The phosphatidylinositol 3-kinase (PI3K) signaling pathway has been associated with a variety of cellular functions ranging from cell cycle regulation to tissue development. Although years of research have extensively characterized this signaling pathway, little is known as to how specific cellular events are coordinated by its activation. Here we demonstrate that Dapr (differentiation-associated protein), a novel protein, appears to focus one aspect of this pathway by acting as a putative scaffold protein during skeletal muscle differentiation. We present for the first time a description of this protein using in silico analysis. dapr was discovered through a previous study employing chromatin immunoprecipitation and CpG microarray analysis experiments as being regulated by myocyte-enhancing factor 2, a key transcription factor involved in the differentiation of skeletal muscle tissue. In this study we show that during the course of differentiation, Dapr binds to the PI3K signaling pathway member protein kinase B (PKB). In C2C12 myoblast cells before differentiation Dapr is localized to the cytosol, migrating with PKB to the membrane after initiation of muscle differentiation. Knockdown of Dapr by RNAi resulted in inhibition of myotube formation. Our findings indicate that Dapr is a key component required by myoblasts for orchestrating their differentiation during myogenesis. Furthermore, it appears that Dapr is involved in the PI3K signaling cascade, potentially acting as a scaffold protein for PKB and coordinating its compartmentalization during differentiation.

In the transformation of myoblasts into myotubes, PI3K signaling is one of the key pathways (1). Activation of this pathway typically begins with signaling through the activated surface receptor by insulin growth factor 1 (2). Downstream, PI3K-targeted phosphorylation produces the lipid products phosphatidylinositol 3,4-diphosphate and phosphatidylinositol 3,4,5-trisphosphate. These phosphoinositides provide membrane anchoring points for pleckstrin homology domain-containing proteins with binding motifs to them. Protein kinase B (PKB, also known as AKT) is one such protein (3). The targeted localization of PKB and other kinases to the plasma membrane is an important part of their activation (4, 5). Indeed, the compartmentalization of kinases within the cell has come to be known as a key regulatory mechanism of signaling cascades. A kinase-associated protein (6) is an example of one of the first proteins identified to function in this manner. Not only does A kinase-associated protein localize kinases to specific parts of the cell, it also brings proteins together within proximity of one another; in this case, protein phosphatase 2 and protein kinase A (7).

Among its many important roles as a signal transducer, including insulin-mediated signaling, PKB has also been shown to play a pivotal role in cellular differentiation (1, 8, 9). Its role in the development of myotubes has been well established. Initial work demonstrated that PI3K inhibition causing disrupted myoblast differentiation could be countered by expression of constitutively active PKB (1). Subsequent work revealed a positive feedback mechanism, with PKB translocation regulated by MYOD, a muscle-specific transcription factor, and a subsequent effect on MYOD and MEF2 transactivation (10). However, there are still many unanswered questions regarding the exact nature and coordination of the process of muscle cell differentiation. One of these is how a protein involved in so many different signaling pathways, such as PKB, is focused into a particular biological task like cellular differentiation. Here, we have identified a novel gene, Dapr (differentiation-associated protein), which we propose as a possible candidate fulfilling this role. We will provide evidence that Dapr is a previously unknown and uncharacterized protein that seems to be involved in the localization of a key component in the PI3K signaling pathway during skeletal muscle differentiation. We will demonstrate that the regulation of this physiological process occurs through a specific chain of events that involves Dapr and that its presence is essential for myoblast differentiation to occur.

**EXPERIMENTAL PROCEDURES**

Cloning of dapr Gene and DNA Constructs—Myristoylated and HA-PKBpcDNA3 were generously provided by Dr. J. Woodgett (University of Toronto). Full-length dapr was origi-
nally cloned from a cDNA library using primers designed to include the full open reading frame (5'-ATGGAGCTTTGGAAG-3' and 5'-GTGATTTGCTTTGCTTGA-3'). Amino-terminal Myc-tagged Dapr was generated by incorporating the Myc epitope into the sense primer. For expression into mammalian cells, Dapr was subcloned into the pcDNA3 vector (Invitrogen). This was then used as the template to generate all deletion mutants. GST fusion proteins were generated by subcloning directly into the pGEX4T-1 vector (GE Healthcare) and expressed bacterially.

