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Insulin Receptor Substrate 1 Binds Two Novel Splice Variants of the Regulatory Subunit of Phosphatidylinositol 3-Kinase in Muscle and Brain

DAVID A. ANTONETTI, PETRA ALGENSTAEDT, AND C. RONALD KAHN*

Research Division, Joslin Diabetes Center, and Department of Medicine, Harvard Medical School, Boston, Massachusetts 02215

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We have identified two novel alternatively spliced forms of the p85α regulatory subunit of phosphatidylinositol (PI) 3-kinase by expression screening of a human skeletal muscle library with phosphorylated baculovirus-produced human insulin receptor substrate 1. One form is identical to p85α throughout the region which encodes both Src homology 2 (SH2) domains and the inter-SH2 domain/p110 binding region but diverges in sequence from p85α on the 5′ side of nucleotide 953, where the entire break point cluster gene and SH3 regions are replaced by a unique 34-amino-acid N terminus. This form has an estimated molecular mass of ~53 kDa and has been termed p85/AS53. The second form is identical to p85 and p85/AS53 except for a 24-nucleotide insert between the SH2 domains that results in a replacement of aspartic acid 605 with nine amino acids, adding two potential serine phosphorylation sites in the vicinity of the known serine autophosphorylation site (Ser-608). Northern (RNA) analyses reveal a wide tissue distribution of p85α, whereas p85/AS53 is dominant in skeletal muscle and brain, and the insert isoforms are restricted to cardiac muscle and skeletal muscle. Western blot (immunoblot) analyses using an anti-p85 polyclonal antibody and a specific anti-p85/AS53 antibody confirmed the tissue distribution of p85/AS53 protein and indicate a ~7-fold higher expression of p85/AS53 protein than of p85 in skeletal muscle. Both p85 and p85/AS53 bind to p110 in coprecipitation experiments, but p85α itself appears to have preferential binding to insulin receptor substrate 1 following insulin stimulation. These data indicate that the gene for the p85α regulatory subunit of PI 3-kinase can undergo tissue-specific alternative splicing. Two novel splice variants of the regulatory subunit of PI 3-kinase are present in skeletal muscle, cardiac muscle, and brain; these variants may have important functional differences in activity and may play a role in tissue-specific signals such as insulin-stimulated glucose transport or control of neurotransmitter secretion or action.

Insulin binding of the insulin receptor leads to the tyrosine phosphorylation of insulin receptor substrates 1 and 2 (IRS-1 and IRS-2) and Shc, which can then interact with and modulate the activity of a number of proteins via Src homology 2 (SH2) domains, including phosphatidylinositol (PI) 3-kinase, the protein tyrosine phosphatase SHPTP-2, and Grb2, which, acting through Sos can stimulate Ras GTPase activity (reviewed in references 6, 36, and 37). Recent studies have indicated an important role for PI 3-kinase, especially in insulin stimulation of glucose transport (18, 20, 22, 28, 39), p70 S6 kinase (20, 28), glycogen synthesis (27), and antilipolysis (22). PI 3-kinase is a heterodimer composed of an 85-kDa regulatory subunit (p85α) and a 110-kDa catalytic subunit (p110α). Isoforms of p85α and p110α have been identified and termed p85β (23) and p110β. In addition, a G-protein-activated form of PI 3-kinase, termed p110γ, which is not dependent on the 85-kDa regulatory subunit has been found (30). Although p85α is generally considered a regulatory subunit only, recent evidence suggests an essential role of p85 in catalysis as well (15).

The p85 molecule is composed of a number of modular domains which dictate its activity and regulation. There are two SH2 domains in p85 at the center and C terminus of the molecule (amino acids 333 to 430 and 624 to 720) which bind to specific phosphotyrosines in IRS-1, IRS-2, and several growth factor receptors, leading to the activation of PI 3-kinase (10, 11, 17, 21). At the N terminus (amino acids 10 to 80), there is an SH3 domain whose exact function is unknown but which presumably is involved in additional protein-protein interactions (29). The inter-SH2 region contains the p110α binding site (7), and the fusion of amino acids 466 to 567 from this region to p110 yields a constitutively active p110 molecule (12). Finally, the region between the SH3 domain and the more N-terminal SH2 domain constitutes a break point cluster gene (bcr) homology region. This region is also homologous to Rho–GTPase-activating protein and binds Cdc42Hs, a small G protein in the Rho family leading to the activation of PI 3-kinase (40).

At least two other genes with homology to p85α have been identified and cloned. One, termed p85β, is similar in structure to p85α but is only 62% identical on the amino acid level (23). Furthermore, p85β does not transduce the insulin signal leading to increased PI 3-kinase activity in experiments using transfected cells (5). Recently, Pons et al. (24) have described another regulator of PI 3-kinase which they termed $p55^{PIK}$. This molecule is the product of a separate gene and is ~70% identical to p85α in the two SH2 domains and the inter-SH2 region. In contrast to p85α and -β, this molecule does not encode the SH3 or bcr region and is more abundant in testes, brain, and, to a lesser extent, adipose tissue (24).

Since skeletal muscle is the predominant tissue involved in insulin-stimulated glucose disposal and the major site of insulin resistance in non-insulin-dependent diabetes mellitus, we recently set out to identify novel IRS-1-binding proteins by screening a human skeletal muscle cDNA expression library

* Corresponding author. Mailing address: Joslin Diabetes Center, One Joslin Pl., Boston, MA 02215. Phone: (617) 732-2635. Fax: (617) 732-2593.

with baculovirus-produced human IRS-1. This report describes the identification and characterization of two novel splice variants of the p85α gene uncovered in this screening; one has the SH3 region and the bcr region replaced with a 34-amino-acid leader, and the other possesses a 24-nucleotide insert in the inter-SH2 domain which adds new potential serine phosphorylation sites. The specific tissue distribution of these splice variants and their structural features suggest novel roles in signal transduction and raise the possibility that other tissue- or pathway-specific splice variants of p85α exist.

MATERIALS AND METHODS

Baculovirus-produced IRS-1 protein. PCR was used to introduce a *Bam*HI site at the 5′ untranslated end of human IRS-1 (3), which was then subcloned into pBlueBac III (Invitrogen, San Diego, Calif.) in two fragments, using the *Bam*HI and *Nco*I sites of the vector. The 5′ fragment was from the *Bam*HI site (directly before the initiation ATG, nucleotide position 1021) to the *Sfi*I site (nucleotide position 1792), and the 3′ fragment was from the *Sfi*I site to the *Bsp*HI site (nucleotide position 4840). The plasmid was introduced into baculovirus, and a baculovirus stock containing human IRS-1 was made according to the manufacturer's protocol. Production of IRS-1 protein and purification over a Sephacryl 300 HR column were carried out as described for rat IRS-1 protein (31). Purification of the protein was verified by Western blot (immunoblot) analysis using a C-terminal antibody to human IRS-1.

