Molecular and Genetic Studies Imply Akt-mediated Signaling Promotes Protein Kinase CβII Alternative Splicing via Phosphorylation of Serine/Arginine-rich Splicing Factor SRp40*

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From the †Department of Biochemistry and Molecular Biology and Internal Medicine and the ‡Department of Pathology, University of South Florida College of Medicine, Tampa, Florida 33612, the ¶J. A. Haley Veterans Hospital, Tampa, Florida 33612, and the ‖Howard Hughes Medical Institute, Department of Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

Insulin regulates alternative splicing of PKCβII mRNA by phosphorylation of SRp40 via a phosphatidylinositol 3-kinase pathway (Patel, N. A., Chalfant, C. E., Watson, J. E., Wyatt, J. R., Dean, N. M., Eichler, D. C., and Cooper, D. C. (2001) J. Biol. Chem. 276, 22648–22654). Transient transfection of constitutively active Akt2 kinase promotes PKCβII exon inclusion. Serine/arginine-rich (SR) RNA-binding proteins regulating the selection of alternatively spliced exons are potential substrates of Akt kinase because many of them contain RXRXX(S/T) motifs. Here we show that Akt2 kinase phosphorylates SRp40 in vitro and in vivo. Mutation of Ser56 on SRp40 blocked in vitro phosphorylation. In control Akt2(+/+) fibroblasts, insulin treatment increased the phosphorylation of endogenous SR proteins, but their phosphorylation state remained unaltered by insulin in fibroblasts from Akt2(−/−) mice. Levels of PKCβII protein were up-regulated by insulin in Akt2(+/+) cells; however, only very low levels of PKCβII were detected in Akt2(−/−) cells and did not change following insulin treatment. Endogenous PKCβII and βII mRNAs in Akt2(+/+) and Akt2(−/−) gastrocnemius muscle tissues were compared using quantitative real-time PCR. The results indicated a 54% decrease in the expression of PKCβII levels in Akt(−/−), whereas PKCβ levels remained unchanged in both samples. Further, transfection of Akt2(−/−) cells with a PKCβII splicing minigene revealed defective βII exon inclusion. Co-transfection of the mutated SRp40 attenuated βII exon inclusion. This study provides in vitro and in vivo evidence showing Akt2 kinase directly phosphorylated SRp40, thereby connecting the insulin, PI 3-kinase/Akt pathway with phosphorylation of a site on a nuclear splicing protein promoting exon inclusion. This model is upheld in Akt2-deficient mice with insulin resistance leading to diabetes mellitus.

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Insulin regulates splicing of pre-mRNA for protein kinase CβII (PKCβII)1 by enhanced exon inclusion in rat skeletal muscle myotubes (L6) (1, 2). Alternative splicing of nuclear pre-mRNA allows for the formation of multiple RNA isoforms from a single primary RNA transcript. It is a widespread mechanism controlling gene expression in higher eukaryotes (3), and an estimated 30,000 human genes generate over 100,000 protein isoforms with distinct functions by alternative splicing (4, 5). Alternative splicing can be controlled developmentally or by tissue specificity (6–9) and is a well studied mechanism. Our laboratory has demonstrated hormonal regulation of PKCβII alternative splicing, and mechanisms for exon recognition involve the regulated phosphorylation of the serine/arginine-rich (SR) protein SRp40 (10, 11).

A family of splicing factors, SR proteins, are highly phosphorylated RNA-binding proteins involved in the alternative splicing process (12–14). Ten highly conserved SR proteins consist of one or two N-terminal ribonucleoprotein-type RNA-binding domains and a C-terminal SR domain that promotes protein-protein interactions within the splicing complex. SR proteins are required for constitutive splicing for the formation of the early pre-sarcosomal complex to stabilize U1 small nuclear ribonucleoprotein at the 5′ splice site and in spliceosome formation to mediate interactions between U1 small nuclear ribonucleoprotein and U2AF, bound to the 5′ and 3′ splice sites (15–17). SR proteins also function in alternative splicing in vivo in a concentration-dependent manner by modulating 5′ splice site choice (18, 19). The SR domains direct SR proteins to localize to speckles within the nucleus (20), and some SR proteins can “shuttle” between the nucleus and cytoplasm depending on their phosphorylation state (21). The function of SR proteins in hormone-regulated alternative splicing has not been elucidated.

Our studies focused on SRp40 because its expression was known to be regulated by insulin (22). Predictions by SELEX and reports by other groups identified a binding site for SRp40 in the downstream intron of the rat PKCβ gene (23). We found that modified antisense oligonucleotides (RNase H-resistant) blocked the interaction of SRp40 with the PKCβ intron and

1 The abbreviations used are: PKC, protein kinase C; PI, phosphatidylinositol; SR, serine/arginine-rich; MEM, minimum essential medium; HA, hemagglutinin; RT, reverse transcription; TBP, TATA-binding protein; CA, constitutively active; SD, splice donor exon; SA, splice acceptor exon.

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inhibited 5′ splice site choice, resulting in splicing of PKCβI. Further, increases in the phosphorylation state of SRp40, independent of alterations in its concentration, modulated PKCβII 5′ splice site choice in vitro (24).

