A Novel 55-kDa Regulatory Subunit for Phosphatidylinositol 3-Kinase Structurally Similar to p55PIK Is Generated by Alternative Splicing of the p85α Gene*

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Phosphatidylinositol 3-kinase, which is composed of a 110-kDa catalytic subunit and a regulatory subunit, plays important roles in various cellular signaling mechanisms. We screened a rat brain cDNA expression library with 32P-labeled human IRS-1 protein and cloned cDNAs that were very likely to be generated by alternative splicing of p85α gene products. These cDNAs were demonstrated to encode a 55-kDa protein (p55α) containing two SH2 domains and an inter-SH2 domain of p85α but neither a bcr domain nor a SH3 homology domain. Interestingly, p55α contains a unique 34-amino acid sequence at its NH2 terminus, which is not included in the p85α amino acid sequence. This 34-amino acid portion was revealed to be comparable with p55PIK (p55γ) in length, with a high homology between the two. According to the report by Asano et al. (10), oligonucleotides were synthesized as follows: TCAATGCCTGACACAGCATGA as a forward primer and TCAGTGGCAGTCCTCCTGTCT as a reverse primer. PCR amplification was performed using these primers, and a 321-bp fragment was obtained from human genomic DNA. A human genomic library (a generous gift from Dr. H. Hirai, Third Department of Internal Medicine, Faculty of Medicine, University of Tokyo) was screened using a 32P-labeled 321-bp PCR fragment, and one positive clone was isolated. The coding region of human IRS-1 genomic DNA was subcloned into pBacPAK9 transfer vector, a baculovirus vector (Invitrogen), and the baculovirus was produced according to the manufacturer’s instructions. The purification of IRS-1 from SF-9 cells infected with the baculovirus containing IRS-1 DNA was performed as described previously (11). The insulin receptor was partially purified from human placenta on wheat germ agglutinin agarose as described previously (13). The 32P-labeled IRS-1 probe was prepared by the incubation of IRS-1 with activated insulin receptor in the presence of MNa2 and 32P-labeled γ-ATP (11).

Expression Screening with Human [32P]IRS-1 Protein—An oligo(dT)-primed rat brain cDNA library was prepared in UNI-ZAP XR (Stratagene) according to the manufacturer’s instructions. Sixty 15-cm diameter plates representing 3,000,000 independent plaques were plated and incubated for 7 h at 37 °C. Then, the plates were overlaid with nitrocellulose filters that had been impregnated with 10 ml isopropyl-β-D-thiogalactopyranoside and incubated for 8 h at 37 °C. Hybridization of the filters with the [32P]IRS-1 probe and washing were performed per the method described above. The cDNA inserts in pBluescript were prepared by in vivo excision according to the manufacturer’s instructions (Stratagene). The nucleotide sequences were determined using an ABI automatic sequencer.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EMBL Data Bank with accession number(s) D64045 (rat p85α), D64046 (rat p85γ), D64047 (rat p55γ), and D64048 (rat p55α).

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‡ The abbreviations used are: PI 3-kinase, phosphatidylinositol 3-kinase; PCR, polymerase chain reaction; bp, base pair(s); kb, kilobase(s); SH2, Src homology 2; SH3, Src homology 3; PAGE, polyacrylamide gel electrophoresis; HA, hemagglutinin; bcr, breakpoint cluster region.
**RESULTS AND DISCUSSION**

A human IRS-1 gene was successfully cloned from a human genomic library, and the complete nucleotide sequence of its coding region was determined. In comparison with the sequence reported by Araki et al. (12), two nucleotides were revealed to be different in our IRS-1 nucleotide sequence (C to G at 2166 bp and A to G at 3432 bp). The C to G change at 2166 bp caused a change in the amino acid sequence (C to W at 382). As these differences were thought to be due to polymorphism, we prepared recombinant IRS-1 protein using a baculovirus containing this IRS-1 DNA.