Screening human skeletal muscle cDNA expression library with IRS-1 protein. A human skeletal muscle cDNA expression library was made in the λEXlox system (Novagen, Madison, Wis.), and isopropylthiogalactopyranoside (IPTG)-induced proteins were bound to nitrocellulose filters as described previously (41). The IRS-1 protein was phosphorylated by wheat germ agglutinin-purified insulin receptor (35) in vitro, using [γ-^{32}P]ATP as previously described (38) except that IRS-1 protein was added simultaneously with the [γ-^{32}P]ATP. The phosphorylated IRS-1 was separated from free [γ-^{32}P]ATP by use of a NAP-10 column (Pharmacia Biotec, Piscataway, N.J.). Approximately 10^5 plaques of the human skeletal muscle expression library were screened with the phosphorylated IRS-1 protein. The filters of the expressed proteins were washed in wash buffer 1 (10 mM Tris [pH 7.5], 150 mM NaCl, 0.05% Tween 20, fresh 10 mM 2-mercaptoethanol) at room temperature and then placed in blocking buffer (10 mM Tris [pH 7.5], 250 mM NaCl, 5% dry milk, fresh 10 mM 2-mercaptoethanol) for 4 to 5 h at 4°C. Blocking buffer was removed and replaced with blocking buffer containing the labeled IRS-1 protein probe, and the filters were incubated overnight at 4°C. The filters were then washed in wash buffer 2 (10 mM Tris, pH 7.5, 150 mM NaCl, 0.01% Tween 20, fresh 10 mM 2-mercaptoethanol) three times for 15 min each at room temperature, wrapped in plastic wrap, and subjected to autoradiography. Positive plaques were identified, isolated from the plates, and purified by additional screening. A plasmid (pEXlox) containing the insert was obtained by Cre-mediated excision according to Novagen's instructions and sequenced by using a PCR-based automatic sequencing with fluorescent dyes and a model 373 automatic sequencer (Applied Biosystems, Foster City, Calif.).

Southern and Northern (RNA) blot analysis. The Southern blots of genomic DNA and the genomic clone of the p85α gene (see below) were made according to standard laboratory techniques, using capillary transfer (4, 26). A human multiple-tissue Northern blot was purchased from Clontech (Palo Alto, Calif.). A probe specific for p85/AS53 was produced by PCR, using a 5′ vector primer and a p85/AS53-specific primer directed to the 3′-most end of the unique region. A probe specific for p85α (29) was made by digesting p85 with *Sca*I and isolating the 830-bp fragment which is specific for the unique region of p85α. Two probes were made for the region of AS53 homologous to p85 by restriction enzyme digestion. Homologous probe 1 stretched from the *Xho*I site at 1063 bp to bp ~1560 (numbering according to the p85 sequence) which was the insert-vector junction of one of the p85/AS53 partial clones; homologous probe 2 was a fragment of a p85/AS53 clone stretching from the *Bsp*HI site (bp 1900) to the *Pst*I site (bp 2315) (numbering according to matching p85 sites). The probes were labeled by a random-primed DNA labeling reaction (Amersham, Arlington Heights, Ill.) and separated from free nucleotides by the use of a NICK column (Pharmacia Biotec). Hybridization and washing for both the Northern and Southern blots were carried out as specified in the Clontech manual for multiple-tissue Northern blotting except that 2× rather than 10× Denhardt's solution was used during hybridization.

Western blot analysis. Sprague-Dawley rats were anesthetized with pentobarbital by intraperitoneal injection, and tissues were excised. Human skeletal muscle tissue samples from either gastrocnemius or quadriceps muscle were obtained from patients undergoing amputation. Both the rat and human tissues were homogenized in a Polytron as previously described (2). Samples were separated on 7.5% polyacrylamide gels and transferred to nitrocellulose. After the blots were blocked, they were probed with primary antibody at room temperature for 2 h and detected with ^{125}I-protein A or with horseradish peroxidase-conjugated goat antibody and visualized by enhanced chemiluminescence (Amersham, Arlington Heights, Ill.). Results were observed with autoradiography.

For immunoprecipitation experiments, rats were starved overnight and stimulated with insulin by portal vein injection. Control rats were injected with insulin diluent only. Quadriceps muscles were isolated and lysed as described above. Specific antibodies to either IRS-1 (JD63) or the p110 subunit of PI 3-kinase (N20 or S19; Santa Cruz Biotechnology) was used to generate immune complexes which were pelleted by using protein A–Sepharose beads. The beads were washed two times with IP (immunoprecipitation) wash buffer A (50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.4], 150 mM NaCl, 100 mM NaF, 1% Triton, 0.1% sodium dodecyl sulfate, 2 mM vanadate, 2 mM phenylmethylsulfonyl fluoride) and one time with IP wash buffer B (same as IP wash buffer A but without NaCl). Proteins were solubilized in 2× Laemmli sample buffer with boiling and separated on 7.5% polyacrylamide gels. Nitrocellulose blots were made and probed with a monoclonal antibody to the N-SH2 domain of p85. The blots were then probed with a horseradish peroxidase-conjugated anti-mouse immunoglobulin G antibody (Cappel, West Chester, Pa.), and signal was visualized with enhanced chemiluminescence using LumiGLO (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) and autoradiography.

Genomic clone isolation. The genomic clone of p85α was isolated from a human genomic library in the λBlueStar-1 system (Novagen) according to the manufacturer's protocol. The homologous probe 1 was used to screen ~800,000 plaques, from which two positive clones were obtained through secondary screening. Cre-mediated excision was used to obtain a plasmid clone of the virus containing the p85α gene. One of these two clones, termed 3a, had an insert of ~13 kb and was used for Southern analysis and sequencing. The 3a clone was also subcloned as fragments into the pBluescript II vector (Stratagene, La Jolla, Calif.) to aid in sequencing.

Oligonucleotides, peptides, and antibodies. The oligonucleotides used were made by Oligos Etc. (Wilsonville, Oreg.) or were made at our core facility with a model 394 DNA/RNA synthesizer (Applied Biosystems). Peptides were generated by the Joslin DERC Peptide Core Facility. Antibodies were raised against the peptides linked to keyhole limpet hemocyanin at HRP Inc. (Denver, Pa.). For antibodies specific for p85/AS53, the peptide sequence used was DLDLEY AKTDIN; for antibodies to the insert region, the peptide sequence used was EENFLSCLPSQ. An anti-p85 polyclonal antibody mixture directed against a full-length p85–glutathione *S*-transferase fusion protein and N-terminal SH2 domain–glutathione *S*-transferase fusion protein was purchased from Upstate Biotechnology, Inc. (Lake Placid, N.Y.).

