

# Cloning of cDNA Encoding a Regeneration-Associated Muscle Protease Whose Expression Is Attenuated in Cell Lines Derived from Duchenne Muscular Dystrophy Patients

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**In the dystrophin-mutant *mdx* mouse, an animal model for Duchenne muscular dystrophy (DMD), damaged skeletal muscles are efficiently regenerated and thus the animals thrive. The phenotypic differences between DMD patients and the *mdx* mice suggest the existence of factors that modulate the muscle wasting in the *mdx* mice. To identify these factors, we searched for mRNAs affected by the *mdx* mutation by using cDNA microarrays with newly established skeletal muscle cell lines from *mdx* and normal mice. We found that in the *mdx* muscle cell line, 12 genes, including L-arginine:glycine amidinotransferase and thymosin  $\beta_4$ , are up-regulated, whereas 7 genes, including selenoprotein P and a novel regeneration-associated muscle protease (RAMP), are down-regulated. Northern blot analysis and *in situ* hybridization revealed that RAMP mRNA is predominantly expressed in normal skeletal muscle and brain, and its production is enhanced in the regenerating area of injured skeletal muscle in mice. RAMP expression was much lower in individual muscle cell lines derived from biopsies of six DMD patients compared to a normal muscle cell line. These results suggest that RAMP may play a role in the regeneration of skeletal muscle and that its down-regulation could be involved in the pro-**

**gression of DMD in humans. (Am J Pathol 2004, 164:1773–1782)**

Point mutations or out-of-frame deletions in the dystrophin gene on the X-chromosome are known to cause Duchenne muscular dystrophy (DMD).<sup>1</sup> This disease occurs with a frequency of 1 of 3500 newborn males, which makes it the most common lethal myopathy. Dystrophin is a large membrane-associated protein that plays an important role in linking the intracellular cytoskeletal actin filaments to the sarcolemmal membrane.<sup>2</sup> In addition, it forms a multicomponent complex denoted as dystrophin-associated protein complex, which contains dystroglycans, sarcoglycans, syntrophins, and nitric oxide synthase.<sup>3,4</sup> Thus, dystrophin not only mechanically protects the sarcolemma from muscle contraction-induced tension,<sup>5</sup> it also affects intracellular signaling pathways, particularly in the Ca<sup>2+</sup>-dependent enzymatic cascade.<sup>6</sup>

The *mdx* mutant mouse strain carries a nonsense mutation at position 3185 of the murine dystrophin gene.<sup>7,8</sup> However, despite the lack of subsarcolemmal dystrophin protein in these mice, their skeletal muscle degeneration is less severe than it is in DMD patients as after an initial period of skeletal muscle necrosis at 3 to 4 weeks of age, regenerative activity in the *mdx* mice gradually compensates for the muscle damage in the hindlimb.<sup>9</sup> As a result, the adult *mdx* mice show little functional disability. In contrast, in DMD patients, there is an imbalance between muscle degeneration and repair that leads to the loss of muscle fibers and increased fibrosis.<sup>10</sup> Consistent with these observations is that recent DNA microarray analyses revealed that mRNAs encoding proteins related to the muscle regeneration process are more abundantly

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Supported by grants-in-aid for Research on Nervous and Mental Disorders (13B-1) and for Research in Brain Science (H12-brain-028) from the Ministry of Health, Labor, and Welfare of Japan; a grant-in aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (14770106); and a grant-in-aid from the Nakatomi Foundation.

Accepted for publication January 29, 2004.

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expressed in the skeletal muscle of *mdx* mice than in the skeletal muscle of normal control mice.<sup>10–13</sup> Examples of these muscle-regenerating proteins are insulin-like growth factor-2, transforming growth factor  $\beta$ , procollagens, and osteopontin. The down-regulation of myostatin mRNA in the skeletal muscle of the *mdx* mouse is also related to its higher regeneration capacity.<sup>10,12</sup> Intriguingly, recent reports demonstrated that transgenic overexpression of insulin-like growth factor-1 in muscle<sup>14</sup> or administration of anti-myostatin neutralizing antibody<sup>15</sup> blocked the degeneration and fibrosis in the diaphragm in *mdx* mice, suggesting that the enhancement of muscle regenerative capacity may be a promising therapeutic approach for DMD.

Although the extensive gene profiling of DMD patient biopsies *versus* normal muscle samples has provided many clues about the secondary loss of or changes in DMD muscle,<sup>16,17</sup> it is difficult to be sure that net change observed in the gene expression of DMD muscle reflects an altered genetic program in the muscle cells because the necrotic DMD muscle areas are filled with many macrophages and other inflammatory immune cells. Intact muscle biopsies from young patients also contain many blood cells. Thus, it is difficult to be sure that a net change observed in the gene expression of DMD muscle reflects an altered genetic program in the muscle cells. To overcome this problem, we first immortalized skeletal muscle cells from *mdx* and control mice and compared their expression of several gene sets by using cDNA microarrays. We also established muscle cell lines from biopsies taken from DMD patients, Becker muscular dystrophy (BMD) patients, and an unaffected person to investigate the behavior of the genes whose expression patterns were found to be altered in the *mdx* muscle cell line. In this study, we report that, relative to the control murine muscle cell line, the transcription of 12 genes in the *mdx* muscle cell line is up-regulated while mRNA levels of 7 other genes is down-regulated. Among the down-regulated genes was a novel gene that we found encoded a secreted protease termed regeneration-associated muscle protease (RAMP). We found that RAMP mRNA levels are also often decreased in the muscle cell lines derived from the DMD and BMD patients.