A rat brain cDNA expression library was screened with 32P-labeled recombinant IRS-1, and 81 positive independent clones were isolated after three or four rounds of screening. They included cDNAs containing complete coding regions of p85α, p85β, and p55γ, of which nucleotide sequences were determined. In addition, we obtained three independent cDNAs containing the nucleotide sequence coding for the NH2-terminal SH2 domain of p85α and previously undocumented 166-nucleotide sequence at its 5'-upstream side. These cDNAs contained an open reading frame of 1362 nucleotides, and the deduced amino acid sequence is shown in Fig. 1. The presence of this mRNA in rat brain was confirmed by reverse transcription PCR using the 5'-primer in the newly identified nucleotide sequence and the 3' primer in the nSH2 domain or in the cSH2 domain found in p85α DNA (not shown). We designated this putative protein p55α on the basis of its molecular weight. p55α contains two SH2 domains and an inter-SH2 domain, which are identical to those of p85α. Thus, p55α mRNA is comparable in length to the corresponding NH2-terminal portion of p55γ (11). 16 of the 34 amino acids are identical in the two peptides. These conserved sequences suggest that their unique NH2-terminal portion may have a specific functional role, which p85α does not. Further study is needed to resolve this issue.

The levels of expression of p85α, p55α, p55γ, and p85β mRNAs in various rat tissues were investigated, and the results are shown in Fig. 2. Northern blotting with a 5'-unique 159-nucleotide sequence located in the 5'-untranslated region

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**Northern Blotting**—Northern blotting was performed using a commercially available filter made by Clontech (Palo Alto, CA). The 1–663-nucleotide sequence of p85α, 1011–2175 of p85α, 1–2170 of p85β, 96–1381 of p55γ, and 1–159 of p55α were labeled with [32P]dCTP and used as probes. The filter was hybridized and washed according to the manufacturer's instructions (Clontech). Autoradiography was performed at −80°C for 12–48 h, and the radioactivities of the bands obtained were measured using a BAS2000 (Fuji).

Preparation of the Antibodies—An antibody against the whole p85α molecule (p85αSM+) was purchased from UBI. An anti-p85α specific antibody (p85αSM−) was prepared by immunizing rabbits with a 26-amino acid synthetic peptide based on the SH3 domain of p85α (HLG-DILTVNKGSLVALGFSDGQEARPEDIGWLNGYN, amino acid residues 11–28). These peptides were coupled to keyhole limpet hemocyanin, and rabbits were then inoculated with the peptides. The antiserum was affinity-purified with Affigel 10 covalently coupled with the corresponding peptides (14).

To confirm the specificity of these antibodies, p85α, p85β, and p55γ were expressed in Sf-9 cells using the baculovirus system. These cDNAs coding the full amino acid sequence and the HA tag amino acid synthetic peptide in its NH2-terminal portion (DDAD-DILTVNL-AK) was prepared by immunizing rabbits with a 36-amino acid synthetic peptide based on the SH3 domain of p85α. The antibodies specific to this 34-residue region of p55α were prepared after three or four rounds of screening. They included cDNAs containing complete coding regions of p85α, p85β, and p55γ, of which nucleotide sequences were determined. In addition, we obtained three independent cDNAs containing the nucleotide sequence coding for the NH2-terminal SH2 domain of p85α and previously undocumented 166-nucleotide sequence at its 5'-upstream side. These cDNAs contained an open reading frame of 1362 nucleotides, and the deduced amino acid sequence is shown in Fig. 1. The presence of this mRNA in rat brain was confirmed by reverse transcription PCR using the 5'-primer in the newly identified nucleotide sequence and the 3'-primer in the nSH2 domain or in the cSH2 domain found in p85α DNA (not shown). We designated this putative protein p55α on the basis of its molecular weight. p55α contains two SH2 domains and an inter-SH2 domain, which are identical to those of p85α. Thus, p55α mRNA appears to be transcribed by alternative splicing from the p85α gene. The SH2 domain and bcr homology domain found in p55α are replaced in p55γ by a unique 34-residue NH2-terminal followed by a conserved proline-rich motif (PPPLPVPQPKP). Interestingly, this 34-residue region of p55α is comparable in length to the corresponding NH2-terminal portion of p55γ (11). 16 of the 34 amino acids are identical in the two peptides. These conserved sequences suggest that their unique NH2-terminal portion may have a specific functional role, which p85α does not. Further study is needed to resolve this issue.