RESULTS

Novel forms of the regulatory subunit of PI 3-kinase were identified from a human skeletal muscle library. A human skeletal muscle cDNA expression library was created by using the λEXlox system and screened with partially purified, phosphorylated human IRS-1 protein as described in Materials and Methods (Fig. 1A). Ten plaques producing IRS-1-binding proteins were identified in 100,000 clones, purified through additional screening, and subjected to Cre-mediated excision. Sequencing of these clones revealed four with a high degree of homology to the p85α regulatory subunit of PI 3-kinase. Three of the clones were identical to the 3′ half of p85α from nucleotide 953 into the 3′ untranslated region but had a unique 5′ end replacing the SH3 domain and bcr region of p85 with a unique 100-nucleotide sequence (Fig. 1C and 2). This resulted in a novel protein with a predicted molecular mass of 53 kDa and 34 unique amino acids at the N terminus linked to the C-terminal half of p85α, including both SH2 domains and the entire inter-SH2 region (Fig. 1B and 2). This clone, which was subsequently shown to result from alternative splicing of p85α (see below), was termed p85/AS53. Although they are products of separate genes, p85/AS53 is similar in overall structure to the recently identified PI 3-kinase-related protein termed p55^{PIK} identified in screens of adipocyte libraries (24). p55^{PIK} has a 34-amino-acid N terminus which is 62% identical to that of p85/AS53; the remainder of the protein, including the two SH2 domains and the inter-SH2 region, however, is only about 70% identical to p85α, while AS53 is 100% identical in these regions (Fig. 2). An amino acid sequence comparison of p85, p85/AS53, and p55^{PIK} is shown in Fig. 3.

The fourth clone contained a partial sequence including part of the inter-SH2 domain of p85α and the C-terminal SH2 domain. This clone was identical to p85α except for a 24-nucleotide insert that broke the codon for aspartic acid 605 and replaced it with nine amino acids (Fig. 4). Since p85α and

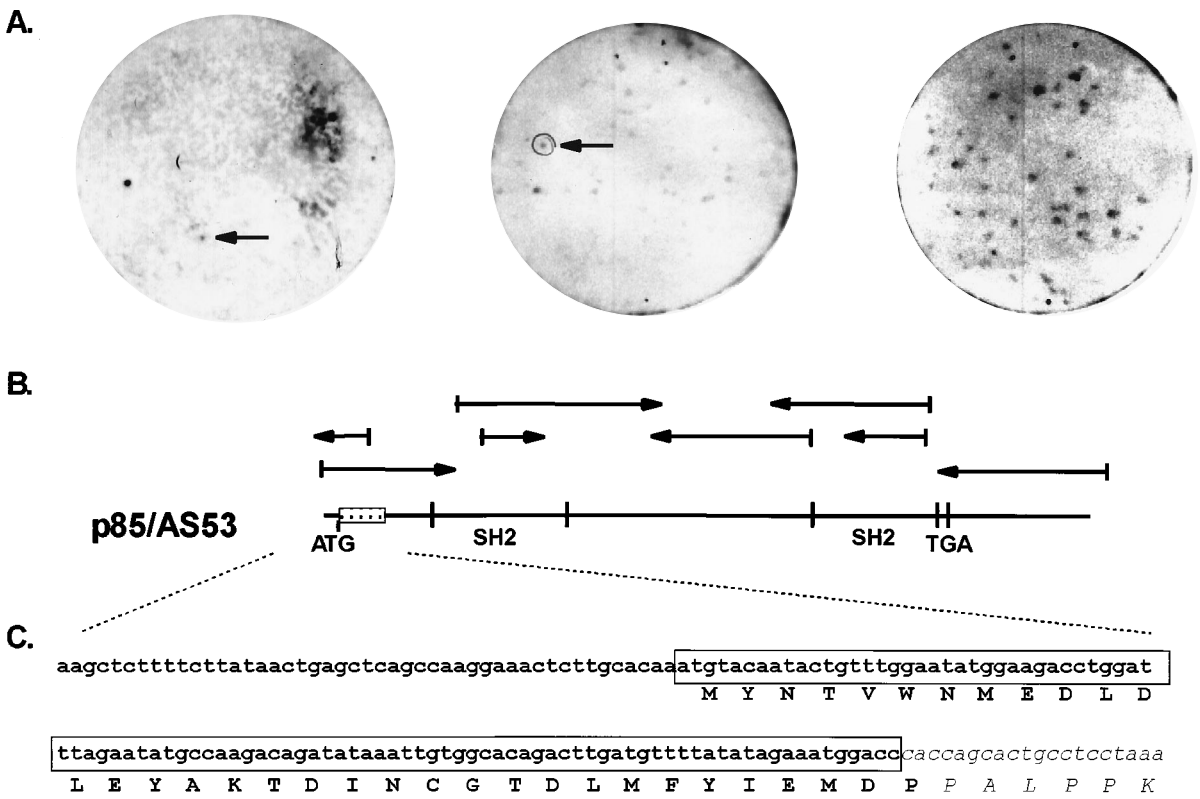

FIG. 1. (A) Primary, secondary, and tertiary screening of skeletal muscle expression library with radiolabeled IRS-1. (B) Schematic representation of the full-length clone of p85/AS53 used for sequencing. The arrows represent individual sequencing reactions. (C) 5′ end of the AS53 cDNA. The unique region of the p85/AS53 cDNA is shown in heavier lettering. The predicted translated region is boxed, with the single-letter amino acid given below the nucleotide sequence and beginning with the first open reading frame, Met.

p85/AS53 are identical over the region covered by this clone, it was impossible to be certain if it represented a p85 isoform or a p85/AS53 isoform.

p85/AS53 and p85 are splice variants of a single gene. Since the p85/AS53 sequence was identical to that of the C-terminal half of p85 including the 3′ untranslated region, it seemed likely that these were products of alternative splicing of the same gene. To test this possibility, we first performed duplicate Southern blot analyses on human genomic DNA, using as probes a region of p85/AS53 which was homologous to p85 and a region which was unique to p85/AS53 (Fig. 5). The homologous probe yielded only one band in each lane, consistent

