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Differential Expression of MARCKS in C2C12 Myoblasts and Myotubes Constitutively Expressing P67

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Abstract: *During skeletal muscle differentiation the level of eukaryotic initiation 2 (eIF2)-associated glycoprotein, p67 gradually increases and its level peaks at the time of fusion of myoblasts into multinucleated myotubes. During the course of differentiation of C2C12 myoblasts, p67 dissociates from eIF2 that leads to increased eIF2α phosphorylation and suppression of global protein synthesis. At the same time, it associates with extracellular signal-regulated kinases 1 and 2 (ERK1/2) to inhibit the activation and activity of the later kinases that ultimately inhibits growth promoting signal to myotubes. To examine whether p67 has any role(s) in actin cytoskeleton dynamic and migration of myoblasts when fusing into myotubes, we investigated the levels of Myristoylated-alanine rich C kinase substrate (MARCKS) in C2C12 myoblasts cell lines constitutively expressing rat p67 and some of its mutants. We found that p67 is involved in higher level expression of MARCKS in myoblasts and its N-terminal lysine-rich domains I & II are required for such activity. On the other hand, in myotubes constitutively expressing rat p67 the level of MARCKS is very low and p67's N-terminal lysine-rich domains I & II along with conserved D251 and H331 residues play important roles in this process. In addition, MARCKS is degraded in rat p67-expressing C2C12 myoblasts and p67's conserved D251 residue and lysine residue-rich domains I & II are involved in this degradation. Altogether, our data suggest that p67 is possibly involved in the inhibition of myobalsts' migration while growing in growth medium whereas, it promotes myoblasts' migration and motility when myoblasts are fusing into myotubes.*

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, a p67 point mutant, which has alanine substitution for aspartic acid at 251 amino acid residue; 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; D6/2+D251A, a D6/2 mutant containing a second site point mutation, where its 251 aspartic amino acid residue has been changed to alanine; D6/2+H331A, a D6/2 mutant containing a second site point mutation, where its histidine amino acid residue at position 331 has been changed to alanine; MARCKS, Myristoylated alanine-rich C kinase substrate; p-MARCKS, the phosphorylated form of MARCKS; and V, vector expressing enhanced green fluorescence protein (EGFP).*

Keywords: *eIF2-associated glyoprotein, p67; MARCKS and p-MARCKS; C2C12 myoblasts and myotubes; p67-mutants*

1. INTRODUCTION

Differentiation of skeletal muscle cells into multinucleated myofibers needs several cellular events for example, (i) inhibition of growth promoting signals including suppression of global protein synthesis to withdraw myoblasts permanently from cell cycle, (ii) expression of muscle-specific proteins like myogenic family of proteins, (iii) detachment of anchored myoblasts from extracellular matrix, (iv) survival, migration and alignments of myoblasts, (v) regulation of actin cytoskeleton dynamics to form filopodia and lamillopodia, (vi) fusions of myoblasts into multinucleated myotubes, (vii) promote motility by preventing apoptosis, and finally (viii) to form myofibers for contractile apparatus. Several proteins are invloved in the above multi-step events. One such protein, p67, which is eukaryotic initiation factor 2 (eIF2)-associated glycoprotein (1), gradually increases its level during C2C12 myoblasts differentiation and peaks at the time of fusion of myoblasts into myotubes (2). During the same time frame of differentiation, p67 dissociates from eIF2 (3) and associates with ERK1/2 MAP kinases (4). These events lead to higher levels of phosphorylation of eIF2α that suppresses rates of global protein synthesis initiation (3) and lower level of ERK1/2 phopshorylation that leads to the inhibition of cell growth and proliferation (4).

Rat p67 has 480 amino acid residues that could be divided into two segments – the N-terminal 1-107 amino acid long segment or p26 (based on its migration near 26 kDa on SDS-PAGE) and p52 segment that contains downsteram 108-480 amino acid residues. In p26 segment, there are two lysine-rich domains I & II separated by an acidic residue-rich domain (1, 5-6). These all three domains make two salt-bridges – one with lysine-rich domain I and acidic domain and the other with acidic residue-rich domain and lysine-rich domain II (7). The lysine-rich domain I along with the O-glycosylation site at $_{60}$SGTS$_{63}$ are required for blocking phosphorylation at serine-51 of eIF2α (8) while lysine-rich domain II and downstrem 310-430 amino acid region bind to eIF2γ-subunit (9). On the other hand, the whole downstream sequences starting from 211 to 480 amino acid residues are needed for binding to ERK1/2 (10) and lysine-rich domain I & II at the p26 segment are required for blocking the phosphorylation of ERK1/2 (1). In p52 segment, there are five conserved amino acid residues – D251, D262, H331, E364, and E459. After folding, the p52 segment creates a shallow groove, where these five amino acid residues come close together to bind to either its p26 segment or other target proteins containing stretches of lysine-rich residues, which coordinate with the above four acidic residues (1). This substrate binding pocket is very close to its catalytic site H231, which cleaves substrate(s) intermolecularly (11) or possibly intramolecularly.

