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Subtilisin-like Proprotein Convertase PACE4 is Required for Skeletal Muscle Differentiation

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Most growth factors stimulate myoblast proliferation and prevent differentiation, whereas insulin-like growth factors (IGFs) promote myoblast differentiation through the phosphatidylinositol 3-kinase (PI3K) pathway. Subtilisin-like proprotein convertases (SPCs) are involved in cell growth and differentiation via activation of pro-growth factors. However, the role of SPCs in myogenesis remains poorly understood. Here we show that PACE4, a member of the SPC family, plays a critical role in myogenic differentiation of C2C12 cells. PACE4 mRNA levels increased markedly during myogenesis, whereas the expression of other member of SPC family, furin and PC6, remained unchanged. The expression pattern of pro-IGF-II, which is processed extracellularly by SPCs, was similar to that of PACE4. The expression of shRNA targeting PACE4, but not furin, suppressed the expression of the muscle-specific myosin light chain (MLC). Interestingly, reduced expression of MLC was restored following treatment with recombinant mature IGF-II. Finally, we demonstrated that the PI3K inhibitor LY294002 blocked the induction of PACE4 mRNA, a result not observed when another myogenic differentiation inhibitor, SB203580 (p38 MAP kinase inhibitor), was employed, indicating the presence of a positive feedback loop regulating PACE4 expression. These results suggest that PACE4 plays an important role in myogenic differentiation through its association with the IGF-II pathway.

Key words: insulin-like growth factor (IGF), myogenic differentiation, PACE4, processing, subtilisin-like proprotein convertase (SPC).

Abbreviations: α1-AT, α1-antitrypsin; α1-PDX, α1-antitrypsin variant Portland; bHLH, basic helix-loop-helix; dec-RVKR-CMK, decanoyl-Arg-Val-Lys-Arg-chloromethylketone; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; FoxO, forkhead box protein O; GFP, green fluorescent protein; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; IGFBP, insulin-like growth factor-binding protein; IGF(s), insulin-like growth factor(s); MAP kinase, mitogen-activated protein kinase; MHC, myosin heavy chain; MLC, myosin light chain; MMP11, matrix metalloproteinase 11; PI3K, phosphatidylinositol 3-kinase; RT-PCR, reverse transcriptase-PCR; SEAP, secreted alkaline phosphatase; shRNA, short hairpin RNA; SPC(s), subtilisin-like proprotein convertase(s); TGF-β, transforming growth factor β.

The development of skeletal muscle is a multistep process that involves the determination of pluripotent mesodermal cells to give rise to myoblasts, the withdrawal of myoblasts from the cell cycle and differentiation into muscle cells, and finally the growth and maturation of skeletal muscle fibers. Several growth factors such as basic fibroblast growth factor 2 and transforming growth factor β1 (TGF-β1) can stimulate myoblast proliferation and prevent differentiation (*1*). On the other hand, insulin-like growth factors (IGF-I and IGF-II) can promote muscle differentiation. Many reports have documented that the IGF signalling pathway requiring the phosphatidylinositol 3-kinase (PI3K)-Akt and mitogen-activated protein kinase (MAP kinase) pathways plays an important role during skeletal muscle differentiation (*2*, *3*). However, the mechanism pertaining to the post-transcriptional regulation of IGF-II in myoblasts remains poorly understood.

Some peptide growth factors including IGFs are activated by subtilisin-like proprotein convertases (SPCs), which cleave precursors of polypeptides at single or paired basic residues. In mammals, seven members of the SPC family (furin, PC1/3, PC2, PACE4, PC4, PC5/6 and PC7/8/LPC) have been identified (*4*). SPCs share a conserved N-terminal domain in the same order (a signal peptide, and prodomain, catalytic and HomoB domains), but differ in their C-terminus. These convertases vary in terms of tissue distribution and organelle localization. PC1 and PC2 are located in secretory granules of endocrine cells, and play an important role in the maturation of propeptide hormones. PC4 is expressed in reproductive tissues, but little is known about its subcellular localization. Furin and PC8 are localized in the *trans*-Golgi network in the form of a membrane-bound enzyme, and are expressed ubiquitously. Both PACE4 and PC6 are

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secreted enzymes, and are localized at the extracellular matrix through binding to heparan sulphate proteoglycans (*5*). The expression of PACE4 is highly regulated during cell differentiation, which differs from that of furin (*6–8*). The 5′-flanking region of the human *PACE4* gene contains several E-box elements, which are the binding sites for members of the basic helix–loop–helix (bHLH) transcription factor. Transcription of the *PACE4* gene seems to be regulated via these E-box elements. On the other hand, the PI3K signaling pathway has been shown to regulate PACE4 transcription in human megakaryoblastic Dami cells (*9*).

Possible substrate candidates for SPCs, such as IGFs, ADAM12 and myostatin, are known to control myogenic differentiation. ADAM12 has been shown to be required for the fusion of C2C12 myoblasts into multinucleated myotubes (*10*). Myostatin, a TGF-β superfamily member, is transcriptionally regulated by MyoD via E-box sites in its promoter, and acts as a negative regulator of muscle growth (*11*, *12*). A more recent study reported that myostatin is localized at the extracellular space as pro-myostatin, where extracellular SPCs are involved in its processing (*13*). Furthermore, membrane-type matrix metalloproteinase-1 (MT1-MMP), which requires SPCs for its activation, also controls myotube formation (*14*). However, the precise role of SPC family members in skeletal muscle differentiation remains poorly understood.

