Multiple forms of p55PIK, a regulatory subunit of phosphoinositide 3-kinase, are generated by alternative initiation of translation

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A cDNA encoding p55PIK, one of the regulatory subunits of phosphoinositide (phosphatidylinositol) 3-kinase, was cloned from a cDNA library derived from the mouse mammary epithelial cell line C57MG. The cDNA coding for full-length p55PIK was transiently expressed in COS-7 cells. Western blot analysis of p55PIK expression using a specific antibody against p55PIK revealed that multiple protein products with different molecular masses were detected in COS-7 cell extracts. Experiments presented here demonstrate that multiple forms of p55PIK detected in COS-7 cells were produced by alternative initiation of translation. We also show that at least two in-frame start codons (AUG#2 and AUG#5) in p55PIK mRNA are used in COS-7 cells for the initiation of translation of p55PIK into proteins of 54 kDa and 50 kDa respectively. p55PIK mRNA was also alternatively translated into two proteins in PC cells, a mouse teratoma cell line, indicating that the alternative initiation of translation of p55PIK is not restricted to COS-7 cells. Results from immunoprecipitation and Western blot analysis showed that two forms (54 kDa and 50 kDa protein species) of p55PIK were detected in C57MG cells. Interestingly, when C57MG cells were treated with insulin, only p55PIK, but not p50PIK, bound to insulin receptor substrate-1 protein, providing evidence that different forms of p55PIKs may have specific distinct roles in signal transduction pathways.

Key words: AUG usage, insulin receptor substrate-1, insulin signalling, p50PIK.

INTRODUCTION

Heterodimeric phosphoinositide (phosphatidylinositol) 3-kinase (PI 3-kinase) is composed of a catalytic subunit, which is a 110 kDa protein (p110), and a regulatory subunit [1]. PI 3-kinase phosphorylates phosphatidylinositol at the D-3 position of the inositol ring of PtdIns(4,5)P_2 and PtdIns4P in response to stimuli from a variety of growth factors and hormones [2–4]. However, the role of this lipid product in cellular regulation remains unclear. In addition, PI 3-kinase was also shown to possess serine/threonine kinase activity [5,6].

PI 3-kinase has been found to play an essential role in the regulation of various cellular activities, including proliferation [7–9], differentiation [10,11], membrane ruffling [12,13] and prevention of apoptosis [14]. Several reports suggest that the activation of PI 3-kinase leads to the activation of c-Akt, Rac, the protein kinase C-δ isoform and p70^{s6} kinase [15–18]. Furthermore, PI 3-kinase is essential in the insulin-induced translocation of the glucose transporter GLUT4 and for anti-lipolytic activity in adipocytes [19,20].

Several regulatory subunits have been characterized and cloned in PI 3-kinase. p85α was the first regulatory subunit of PI 3-kinase to be cloned. p85α contains two Src homology 2 (SH2) domains, and a domain (inter-SH2) responsible for binding to p110, which is located between the two SH2 domains. In addition, other domains have been identified, such as two proline-rich motifs, an SH3 domain and a break-point cluster region (bcr). Phosphorylated tyrosine residues in receptors and non-receptor proteins such as insulin receptor substrate 1 (IRS-1) interact with the SH2 domains in the regulatory subunit of PI 3-kinase, resulting in the activation or recruitment of the enzyme [2,4]. To date, at least two other genes with identity to p85α have been identified and cloned. One, termed p85β, is similar in structure to p85α, but the products are only 62% identical at the amino acid level [21]. Recently, another putative regulator of PI 3-kinase (termed p55PIK) was described by Pons et al. [22]. This molecule is the product of a distinct gene, and is about 70% identical to p85α in the two SH2 domains and the inter-SH2 region. In contrast with p85α and p85β, this molecule does not contain an SH3 domain or a bcr.

In the present study, we examined the expression of p55PIK protein in several cell lines. The presence of two proteins recognized by an anti-p55PIK antibody prompted us to investigate the translational control of p55PIK. We show here that the first in-frame AUG is not used for the initiation of translation, and that multiple forms of p55PIK are generated by alternative initiation of translation. Furthermore, there is a significant difference in the binding of p55PIKs to IRS-1 and IRS-2 on stimulation by insulin in cultured cells, suggesting that multiple forms of p55PIK may have different roles in signal transduction.

