Specific Increase in p85α Expression in Response to Dexamethasone Is Associated with Inhibition of Insulin-like Growth Factor-I Stimulated Phosphatidylinositol 3-Kinase Activity in Cultured Muscle Cells*

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The stimulation of phosphatidylinositol (PI) 3-kinase by insulin-like growth factor I (IGF-I) in L6 cultured skeletal muscle cells is inhibited by the glucocorticoid dexamethasone. The objective of this study was to investigate the mechanism of dexamethasone action by determining its effects on the expression of the p85α and p85β regulatory subunit isoforms of PI 3-kinase, their coupling with the p110 catalytic subunit, and their association with insulin receptor substrate 1 (IRS-1) in response to IGF-I stimulation. Dexamethasone induced a 300% increase in p85α protein content in the L6 cultured myoblast cell line, whereas it increased p110 content by only 38% and had no effect on p85β. The increase in p85α protein was associated with a coordinate increase in p85α mRNA. Stimulation with IGF-I induced the association of p85α and p85β with IRS-1, and this was accompanied by increased amounts of the p110 catalytic subunit and markedly increased PI 3-kinase activity in IRS-1 immunoprecipitates. In cells treated with dexamethasone, greater amounts of p85α and lower amounts of p85β, respectively, were found in IRS-1 immunoprecipitates, such that the α/β ratio was markedly higher than in control cells. In spite of the increase in both total and IRS-1-associated p85α following dexamethasone treatment, IRS-1-associated p110 catalytic subunit and PI 3-kinase activity were decreased by approximately 50%. Thus, dexamethasone induces a specific increase in expression of the p85α regulatory subunit that is not associated with a coordinate increase in the p110 catalytic subunit of PI 3-kinase. As a consequence, in dexamethasone-treated cells, p85α that is not coupled with p110 competes with both p85α/p110 and p85β/p110 complexes for association with IRS-1, leading to increased p85α but decreased p85β, p110, and PI 3-kinase activity in IRS-1 immunoprecipitates.

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Growth factor activation of transmembrane tyrosine kinase receptors results in rapid recruitment of phosphatidylinositol (PI) 3-kinase activity to tyrosine-phosphorylated proteins. In intact cells, PI 3-kinase catalyzes the phosphorylation of PI 4,5-bisphosphate (PI-4,5-P2) at the 3′-position of the inositol ring, thus leading to elevation in intracellular PI 3,4,5-trisphosphate (PI-3,4,5-P3) (reviewed in Ref. 1). Unlike the products of PI kinases in the classical PI cycle (PI-4-P and PI-4,5-P3), 3-phosphorylated phosphoinositides are not cleaved by phospholipase C-γ (2, 3), and it has been suggested that they may serve as intracellular second messengers for yet unidentified in vivo targets (1, 4). Extensive experimental evidence has established a key role for PI 3-kinase in the signal transduction mechanisms of a number of peptide growth factors, including epidermal growth factor, platelet-derived growth factor (PDGF), insulin, and insulin-like growth factor-I (IGF-I). PI 3-kinase has thus been implicated in the regulation of multiple general and specialized cellular processes, including membrane ruffling (5), receptor endocytosis (6), mitogenesis (7, 8), cell differentiation (9), and insulin stimulation of glucose transport (10–12) and glycogen synthesis (13, 14).

Mammalian PI 3-kinase is a heterodimer composed of an 85-kDa (p85) regulatory subunit and a 110-kDa (p110) catalytic subunit (15, 16). Two distinct and closely related 85-kDa protein isoforms, p85α and p85β, have been cloned and shown to be the products of separate genes (17–20). Both of these p85 proteins have the capacity to form stable high affinity complexes with the p110 component of PI 3-kinase (21, 22). The two p85 isoforms have a multidomain structure, containing two SH2 (src homology 2) domains, one SH3 domain, and a region with significant sequence similarity to a GTPase-activating protein domain of the product of the breakpoint cluster region gene (23). The presence of several functional domains suggests that the p85 proteins may have multiple interactive and regulatory roles. At present, functional differences between the p85α and p85β protein isoforms have not been established.