Myristoylated alanine-rich C kinase substrate (MARCKS), which is an actin-binding protein, regulates the cytoskeleton dynamics by crosslinking the actin filaments in the plasma membrane dynamics and migration and motility of myoblasts during their fusion into multinucleated myotubes (12-17). Proteolysis of MARCKS by calpain and possibly other proteinases activates its actin-binding activity that modulates actin dynamics and cell migration (13-17). This proteolysis seems to be PKCα dependent (18) because MARCKS is one of the major substrates of PKC, which when phosphorylates this substrate, inhibits its membrane attachment (18-19). During myogenesis, the phosphorylation of MARCKS declines and this correlates with its decreased level in the cytosol (12). In addition, the low level of phosphorylation of MARCKS is closely associated with the membrane fusion of myogenic cells (17).

To inverstigate whether p67 has any role(s) during migration and motility of myoblasts while fusing into myotubes, we examined the expression of MARCKS in C2C12 myoblasts and myotubes, which are constitutively expressing rat p67 and some of its mutants. Our results show that p67 is involved in higher level expression of MARCKS in myoblasts possibly to inhibit their migration and fusion while growing in growth medium and its N-terminal lysine-rich domains I & II are required for such activity. On the other hand, the lower levels of MARCKS and its phosphorylated form in myotubes constitutively expressing rat p67 indicates its involvement in myoblasts migration and motility while fusing into myotubes. P67's N-terminal lysine-rich domains I & II along with conserved D251 and H331 residues play important roles in this process. In addition, our data also show that MARCKS is degraded in C1C2 myoblasts overexpressing rat p67 and its lysine-rich domains I & II along with its conserved D251 residue are involved in this degradation. Together, our data suggest that p67 is involved in actin dynamics and migration and motility of myoblasts during their fusion into multinucleated myotubes by modulating the expression and activity of MARCKS.

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 BioRad.

2.1. Antibodies

Polyclonal antibodies specific to MARCKS (Sc-6454) and its phospho-specific polyclonal antibody (Sc-12971) 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 (20). 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 (21). 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 (21-24) or followed from molecular cloning, a laboratory manual (25), and current protocols in molecular biology (26).

2.3. Cell Culture and Generation of Stable Cell Lines

C2C12 mouse myoblasts (ATCC) cultures were maintained in growth medium as described (2-4, 22). 50-60% confluent cultures of C2C12 cells were transfected with plasmids complexed with Superfect following protocols described by the manufacturer (Qiagen). 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 (2). C2C12 myoblasts constitutively expressing EGFP, or its various in-frame fusion of rat p67 and its specific mutants were grown in growth medium to confluency and then allowed to differentiate into myotubes for 96 h in differentiation medium following the procedures as described (2).

2.4. Cell Lysate Preparation and Western Blot Analysis

Procedures for preparation of cell lysates either from C2C12 myoblasts or myotubes and Western blots were essentially the same as described (2). Protein bands in Western blots were scanned and their intensities were quantitatively measured by NIH Image 162 software program.