The levels of expression of p85α, p55α, p55γ, and p85β mRNAs in various rat tissues were investigated, and the results are shown in Fig. 2. Northern blotting with a 5'-unique 159-nucleotide sequence located in the 5'-untranslated region...
and a coding region for the NH$_2$-terminal 25-amino acid sequence in the NH$_2$-terminus of p55$_{\alpha}$, neither of which is included in the p85$_{\alpha}$ cDNA nucleotide sequence, revealed three mRNA species of 6.0, 4.2, and 2.8 kb in the brain (Fig. 2B). Among them, the 4.2-kb band was also detected in all of other tissues examined. Northern blotting with nucleotides coding for the p85$_{\alpha}$ SH3 domain revealed two mRNA species of 7.7 and 4.2 kb (Fig. 2A). In addition, the cDNA probe coding for the p85$_{\alpha}$/p55$_{\alpha}$ nSH2 domains was also used for Northern blotting, and four mRNA species of 7.7, 6.0, 4.2, and 2.8 kb were observed (Fig. 2C). The 4.2-kb band was detected on all Northern blots, and the intensities of this band were compared in various tissues. The amount of the 4.2-kb mRNA detected with Northern blotting using a cDNA probe coding for the p85$_{\alpha}$/p55$_{\alpha}$ nSH2 domain is thought to be the sum of the amounts of the p55$_{\alpha}$ and p85$_{\alpha}$ mRNAs. The intensity of the 4.2-kb band among various tissues observed in blotting utilizing a cDNA probe coding for the p85$_{\alpha}$/p55$_{\alpha}$ nSH2 domain was revealed to be similar to that obtained with a cDNA probe coding for the p85$_{\alpha}$ SH3 domain and differed significantly from that obtained with the p55$_{\alpha}$ 5'-unique cDNA probe. This result may suggest that p85$_{\alpha}$ mRNA is expressed more abundantly than p55$_{\alpha}$ mRNA in most tissues, with the apparent exceptions of brain and skeletal muscle. However, it should be noted that in skeletal muscle the p85$_{\alpha}$ mRNA expression level is almost undetectably low, while p55$_{\alpha}$ mRNA can be readily detected. In muscle, the activation of PI 3-kinase is presumed to be involved in insulin-stimulated glucose uptake through the translocation of GLUT4 to the plasma membrane (3). Therefore, it might be possible that p55$_{\alpha}$ plays a more important role than p85$_{\alpha}$ in the stimulation of glucose uptake by skeletal muscle.

In brain, both p55$_{\alpha}$ and p55$_{\beta}$ mRNAs are expressed abundantly, as are those of p85$_{\alpha}$, suggesting that these regulatory subunits have neither bcr homology nor SH3 domains may have a function(s) different from that of p85. PI 3-kinase appears to be important, first, in that its activation is essential for neurite elongation of rat PC-12 cells, and in addition, VPS34, a yeast PI 3-kinase homologue, was shown to be involved in vacuolar protein sorting (15). Thus, PI 3-kinase may play an essential role in the secretion of neurotransmitters via regulation of vesicle sorting in the brain. Taken together, one or more of these four regulatory subunits might be essential for neuronal differentiation, while the others may be involved in the secretion of neurotransmitters.

