{PIK}$ amounts (panel C), IGF-I stimulated association of $p55^{PIK}$ with both IRS-1 and IGF-IR β-subunit, as visualized by blots with antibodies to phosphotyrosine (panels A and B). Importantly, addition of wortmannin increased the amount of IGF-I-stimulated $p55^{PIK}$-associated proteins, *i.e.* IRS-1 (150–200% increase) and IGF-IR (300–400% increase). These results are in agreement with a recent study by Rameh *et al.* (3), who observed a similar phenomenon, but related to the association of p85 PI 3-kinase with IRS-1 and with the insulin receptor in hormone-stimulated CHO-HIR cells. Further, we found that wortmannin alone enhanced association of IRS-1 to $p55^{PIK}$ in the absence of IGF-I. Taken together, our data suggest that PI 3-kinase negatively regulates association of $p55^{PIK}$ to IGF-I signaling molecules, such as IRS-1 and IGF-IR.

Construction of a p55PIK Mutant Unable to Bind PI 3-Kinase p110α and Having a Dominant-Negative Action on Insulin-Stimulated Glucose Transport

Increased association between p55PIK, IRS-1, and IGF-IR seen with wortmannin could be due to inhibition of p110 PI 3-kinase or of related kinases in the cells. To investigate whether the effect of the drug was actually due to repression of PI 3-kinase associated with p55PIK, we engineered a p55PIK mutant expected to be unable to bind p110. In a previous study, Waterfield and associates (29) showed that deletion of amino acids 478–492 in p85α IS domain leads to a loss of p85α binding to p110α (29). Based on sequence homology between p55PIK and p85α, we deleted the corresponding region in p55PIK IS domain, *i.e.* amino acids 203–217, and produced Δ203–217 p55PIK mutant.

First, we looked at the interaction of Δ203–217 p55PIK in the yeast two-hybrid system. We fused the mutant with GAD in the pACT II vector and coexpressed it, as well as wild type p55PIK, with Ras, IGF-IR, or p110α fused to LexA. After growth in appropriate medium, we measured β-galactosidase activity for each cotransformant (Fig. 7A). As expected, neither p55PIK protein produced β-galactosidase activity when coexpressed with Ras, indicating that no interaction occurred between the proteins. Similar to what we showed in Figs. 1 and 2, p55PIK interacted strongly with p110α. In contrast, Δ203–217 p55PIK did not bind to p110α, indicating that the deleted 15 amino acids are essential. To be sure that the loss of interaction seen with p55PIK deletion mutant was not due to a lack of expression, we tested its ability to bind to IGF-IR. As shown in Fig. 7A, Δ203–217 p55PIK was as efficient as wild type p55PIK in binding IGF-IR. It is concluded that amino acids 203–217 of p55PIK are necessary for its interaction with p110α, the catalytic subunit of PI 3-kinase.

Next we wished to determine whether Δ203–217 p55PIK was able to inhibit a biological effect requiring PI 3-kinase such as glucose transport. Indeed, insulin-sensitive glucose transport in adipocytes has been shown to be dependent on PI 3-kinase, based on experiments with pharmacological inhibitors and with constitutively active enzyme mutants (5–9). Therefore, we subcloned wild type and Δ203–217 p55PIK in the eukaryotic expression vector pcDNA3 and studied their effect on translocation of epitope-tagged Glut4 (Glut 4 myc was subcloned into pCIS vector). To do so, isolated rat adipocytes were cotransfected with pCIS-Glut 4 myc and pcDNA3-p55PIK or pcDNA3-Δ203–217 p55PIK or empty expression vector (mock conditions) (Fig. 7B). When Glut 4 myc was expressed alone, insulin stimulated by about 4-fold the amount of Glut 4 myc at the cell surface, compared with the basal state. Regardless of the nature of p55PIK protein expressed in adipocytes, basal Glut 4 myc translocation was unchanged. A slight but significant decrease (15%, $P < 0.01$) was seen when wild type p55PIK was coexpressed with Glut 4 myc, compared with translocation in insulin-stimulated mock adipocytes. This