**Cell Culture—**C2C12 murine skeletal muscle cells were obtained from the ATCC and grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 1% antibiotic (penicillin and streptomycin) in the presence of 5% CO2. Induction of differentiation involved growing cells to 80% confluence then replacing the growth media with differentiation media (Dulbecco’s modified Eagle’s medium supplemented with 1% horse serum, 1% antibiotic and 10 ng/ml insulin-like growth factor 1). Transient transfection of the cell lines was accomplished by incubating cells (~60% confluence) with Lipofectamine 2000 (Invitrogen) and the appropriate expression vector. Expression of the protein product was allowed to proceed for the next 48 h. At this time, the cells were rinsed twice with 1× PBS and then harvested for subsequent analysis.

**GST Fusion Protein Preparation and Association—**GST fusion proteins were expressed using the GST Gene Fusion System (GE Healthcare). After overnight induction at 30 °C with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (Sigma) 1 liter of bacterial culture was pelleted and resuspended in 50 ml of PBS plus 1 mM EDTA with protease inhibitors (Roche Applied Science). The cells were sonicated using a Branson 450 Sonifier (Branson Ultrasonics Corp., Danbury, CT), and the proteins were solubilized by the addition of 1% Triton X-100 for 30 min at 4 °C. Cellular debris was pelleted by centrifugation at 9000 × g for 30 min at 4 °C. The lysate was bound to 500 μl of glutathione-agarose beads prepared as per the manufacturer’s protocol (Sigma) at 4 °C overnight. The beads were then pelleted at 1000 × g for 5 min at 4 °C, washed with PBS plus 0.1% Triton X-100 with protease inhibitors, and resuspended to a 50% slurry. Aliquots of each were run on SDS-PAGE and stained with Coomassie Blue to confirm protein production (supplemental Fig. 4). C2C12 cells were washed with ice-cold PBS plus 1 mM EDTA and lysed by incubating on ice for 10 min in 1.5 ml of Gentle Soft Lysis Buffer (10 mM NaCl, 20 mM PIPES, 0.5% Nonidet P-40, 0.05% β-mercaptoethanol, 5 mM EDTA, 50 mM NaF, and 100 μM Na3VO4) and protease inhibitors. Harvested cell lysate was passed through a 21-gauge needle, and debris was removed by pelleting at 16000 × g for 10 min at 4 °C. The supernatant was precleared using 50 μl of a 50% slurry of glutathione-agarose beads alone for 15 min at 4 °C with rocking. Pre cleared lysate was transferred to a fresh 2-ml tube, and 100 μl of the 50% slurry of GST fusion protein bound to agarose beads was added. Samples were incubated at 4 °C with rocking for 1 h and then washed with Gentle Soft Lysis buffer containing protease and phosphatase inhibitors. The supernatant was removed, and the beads were boiled in 6× protein loading buffer for 5 min.

**Differential Centrifugation—** All steps were carried out on ice or at 4 °C. At the appropriate time points, C2C12 cells were washed with PBS (pH 7.4) containing 0.5 mM EDTA and harvested with PBS/EDTA supplemented with protease inhibitor mixture (Roche Applied Science). Cells were pelleted by centrifugation at 2000 rpm for 5 min and flash-frozen in liquid nitrogen for storage at −80 °C. Pellets were resuspended in 300 μl HES buffer (250 mM sucrose, 20 mM HEPES-NaOH (pH 7.4), 1 mM EDTA, 50 mM NaF, 6 mM Na3VO4, and protease inhibitor mixture) and passed through a 21-gauge needle. Next, the cell lysate was centrifuged at 19,000 × g for 20 min. The pellet (P1) was then resuspended in 300 μl of HES buffer for subsequent processing and the supernatant centrifuged at 40,000 × g for 20 min. The resulting supernatant from this spin was then transferred to a new tube and centrifuged again at 180,000 × g for 1.5 h resulting in a cytosolic fraction (supernatant). Cytosolic fractions were concentrated by spinning at 1.5 × g for 1 h to a final volume of 250 μl using an Amicon Ultra centrifugal filter devices (Millipore, Billerica, MA). To isolate plasma membrane, the P1 pellet was layered onto a 1.12 m sucrose cushion and centrifuged at 100,000 × g for 1 h. Material from the interface containing the plasma membrane fraction was collected, and the final volume was adjusted to 2 ml in HES buffer. The membrane was pelleted by spinning the samples at 40,000 × g for 20 min. The resulting plasma membrane pellet was resuspended in 50 μl of PBS plus EDTA with protease inhibitors and 10 μl of 6× protein loading buffer and boiled for 5 min at 95 °C.