3. RESULTS AND DISCUSSION

C2C12 myoblasts constitutively expressing rat p67 and its certain mutants were grown in the presence of G418. Cell extracts from control cells expressing EGFP and its fusions of rat p67 or p67 mutants were analyzed for the levels of MARCKS on a Western blot (Fig. 1). Our results show more than 4-fold increase in MARCKS's level in rat p67-expressing cells as compared to control cells (Fig. 1, compare lane 2 with lane 1). This level however reduced to 2.5-3.0 folds when p67's point mutant D251A was expressed (Fig. 1, lane 3). Dramatic decrease near undetectable MARCKS's level in p67's block mutant, D6/2-expressing cells was prominent (Fig. 1, lane 4), while p67's another double block mutant, K1K2, where both of its N-terminal lysine/arginine residue-rich sequences were substituted with neutral amino acids (21), did not show any change of MARCKS level as compared to control cells (Fig. 1, compare lane 5 with lane 1). When a double mutant, D6/2+D251A, where aspartic acid residue at 251 location is substituted with alanine in D6/2 mutant of p67, was constitutively expressed in C2C12 myoblasts, MARCKS level came back to normal like control cells from undetectable level in D6/2 mutant-expressing cells (Fig. 1 compare lane 6 with 1 and 4). Similarly, another compansatory double mutant, D6/2+H331A, where histidine residue at 331 location is substituted with alanine in D6/2 mutant of p67, was constitutively expressed in C2C12 myoblasts, MARCKS level appeared to be 2-3 fold higher as compared to control cells and several folds (>10) higher from undetectable level in D6/2 mutant-expressing cells (Fig. 1, compare lane 6 with 1 and 4). In rat p67-expressing C2C12 cells, in addition to the detection of a doublet running around 25-30 kDa, we have also detected several faster migrating degraded MARCKS protein running close to the full-length MARCKS, which runs around 63 kDa region of the 15% SDS-PAGE (Fig. 1, lane 2). Some of these faster migrating bands started disappearing in D251A-expressing cells (Fig. 1, lane 3), while in D6/2-expressing cells, everything disappeared except the faster migrating dublet (Fig. 1, lane 4). The slower migrating bands as compared the faster migrating doublet were near undetectable in K1K2- and D6/2+D251A-expressing cells (Fig. 1, lanes 5 and 6). In addition to the faster migrating doublet, some of the comparably slower migrating bands were detected in D6/2+H331A-expressing cells (Fig. 1, lane 7). Together, these data suggest that p67 may be invloved in higher level expression of MARCKS and its conserved D251 residue, H331 residue, N-terminal lysine/arginine resdiue-rich sequences, and acidic residue-rich sequences may have role(s) in this upregulated epression of MARCKS. The N-terminal acidic residue-rich sequences possibly play most significant role(s) in this process.

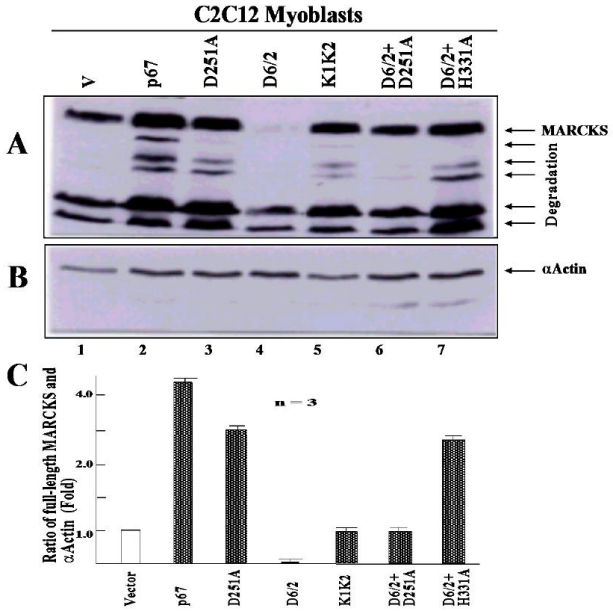

Fig1. ***Levels of MARCKS in C2C12 myoblasts constitutively expressing EGFP vector and its in-frame-fusions of rat p67 and its mutants D251A, D6/2, K1K2, D6/2+D251A, and D6/2+H331A.*** *Stable cell lines constitutively expressing EGFP expresion vector, and its in-frame fusions of rat p67 or its various mutants were generated. Cell lysates from these cell lines were made and the levels of myristoylated alanine-rich C kinase substrate (MARCKS) and αActin were measured on Western blots with polyclonal antibody specific to MARCKS (A) and monoclonal antibody specific to αActin (B). The ratios of full-length MARCKS and αActin were calculated after scanning the corresponding band intensities and the results are plotted in Figure C. Several faster migrating protein bands that were detected by the MARCKS antibodies are labeled as degradation products of MARCKS. The results are from three independent experiments (n=3).*