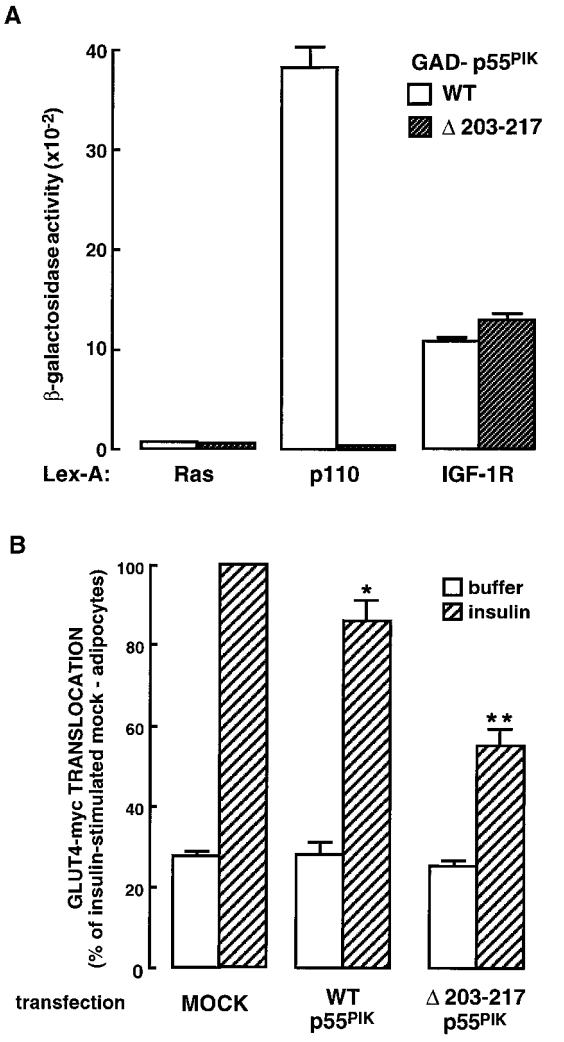

Fig. 7. Comparison of Δ203–217 p55PIK with Wild-Type p55PIK for Its Binding to p110α in the Yeast Two-Hybrid System (A), and for Its Effect on Insulin-Induced Glut 4 myc Translocation in Rat Adipocytes (B)

A, LexA fused to Ras, IGF-R cytoplasmic domain, or PI 3-kinase p110α was coexpressed with GAD-p55PIK or GAD-Δ203–217 p55PIK in the yeast strain L40, and β-galactosidase activity was measured as described in *Materials and Methods*. One experiment of three is shown, and data represent the average ± SE of three isolated colonies. B, Isolated rat adipocytes were transfected, as previously described (9), with 2 μg of pCIS Glut 4 myc and 8 μg of pCIS vector (mock) or 6 μg of pcDNA3-p55PIK or pcDNA3-Δ203–217 p55PIK adjusted to 10 μg total DNA using pCIS vector. Twenty four hours later, cells were stimulated (*hatched bars*) or not (*open bars*) with 10^{-7} M insulin before the quantification of myc at the cell surface with antibodies. Results are expressed as percentages of insulin-stimulted Glut 4 myc translocation in mock conditions and represent the mean ± SE of three independent experiments done in triplicate. *, $P < 0.01$; **, $P < 0.0005$.

decrease reached 45% with Δ203–217 p55^{PIK}, indicating that p55^{PIK} deletion mutant was able to inhibit translocation of Glut 4 myc to the cell surface in response to insulin. Thus, our p55^{PIK} mutant seems to behave in a dominant-negative fashion, since it inhibits the stimulating effect of insulin on PI3-kinase activity and subsequent Glut 4 translocation in intact cells.

Increased Coimmunoprecipitation of IRS-1 and IGF-IR with Δ203–217 p55^{PIK} Compared with p55^{PIK}