**Co-immunoprecipitation—** Approximately 1 × 106 cells were lysed by rocking for 20 min at 4 °C with 400 μl of EBC lysis buffer (50 mM Tris (pH 8.0), 120 mM NaCl, 0.5% IGEPAL). Debris was removed by centrifugation at 9000 × g for 10 min. The resulting supernatant was incubated 1.5 h at 4 °C with a 50% protein A bead slurry pre-blocked in 4% bovine serum albumin and 5 μg of a monoclonal HA antibody (Upstate, Bil-lerica, MA) or 50 μl of Myc hybridoma. After 5 washes with NETN (0.5% IGEPAL, 20 mM Tris (pH 8.0), 100 mM NaCl, 1 mM EDTA) buffer at room temperature, beads were boiled for 5 min at 95 °C in 2× protein loading buffer.

**Western Blot Analysis—** Samples were run on 10% SDS-polyacrylamide gels and transferred (semidyed electroblotter, GE Healthcare) onto PVDF membranes (Hybond-C, GE Healthcare). Blots were probed with antibodies directed against PKB, PI3K, MKK6, β-tubulin (Cell Signaling Technologies, Danvers, MA), sarcomeric actin (Sigma), HA (Upstate), and Myc (gift from J. Woodgett). The appropriate secondary antibodies conjugated to horseradish peroxidase were used against each respective primary antibody. Blots were incubated in ECL reagent (Western Lightning Chemiluminescence reagent, PerkinElmer Life Sciences) and exposed to film for visualization.

**Real-time PCR—** Triplicate reactions were carried out on each sample of cDNA generated from total RNA. Reactions were carried out as per manufacturer’s instructions using the Full velocity SybrGreen qPCR Master Mix (Stratagene, La Jolla, CA). A final concentration of 0.1 μM for both the forward and reverse primers was found to provide the clearest signal. PCR primer sequences used were as follows: dapr forward 5’-CACCGTTGTACGGCTTTCGA-3’ and reverse 5’-CCAAGAATG-
GAGCCAGCTTC-3'; cyclophilin A forward 5'-ATGCTGG-ACCAACACAA-3' and reverse 5'-CATGGCTTCCACAA-TGTT-3'. All reactions were run on a Stratagene MX3000P using the following program: 95 °C for 5 min, 95 °C for 10 s, then 30 s at 55 °C for 40 cycles. Results were analyzed using the accompanying software.

siRNA Knockdown—C2C12 cells were propagated into 6-well dishes (Nunc, Rochester, NY) in 3 ml of propagation medium (Dulbecco's modified Eagle's medium plus antibiotics fortified with 10% fetal bovine serum (Invitrogen)) and allowed to settle overnight. The cells were transfected at ~20% confluence and then 24 h later at ~50% confluence as follows; 40 nM siRNA oligo (Integrated DNA Technologies, Coralville, IA) and 5 μl of Lipofectamine 2000 reagent (Invitrogen) were combined in 500 μl of Opti-MEM medium (Invitrogen). siRNA oligo was then incubated with cells at 37 °C for 4 h at which time the Opti-MEM was replaced with propagation medium, and cells were allowed to recover overnight. siRNA oligo sequence pairs were as follows: 5'-UAUAAUUGCAACCUCUUGGCUUUCC-3', 5'-GGAAAGGcCAGGUGUAGCGAUAUAUA-3'. A medium GC content oligonucleotide (Invitrogen) was used as a control. Two other oligos were tested for their ability to knockdown Dapr protein expression (data not shown). All three oligos were found to be equally capable of reducing Dapr protein expression.

