Characterization of a Novel Scaffold Protein Involved in Skeletal Muscle Differentiation

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ABSTRACT

The phosphatidylinositol 3-kinase (PI_3K) signaling pathway is associated with a variety of cellular functions, ranging from cell cycle regulation to tissue development. Altered or deviant signaling through this cascade is associated with a number of cancers and disorders. Although years of research have extensively characterized this signaling pathway, not all aspects of it have been fully elucidated. Here we show that a novel pleckstrin homology domain containing protein is also involved in this pathway. This protein was identified during a screen of MEF2 transcription factor binding sites during skeletal muscle differentiation. It is a well-conserved, though previously uncharacterized, protein found in the sequenced genomes of a variety of Tetrapods. Association analysis demonstrated that it binds specifically to the PI_3K signaling pathway members PDK1, PKB, and GSK3 . In C_2C_{12} myoblast cells prior to differentiation, the protein was localized to the cytosol, migrating to the membrane following initiation of muscle differentiation. Knockdown analysis by RNAi resulted in inhibition of myotube formation. Finally, mRNA levels were found to be differentially regulated across a number of tumour pathologies.

INTRODUCTION

Signaling cascades linked to membrane receptors are key mechanisms for coordinating intra- and extra-cellular events involved in terminal cellular differentiation. In skeletal muscle C_2C_{12} cell lines, the process of determining myoblast cell fate involves a number of known signaling pathways. Included are those such as the phosphatidylinositol 3-kinase (PI_3K), the mitogen activated protein kinase p38, calcineurin-NFAT signaling, ERK and the Wnt signaling pathways. Of these, the PI_3K signaling pathway has been shown to play a pivotal role during the transformation of myoblasts into myotubes. Downstream of PI_3K are the lipid products PI-3,4,5-P_3 and PI-3,4-P_2 which provide membrane anchoring points for pleckstrin homology containing proteins 3-phosphoinositide-dependent kinase 1 (PDK1) and protein kinase B (PKB, also known as AKT). Localization of these kinases to the plasma membrane is an important part of their activation. Indeed, the compartmentalization of kinases within the cell is a key regulatory mechanism of signaling cascades. AKAP (A Kinase Associated Protein) was one of the first proteins identified to fulfill such a role. Not only does AKAP localize kinases to specific parts of the cell, it also brings proteins together within proximity of one another: for example, protein phosphatase 2 and PKA. Here, we identify a novel gene DASP (Differentiation Associated Scaffold Protein) a scaffold protein involved in coordinating activation of the PI_3K signaling pathway. DASP was initially discovered in a screen of MEF2 transcription factor targets during skeletal muscle differentiation using chromatin immunoprecipitation combined with CpG microarray analysis.

METHODS

DNA constructs. HA-PKBpcDNA$_3$, FLAG-PDK1 pcDNA$_3$ and HA-GSK3 were generously provided by Dr. Jim Woodgett. Amino- or carboxy-terminal MYC tagged DASP was generated by incorporating the MYC epitope into either the sense or antisense primer respectively.

For expression into mammalian cells, DASP was subcloned into the pcDNA$_3$ 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.

Differential centrifugation. C_2C_{12} cells were washed and harvested, and pelleted by centrifugation for 5 minutes. Pellets were resuspended in 300 l lysis buffer (250 mM sucrose, 20 mM HEPES-NaOH (pH 7.4), 1 mM EDTA, 50 mM NaF, 6 mM Na_2VO_4 and protease inhibitor cocktail) and passed through a 21-gauge needle. The cell lysate from the initial spin was centrifuged at 19000xg for 20 minutes and the resulting pellet (P1) was then re-suspended in 300 ul lysis buffer. The supernatant from the second spin was centrifuged at 40000xg for 20 minutes to yield a high-density microsomal pellet (P2). The supernatant from this spin was transferred to a new tube and spun again at 180000xg for 1.5 hours resulting in the cytosolic fraction (supernatant) and a low-density microsome (P3) pellet. The P1 pellet was layered onto a 1.12 M sucrose cushion and centrifuged at 100000xg for 1 hour. Pelleted material containing the nuclear/endoplasmic reticulum fractions was re-suspended in 100 l RIPA buffer (50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 1% IGEPAL, 0.5% sodium deoxycholate, 0.1% SDS) with protease inhibitors. The interphase material containing the plasma membrane fraction, was collected and the final volume adjusted to 2 ml in lysis buffer and was pelleted by spinning at 40000xg for 20 min. Plasma membrane, P2 and P3 pellets were then re-suspended in 50 l PBS and 6x protein loading buffer and boiled for 5 minutes at 95 C.