To examine the level of phosphorylation of MARCKS in the above cell lines, we used the same cell extracts that were used for detecting the total MARCKS's level (Fig. 1) and analyzed on a Wetern blot using a phospho-specific monoclonal antibody for MARCKS (Fig. 2). We detected an intensed doublet band running around 30 kDa but not around the 63 kDa region, where usually the full-length MARCKS runs on SDS-PAGE. The relative intensities of this doublet in control cells, rat p67-expressing cells, and some of p67 mutant-expressing cells did vary significantly except in D6/2+D251A-expressing cell, where the intensity of this doublet is 2.5-3.0 folds lower as compared to the control cells (Fig. 2, see lanes 1-7 and compare lane 6 with lane 1). We also detected very low level of full-length phosphorylated form of MARCKS in p67-expressing cell and this level decreased to about 3-fold in D251A-expressing cells (Fig. 2, see lanes 2 & 3). However, we did not detect this phosphorylated form of MARCKS either in control cells or in C2C12 myoblasts cosntitutively expressing p67 mutants - D6/2, K1K2, D6/2+D251A, and D6/2+H331A (Fig. 2, see lanes 1 and 4-7). Therefore, these results indicate that p67 may be involved in the increased phopshorylation of MARCKS but at a very low level.

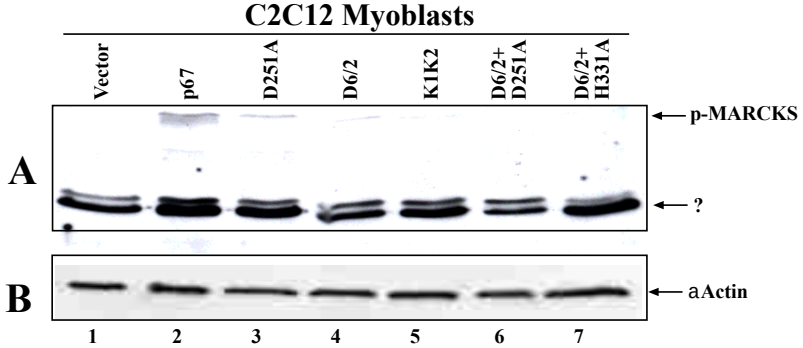

Fig2. ***Phosphorylation levels of MARCKS in C2C12 mouse myoblasts constitutively expressing p67 and its mutants D251A, D6/2, K1K2, D6/2+D251A, and D6/2+H331A.*** *Cell lysates from the cell lines mentioned in legend to Figure 1 were analyzed for the levels of the phosphorylated form of MARCKS on Western blot using phospho-specific antibody to this proteins (A). As a loading control, the levels of αActin were also measured on Western blots using monoclonal antibody specific to αActin (B). Faster migrating protein bands appeared as a doublet and stained heavily with the phospho-specific antibodies of MARCKS are marked as (?).*

We then moved to analyze the levels of total MARCKS (Fig. 3) and its phosphorylated form (Fig. 4) in C2C12 myotubes expressing EGFP or its fusions of rat p67 and its mutants, D251A, D6/2, K1K2, D6/2+D251A, and D6/2+H331A. Total protein samples from these myotubes were used for the analyses on Western blots using polyclonal antibodies specific to MARCKS (Fig. 3) and a monoclonal antibody specific to phosphorylated form of MARCKS (Fig. 4). We found that rat p67-expressing myotubes have similar level of MARCKS like control myotubes (Fig. 3, lanes 1 & 2), its level is 6-7 fold higher in D251A-expressing myotubes (compare lane 3 with lane 2), very similar level in D6/2-expressing myotubes (compare lane 4 with lane 1), around 8-fold higher in K1K2-expressing myotubes (compare lane 5 with lane 1), about 50% increase in D6/2+D251A-expressing myotubes (compare lane 6 with lane 1), and about 14-fold higher in D6/2+H331A-expressing myotubes (compare lane 7 with lane 1). These results suggest that p67 is involed in keeping MARCKS level low in myotubes and its D251 residue, N-terminal lysine/arginine-rich domains I & II, and together N-terminal acidic residue-rich sequences and H331 residue are involved in this process.