Kinases that phosphorylate SR proteins are generally thought to be constitutively active kinases such as SRPK1, SRPK2, and Ctk/Ste (25–28). However, in the case of alternative splicing of PKCβII mRNA, insulin enhances 5′ splice site selection via activation of phosphatidylinositol 3-kinase (2, 24).

Activation of phosphatidylinositol 3-kinase is an obligate intermediate event in transducing the metabolic, anti-apoptotic, and mitogenic effects of insulin. PI 3-kinase phosphorylates specific phosphoinositides to generate the phosphorylated lipids phosphatidylinositol 3,4,5-trisphosphate, which recruit downstream effector enzymes such as phosphoinositide-dependent kinase 1 and Akt kinase via their pleckstrin homology domains. Phosphoinositide-dependent kinase 1 is a serine/threonine kinase that phosphorylates downstream kinases such as Akt on regulatory residues dependent of alterations in its concentration, modulated Akt in L6 rat skeletal muscle cells, a model that mimics in vivo (24).

A number of laboratories have sought to identify in vivo substrates of Akt to further extend its roles in signaling (for reviews see Refs. 34 and 35). The growing list of substrates include BAD (36), glycogen synthase kinase 3 (37), 6-phosphofructo-2-kinase (38), IkB kinase (39, 40), Raf-1 kinase (41), breast cancer susceptibility gene 1 (BRCA1) (42), forkhead transcription factors (43), and WNK1 (with no K (lysine) protein kinase) (44). Analysis of its substrates defined the minimal phosphorylation motif as Arg-Xaa-Arg-Yaa-Zaa-(Ser/Thr)-Hyd, preferably small residues other than glycine, and Hyd is a bulky hydrophobic residue (45). This approach identified splicing factors hknPA1, snNP1, RNP S1, as well as SR proteins as potential substrates, and GenPept data base searches using this consensus motif scored spliceosomal proteins involved in RNA processing and previously identified as substrates of SR kinases as potential substrates for Akt. However, the activation of a PI 3-kinase/Akt pathway targeting a specific SR protein with a role in RNA splicing and metabolic functions has not been identified.

Previously, we demonstrated a role for Akt in mediating the 5′ splice site selection of PKCβII mRNA in the pSPL3-βII minigene in L6 myotubes (11) and have shown insulin activation of Akt in L6 rat skeletal muscle cells, a model that mimics many of the properties of human skeletal muscle cells in culture (2). In this study we provide biochemical and genetic evidence that Akt2 kinase phosphorylates SRp40 in vivo and in vitro. In immortalized fibroblasts from mice lacking Akt2 (46), SRp40 phosphorylation is not regulated by insulin, and PKCβII levels are low compared with cells from wild type mice. This report is novel because it links the binding of an extracellular ligand, insulin, to a kinase pathway involved in post-transcriptional processing of pre-mRNA that alters gene expression by regulating the phosphorylation of SR proteins. Although this had been predicted based on in vitro studies, this observation further extends the function of Akt2 to post-transcriptional processing of pre-mRNA and demonstrates that in addition to known SR protein kinases, Akt2 phosphorylation of splicing factors plays a critical role in exon inclusion.

EXPERIMENTAL PROCEDURES

Materials—Tissue culture medium was purchased from Invitrogen. Fetal bovine serum was purchased from Atlanta Biologicals (Norcross, GA). Porcine insulin was obtained from Sigma. The reagents used for polyacrylamide gel electrophoresis were from Bio-Rad (Richmond, CA). Anti-SRP40 (polyclonal antibody) used for the in vivo assay was provided by Dr. Taub (University of Pennsylvania). SRp40 polyclonal antibody was raised in rabbits to the synthetic peptide NH2-(GC)-EVYTFADAHPRKLNE-COOH (residues 133–146, RNA-binding domain), whereas ASP/SRF2 polyclonal antibody was raised in rabbits to the synthetic peptide NH2-(GC)-KEDMTYAVRKLDTNK-COOH (residues 165–179, RNA-binding domain) by Bio-Synthesis, Inc. (Louisville, KY). The antibodies were characterized alongside unreactive preimmune sera and were shown to recognize both the long and short forms of SRp40 in mouse and rat call lines. Antibody to the phosphoepitope of all SR proteins (mAb104) was obtained from hybridoma cells (CRL 2067; ATCC). Anti-PKCβII (polyclonal antibody), anti-rabbit, and anti-mouse IgG antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-Akt (T308) and Akt2 antibody were from Cell Signaling (Beverly, MA). ECL reagents were from Amersham Biosciences. PCR primers were synthesized by MWG BIOTECH, Inc. (High Point, NC). Primers for β-actin were from Clontech (Palo Alto, CA). Superscript II reverse transcriptase was from Invitrogen. Teg Platinum polymerase was from PerkinElmer Life Sciences. Lipofectin was from Promega. All other biochemicals and reagents were purchased from the usual vendors.

Cell Culture—Rat L6 skeletal myoblasts (obtained from Dr. Amira Klip, The Hospital for Sick Children, Toronto, Canada) were grown α-MEM supplemented with 10% fetal bovine serum to confluency in 100-mm or 6-well plates. The cells used for transient transfection were grown to a visual density of 60%. The cells were fused into myotubes by changing the medium to α-MEM supplemented with 2% fetal bovine serum for 4 days. The extent of cellular differentiation was established by observation of multi-nucleation of 55–90% of cells. For experiments, myotubes were incubated in α-MEM with 0.1% bovine serum albumin for 6 h and placed in phosphate-buffered saline with 0.1% bovine serum albumin just prior to treatment with insulin.