In this study, we initially investigated the role of SPCs in myogenic differentiation. The mouse skeletal muscle cell line C2C12 is well characterized and commonly used as a model in investigations concerning the cellular and molecular mechanisms involved in muscle differentiation. Two SPC inhibitors, decanoyl-Arg-Val-Lys-Arg-chloromethylketone (dec-RVKR-CMK) and α1-anti-trypsin variant Portland (α1-PDX), blocked the differentiation of C2C12 myoblasts. During differentiation, only PACE4 mRNA transcript levels increased markedly of all the SPC family members examined. It was further shown using RNA interference that PACE4 is a key factor in the differentiation of C2C12 myoblasts, and that IGF-II rescued the suppression of myogenic differentiation following PACE4 knockdown. These results suggested that PACE4 plays an important role in myogenic differentiation through activation of IGF signalling.

MATERIALS AND METHODS

Materials—The SPC inhibitor dec-RVKR-CMK, PI3K inhibitor LY294002 and p38 MAP kinase inhibitor SB203580 were obtained from Calbiochem. Recombinant mouse IGF-II was purchased from PeproTech. All other reagents used were of the highest grade available.

Construction of Plasmid—A full-length cDNA encoding mouse MyoD was obtained by PCR using C2C12 cell cDNA and a PCR primer set (5′-GGATATGGAGCTTCT ATCG-3′ and 5′-CTGCTGCTGCAGTCGATCTC-3′). The amplified product was cloned into TA-cloning vector pCR2.1-TOPO (Invitrogen), yielding pCR2.1-mMyoD, and the fidelity of the inserted cDNA sequence was confirmed by DNA sequencing. The *Eco*RV and *Bam*HI fragment of pCR2.1-mMyoD was subcloned into the mammalian expression vector pFLAG-CMV2 (Sigma), generating pFLAG-mMyoD. Rat wild-type α1-antitrypsin (α1-AT) ($AVPM^{352}$, P1 site is Met^{352}) and α1-PDX ($RVPR^{352}$) were subcloned into the pcDNA3 vector (Invitrogen) as previously described (*15*).

For the stable expression of short-hairpin RNA (shRNA) utilized for the targeting of PACE4, 5′-gatccG CTTTCGAGTATGGCATTAAAttcaagagaTTTAATGCCAT ACTCGAAAGCttttttggaaa-3′ and 5′-agcttttccaaaaaaGCT TTCGAGTATGGCATTAAAtctcttgaaTTTAATGCCATACT CGAAAGCG-3′ [mouse PACE4; accession number D50060 (nucleotide number 901–921)] and 5′-gatccGCG AAGTGACTCTCTTTATTttcaagagaAATAAAGAGAGTCA CTTCGttttttggaaa-3′ and 5′-agcttttccaaaaaaCGAAGTG ACTCTCTTTATTtctcttgaaAATAAAGAGAGTCACTTCG CG-3′ (nucleotide number 388–407) were annealed and ligated into the *Bam*HI and *Hind*III sites of pSilencer 3.1-neo (Ambion), generating pSilencer-neo-mPACE4 Site 1 and Site 2, respectively. Similarly, the shRNA expression plasmid utilized for the targeting of furin was produced using the following oligonucleotides: 5′-gatccGACCAAGTTCACTCTCGTTCTGttcaagagaCAGA ACGAGAGTGAACTTGGTCttttttggaaa-3′ and 5′-agcttttcc aaaaaaGACCAAGTTCACTCTCGTTCTGtctcttgaaCAGAA CGAGAGTGAACTTGGTCG-3′. The target sequence chosen was one containing no sequence similarity with other SPC family members and low sequence homology with non-target mouse sequences.

Cell Culture, Transfection and Construction of Stable Cell Lines—Mouse C2C12 skeletal myoblasts and mouse C3H10T1/2 embryonic fibroblasts (RIKEN CELL BANK, Tsukuba, Japan) were routinely maintained in a growth medium consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C under 5% CO_2.

To express the various cDNA constructs, C2C12 cells were transfected using Lipofectamine 2000 as outlined by the manufacturer's protocol (Invitrogen). The transfectants were then selected using medium containing 400 μg/ml G418 for 2 weeks. Non-transfected control cells in 400 μg/ml G418 failed to survive after 2 weeks. Polyclonal stable transfectants were employed to avoid potential artifacts associated with the selection of individual clones from single transfected cells.

To introduce myogenesis, C2C12 cells were grown to confluence in medium containing 10% FBS and then the medium was replaced with 2% horse serum in DMEM (differentiation medium) without or with inhibitor and/or IGF-II.