Since we obtained a p55^{PIK} mutant having features corresponding to a dominant-negative form for PI 3-kinase, we examined its association with IGF-IR-signaling proteins. First, we checked that PI 3-kinase activity associated with Δ203–217 p55^{PIK} expressed in 293 cells was decreased compared with that seen with wild type p55^{PIK} (not shown). For the association experiments, 293 cells were transfected with pcDNA3-p55^{PIK} or pcDNA3-Δ203–217 p55^{PIK} and stimulated or not with IGF-I. Cell lysates were subjected to immunoprecipitation with antibodies to p55^{PIK}, and Western blotting using several antibodies was performed (Fig. 8). In IGF-I-stimulated cells showing the same levels of expressed proteins (panel D), Δ203–217 p55^{PIK} associated with more tyrosine-phosphorylated IGF-IR proteins (panels C, 150–250% increase), compared with wild-type p55^{PIK}. Moreover, increased binding of p55^{PIK} to IGF-IR was also seen in unstimulated cells. We found similar results concerning association of IRS-1 to Δ203–217 p55^{PIK} (not shown). Interestingly, immunoblot with antibodies to IGF-IR β-subunit (panel C) revealed that the levels of tyrosine (panel A) and serine phosphorylation (panel B) of IGF-IR in basal and IGF-I-stimulated conditions reflected the amount of associated-IGF-IR to p55^{PIK} proteins and was independent of associated-PI 3-kinase activity. Identical results were found concerning the phosphorylation state of IRS-1 coimmunoprecipitated with wild type or mutant p55^{PIK} (not shown). Thus, we showed that Δ203–217 p55^{PIK} was more efficient than the wild type protein in associating IRS-1 and IGF-IR. These results strengthen those found with the PI 3-kinase inhibitor, wortmannin (see Fig. 6). Our data would indicate that, when PI 3-kinase is activated by association of p55^{PIK} to tyrosine-phosphorylated proteins, it exerts in return a negative control on these associations, probably through its lipid kinase activity.

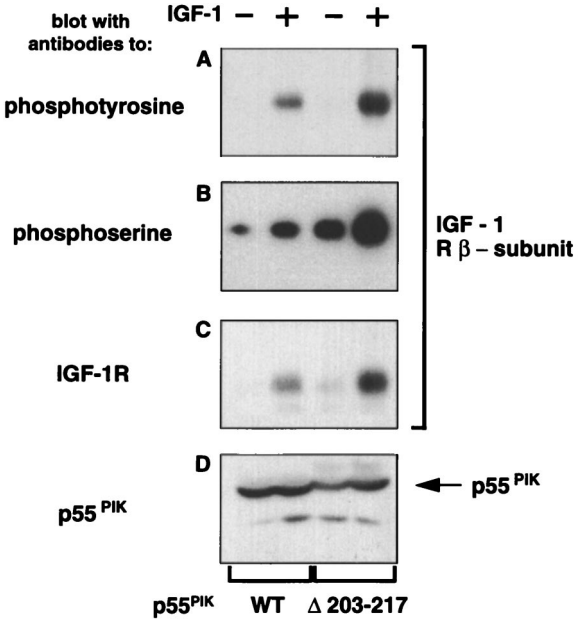

Fig. 8. Association of Δ203–217 p55^{PIK} with IGF-IR-Signaling Proteins in Mammalian Cells

293 EBNA cells expressing wild type p55^{PIK} or Δ203–217 p55^{PIK} were stimulated or not with 10^{-7} M IGF-I for 5 min. Cell lysates were subjected to immunoprecipitation with antibodies to p55^{PIK}; the proteins were separated by SDS-PAGE and transferred to membranes that were blotted with antibodies to phosphotyrosine (A), phosphoserine (B), IGF-IR β-subunit (C), and p55^{PIK} (D). The autoradiograms are representative of three independent experiments.

DISCUSSION

In this report, we investigated the interaction between the newly identified regulatory subunit of PI 3-kinase, p55^{PIK} (14), and several proteins involved in signal transduction. p55^{PIK} is highly homologous to the C terminus part of p85, especially at the level of the IS region flanked by two SH2 domains. The most striking difference is seen in the N terminus of the protein. Indeed, the SH3 domain, the first proline-rich motif, and the Bcr region found in p85 are replaced in p55^{PIK} by a unique 34-amino acid sequence. Using the yeast two-hybrid system, we have demonstrated here a direct interaction of p55^{PIK} with p110α, and we have identified in both proteins the implicated domains. We found that, like for p85α, the IS domain of p55^{PIK} binds to the N terminus of p110α (29, 30). Moreover, deletion of the 200 carboxy-terminal amino acids of p110α decreases binding, suggesting that they could participate in this interaction. It is also possible that deletion of this region induces conformational changes in the global structure of the protein, such that the N terminus of p110α is less able to recognize the IS domain of p55^{PIK}. Experiments based on further deletions in the IS domain led us to conclude that p55^{PIK} amino acids 203–217, corresponding to amino acids 478–492 of p85α, are essential for p110α binding. Similarly, it was shown by Waterfield and associates (29) that this sequence participates in formation of a p110α binding site on p85α. Further, this sequence is included in the typical structure of IS domain forming an antiparallel coiled coil of two 70-residue long α-helices. Disruption in the 478–492 sequence profoundly alters the structure of the binding site, so that interaction of p85α with