MATERIALS AND METHODS

Materials

Phosphatidylinositol, horseradish peroxidase (HRP) and calf serum were purchased from Sigma (St. Louis, MO, U.S.A.), pQE31 vector was from Qiagen (Santa Clarita, CA, U.S.A.) and pcDNA3 vector was from InVitrogen (Plymouth Meeting, PA, U.S.A.). Rabbit anti-p85, anti-IRS-1 and anti-IRS-2 antisera, human fibronectin and human recombinant basic fibroblast growth factor were from UBI (Lake Placid, NY, U.S.A.). Antibodies against the PI 3-kinase catalytic subunits p110α and p110β were purchased from Santa Cruz Biotechnology (Santa

Abbreviations used: bcr, break-point cluster region; eIF, eukaryotic initiation factor; HRP, horseradish peroxidase; IRS, insulin receptor substrate; ORF, open reading frame; PI 3-kinase, phosphoinositide 3-kinase; SH2(3), Src homology 2(3).

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Cruz, CA, U.S.A.). Dulbecco's modified Eagle's medium, Ham's F12 medium, Lipofectin, G418 and fetal bovine serum were from Life Technologies (Gaithersburg, MD, U.S.A.). Protein A–Sepharose and CNBr-activated Sepharose were from Pharmacia (Piscataway, NJ, U.S.A.). Oligonucleotide primers were synthesized by the Biopolymer Laboratory of the University of Maryland School of Medicine. C57MG, a mouse mammary epithelial cell line, was kindly provided by Dr. Daniel Sussman (University of Maryland). COS-7 cells were purchased from the American Type Culture Collection (Rockville, MD, U.S.A.).

Generation of anti-p55PIK antisera

For developing a rabbit polyclonal anti-p55PIK antibody, we prepared as antigen recombinant His-tagged p55PIK fusion protein cloned into pQE31 and expressed in *Escherichia coli* host strain M15. The His_6-tagged fusion protein (His-p55PIK) was purified from the bacterial lysate using Ni^+/nitrilotriacetic acid–agarose according to the instructions provided by the manufacturer (Qiagen). The antiserum was generated in New Zealand White rabbits by Lampire Biologicals (Pipersville, PA, U.S.A.). To purify the anti-p55PIK antibody, His-p55PIK proteins were also conjugated to CNBr-activated Sepharose. The antiserum was purified by affinity chromatography on a His-p55PIK–Sepharose gel. HRP was conjugated to the purified antibody as described previously [23].

Western blot analysis of cellular extracts indicated that the anti-p55PIK antibody also recognized p85α in tissue extracts.

Site-directed mutagenesis in the p55PIK open reading frame (ORF)

The following oligomers were used in PCR to obtain the cDNA encoding the whole putative p55PIK protein (numbering is according to the data published in Pons et al. [22]): p55N1 (5′ TTTTTGAATTC**TCAGATAAAAGATATATA**; nucleotides 1312–1329, the cDNA primer, are in bold); p55C1 (5′ TTTTTGCGGCCGC**AGAGTGTGTCTCTTCCAC**; nucleotides 2763–2780 on the antisense strand, the cDNA primer, are in bold). The resultant 1.4 kb fragment was digested by *Eco*RI and *Not*I and ligated into pcDNA3 vector previously digested with *Eco*RI and *Not*I. A clone named pc3-p55-a2 was selected and its nucleotide sequence was verified. This construct was the parental plasmid for all the other p55PIK constructs described in this paper.

Constructs in which an AUG codon or the first CUG codon from pc3-p55-a2 was deleted were generated by PCR using appropriate primers as follows. The sense primer for making the AUG#1 deletion (p55-ΔAUG#1) was 5′ TTTTTTGAATTCTACAATACGGTGTGGA; the primer for making the AUG#2 deletion (p55-ΔAUG#2) was 5′ TTTTTTGAATTCGACCGCGATGACGCAGACTG; the primer for the AUG#3,4 deletion (p55-ΔAUG#3,4) was 5′ TTTTTGAATTCCCCTATTCGTCAGAACTGATATT; the primer for the CUG#1 deletion (p55-ΔCUG#1) was 5′ TTTTTGAATTCGATATTTTATATTGAAATGG. Oligomer p55C1 (described above) was used as the common primer for the antisense strand in all the PCR reagents. The fragments generated by PCR amplification were digested by *Eco*RI and *Not*I. The insert was purified from the agarose gel after electrophoresis and ligated into the pcDNA3 vector.

A pc3-p55Δ*Bam*HI construct containing the cDNA encoding p55PIK but with deletion of the putative first 32 N-terminal amino acids was generated by digesting pc3-p55-a2 with *Bam*HI and re-ligating it into the pcDNA3 plasmid.

The mutagenic primer used to change AUG#2 into AUC (p55-M2 → I) was 5′ TTTTTGAATTCGGTGTGGAGT**ATC**GACCGCGATGAC. The mutagenic primers used to change AUG#5 into AUC (p55-M5 → I) were 5′ TATATTGAA**ATC**GATCCTCCAGC and 5′ GCTGGAGGATC**GAT**TTCAATATA (on the antisense strand). The bold sequence corresponds to the three nucleotides that were substituted to generate a codon change (Met to Ile). The site-directed mutagenesis reaction was carried out as described in [24]. All constructs were confirmed by DNA sequencing.