RNA Extraction and RT–PCR Analysis—Total RNA was isolated using ISOGEN solution (Nippon Gene, Japan) according to the manufacturer's protocol. One microgram of each total RNA sample was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase using a random hexamer. PCR was carried out through 25–35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, followed by extension at 72°C for 1 min with specific primers (Table 1). PCR products were subjected to 1.5% agarose gel electrophoresis and

Table 1. **Primer sequences used for RT–PCR and expected product size.**

Gene	Primer sequence	Product size (bp)
PACE4A	Fw: 5′-CCCTCTGGAACCAAGTCTCAACTT-3′	414
	Rv: 5′-ACCACACTCCGGATGGCACACACT-3′	
Furin	Fw: 5′-TATGGCTACGGGCTGTTGGA-3′	398
	Rv: 5′-CTCGCTGGTATTTTCAATCTCT-3′	
PC6	Fw: 5′-CTGCTGGTTTTAAGGTGAGCCA-3′	403
	Rv: 5′-TCCCCAGCAGCCCGTTCTCC-3′	
Myogenin	Fw: 5′-CACCTGATGGAGCTGTATGAG-3′	750
	Rv: 5′-CTGCTACAGAAGTGATGGCTT-3′	
pro-IGF-II	Fw: 5′-CCAATGGGGATCCCAGTGGGGA-3′	546
	Rv: 5′-TCACTGATGGTTGCTGGACATC-3′	
MLC	Fw: 5′-ATGGCACCCAAGAAGGCCAA-3′	510
	Rv: 5′-CTATTCCTGGTCCTTAGCATC-3′	
MMP11	Fw: 5′-GCACTGCTGGAGCCGGAAAC-3′	550
	Rv: 5′-GGACCTTCACCTTCACGGCA-3′	
G3PDH	Fw: 5′-GGTGAAGGTCGGAGTCAACGGATTTGG-3′	985
	Rv: 5′-CATGTGGGCCATGAGGTCCACCAC-3′	

DNA fragments were detected using ethidium bromide staining.

Quantitative Real-time RT–PCR—Real-time PCR was conducted using an ABI Prism 7000 Sequence Detection System (Applied Biosystems) with the Power SYBR Green PCR Master Mix (Applied Biosystems) as previously described (*16*). The following primers were used: myosin heavy chain (MHC), sense 5′-GCCAGGATGGGA AAGTCACTGTGG-3′ and antisense 5′-GGGCTCGTTCA GGTGGGTCAGC-3′; and glyceraldehyde-3-phosphate dehydrogenase (G3PDH), sense 5′-GTGTCCGTCGTGGA TCTGA-3′ and antisense 5′-CCTGCTTCACCACCTTCT TG-3′.

Real-time PCR conditions comprised initial heating at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. MHC mRNA transcript levels were normalized with respect to G3PDH mRNA transcript levels. Specific transcripts were confirmed by melting curve analysis at the end of each PCR, and the specificity of the PCR was further verified by subjecting the amplification products to agarose gel electrophoresis.

Secreted Alkaline Phosphatase assay—Secreted alkaline phosphatase (SEAP) assay was performed as previously described (*16*). C2C12 cells stably transfected with either pSEAP/neo-hPACE4p -649/-1 or pSEAP/neo-basic, were cultured in 35 mm dishes containing phenol red-deficient DMEM with 10% FBS at 2×10^5 cells/dish. After 24 h, the medium was replaced with phenol red-free DMEM containing 2% horse serum (day 0). At the indicated days, the conditioned medium was collected for the SEAP assay. The supernatant of the medium was incubated for 30 min at 65°C to inactivate endogenous alkaline phosphatase activity derived from the serum. The sample was then incubated in duplicate at 37°C in 1 M diethanolamine (pH 9.8), 1 mM $MgCl_2$, 10 mM L-homoarginine and 12 mM *p*-nitrophenyl phosphate. The absorbance at 405 nm was then determined using a microtitre plate reader.

Immunocytochemistry—C2C12 cells were plated onto glass coverslips coated with poly-L-lysine in 35-mm tissue culture dishes at 1×10^5 cells/dish in growth medium. After 24 h, the culture medium was replaced with differentiation medium. At the indicated time points, the cells were washed in PBS, fixed in 4% paraformaldehyde for 20 min at room temperature, and then permeabilized using 0.1% Triton X-100 for 5 min at room temperature. Cells were then incubated in PBS containing 5% BSA for 30 min to decrease nonspecific binding. Following this, cells were incubated with mouse monoclonal antibody directed against skeletal MHC (MY-32, Sigma), at 1:200 dilution in PBS containing 5% BSA for 2 h at room temperature and subsequently washed three times with PBS. The primary antibody was visualized using FITC-conjugated anti-mouse IgG (Vector Laboratories) at 1:200 dilution in PBS containing 5% BSA for 2 h in the dark at room temperature. Cells were then washed three times with PBS. Coverslips were mounted on glass slides using VECTASHIELD (Vector Laboratories). MHC-positive cells were detected using a confocal laser-scanning microscope (Radiance; Bio-Rad Laboratories).

