Insulin regulates the inclusion of the exon encoding protein kinase C (PKC) βII mRNA. In this report, we show that insulin regulates this exon inclusion (alternative splicing) via the phosphatidylinositol 3-kinase (PI 3-kinase) signaling pathway through the phosphorylation state of SRp40, a factor required for insulin-regulated splice site selection for PKCβII mRNA. By taking advantage of a well known inhibitor of PI 3-kinase, LY294002, we demonstrated that pretreatment of L6 myotubes with LY294002 blocked insulin-induced PKCβII exon inclusion as well as phosphorylation of SRp40. In the absence of LY294002, overexpression of SRp40 in L6 cells mimicked insulin-induced exon inclusion. When antisense oligonucleotides targeted to a putative SRp40-binding sequence in the βII-βI intron were transfected into L6 cells, insulin effects on splicing and glucose uptake were blocked. Taken together, these results demonstrate a role for SRp40 in insulin-mediated alternative splicing independent of changes in SRp40 concentration but dependent on serine phosphorylation of SRp40 via a PI 3-kinase signaling pathway. This switch in PKC isoform expression is important for increases in the glucose transport effect of insulin. Significantly, insulin regulation of PKCβII exon inclusion occurred in the absence of cell growth and differentiation demonstrating that insulin-induced alternative splicing of PKCβII mRNA in L6 cells occurs in response to a metabolic change.

Insulin regulates levels of protein kinase C (PKC) βII mRNA in skeletal muscle by a novel mechanism that signals the activation of splice sites in the last intron of the pre-mRNA. Exon inclusion results in an mRNA that now encodes the C-terminal region of PKCβII affecting subcellular localization and substrate specificity of the kinase. The terminal PKCβII-specific exon with its 3'-untranslated region is spliced to the PKCβII-specific exon via exon inclusion such that a stop codon is introduced at the splice site, and as a result, the PKCβII exon becomes part of an extended 3'-untranslated region of PKCβII mRNA (1, 2). Therefore, PKCβII and PKCβI differ only by their C-terminal 52–50 amino acids, respectively. In contrast to PKCβII, increased expression of PKCβII results in activation/inactivation of the mitogen-activated kinase cascade (3), glycogen kinase synthase 3β (4), TLS/Fus (5), insulin receptor signaling (6), cyclin-dependent kinase (CDK)-activating kinase, as well as cell proliferation (8–10), protein trafficking (11), apoptosis, and glucose transport (12, 13).

Pre-mRNA splicing occurs on nuclear spliceosomes, a macromolecular complex consisting of small nuclear ribonucleoproteins, proteins associated with heterogeneous nuclear RNA, and other splicing factors including serine-arginine-rich (SR) proteins (14, 15). Exon splicing is highly regulated, and numerous consensus sequences that bind specific factors participate in the control of tissue-specific or developmentally controlled splicing via SR protein-RNA and protein-protein interactions (16). SR and SR-like proteins are characterized by a modular composition with one or more RNA recognition motifs and an arginine and serine domain (RS domain) in which the serine residues can be highly phosphorylated. The RS domain is responsible for protein-protein interactions and nuclear localization (17–19). SR and SR-like proteins have been implicated in 5'-splice site recognition and in the communication of splice sites caused by a network of SR proteins (20). They can bind to exon enhancer motifs that are often purine-rich sequences that promote the use of suboptimal splice sites (21). Their interaction with exon enhancers results in a concentration-dependent influence on alternative splicing (22–25). Several SR protein kinases have been reported, including a U1 snRNP 70K-associated kinase, SR protein kinase (SRPK1), lamin B receptor kinase, and a family of CDC2-like kinases (10, 26–28). Both hyper- and hypophosphorylation of SR proteins has been shown to influence splicing (29–31), and the interaction of SR protein kinases with SR proteins can also influence their subcellular localization (32, 33). However, at this time the regula-

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**Abbreviations**

- PKC: protein kinase C
- PI 3-kinase: phosphatidylinositol 3-kinase
- SR: serine-arginine-rich
- HRS: hepatic Arg-Ser protein
- RT-PCR: reverse transcriptase-polymerase chain reaction
- AS: antisense oligonucleotides
- BSA: bovine serum albumin
- PAGE: polyacrylamide gel electrophoresis
- MEM: minimal Eagle's medium
- bp: base pairs
- PCR: polymerase chain reaction

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**Conflicts of interest**

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tion of SR protein kinases by peptide hormone-activated signal transduction pathways has not been demonstrated to our knowledge.