The immortalized murine fibroblasts were derived as described (46) and were maintained in Dulbecco’s modified Eagle’s medium, high glucose, supplemented with 15% fetal calf serum, 2 mM L-glutamine, and penicillin-streptomycin (1 mg/ml) and kept at 37 °C in a humidified 5% CO2, 95% air atmosphere.

Plasmid Constructs—The constitutively active hemagglutinin (HA) epitope-tagged Akt2, pcDNA3-m/p-HA-Akt2, was prepared from a PCR product by adding 12 amino acids derived from the N terminus of the Lck tyrosine kinase to the N terminus of HA-Akt2. This PCR fragment was subcloned as described (47). The SRp40 cDNA construct used for in vitro analysis was kindly provided by Dr. Rebecca Taub (48). The myc-SRP40 fusion protein was cloned as follows. Full-length SRp40 cDNA was amplified from L6 myotubes using sense (5′-AGGTATCCA-TGAGTGCTGTCCAGGAGTT-3′) and (5′-AAGCTGATCCGGACAA-ACTCTGGCACTGACTTTGAAGTGATATC-3′) antisense primer. The product was ligated then into pcDNA3/myc-His vector. The resulting clone was sequenced and demonstrated to express a fusion protein in L6 myotubes.

Transient Transfection of Plasmid DNA—Plasmid DNA (1 µg/well) was transfected into cells at 60% confluency using Lipofectin™ for 3 h as per the manufacturer’s instructions. The cells were then placed in α-MEM with 2% fetal bovine serum overnight prior to insulin addition. Overexpression was demonstrated by Western blot analysis using anti-Akt2 (Cell Signaling). Total RNA was isolated using Stat-80, and RT-PCR analysis was performed as described below. The transfectivity of L6 myotubes was routinely shown to be >60% (49).

Transient Transfection of the pSPL3-βII Minigene—L6 cells were co-transfected with the 2 µg of heterologous splicing minigene pSPL3-βII (11) alone or along with the mutated SRp40™ using Lipofectin™ for 3 h as per the manufacturer’s instructions. The cells were then placed in α-MEM with 2% fetal bovine serum overnight prior to insulin addition (100 nm) for 30 min. Total RNA was isolated, and RT-PCR was performed as described below.

RT-PCR Analysis for PKCβII and βII and pSPL3-βII Minigene—Total RNA (1 µg) was used to synthesize first strand cDNA using an oligo(dT) primer and Superscript II reverse transcriptase. Inclusion of the βII-specific exon was detected using primers corresponding to the C4 kinase domain, common to both PKCβII and βII (5′-GTTGGGGCTGAAAGGGAACG-3′), and an antisense primer to the βV5 exon common to both transcripts, (5′-TGCTCTGGA-AAACTTCTTGTG-3′). The PCR products for PKCβII are 159 and 374 bp for PKCβII. This assay allows for a relative comparison of both PKCβII and PKCβII mRNA levels in the same reaction. The pSPL3-βII minigene was amplified using sense primers for splice donor exon (SD)
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FIG. 1. Constitutively active Akt2 kinase mimics insulin-induced endogenous PKCβII splicing. a, the figure depicts alternative splicing of PKCβII mRNA regulated by insulin. Upon insulin treatment, SRp40 is phosphorylated which then mediates PKCβII exon inclusion to produce mature PKCβII mRNA. b, L6 cells were transfected with CA-Akt2 or SRp40 and treated with insulin for 30 min. Total RNA was isolated, and RT-PCR was performed using sense primers for C4 and antisense primer for βII. 5% of products were resolved on 6% PAGE gels and detected by silver staining. The experiments were repeated three times to ensure reproducibility. Splicing efficiency was quantified by densitometric scanning of the silver-stained gels from three experiments and represented on the graph. An arbitrary value of 100% splicing efficiency is attached to β exon in the control (t0) sample and 100% βII splicing efficiency in the insulin-treated (t30) sample.

(5’-TCTCAATGTCCGTGCCACTC-CAAC-3’) and antisense primer for splice acceptor exon (SA) (5’-CCACCACACACACCACCTCT-3’). The expected products are shown in Fig. 4. Following 35 cycles of amplification in a Biotera Trioblock thermocycler (for C4-βI/βV the cycling conditions were: 95 °C for 30 s, and 68 °C for 2 min; SD-SA: 94 °C for 1 min, 58 °C for 1 min; and 72 °C for 3 min) using Taq Platinum DNA polymerase (PerkinElmer Life Sciences), 5% of the PCR reaction was resolved by 6% PAGE gels and detected by silver staining. Densitometric analyses of the bands were done using the Scan Analysis Software.

In Vivo Labeling of Cells with [32P]Orthophosphate—Rat skeletal muscle (L6) cells were transfected with myc-Akt2 CA (constitutively active) (50) and labeled with [32P]orthophosphate (0.5 mCi/ml) in α-MEM without phosphate for 2 h either with insulin (100 nM) or without insulin. SRp40 was immunoprecipitated using the SRp40-specific antibody. The immunoprecipitates were then separated on SDS-PAGE, and phosphorylated SRp40 was detected by autoradiography.