RESULTS

Effect of SPC-specific Inhibitors During Skeletal Muscle Differentiation—In an effort to investigate the possible involvement of SPC family members in skeletal muscle differentiation, C2C12 cells were treated with the SPC family-specific inhibitor dec-RVKR-CMK during differentiation. After 6 days of differentiation, cells were fixed and examined by immunofluorescence microscopy following treatment with anti-MHC antibody. As shown in Fig. 1A, many elongated myotube-like cells were detected in the absence of dec-RVKR-CMK. The immunofluorescence analysis showed that these elongated cells were MHC-positive and contained multiple nuclei. In contrast, myotube formation was distinctly blocked when dec-RVKR-CMK was added at 50 μM to cells cultured in the differentiation medium (Fig. 1A, +CMK). Additionally, treatment with this inhibitor resulted in marked reduction of MHC protein expression. Furthermore, real-time PCR analysis demonstrated that

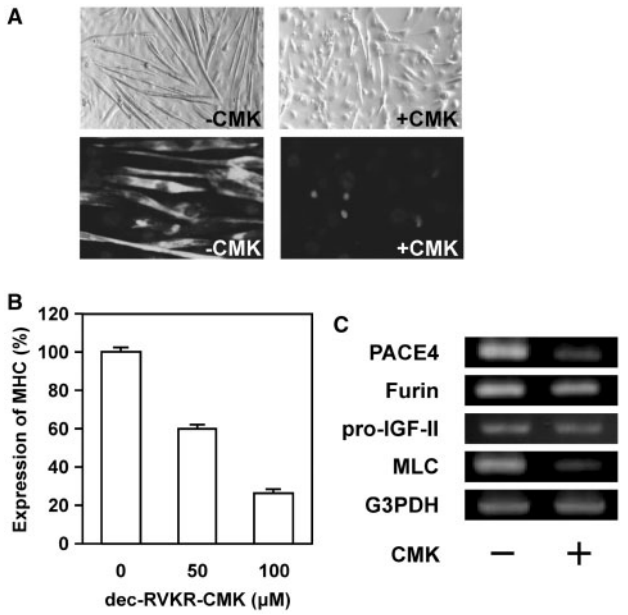

Fig. 1. **Effect of SPC-specific inhibitor dec-RVKR-CMK during skeletal muscle differentiation.** (A) C2C12 cells were cultured in medium containing 10% FBS until confluent. To initiate myogenesis, the medium was replaced with differentiation medium containing 2% horse serum in the absence (−*CMK*) or presence (+*CMK*) of 50 μM dec-RVKR-CMK. After 6 days, cells were fixed and subjected to immunofluorescence analysis using anti-MHC antibody. (B and C) Confluent C2C12 cells were treated with different concentrations of dec-RVKR-CMK in differentiation medium for 6 days. Total RNA was isolated and analysed using SYBR Green-based real-time PCR (B) and conventional RT–PCR (C). In the real-time PCR analysis, MHC expression is shown as the ratio of MHC/G3PDH and the value in untreated cells is shown as 100%. The data are shown as the means ± SD of three independent experiments.

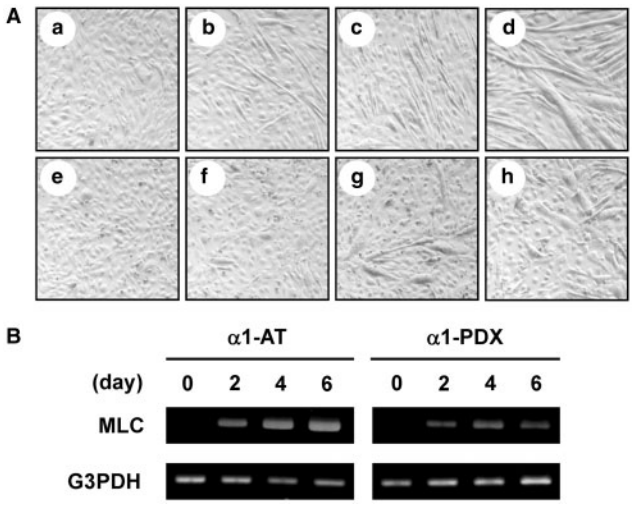

Fig. 2. **Inhibition of skeletal muscle differentiation by α1-PDX, an engineered derivative of α1-AT.** C2C12 cells were transfected with either pcDNA3-α1-AT or pcDNA3-α1-PDX, and then selected using medium containing 400 μg/ml G418. G418-resistant cells that had reached confluence were induced to differentiate using differentiation medium. (A) Changes in cell morphology in α1-AT-expressing cells (a–d) and α1-PDX-expressing cells (e–h) were observed by light microscopy at day 0 (a and e), 2 (b and f), 4 (c and g), 6 (d and h) following induction of myogenic differentiation. (B) Total RNA was isolated from the respective cells at the indicated times and analysed by RT–PCR.

dec-RVKR-CMK decreased the amount of MHC mRNA transcripts in a dose-dependent manner (Fig. 1B). As shown in Fig. 1C, the inhibitor reduced the level of MLC and PACE4 mRNA transcripts, although the level of furin mRNA transcripts remained unchanged, suggesting that PACE4 expression is regulated through a positive feedback loop. These results revealed that SPC activity was required for myogenic differentiation in C2C12 cells.