The precise mechanisms by which SR proteins govern alternative splicing are under investigation in many laboratories. One model proposes that different concentrations of spliceosomal proteins in different cell types cause alternative processing of pre-mRNAs. Evidence for this mechanism is based on the variable expression levels of some SR proteins in tissues as a function of cell growth or differentiation (24, 34–36). Another model proposes the existence of cell and/or developmental specific splicing factors that modulate splice site selection. For example, the female-specific expression of Drosophila transformer protein determines the sexual fate of the fruit fly by directing splicing decisions (37, 38). In addition, our recent finding that insulin regulated 5′-splice site selection of the PKCβII-exon within minutes after it binds to cell surface receptors suggested a third possibility. SR proteins could regulate alternative splicing via a receptor-linked signaling pathway responding to metabolic change rather than to a change in growth or development (39, 40).

It is well known that insulin binding to its receptor activates at least three kinase pathways that can signal to the nucleus (41, 42). Insulin-induced mitogen-activated protein kinase (MAPK) activation is associated with mitogenic signaling of insulin, and insulin-induced signal transducers and activators of transcription or JAK/signal transducers and activators of transcription pathways lead to nuclear transcriptional activator and repressor activation involved in cell differentiation (43, 44). In contrast, insulin activation of the phosphatidylinositol 3-kinase pathway is associated with metabolic signaling, consistent with the observation that insulin regulates PKCβII exon inclusion independent of cell growth and differentiation. Therefore, the possibility that a PI 3-kinase-dependent signaling pathway could alter the phosphorylation of post-transcriptional regulatory factors such as SR proteins as a step in the regulation of PKCβII expression was examined.

Our studies focused initially on SRp40 for the following reasons. One, it was first described as an early response gene in PKC increases in its concentration. This suggested that if SRp40 was involved in the regulation of PKC, then insulin-influenced changes in concentration of SR protein kinases by peptide hormone-activated signal transduction pathways has not been demonstrated to our knowledge.

**Preparation of Nuclear Extracts**—L6 myoblast nuclear extract was prepared from cells treated with or without insulin for 30 min as described by Dignam et al. (47).

**Immunoprecipitation of SRp40**—L6 myotubes were collected by centrifugation, and pellets were lysed in 20 volumes of 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0, with protease inhibitors as follows: benzamidine HCl, 16 μg/ml; aprotinin, 10 μg/ml; leupeptin, 10 μg/ml; phenylmethylsulfonyl fluoride, 1 mM. Cells were placed on ice for 30 min, and insoluble material was pelleted. An aliquot (50 μl) of cytosolic lystate was incubated at a final concentration of 1 μg/ml with anti-SRp40 polyclonal 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, beads were washed twice with 1 ml of lysis buffer containing 0.5 M 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, centrifuged at 1000 rpm for 5 min before loading on the gel, followed by Western blot analysis.

**Western Blot Analysis**—L6 muscle cell cytosolic (40 μg) or immunoprecipitates were subjected to 9% SDS-polyacrylamide gel electrophoresis (PAGE) (48). Proteins were electrophoretically transferred to nitrocellulose membranes, blocked with 0.1% Tween with Tris-buffered saline 0.1% Tween 20 containing 5% nonfat dried milk, washed, and incubated with a polyclonal antibody against SRp40 or monoclonal antibody against the phosphoepitope of SR proteins, mAb104 (49), or anti-PKCα antibody. Following incubation with anti-rabbit IgG or anti-mouse IgM horsed-ish peroxidase, detection was performed using enhanced chemiluminescence (Amersham Pharmacia Biotech).