Two forms of p110 also have been cloned, one from bovine brain designated p110α (20), and a second human variant designated p110β (24). Expression studies have demonstrated that both p110 proteins have intrinsic PI 3-kinase activity and can associate with the p85 component in intact cells (20, 24). The domains in p85 and p110 required for subunit interaction have been identified and mapped to an amino acid sequence between 1 The abbreviations used are: PI, phosphatidylinositol; IGF-I, insulin-like growth factor-I; PDGF, platelet-derived growth factor; IRS-1, insulin receptor substrate-1; SH, src homology; p85, regulatory subunit of PI 3-kinase of 85 kDa; p110, catalytic subunit of PI 3-kinase of 110 kDa; MEM, minimal essential medium; PY, phosphotyrosine.
the two SH2 domains of p85 and an NH₂-terminal amino acid sequence of p110, respectively (20, 25–27). These studies have established a structural model of the PI 3-kinase complex, in which the p110 subunit contains catalytic activity and is tightly associated with the p85 subunit, which acts as an adapter and/or regulatory subunit. Integrity of the p85-p110 complex appears to be necessary for p110 catalytic activity (26). Thus, overexpression of the p85 subunit or a portion of the p85 protein, such as an intact p85 SH2 domain, through transfection or microinjection of cells results in inhibition of PI 3-kinase activation and cell signaling (28, 29). The physiological occurrence of selective up-regulation of p85 expression as a mechanism of inhibition of PI 3-kinase activity has not been investigated.

The best characterized mode of PI 3-kinase activation in response to peptide growth factors involves changes induced in the p85 protein upon binding to certain phosphorylated tyrosine residues, which are then transmitted to the associated p110 catalytic subunit and cause its activation. In the case of the epidermal growth factor and PDGF receptors, the p85 protein binds directly to phosphorylated tyrosines in the receptor molecule through its SH2 domains (reviewed in Ref. 1). In the epidermal growth factor and PDGF receptors, the p85 isoform with catalytically active p110 subunits.

The line of L6 rat skeletal muscle cells has been described previously (36). Cells were cultured in MEM supplemented with 10% donor calf bovine serum, 2 mM glutamine, and nonessential amino acids in a 5% CO₂ atmosphere at 37 °C. Cells were plated in MEM containing 10% donor calf bovine serum in 150-mm culture dishes. On day 4 after plating, when the cells were 50–60% confluent, the medium was replaced with serum-free MEM containing 0.5% bovine serum albumin, and cells were incubated in the presence or absence of 1 μM dexamethasone (concentration confirmed by absorbance at 242 nm using a molar absorbance coefficient of 1.5 × 10⁻⁶ mol⁻¹ cm⁻¹) for the indicated times. All experiments were carried out with undifferentiated myoblasts.

**Analysis of p85a and p85b mRNA Content**—L6 cell monolayers were rinsed twice with ice-cold phosphate-buffered saline and solubilized directly on the tissue culture plates with 4 M guanidinium isothiocyanate, 0.1 M Tris-HCl, pH 7.5, 0.66% Na₂-laurylsarcosine, and 5% β-mercaptoethanol. Total cellular RNA was isolated by low temperature 4 M guanidinium isothiocyanate-phenol-chloroform extraction, followed by LiCl precipitation (39), and quantitated by spectrophotometry at 260 nm. In all samples, intact ribosomal RNA bands were visualized after electrophoresis. To quantitate p85a and p85b mRNA content in L6 cells, a polymerase chain reaction (PCR) amplification method was used with oligonucleotide primers complementary to sequences that are identical or highly homologous in p85a and p85b mRNA but flank regions of different sizes, such that amplified cDNA fragments from the two p85 isoforms could be separated by polyacrylamide gel electrophoresis (40). Specific primer sequences and cDNA copies of p85a and p85b mRNAs were synthesized using 100 units of Moloney murine leukemia virus-reverse transcriptase in a 20-μl reaction volume containing 3–5 μg total cell RNA and 0.75 μM reverse primer 5'-GTACAGGTT- ATGAGGCTC-3' at 42 °C. This primer is complementary to a nucleotide sequence that is identical in bovine p85a and p85b (nucleotides 2064–2047 and 2046–2029 in p85a and p85b DNA sequences, respectively) and bovine and mouse p85 (17, 19). The reverse transcription products were combined with 2.5 units of Ampli-Taq, 1 × PCR buffer, 100 μM dNTPs, 4 mM MgCl₂, and 0.15 μg oligonucleotide primers in a 100-μl final reaction volume for PCR amplification. The sense primer for PCR amplification was a (1:1) mixture of the oligonucleotides 5'-GACAAACGCATGAACAG-3' and 5'-GACAGCGCATGAGACG-3', corresponding to nucleotides 1678–1694 and 1657–1693 of bovine p85a and bovine p85b cDNAs, respectively. The oligonucleotide sequence 5'-GACAAAACCATGAGACG-3' is identical in bovine and mouse p85a (17, 19). The antisense primer was the same one used for reverse transcription. Based on p85a and p85b DNA sequences, PCR amplification with these primers should generate cDNA products of 386 and 389 bases for p85a and p85b, respectively. 30 cycles of PCR were performed with denaturation at 95 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 1 min. PCR products were 32P-labeled by using a sense primer phosphorylated with T4 polynucleotide kinase (Life Technologies, Inc.) in the presence of [³²P]ATP. Amplified cDNA fragments were precipitated with ethanol, resolved on a 6% denaturing polyacrylamide gel, and visualized by autoradiography. Scanning optical densitometry (Molecular Dynamics, Sunnyvale, CA) was performed to quantify the amplified cDNAs.