Transfection of COS-7 cells and PC cells

COS-7 cells were cultured in Dulbecco's modified Eagle's medium/Ham's F12 medium supplemented with 10 % (v/v) calf serum. Before transfection, subconfluent cells were washed twice with PBS. Transient transfection of COS-7 cells was carried out using the DEAE-dextran method as described in [24]. After transfection, the cells were cultured in Dulbecco's modified Eagle's medium/Ham's F12 containing 10 % (v/v) calf serum for 48–72 h and then a cell lysate was prepared as described below.

PC cells were cultured in defined medium as described in [25]. The transfection was carried out using Lipofectin using the protocol provided by the manufacturer (Life Technologies). Stable pc3-p55-a2 transfectants were selected from G418-resistant cell clones and amplified in defined medium containing 400 μg/ml G418. RNA from transfected cells was extracted for measuring the expression of p55PIK mRNA by Northern blot analysis. The mRNA transcribed from pc3-p55-a2 was about 1.5 kb in size, and was easily distinguished from endogenous p55PIK mRNA ($\sim$ 5.8 kb). Cells showing the highest expression of pc3-p55-a2 and cells transfected by empty vector were used for the preparation of cell lysates and analysed by Western blotting.

Immunoprecipitation and Western blot analysis

For the preparation of cell lysates, cells were washed twice with PBS and incubated with extraction buffer (20 mM Tris/HCl, pH 7.4, 10 % glycerol, 1 % Nonidet P40, 170 mM NaCl, 1 mM PMSF and 10 μg/ml leupeptin) at 4 °C for 30 min. The extracted cell lysates were collected in Eppendorf tubes and centrifuged at 12000 ***g*** for 15 min. The supernatants were used for immunoprecipitation. In some experiments, the supernatants were mixed with 3× SDS/PAGE sample buffer [26] and used for direct Western blotting analysis without immunoprecipitation. The protein concentration of cell lysates was determined. A portion of 2 μg of anti-p55PIK antibody was added to an aliquot of cell lysate (1 mg of protein) and incubated at 4 °C for 4 h with shaking. The immune complex was collected by incubation with Protein A–Sepharose at 4 °C for 4 h followed by centrifugation. The pellets were resuspended in SDS/PAGE sample buffer and separated by SDS/PAGE as described by Laemmli [26] on 10 % (w/v) polyacrylamide gels. Proteins were transferred to an Immobilon membrane (Millipore) in buffer consisting of 15 % (v/v) methanol, 200 mM glycine and 25 mM Tris, pH 8.3. Following transfer, the filters were blocked with 5 % (w/v) non-fat dried milk in PBS containing 0.02 % Tween 20 for at least 4 h at room temperature. The blots were incubated with 0.5 μg/ml HRP-conjugated antibody in PBS/0.02 % Tween 20 containing 1 % (w/v) non-fat dried milk for 2 h. The proteins were detected by an ECL® chemiluminescence system (Amersham).

PI 3-kinase assay

COS-7 cells were transiently transfected with plasmid DNA from various constructs. Cell lysates were prepared as described above.

The expression of p55PIK was checked by Western blotting using anti-p55PIK antibody. For assays of PI 3-kinase [27], cell lysates containing equal amounts of expressed p55PIKs were incubated with 1 μg of anti-p55PIK antibody and immunoprecipitated. The immune complex was washed several times with extraction buffer and twice with assay buffer (40 mM Tris/HCl, pH 8.4, 1 mM EGTA, 5 mM $MgCl_2$). A 30 μl portion of assay buffer supplemented with 20 μg of phosphatidylinositol and 1 μM [γ-^{32}P]ATP (5 μCi) was added to the immune complex and incubated at 30 °C for 15 min. The reaction was quenched by 50 μl of 4 M HCl, and lipid was extracted with 100 μl of chloroform/methanol (1:1, v/v). A 25 μl portion of the organic extract was loaded on a TLC plate and separated with chloroform/methanol/25 %-satd. NH_4OH/water (20:14:5:3, by vol.) solvent mixture. The plate was dried and exposed to X-ray film.

RESULTS

Cloning of p55PIK cDNA

During experiments involving cloning of growth-related genes in mammary epithelial cells, a cDNA clone with a 5.4 kb insert was obtained by screening a cDNA library derived from the mouse mammary epithelial cell line C57MG. Partial sequence data showed very high identity between this clone and the 5′ non-coding region of the reported p55PIK mRNA [22]. By Northern blot analysis, the cDNA probe detected a single mRNA species with a size of approx. 5.8 kb in several cell lines (results not shown), consistent with the reported size of p55PIK mRNA in mouse tissues. These results suggested that the cDNA clone obtained corresponded to p55PIK mRNA. Nucleotide sequencing of the insert confirmed that the cDNA corresponded to p55PIK mRNA. When compared with the reported sequence, two substitutions were found in the sequence corresponding to the coding region (nt 1518, C → T; nt 2385, T → C). These are believed to be polymorphic changes that did not result in any amino acid change.