**Overexpression of SRp40 in L6 Rat Skeletal Muscle Cells**—L6 myoblasts were stably transfected with pCMV5 (empty vector control) or HRS/SRp40 in pCMV5 (46), using a calcium phosphate/DNA precipitate (48). Cells were stably transfected with pCMV5 or mAb104 (49), or anti-PKCα antibody. Following incubation with anti-rabbit IgG or anti-mouse IgM horseradish peroxidase, detection was performed using enhanced chemiluminescence (Amersham Pharmacia Biotech).

Our studies focused initially on SRp40 for the following reasons. One, it was first described as an early response gene (HRS/SRp40). Two, SRp40 concentrations were increased by insulin in the regenerating liver where it is induced as a delayed early gene. Three, SRp40 levels are transcriptionally up-regulated by insulin. Four, SRp40 effects on exon inclusion have been demonstrated previously for the alternative splicing of fibronectin mRNA (45). Finally, increased SR protein concentrations during development, cell differentiation, and cell proliferation determine alternative splicing decisions (24, 25, 46).

In our case, however, insulin regulation of PKCβII exon inclusion in BC3H-1 myocytes and L6 myotubes occurs within 15 min, prior to SRp40 transcriptional up-regulation and increases in its concentration. This supported the idea that SRp40 was involved in the insulin-induced alternative splicing that results in PKCβII mRNA, thereby altering the efficiency of alternative splicing to the C4 kinase domain common to both PKCβII and -βIII (5′-GTTGTTGGCCCTGGAAGG-GAAC-3′) and an antisense primer to the βIVS exon common to both transcripts, (5′-TGGCTTGGTAACCTTGTGACG-3′). The PCR products would be 159 bp for PKCβII, 374 bp for PKCβIII (where the first splice site was activated, SSII), and 510 bp for PKCβIII (where the second splice site, SSIII, was activated). We found that insulin activated two 5′-splice sites in a time-dependent manner in some experiments (39). This assay allows for relative comparison of both PKCβII versus PKCβIII mRNA levels in the same reaction. Following 35 cycles of a two-step PCR amplification program (95°C, 30 s, and 58°C, 2 min) using Taq platinum DNA polymerase (PerkinElmer Life Sciences), 50% of the PCR was resolved by electrophoresis on 1.2% agarose gels containing 1 μg/ml ethidium bromide at 120 V for 60 min. An additional set of primers was also used to evaluate only PKCβII mRNA in some experiments. A sense primer corresponding to the coding region of the βII exon (5′-CAGGCCGCAATCCAGGACCT-3′) was used with antisense corresponding to -βIVS as described above. The resulting products would be 227 bp. This assay offers increased sensitivity for detecting the βII exon since PKCβII mRNA was not amplified. For each experiment, β-actin was determined to compare RNA levels between samples. PCR
Insulin Regulates SR Splicing Proteins

RESULTS

Insulin Activation of PI 3-Kinase Results in Exon Inclusion—Since insulin regulates the alternative splicing of PKCβII mRNA independent of growth and differentiation, we proposed that an insulin-stimulated PI 3-kinase pathway was involved. An insulin-sensitive p85/p110 PI 3-kinase is recruited to protein phosphotyrosine residues in response to activation of the insulin receptor tyrosine kinase producing phosphatidylinosititol 3,4,5-trisphosphate which is necessary for metabolic and some mitogenic actions of insulin (41, 53), and several protein serine/threonine kinases downstream of PI 3-kinase are implicated in the regulation of glucose transporter recruitment, glycogen synthesis, protein synthesis, and gene transcription, making this pathway a likely candidate for regulating post-transcriptional events such as alternative splicing (55).