Expression of myc-SRp40 in L6 Cells—The cDNA construct encoding myc-SRp40 was transfected into L6 myotubes (100 mm dishes) as described using Lipofectin (24). Post-transfection (36 h), the cells were serum-starved for 24 h in 0.5 ml of ice-cold lysis buffer, centrifuged at 4 °C for 30 min at 26,000 × g, and the myc fusion proteins were purified by protein A-agarose using c-myc antibody (Santa Cruz). The blot was probed with SRp40 antibody.

Akt2 II splicing in L6 Cells—The HA-tagged construct containing HA-Akt2 CA was transfected into L6 myotubes as described above. The cells were transfected and serum-starved in the same manner, and the cell lysate and HA-antibody (Santa Cruz) were incubated with protein A-agarose. Isolation of the HA fusion protein was identical to that described above.

Immunoprecipitation of SRp40 and Akt2—L6 myotubes were collected by centrifugation, and the pellets were lysed in 20 volumes 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0, with protease inhibitors: benzamidine HCl, 16 μg/ml; aprotinin, 10 μg/ml; leupeptin; 10 μg/ml; phenylmethylsulfonyl fluoride, 1 mM. The cells were placed on ice for 30 min, and insoluble material was pelleted at 12,000 × g for 10 min at 4 °C. An aliquot (50 μl) of lysate was incubated at a final concentration of 1 μg/ml with anti-SRp40 polyclonal antibody or anti-Akt2 kinase antibody followed by agitation at 4 °C for 2 h. A 40-μl aliquot of protein A-Sepharose beads in a 1:1 suspension with the lysis buffer was added and incubated again for 1 h. After centrifugation at 10,000 × g, the beads were washed twice with 1 ml of lysis buffer containing 0.5% NaCl, followed by a wash in lysis buffer with no NaCl. After adding 50 μl of SDS-PAGE sample buffer, the precipitate was boiled for 5 min and centrifuged at 1000 rpm for 5 min before loading on the gel followed by Western blot analysis.

In Vitro Akt2 Kinase Assay—Constitutively active HA-Akt2 and myc-SRp40 were expressed in L6 cells and immunoprecipitated as described above. The immunoprecipitates were incubated with 10 μCi of [γ-32P]ATP (ICN Biomedicals, Irvine, CA) for 30 min in 25 μl of kinase buffer (20 mM HEPES, 10 mM MgCl2, 10 mM MnCl2, 1 mM diithiothreitol, 5 mM ATP) at 25 °C using myc-SRp40 as substrate. The reactions were terminated using Laemml SDS sample buffer. Incorporation of phosphate was determined following electrophoresis of samples on a 12% SDS-PAGE gel and phosphorus imaging (Bio-Rad) of the gel. The Akt kinase was also assayed using CRESTide (GRPRTPSSAPGC, 30 μM) or Kemptide (LRRASLG, 30 μM) in parallel assays, and incorporation of 32P radioactivity into these peptides was determined using P81 phosphocellulose (data not shown).

smMutation of the Akt2 Phosphorylation Site on SRp40—The Akt2 site was predicted based on the sequence (RIIXX(XST/I)) (35). One site (Ser244) is located in the RS domain of SRp40. This was mutated to Ala in the construct using site-directed mutagenesis (Stratagene) and is denoted as SRp40*. Expression of the mutated protein was verified by immunoprecipitation using myc antibody followed by detection with SRp40 antibody.

Cross-linking of Akt2 and SRp40 Antibodies and Immunoprecipitation—Purified IgG fractions were cross-linked to protein A magnetic beads (New England Biolabs) using 0.2 mM triethanolamine with 25 mM dimethyl pimelidate dihydrochloride and blocked in 0.1 M ethanolamine. IgG cross-linked beads were used to immunoprecipitate Akt2 and SRp40 from cell lysates.

Western Blot Analysis—L6 muscle cell lysates (40 μg) or immunoprecipitates were separated on 10% PAGE-SDS. Proteins were electrophoretically transferred to nitrocellulose membranes, blocked with 5% BSA and 0.1% Tween 20 containing 5% nonfat dried milk, washed, and incubated with a polyclonal antibody against SRp40, ASP/SP2, or monoclonal antibody against the phosphoepitope (RS domain) of SR proteins, mAb104 (51), or Akt2 (Cell Signaling Laboratories, Beverly, MA). Following incubation with anti-rabbit or anti-mouse IgG horseradish peroxidase, detection was performed using enhanced chemiluminescence (Pierce).