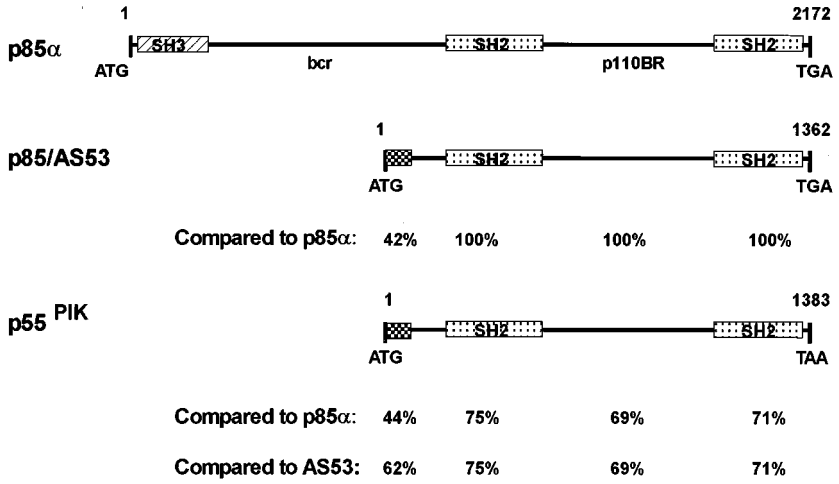

FIG. 2. Schematic comparison of the structural features of p85, p85/AS53, and $p55^{PIK}$. The 100-nucleotide unique translated region of p85/AS53 and the corresponding region in $p55^{PIK}$ are depicted by the cross-hatched box at the N terminus. The cDNA sequence of p85/AS53 was broken into four regions for comparisons: the unique region, the first SH2 region, the inter-SH2 region, and the second SH2 region. Nucleotide comparisons of these regions are shown. p110BR, p110 binding region.

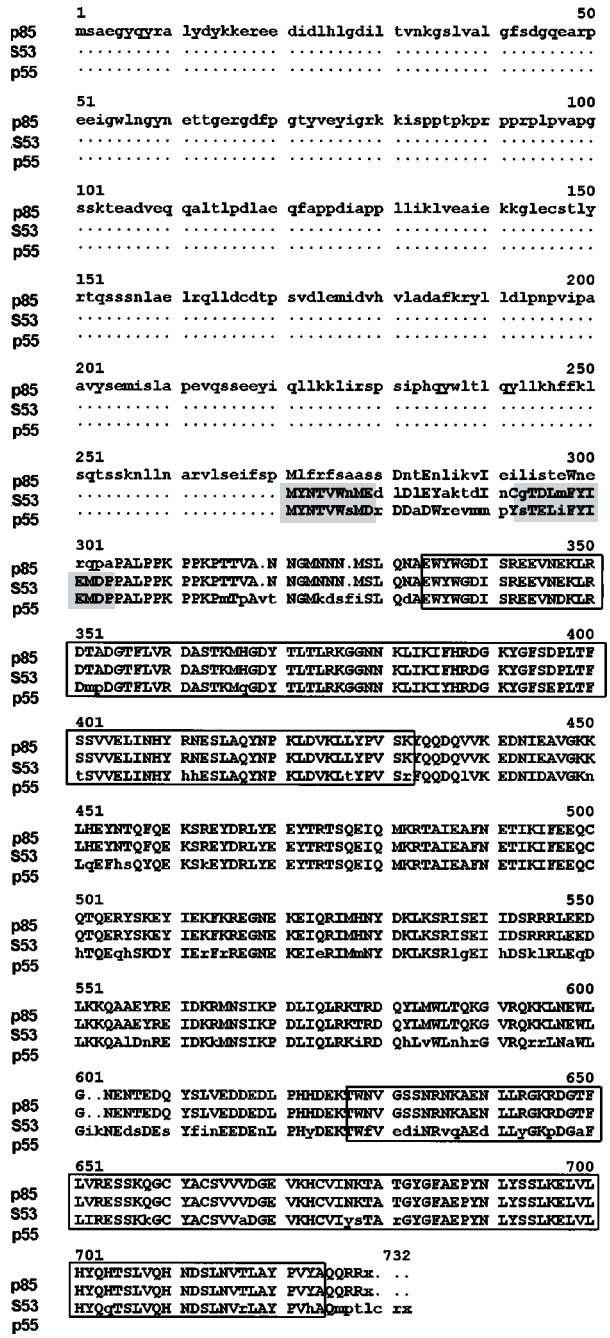

FIG. 3. Comparison of the amino acid sequences of p85, p85/AS53, and $p55^{PIK}$. The sequence of the unique region of p85/AS53 was confirmed in all three clones of p85/AS53 obtained and also with second-strand sequencing of one of the clones. For the homologous region, our sequence was ~99% identical to that obtained previously by Skolnik et al. for human p85α (29); therefore, the sequence of Skolnik et al. was used for this region. The two regions of homology between p85/AS53 and $p55^{PIK}$ in the unique 34-amino-acid N-terminus region are highlighted by stippled boxes. The two SH2 domains are highlighted by unstippled boxes.

with the hypothesis that the identical regions of p85/AS53 and p85 are derived from the same gene. By contrast, following *Eco*RI digestion, the p85/AS53U probe revealed a distinct fragment smaller than that revealed by homologous probe 1. Since there were no *Eco*RI sites in either the p85 or AS53 cDNA between these probes, this result indicates the presence of an intron containing an *Eco*RI site between exons encoding the p85/AS53 unique region and the homologous region.

To obtain the genomic clone of the p85α gene, a human genomic library in the λBlueStar-1 vector system was screened by using homologous probe 1. Two positive clones were isolated by plaque purification, and plasmids were obtained through Cre-mediated excisions. Southern blotting identified one clone of ~13 kb which contained all of the exons of the translated regions for the p85/AS53 and p85 mRNA. Figure 6 reveals the results of replicate Southern blot analyses of the genomic clone hybridized with probes for the p85/AS53 unique region, the p85 unique region, the 5′ side of the homologous region (homologous probe 1), and the 3′ side of the homologous region (homologous probe 2). With additional Southern blotting, we were able to identify the AS53 unique region as 5′ to the p85 unique region. Partial sequencing of the genomic clone also defined the 3′ exon boundary of the AS53 unique portion of the gene and demonstrated that the 24-nucleotide insert was present as a 5′ extension to an exon encoding part of the inter-SH2 domain (Fig. 6B). Together, these data indicate that the p85 gene can be alternatively spliced to form p85 or p85/AS53, as well as the insert isoforms of these splice variants.