p110α is completely abolished. The peptide corresponding to amino acids 478–492 in p85α shows 100% identity with the one found in p55PIK, reflecting very likely the biological importance of this region. This means that interaction of p110 with different regulatory subunits, such as p85 and p55PIK, involves recognition of highly conserved regions on the two proteins.

Next we showed that both SH2 domains of p55PIK bind to the carboxy-terminal tyrosine 1316 of the activated IGF-IR. However, with IGF-IR mutated on tyrosine 1316 we observed a weak, but detectable, interaction with p55PIK. We reported the same observation in a previous work concerning interaction between p85α and insulin receptors, and IGF-IRs (20). Taken together, these results suggest that both PI 3-kinase-regulatory subunits are able to recognize determinants other than the receptor carboxy-terminal autophosphorylation site. The identity of this (these) additional site(s) is presently unknown. Despite the similarities between p55PIK and p85α in their interaction with IGF-IR, we revealed a difference that could contribute to generation of specificity in IGF-I signaling. Indeed, in previous studies on p85α, we and others showed that only the carboxy-terminal SH2 domain of p85α was able to bind to IGF-IR, while both p85α SH2 domains bound to the insulin receptor (20, 21). In contrast to p85α, both the NH_2- and COOH-terminal SH2 domains could link p55PIK to the IGF-IR. Moreover, it has been shown that binding of p85 to insulin or IGF-IRs could lead to PI 3-kinase activation *in vitro* (18, 31). Studies from Cantley and associates (32) and Gibbs and co-workers (33) have shown that doubly phosphorylated peptides (corresponding to platelet-derived growth factor receptor or IRS-1 sequences containing pYXXM motifs) bind with high affinity to the two SH2 domains of p85 and are more efficient in stimulating PI 3-kinase activity, compared with singly phosphorylated peptides that have low binding affinity. Thus, the monovalent (one SH2 domain in case of p85α) *vs.* bivalent (two SH2 domains in case of p55PIK) binding to SH2 domains would result in PI 3-kinase stimulation having variable intensity and/or duration. Hence this is likely to modulate biological responses induced by PI 3-kinase. Our data could also indicate that, compared with p85α, p55PIK could be the preferential regulatory subunit for PI 3-kinase activation through the IGF-IR itself. If this were the case, it is tempting to think that, for activation of PI-3 kinase, the IGF-IR is using a direct pathway rather than relying on IRS-1, which appears to be the preferred partner used by the insulin receptor to activate PI 3-kinase. One intriguing implication of such a view is that targeting of PI 3-kinase by IRS-1 to certain intracellular compartments would occur more easily with the insulin receptor than with the IGF-IR. This might explain, at least in part, the specific effect of insulin on translocation of glucose transporters, which is thought to involve targeting of PI 3-kinase to the Glut4-containing low density microsomes compartment (34).

Our observation in the yeast two-hybrid system, showing a direct interaction between p55PIK and IGF-IR, was strengthened by the demonstration of a direct association between p55PIK synthesized by *in vitro* transcription/translation, and WGA-purified autophosphorylated IGF-IR. In addition, we found that p55PIK coimmunoprecipitated with IGF-IR in lysates from IGF-I-stimulated 293 cells. Since IRS-1 was present in the same immunoprecipitates, p55PIK could form either a ternary complex with IRS-1 and IGF-IR and/or two types of binary complexes including p55PIK/IRS-1 and p55PIK/IGF-IR. We would expect that part of p55PIK interacts directly with IGF-IR in intact cells, in view of our results using *in vitro* binding experiments and yeast two-hybrid system. Moreover, we showed that p55PIK expressed in 293 cells is tyrosine phosphorylated in response to IGF-I by endogenous IGF-IRs, suggesting that this phosphorylation could occur in a physiological set-up. White and associates (14) showed that, in CHOIR/p55PIK cells, insulin induced phosphorylation of p55PIK on tyrosine 341, located in a YFIN motif. Given the close or identical specificity of the insulin and IGF-IR tyrosine kinase, we anticipate that the same site is phosphorylated upon IGF-I stimulation.