RNA interference. C_2C_{12} cells were propagated into 6-well dishes (Nunc) in 3ml propagation medium: DMEM with 10% Fetal Bovine Serum (Invitrogen). The cells were initially transfected at approximately 20% confluence then again 24 hours later at approximately 50% confluence.

GST Fusion Protein Preparation and Association. GST-fusion proteins were expressed using the GST Gene Fusion System (G.E. Healthcare). At the appropriate logarithmic growth, bacterial cultures were induced overnight at 30C with 0.5 mM IPTG (Sigma). Cells were sonicated and protein solubilized by addition of 1% Triton X-100 for 30 min at 4C. Debris were pelleted by centrifugation and the lysate containing the fusion protein bound to 500 l of glutathione agarose beads (Sigma) overnight at 4C. Beads were then pelleted, washed and resuspended to a 50% slurry. C_2C_{12} cells were washed with ice cold PBS and lysed by incubating on ice for 10 minutes in 1.5 ml of lysis buffer (10 mM NaCl, 20 mM PIPES, 0.5% NP40, 0.05% -mercaptoethanol, 5 mM EDTA) with protease inhibitors, 50 mM NaF and 100 M Na_2VO_4. Harvested cell lysate was passaged through a 21-gauge needle and debris removed by centrifugation. The pre-cleared lysate was incubated with 100 l of bead slurry bound to the GST fusion protein. Samples were incubated at 4C with rocking for 1 hour and then washed and the bead complex boiled in 6x protein loading buffer for 5 minutes.

Co-Immunoprecipitation. Harvested cells were lysed by rocking 20 minutes at 4C with 400 l lysis buffer (50 mM Tris (pH 8.0), 120 mM NaCl, 0.5% IGEPAL). Debris was pelleted by centrifugation and the resulting supernatant incubated with Protein A beads and antibodies for 1.5 hours at 4C. Following repeated washes, beads were boiled for 5 minutes at 95C in 2x protein loading buffer.

Western blot analysis. Cell lysates equalized for protein were *run on 10% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Hybond-C, GE Healthcare*). Blots were probed with antibodies directed against PDK1, phospho-PDK1 (ser-241), PKB, phospho-PKB (ser-473), GSK3 , phospho- GSK3 (ser-9), PI_3K, MKK6, glycogen synthase, -tubulin (Cell Signaling Technologies), sarcomeric actin (Sigma), anti-HA (Upstate), anti-Myc and anti-FLAG antibodies were generous gifts from J. Woodgett. Secondary antibodies conjugated to horseradish peroxidase were used. Blots were incubated in ECL reagent (Western Lightning Chemiluminescence reagent, Perkin Elmer) and exposed to film for visualization.

RESULTS

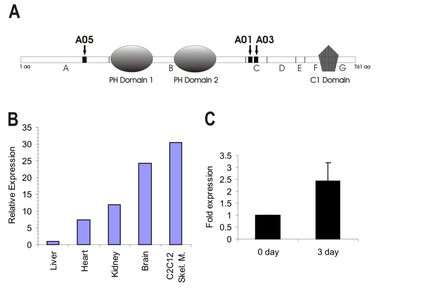


Figure 1 Characterization of DASP: A, An illustration of DASP showing *in silico* identified domain structures and the locations of siRNA oligos (A01, A03, A05). Alphabetic labels indicate the positions of separate exons. **B**, Expression of DASP mRNA in mouse tissues. Levels were determined by real-time PCR and quantified relative to that found in liver. **C,** DASP mRNA expression changes during differentiation. Steady-state mRNA level of DASP was quantified from both undifferentiated and 3 day differentiated C_2C_{12} mouse skeletal muscle myocytes.