Production of anti-p55PIK antiserum and overexpression of p55PIK in COS-7 cells

Analysis of the deduced amino acid sequence of p55PIK indicated that p55PIK was different from p85α, since the SH3 and bcr domains of p85α were replaced by a putative unique 34-amino-acid N-terminal sequence with no known function [22]. An antiserum raised against the synthetic peptide MYNTVWSMD-RDDWDW derived from the unique N-terminal sequence of p55PIK failed to recognize p55PIK (results not shown). For the production of antibody against p55PIK, a construct expressing a His_6-tagged p55PIK fusion protein was made in pQE31 vector and introduced into *E. coli* cells. The purified fusion protein was injected into rabbits. The antibody was purified as described in the Materials and methods section. The purified antibody (anti-p55PIK) detected p55PIK protein expressed in bacteria, and also in cell lysates of the teratoma-derived cell lines 1246 and PC (results not shown). The level of endogenous protein in the cells was low, and could barely be detected by direct Western blotting without prior immunoprecipitation with anti-p55PIK. In subsequent experiments, we analysed the translation of p55PIK by transfecting cultured cells with plasmids encoding individual proteins.

Using this antibody, we examined the p55PIK protein expressed in COS-7 cells transfected with a cDNA construct encoding full-length p55PIK (pc3-p55-a2). Two distinct bands were specifically detected by anti-p55PIK on Western blots (Figure 1), indicating the presence of two proteins with apparent molecular masses of 54 kDa and 50 kDa respectively. These proteins were absent from cell lysates prepared from COS-7 cells transfected with empty vector. Cell lysates from COS-7 cells expressing p55PIK cDNA were immunoprecipitated, and the immune complex was treated with alkaline phosphatase. This treatment did not change the migration pattern of p55PIK on SDS/PAGE, indicating that the two bands detected by Western blotting did not result from differential phosphorylation of the expressed proteins.

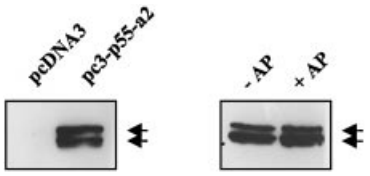

Figure 1 Multiple bands detected by anti-p55PIK antibody in COS-7 cells

pc3-p55-a2 plasmid DNA was transfected into COS-7 cells by the DEAE-dextran method, as described in the Materials and methods section. After 48 h cell lysates were prepared, and portions of 20 μg of protein from cellular extracts were used for Western blotting. As a control, COS-7 cells were transfected by empty vector pcDNA3 plasmid DNA. Two bands were detected by anti-p55PIK antibody in pc3-p55-a2-transfected cells (arrows). The expressed p55PIKs were immunoprecipitated and the immune complex was treated with alkaline phosphatase (AP). This treatment did not change the migration pattern of p55PIKs on SDS/PAGE, showing that differential phosphorylation does not explain the difference in molecular mass of the two proteins.

At least two hypotheses could explain the presence of two protein species recognized by anti-p55PIK. First, the p55PIK protein could be cleaved proteolytically to generate a product that is 4 kDa shorter than the full-length protein. Secondly, translation of p55PIK mRNA could be initiated at methionine or leucine codons located at two or more sites within the p55PIK gene, resulting in proteins with different molecular masses. Nucleotide sequencing of p55PIK cDNA indicates the presence of several putative initiation codons.

Alternative initiation of translation of p55PIK in COS-7 cells

To determine if the multiple bands observed in COS-7 cells expressing p55PIK cDNA were due to the usage of alternative translational initiation sites, we selectively eliminated potential initiation sites from p55PIK cDNA, as described in the Materials and methods section, and examined the resulting expressed proteins in transfected COS-7 cells. Since the difference in molecular mass between the two protein species was about 4 kDa, it was expected that the putative start codons were located within the first 40 codons of p55PIK mRNA. As shown in Figure 2(A), there are six possible initiation codons in this region: five AUGs [AUG#1 (codon 1), AUG#2 (codon 8), AUG#3,4 (codons 19,20) and AUG#5 (codon 32)] and one CUG (codon 26). Several constructs were made that encoded p55PIK proteins in which the possible initiation codon(s) were deleted, and these were introduced into COS-7 cells. Cell lysates were prepared in order to examine the proteins expressed by Western blot analysis using anti-p55PIK antibody (Figure 2B). The results clearly showed that AUG#2 (not AUG#1) was used as a start codon in COS-7 cells for translation into the 54 kDa species of p55PIK (Figure 2B, p55ΔAUG#1 and p55ΔAUG#2). Moreover, AUG#5 (codon 32) could also be used as a start codon, because deletion of this AUG eliminated the translation of both protein species (Figure 2B, p55-Δ*Bam*HI).