Immunofluorescence Microscopy—C2C12 cells were propagated in 12-well Falcon dishes on washed, autoclaved 18-mm glass coverslips coated with 25 μg/ml poly-1-lysine (Sigma). After 2 washes in PBS, cells were fixed for 15 min in 3% paraformaldehyde and 0.02% glutaraldehyde. Cells were then permeabilized by dipping coverslips in methanol stored at ~20 °C for 15 s and then quenched for three 10-min incubations in 0.5 mg/ml NaBH4. Cells were then washed 3 times in PBS and then incubated in 1% bovine serum albumin (Sigma) for 1 h at room temperature. Primary antibody incubations for Dapr and α-sarcomeric actin (Sigma) were performed by inverting coverslips over a 30-μl droplet of antibody in 1% bovine serum albumin, PBS and incubating overnight at 4 °C. Cells were then washed 3 times for 10 min each in PBS before incubation in Alexa 488- or 594-conjugated anti-rabbit or mouse IgG secondary antibodies (Invitrogen) in 1% bovine serum albumin, PBS for 1 h at room temperature. Coverslips were washed 3 times for 10 min each in PBS, rinsed in water, and mounted on microscope slides using Vectashield (Burlingame, MA) hardening mounting medium. Slides were allowed to dry overnight at 4 °C. C2C12 cells that were imaged for Golgelle Nights Golgi-GFP kit (Invitrogen). Cells were then incubated with anti-Dapr antibody and prepared as per above for imaging. Expression of the Golgi protein N-acetylgalactosaminyltransferase-2 was carried out as outlined by the manufacturer (Invitrogen). All fluorescence images were acquired using an Olympus 1X81-DSU Spinning Disk Confocal microscope with a UPlanSapo 100×/1.4 oil immersion objective.

dapr Antibody Production—Rabbit polyclonal antibodies were raised against a synthesized peptide corresponding to a carboxyl-terminal epitope of Dapr (Quality Controlled Biochemicals, Hopkinton, MA). Specificity of the Dapr antibody was tested by immunoprecipitation and Western blotting (data not shown).

RESULTS

Identification and in Silico Analysis of dapr—In a previous study we combined chromatin immunoprecipitation and CpG microarray analysis to search for genes regulated by the muscle related transcription factor MEF2 during skeletal muscle differentiation (11). Using chromatin immunoprecipitation-PCR we confirmed MEF2 binding proximal to a number of genes, peaking 2 days after initiation of differentiation. From those positive targets, we chose to characterize and have named a novel gene based on bioinformatics analysis: differentiation-associated protein (dapr, also known as PLEKH3 and previously known as 9430067K14Rik in mouse and LOC389072 in human). To our knowledge this is the first characterization of this protein. The full-length REFSEQ sequence for dapr (NM_001039493) is 8562-bp long. BLAT (12), searching the mouse genome, indicated that it spanned ~167 kilobases on chromosome 1 (6472397–64891100, February 2006 assembly). The predicted protein coding region (NP_001034582) is 761 amino acids long (86 kDa) and occurs between base pairs 361 and 2646 (supplemental Fig. S1). There was a 49% similarity to PLEKH1 (BL2SEQ (13), E-value = 1e-64) over 68% of the protein and no similarity to PLEKH2.