To examine involvement of the insulin-stimulated PI 3-kinase pathway, L6 myotubes were pretreated with LY294002, a specific PI 3-kinase inhibitor (52), prior to insulin addition. RT-PCR analysis was used to evaluate changes in PKCβII mRNA expression post-transcriptional events such as alternative splicing (Fig. 1). A PI 3-kinase inhibitor, LY294002, blocked insulin effects on exon inclusion. A schematic diagram of the relevant portion of PKCβ pre-mRNA along with the positions of the primers used for detection of PKCβII mRNA, C4 represents the last exon (indicated as a box) common to both βII and β. Lines represent introns. B, RT-PCR assay of L6 myotubes treated with or without insulin (100 nM, 30 min) and LY294002 (100 nM, 1 h pretreatment); M indicates the 100-bp DNA ladder included for size determination. β-Actin levels in corresponding samples are shown in the lower panel. β-Actin levels in corresponding samples are shown in the lower panel. Detection of PKCβII mRNA was negligible in cells treated with LY294002. The experiment was repeated on four occasions with the same results.

Table I

| βII exon | AS 35 (control, 100 nM) | AS 35 (control, 100 nM) |
|---|---|---|
| Cyclotropin | 686 ± 65 | 379 ± 26 |
| SRp40 | 681 ± 67 | 681 ± 67 |
| SRp55 | 680 ± 70 | 680 ± 70 |
| SRp55 | 1190 | 1190 |
| SRp55 | 34 | 29 |
| SRp55 | 1190 | 1190 |
| SRp55 | 34 | 29 |
| SRp55 | 34 | 29 |

**Table I**

Insulin effects on 2-deoxyglucose uptake in L6 myotubes

L6 myotubes were grown in 24-well plates until confluent and differentiated into myotubes as described. Cells were serum-starved overnight prior to pretreatment with inhibitors for 1–4 h prior to addition of insulin for 30 min.

| [3H]2-Deoxyglucose uptake | Basal | Insulin (100 nM) |
|---|---|---|
| Empty vector control | 644 ± 35 | 1396 ± 70 |
| LY294002 (10 μM) | 544 ± 31 | 636 ± 39 |
| Cycloheximide (10 μg/ml) | 526 ± 28 | 744 ± 38 |
| SRp40 cDNA | 1140 ± 50 | 1190 ± 45 |
| SRp55 | 681 ± 67 | 729 ± 27 |
| Empty vector control | 686 ± 65 | 1421 ± 49 |
| SRp40 (control, 100 nM) | 689 ± 40 | 872 ± 32 |

Significant difference (p < 0.05) from glucose transport activity in empty vector control. Data are expressed as the mean ± S.E. (pmol/mg of protein) for triplicate determinations. Experiments were repeated on two or more occasions with similar results.

**Fig. 1.** A PI 3-kinase inhibitor, LY294002, blocked insulin effects on exon inclusion. A, schematic diagram of the relevant portion of PKCβ pre-mRNA along with the positions of the primers used for detection of PKCβII mRNA. C4 represents the last exon (indicated as a box) common to both βII and β. Lines represent introns. B, RT-PCR assay of L6 myotubes treated with or without insulin (100 nM, 30 min) and LY294002 (100 nM, 1 h pretreatment); M indicates the 100-bp DNA ladder included for size determination. β-Actin levels in corresponding samples are shown in the lower panel. Detection of PKCβII mRNA was negligible in cells treated with LY294002. The experiment was repeated on four occasions with the same results.
Insulin Regulates SR Splicing Proteins

not dividing and represented fully differentiated insulin-responsive skeletal muscle cells, it was possible that insulin increased SRp40 phosphorylation state as well as SRp40 concentration. To examine this possibility, SRp40 was immunoprecipitated in lysates from cells treated with insulin and LY294002 using a polyclonal antibody developed for HRS/SRp40 (46). The immunoprecipitates were analyzed by Western blot analysis probed with mAb104. As shown in Fig. 3A, following insulin treatment for 15 and 30 min, SRp40 phosphorylation increased. Pretreatment of cells with LY294002 blocked insulin effects on SRp40 phosphorylation. The cell lysates were also analyzed directly by Western analysis using anti-SRp40 antibody. In this, insulin treatment for up to 30 min was shown to have no discernible effect on SRp40 protein levels (Fig. 3B). SRp40 protein levels were also unaltered by LY294002 pretreatment.

