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The N-Terminal Acidic Residue-Rich Domain and Lysine-Rich Domains I and II of P67 are Required for Stable Expression of Focal Adhesion Kinase (FAK)

Bansidhar Datta*, Rekha Datta

Department of Chemistry and Biochemistry, Kent State University, Kent, OH, USA
bdatta@kent.edu

Abstract: *Differentiation of skeletal muscle cells into multinucleated myotubes needs several cellular events including shutting off the growth promoting signals mediated by several growth factors. Integrin-mediated growth signal is transduced to the downstream effectors through the higher levels of expression and activity of focal adhesion kinase (FAK), which seems to have strong proliferative signal almost in all tissues that leads to tumorigenesis. During myogenesis, the level of eukaryotic initiation factor 2 (eIF2)-associated glycoprotein p67 gradually increases and its level peaks at the time of fusion of C2C12 myoblasts into myotubes. This increased level binds to extracellular signal-regulated kinases (ERK1/2) to inhibit their activation and activity when C2C12 myoblasts are differentiating into myotubes. To investigate whether p67 is involved in the inactivation of FAK during the differentiation of C2C12 myoblasts, we examined the level of FAK. We found that degradation of FAK peaked at the time of fusion of C2C12 myoblasts into myotubes and at the same time the level of p67 is also high. In addition, p67's N-terminal lysine-rich domains I & II and acidic residue-rich domain are involved in this degradation of FAK possibly by increasing the cellular concentration of p52 segment of p67 that has intermolecular proteolysis activity. We also found that the level of the Akt1/2 kinase, one of the several downstream effectors of FAK, gradually increased during the differentiation of C2C12 myoblasts, possibly to prevent differentiated C2C12 myoblasts from apoptosis and this process may be independent of FAK.*

Abbreviations Used: *p67, a 67 kDa glycoprotein that binds to both eukaryotic initiation factor 2 (eIF2) and extracellular signal-regulated kinases 1 & 2 (ERK1/2); D251A and D262A, two p67 point mutants, which have alanine substitutions for aspartic acid residues at 251 and 262 positions; D6/2, a p67 block mutant, where a stretch of N-terminal acidic amino acid residues has been replaced with uncharged amino acids; K1K2, a p67 block mutant, where both of its N-terminal lysine/arginine-rich stretches of amino acid residues have been replaced with uncharged amino acids; FAK, Focal adhesive kinase; Akt1/2, the serine/threonine protein kinase or protein kinase B (PKB); and Vector, vector expressing enhanced green fluorescence protein (EGFP).*

Keywords: *eIF2-associated glycoprotein p67, p67-mutants, Focal adhesive kinase, Cdc42*

1. INTRODUCTION

Skeletal muscle differentiation is a well-orchestrated process where several events take place. These include permanent withdrawal from cell cycle, expression of several muscle-specific proteins, migration and alignment of myoblasts, and fusion of myoblasts to form multinucleated myotubes (1). Proliferation and differentiation of cells are regulated by extracellular signals from growth factors and extracellular matrix (ECM) proteins (2-3). The Integrin-mediated adhesion to ECM controls proliferation and differentiation of myoblasts (4). Upon Integrin binding to ECM proteins, focal adhesion kinase (FAK) is activated and interacts with several downstream effectors to propagate signals that regulate cellular processes such as proliferation, migration, survival, and differentiation (5-6). Both Integrin and FAK are essential for fusion of myoblasts into myotubes (7-8). In fact, during fusion of myoblasts FAK is transiently upregulated and possibly proteolytically degraded either in the proteasome or by cellular proteinases like calpain and others (9). This degradation is essential to inhibit the activity of FAK, which has strong growth promoting effects in many cells including almost all tumor cells (10).

Eukaryotic initiation factor 2 (eIF2)-associated glycoprotein p67 when not bound to either eIF2 or extracellular signal-regulated kinases 1 and 2 (ERK1/2), shows auto-proteolysis activity that generates its N-terminal p26 segment and downstream p52 segment (11-14). The later segment itself has auto-proteolysis activity (13) and this activity may have been extended intermolecularly to cleave other

cellular proteins. In fact, it is involved in the degradation of MARCKS (15), certain cyclin-dependent kinases (16), and proteolytic processing of "pro" forms of certain cyclins (16) and Rho GTPase family members like Cdc42 (17).