To further characterize dapr in silico, we used HMMER (14) with the PFAM (Version 19) (15) and SMART (Version 5.0) (16) PSSM scoring matrices to find conserved domain structures. PFAM matrices predicted (p < 0.005) two pleckstrin homology (PH) domains found centrally from amino acid positions 213 to 309 and 362 to 465 (Fig. 1A). The SMART data base search predicted (p < 0.05) the same two PH domains along with a C1 (protein kinase C conserved region 1) binding domain located from amino acids 670 to 720 near the carboxy-terminal end. We did not find any predicted kinase domains. Because this was a novel gene, we searched for homologous sequences in the various public databases available at NCBI (13) and University of California Santa Cruz (17). We were able to find homologous genes or gene fragments for dapr across all sequenced Vertebrata (see supplemental Fig. S2 for an example alignment), none of which have been characterized. Dapr homology is greatest within the sequenced Tetrapoda, with similarity (>80%) over all exons. Outside of the Tetrapoda, sequence similarity to other Vertebrata (such as the Chondrichthyes and Petromyzontiformes) is confined to exons 4, 5, and 6 near the carboxy-terminal end.

Expression and Subcellular Localization of Dapr—To begin characterizing Dapr, we used real-time PCR to determine its steady-state mRNA levels in a variety of tissue types. Dapr was found to be expressed in all tissues profiled (Fig. 1B). When normalized to the tissue with the lowest expression levels (liver), skeletal muscle and heart were found to demonstrate the greatest quantities of dapr mRNA transcript. In C2C12 cells, levels rose 2.4 ± 0.7-fold (mean ± S.E., n = 3) above predifferentiation expression levels (Fig. 1C). Using an antibody raised against Dapr, we then examined protein expression in control and 3-day-differentiated C2C12 cells (Fig. 1D). No discernible change was observed in the level of protein between these time
Dapr-Mediated Muscle Differentiation

Binding of PKB to Dapr—Understanding the importance of the PI3K signaling pathway in muscle differentiation, we looked at one of its key proteins, PKB, to see if there was any association with Dapr. To test the specificity of Dapr binding to endogenous PKB, we generated a GST fusion protein with the full-length protein (Fig. 2A and supplemental Fig. S3). The GST fusion protein was then incubated with cell lysates from C2C12 myoblast cells and 3-day differentiated cells. Dapr was found to selectively bind to endogenously expressed PKB in vitro. Non-specific binding was not observed to either the glutathione-agarose beads or to the GST protein. Furthermore, no signal was detected when the samples were probed for the controls PI3K or MKK6, a mitogen-activated kinase kinase from the SAPK (stress-activated protein kinase) pathway (Fig. 2A). Signaling through PKB is one of the key routes for insulin-mediated glucose uptake and glycogen synthesis (19), with glycogen synthase being the primary downstream-regulated target in skeletal muscle (20). We, thus, performed GST pulldown assays to test for any association between glycogen synthase and Dapr, but no association was found. Binding intensity of PKB to Dapr was comparable with glycogen synthase kinase 3β (supplemental Fig. S4).

To further ascertain PKB and Dapr association, we performed a series of co-immunoprecipitations (Fig. 2B). HeLa cells were transfected with vectors expressing Dapr and PKB. Immunoprecipitation of HA-tagged PKB resulted in detection of Myc-tagged Dapr. This clearly demonstrates that PKB binds to Dapr. We also attempted to immunoprecipitate Myc-tagged (amino- and carboxyl-terminal) Dapr but were unable to detect PKB. This is undoubtedly due to the inaccessibility of the Myc epitope during immunoprecipitation as compared with on a denaturing gel. To narrow down the potential binding region of this association, we divided Dapr into four arbitrary segments (see the legend to Fig. 2C for explicit boundaries) using the edges of the two PH domains as a rough initial guideline. PKB demonstrated no associations with any of the fragments in isolation (data not shown). However, when com-

points. Utilizing the online resource genepaint (18) we found RNA in situ hybridization probes to dapr were expressed ubiquitously in developing E14.5 stage mouse embryos. Furthermore, we found Dapr protein was expressed in mouse embryonic stem cells (data not shown).