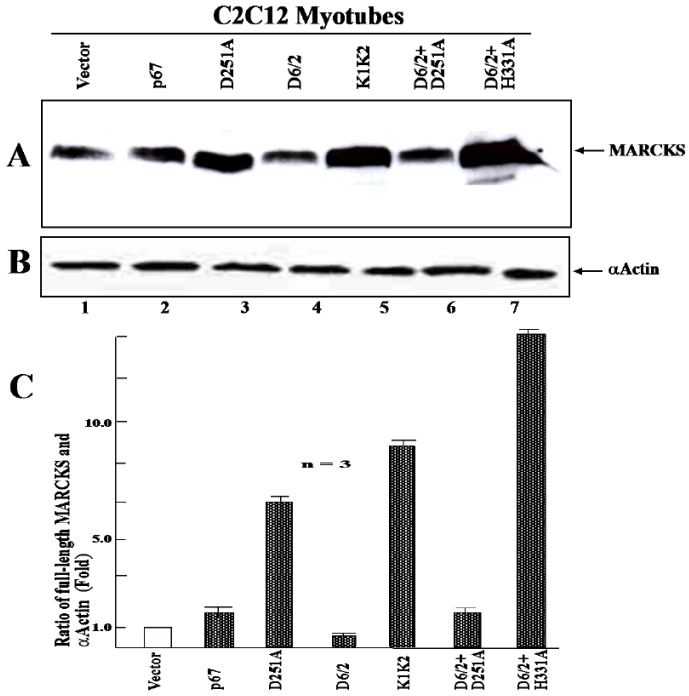

Fig3. ***Levels of MARCKS in C2C12 mouse myotubes constitutively expressing EGFP vector and its in-frame-fusions of rat p67 and its mutants D251A, D6/2, K1K2, D6/2+D251A, and D6/2+H331A.*** *C2C12 myoblasts constitutively expressing EGFP vector or EGFP-fusions of p67, D251A, D6/2, K1K2, D6/2+D251A, and D6/2+H331A mutants were allowed to differentiate into mytubes in differentiation medium for 96 h. Lysates were prepared after harvesting myotubes and analyzed for the levels of total MARCKS on Western blots using antibodies specific to MARCKS (A) and αActin as a loading controls (B). The ratios of full-length MARCKS and αActin were calculated after scanning the corresponding band intensities and the results are plotted (C). The results are from three independent experiments (n=3).*

In terms of the phosphorylated form of full-length MARCKS, we could not detect any in control myotubes, D251A-expressing myotubes, and K1K2-expressing myotubes (Fig. 4, lanes 1, 3, and 5). However, p-MARCKS level was very low in rat p67-expressing and D6/2+D251A- expressing myotubes (lane 2). This level increased to 2.5 fold in D6/2-expressing myotubes (compare lane 4 with lane 2), and about 30-fold in D6/2+H331A-expressing myotubes (compare lane 7 with lane 2 in Fig. 4). In addition to the detection of differential phosphorylation of the full-length MARCKS in myotubes expressing rat p67 or its various mutants mentioned in Figure 4, a highly intensed faster migrating doublet that lighted up with p-MARCKS antibody was also detected in a very high level. This doublet could be the degraded product of the full-length p-MARCKS. Similar doublet was also detected in myoblasts expressing rat p67 and the same set of p67 mutants although, in a low level (Fig. 2). Altogether, our data indicate that p67 has some positive effect on MARCKS phosphorylation, its N-terminal acidic residue-rich sequences has inhibitory effect on this phosphorylation, and together with H331 residue and the acidic residue sequences exihit most significant inhibitory effect.

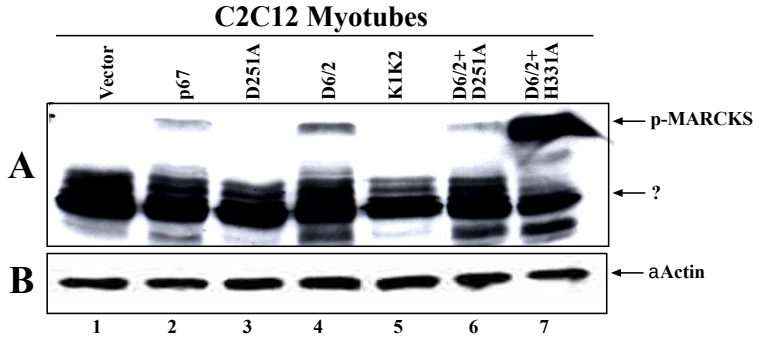

Fig4. ***Phosphorylation levels of MARCKS in C2C12 mouse myotubes constitutively expressing p67 and its mutants D251A, D6/2, K1K2, D6/2+D251A, and D6/2+H331A.*** *Lysates from the myotube lines mentioned in legend to Figure 3 were analyzed for the levels of the phosphorylated form of MARCKS on Western blot using phospho-specific antibody to this proteins (A). As a loading control, the levels of αActin were also measured on Western blots using monoclonal antibody specific to αActin (B). A faster migrating protein band stained heavily with the phospho-specific antibodies of MARCKS is marked as (?).*