To confirm the importance of SPC in myogenic differentiation, we examined the effect of α1-PDX, an SPC specific inhibitor of the α1-AT variant (*17*). α1-PDX contains a minimal consensus sequence (Arg-X-X-Arg) in its reactive site loop which is efficiently cleaved by SPCs and inhibits furin, PACE4 and PC6 (*15*). C2C12 cells were transfected with either pcDNA3-α1-PDX or pcDNA3-α1-AT, and transfected cells were selected in medium containing G418 for 2 weeks. Polyclonal G418-resistant cells that had reached confluence were induced to differentiate. The changes in cellular morphology during myogenic differentiation are shown in Fig. 2A. Many fused myotube-like cells were observed in wild-type α1-AT-expressing cells, just as in the parent C2C12 cells. On the other hand, α1-PDX effectively blocked myotube formation despite the similar culture conditions. Furthermore, the expression patterns of MLC transcripts during the differentiation of both cells were examined by RT–PCR analysis. MLC mRNA transcript levels in α1-AT-expressing cells gradually increased during myogenic differentiation, whereas the induction of MLC was diminished in α1-PDX-expressing cells (Fig. 2B). Thus, SPC inhibitors were able to block the myogenic differentiation of C2C12 cells, supporting the view that SPCs are crucial for myogenesis.

The Expression Patterns of SPC Family Members During Myogenic Differentiation of C2C12 Cells—In an effort to determine which SPC family members are affected during myogenic differentiation, the expression patterns of SPC family members (furin, PACE4 and PC6) were examined by RT–PCR analysis. As shown in Fig. 3A, the gene expression of myogenin and MLC, which are skeletal muscle-specific markers, increased during differentiation, in agreement with previous reports (*18*). The level of PACE4 mRNA transcripts gradually increased during culturing in differentiation medium and reached a maximum at 48 h. On the other hand, the levels of furin and PC6 mRNA transcripts remained relatively unchanged. Furthermore, the expression patterns of pro-IGF-II and matrix metalloproteinase 11 (MMP11), which are considered as target candidates for PACE4 (*19*, *20*), were simultaneously assessed by RT-PCR analysis. Both pro-IGF-II and MMP11 expression progressively increased during differentiation. Interestingly, treatment with dec-RVKR-CMK led to a reduction in MLC mRNA transcript levels, but had no effect on pro-IGF-II mRNA transcript

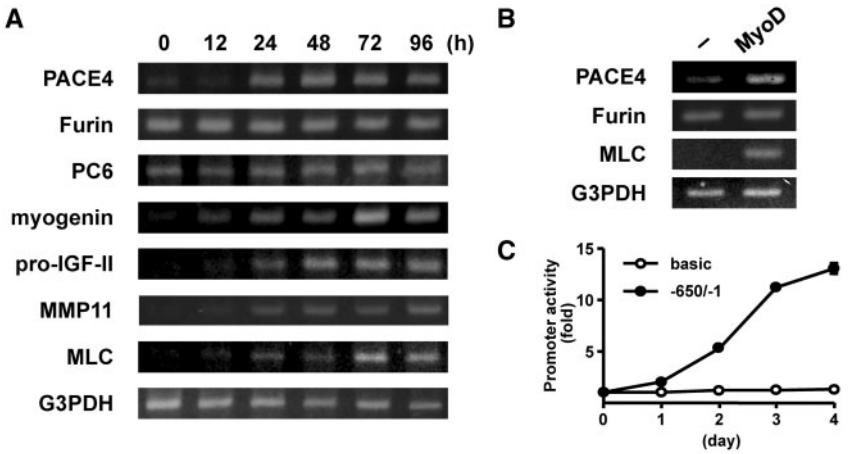

Fig. 3. **Expression patterns of SPC mRNA during myogenic differentiation.** (A) C2C12 cells were induced to differentiate into myotubes by incubating cultures in differentiation medium. At the indicated times, total RNA was isolated from C2C12 cells and analyzed by RT–PCR. G3PDH was amplified as an internal control. (B) Mouse fibroblast C3H10T1/2 cells transiently transfected with pFLAG-MyoD (*MyoD*) and pFLAG empty vector (–) were induced to differentiate in differentiation medium for 48 h. Total RNA was isolated and analysed by RT–RCR. (C) The 5′-flanking region (−1 to −649) of the human *PACE4* gene was ligated into the SEAP reporter vector pSEAP-neo-basic (*basic*), yielding pSEAP-neo-hPACE4 −649/−1 (*−649/−1*), and the construct was stably transfected into C2C12 cells. Cells were grown to confluence, and then placed in phenol red-free differentiation medium to induce differentiation. The conditioned medium was collected every 24 h and used for the SEAP assay. The SEAP activity was expressed relative to the SEAP activity of the conditioned medium of cells stably transfected with pSEAP-neo-basic at day 0. The data are the means ± SD of three separate determinations.

levels (Fig. 1C). Using another model of myogenic differentiation, the expression pattern of PACE4 during myogenic differentiation was analysed. Mouse pluripotent C3H10T1/2 cells were transiently transfected with a plasmid expressing MyoD. As shown in Fig. 3B, the forced expression of MyoD in C3H10T1/2 cells resulted in marked induction of MLC expression, consistent with previous reports (*18*). These cells transfected with MyoD also induced the expression of PACE4 mRNA transcripts, but not of furin mRNA. These results revealed that transcriptional expression of PACE4 was highly regulated during myogenic differentiation.