These results indicated that in non-dividing L6 myotubes, insulin affects SRp40 function via phosphorylation rather than increasing concentrations of the factor, and SRp40 phosphorylation was blocked by an inhibitor of PI 3-kinase.

SRp40 Overexpression Mimicked Insulin Effects on Splice Site Selection—To establish further SRp40 involvement, SRp40 overexpression experiments were carried out (46). The co-transfection of HRS/SRp40 and a fibronectin minigene in H35 cells was correlated to HRS-mediated regulation of EIIIB exon inclusion, and this correlated to induction of HRS protein and fibronectin EIIIB+ transcripts in developing liver. By analogy, we stably overexpressed SRp40 cDNA in L6 myoblasts to determine whether overexpression would mimic insulin effects on splicing. As shown in Fig. 4, A and B, increased SRp40 levels resulted in the inclusion of the PKCβII-specific exon as determined by RT-PCR analysis, and the effect was analogous to that observed for insulin treatment. Basal levels of PKCβII mRNA were negligible in serum-starved L6 myotubes, but upon stimulation with insulin for 30 min, inclusion of the 216-bp PKCβII exon was evident. Transient overexpression of SRp40 also mimicked insulin-induced PKCβII exon inclusion, and the splicing observed in the presence of transient SRp40 expression was also blocked by LY294002 (Fig. 4C).

Protein levels of SRp40 and PKCβII were also analyzed to determine that the transfected cDNA was expressed and to determine whether the increase in PKCβII mRNA resulted in newly synthesized protein. The transfection of cells with SRp40 constructs increased levels of the protein, >5-fold, over endogenous SRp40 levels (Fig. 5A). The phosphorylation of SRp40 in response to insulin also increased 5-fold over non-insulin-stimulated levels as determined using mAb104 to detect phospho-SRp40. Predicted splice products and their lengths are indicated. B, RT-PCR analysis of PKCβII exon inclusion in L6 myotubes stably expressing either the empty vector or pCMV-SRp40. As a control, β-actin was amplified. C, RT-PCR analysis of PKCβII exon inclusion in L6 myotubes transiently transfected with SRp40 cDNA. The experiments were repeated to ensure reproducible results.

Effect of Antisense Oligonucleotide Targeted to Putative SRp40-binding Site in the Intron Spanning βII–βIII Exons—Since SRp40 mimicked insulin to enhance exon inclusion, the cis-elements involved in the regulation are likely to occur in the exon or intron sequence proximal to the splice site. An SRp40-binding motif, TGGGACCTTCGCTTGCAG, downstream from the PKCβII exon 5’-splice site was identified from sequence analysis. This site is similar to a cis-element predicted earlier, TGGGACNNRNGCTCGY, with a 2-bp difference at the 3′-end (58). To determine if this sequence might be involved in insulin-stimulated splicing, an antisense oligonucleotide was designed. The modification used to synthesize the oligonucleotide ensured that it was RNase H-resistant and would not result in destabilizing the pre-mRNA. A 2′-O-methoxymethyl oligonucleo-

![Fig. 2. Identification of SR proteins in nuclear extracts from L6 myotubes treated with insulin.](image-url)

![Fig. 3. Insulin increased the phosphorylation state of SRp40 in L6 myotubes without altering concentrations of the factor.](image-url)

![Fig. 4. Exon inclusion in PKCβII mRNA in the presence of insulin and SRp40 cDNA.](image-url)
Effects of Cycloheximide, SRp40 Overexpression, and Antisense Oligonucleotides on Cellular 2-Deoxyglucose Uptake—To show that the functional consequences of SRp40 overexpression correlated to PKCβII-mediated metabolic changes induced by insulin action (51), PKCβII-mediated response on glucose uptake by L6 cells was examined.