Expression of the novel splice variants is tissue specific. The expression of p85, p85/AS53, and the insert isoforms of these proteins was determined by probing a multiple-tissue human Northern blot. Hybridization with a probe specific for the p85 mRNA splice variant revealed two bands, one at ~7.5 kb and another at ~4.4 kb, with a wide tissue distribution. These results are consistent with the previously published expression of p85 mRNA (29). When the blot was hybridized with a probe specific for the AS53 splice variant, a band was identified at ~2.4 kb in skeletal muscle and brain tissue. A low signal at this same position could also be observed in kidney and cardiac muscle. We observed in brain tissue an additional band at ~5.7 kb which probably represents cross-hybridization with the mRNA for $p55^{PIK}$, which is highly expressed in brain tissue and is about 62% identical with the probe (24). When the same blot was hybridized with an oligonucleotide probe specific for the insert region, specific signals were observed over the 2.4-kb p85/AS53 band of skeletal muscle, the 4.4-kb p85 band of cardiac muscle, and, to a much lesser extent, the 4.4-kb p85 band of human skeletal muscle (Fig. 7). Using a nested PCR strategy, we generated AS53- or p85-specific fragments by PCR and used them in a second PCR to test for the presence of the 24-nucleotide insert. This analysis confirmed the Northern blot analysis as to the presence of the insert in both AS53 and p85 mRNA in human skeletal muscle (data not shown).

The Northern blot was also hybridized with a probe for the homologous region of p85/AS53. This analysis allowed for a determination of the quantities of the p85/AS53 and the p85 mRNAs. As is apparent in Fig. 8, the 2.4-kb band corresponding to the p85/AS53 mRNA was more abundant than both the 7.5- and 4.4-kb forms of the p85 mRNA in skeletal muscle and brain. This blot also more clearly reveals the presence of the p85/AS53 mRNA in kidney and cardiac muscle tissue.

Expression of p85/AS53 and p85/AS53 with insert proteins. To determine the expression of these alternatively spliced variants of p85 at the protein level, we generated antibodies to a 12-amino-acid segment of the p85/AS53 unique region and to an 11-amino-acid peptide, including the 9 amino acids encoded by the insert. Western blot analysis with the anti-p85/AS53 antiserum revealed a specific band with an apparent molecular mass of 49 kDa in human and rat skeletal muscle tissue. Blotting with antiserum to the insert peptide also yielded a specific

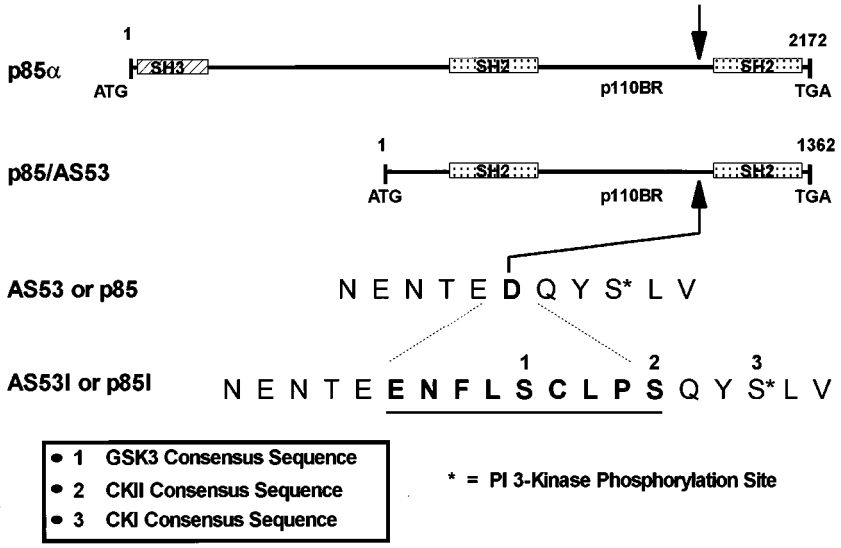

FIG. 4. Schematic of the 24-nucleotide insert in p85 and p85/AS53. The codon for aspartic acid 605 is disrupted with a 24-nucleotide insert, yielding the nine amino acids shown. The insert makes Ser-608 (Ser-3 in the diagram) a casein kinase I consensus phosphorylation site. Ser-2 within the insert is in a casein kinase II consensus sequence. Finally, Ser-1 is in a consensus sequence for glycogen synthase kinase 3. Also note that Ser-608 is the site of autophosphorylation by PI 3-kinase, as indicated by the asterisk. p110, p110 binding region.

band at ~49 kDa in human skeletal muscle but no reaction with rat skeletal muscle proteins in a parallel lane (Fig. 9). Whether this result indicates a difference in sequence for the insert in rats versus humans or the lack of this isoform in the rat is unknown.

The tissue distribution of the p85/AS53 protein was determined by duplicate Western blot analyses of rat tissues blotted with the anti-p85/AS53 antibody and anti-p85 polyclonal antibody. The anti-p85/AS53 antiserum revealed a band at ~49 kDa in rat gastrocnemius/soleus and quadriceps muscles and, to a lesser extent, in rat cardiac muscle. Rat brain, on the other hand, showed a strong band at ~55 kDa and only a weak band at ~49 kDa (Fig. 10A). Most other tissues also showed a weak to moderate band at ~55 kDa with this antiserum. With the anti-p85 polyclonal antibody, the p85 protein was found to be widely expressed, in accord with the tissue distribution of the p85 mRNAs. The absence of a strong p85 band with this antibody, however, is notable in both skeletal muscle samples (Fig. 10B, lanes 1 and 2). Assuming that the reactivity of the

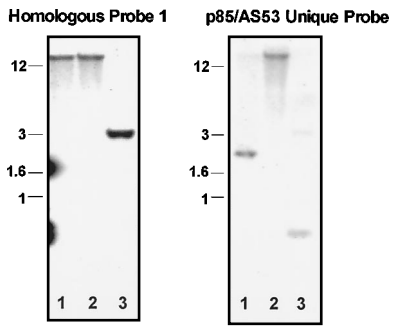

FIG. 5. Southern blot analysis of human DNA. Genomic analysis was performed with probes for the homologous region of p85/AS53 and p85 (homologous probe 1) and for the unique region of p85/AS53 (p85/AS53 unique probe). The DNA was digested with *Eco*RI (lane 1), *Bam*HI (lane 2), or *Pst*I (lane 3) and was separated on a 0.8% agarose gel. The DNA was transferred to nitrocellulose, UV cross-linked, hybridized with the radiolabeled probes, and visualized by autoradiography.