**Cell Lysis and Immunoprecipitation of PI 3-Kinase**—After incubation in serum-free medium without or with dexamethasone for the indicated times, cells were left untreated or stimulated with IGF-I (100 nm) for 10 min, washed once with ice-cold phosphate-buffered saline containing 100 μM sodium orthovanadate and twice with 20 mM Tris-HCl (pH 7.6) containing 157 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, and 100 μM sodium fluoride.
orthovanadate (Buffer A). The cells were lysed in Buffer A (1 ml/150-mm dish) containing 1% Nonidet P-40, 10% glycerol, 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 4 μg/ml leupeptin, and 2 mM sodium orthovanadate (lysis buffer). Insoluble material was removed by centrifugation at 12,000 × g for 10 min at 4 °C; the protein concentration in the resulting supernatant was determined with the Bradford dye binding assay (41), and the final protein concentration was adjusted to 2 mg/ml with lysis buffer.

Immunoprecipitation was carried out by incubation of the cell lysate overnight at 4 °C with anti-IRS-1 antibodies and adsorption of resulting immune complexes to protein A-Sepharose beads for 2 h at 4 °C. The pelleted beads were washed successively in phosphate-buffered saline containing 1% Nonidet P-40 and 100 μg/ml sodium orthovanadate (three times), 100 mM Tris-HCl, pH 7.6, containing 500 mM LiCl and 100 mM sodium orthovanadate (three times), and 10 mM Tris-HCl, pH 7.6, containing 100 mM NaCl, 1 mM EDTA, and 100 mM sodium orthovanadate (twice).

**PI 3-Kinase Assay**—The washed immunoprecipitates were resuspended in 50 μl of 10 mM Tris-HCl, pH 7.6, containing 100 mM NaCl, 1 mM EDTA, and 100 μM sodium orthovanadate, and then combined with 10 μl of 100 mM MgCl2 and 10 μl of 2 μg/ml phosphatidylinositol (Avanti) that had been sonicated in 10 mM Tris-HCl, pH 7.6, containing 1 mM EGTA. The PI 3-kinase reaction was started by the addition of 10 μl of 100 mM ATP containing 30 μCi of [γ-32P]ATP. After 10 min at 22 °C with constant vortexing, the reaction was stopped by the addition of 20 μl of 8 N HCl and 160 μl of chloroform/methanol (1:1, v/v). The mixture was vigorously mixed and centrifuged to separate the phases, and the lower organic phase was removed and applied to a silica gel TLC plate precoated with 1% potassium oxalate (Merck, Darmstadt, Germany). The plates were developed in chloroform/methanol/water/ammonia (60: 47: 11.3: 2, v/v), as described previously (36). The PI-3 product was identified by its comigration with a PI-4 standard and quantitated by scanning densitometry.

**Immunoblotting**—For identification and quantification of specific proteins, cell lysates were prepared with 1% Nonidet P-40 as described above and analyzed by immunoblotting either directly or following immunoprecipitation with anti-IRS-1, anti-p85, or anti-p110 antibodies, as indicated. Immunoprecipitates or total cell lysates for immunoblotting were boiled in Laemmli buffer with 100 mM dithiothreitol for 4 min, electrophoresed on 8% SDS-polyacrylamide gels, and transferred by electroblotting onto nitrocellulose sheets (Schleicher & Schuell) as described previously (37). The transfer buffer used was 10 mM Tris, 192 mM glycine, 20% methanol (v/v), and 0.02% SDS, and blotting was carried out at 50 volts for 2.5 h. The membranes were incubated with anti-IRS-1, anti-p85, anti-p85β, or anti-p110 antibodies as appropriate under conditions reported previously (37). Specific protein bands on the membranes were visualized by autoradiography using AmplifyTM (Amersham Int., Little Chalfont, United Kingdom), dried at 80 °C under vacuum, and exposed to Kodak XAR-5 film at −80 °C for 6–36 h.