Levels of p67 gradually increase during differentiation of C2C12 myoblasts and its level peaks at the time of myoblasts' fusion into multinucleated myotubes (18). During the period of C2C12 myoblasts differentiation, p67 dissociates from eIF2, leading to the increased phosphorylation of eIF2α and thus suppression of rates of global protein synthesis (19), and associates with ERK1/2 MAP kinases to inhibit their activation and activity (11, 18-20). The later event leads to the inhibition of cell cycle progression and possibly permanent withdrawal from the cell cycle. These events along with several others are absolutely necessary for myoblasts' fusion into myotubes. During fusion of myoblasts' into myotubes, growth-promoting signals must be inhibited as well (3, 21). FAK provides potent growth promoting signals to almost all cells including tumor cells (10). Therefore, we asked whether p67 is involved in the degradation FAK while myoblasts are fusing into myotubes.

In this study, we examined the expression of FAK during the period of differentiation of C2C12 myoblasts. We found that its level gradually increased while C2C12 myoblasts were differentiating into myotubes and at the same time, it was proteolytically degraded. At the time of fusion, its cleavage was much higher as compared to the beginning of the differentiation. The expression of one of the downstream effectors of FAK, Akt1/2 is also gradually increased while C2C12 myoblasts were differentiating into myotubes. Since p67 level also peaks at the time of myoblasts' fusion, we examined the level of FAK in KRC-7 cells constitutively expressing rat p67 and some of its mutants. Our results show that there is slight change but not reproducible in FAK level in p67-expressing cells and it is completely degraded in p67 mutants, D6/2- and K1K2-expressing cells but not in D25A- and D262A-expressing cells. These results thus suggest that p67's acidic residue-rich domain and lysine residue-rich domains I & II are involved in stabilizing FAK, whereas, its conserved D251 and D262 residues are not involved in this process.

2. MATERIALS AND METHODS

All chemicals used in this study were obtained from Sigma Chemicals (St. Louis, MO), Merck (Darmstadt, Germany), ICN Biomedicals, Inc. (Aurora, Ohio), Fisher Chemicals (New Jersey), or GIBCO-BRL (Rockville, MD). All enzymes used in this study were purchased from New England Biolabs (Beverly, MA). Molecular mass markers were purchased from Bio-Rad.

2.1. Antibodies

Monoclonal antibodies specific to FAK (Sc-558) and Akt1/2 (Sc-8312) were purchased from Santa Cruz Biotechnology. A monoclonal antibody specific to αActin was purchased from Sigma (Sigma Biochemicals, St. Louis, MO). Tagged secondary antibodies for Western blots were obtained with the ECL kit (Perkin Elmer).

2.2. Site-Directed Mutagenesis and Subcloning into Mammalian Expression Vector

A ~1.4 kb cDNA insert encoding the entire p67 coding region was produced by PCR using appropriate forward and reverse primers (5' TCCCCCGGGTGATGGCGGGCGTGGAAGAG 3' and 5' TCCCCCGGGAAGTTTTAATAGTCATCTCCTC 3' respectively) with the pGEM-p67 template (22). The resulting DNA fragment was gel purified, digested with *Sma*I, and ligated in M13mp18 (Stratagene) at the *Sma*I site. A single-stranded uracil template was made, and annealing reactions were performed with mutant oligonucleotides. A detailed description for the generation of specific p67 mutants and their sequence variations as compared to wild type rat p67 was reported (23). The cDNA inserts for p67 with mutations at specific domains or sites were isolated from the RF form of M13mp18, digested with *Xma*I, and the DNA inserts were ligated at the *Xma*I site of pEGFP-C3 vector (Clontech, Palo Alto, CA). Plasmids with sense orientation were selected for further analysis. The specific fusion junction between mutant cDNA of p67 and enhanced green fluorescent protein (EGFP) was confirmed by DNA sequencing. All procedures for manipulation of recombinant DNA were either published earlier (13, 18, 23) or followed from molecular cloning, a laboratory manual (24), and current protocols in molecular biology (25).