Analysis of PACE4 Promoter Activity During Skeletal Muscle Differentiation—Changes in promoter activity of the *PACE4* gene during myogenic differentiation were investigated. The 5′-flanking region (from −649 to −1) of the human *PACE4* gene was ligated into the SEAP reporter plasmid (pSEAP-basic), yielding pSEAP −649/−1 (*16*). To facilitate selection of stable transfectants, the neomycin resistance gene cassette was introduced into pSEAP-hPACE4p −649/−1 to generate pSEAP/neo-hPACE4p −649/−1. C2C12 cells were transfected with either pSEAP/neo-hPACE4p −649/−1 or pSEAP/neo-basic, and stably transfected cells were selected in medium containing G418. Transfected cells showed no morphological differences compared with parent cells (data not shown). The medium was changed to differentiation medium (day 0), and the conditioned medium was collected every 24 h for the SEAP reporter assay. The SEAP activity in the conditioned medium of these cells reflected promoter activity of the *PACE4* gene. In the conditioned medium of C2C12 cells stably transfected with pSEAP/neo-hPACE4p −649/−1, the SEAP activity at day 3 and 4 was ca. 10-fold higher compared with that of myoblasts (day 0) (Fig. 3C). On the other hand, the SEAP activity of pSEAP/neo-basic-transfected cells displayed no change under the same conditions. These observations revealed that the *PACE4* gene was upregulated during myogenic differentiation.

Effect of PACE4 Knockdown on Myogenic Differentiation—In an effort to examine the functional role of PACE4 during myogenic differentiation, an RNA interference approach was employed. Two different PACE4-specific oligonucleotides for RNA interference (Site 1 and Site 2) were designed and ligated into the shRNA expression vector pSilencer 3.1, yielding pSilencer-neo-mPACE4 Site 1 and Site 2, respectively. As a negative control, pSilencer-neo-GFP which targets the green fluorescent protein (GFP) mRNA was used. C2C12 cells were transfected with pSilencer-neo-mPACE4 Site 1, pSilencer-neo-mPACE4 Site 2 or pSilencer-neo-GFP, and selected in medium containing 400 μg/ml G418. G418-resistant cells that had reached confluence were induced to differentiate. After 6 days, total RNA was isolated from the respective cells and analysed by RT–PCR. In GFP shRNA-expressing C2C12 cells, PACE4 mRNA transcript levels increased as myogenic differentiation progressed, just as with the parent cell line (Fig. 4A). On the other hand, both shRNAs targeting different regions of the *PACE4* gene effectively blocked the expression of PACE4 mRNA, but had no effect on furin mRNA transcript levels, indicating that these shRNAs were specific for the *PACE4* gene. The expression of MLC mRNA transcript was markedly reduced in both PACE4 knockdown cells compared with GFP shRNA-expressing cells. The reduction in MLC mRNA transcript levels by Site 2 was slightly greater than that observed by Site 1. Furthermore, we established furin knockdown cells in the same manner, and examined the role of furin in myogenesis.

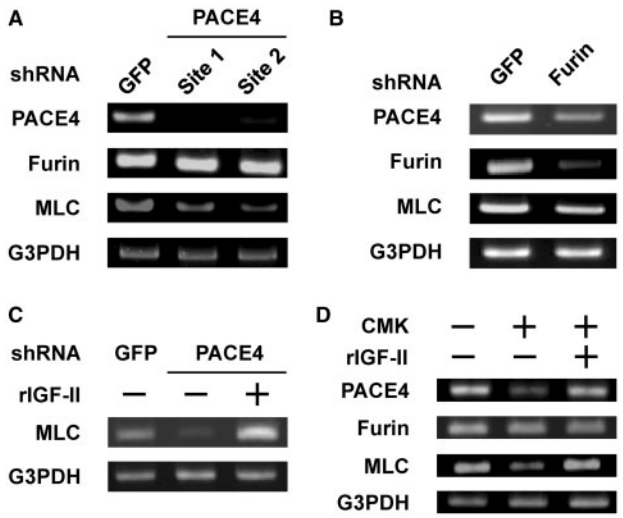

Fig. 4. **Effect of PACE4 knockdown during myogenic differentiation.** (A) Two different oligonucleotides corresponding to the *PACE4* gene were designed and ligated into the shRNA expression vector pSilencer 3.1, yielding pSilencer-neo-mPACE4 Site 1 and pSilencer-neo-mPACE4 Site 2, respectively. C2C12 cells were transfected with pSilencer-neo-mPACE4 Site 1, pSilencer-neo-mPACE4 Site 2 or pSilencer-neo-GFP and then selected using medium containing 400 μg/ml G418. Stably transfected cells that had reached confluence were placed in differentiation medium. After 6 days, total RNA was isolated from the cells and analysed by RT–PCR. (B) Furin-knockdown C2C12 cells were induced to differentiate using differentiation medium. At day 8, total RNA was isolated and analyzed by RT–PCR. (C) PACE4 shRNA-expressing C2C12 cells were grown to confluence and cultured in differentiation medium in the absence or presence of 30 nM recombinant mature IGF-II (*rIGF-II*). After 6 days, total RNA was isolated and analysed by RT–PCR. (D) Confluent C2C12 cells were placed in differentiation medium in the absence or presence of 50 μM dec-RVKR-CMK (*CMK*), in combination with 30 nM mature IGF-II. After 6 days, total RNA was isolated and analysed by RT–PCR.