**Statistical Analysis**—Data are presented as mean ± S.E. Statistical analysis was performed by paired and unpaired Student’s t tests as appropriate.

**RESULTS**

**Identification of p85α and p85β Isoforms of PI 3-Kinase in L6 Myoblasts**—Two isoforms of the p85 regulatory subunit of PI 3-kinase have been described, p85α and p85β, that are expressed in several, although not all, cell types (16, 19). Previous work has demonstrated that p85 is expressed in rat skeletal muscle, but the isoform pattern in this tissue is not known (42). To investigate the expression of p85 isoforms in rat L6 skeletal muscle cells, we employed polyclonal antibodies to the full-length 85-kDa subunit of p85α that detect both p85α and p85β (anti-p85α) and a monoclonal antibody raised against a 19-kDa fragment corresponding to the COOH terminus of p85α that specifically recognizes p85αβ (anti-p85β). As shown in Fig. 1A, two distinct protein species of 85 and 87 kDa were detected by direct immunoblotting with the isoform-nonspecific anti-p85 antibodies. The apparent molecular weights of these two protein species are similar to the reported size of p85α and p85β expressed in a reticulocyte lysate expression system (19). Immunoprecipitation of Nonidet P-40 extracts of L6 cells with anti-p85α prior to immunoblotting produced similar results, although the ratio of immunoreactive p85α to immunoreactive p85β was considerably greater as compared to direct immunoblotting (Fig. 1A), likely reflecting the relatively higher affinity of anti-p85 antibodies for native than denatured p85α. Immunoblotting of these immunoprecipitates with the α isoform-specific antibody anti-p85α confirmed the identity of the lower 85-kDa protein as p85α (Fig. 1B). These data indicate that both p85α and p85β are expressed in rat L6 myoblasts, migrating as 85- and 87-kDa proteins, respectively, on SDS-polyacrylamide gels.

**Regulation of p85α, p85β, and p110 Expression**—To investigate the effects of the glucocorticoid dexamethasone on the expression of the catalytic subunit p110, respectively, were not coordinate.
correspond to the PCR products obtained by amplification of p85α and p85β mRNAs, respectively. The products of 386 and 389 bases were co-amplified in the same PCR reaction by using primers designed to be complementary to identical or highly homologous nucleotide sequences in the p85α and p85β genes that flank fragments of different length. After reverse transcription and PCR amplification, the resulting 35S-labeled PCR products were resolved on a denaturing acrylamide gel and visualized by autoradiography, as shown in A. The correct size of the PCR fragments was confirmed by comparison with a sequencing reaction which was run on the same gel (not shown). The products of 386 and 389 bases correspond to the PCR products obtained by amplification of p85α and p85β mRNA, respectively. B, quantitation of p85α and p85β mRNAs in multiple experiments (n = 3). Open bars, control cells; shaded bars, cells treated with 1 µM dexamethasone for 48 h. *, p < 0.05 versus control cells, paired t test.

PI 3-Kinase Activity Associated with IRS-1—The mechanism of PI 3-kinase activation in L6 cells stimulated with IGF-I involves association of the p85 subunit to tyrosine-phosphorylated residues of the substrate IRS-1 and subsequent activation of the lipid kinase intrinsic to the p110 subunit. Although IGF-I also increases the amount of PI 3-kinase activity recoverable in anti-IGF-I receptor immunoprecipitates, receptor-associated activity represents a minor fraction compared to IRS-1-associated PI 3-kinase activity in L6 myoblasts (36). Since dexamethasone treatment induced a marked increase in the amount of recoverable PI 3-kinase activity, in vitro kinase assays were performed using radiolabeled substrates. The results are consistent with the observed effects of dexamethasone on p85α and p85β protein levels, indicating that the specific augmentation of p85α protein content in L6 myoblasts can be explained by increased levels of p85α mRNA.