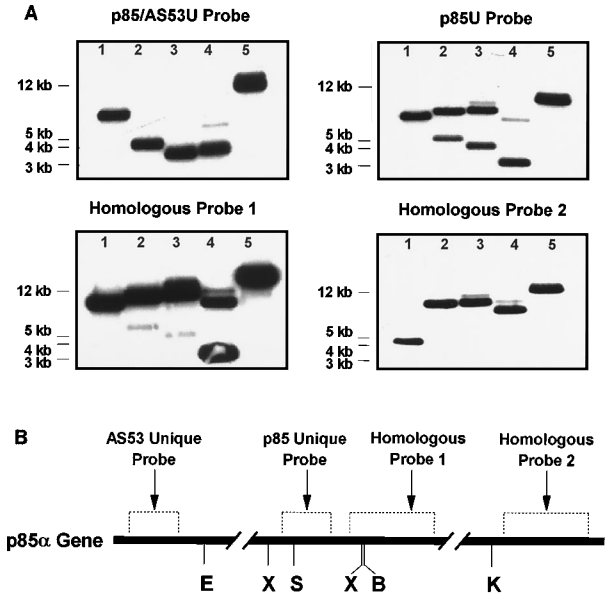

FIG. 6. Analysis of the genomic clone for p85α. (A) The genomic clone for p85α was obtained by screening a human genomic library with homologous probe 1. To determine if this clone had exons encoding the p85/AS53 unique region, the p85 unique region, and the homologous region, Southern analyses were performed. The clone was digested with *Kpn*I and *Sac*II (lane 1), *Sma*I (lane 2), *Xho*I and *Sac*II (lane 3), *Xba*I and *Sac*II (lane 4), and *Sal*I and *Sac*II (lane 5). *Sac*II was previously determined to cut the genomic clone from the vector without further digesting the genomic clone. Replicate Southern blots were made on nitrocellulose, and the blots were probed with a probe specific for the p85/AS53 unique region (p85/AS53U probe), a probe specific for p85 (p85U probe), a probe specific for the 5′ portion of the homologous region (homologous probe 1), and a probe specific for the 3′ portion of the homologous region (homologous probe 2). (B) The Southern analyses depicted in Fig. 6, along with additional Southern analyses and partial sequencing of the genomic clone, were used to develop the working map of the p85 gene. Abbreviations: E, *Eco*RI; B, *Bam*HI; K, *Kpn*I; X, *Xba*I; S, *Sma*I. The distances between restriction sites are drawn to scale, and gaps are used when the precise distance could not be determined. Fragments recognized by the specific probes are indicated.

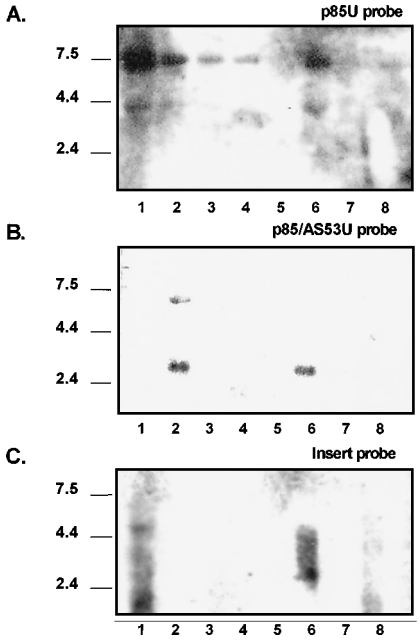

FIG. 7. Tissue distribution of p85 splice variants. A human multiple-tissue Northern blot was probed with probes specific for the p85 unique region (p85U probe), the p85/AS53 unique region (p85/AS53U probe), and an oligonucleotide probe specific for the 24-nucleotide insert. The tissues tested were heart (lane 1), brain (lane 2), placenta (lane 3), lung (lane 4), liver (lane 5), skeletal muscle (lane 6), kidney (lane 7), and pancreas (lane 8). Sizes are indicated in kilobases.

anti-p85 polyclonal antibody with the p85/AS53 protein is similar to that with the p85 protein, the relative amounts of the p85/AS53 and p85 proteins can be estimated by a comparison of the intensities of the signals on this blot. The quantity of p85/AS53 relative to p85 protein matches very well with the results obtained by Northern blot analysis in that p85/AS53 is expressed to a much greater extent than p85 in skeletal muscle. Note also that p85/AS53 does not appear to be present in fat tissue (Fig. 10).

p85/AS53 coprecipitates with p110 and IRS-1. Given that the splice variant p85/AS53 retained the two SH2 domains and the inter-SH2 domain including the p110 binding region, we carried out immunoprecipitation experiments to determine if p85/AS53 bound to IRS-1 and p110. Rats were starved overnight and stimulated with insulin by portal vein injection. Control rats received injections of insulin diluent only. At specific time points after insulin stimulation, the quadriceps muscles were removed. The muscles were homogenized, and the lysates were used for immunoprecipitation with anti-p110 and anti-IRS-1 antibodies and protein A-Sepharose. Coprecipitated proteins were separated on 7.5% polyacrylamide gels, and blots of the gels were probed with a monoclonal antibody specific for the SH2 domain of p85. Figure 11A reveals that both p85/AS53 and p85 coprecipitated with p110 in an insulin-independent manner. Furthermore, the ratio of AS53 to p85 which coprecipitated with p110 matched the ratio of AS53 to p85 in total lysate (2.97 and 2.15, respectively), suggesting that AS53 bound to p110 as well as p85. Figure 11B reveals that both p85/AS53 and p85 also coprecipitated with IRS-1. However, when quantitated with respect to the amount of these proteins in the total lysate, p85 coprecipitated with IRS-1 to a much greater extent than AS53.

DISCUSSION

Over the past decade, a large number of the molecules that transduce the insulin signal have been identified by both functional assays and cDNA cloning, yielding a wealth of information about the mechanisms by which insulin regulates cell growth and metabolism. One important unanswered question is how insulin signaling may differ in various tissues, yielding the unique pattern of responses which is observed following administration of this hormone. To help identify components which might be involved in the skeletal muscle-specific response to insulin, we screened a human skeletal muscle cDNA expression library with baculovirus-produced IRS-1 which had been tyrosine phosphorylated with the insulin receptor in vitro. In this tissue, we identified two novel splice variants of the p85α regulatory subunit of PI 3-kinase. The first of these variants had the amino-terminal half of p85 from amino acids 1 to 304, including the SH3 domain and the bcr region, replaced by 34 amino acids. We termed this molecule p85/AS53. The second splice variant, which was found to be present in both p85/AS53 and p85, was represented by a 24-nucleotide insert in the inter-SH2 region resulting in replacement of an Asp by nine novel amino acids near the Ser site of autophosphorylation in p85. To our knowledge, this is the first evidence for alternative splicing of the p85α gene. Genomic cloning of the p85α gene revealed an intron-exon structure which confirmed the formation of p85/AS53, p85, and the insert isomers as splice variants. Although we were able to identify distinct exons for the AS53 unique region and the p85 unique region, we do not know whether the alternative forms are produced solely by alternative splicing or if alternative promoters are also employed as is the case for the genes for glucokinase (32), the