We further investigated coimmunoprecipitation of p55PIK with tyrosine-phosphorylated proteins in 293 cells and showed that PI 3-kinase negatively regulated these associations. Indeed, the inhibitor of PI 3-kinase, wortmannin, induced increased association of both IRS-1 and IGF-IR with p55PIK. This increase was seen in basal and IGF-I-stimulated conditions and could be explained by at least the following two hypotheses: 1) a decreased lipid kinase activity, 2) and/or a decreased serine kinase activity of PI 3-kinase. To further address this phenomenon, we used Δ203–217 p55PIK mutant. Indeed, p55PIK deleted in these 15 amino acids in the IS domain is unable to bind to p110α, but still associates with IGF-IR in a yeast two-hybrid system. First, we showed that Δ203–217 p55PIK acts as a dominant-negative mutant of PI 3-kinase, since its overexpression in adipocytes inhibited a biological function requiring PI 3-kinase activity, *i.e.* insulin-stimulated translocation of Glut 4 glucose transporters. This inhibitory action of Δ203–217 p55PIK could be explained by the fact that this mutant could compete with endogenous p85 for binding to phosphoproteins, such as IRS-1. Since Δ203–217 p55PIK does not bind to the p110α catalytic subunit, this would result in less PI 3-kinase associated with and activated by IRS-1 and, consequently, in less insulin-stimulated glucose transport. Such a phenomenon was previously described by Quon *et al.*, who used a p85α mutant deleted in IS amino acids 479–513. This region comprises a sequence of amino acids 478–492, which is necessary for p110α binding (35). We also observed a slight decrease in Glut 4 myc translocation in response to insulin, when adipocytes expressed wild type p55PIK, compared with mock conditions. This could be due to the presence of an excess of regulatory sub-

units of PI 3-kinase in these cells, compared with the amount of endogenous p110 catalytic subunit. Therefore, IRS-1-binding sites would be occupied not only by functional PI 3-kinase, comprising both subunits, but also by p85 or $p55^{PIK}$ alone. This will lead to decreased PI 3-kinase associated with and activated by IRS-1, and hence to decreased Glut 4 myc translocation.

We used Δ203–217 $p55^{PIK}$ for coimmunoprecipitation experiments in 293 cells and found that, compared with wild type $p55^{PIK}$, both associations with IRS-1 and IGF-IR were increased. These findings substantiate those seen with wortmannin and clearly demonstrate that inhibition of PI 3-kinase associated with $p55^{PIK}$ leads to stronger interaction between $p55^{PIK}$ and tyrosine-phosphorylated proteins.

Since PI 3-kinase has been shown to be able to exert serine kinase activity toward some proteins including IRS-1 (2, 36, 37), and since serine phosphorylation has been implicated in negative regulation of interaction between PI 3-kinase and IRS-1 (38, 39), we were interested to determine whether increased association of IRS-1 and IGF-IR with $p55^{PIK}$ could be due to a change in serine phosphorylation of the associated protein. However, using antibodies to phosphoserine, we found that the phosphoserine content of IGF-IR was unchanged, suggesting that increased association was probably not due to decreased serine phosphorylation, and hence that p110 serine kinase activity was not involved in regulation of this phosphoprotein association. Another mechanism, which might explain enhanced association of $p55^{PIK}$ with IRS-1 and with IGF-IR, would be that PI 3-kinase causes recruitment of a tyrosine phosphatase that decreases tyrosine phosphorylation of IRS-1 and IGF-IR. This would result in decreased binding of PI 3-kinase to IRS-1 and IGF-IR. However, this hypothesis can also be discarded, since blotting with antibodies to phosphotyrosine did not reveal changes in tyrosine phosphorylation of IRS-1 or IGF-IR. Taking these observations as a whole, we would like to conclude that changes in interaction between SH2 domain-containing proteins and their target can be modulated by the phospholipid products generated by PI 3-kinase. Using a different approach, Rameh and co-workers reached a similar conclusion in their study in which they showed that phosphatidylinositol (3,4,5)-trisphosphate was able to compete with tyrosine-phosphorylated proteins for their association with SH2 domains of p85 PI 3-kinase. An interesting implication of our observation is that, after its activation by binding to specific phosphorylated tyrosines, PI 3-kinase could be able to down-regulate itself by producing phospholipids that would act as inhibitors of PI 3-kinase binding to its activating proteins. This mechanism would provide a negative feedback loop for PI 3-kinase. Another possible function of lipid-mediated modulation of PI 3-kinase binding to SH2 domains could consist in the disengagement of the enzyme from its activating structures and its subsequent relocation.