By serum-depriving L6 myotubes for longer periods (up to 18 h) before measuring glucose uptake, PKCβII mRNA and protein levels were demonstrated to be low (Fig. 5C). Under these conditions, glucose uptake reflects a requirement for new protein synthesis if the rapid effect of insulin on splicing of PKCβII is relevant to a physiological event. To demonstrate this requirement, L6 myotubes were serum-starved for 18 h prior to 2-deoxyglucose uptake (56). As shown in Table I, cycloheximide pretreatment blocked insulin effects on PKCβII mRNA in L6 myotubes also mimicked insulin effects by increasing basal glucose uptake, and there was no further stimulation in the presence of insulin. Basal glucose uptake was also highly elevated in cells stably transfected with SRp40, and insulin had no further stimulatory effect (data not shown.)

To demonstrate further the importance of the switch in PKCβ isoforms, cells were transiently transfected with the antisense oligonucleotide (AS 34) shown to block splicing of the pre-mRNA (Fig. 6). At a concentration shown to block exon inclusion and the switch to PKCβII, insulin effects on glucose transport were totally blocked. The control antisense oligonucleotide with the 4-bp mismatch (AS35) had no effect on glucose transport. Hence, under conditions where insulin stimulation of alternative splicing of PKCβII mRNA was controlled, glucose uptake was linked to the regulation of SRp40 phosphorylation and PKCβII splicing by insulin.

As a control, L6 myotubes were pretreated for 2 h with LY379196, which inhibits PKCβ (IC50 30 nM). At 30 nM, glucose uptake was blocked 50% of the full insulin effect consistent with the involvement of PKCβII in insulin-stimulated glucose transport and previous studies demonstrating the effects of PKCβ inhibitors (51, 65).

DISCUSSION

Insulin is known to activate PI 3-kinase (41), and several downstream kinases are activated in a PI 3-kinase-dependent manner including Akt, PKCζ, and PKCβII in skeletal muscle (51, 65–67). Insulin signaling to the nucleus by PI 3-kinase is not as well studied, but it is associated with activation of gene transcription in addition to its roles in glycogen and protein synthesis and glucose transport (55). Our study found increased phosphorylation of SR proteins by insulin-dependent PI 3-kinase pathways. This indicated that factors involved in 5’-splice site selection could be regulated by insulin signaling mechanisms.

The C-terminal portion of SR proteins contains a domain rich in serine and arginine residues that is highly phosphorylated. At least seven members of the SR family, including SRp40 and SR2/ASF, contain phosphoepitopes that are recognized by mAb104 (68). The finding that insulin treatment increased the phosphorylation state of at least seven proteins in nuclear extracts from skeletal muscle cells is consistent with the observation that the RS domains are highly phosphorylated in vivo (10, 69).

We focused on HRS/SRp40, a splicing factor with a molecular mass of about 40 kDa that was originally shown to be induced by insulin in rat hepatocytes (71). By using immunoprecipitation of SRp40 followed by detection with an antibody to examine serine phosphorylation specifically, it became evident that SRp40 was targeted to bind to the potential SRp40-binding site in the intron spanning βII–βI exons as shown in Fig. 6A. This antisense sequence blocked insulin-stimulated exon inclusion in a dose-dependent manner (Fig. 6, B and C). A control oligonucleotide containing a 4-bp mismatch failed to block exon inclusion in the presence of insulin and confirmed the specificity of the antisense for the target sequence. Thus, by blocking SRp40 protein interaction with the element with the 2′-O-methoxyethyl oligonucleotide, insulin-induced splicing was directed away from exon inclusion to the alternative product, PKCβ mRNA.
insulin treatment increased the phosphorylation state of the SR protein rather than its concentration. The phosphorylation was blocked by pretreatment with LY294002 as was splicing. This indicated a role for PI 3-kinase in alternative splicing, and to our knowledge, this is the first report of a hormone signaling the phosphorylation of an SR protein.

Since the overexpression of trans-factors has also been useful in establishing their role in splicing, SRp40 cDNA was expressed in differentiated myotubes and resulted in exon inclusion. This was used as evidence to link the regulation of SR concentration to splice site selection in previous studies (46). Here, the overexpression was linked to increased RS domain phosphorylation, increased PKCβII mRNA, increased PKCβII protein, and to increased glucose transport. It is premature to suggest which downstream kinases or phosphatases are activated or inhibited by PI 3-kinase to result in increased SR protein phosphorylation. It is possible that insulin-activated kinases such as Akt or PKC could phosphorylate SR proteins (72).