2.3. Cell Culture and Generation of Stable Cell Lines

C2C12 mouse myoblasts (ATCC) cultures were maintained in growth medium as described (18-20, 26). When cells reached confluence in growth medium, the medium was replaced with differentiation medium and allowed to differentiate into myotubes for 120 h. Rat tumor hepatoma (KRC-7) cells

were grown, transfected with pEGFP-C3 vector for the expression of EGFP alone or its in-frame fusion proteins with rat p67 and its mutants (D6/2, K1K2, D251A, and D262A). Cells constitutively expressing either EGFP or EGFP-fusions of p67 or its specific mutants were selected by treating cells with G418 following the procedures as described (18-20, 26).

2.4. Cell Lysate Preparation and Western Blot Analysis

Procedures for preparation of cell lysates either from C2C12 myoblasts, myotubes, or KRC-7 cells stable expressing various fusion proteins and Western blots were essentially the same as described (13, 27). Protein bands in Western blots were scanned and their intensities were quantitatively measured by NIH Image 162 software program.

3. RESULTS AND DISCUSSION

FAK mediates signals from integrins and plays an essential role in myotube formation (6). During myogenesis, FAK is degraded either at the proteasome or by other proteinases (9). To examine whether p67 has any role(s) in modulating the expression of FAK in C2C12 myoblasts or myotubes, we first examined the level of FAK at different time intervals of C2C12 myoblasts' differentiation into myotubes. C2C12 myoblasts were grown to confluence in growth medium, replaced the medium with differentiation medium, and allowed cells to differentiate for 120 h. Cell lysates were prepared in every 24 h of differentiation and freezed at -80°C for further analysis. Total protein samples from different time points of differentiation were analyzed on Western blots for the levels of FAK (Fig. 1A) and αActin (Fig. 1B). Our data show a gradual increase in FAK level as C2C12 were differentiating into myotubes and its level peaked upto 6-fold at 96 h and slightly decreased (~3-fold) at 120 h of differentiation. When C2C12 myoblasts were grown in differentiation medium after their confluency in growth medium, they usually take 96 h to fuse into myotubes (18). While the full-length FAK gradually increased during differentiation, it started cleaving into two closely migrating proteins and a faster migrating degraded product. This proteolysis peaked at 96 h of differentiation followed by a decrease in degradation and the level of the full-length at 120 h (see the faster migrating FAK bands in Figure 1A). During the differentiation of C2C12 myoblasts into myotubes, p67 level gradually increases and its level peaks at 96 h of differentiation when myoblasts fuse into myotubes (18). These results thus provide a correlation between the increase in p67 level and the proteolytic cleavage of FAK during differentiation of C2C12 myoblasts into myotubes.

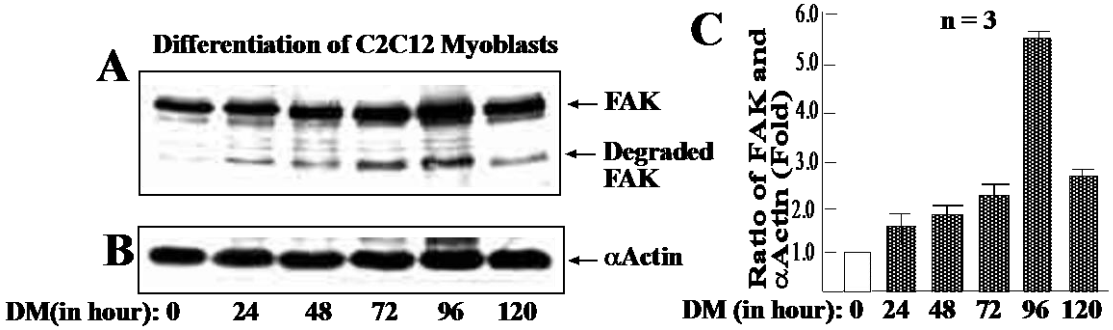

Fig1. ***Levels of FAK in C2C12 mouse myoblasts undergoing differentiation into myotubes****. C2C12 myoblasts were grown to confluency and then allowed to grow in differentiation medium for five days. Cells were harvested and lysates were prepared every 24 h intervals and stored frozen at -80℃. At the end of differentiation periods total proteins from different time intervals were analyzed on Western blots for the levels of FAK (A) and αActin (B) using specific antibodies to these proteins. These experiments were repeated three times and a representitative result is shown.*