Real-Time Quantitative PCR—Total RNA was isolated from control C57BL/6J mouse Akt2 (+/+) and Akt2 (−−) gastrocnemius tissue from 16-week-old animals (46) using TriReagent (Sigma). The acquisition, care, housing, use, and disposal of animals were in compliance with applicable federal, state, local, and institutional laws and the regulations of the University of Pennsylvania. Total RNA (1 μg) was reverse transcribed using a high capacity cDNA archive kit (product 4322171; Applied Biosystems) according to the manufacturer’s instructions. Real time quantitative PCR was performed with an ABI PRISM 7900 sequence detection system (PE Applied Biosystems, Foster City, CA) to quantify the relative levels of mRNA in the samples. Real time quantitative PCR was performed to amplify PKCβ, and βII and TATA-binding protein (TBP) as the endogenous control. A 2-μl sample of cDNA was amplified by real time PCR using Assays-by-Design service from Applied Biosystems, consisting of a 20-fold mix of unlabeled PCR primers (obtained from Applied Biosystems) for PKCβ, PKCβIII, and TBP. TaqMan® MGB probes (FAM dye-labeled) were utilized. The primers designed for PKCβ were at the junction of the last common domain, C4, and the −β exon, whereas that for PKCβIII were at the junction of C4 and βII exon. Each sample was run in triplicate for PKCβ, PKCβIII, and TBP in a 20-μl reaction using TaqMan® Universal
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Akt2 Kinase Phosphorylates SRp40 Promoting PKCβII Exon Inclusion—We previously found increased phosphorylation of SRp40 by a PI 3-kinase-dependent pathway following insulin treatment (24), although the specificity of the kinases involved and the phosphorylation

Transfection of cells with constitutively active Akt2 kinase mimics insulin-induced splicing of PKCβII. Insulin-stimulated inclusion of the exon specifying the C-terminal V5 region of PKCβII (1, 2) and insulin-regulated splicing of the βII exon involved phosphorylation of SRp40 by a PI 3-kinase-dependent signaling pathway (24). It has been shown that the physiological responses of insulin such as glucose uptake and Glut4 translocation are preferentially mediated by Akt2 kinase (46). We hypothesized that insulin activation of Akt would also result in the increased phosphorylation of SRp40 because SR proteins are potential i

RESULTS

Transfection of cells with constitutively active Akt2 kinase immunoprecipitates were incubated with 10 mCi of [γ-32P]ATP (ICN Biomedicals) and myc-SRp40 (or mutated myc-SRp40) immunoprecipitates for 30 min in 25 ml of kinase buffer (20 mM HEPES, 10 mM MgCl2, 10 mM MnCl2, 1 mM dithiothreitol, 5 mM ATP) at 25 °C. The reactions were terminated using Laemmli SDS sample buffer. The proteins were separated on 12.5% SDS-PAGE, and phosphorylation was visualized by phosphorus imaging (Bio-Rad). The experiments were repeated five times with similar results. b, the myc-SRp40 construct was validated in separate experiments. The construct was transfected into L6 cells, and the lysate was immunoprecipitated with using anti-myc antibody. The SRp40 antibody was then used for detection. In a separate experiment performed simultaneously, the lysate was immunoprecipitated with SRp40 antibody, and anti-myc was used for detection. Lane 1, mock transfection; lane 2, insulin at 0 min; lane 3, insulin at 30 min. IP, immunoprecipitation; IB, immunoblot.

assessed by Student's t test. A level of p < 0.05 was considered statistically significant. Significance is determined after three or more separate experiments.

FIG. 3. a, in vitro Akt2 kinase assay. Constitutively active Akt2 kinase immunoprecipitates were incubated with 10 mCi of [γ-32P]ATP (ICN Biomedicals) and myc-SRp40 (or mutated myc-SRp40) immunoprecipitates for 30 min in 25 ml of kinase buffer (20 mM HEPES, 10 mM MgCl2, 10 mM MnCl2, 1 mM dithiothreitol, 5 mM ATP) at 25 °C. The reactions were terminated using Laemmli SDS sample buffer. The proteins were separated on 12.5% SDS-PAGE, and phosphorylation was visualized by phosphorus imaging (Bio-Rad). The experiments were repeated five times with similar results. b, the myc-SRp40 construct was validated in separate experiments. The construct was transfected into L6 cells, and the lysate was immunoprecipitated with using anti-myc antibody. The SRp40 antibody was then used for detection. In a separate experiment performed simultaneously, the lysate was immunoprecipitated with SRp40 antibody, and anti-myc was used for detection. Lane 1, mock transfection; lane 2, insulin at 0 min; lane 3, insulin at 30 min. IP, immunoprecipitation; IB, immunoblot.

PCR Master Mix according to the manufacturer's instructions (Applied Biosystems). The reactions were performed in an ABI PRISM® 7900 sequence detector system (PE-ABI). All of the standards and samples were assayed in triplicate. Thermal cycling was initiated with an initial denaturation at 50 °C for 2 min and 95 °C for 10 min. After this initial step, 40 cycles of PCR (95 °C for 15 s; 60 °C for 1 min) were performed. Statistical analysis of the quantitative real time PCR was obtained using the (2 \(-\Delta\Delta C_t\)) method (52, 53), which calculates relative changes in gene expression of the target (β or βII) normalized to an endogenous reference (TBP) and relative to a calibrator (Akt 

where inclusion of the exon specifying the C-terminal V5 region of PKCβII (1, 2) and insulin-regulated splicing of the βII exon involved phosphorylation of SRp40 by a PI 3-kinase-dependent signaling pathway (24). It has been shown that the physiological responses of insulin such as glucose uptake and Glut4 translocation are preferentially mediated by Akt2 kinase (46). We hypothesized that insulin activation of Akt would also result in the increased phosphorylation of SRp40 because SR proteins are potential in vitro substrates for Akt (45), and SRp40 overexpression increased glucose uptake (24). To demonstrate this relationship, skeletal muscle (L6) cells were either treated with insulin for 30 min or left untreated (control), or cells were transiently transfected with constitutively active (CA) Akt2 cDNA construct (55). Simultaneously, separate wells were transiently transfected with SRp40. The endogenous splicing of PKCβII in cells with CA-Akt2 was compared with insulin-treated cells and to cells transfected with SRp40 cDNA where inclusion of the βII exon was detected without insulin treatment as reported earlier (24). In cells transiently transfected with constitutively active Akt2, PKCβII exon inclusion was detected without insulin treatment (Fig. 1), suggesting that Akt2 kinase acted downstream of PI 3-kinase.

