Regulation of Skeletal Muscle Differentiation by DAPR Through CyclinD1 Phosphorylation

James Paris, Carl Virtanen and Mark Takahashi

University Health Network Microarray Centre, MaRS Centre, Toronto Medical Discovery Tower, 101 College Street, 9-301, Toronto, Ontario, M5G 1L7, Canada

Signaling cascades linked to membrane receptors are key mechanisms for coordinating intra and extra-cellular events. One such pathway, the phosphatidylinositol 3-kinase (PI3K) signaling pathway has been associated with a variety of cellular functions, ranging from cell cycle regulation to tissue development. Recently, we have identified a novel signaling molecule, DAPR, which focuses one aspect of this pathway by acting as a scaffold for key proteins involved in PI3K signalling. Here, we show that DAPR binds PKB and the dual tyrosine kinase MIRK/DYRK1B. Activation or inhibition of these kinases has been associated with the regulation of cellular proliferation. The withdrawal of cells from the cell cycle is especially relevant in developing cells as they leave the mitotic cell cycle in order to terminally differentiate. Cyclins family of proteins play a key regulatory role in the progression of cells through cell cycle. One of the first of these proteins to appear is cyclinD1. It is instrumental in the advancement of cells to move from the G1 to S phase. Regulation of cyclinD1 can have a profound effect on the progression of cells through the cell cycle. MIRK/DYRK1B phosphorylates cyclinD1, targeting it for subsequent degradation in the proteasome during the G0 phase. This halts cell cycle progression allowing for terminal differentiation to proceed. In C2C12 mouse myoblast cells, we demonstrate through knockdown of DAPR by siRNA an increased stability of cyclinD1 protein and the inhibition of terminal myotube differentiation. Our findings suggest a role for DAPR in which regulatory kinases are brought together and co-localized to the appropriate downstream target. In this case, MIRK/DYRK1B acting to phosphorylate cyclinD1. Interestingly, MIRK/DYRK1B is also known to decrease class II histone deacetylases which inhibit the transcription factor MEF2. As DAPR is regulated by MEF2, this opens the possibility of a positive feedback loop.

INTRODUCTION

Terminal differentiation of cells committed to a specific tissue type requires a number of events to occur. Of paramount importance at the cellular level is the coordination and transmission of the signal to trigger differentiation. This series of events leading to cellular determination relies extensively upon the complex network of proteins involved in signal transduction. The careful orchestration of these signaling molecules is key to the outcome. Partnering to one molecule may lead to the necessary series of events occurring while binding to another may lead to cell death. We previously identified a new protein, DAPR, that is involved in the coordination of the PI3 kinase signaling cascade. This signaling pathway is instrumental in the regulation of skeletal muscle differentiation. DAPR binds to and co-localizes with PKB to the cell membrane of differentiating skeletal muscle cells. Loss of DAPR inhibits terminal differentiation of C2C12 mouse myoblast cells. From these early findings it can be seen that DAPR is an integral component in the differentiation machinery of skeletal muscle cells. However, it still remains unclear as to the mechanism through which DAPR is involved in regulating differentiation.

In order for cells to undergo the process of cellular differentiation, they must first exit the cell cycle. The mitotic process of cellular division and proliferation is itself regulated by a number of proteins called cyclins. These proteins in turn regulate kinases and other proteins that are necessary for the cell to progress through cellular division. One of the first cyclins involved in this process is cyclin D1. The movement of cells out of the G1 phase begins with the cyclin D1 phosphorylating the cyclin dependent kinases 4 and 6. These in turn will phosphorylate and inhibit the actions of the retinoblastoma protein, allowing the cell cycle to proceed.

Here, we present preliminary evidence for a proposed means by which DAPR plays an integral role in regulation of cell cycle progression. We hypothesize that DAPR is involved in the activation of the upstream kinase of cyclin D1, MIRK/DYRK. We propose that DAPR is involved as a scaffolding protein into which PKB and MIRK/DYRK bind and become activated. Through this association of proteins the stability of cyclin D1 is compromised and targeted for destruction. Thus, the progression of cell cycle is halted and the cells may then proceed toward terminal differentiation.