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methylasone for 72 h, there was no difference in the basal level of PI 3-kinase activity associated with IRS-1, but IGF-1 stimulation induced only a 23-fold increase in IRS-1-associated PI 3-kinase activity (Fig. 5A). Thus, in comparison with control cells, dexamethasone reduced by 45% the level of IGF-1-stimulated PI 3-kinase activity associated with IRS-1 (Fig. 5B).

Tyrosine Phosphorylation and Cellular Content of IRS-1—The reduced levels of IRS-1-associated PI 3-kinase activity despite the marked increase in p85α protein content in dexamethasone-treated L6 myoblasts could be explained by several mechanisms, including decreased tyrosine phosphorylation and/or cellular content of IRS-1 protein, impaired association between p85 proteins and tyrosine-phosphorylated IRS-1, altered “coupling” between p85 and the catalytic subunit p110, or a combination of these factors. To investigate the tyrosine phosphorylation and cellular content of IRS-1, Nonidet P-40 extracts from control and dexamethasone-treated L6 myoblasts were resolved on 7% polyacrylamide gels, transferred to nitrocellulose, and subjected to immunoblotting with anti-PY or anti-IRS-1 antibodies, as described under “Experimental Procedures.” The autoradiograph in Fig. 6A shows a representative experiment performed with IGF-1 stimulation of intact cells for 10 min. The position of PI 3-phosphate is indicated. The bar graph in B shows the quantitation of multiple experiments (n = 4). Open bars and shaded bars represent control and dexamethasone-treated cells, respectively. *, p < 0.05 versus control cells, paired t test.

FIG. 5. Dexamethasone effect on IRS-1-associated PI 3-kinase activity. L6 myoblasts were incubated in the presence or absence of 1 μM dexamethasone (Dex) for 72 h, and then stimulated with 100 nm IGF-1. PI 3-kinase activity was measured in anti-IRS-1 immunoprecipitates by in vitro assay with [32P]ATP and phosphatidylinositol, followed by analysis of phosphorylated lipids by thin layer chromatography as described under “Experimental Procedures.” The autoradiograph in A shows a representative experiment performed with IGF-1 stimulation of intact cells for 10 min. The position of PI 3-phosphate is indicated. The bar graph in B shows the quantitation of multiple experiments (n = 4). Open bars and shaded bars represent control and dexamethasone-treated cells, respectively. *, p < 0.05 versus control cells, paired t test.

FIG. 6. Effects of dexamethasone on IRS-1 tyrosine phosphorylation and protein content. L6 cells were incubated in the presence or absence of 1 μM dexamethasone (Dex) for 72 h and then stimulated with 100 nm IGF-1 for 10 min or left untreated. Total cell lysates were resolved on 7% polyacrylamide gels and subjected to immunoblotting with either anti-PY or anti-IRS-1 antibodies, as described under “Experimental Procedures.” The autoradiograph in A shows a representative immunoblot of tyrosyl phosphoproteins from control and dexamethasone-treated L6 myoblasts. Quantitation of IRS-1 tyrosine phosphorylation and protein content from multiple experiments is shown in B and C (left panel), respectively. The bar graph in C (right panel) illustrates the ratio of tyrosine phosphorylated to total IRS-1 in control and dexamethasone-treated cells. Open bars and shaded bars represent control and dexamethasone-treated cells, respectively. *, p < 0.05 versus control cells, paired t test.

FIG. 7. Association of metabolically labeled proteins with immune complexes of IRS-1. L6 myoblasts were metabolically labeled with [35S]methionine in the presence or absence of 1 μM dexamethasone (Dex) for 20 h. Prior to lysis, cells were stimulated with 100 nm IGF-I for 10 min or left untreated. Proteins were then immunoprecipitated from cleared lysates using anti-IRS-1 antibody, as described under “Experimental Procedures.” Immune complexes were resolved by 8% SDS-PAGE and subjected to fluorography. A representative of three experiments is shown.
FIG. 8. Effects of dexamethasone on IRS-1/p85 complex formation. Total lysates from L6 myoblasts were subjected to immunoprecipitation with either anti-IRS-1 (panels A and B) or anti-p85 antibodies (panel C). Immunoprecipitates (IP) were resolved on 8% polyacrylamide gels and subjected to immuno blotting with the indicated antibodies, as described under “Experimental Procedures.” In each blot, lane numbers represent the following conditions: 1, control cells in the unstimulated state; 2, cells treated with 1 μM dexamethasone for 72 h in the unstimulated state; 3, control cells stimulated with 100 nM IGF-I for 10 min; 4, cells treated with 1 μM dexamethasone for 72 h and subsequently stimulated with 100 nM IGF-I for 10 min.