RT–PCR analysis confirmed that furin mRNA transcript levels were reduced in furin shRNA-expressing cells (Fig. 4B). However, furin knockdown did not affect the level of MLC mRNA transcripts. These findings showed that the SPC family member PACE4 plays a key role in skeletal muscle differentiation.

Effect of Recombinant Mature IGF-II in PACE4 Knockdown Cells—Previous reports have suggested that the processing of pro-IGF-II appears to occur very late in the secretory pathway or in the extracellular space (*21*). In fact, pro-IGF-II protein has been detected in human serum and in the conditioned media from some cell lines (*22*, *23*). Given that PACE4 efficiently processes pro-IGF-II in the 293 expression system (*20*), pro-IGF-II is a strong candidate as a PACE4 substrate. In an effort to determine whether PACE4 functions in myogenesis via the processing of pro-IGF-II, a rescue experiment using C2C12 cells stably expressing PACE4 Site 2 shRNA and recombinant mature IGF-II was performed. Stably transfected cells were induced to myogenic differentiation in the absence or presence of 30 nM recombinant mature IGF-II. After 6 days, total RNA was isolated and analysed by RT–PCR. As shown in Fig. 4C, the reduced

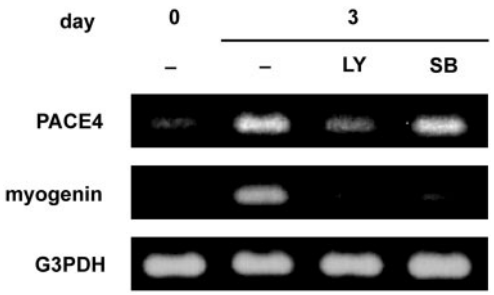

Fig. 5. **Expression of PACE4 transcripts through the PI3K pathway during myogenic differentiation.** C2C12 cells that had reached confluence were treated with the PI3K inhibitor LY294002 (*LY*) or the p38 MAPK inhibitor SB203580 (*SB*) at 10 μM in differentiation medium. After 3 days, total RNA was isolated from the C2C12 cells and analysed by RT–PCR.

level of MLC mRNA transcripts by PACE4 knockdown was restored following treatment with exogenous mature IGF-II. Additionally, a similar rescuing effect of mature IGF-II was observed with the diminished expression of MLC transcripts in C2C12 cells treated with dec-RVKR-CMK (Fig. 4D). The transcriptional level of PACE4 was also restored following treatment with mature IGF-II, indicating that PACE4 expression is regulated via a positive feedback loop during myogenic differentiation.

Up-regulation of PACE4 Transcripts was Mediated Through the PI3 Kinase Pathway—IGF activates both PI3K and p38 MAP kinase, and their respective signalling pathways are required for skeletal muscle differentiation (*2*, *3*). Finally, in an effort to investigate which pathway is involved in a positive feedback loop regulating PACE4 expression during myogenesis, the two inhibitors LY294002 (a PI3K inhibitor) and SB203580 (a p38 MAP kinase inhibitor) were employed. As shown in Fig. 5, inhibition of PI3K activity resulted in inhibition of the upregulation of PACE4 and myogenin mRNA. On the other hand, inhibition of p38 MAP kinase resulted in no changes in PACE4 mRNA transcript levels, although elevation of myogenin mRNA transcript levels was blocked. Thus, transcriptional expression of PACE4 appears to be positively regulated by the PI3K signalling pathway.

DISCUSSION

This study demonstrated that PACE4 plays a pivotal role in the myogenic differentiation of C2C12 cells. During myogenic differentiation, of all SPC family members, the transcript levels of only PACE4 increased markedly. The 5′-flanking region of the human *PACE4* gene contains 12 E-box elements within 1 kb upstream of the transcription initiation site (*6*). The E-box is a specific binding sequence for the bHLH transcription factor. Previous data revealed that bHLH transcription factors Hash1 and Hash2 negatively regulate expression of the *PACE4* gene in neurons and placenta, respectively (*7*, *8*). In skeletal muscle, many bHLH transcription factors, such as MyoD and myogenin, have been identified. We showed that in MyoD-expressing C3H10T1/2 cells,

PACE4 mRNA transcript levels were elevated during the course of differentiation. However, overexpression of MyoD and myogenin in C2C12 cells had no effect on promoter activity of the *PACE4* gene (data not shown). Many bHLH transcription factors other than MyoD and myogenin have been identified in skeletal muscle, and have been shown to be controlled by MyoD during myogenic differentiation. Taken together, MyoD may indirectly control the transcriptional regulation of PACE4 via other bHLH transcription factors.