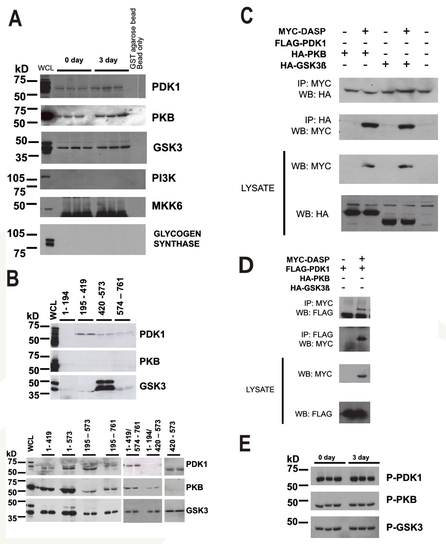


Figure 2 *In vivo* associations between DASP with PDK1, PKB and GSK3 : A, GST-DASP bound to glutathione agarose beads were incubated with whole cell lysates from either undifferentiated (0 day) or three day differentiated (3 day) C_2C_{12} cells. **B,** Binding of PDK1, PKB and GSK3 were assessed following incubation with GST fusion proteins corresponding to a variety of DASP fragments. Boundaries of each fragment are indicated by the residue number. **C,** HeLa cells were transfected with HA tagged PKB and GSK3 in the presence or absence of MYC tagged DASP. HA or MYC immunoprecipitates were probed with either a MYC or HA antibody to detect the presence of DASP or PKB and GSK3 , respectively. **D,** Co-immunoprecipitations of transfected FLAG tagged PDK1 and MYC tagged DASP in HeLa cells. **E,** Phosphorylation status of each protein was established by immunoblotting using phospho-specific antibodies raised against specific phosphorylation sites for each kinase.

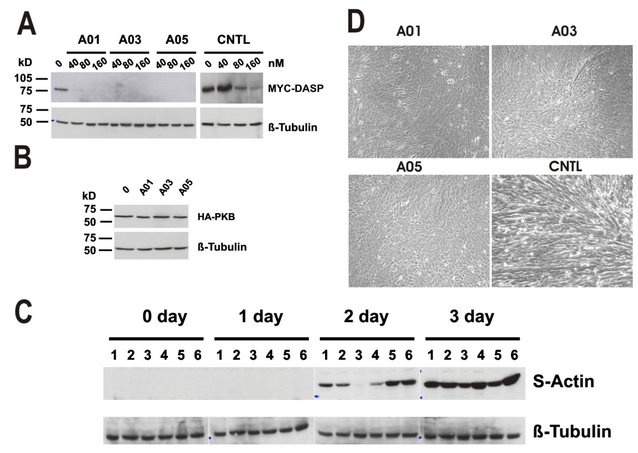


Figure 4 RNA interference of DASP: A, Three siRNA oligo sets designed specifically for DASP were co-transfected at a variety of concentrations into HeLa cells along with MYC-tagged DASP. Cells were also transfected with a negative control oligo set (CNTL). **B,** Influence of siRNA (40 nM each) on transfected HA-tagged PKB in HeLa cells. **C,** Undifferentiated C_2C_{12} cells were transfected with the three siRNA (0 or 40 nM) to knockdown DASP expression in pre-differentiating and differentiating cells. Sarcomeric actin (S-actin) and -tubulin protein expression were determined at each time point by western blot analysis; (1) No siRNA, (2) A01, (3)A03, (4) A05, (5) control oligo, (6) no transfection. **D,** Phase micrographs of C_2C_{12} cells transfected with siRNA following three days of differentiation.

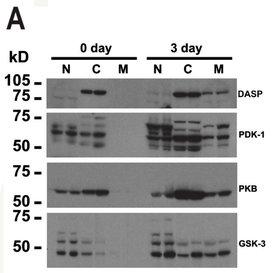


Figure 3 Sub-cellular localization of DASP: A, Lysates from both non-differentiating (0 day) and 3 day differentiating C_2C_{12} cells were subjected to ultracentrifugation through a sucrose cushion and subcellular compartments separated (N-nuclear, C-cytosol, M-plasma membrane). Equal volumes of each fraction were run out on denaturing acrylamide gels and probed for the presence of DASP, PDK1, PKB and GSK3 . **B,** Deletion mutants corresponding to the pleckstrin homology domains were constructed and fused to GST. *In vitro* association of PDK1, PKB and GSK3 to the GST tagged deletion mutants was assessed following incubation of the whole cell lysates (WCL) from C_2C_{12} cells. Western blot analysis was carried out to determine presence of the three kinases associated with the GST tagged proteins.

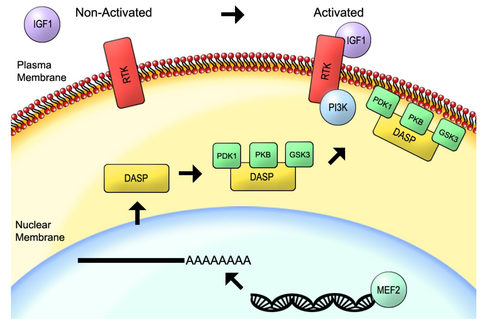
CONCLUSIONS

1) We have identified and cloned a novel signaling molecule DASP, which is involved in the PI_3kinase signaling pathway.

2) Transcription of Dasp mRNA is under the regulation of MEF2 and increases in expression during skeletal muscle differentiation.

3) DASP binds to PDK1, PKB and GSK3 and becomes membrane localized following the initiation of muscle differentiation, suggesting a role as a scaffold protein in this pathway.

4) DASP is a key component involved in the regulation of C_2C_{12} skeletal muscle differentiation.



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