In this study, we found that p67 is involved in the upregulated expression of MARCKS and possibly in the maintenance of its low level of phosphorylation in C2C12 myoblasts. P67's N-terminal lysine-rich domains I & II are required for such activity (Figs. 1 & 2). On the other hand, in C2C12 myotubes p67 is involved in the downregulation of MARCKS and its N-terminal lysine-rich domains I & II along with conserved D251 and H331 residues play important roles in this process (Fig. 3). In addition, p67's N-terminal acidic residue-rich sequences and conserved H331 residue but not D251 residue are involved in the suppression of MARCKS's phosphorylation (Fig. 4). Phosphorylation of MARCKS is mediated by PKC and this causes the inhibiiton of actin-binding to MARCKS. This leads to the inhibition of myoblasts' differentiation into myotubes (12, 17). During fusion of myoblasts into myotubes, migration and mobility of myoblasts are extremely important. High levels of MARCKS inhibits such migration and motility of myoblasts (12-14). Therefore, p67 is possibly involved in keeping myoblasts in their cycling state in growth medium by upregulating the level of MARCKS in myoblasts. In differentiation medium however, p67's lysine-rich domains I & II and its D251 and H331 residues are involved in myoblasts' migration and motility to fuse into myotubes. We have also examined the p67's effect on expression of PKC both in myoblasts and myotubes (27). MARCKS is one of the major substrates of PKC and phosphorylated MARCKS has negative role in membrane attachment via myristoyl group (12, 18). Although, there is a 10-fold increase in PKC level in p67-expressing C2C12 myoblasts (27), the level of MARCKS's phosphorylation is very low (Fig. 2), suggesting that p67 may be involved in lowering the phosphorylation of MARCKS in these cells directly or indirectly.

P67 has two stretches of lysine residue-rich sequences known as domain I & domain II (1). Among these domains, lysine-rich domain I plays important role in p67's binding to either eIF2α (9) and ERK1/2 kinases (10). Both lysine-rich domains I & II are invloved in p67's auto-proteolysis (28). The downstream 108-480 amino acid segment of p67 folds to create a shallow groove, where the conserved amino acid residues, D251, D262, H331, E364, and E459 come together to coordinate with the lysine-rich domain II via salt-bridge formation (1). This brings the catalytic H231 residue in close proximity to cleave after the arginine107 and this auto-proteolysis generates several fragments - the most stable p26 segment contains the N-terminal 1-107 amino acid residues, and p52, which contains downstream 108-480 amino acid segment of p67 (28). This p52 segment also shows auto-proteolysis (28) and possibly it may have intermolecular proteolytic activity that can cleave other proteins. This p52 segment of p67 creates the shallow groove, where other target proteins may fit in and have the ability to form salt-bridge with its conserved mainly with acidic residues mentioned above. This coordination will bring the targets close to p52's catalytic site H231 residue and get proteolytically degraded. MARCKS along with a group of proteins such as the cortexillin family of proteins that include cortexillin I & II, Gelsolin, Villin, and gCap39 share a common C-terminal nonapeptide sequence containing nine lysine residues. Our examination of the C-terminal amino acid sequence of MARCKS show $_{151}$PKKKKKRFSFKKSFKLSGPSFKK$_{173}$, which contains the stretch of lysine-rich sequence (12).

In rat p67-expressing myoblasts, MARCKS is degraded and p67's lysine residue-rich domain I & II are possibly involved in this degradation. This is due to the fact that p67's acidic residue-rich domain

is closely associated with these two domains through salt-bridge formation (7). Exposing these basic domains by substituting acidic residues to neutral amino acids in its N-terminal acidic residue-rich domain, resulted in a much higher level of MARCKS degradation and this is inhibited when both lysine residue-rich sequences in domains I & II were substituted with neutral amino acids (Fig. 1). In addition, p67's conserved D251 residue is possbily invloved in coordination with the basic lysine-rich domains I & II at its substrate-binding pocket because alanine substitution of this residue reversed MARCKS's significant degradation (Fig. 1). Together, when lysine-rich domains I & II are binding to p67's substrate-binding pocket, it generates p52 segment through its auto-proteolysis. This p52 segement is possibly involved in the degradation of MARCKS in C2C12 myoblasts either directly or inderectly. The lysine-rich sequences at the C-terminal of MARCKS (12) possibly binds at the substrate-binding pocket of p52 and causes its degradation directly. During myogenesis, MARCKS's degradation is essential and Ca^{2+}-dependent proteinase, calpain has been shown to be involved in this process (13-14). In this study, we show that p67 is also involved in the degradation MARCKS in C2C12 myoblasts suggesting p67's roles in actin dynamics and migration of C2C12 myoblasts during myogenesis.

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