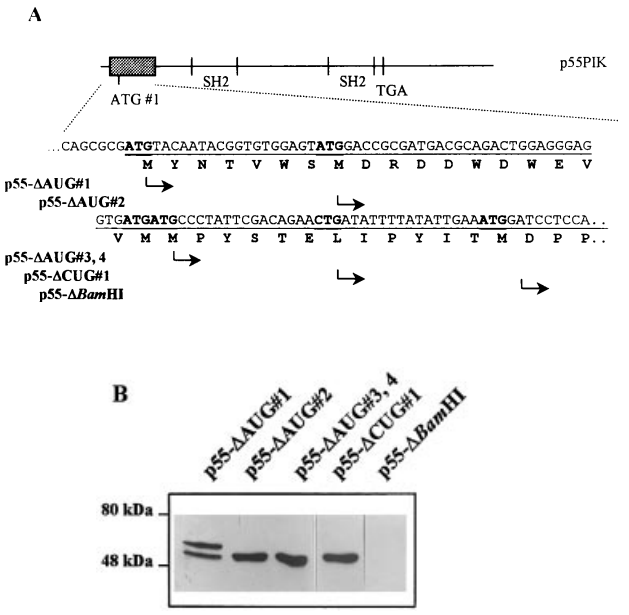

Figure 2 Alternative initiation of translation of p55PIK in COS-7 cells

(**A**) Five AUGs and one CUG represent possible start codons in the sequence of p55PIK cDNA encoding the N-terminal 40 amino acid residues. Constructs in which suspected start codons were deleted were introduced into COS-7 cells. Western blot analysis was performed to determine the protein products in COS-7 cells. (**B**) Results indicated that the first start codon is AUG#2 (compare p55-ΔAUG#1 and p55-ΔAUG#2). In addition, AUG#5 represents another possible start codon used in COS-7 cells (p55-Δ*Bam*HI).

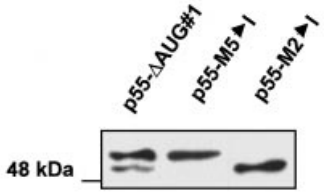

Figure 3 Identification of the start codons used for translational initiation of p55PIK in COS-7 cells

AUG#2 and AUG#5 were changed to AUC (Ile) by site-directed mutagenesis. Two constructs (p55-M5 → I and p55-M2 → I) were introduced into COS-7 cells. Cell lysates were prepared and Western blotting analysis using anti-p55PIK antibody was carried out as described in the Materials and methods section. The elimination of p55PIK in p55-M2 → I-transfected cells and of p50PIK in p55-M5 → I-transfected cells showed that AUG#2 and AUG#5 are the start codons for the initiation of p55PIK in COS-7 cells.

Next, we used site-directed mutagenesis to determine the exact start codons for the initiation of p55PIK mRNA translation in COS-7 cells. As expected, mutation of AUG#2 (p55M2 → I) and AUG#5 (p55M5 → I) resulted in the elimination of the production of the two proteins (Figure 3), showing that these two in-frame AUGs are the major start codons used in COS-7 cells for the translational initiation of p55PIK. The two protein products are referred to subsequently as p55PIK and p50PIK. The unexpected internal initiation of p55PIK and the usage of AUG#2 as a start codon may explain in part our inability to detect p55PIK with our original anti-(N-terminal peptide) antiserum.

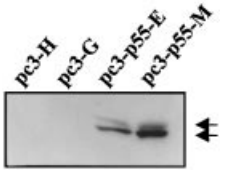

Figure 4 Multiple forms of p55PIKs are produced in PC cells

PC cells were transfected by pc3-p55-a2 and stable transfectants were selected. Two clones with a high level of expression of pc3-p55-a2 mRNA were investigated (pc3-p55-E and pc3-p55-M). The cell extracts were prepared as described in the Materials and methods section and used in Western blotting. The cell extracts from empty-vector-transfected cells (pc3-H and pc3-G) were used as controls.

Alternative initiation of translation of p55PIK is a widespread occurrence

The discovery of alternative initiation of translation of p55PIK in COS-7 cells prompted us to examine the translation of p55PIK in other cells. PC cells, derived from mouse teratoma [25], were stably transfected with pc3-p55-a2 plasmid DNA. The cell lysates from two separate transfectants were prepared and analysed directly by Western blotting. Figure 4 shows that p55PIK mRNA was translated into p55PIK and p50PIK in these cells. Lysates of PC cells transfected with empty pcDNA3 vector prepared under the same conditions did not show the presence of p55PIK proteins. Furthermore, the two protein species with molecular masses of 54 and 50 kDa were also observed in CHO cells transfected with pc3-p55-a2 (results not shown). These results showed that alternative initiation of translation of p55PIK is not restricted to COS-7 cells.