On the other hand, elevation of PACE4 mRNA transcript levels during myogenic differentiation was blocked by use of a PI3K inhibitor, but not by a p38 MAP kinase inhibitor. Furthermore, reduced expression level of PACE4 was restored by the addition of active IGF-II, indicating that a positive feedback loop controls transcription of the *PACE4* gene. A previous report showed that platelet-derived growth factor-induced PACE4 expression is also blocked by the two PI3K inhibitors LY294002 and wortmannin, but not by an MAP kinase inhibitor, in human megakaryoblastic Dami cells (*9*). The PI3K signalling pathway has been shown to be an important during skeletal muscle differentiation (*2*, *3*). IGFs promote myoblast differentiation through the PI3K-Akt signalling pathway. Studies have demonstrated that the forkhead transcription factor forkhead box protein O1 (FoxO1) is a strong candidate as a substrate for Akt in skeletal muscle (*24*, *25*). Phosphorylated FoxO1 translocates from the nucleus to the cytoplasm, where it is inactivated (*26*). Furthermore, a transcriptionally inactive FoxO1 mutant partially rescued the inhibition of myogenic differentiation induced by a PI3K inhibitor, indicating that FoxO negatively regulates skeletal myocyte differentiation (*24*). Since FoxO1 is shown to inhibit IGF-II expression at the transcription level (*25*), the transcriptional regulation of the *PACE4* gene may also be controlled by FoxO. Further studies are required to identify the transcription factors responsible for the regulation of PACE4 expression in skeletal muscle.

Use of dec-RVKR-CMK, a specific inhibitor of SPC family members, caused a delay in myotube formation of C2C12 cells. Interestingly, although dec-RVKR-CMK blocked the expression of MLC and MHC, the inhibitor had no effect on pro-IGF-II expression, suggesting that SPC family is involved in myogenesis via the processing of pro-IGF-II but not its transcription. Furthermore, we demonstrated that recombinant mature IGF-II reversed the inhibition of C2C12 differentiation induced by dec-RVKR-CMK and PACE4 knockdown. Taken together with a previous study which showed that PACE4 efficiently mediates the processing of pro-IGF-II (*20*), these observations suggest that the processing of pro-IGF-II by PACE4 after the induction of myogenesis accelerates the progression of myogenic differentiation. To determine whether pro-IGF-II is a substrate for PACE4 during myogenesis, we examined the influence on the processing of pro-IGF-II in dec-RVKR-CMK-treated and PACE4-knockdown cells by immunoblot analysis. Unfortunately, we were unable to detect either pro- or mature IGF-II protein in the conditioned medium from C2C12 cells due to the lack of suitable commercially available anti-IGF-II antibody (data not shown).

In addition to IGF-II, proteins comprising the IGF signaling pathway such as IGF-I, IGF-I receptor and insulin receptor, are also processed by SPC family members. Like IGF-II, IGF-I promotes myogenic differentiation, although its effect is less marked than that of IGF-II (*27*). A previous report showed that furin-deficient RPE.40 and Lovo cells can process pro-IGF-I, suggesting that an SPC family member other than furin acts as a processing protease involved in IGF-I maturation (*28*). Since LoVo cells express PACE4 and PC7 (*29*, *30*), PACE4 may be involved in the proteolytic processing of IGF-I. Moreover, several proteins other than those involved in the IGF signalling pathway, which are cleaved by the SPC family, have been reported to regulate myogenic differentiation. For example, ADAM12 is required for the fusion of myoblasts into multinucleated myotubes (*10*), and its mRNA transcript levels increase transiently during C2C12 myogenic differentiation (*31*). MT1-MMP mRNA transcript levels slowly increase during C2C12 differentiation (*32*), and the inhibition of MT1-MMP expression by RNA interference impairs the formation of myotubes (14). Furthermore, it was recently reported that myostatin, which negatively regulates skeletal muscle growth, is secreted as pro-myostatin and is cleaved extracellularly by SPC family members (*13*). Cleavage of pro-myostatin by PACE4 in the extracellular pool may generate mature myostatin which can then control muscle growth. Additionally, the present study demonstrated that MMP11 mRNA transcript levels increased during C2C12 myogenic differentiation, like the case with PACE4. The maturation of MMP11 may also occur extracellularly, suggesting MMP-11 as a potential substrate of PACE4 (*19*). Interestingly, MMP11 activates PI3K activity via cleaving insulin-like growth factor-binding protein-1 (IGFBP-1) (*33*). IGF activity is also controlled by several high-affinity extracellular binding proteins (IGFBPs). The high affinity of IGFBPs for IGFs leads to the effective inhibition of IGFs. Proteolytic cleavage of IGFBPs by several MMPs including MMP11 increases IGF activity. Thus, maturation of MMP11 by PACE4 may be involved in myogenic differentiation as an additional mechanism for increasing the activity of the IGF pathway through IGFBP degradation.

In conclusion, we revealed that SPC family members, and particularly PACE4, play a pivotal role in the myogenic differentiation of C2C12 cells, and that PACE4 expression is regulated via a positive feedback loop. Further studies are required to clarify the role of the PACE4-IGF-PI3K signalling pathway in myogenesis.

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CONFLICT OF INTEREST

None declared.

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