To determine whether the expression or degradation of FAK is affected by overexpressing rat p67, we analyzed FAK's level in Western blots taking cell extracts from rat tumor hepatoma (KRC-7) cell lines constitutively expressing EGFP and EGFP fusions of rat p67 and its mutants D6/2, K1K2, D251A, and D262A. These cell lines were maintained in growth medium containing low level of G418. Total protein samples from these lines were prepared and analyzed for the expression of FAK and αActin on Western blots (Fig. 2). Our results show that FAK level increased slightly in p67-expressing cells as compared to control EGFP-expressing cells (Fig. 2A & C, compare lane 2 with lane 1). However, full-length FAK was completely degraded in cells expressing either D6/2 or K1K2 mutants (Fig. 2A, lanes 3-4). This degradation was not detected in either D251A or D262A mutant-expressing KRC-7 cells (Fig. 2C, lanes 3-4). Together, these results suggest that p67's acidic residue-

rich domain and its lysine-rich domains I & II are involved in the stabilization of FAK whereas, its conserved D251 and D262 residues are not involved in this process.

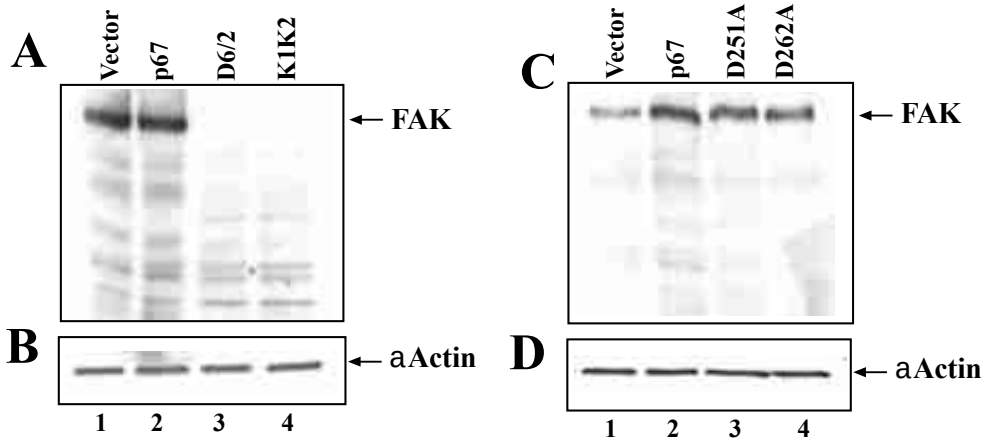

Fig2. ***Levels of FAK in rat tumor hepatoma cells constitutively expressing EGFP and in-frame EGFP fusions of rat p67 and its mutants D6/2, K1K2, D251A, and D262A***. *Stable cell lines constitutively expressing EGFP expresion vector, and its in-frame fusions of rat p67 or its various mutants were generated. Cell lysates from these cell lines were made and analyzed for the levels of FAK (A & C) and αActin (B & D) on Western blots using antibodies specific to these proteins. These experiments were repeated three times and a representitative result is shown.*

Published report also suggests that serine/threonine kinase B (PKB)/Akt1/2 could be activated by FAK and this activation leads to the protection of cells against apoptosis (28-29). We therefore examined the levels of Akt1/2 in C2C12 myoblasts differentiating into myotubes (Fig. 3). Cell extracts from different time intervals during differentiation were analyzed for the levels of Akt1/2 (Fig. 3A) and αActin (Fig. 3B) on Western blots. We found that there was a gradual increase of Akt1/2 level while C2C12 myoblasts were differentiating into myotubes and its level was more than 3-fold higher at 120 h of differentiation as compared to 0 h of differentiation (Fig. 3A, compare lane 6 with lane 1). It is therefore possible that increased level of Akt1/2 in myotubes may be involved in protecting myotubes against apoptosis and this process may or may not involve FAK.