While the precise impact of $p55^{PIK}$, and of PI 3-kinase in general, on IGF-I signaling is not known, the present demonstration of direct interaction between $p55^{PIK}$ and molecules involved in IGF-I action provides the basis for such a role. Further, our present study suggests that the activity of PI 3-kinase is likely to be the subject of modulations through mechanisms interfering with binding of its adapter subunits, such as $p55^{PIK}$ or p85α. One mechanism would involve the lipid kinase activity of PI 3-kinase itself, which appears to be able to reduce the interaction. Whether this process is involved only in regulating the interaction between the adapter subunits of PI 3-kinase and certain molecules, and consequently the enzymatic activity, or whether they play an additional role in releasing and therefore targeting PI 3-kinase to cellular compartments, remains to be investigated.

MATERIALS AND METHODS

Materials

Yeast strain L40 (MATa, trp1, leu2, his3, LYS2::lexA-HIS3, URA3::lexA-lacZ) and yeast two-hybrid expression vector pBTM116 were provided by A. Vojtek (Seattle, WA), and the plasmid pACTII was provided by S. Elledge (Houston, TX). Human IGF-IR cDNA was a gift from P. De Meyts (Copenhagen, Denmark). Full-length, BX, R1, and ΔR1 constructs of p110α, and the p85α cDNA into the pGBT9 vector were a gift from J. E. Pessin (Iowa City, IA). Oligonucleotides were purchased from Eurogentec (Seraing, Belgium), restriction enzymes were from New England Biolabs (Beverly, MA), Pwo DNA polymerase was from Boehringer Mannheim (Meylan, France), and synthetic defined dropout yeast media lacking the appropriate amino acids were from Bio101 (La Jolla, CA). Cell culture media and Geneticin were from Life Technologies, Inc. (Paisley, Scotland). All chemical reagents used were from Sigma (St Louis, MO), except protein A-Sepharose, which was from Pharmacia Biotech Inc. (Uppsala, Sweden); 2-mercaptoethanol and (luminol) 3-aminophtalhydrazide were purchased from Fluka (Buchs, Switzerland). IGF-I was a generous gift from Lilly Research Laboratories (Indianapolis, IN). [^{35}S]Methionine and [^{125}I]Ig against mouse Igs were from Amersham (Buckinghamshire, UK). Antibodies to phosphotyrosine and phosphoserine were from Upstate Biotechnology, Inc. (Lake Placid, NY) and Zymed Laboratories, Inc. (San Francisco, CA), respectively; rabbit polyclonal antibody to IGF-IR β-subunit (C-20), and monoclonal antibody to myc (9E10) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); antiserum to $p55^{PIK}$ was produced by M. F. White (Boston, MA); antibodies to IRS-1 were made by U145 and were raised to the C-terminal (1223–1235) peptide of rat IRS-1.

Plasmid Constructions

Complementary DNA corresponding to the intracellular part of the IGF-IR β-subunit (amino acids 993-1337) was inserted into the pBTM116 yeast expression vector as previously described (23). The fusion protein, which corresponded to the complete LexA bacterial repressor (containing a DNA-binding domain) fused to the IGF-IR β-subunit was named LexA-IGF-IRβ. All mutants derived from this fusion protein