Unlike p85$_{\alpha}$ and p55$_{\alpha}$, p55$_{\gamma}$ and p85$p$ genes generate only

- **55-kDa Regulatory Subunit for PI-3 Kinase from p85$\alpha$ Gene**

**Fig. 2. Northern blotting of p85$\alpha$, p55$\alpha$, p55$\gamma$, and p85$p$ mRNA species in various rat tissues.** Rat multiple tissue Northern blot was obtained from Clontech and used for the detection of mRNA. 32P-Labeled cDNA probes encoding nucleotides 1–2170 of p85$_{\alpha}$ (panel A), 1–159 of p55$_{\gamma}$ (panel B), 1011–2175 of p85$_{\alpha}$ (same as nucleotides 201–1365 of p55$_{\alpha}$) (panel C), 96–1381 of p55$_{\alpha}$ (panel D), and nucleotides 1–2170 of p85$_{\beta}$ (panel E) were hybridized and washed according to the manufacturer's instruction (Clontech). The intensity of the 4.2-kb bands was measured by using a BAS2000 (Fujifilm).
one mRNA species each, of 5.8 and 3.4 kb, respectively (Fig. 2, D and E), suggesting that no other mRNAs are generated by alternative splicing of p55γ and p85β gene products.

In order to detect the endogenous p85x, p55x, and p55γ proteins in rat tissues, we prepared specific antibodies against each of the three. These antibodies did not recognize different isoforms of regulatory subunits, produced in the SF-9 cell experiment using the baculovirus expression system (Fig. 3). As shown in Fig. 3A, by immunoblotting using the anti-HA antibody (12CA5), the electrophoretic mobility of p55x is essentially the same as that of p55γ. The rat brain lysates immunoblotted by the beads covalently coupled with αp85PAN-UBI, which recognize all p85x, p55x, p55γ, and p85β expressed in SF-9 cells because of the highly conserved amino acid sequence of these peptides (data not shown), were subjected to SDS-PAGE and immunoblotted with control antibody, αp85PAN-UBI, αp85xSH3, αp55x, and αp55γ (Fig. 3E). The immunoblot obtained with αp85PAN-UBI revealed the two bands of 85 and 55 kDa, while that obtained with αp85xSH3 showed only the 85-kDa band. In contrast, αp55x and αp55γ both showed the 55-kDa band. These results indicate the expression of these isoforms in brain.

Finally, to determine whether or not p55x is associated with PI 3-kinase activity, as in the case of p85x, we immunoprecipitated the rat brain soluble fraction with each control antibody, αp85PAN-UBI, αp85xSH3, or αp55x. PI 3-kinase activities in these immunoprecipitates were measured (Fig. 3F). The immunoprecipitates obtained with αp85PAN-UBI, αp85xSH3, or αp55x were demonstrated to contain significant PI 3-kinase activity, as compared with the control antibody immunoprecipitate. This result strongly suggests that p55x also exists as a heterodimer with a p110 catalytic subunit and that it functions as a regulatory subunit of PI 3-kinase.

In this study, we showed that there are at least four isoforms of the regulatory subunit for PI 3-kinase. All of the four isoforms contain two SH2 domains and the binding site for association with the p110 catalytic subunit, suggesting that these regulatory subunits of PI 3-kinase interact with phosphotyrosine residues on the receptors or receptor substrates through one or both of their SH2 domains, resulting in activation of the p110 catalytic subunit. However, SH3 and SHC homology domains found in p85x or β are replaced in p55x or γ by unique 34-residue NH2 termini. Although the functional roles of SH3 domain have not been understood yet, the association between SH3 domain and proline-rich segments in various signaling proteins (i.e. dynamin (16), paxillin (17), hSOS1 (18), p85 subunit of PI 3-kinase (19)) is reported. Thus, the differences in the NH2-terminal region observed among the regulatory subunit isoforms may contribute to differences in subcellular distributions and/or to varying degrees of PI 3-kinase activation in response to various growth factor receptors and oncogenic products.

In summary, we have identified a novel alternatively spliced regulatory subunit, which may have important functions in brain and muscle. Our future studies will focus on the variety of possible functions mediated by differences in the NH2-terminal portions of the regulatory subunits of PI 3-kinase.

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