Effects of dexamethasone on IRS-1/p110 complex formation. Total lysates were prepared from control or dexamethasone (Dex)-treated L6 myoblasts and subjected to immunoprecipitation with anti-IRS-1 antibody. Immunoprecipitates were then resolved on 8% polyacrylamide gels and subjected to immunoblotting with anti-p110 antibody, as described under “Experimental Procedures.” Panel A shows a representative experiment, and the bar graph in panel B shows the quantitation of p110 in IRS-1 immunoprecipitates from multiple experiments (n = 3). Open bars represent control cells and shaded bars represent cells treated with 1 μM dexamethasone for 72 h. *, p < 0.05 versus control cells, paired t test. In panel C, lysates from control and dexamethasone-treated L6 myoblasts were subjected to immunoprecipitation with anti-p110 antibody followed by immunoblotting with anti-IRS-1 antibody.

IRS-1 in dexamethasone-treated L6 myoblasts (Fig. 7, third and fourth lanes). However, the amounts of IRS-1 associated 35S-labeled p85α and 35S-labeled p85β in IRS-1 immunoprecipitates were approximately 200 and 70%, respectively, in L6 myoblasts treated with dexamethasone compared to control cells, such that the p85α/p85β ratio was increased. In spite of the increase in IRS-1-associated p85α, the amount of 35S-labeled p110 was found to be decreased by 30% in cells treated with dexamethasone (Fig. 7, fourth lane). Thus, the increase in IRS-1-associated p85α was not associated with a coordinate increase in IRS-1-associated p110.

The levels of IRS-1-associated p85α and p85β in control and dexamethasone-treated L6 myoblasts also were determined by immunoblotting with anti-p85 antibody (Fig. 8A, left panel). To specifically detect p85α, similar immunoprecipitates were also analyzed with a monoclonal antibody to p85α (Fig. 8A, right panel). Quantification of the two p85 isoforms in IRS-1 immunoprecipitates is presented in Table I. Small amounts of both p85α and p85β were associated with IRS-1 in the basal state, and the amount of immunoreactive p85β was approximately...
TABLE I
Quantitation of p85α and p85β subunits of PI 3-kinase in IRS-1 immunoprecipitates

|             | Basal control | IGF-I control | Fold increase control | Basal dexamethasone | IGF-I dexamethasone | Fold increase dexamethasone |
|-------------|---------------|---------------|-----------------------|---------------------|---------------------|-----------------------------|
| α           | 0.50          | 4.70^a        | 9.4                   | 2.15^a              | 9.50^bc             | 4.4^a                       |
| β           | 1.00          | 5.50^a        | 5.5                   | 0.78^a              | 3.30^bc             | 4.1                         |
| α/β         | 0.50          | 0.85^a        |                       | 2.69^a              | 2.88^ab             |                             |

^a p < 0.05 versus basal control.
^b p < 0.05 versus basal dexamethasone.
^c p < 0.05 versus IGF-I control.
^d p < 0.05 versus fold increase control.

This study demonstrates that both the cellular amounts of the various subunits constituting the PI 3-kinase enzyme complex and PI 3-kinase subunit association with tyrosine-phosphorylated IRS-1 are differentially regulated by the glucocorticoid dexamethasone in undifferentiated L6 skeletal muscle cells. Dexamethasone markedly increased p85α in L6 myoblasts, but did not alter the levels of p85β and induced only a modest increase in cellular p110 content. Under these conditions, a greater amount of p85α and reduced amounts of both p85β and p110 were recruited to the IGF-I receptor substrate IRS-1 upon hormone stimulation. In addition, the activity of PI 3-kinase measured in IRS-1 immune complexes was significantly decreased by dexamethasone, likely reflecting the reduced amounts of IRS-1-associated p110 catalytic subunit.