PI 3-kinase activity of p55PIK and p50PIK

As shown above, translation of p55PIK mRNA can be initiated from at least two start codons to generate p55PIK and p50PIK. It is reported that p55PIK has the ability to bind the catalytic subunit of PI 3-kinase [22]. Thus we examined whether either p55PIK or p50PIK expressed in COS-7 cells bound the catalytic subunit and showed PI 3-kinase activity. For this purpose, constructs expressing either p55PIK or p50PIK (p55M5 → I and p55M2 → I respectively) were transiently transfected into COS-7 cells, and proteins were immunoprecipitated by anti-p55PIK antibody. The immune complex was analysed by Western blotting using anti-p110α and anti-p110β antibodies, or was assayed as described in the Materials and methods section to measure PI 3-kinase activity. As shown in Figure 5, both p55PIK and p50PIK bound to p110α (but not to p110β; results not shown), and had PI 3-kinase activity.

Effects of insulin on binding of p55PIK and p50PIK to IRS-1 and IRS-2

Next we used immunoprecipitation and Western blotting analysis to examine the possible different functions of p85, p55PIK and p50PIK in signalling pathways. For this purpose, we used mouse mammary epithelial C57MG cells treated with insulin, since it is known that these cells respond to this hormone. It is well established that insulin stimulates the tyrosine-phosphorylation of IRS-1 and IRS-2, two major target proteins of insulin receptors. Phosphorylated IRSs act as docking proteins to bind numerous molecules, including PI 3-kinase, that mediate the insulin signalling pathway (reviewed in [27]). In C57MG cells, insulin treatment induced the tyrosine-phosphorylation of IRS-

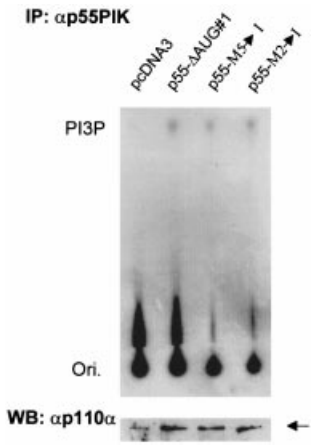

Figure 5 p55PIK and p50PIK have PI 3-kinase activity

pc3-p55-a2, p55-M2 → I and p55-M5 → I plasmid DNAs were transfected into COS-7 cells. Aliquots of cellular extracts containing the same amount of expressed proteins were immunoprecipitated (IP) by anti-p55PIK antibody (αp55PIK) and the immune complex was separated on SDS/PAGE. After protein transfer, the blots were analysed by Western blotting (WB) using anti-p110α antibody (αp110α) (the arrow indicates the position of the p110α subunit of PI 3-kinase). Aliquots of the immune complex were also assayed for PI 3-kinase activity, as described in the Materials and methods section. As a control, COS-7 cells were transfected with pcDNA3 plasmid. Ori, origin; PI3P, PtdIns3*P*.

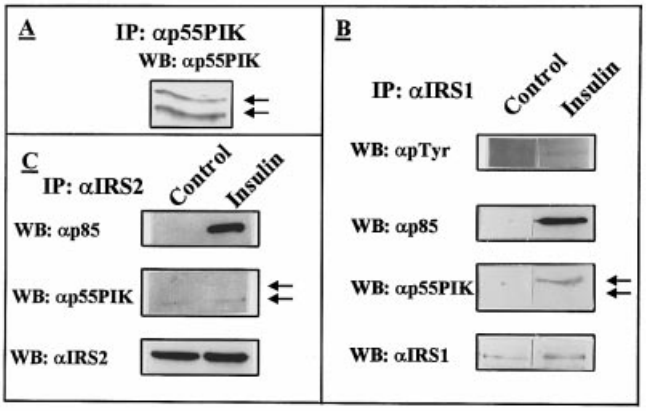

Figure 6 p55PIK preferably binds phosphorylated IRS-1 in C57MG cells in response to insulin

C57MG cells were cultured as described in the Materials and methods section and treated with insulin (5 μg/ml for 10 min at 37 °C). A cellular extract was prepared, and aliquots (1 mg of protein) were immunoprecipitated (IP) with 2 μg of anti-p55PIK antibody (**A**), 4 μg of anti-IRS-1 antibody (**B**) or 4 μg of anti-αIRS-2 antibody (**C**). Immune complexes were separated on SDS/PAGE and proteins were transferred to PVDF membranes. Western blots (WB) were incubated with HRP-conjugated anti-p55PIK antibody (**A**) or other antibodies as indicated: anti-(phosphorylated tyrosine) (αpTyr), anti-(85 kDa subunit of PI 3-kinase) (αp85), anti-p55PIK (αp55PIK), anti-IRS-2 (αIRS-2) and anti-IRS-1 (αIRS1). ECL was used to detect the signals on blots. Arrows indicate the positions of p55PIK and p50PIK in C57MG cell extracts.