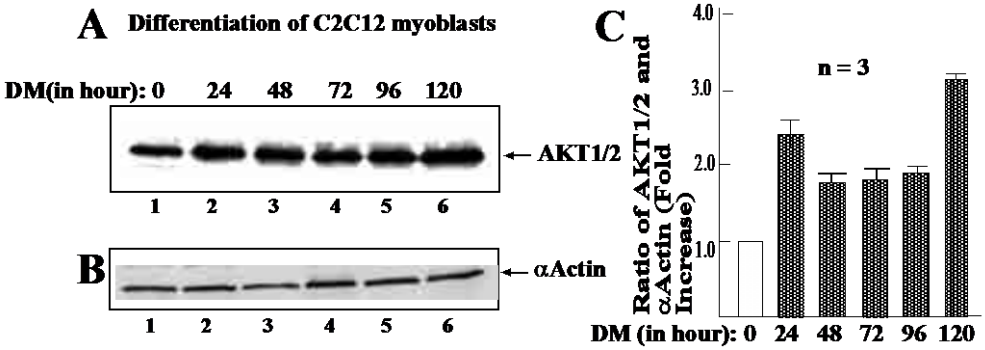

Fig3. ***Levels of Akt1/2 in C2C12 mouse myoblasts undergoing differentiation into myotubes***. *C2C12 myoblasts were grown to confluency and then allowed to grow in differentiation medium for five days. Cells were harvested and lysates were prepared every 24 h intervals and stored frozen at -80℃. At the end of differentiation periods total proteins from different time intervals were analyzed on Western blots for the levels of Akt1/2 (A) and αActin (B) using specific antibodies to these proteins. After scanning the band intensities from panel A and panel B, the ratios of Akt1/2 and αActin were calculated and plotted on a graph (C). The results are from three independent experiments (n=3).*

A cytoplasmic protein tyrosine kinase, FAK is over-expressed and activated in several advanced–stage solid tumors (10). FAK activation involves integrin receptor clustering upon cell binding to ECM proteins, which may involve FAK dimerization followed by auto-phosphorylation at Y397. Src-family of kinases binds to this phosphorylated site and forms an activated FAK-Src complex (10, 30-31). This activation promotes cell movement, invasion, motility, survival, and proliferation through kinase-dependent and -independent mechanisms (10, 29-30). During myogenesis FAK has been shown to be degraded at the proteasome (9). It is also degraded by other proteinases like calpain (9). Given the facts that FAK has a strong growth promoting signals that lead to tumorigenesis in almost

all tissues and growth inhibition of C2C12 myoblasts are essential to withdraw permanently from cell cycle for their fusion into myotubes, it imperative to degrade FAK during myogenesis. During differentiation, C2C12 myoblasts fuse into multinucleated myotubes and p67's level peaks at the same time of fusion (18). In this study, we noticed the increased degradation of FAK and possibly its inactivation at the time of myoblasts' fusion into myotubes (Fig. 1) and over-expression of rat p67 mutants such D6/2 and K1K2 in rat tumor hepatoma cells degraded FAK completely (Fig.2). These results therefore suggest that p67's N-terminal acidic residue-rich domain and lysine-rich domains I & II are involved in this process. Mutation to these domains possibly increases the cellular concentration of p52 segment of p67 and that in term may have intermolecular proteolysis activity, which degrades FAK to allow C2C12 myoblasts fuse into multinucleated myotubes.

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AUTHOR'S BIOGRAPHY

Bansidhar Datta, Ph.D, is working as an Associate Professor in Department of Chemistry & Biochemistry, Kent State University,Kent, OH 44242

Rekha Datta, is a Research Associate, Department of Chemistry & Biochemistry, Kent State University, Kent, OH 44242