Two consensus sequences have been proposed for SRp40-binding sites (58, 73). Both are present in the introns flanking the βI-specific exon. The first sequence occurs prior to the 3′-pyrimidine tract (ACDGS). The second sequence we identified by sequence analysis is longer, occurs about 350 bp after the first 5′-splice site, and corresponds closely to one described (58). When the second site was targeted using antisense oligonucleotides, exon inclusion was inhibited. The use of antisense oligonucleotides for down-regulating gene expression is well documented where sequences are targeted to block translation or lead to destabilization of the message by RNase H or inhibit transcription by forming triplex structures within the promoter regions of DNA. Antisense oligonucleotides have also been used to restore splicing of mutated pre-mRNA in thalassemic β-globin and to redirect splice site selection for Bcl-xS versus Bcl-xl independent of down-regulating gene expression (59, 60, 74). Here, blocking one site for SRp40-RNA interaction provided mutual dependence of a downstream sequence with insulin-induced changes in SRp40 phosphorylation. This finding is analogous to studies where antisense toward SP2/ASF-binding sites blocked splicing of bGH pre-mRNA in vitro (61).

The effect of the PI 3-kinase inhibitor, LY294002, to block glucose uptake has been reported (57), and its effect is consistent with a role for PI 3-kinase in insulin action (53) since this signaling pathway directly links downstream kinases with the recruitment of glucose carriers to the plasma membrane and results in increased glucose uptake. Next, we evaluated the effect of newly synthesized PKCβII on insulin-stimulated glucose uptake. Cycloheximide blocked the recruitment of glucose carriers in adipocytes (62, 63). Although there is a conflicting report in adipocytes (64), differences in the preparation and pretreatment of cells could be involved. In this study, cycloheximide treatment blocked insulin effects in serum-depleted myotubes.

We demonstrated that overexpression of SRp40 mimicked insulin to increase basal glucose uptake. This is consistent with the effects of increased PKCβII concentrations that occur following SRp40 overexpression. Since increased SRp40 concentrations may be altering alternative splicing of other pre-mRNA in a nonspecific manner, this correlation should be interpreted tentatively. For example, the insulin receptor is also alternatively spliced, and the B form of the receptor is thought to signal more effectively (76). However, the ability of antisense oligonucleotides targeting the SRp40-binding site to block insulin effects on glucose transport suggests that the alteration in splicing alone is responsible for the increase in transport. Finally, LY379196, a PKCβ inhibitor which blocks glucose uptake in primary mouse myotubes (65), also inhibited insulin effects on glucose uptake.

Our studies in cells support in vitro observations for an SRp40 role in splice site selection where addition of one or more SR proteins to in vitro deficient splicing extracts restored splice site selection in a concentration-dependent manner (7, 70). The identification of SRp40 as a component of insulin-regulated splicing was defined by the following criteria: its ability to mimic insulin effects on PKCβII splicing, inhibition of its phosphorylation state by LY294002, a compound that blocks insulin activation of PI 3-kinase, and the ability of 2′-O-methoxyethyl antisense oligonucleotides directed to a putative SRp40 site to block insulin effects on splicing as well as to block insulin effects on glucose transport. The demonstration of SRp40 as a factor regulated by a PI 3-kinase signaling cascade provides an additional mechanism for regulating alternative splicing. SRp40 phosphorylation correlates to alternative splicing of the βI exon in a manner analogous to insulin treatment and links a signaling pathway to exon inclusion events in vivo. Unlike systems of tissue-specific alternative splicing, however, the concentration of SRp40 did not change with insulin treatment, rather its phosphorylation state increased. Taken together, the multiple strategies used here to investigate SRp40 interactions in intact cells indicate a pivotal role for this trans-factor and PI 3-kinase in insulin-stimulated alternative splicing of PKCβ pre-mRNA and subsequent effects of insulin on glucose transport.

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