were obtained by site-directed mutagenesis of double-stranded DNA using the Transformer kit (CLONTECH, Palo Alto, CA). The mutations were verified by DNA sequence analysis. Complementary DNAs of full-length p55PIK and its subdomains were amplified by PCR using pairs of primers described in Table 1. They were then subcloned in frame into the polylinker of pACTII, using the *Nco*I (5′ side) and *Eco*RI (3′ side) restriction sites. The fusion proteins obtained corresponded to the GAL4 activation domain (1–147) fused to different p55PIK regions and were tagged with the hemagglutinin peptide. The junction of each fusion cDNA was checked by sequencing with a primer corresponding to a sequence of pACTII (5′- taccactacaatggatg-3′). Sequence analyses were performed using the T7 sequencing kit (Pharmacia Biotech, Uppsala, Sweden). GAD-P55PIK deleted from amino acids 203 to 217 (GAD-Δ203–217 p55PIK) was obtained by using the site-directed mutagenesis kit purchased from CLONTECH, and the deletion was checked by sequence analysis. The complete p55PIK and deletion mutant Δ203–217 p55PIK cDNAs were also subcloned into the pcDNA3 eukaryotic expression vector by amplifying the cDNA using the following pair of primers: sense, (5′-ccggaattcgaccgcgatgacgcagactg-3′)/anti-sense (5′- ctagtctagattatctgcagagcgtaggc-3′). The obtained PCR product was cut with *Eco*RI and *Xba*I and ligated into pcDNA3 polylinker. The insertion of p55PIK cDNA was checked by sequencing with a primer corresponding to a sequence of pcDNA3 (5′-gcgtgtacggtgggaggtc-3′). Amplification by PCR using the following primers (5′ to 3′): 1) ccccccggggatgcctccaagaccatcatcagg; 2) ccggaattcgatatg-gttaaagatccag; 3) cgcggatcctcagttcaaagcatgctgc yielded full-length p110α cDNA and p110ΔR1 cDNA with primer sets 1 and 3 and 2 and 3, respectively. Both cDNAs were subcloned in-frame into the pBTM116 vector. The obtained plasmids encoded for the fusion proteins LexA-p110α and LexA-p110ΔR1 (p110α lacking amino-terminal amino acids 1–127), respectively. The LexA-p110BX (LexA fused to amino acids 1–576 of p110α) and LexA-p110R1 (LexA fused to amino acids 1–127 of p110α) constructs were obtained by digestion of pGBT9-p110BX with *Bam*HI-*Pst*I and pGBT9-p110R1 with *Eco*RI, respectively, and then in-frame religation into the polylinker of pBTM116. The LexA-p110BP construct, corresponding to amino acids 1–870, was obtained by digestion of pBTM116-p110α by *Pst*I to remove C-terminal 870-1068 amino acids of p110α, and religation of the vector. LexA-p85α was obtained by PCR amplification of p85α cDNA using the following set of primers: sense (5′-gccgaggggtac-gaattccgggcgctg-3′)/antisense (5′-atcgcctcggatccgcgta-cactgggtagg-3′), and then subcloning of the PCR product in-frame into the pBTM116 cloning site, using the *Eco*RI and *Bam*HI restriction sites. The junction of fusion between LexA, p110α constructs, and p85α cDNAs was checked by sequencing with a primer corresponding to a sequence of LexA (5′-cttcgtcagcagagcttc-3′).

Yeast Transformation and Reporter Gene Activity

The yeast strain L40 was cotransformed with pBTM116 and pACTII plasmids expressing hybrid proteins of interest, using the lithium acetate method (40). L40 were grown for 48 h on plates containing Trp^-, Leu^- synthetic complete (SC) medium to select the clones containing both plasmids (pBTM116 and pACTII carry the Trp^+ and Leu^+ selection markers, respectively). The histidine reporter gene was tested by replicating the clones expressing the different sets of plasmids on plates containing SC medium without tryptophan, leucine, and histidine and growing them at 30 C for 48 h. Double transformants were also assayed for β-galactosidase activity, using a color filter assay as previously described (41) or a liquid culture assay. Briefly, three clones of each transformation were grown for 24 h in Trp^-, Leu^- SC medium, then diluted 10 times in 2 ml of the same SC medium. After 24 h of additive growth, 1 ml of cells was used for determination of absorbance at 600 nm; 100–500 μl of cells were used for colorimetric assay at 574 nm. Cells were pelleted, resuspended in 500 μl Z buffer/25 μl chloroform, and vortexed for 15 sec. After 10 min incubation at 30 C, 100 μl of the chromogenic substrate, chlorophenol red-β-D-galactopyranoside, at 50 mM was added. The reaction was performed at 30 C, and β-galactosidase activity was measured according to Miller's method (42). One unit of β-galactosidase activity was defined as follows: $(A_{574} \times 1{,}000)/[A_{600} \times \text{volume (ml)} \times \text{time (min)}]$.