1 and increased the binding of PI 3-kinase p85 subunits to IRS-1, showing that C57MG cells were responding to insulin. In the C57MG cells, p55PIK and p50PIK were expressed in almost equal amounts (Figure 6A). No tyrosine-phosphorylation of p55PIK or p50PIK was detected before or after treatment with insulin (results not shown). Interestingly, insulin only stimulated the binding of p55PIK, and not of p50PIK, to IRS-1 (Figure 6B). IRS-2 is another target protein of the activated insulin receptor [28,29]. Although insulin stimulated the binding of p85 to IRS-2 in C57MG cells, no increase in the binding of p55PIK or p50PIK to IRS-2 was detected in the IRS-2 complex (Figure 6C). It seems that there was a weak level of association between p50PIK and IRS-2, and that this was not affected by insulin treatment (Figure 6C). The mechanism and physiological significance of the interaction between p50PIK and IRS-2 requires further investigation. These results provide evidence that p85, p55PIK and p50PIK associate differently with IRS-1 and IRS-2 proteins, and may play different roles in the signal transduction pathways of insulin in these cells.

DISCUSSION

In the present study, we examined the expression of p55PIK proteins in cultured cells, using an antibody raised against recombinant p55PIK protein developed in our laboratory. We provide evidence that the alternative initiation of translation of p55PIK mRNA leads to the production of two proteins, p55PIK and p50PIK, in several cell lines. Studies in which deletion or site-directed mutagenesis was used to alter several putative start codons of p55PIK demonstrated that the translation of p55PIK and p50PIK proteins is initiated from AUG#2 and AUG#5 respectively. More interesting, experiments carried out with the insulin-responsive mammary epithelial cell line C57MG indicated that there is a significant difference in the binding of p55PIK and p50PIK to IRS-1 and IRS-2 upon insulin stimulation. These data suggest that the two protein species p55PIK and p50PIK translated from p55PIK mRNA may have different roles in signal transduction pathways.

Alternative initiation of translation can greatly amplify the potential functions of a gene, because the alternative forms of the protein so produced may have co-operative, alternative or even opposite effects (reviewed in [30]). Alternative forms of human fibroblast growth factor 2 have different cellular localizations and properties [31]. The quantitative differences in two forms of Frequency (FRQ), the central components of the *Neurospora* circadian clock, may be required for the completion of the feedback cycle that constitutes the clock [32,33]. Furthermore, the two forms of FRQ may differently regulate the function of the clock [33].

PI 3-kinase plays a major role in various cellular processes. It is anticipated that the p55PIK and p50PIK proteins play different roles in signalling pathways. The finding that p55PIK protein preferably binds tyrosine-phosphorylated IRS-1 upon stimulation with insulin provides strong evidence to support this notion. Although no specific function for the unique 24-amino-acid stretch (codon 8 to codon 32) found in p55PIK, but not p50PIK, is known at this time, it has been reported that Tyr-22 (codon 30) is a potential phosphorylation site in the YXXM motif [22]. This residue, Tyr-22, is located near negatively charged amino acids, making it a likely phosphorylation site. This YXXM motif is particularly interesting, as it has the potential to bind to the SH2 domains in p55PIK. This site could constitute an intramolecular regulatory mechanism analogous to that described for Src kinases. An intramolecular association between Tyr-22 and one of the SH2 domains may block activation by heterologous molecules such as IRS-1. In contrast, Tyr-22 may provide an intramolecular mechanism for PI 3-kinase activation. The absence of such a structure in p50PIK protein may confer to the two proteins different properties in regulating the catalytic activity of PI 3-kinase. It is also possible that the 24 N-terminal residues form a new unidentified domain which provides interaction sites for other proteins or results in localization of

p55PIK to specific cell compartments. Future work with yeast two-hybrid systems may be useful in identifying the proteins that bind p55PIKs.

At least five isoforms of the regulatory subunit for PI 3-kinase have been reported: two p85 subunits (p85α and p85β) [1,21], two p55 subunits (p55α and p55PIK) [22,34] and one p50 subunit (p50α) [34]. Among these, p55α and p50α seem to be derived from splicing variants of p85α gene [34].