Using immunohistochemistry, subcellular localization of Dapr within undifferentiated C2C12 myoblasts was found to be primarily in the Golgi apparatus (Figs. 1E and 5A). After differentiation, Dapr moved away from this organelle and localized to the cytoplasm and cell membrane (see Fig. 5C).
Dapr-Mediated Muscle Differentiation

FIGURE 3. Subcellular localization of Dapr. Lysates were harvested from non-differentiating (0 day), and 3-day-differentiated C2C12 cells (A) or C2C12 cells co-transfected with myristoylated PKB and Myc-Dapr or pcDNA3 vector (B). These were subjected to ultracentrifugation through a sucrose cushion, and the subcellular compartments were separated (C, cytosol; M, plasma membrane). Equal amounts of each fraction were run out on denaturing acrylamide gels and probed for the presence of Dapr and PKB. C, aliquots of each fraction were separated by gel electrophoresis and probed with antibodies to proteins located specifically to either the cytosol (β-tubulin) or plasma membrane (Na/K ATPase).

FIGURE 4. RNA interference of Dapr. A, siRNA oligonucleotide (40 nm each) designed specifically for Dapr was transfected into C2C12 cells. Cells were also transfected with a negative control oligo set (CNTL). Lysates were probed for the presence of Dapr, PKB, and β-tubulin by Western blotting. B, subcellular localization of Dapr and PKB were assessed by Western blotting of fractions collected from C2C12 cells transfected with Dapr siRNA oligonucleotide or control oligonucleotide. C, cytosol; M, membrane. C, phase micrographs of C2C12 cells transfected with Dapr RNAi (CNTL) and Dapt RNAi (40 nm). Lysates were harvested at a variety of times (0, 1, 2, and 3 days) from cells undergoing differentiation. Equal quantities of each were separated on gels and expression of S-actin and β-tubulin were determined at each time point by Western blot analysis.

and membrane fractions. When myristoylated PKB was introduced, Myc-tagged Dapr was found to move to the membrane (Fig. 3B).

Knockdown of Dapr Inhibits Differentiation of C2C12 Myoblasts—We next investigated the impact that a loss of Dapr would have on the process of differentiation using RNA interference. C2C12 cells were transfected with siRNA oligos (see “Experimental Procedures” for sequence and transfection protocol). Cell lysates and immunohistochemically stained whole cells were then characterized for Dapr protein expression. Exposure to siRNA oligos resulted in significant reduction of Dapr protein at the time of differentiation (Figs. 4A and 5, B and D). No changes were observed in the protein levels of PKB or β-tubulin, indicating that the influence of the siRNA oligo was specific only to Dapr.

To better understand the impact that a loss of Dapr has on differentiating C2C12 cells in regard to the subcellular localization of PKB, we examined the activity of Dapr and PKB using differential centrifugation on control and siRNA treated cells. After initiation of differentiation in control cells, PKB and Dapr were found to be in the membrane fraction (Fig. 4B). However, knockdown of Dapr resulted in the complete absence of PKB in the membrane fraction.

On a more global level, knockdown of Dapr had a significant effect on the resultant phenotype of cultures of differentiating myoblasts, with fewer formations of myotubes present (Fig. 4C). As a functional marker to measure the fusion of myoblasts into myotubes, levels of sarcomeric actin (S-actin) were measured. During the normal course of differentiation, S-actin is fully elevated in expression after 2 days. Knockdown of Dapr expression resulted in a delay of S-actin expression to normal levels at 3 days of differentiation (Fig. 4D). The fact that S-actin levels do reach normal levels is undoubtedly due to the transient nature of the RNAi protocol and the fact that transfection rates are not 100%. In other words, cells that did not become transfected with RNAi oligos may overcome any inhibition presented by the siRNA in neighboring cells, eventually forcing fusion and expression of the proteins. Indeed, immunofluorescence showed that after Dapr knockdown those cells that did not express Dapr also lacked S-actin expression (Fig. 5, B and D) after 2 days. This is compared with control stained cells that fully form myotubes (Fig. 5, A and C).