Akt2 Kinase Phosphorylated SRp40 in Vivo—We previously found increased phosphorylation of SRp40 by a PI 3-kinase-dependent pathway following insulin treatment (24), although the specificity of the kinases involved and the phosphorylation
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A hormone-signaling pathway to the phosphorylation of a specific SR protein resulting in regulation of a splicing event was lacking. To demonstrate that Akt2 kinase phosphorylates SRp40, L6 myotubes that had been transfected with CA-Akt2 or treated with insulin were labeled with [γ-32P]orthophosphate prior to immunoprecipitation with SRp40 antibody. Following SDS-PAGE separation of immunoprecipitates, the autoradiogram revealed that SRp40 in the basal state was phosphorylated. CA HA-Akt2 transfection increased its phosphorylation 2.5-fold compared with the empty vector control.

Insulin treatment of cells transfected with the empty vector resulted in a 3-fold increase in SRp40 phosphorylation (Fig. 2). Levels of SRp40 remained unchanged during insulin treatment and transfection with Akt2 (Fig. 2).

**SRp40 Is an Akt2 Substrate**—Akt2 regulates PKCβII exon inclusion as shown above in Fig. 1. Also, phosphorylation of SRp40 via insulin and its downstream kinases is essential for PKCβII exon inclusion (24). Further, protein sequence analysis revealed a consensus Akt2 phosphorylation sequence (RXRXXS) at Ser residue 86. Hence, to examine whether Akt2 phosphorylated SRp40 in vitro, an Akt kinase assay was performed. L6 cells were transfected with either myc-SRp40 or HA-Akt2 CA. Following immunoprecipitation with Myc antibody and HA antibody, respectively, the assay was performed in the kinase reaction buffer for 30 min in the presence of [γ-32P]ATP. The samples were separated on SDS-PAGE and analyzed by phosphorus imaging (Bio-Rad). The results indicated that Akt2 phosphorylated SRp40 (Fig. 3a). Further, when Ser86 was mutated to alanine (SRP40*), the mutation abolished Akt phosphorylation of SRp40. The constructs were validated in separate experiments (Fig. 3b) using anti-Myc and anti-SRp40 antibodies.

**Mutation of Akt2 Phosphorylation Site in SRp40 Attenuates PKCβII Exon Inclusion**—We surmised that phosphorylation at Ser86 was a key regulatory factor in splice site selection. A heterologous minigene pSPL3–32 (11) was developed to study insulin regulation of in vivo splice site selection of PKCβII exon. In this system, SD splices on to SA constitutively, but inclusion of PKCβII exon and activation of its splice sites occurs only upon insulin treatment. The mutated SRp40, SRp40*, was co-transfected in L6 cells with the splicing minigene pSPL3-βII and treated with insulin for 30 min. RT-PCR was performed using primers for SD and antisense primer for SA. 5% of products were resolved on 6% PAGE gels and detected by silver staining. Constitutive splicing of SD to SA occurs on basal state in both cell types. PKCβII exon inclusion and splice site activation occurs upon insulin treatment in Akt2(+/+) cells. SSII, splice site I; SSIII, splice site II. The results are representative of two experiments with similar results.

**Fig. 4.** a, mutation of Akt2 phosphorylation site on SRp40 attenuates PKCβII exon inclusion. The PKCβII exon in cloned between the splice donor and splice acceptor of pSPL3 to generate the pSPL3-βII splicing minigene (11). L6 cells were co-transfected with the heterologous splicing minigene pSPL3-βII alone or along with the mutated SRp40* and treated with insulin (100 nM) for 30 min. Total RNA was isolated and RT-PCR performed using sense primers for SD (5′-TCTCAATCCTGACCTGAAACC-3′) and antisense primer for SA (5′-CCACACGCCACACCTCTT3′). 5% of products were resolved on 6% PAGE gels and detected by silver staining. PKCβII exon inclusion and splice sites I and II activation occurs upon insulin treatment in L6 cells transfected with pSPL3-βII (control), whereas those co-transfected with pSPL3-βII and the mutated SRp40 (SRp40*) showed decreased exon inclusion. Constitutive splicing of SD to SA was observed in all of the samples. The results represent two separate experiments performed with similar results. b, fibroblasts from Akt2(+/−) mice show defective PKCβII minigene splicing. Immortalized mouse fibroblasts derived from wild type (+/+ or Akt2-deficient (−/−) mice were transfected with the PKCβII splicing minigene. Total RNA was isolated, and RT-PCR was performed using sense primers for SD and antisense primer for SA. In the treated samples, 5% of products were resolved on 6% PAGE gels and detected by silver staining. Constitutive splicing of SD to SA occurs in both cell types. PKCβII exon inclusion and splice site activation occurs upon insulin treatment in Akt2(+/+) cells. SSII, splice site I; SSIII, splice site II. The results are representative of two experiments with similar results.