The translation initiation site for p55α has not yet been determined. Deduced amino acid sequences of p55α show a high degree of identity in the N-terminal region with p55PIK. Since the results in the present paper show that AUG#2 in the p55PIK mRNA is used as a start codon, this suggests that the translation of p55α may also be initiated at AUG#2. This possibility is supported by the presence of a Kozak sequence preceding AUG#2 in p55α mRNA [34]. The structure of p50α is very similar to the p50PIK protein species produced by alternative initiation of translation from p55PIK mRNA. Considering all the results, it seems that 85 kDa, 54 kDa and 50 kDa proteins constitute three subfamilies of PI 3-kinase regulatory subunits. Until now, most functions linked to PI 3-kinase have been established for the p85 subunit (especially p85α). The role of the 54 and 50 kDa proteins in the regulation of PI 3-kinase activity is largely unknown. All the isoforms contain two SH2 domains and a binding site for association with the p110 catalytic subunit, suggesting that the regulatory subunits of PI 3-kinase interact with phosphotyrosine residues on receptors or other signalling proteins through one or both of their SH2 domains, resulting in activation of the p110 catalytic subunit. However, SH3 and bcr homology domains found in p85α or p85β are replaced by a unique 24-residue N-terminus in p55α and p55PIK, and this unique sequence is absent from the p50 species. Thus the differences in the N-terminal region observed among the regulatory subunit isoforms may contribute to differences in their subcellular distribution and/or to varying degrees of PI 3-kinase activation in response to various growth factor receptors and oncogenic products. More work is needed to address the question of how the different subfamilies of regulatory subunits, including different isoforms of p55, p50 and p85, co-operatively regulate the catalytic activity of the p110 subunit of PI 3-kinase.

We show here that p55PIK and p50PIK differentially associate with IRS-1 and IRS-2 in response to insulin. Because of the size similarity between p55α and p50α (isoforms of p85α) on the one hand and between p55PIK and p50PIK on the other hand, there may have been concern about the identity of the p55 and p50 proteins that bound to IRS-1, and not to IRS-2, in the C57MG cells and were detected by the anti-p55PIK antibody (Figure 6). The presence of p50α and p55α isoforms in C57MG cells has not been previously reported. In order to investigate this question and rule out a contribution of p50α and p55α in the C57MG cells, we used an anti-p85α monoclonal antibody raised against the SH2 domain of p85α that specifically recognizes p50α and p55α proteins but fails to recognize p50PIK and p55PIK expressed in mammalian cells. By using this antibody, we failed to detect p55α and p50α in immune complexes prepared by incubating C57MG cell extracts with either anti-p55PIK antibody or a polyclonal anti-p85α antibody (results not shown). These data suggest that the endogenous levels of p55α and p50α in C57MG cells, if present at all, are extremely low, and that the p55 and p50 protein species detected by the anti-p55PIK antibody in C57MG cells correspond to p55PIK and p50PIK proteins alternatively translated from p55PIK mRNA.

In eukaryotic cells, the choice of mRNA translation start sites is generally thought to be controlled by a ribosomal-scanning mechanism [35]. The 40 S ribosomal subunit first binds the mRNA 5′ cap structure and scans along the mRNA in a 5′ to 3′ direction, becoming associated with eukaryotic initiation factor 2 (eIF2)·GTP·Met-tRNAi (where i denotes intitiator) in a ternary complex that is competent to recognize the initiation codon. Upon encountering the initiation codon in a favourable context, the 60 S subunit joins the scanning complex to form the 80 S ribosome and to initiate protein synthesis [35,36]. Data from a variety of systems suggest that regulation of translation is achieved mainly at the level of initiation, although trans-acting factors and cis-acting elements, such as the secondary structure of mRNA, the sequence context around the initiation codon, internal ribosome entry sites and short ORFs in the 5′ untranslated region of a transcript, can influence the choice of start site and the amount of protein synthesized [36]. For example, in the control of the eukaryotic transcrption factor GCN4, phosphorylation of eIF2 is influenced by the availability of amino acids, which determines whether initiation occurs at an upstream ORF or at the GCN4 ORF, and thereby sets the amount of GCN4 [37]. Translational regulation of heat-shock protein synthesis is also influenced by temperature-dependent phosphorylation of eIF2 [38]. The translational regulation of p55PIK mRNA seems complicated, and depends on the tissue and cell type. Although the presence of two forms of p55PIK was detected in cultured cells such as PC cells and C57MG cells by immunoprecipitation and Western blot using anti-p55PIK antibody, no p55PIK was detected in several mouse tissues, except testes, where both p55PIK and p50PIK were detected, suggesting that the translational initiation for the production of p55PIK is tightly regulated *in vivo*. This implies that p55PIK may have profound effects on cell proliferation (X. Xia and G. Serrero, unpublished work). The demonstration of multiple forms of p55PIK that may be differentially regulated also suggests the existence of an additional mechanism to control signal transduction pathways. This mechanism may be vital for cells responding to changes in the environment.

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