In Vitro Association Experiment

p55PIK cDNA in pBluescript II SK under the T3 procaryotic phage RNA polymerase promoter was used to synthesize purified and ^{35}S-labeled p55PIK protein by performing the coupled transcription/translation reticulocyte lysate system from Promega (Madison, WI). This protein was incubated with p55PIK antibody prebound to protein-A Sepharose beads or with WGA-purified IGF-IRs preadsorbed on WGA-coupled agarose beads. IGF-IRs were priorly phosphorylated for 20 min at 25 C, with 50 μM ATP, 4 mM $MnCl_2$, and 8 mM $MgCl_2$. After 3 h, protein A- or WGA-coupled agarose beads were washed four times with 30 mM HEPES, 30 mM NaCl, pH 7.4, containing 1% (vol/vol) Triton X-100. Finally, the pellets were resuspended in Laemmli buffer and subjected to SDS-PAGE under reducing conditions.

Cell Surface Epitope-Tagged Glut 4 Measurement in Isolated Rat Adipocytes

Adipocytes from epididymal fat pads of male Wistar rats were isolated by collagenase (Boehringer Mannheim) digestion (43). They were transfected by electroporation, using 2 μg pCIS-Glut 4 myc and 6 μg of pcDNA3-p55PIK or pcDNA3-Δ203–217 p55PIK, as previously described (9). Then, the cells were stimulated or not with 10^{-7} M insulin for 30 min at 37 C, and cell surface binding of antibodies to myc, which reflects the amount of Glut 4 myc translocated to the plasma membrane, was measured as described by Tanti *et al.* (9). Results were normalized by measuring protein concentration in each sample, using BCA (Pierce, Rockford, IL). Statistical analysis of the results was performed using an unpaired Student's *t* test.

Association Experiment in 293 Cells

Murine p55PIK and Δ203–217 p55PIK cDNAs, subcloned into pcDNA3 as described above, were used for transient expression in 293 EBNA cells, using the calcium phosphate precipitation method (44). This cell line, as well as culture conditions, were previously described (39). Dishes (56 cm^2) of 293 EBNA cells transfected with 8 μg pcDNA3-p55PIK or pcDNA3-Δ203–217 p55PIK were incubated with 100 nM wortmannin for 15 min, before a 5-min stimulation with 10^{-7} M IGF-I. The cells were then washed once with buffer A (50 mM HEPES, 150 mM NaCl, 10 mM EDTA, 10 mM $Na_4P_2O_7$, 2 mM sodium orthovanadate, 100 mM NaF, pH 7.5) and lysed on ice for 20 min using buffer A supplemented with 1% (vol/vol) Nonidet-P40 and protease inhibitors: 100 U/ml aprotinin, 1 mM $PhMeSO_2F$, 20 μM leupeptin, 2 μM pepstatin, and 4 mM benzamidine. Immunoprecipitation with antibody to p55PIK was performed for 3 h at 4 C. Samples were then washed three times with buffer A, resuspended in Laemmli buffer, loaded on a 7.5% polyacrylamide gel, and subjected to SDS-PAGE under reducing conditions. Proteins were transferred to an Immobilon membrane (polyvinylidene difluoride; Millipore Corp., Milford, MA). The membrane was blocked with TBS (10 mM Tris-HCl, 140 mM NaCl, pH 7.4) containing 5% (wt/vol) BSA, probed with antibodies to phosphotyrosine or to IGF-IR, and incubated with [^{125}I]protein A. Membranes were also stripped for 30 min at 50 C with stripping buffer

(62.5 mM Tris-HCl, pH 6.7, 100 mM 2-mercaptoethanol, and 2% (vol/vol) SDS), and reprobed with different antibodies followed by addition of ^{125}I-protein A. After incubation with antibody to phosphoserine, the membrane was incubated with a second antibody conjugated with horseradish peroxidase and proteins were revealed using the chemiluminescence detection system. After autoradiography, quantification was performed by scanning the autoradiograms followed by densitometric analysis of the obtained scans.

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