**Akt2 Kinase Association with SRp40 Is Regulated by Insulin**—Although recent reports have indicated nuclear targeting of Akt kinase (56), our studies did not examine shuttling of Akt2 kinase or SRp40. We examined SRp40 immunoprecipitates for the presence of Akt2 kinase. SRp40 was immunoprecipitated from L6 whole cell lysates using SRp40-specific SR proteins in vitro (45). However, the in vivo evidence linking a hormone-signaling pathway to the phosphorylation of a specific SR protein resulting in regulation of a splicing event was lacking. To demonstrate that Akt2 kinase phosphorylates SRp40, L6 myotubes that had been transfected with CA-Akt2 or treated with insulin were labeled with [γ-32P]orthophosphate prior to immunoprecipitation with SRp40 antibody. Following SDS-PAGE separation of immunoprecipitates, the autoradiogram revealed that SRp40 in the basal state was phosphorylated. CA HA-Akt2 transfection increased its phosphorylation 2.5-fold compared with the empty vector control. Insulin treatment of cells transfected with the empty vector resulted in a 3-fold increase in SRp40 phosphorylation (Fig. 2). Levels of SRp40 remained unchanged during insulin treatment and transfection with Akt2 (Fig. 2).
The experiments were repeated thrice with similar results. SRp40 antibody (experiment, L6 lysates were immunoprecipitated using Akt2 antibody, sates prior to Akt2 immunoprecipitation is shown in the anti-Akt2 antibody. Control using nonspecific IgG and protein G-agarose beads, and separated by SDS-PAGE, and endogenous Akt2 was detected with cell lysates were immunoprecipitated with SRp40 antibody as described phosphorylation of SRp40 in wild type (Fig. 5). Both experiments demonstrated that the association is not enhanced by insulin treatment. This association confirms their enzyme-substrate interaction following insulin treatment. Because all SR proteins have an RS domain and hence are potential substrates of Akt2 kinase, we performed Western blots analysis using ASF/SFP2 antibody on the Akt2 immunoprecipitates (Fig. 5c). As expected, Akt2 associates with ASF/SFP2, but this association is not enhanced by insulin treatment. This leads us to suggest that even though Akt2 kinase has the potential to phosphorylate the SR proteins, insulin regulates the Akt2-mediated phosphorylation only of specific SR proteins such as SRp40.

**Fibroblasts from Akt2 (−/−) Mice Do Not Phosphorylate SRp40 in Vivo**—In humans, mutation in AKT2 is linked to severe insulin resistance and type 2 diabetes mellitus (57). Further, because the loss of Akt2 in mice is linked to the development of type 2 diabetes and a reduction in insulin-dependent glucose uptake (46, 58), we hypothesized that cells from these animals would be defective in insulin-dependent phosphorylation of SRp40 and alternative splicing of PKCβII when compared with wild type cells. Immortalized fibroblasts from wild type (+/+ ) or Akt2-deficient (−/−) mice were treated with insulin for 15 or 30 min or treated with vehicle (0 min), and cell lysates were separated on SDS-PAGE. It was then analyzed with mAb104 antibody, which detects the phospho-epitope of SR proteins, anti-SRP40 antibody, or anti-PKCβII antibody. As seen in Fig. 6, insulin treatment increased the phosphorylation of SRp40 in wild type (+/+ ) cells, whereas the Akt2-deficient (−/−) cells did not show increased phosphorylation with insulin stimulation. The levels of SRp40 remained unaltered in both cell types. In Akt2(+/−) cells, PKCβII levels were less than one-half of the levels noted in (+/+ ) cells. In Akt2(+/−) cells, the PKCβII levels represent the synthesis of new enzyme at 30 min as reported earlier for other cell types (2, 59). This provided genetic evidence that Akt2 deficiency resulted in a lack of increased phosphorylation of SRp40 following insulin treatment and resulted in reduced PKCβII protein levels.

**Muscle Tissues from Akt2(−/−) Mice Expressed Less PKCβII mRNA—**Gastrocnemius muscle tissues from 16-week-old Akt2(+/+) and (−/−) mice (46) were examined for levels of PKCβII and PKCβII mRNA by real time quantitative PCR. Statistical analysis of the quantitative real time PCR was obtained using the (2−ΔΔCt) method. Results indicated a 54% decrease in the expression of PKCβII in Akt2(+/−) cells, the PKCβII mRNA levels remained unchanged in both (+/+) and (−/−) tissues, indicating a defect in exon inclusion. This is consistent with the 2-fold difference in proteins noted in fibroblasts.