Glucocorticoids have been reported to increase the amount of p85 protein in rat skeletal muscle (42) and in F442A adipocytes.
In these previous studies, the $p85_a$ isoform pattern was not determined and, therefore, it is not known whether the effects of dexamethasone were isoform-specific. In L6 myoblasts, the increase in $p85_a$ protein content was associated with an increase in $p85_a$ mRNA and no change in $p85_b$ mRNA, suggesting that dexamethasone may act by specifically increasing expression of the $p85_a$ gene. The $p85_a$ and $p85_b$ isoforms possess 62% overall identity at the amino acid level and 58% nucleotide identity and, thus, are thought to be encoded by two distinct but related genes (19). Information on $p85_a$ gene structure is very limited at present and, in future studies, it will be important to identify the gene regulatory elements that dictate the tissue distribution of the two $p85_a$ isoforms as well as the glucocorticoid responsiveness limited to $p85_a$. Our data would indicate that glucocorticoid response elements may be identified exclusively in the $p85_a$ gene (and not in the $p85_b$ gene).

The selective regulation of the $\alpha$ isoform of $p85$ by dexamethasone supports the concept that $p85_a$ and $p85_b$ may have distinct biological roles. Although the related $p85_a$ and $p85_b$ regulatory subunits both have been shown to form stable complexes with the catalytic $p110$ component of PI 3-kinase (21, 22), functional differences of $p85_a$ as compared to $p85_b$ previously have been reported. Studies conducted in T-lymphocytes have demonstrated that the two $p85_a$ isoforms have a different phosphorylation pattern upon T-cell activation (44). Following activation of the CD3 antigen complex in T-cells, rapid serine phosphorylation of $p85_a$ was observed, whereas phosphorylation of $p85_b$ was unchanged. In addition, the catalytic subunit $p110_a$ was shown to undergo rapid threonine phosphorylation when associated with $p85_b$ but not with $p85_a$. It has recently been reported that much larger stimulation of PI 3-kinase is found in $p85_a$ compared to $p85_b$ immunoprecipitates upon insulin stimulation of CHO-T cells, even though both $p85_a$ and $p85_b$ associate with IRS-1/45). This has led to the suggestion that insulin causes recruitment of both $p85_a$ and $p85_b$ regulatory subunits to IRS-1 signaling complexes, but the activity of IRS-1-associated PI 3-kinase is stimulated only in the $p85ap110$ complex, with little or no stimulation of the $p85bp110$-PI 3-kinase complex. The results in the current study demonstrating specific augmentation of $p85_a$ protein content by dexamethasone indicate that the control of $p85_a$ and $p85_b$ expression represents an additional level of differential regulation of the two $p85_a$ isoforms.

IGF-1 stimulation of L6 myoblasts induced a severalfold increase in the association of both $p85_a$ and $p85_b$ isoforms with IRS-1 immune complexes. Both $p85_a$ isoforms have reportedly been shown to associate with IRS-1 signaling complexes upon insulin stimulation in COS-1 cells transiently transfected with the insulin receptor and in CHO-T cells stably overexpressing the insulin receptor (45). In the latter study, the $\alpha/\beta$ ratio in IRS-1 immune complexes generally reflected the $\alpha/\beta$ ratio in the total cell lysate, indicating no preferential recruitment of a given $p85$ isoform to IRS-1 (45). By contrast, our results indicate that $p85_b$ may preferentially associate with IRS-1 in signaling complexes, since the association of $p85_b$ with IRS-1 immune complexes was greater quantitatively than that of $p85_a$ in response to IGF-1 stimulation (Figs. 7 and 8). It is possible that association of $p85_a$ and $p85_b$ with IRS-1 signaling complexes may be modulated in a cell context specific manner and differ in cell lines expressing high levels of insulin receptors in which hyperphosphorylation of IRS-1 may occur. Interestingly, the amount of $p85_a$ in anti-phosphotyrosine immunoprecipitates from PDGF-stimulated L6 myoblasts was significantly higher than the amount of $p85_b$ and thus the $\alpha/\beta$ ratio was much greater than in IRS-1 immune complexes. This suggests that association of $p85_a$ and $p85_b$ with tyrosine-phosphorylated proteins in L6 skeletal muscle cells may differ depending on the specific protein target (i.e. IRS-1 versus the PDGF receptor).