EXPERIMENTAL PROCEDURES

Cell Culture

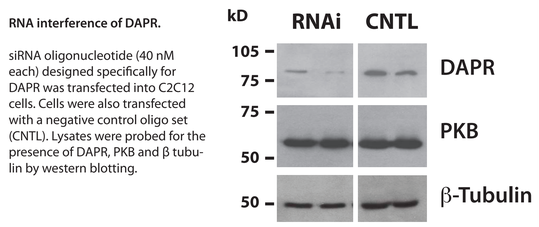
C2C12 murine skeletal muscle cells were obtained from the ATCC and grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% antibiotic (penicillin and streptomycin) in the presence of 5% CO2. Induction of differentiation involved growing cells to 80% confluence then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

GST Fusion Protein Preparation and Association

GST-fusion proteins were expressed using the GST Gene Fusion System (GE Healthcare, Baie d'Urfe, Canada). Following overnight induction at 30°C with 0.5 mM IPTG (Sigma, Oakville, Canada) 1l of bacterial culture was pelleted and resuspended in 50 ml PBS + 1mM EDTA with protease inhibitors (Roche, Mississauga, Canada). The cells were sonicated using a Branson 450 sonifier (Branson Ultrasonics Corporation, Danbury, USA) and the proteins solubilized by the addition of 1% Triton X-100 for 30 min at 4°C. Cellular debris was pelleted by centrifugation at 9,000 x g for 30 min at 4°C. The lysate was bound to 500 µl of glutathione agarose beads prepared as per manufacturer's protocol (Sigma, Oakville, Canada) at 4°C overnight. The beads were then pelleted at 1,000 x g for 5 minutes at 4°C, washed with PBS + 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 Coomasie Blue to confirm protein production. C2C12 cells were washed with ice cold PBS + 1 mM EDTA and lysed by incubating on ice for 10 minutes in 1.5 ml of Gentle Soft Lysis Buffer (10 mM NaCl, 20 mM PIPES, 0.5% NP40, 0.05% B-mercaptoethanol, 5 mM EDTA) with protease inhibitors , 50 mM NaF and 100 µM Na3VO4. Harvested cell lysate was passaged through a 21-gauge needle and debris removed by pelleting at 16,000 x g for 10 minutes at 4°C. The supernatant was pre-cleared using 50 µl of 50% slurry of glutathione agarose beads alone for 15 minutes 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 hour and then washed with Gentle Soft Lysis buffer containing protease and phosphatase inhibitors. The supernatant was removed and the beads boiled in 6x protein loading buffer for 5 minutes.

RNA interference of DAPR.

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.