**DISCUSSION**

Insulin addition to skeletal muscle cells results in the rapid post-transcriptional processing of the PKCβ pre-mRNA transcript resulting in the inclusion of an exon that encodes PKCβII mRNA (1). The expression of PKCβII further enhances insulin action in skeletal muscle cells and results in increased glucose transport activity (2, 49). To further elucidate the signaling pathway involved in the alternative splicing of PKCβII, we initially overexpressed SRp40, an SR protein associated with 5′ splice site selection, and it was determined that the phosphorylation state of the protein rather than its cellular levels was the crucial factor in its function (24). Because the phosphorylation state of SRp40 was modulated by PI 3-kinase, it was hypothesized that Akt could act as an SR kinase and that insulin modulation of its activity was responsible, in part, for regulating exon inclusion. Transfection of cells with a tagged Akt2 adenovirus construct allowed for the identification of phosphorylated SRp40 in cells endogenously labeled with [32P]orthophosphate. This protein is highly phosphorylated in its endogenous state, and the overexpression of Akt2 resulted in a 2.5-fold increase in the incorporation of 32P into the immunoprecipitated protein. Insulin, an activator of Akt2 and other potential SR kinases, increased its phosphorylation by 3-fold. Insulin may also increase the phosphorylation of SRp40.

**Fig. 5.** Co-immunoprecipitation of Akt2 and SRp40. a, L6 whole cell lysates were immunoprecipitated with SRp40 antibody as described and separated by SDS-PAGE, and endogenous Akt2 was detected with anti-Akt2 antibody. Control using nonspecific IgG and protein G-agarose beads (IgG) showed no interaction. L6 lysates prior to SRp40 immunoprecipitation is shown in the bottom panel. b and c, in a separate experiment, L6 lysates were immunoprecipitated using Akt2 antibody, separated by SDS-PAGE, and detected by Western blotting using SRp40 antibody (b) or ASF/SFP2 antibody (c). Control using nonspecific IgG and protein G-agarose beads (IgG) showed no interaction. L6 lysates prior to Akt2 immunoprecipitation is shown in the bottom panel. The experiments were repeated thrice with similar results. *IP*, immunoprecipitation; *IB*, immunoblot.

**Fig. 6.** Mouse fibroblasts from Akt2(−/−) cells do not phosphorylate SRp40 in vivo upon insulin treatment. Mouse embryonic fibroblasts derived from wild type (+/+ ) or Akt2-deficient (−/−) mice (46) were treated with insulin for 0 (control), 15, or 30 min as indicated. a, cell lysates were separated on 12.5% SDS-PAGE and analyzed with mAb104 antibody, which detects phospho-epitopes of SR proteins. Akt2-deficient (−/−) samples do not show increase in phosphorylation of SRp40 upon insulin treatment. b, the levels of SRp40 in the above samples remain unchanged in both the wild type (+/+ ) or Akt2(−/−) cells upon insulin treatment. c, PKCβII increased upon insulin treatment in wild type Akt2(+/+) cells, whereas Akt2-deficient (−/−) cells show decreased PKCβII expression that remains unchanged upon insulin treatment. The experiments were repeated thrice to ensure reproducibility. *Ab*, antibody.
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via kinases such as PKC in addition to Akt. PKC is another potential SR kinase that is modulated during insulin signaling by a PI 3-kinase-dependent mechanism. Further, SR kinases such as Clk/Sty may also phosphorylate SRp40 (25, 60) independent of Akt2 kinase. The phosphorylation of SR proteins most likely facilitates their recruitment to nuclear speckles where they are likely to interact with other spliceosomal proteins (61).

All Akt/PKB isoforms are thought to have similar substrate specificity, although this has not been directly tested. Akt2 expression is the highest in insulin-responsive tissues, including liver, brown fat, and skeletal muscle, suggesting its importance in insulin signaling. The involvement of insulin-activated Akt isoforms in mRNA splicing is predicted because SR proteins contain multiple Akt phosphorylation consensus sequences, RXRX(S/T) (62) within their C-terminal RS domain. SR proteins are optimal substrates for Akt in vitro when solid phase phosphorylation screening identified several spliceosomal proteins (45). Some of these proteins have more than 20 possible Akt phosphorylation motifs in their protein sequences. The present study demonstrated that Akt2 phosphorylated a specific site within SRp40. Mutation of this site significantly reduced PKCβII exon inclusion and splice site activation. The precise role of SRp40 has not been defined in insulin-regulated splice site selection, but antisense oligonucleotides that block the interaction of the SR protein with the mRNA-binding domain demonstrated its requirement for insulin action in L6 muscle tissues because the antisense also blocked insulin effects on glucose uptake (24), and overexpression of the protein, also shown here, mimicked insulin effects on splicing of PKCβII mRNA. In fibroblasts derived from Akt2(−/−) mice, phosphorylation of SRp40, splicing of PKCβII and insulin-induced increases were not detected. Muscle tissue from Akt2(−/−) mice also expressed lower levels of PKCβII mRNA, whereas PKCβI levels remained unchanged. These tissues are also insulin-resistant (29). Hence the phosphorylation state of SR proteins may be important in the development of type 2 diabetes. It is interesting that muscle tissues from diabetic patients was shown to express lower levels of splicing factors, many of which are alternatively spliced (54).

This is a unique study linking Akt2 activation by a hormone, such as insulin, to a specific SR protein that regulates the splicing of an mRNA variant with functional consequences in the action of the hormone. Our study provides a means of relating Akt to nuclear functions that sustain insulin-signaling pathways post-transcriptionally.

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