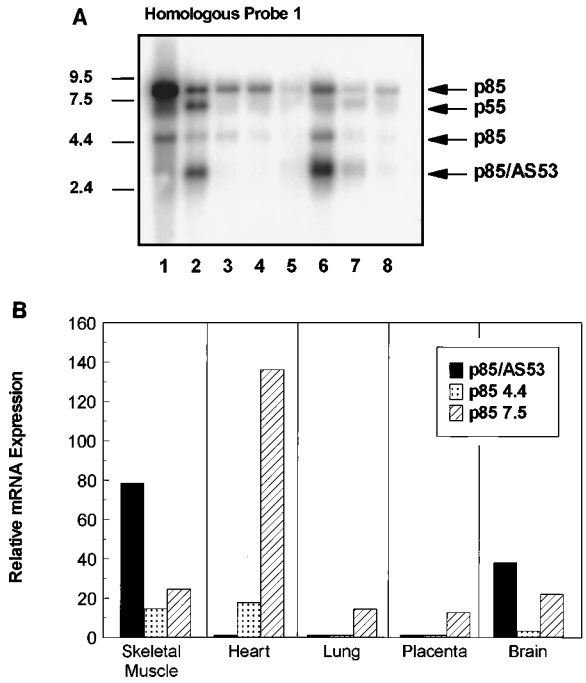

FIG. 8. Tissue distribution of p85 splice variants. (A) The multiple-tissue Northern blot shown in Fig. 7 was hybridized with a probe for the homologous region of p85/AS53 and p85 (homologous probe 1). This allowed a determination of the relative quantity of p85/AS53 mRNA to p85 mRNA in the different tissues tested. Sizes are indicated in kilobases. (B) The relative quantities of p85/AS53 and p85 mRNAs in skeletal muscle, heart, lung, placenta, and brain tissues were determined on a PhosphorImager.

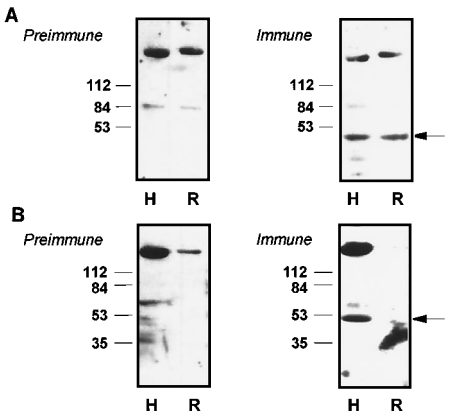

FIG. 9. Immunoblot analysis of p85/AS53 and insert isoforms. (A) An antibody was raised to a 12-amino-acid peptide in the unique region of p85/AS53. This antibody is shown to specifically bind a protein with a molecular mass of ~49 kDa in human (lane 1) and rat (lane 2) skeletal muscle in a Western blot analysis. This antibody does not cross-react with p85. (B) Another antibody directed to the insert region was developed. An 11-amino-acid peptide containing the 9 amino acids of the insert plus the 2 flanking amino acids was used to generate a specific antibody for the insert isoforms of p85/AS53 and p85. The anti-insert antibody specifically binds a protein of ~49 kDa in human (lane 1) but not rat (lane 2) skeletal muscle. We were not able to detect the expected small amount of p85 with the insert in human skeletal muscle. For both anti-p85/AS53 and anti-insert antibodies, a 100-fold dilution of rabbit serum was used for Western blotting, and detection was by enhanced chemiluminescence. Sizes are indicated in kilodaltons.

cyclic AMP phosphodiesterase (13), and dystrophin (1). p85/AS53 is clearly not the same as the previously identified $p55^{PIK}$ (24), which is encoded by a distinct gene (34a) and is only 60 to 70% homologous to p85α and p85/AS53 throughout the length of the molecule.

The tissue distribution of the splice variants of p85α revealed an interesting restricted pattern. By both Northern and Western blot analyses, the molecule p85/AS53 is found to be expressed predominantly in skeletal muscle and brain, with much smaller amounts in heart and kidney. Indeed, in skeletal muscle, the ratio of AS53 to p85α is about 7 to 1, while in lung and fat, the ratios favor p85 by well over 10 to 1. For cardiac muscle, AS53 appears to be about half as abundant as p85. Northern blot analysis and primer-specific PCR indicated that the 24-nucleotide insert isoforms of p85 and p85/AS53 are present primarily in skeletal and cardiac muscle. The distribution of AS53 is distinct from that of $p55^{PIK}$, which is most abundant in brain and testes and also expressed in fat (24). Whether the strong signal at ~55 kDa in immunoblots of brain which we observed with our anti-p85/AS53 antiserum represents a cross-reaction of antibody with $p55^{PIK}$, other alternatively spliced variants of p85, or a shift in mobility of p85/AS53 in the polyacrylamide gel due to a posttranslational modification is unclear.

The precise and complete roles that PI 3-kinase plays in signal transduction are not fully determined. Experiments using inhibitors of PI 3-kinase activity such as wortmannin, LY294002, or specific antibodies indicate that this kinase is involved in the stimulation of mitogenesis (25, 33), middle-T-antigen transformation (19, 33), and p70 S6 kinase activation (20, 28) and, in the case of insulin action, is essential for hormone-induced stimulation of glucose uptake (18, 20, 22, 28, 39), glycogen synthesis (27), and antilipolysis (22). PI 3-kinase phosphorylates PI, PI-4 phosphate, and PI-4,5 diphosphate at the 3 position (9). The major lipid cellular substrate is probably PI-4,5 diphosphate; thus, PI-3,4,5 triphosphate has been postulated to act as an intracellular messenger. However, PI 3-kinase can also phosphorylate some proteins, including IRS-1 (16) and its own p85 regulatory subunit (8), on serine residues, implying a role for protein phosphorylation in PI 3-kinase-mediated signal transduction.

The functional significance of the alternatively spliced variants is under investigation; however, the tissue-specific expression of the splice variants suggests that they may play a part in the specific responses of these tissues to insulin and other growth factors. The p85/AS53 molecule is missing the SH3 domain, as well as the bcr region present in p85. The bcr region has homology to Rho–GTPase-activating protein and binds Cdc42Hs, a small G protein in the Rho family (40). With this region deleted, the ability of Cdc42Hs to regulate PI 3-kinase would presumably be lost. Furthermore, flanking the bcr region are two proline-rich regions which have recently been shown to regulate an interaction of p85 with an SH3 domain of Grb2 (34). The molecule p85/AS53 is missing one of these proline-rich regions, and the splicing of the unique 34-amino-acid leader occurs in the midst of the other proline-rich region. Thus, the proposed p85-Grb2 interaction may be affected in the p85/AS53 splice variant.