Ample experimental evidence has established the concept of IRS-1 acting as a "docking protein" capable of simultaneously binding multiple protein components through specific amino acid motifs containing phosphorylated tyrosine residues. GST fusions of the NHL-terminal SH2 domain of $p85_a$ were found to bind strongly to Tyr608-Met-Pro-Met and Tyr939-Met-Asn-Met and, to a lesser extent, to Tyr461-Ile-Cys-Met and Tyr867-Met-Tyr-Met in IRS-1 (35). At present, it is not known whether the same sites serve also for the two SH2 domains present in $p85_b$. Although both $p85_a$ and $p85_b$ were shown to bind simultaneously to a single IRS-1 molecule in COS-1 cells transiently transfected with the insulin receptor, this was not the case in CHO-T cells stably expressing the insulin receptor (45). In the PDGF receptor, Tyr761 is a common binding site for both Nck and $p85_a$, indicating that SH2 domains of different signaling molecules can compete for binding to the same phosphorylated tyrosine motif (46). In L6 myoblasts treated with dexamethasone, the increase in IRS-1-bound $p85_a$ was associated with a coordinate decrease in IRS-1-associated $p85_b$, which could potentially be explained by $p85_a$ and $p85_b$ competing for the same binding site(s) on tyrosine-phosphorylated IRS-1.

Dexamethasone induced a cellular excess of $p85_a$ and a greater number of IRS-1-$p85_a$ complexes in L6 myoblasts. That the pool of IRS-1-associated $p85_a$ was largely composed of regulatory subunit lacking the $p110$ catalytic subunit is indicated by the detection of reduced amounts of IRS-1-associated $p110$ (demonstrated by both $p110$ immunoblotting and metabolic labeling experiments) and a corresponding decrease in PI 3-kinase activity. Although there was an increase in $p110$ protein in total cell lysates following treatment of L6 myoblasts with dexamethasone, this was much less pronounced than the increase in $p85_a$ protein (38 versus 300%, respectively). In interpreting these results, it is important to note that two $p110$ proteins ($p110_a$ and $p110_b$) have been identified (20, 24). We have used a monoclonal antibody raised against bovine $p110$, which recognizes only the $p110_a$ isoform. Therefore, it is not known whether or not dexamethasone affects cellular levels of $p110_b$ and what fraction of IRS-1-associated $p85_a$ in dexamethasone-treated cells was coupled with $p110_b$. However, the decrease in IRS-1-associated $p110_a$ and IRS-1-associated PI 3-kinase activity in response to dexamethasone were coordinate. In addition, a single $^{35}S$-labeled protein of 110 kDa, possibly representing both $p110_a$ and $p110_b$, was detectable in IRS-1 immunoprecipitates upon IGF-1 stimulation. This band was less intense in L6 myoblasts treated with dexamethasone, confirming that the total amount of IRS-1-associated catalytic $p110$ subunit was decreased.

A $p85$ subunit lacking a $p110$ subunit may have the capacity to bind to the specific tyrosine-phosphorylated motif in target proteins without localizing catalytic activity in the protein-potassium signaling complex. Generation of non-coupled (i.e. monomeric or free) $p85_a$ can be achieved experimentally by transfection and overexpression of the $p85_a$ cDNA in mammalian cells. When this experiment was performed in 293 cells overexpressing the PDGF receptor, overexpression of $p85_a$ was shown to completely abrogate activation of PDGF receptor-associated PI 3-kinase activity (28). In addition, microinjection of the $p85_a$ NH2-terminal SH2 domain into rat 1 fibroblasts overexpressing the insulin receptor was shown to inhibit insu-

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lin- and IQG1-induced DNA synthesis by competing with endogenous PI 3-kinase for binding to IRS-1 (29). These studies support the concept that cellular overexpression of the p85α regulatory subunit of the PI 3-kinase complex can lead to inhibition of PI 3-kinase activity and impairment of cell signaling through activation of this enzyme. Accordingly, the excess of free p85α induced by dexamethasone in L6 myoblasts may compete with both p85α-p110 and p85β-p110 complexes for binding to IRS-1. If there are functional specificities for IRS-1-bound p85α and p85β, respectively, this may have distinct effects by disrupting specific signaling responses not only through IRS-1-p85α-p110 complexes, but also through IRS-1-p85β-p110 complexes.

There is evidence that free p85α binds to tyrosine-phosphorylated proteins with greater avidity than the intact p85α-p110 complexes. This mechanism may not be limited to muscle cells and may be functional as a p85α regulatory subunit relative to the other components of the PI 3-kinase subunit along with inhibition of IRS-1-associated PI 3-kinase activity in response to dexamethasone demonstrated in this study suggests a novel mechanism of PI 3-kinase regulation. This mechanism may not be limited to muscle cells and may occur in other cell types under physiological circumstances and/or in disease states characterized by high levels of endogenous cortisol or exogenously administered glucocorticoids.

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