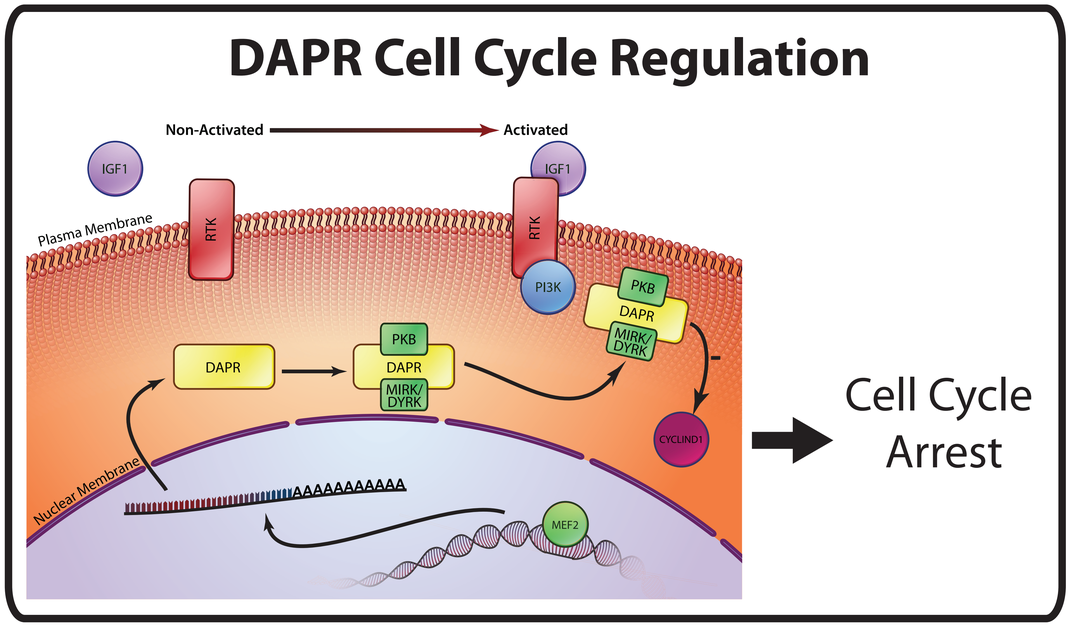


Western Blot Analysis

Samples were run on 10% SDS-polyacrylamide gels and transferred (semi-dry electroblotter, GE Healthcare, Baie d'Urfe, Canada) onto nitrocellulose membranes (Hybond-C, GE Healthcare, Baie d'Urfe, Canada). Blots were probed with antibodies directed against cyclin D1, PKB and β-tubulin (Cell Signaling Technologies, Danvers, USA), sarcomeric actin (Sigma, Oakville, Canada), MIRK/DYRK (Abgent, San Diego, USA). 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, Perkin Elmer, Waltham, USA) and exposed to film for visualization.

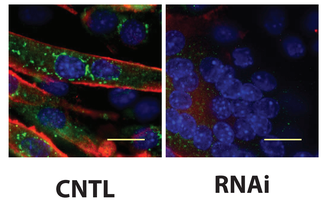
siRNA knockdown

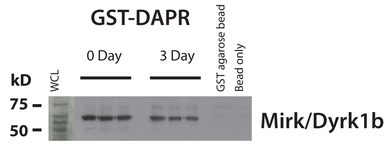
C2C12 cells were propagated into 6-well dishes (Nunc, Rochester, USA) in 3ml propagation medium: DMEM + antibiotics fortified with 10% Fetal Bovine Serum (Invitrogen, Carlsbad, USA) and allowed to settle overnight. The cells were transfected at approximately 20% confluence and 24 hours later at approximately 50% confluence as follows: 40 nM of siRNA oligo (Integrated DNA Technologies, Coralville, USA) and 5µl Lipofectamine 2000 reagent (Invitrogen, Carlsbad, USA) were combined in 500 µl Optimem medium (Invitrogen, Carlsbad, USA). siRNA oligo was then incubated with cells at 37°C for 4 hours at which time the OptiMEM was replaced with propagation medium and cells allowed to recover overnight. siRNA oligo sequence pair were as follows;
5'-UAUAAUUGCACACCUUGGCCUUUCC-3',
5'- GGAAAGGCCAAGGUGUGCAAUUAUA-3'
A medium GC content oligonucleotide (Invitrogen, Carlsbad, USA) was used as a control. Two other oligos were tested for their ability to knockdown DAPR protein expression (see supplementary data). All three oligos were found to be equally capable of reducing DAPR protein expression.



Immunohistochemistry of DAPR.

Immunohistochemistry of C2C12 cells transfected with siRNA control (CNTL) and DAPR (RNAi) following three days of differentiation.



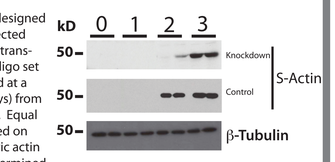


DAPR binds to MIRK/DYRK1B.

Cell lysates from non-differentiated and 3 day differentiated cells were incubated with GST-DAPR and bound proteins were run on SDS-PAGE and analyzed by western blot analysis for the presence of MIRK/DYRK1B.

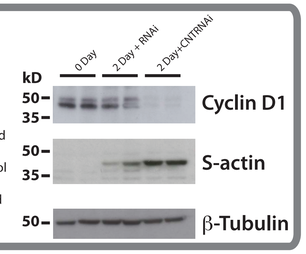
RNA interference of DAPR time course

siRNA oligonucleotide (40 nM) designed specifically for DAPR was transfected into C2C12 cells. Cells were also transfected with a negative control oligo set (control). 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 sarcomeric actin (S-actin) and β-tubulin were determined at each time point by western blot analysis.



Knockdown of DAPR results in the stabilization of cyclin D1.

Presence of cyclin D1, S-Actin and β tubulin were characterized in non-differentiating C2C12 cells (0 day) and 2 day differentiated C2C12 cells exposed to either DAPR siRNA or control (CNTL) siRNA. Lysates were run on SDS-PAGE and proteins characterized by western blot analysis.



UHN Microarray Centre

University Health Network
Toronto General Hospital Toronto Western Hospital Princess Margaret Hospital