DISCUSSION

Identification of the Novel Gene dapr—Much work has been done to dissect out the mechanisms through which muscle precursor myoblast cells differentiate into myotubes. Pivotal to this process is the activation of a number of transcription factors, such as MYOD and MEF2, which bind to DNA and transactivate a number of genes that orchestrate differentiation (21). To understand this process better, we first turned our attention to the transcription factor MEF2. Using chromatin immunoprecipitation and CpG microarray analysis we then sought to
represent 20 other tissue types. In differentiating muscle cells, Dapr expres-
sion more than doubled compared with non-differentiated myoblast cells. This shows that it is regulated during this process. However, when we looked at protein expression during differentiation, no discernible changes were observed. It is unknown whether the difference between this result and that of the previously determined rise in steady-state mRNA expression during differentiation at the same time points is due to a higher protein turnover or reduced or time-lagged rates of protein synthesis.

Immunohistochemical staining revealed that Dapr is localized to the Golgi apparatus before differentia-
tion (Fig. 1E). Recent research suggests new roles for the Golgi apparatus beyond traditional protein packaging for the extracellular matrix. For example, this organelle has been implicated in being involved in mechanisms for signal transduction (23). Although beyond the scope of the current paper, further investigation into the role the Golgi apparatus plays in the trafficking or packaging of Dapr would be of great interest.

**Dapr Involvement in PKB-directed Signaling**—Our *in silico* analysis revealed that Dapr contained two putative PH domains (Fig. 1A), motifs which are commonly associ-
ated with phosphoinositide binding and membrane targeting (24). PKB also contains a PH domain, binds phosphoinositides (3), and is known to be involved in muscle differentiation through its involvement in PI3K signaling (1). Importantly, it is also involved in the regulation of MEF2 (25). We hypothesized that Dapr could be associated with the PI3K signaling cascade, specifically in binding with PKB. We were able to show that Dapr did indeed bind to PKB (Fig. 2A). We, therefore, next sought to gain some initial insights into the possible mechanism through which Dapr may act with PKB in PI3K signaling.

Knowing that PKB activation occurs by phosphorylation (1), we examined its phospo status (supplemental Fig. S4) *in vitro*. We found PKB phosphorylated regardless of the phase during differentiation, and overexpression of Dapr did not alter this (data not shown). This suggests that endogenous Dapr within the cell is not the key trigger for phosphorylation of PKB. Because PKB binds the membrane protein phosphatidylinositol 3,4,5-trisphosphate, we next decided to look at whether Dapr may function as a means to localize PKB to the membrane at the appropriate time during differentiation. The presence of PH domains could suggest a potential role in membrane localization for Dapr (24). C1 domains are also known to target mem-

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**FIGURE 5. Influence of Dapr siRNA on Dapr and S-actin expression within the cell.** A, zero-day negative control-transfected representative C2C12 myoblast. Scale bars represent 10 µm. B, zero-day Dapr siRNA-transfected C2C12 representative myoblast. Scale bars represent 10 µm. C, 2-day differentiated negative control transfected C2C12 myotubes. Scale bars represent 20 µm. D, 2-day Dapr siRNA-transfected C2C12 cells. Scale bars represent 20 µm. Green, Dapr; red, S-actin; blue, nucleus.
Dapr-Mediated Muscle Differentiation

FIGURE 6. Schematic representation showing the proposed function of Dapr. Under non-activated conditions Dapr associates with PKB in the cytosol. After activation of the PI3K pathway, Dapr localizes to the membrane bringing PKB along with it. IGF, insulin-like growth factor; RTK, receptor tyrosine kinase.

that it binds to PKB, and during differentiation, both proteins are shuttled to the plasma membrane (Fig. 6). It may, therefore, be involved in focusing PKB actions during this process. By knocking down the expression of Dapr using RNAi, we have demonstrated an inhibition in the ability of the cells to differentiate properly, illustrating its key role in skeletal muscle differentiation. Another related member of this protein, PLEKHM1, has been shown to be a component of osteoclast formation and is involved in osteopetrosis (22). As it has a similar domain structure to Dapr (with the addition of a RUN domain in the amino-terminal end), we hypothesize that it may also be involved in the same signaling pathway. As this is only an initial characterization of Dapr, the full breadth of its structure and biology has yet to be ascertained. The implications of Dapr being involved in this signaling cascade make it a worthy candidate for future, focused studies.

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