While p85/AS53 loses the SH3 domain and bcr region, it gains an unique 34-amino-acid N terminus which contains two

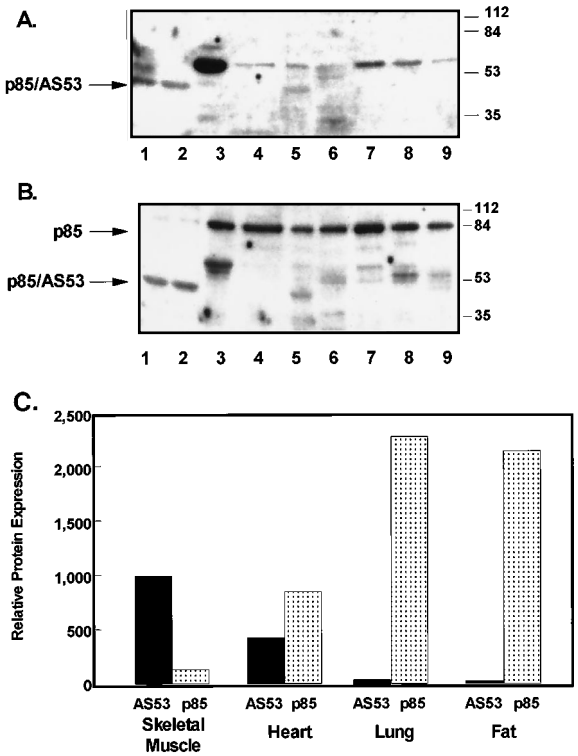

FIG. 10. Tissue distribution of p85 and p85/AS53. Duplicate multiple-tissue rat Western blots were developed to determine the tissue distribution of p85/AS53 protein and the relative quantities of p85/AS53 and p85. The blot contained extracts of gastrocnemius/soleus muscle (lane 1), quadriceps muscle (lane 2), brain (lane 3), fat (lane 4), heart (lane 5), pancreas (lane 6), lung (lane 7), kidney (lane 8), and liver (lane 9) tissues (~100 μg of protein per lane). (A) The blot was probed with the anti-p85/AS53 antibody and detected by ^{125}I-protein A. The specific expression of p85/AS53 in skeletal muscle and to a lesser extent cardiac muscle can be observed. (B) A duplicate Western blot was probed with the anti-p85 polyclonal antibody (Upstate Biotechnology, Inc.). The presence of both p85/AS53 and p85 can be observed. (C) The relative quantities of p85/AS53 and p85 were determined by scanning the autoradiogram and analyzing the image with ImageQuant software.

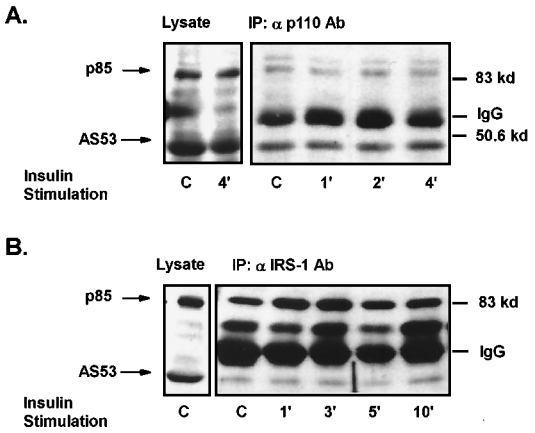

FIG. 11. Coimmunoprecipitation of p85 and p85/AS53 with p110 and IRS-1. Quadriceps muscles were isolated from control (C) and insulin-stimulated rats (stimulation times are indicated in minutes). By using immunoprecipitation (IP) with specific anti-p110 (α-p110) and anti-IRS-1 (α-IRS-1) antibodies (Ab), immune complexes were generated in the quadriceps lysates and pelleted with protein A-Sepharose beads. Proteins were separated on 7.5% polyacrylamide gels and transferred to nitrocellulose, and the blots were probed with a monoclonal antibody to the N-SH2 of p85. Signal was detected by using a horseradish peroxidase-conjugated secondary immunoglobulin G (IgG) antibody and enhanced chemiluminescence. Shown are representative blots of three separate experiments.

regions of 9 and 11 amino acids that are almost completely identical to the same domain in $p55^{PIK}$ (Fig. 3). These regions of identity may indicate an as yet undefined functional significance for the N-terminal 34 amino acids of p85/AS53 and $p55^{PIK}$.

Preliminary data indicate that p85/AS53 behaves differently than p85 with respect to protein-protein interactions. Though the ratio of AS53 to p85 bound to p110 matched the ratio of AS53 to p85 in the total cell lysate, following insulin stimulation much less AS53 than p85 bound to IRS-1. The cause for the relative reduction in binding of AS53 to IRS-1 is uncertain but may involve conformational differences in one or both of the SH2 regions of AS53 due to the presence of the unique 34-amino-acid leader sequence or a difference in compartmentalization of p85 and AS53. Alternatively, the SH2 binding domains of p85/AS53 could be occupied by other phosphotyrosine peptides. Indeed, p85/AS53 includes a potential tyrosine phosphorylation site in a YXXM motif in the 34-amino-acid leader which, if phosphorylated, might allow for homodimerization.

Of perhaps even greater interest is the 24-nucleotide insert which breaks the codon for an aspartic acid and replaces this amino acid with nine new amino acids including two serines. This insert occurs just prior to Ser-608, the Ser which is autophosphorylated by PI 3-kinase, leading to a decrease in the PI 3-kinase activity of the protein (8). Furthermore, the two serines introduced in the insert are in perfect glycogen synthase kinase 3 (GSK-3) and casein kinase II (CK II) consensus sequences, respectively (14). The CK II site is dependent on the prior phosphorylation of Ser-608, and the GSK-3 site is dependent on the phosphorylation of the CK II site; thus, a progressive phosphorylation may occur in which autophosphorylation on Ser-608 leads to the phosphorylation of the CK II site, which in turn leads to the phosphorylation of the GSK-3 site. The specific tissue distribution of the insert isomers may confer an additional level of PI 3-kinase regulation to the skeletal and cardiac muscles.

In summary, two novel splice variants of the regulatory subunit of PI 3-kinase are present in skeletal muscle, cardiac muscle, and brain. These splice variants may have important functional differences in activities or mechanisms of regulation and may play a role in tissue-specific activation of PI 3-kinase, including insulin-stimulated glucose transport or neural control of vesicular trafficking. Whether other splice variants of p85 exist in other tissues or play a role in other specific growth factor signaling pathways remains to be determined. In any case, the finding of alternative splicing of p85α may help our understanding of some of the missing elements in the specificity of insulin and growth factor signaling.

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