## Materials and Methods

### Mice

Breeding pairs of C57BL/10 ScSn-Dmd<sup>mdx</sup> (*mdx*) mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and propagated in a standard pathogen-free animal facility in the institute. C57BL/10 (B10) mice were purchased from Nihon SLC (Hamamatsu, Japan). All animal experiments were based on institutionally approved protocols.

### Primary Culture of Skeletal Muscle and Immortalization

*Ex vivo* culture of skeletal muscle was done according to the published protocol with a slight modification.<sup>18</sup> In,

brief, the hindlimb muscles were taken from 2-month-old B10 and *mdx* mice, thoroughly minced into a coarse slurry, and enzymatically dissociated with 5% trypsin (Difco, Detroit, MI) in phosphate-buffered saline (PBS) at 37°C for 30 minutes. The dissociated muscle tissues were resuspended in Dulbecco's modified Eagle's minimum essential medium-high glucose type (Sigma, St. Louis, MO) supplemented with 20% fetal calf serum and 0.5% penicillin-streptomycin (Sigma), triturated by using a 10-ml pipette, and passed through a 100- $\mu$ m nylon mesh. The cells were cultured in Dulbecco's modified Eagle's medium containing 20% fetal calf serum (JRH Biosciences, Lenexa, KS), 4% Ultrosor G (Biosepra, Cergy-Saint-Christophe, France) and 0.5% penicillin-streptomycin in gelatin-coated flasks for 24 hours. The nonadherent cells were then transferred to new flasks for the subculture of the primary myoblastic cells. Two days later, half of the cells were infected for 1 day with a recombinant retrovirus producing a temperature-sensitive form of the simian virus 40 large T antigen (SV40 tsT)<sup>19</sup> and then cultured at the permissive temperature of 32.5°C until continuously growing cells appeared. The retrovirus was produced as previously described by using the PLAT-E packaging cell line<sup>20</sup> and the pMESVts retrovirus vector<sup>19</sup> (a kind gift from Dr. Drinkwater, University of Wisconsin Medical School). After 1 month of culture, the Ultrosor G was removed from the growth medium and myoblastic subclones were isolated by limiting dilution. For myotube formation, the culture medium was changed to Dulbecco's modified Eagle's medium supplemented with 5% horse serum (Invitrogen, Carlsbad, CA) followed by incubation at 39.5°C for 7 days.

### Immunocytochemistry

Cells grown in a 4-well culture dish (Nunc, Roskilde, Denmark) were treated with 0.1% Triton X-100 (Wako, Osaka, Japan) in PBS for 1 minute and fixed in cold methanol for 2 minutes. After rinsing with PBS, cells were blocked with 2% bovine serum albumin (Sigma) in PBS for 1 hour and incubated with rabbit anti-desmin antibody (Progen, Heidelberg, Germany) (1:100 dilution) for 1 hour. Samples were washed with PBS and incubated with anti-rabbit immunoglobulin conjugated with horseradish peroxidase (Jackson Immunoresearch Laboratories, West Grove, PA) (1:250 dilution) for 1 hour. After rinsing with PBS three times, the peroxidase activity was visualized by incubation with 0.25% diaminobenzidine (Sigma) in PBS containing 0.075% H<sub>2</sub>O<sub>2</sub> and 3.36 mmol/L NiCl<sub>2</sub> (Nakalai, Kyoto, Japan).

### cDNA Microarray Analysis

Poly(A)<sup>+</sup> RNAs were isolated by using the FastTrack 2.0 (Invitrogen). According to the previously published method,<sup>21,22</sup> mouse microarrays carrying ~4000 different cDNAs derived from a mouse fetus at 17.5 days post coitum and an adult mouse brain were hybridized with Cy3- and Cy5-labeled cDNA probes prepared from the mRNAs of the C57BL/10- and *mdx*-derived cell lines cultured in the

**Table 1.** Summary of the Expression Pattern and Primer Sequences of the Genes that Are Up-Regulated and Down-Regulated in *Mdx* Mice

Name of gene/protein	Accession no.	Mdx/B10 cell line*	Mdx/B10 muscle†	Patient/normal human cell	Sense primer sequence (5'-3')	Anti-sense primer sequence (5'-3')	PCR band size (bp)
1 SCHIP-1	NM_01392	↑	→	→	GTCTATCAGACAGAAGTTGGC	GAAGATCAGCGACGGGAGAC	415
2 OX-2	AF004023	↑	N.T.	N.T.			
3 Arg/Gly amidinotransferase	U07971	↑	↑	↑	GGAAGTGATAGTGGCCAGAGC	CAGGATGTCTCGAGGCATTGC	293
4 MAD/MEF2C	L08895	↑	N.T.	N.T.			
5 PC3	M60921	↑	↑	→	(m) CTCGAGATGAGCCACGGGAAGAGAAC (h) ATGAGCCACGGGAAGGAAGGGAAC	(m) GCGGCCCGCTAGCTGGAGACGGCCATCA (h) GGGTCAGCTCGTGGGCAGC	(m) 476 (h) 304
6 mc7	AJ278191	↑	N.D.	→	GACGGCACGTGTGACGAGTG	CCTCGCTGTCTTCTCACTC	422
7 CGI 61	AF151819	↑	→	→	CCTCCTTAGCAAAGCTGAATG (m) CCGGAATTCATGTCTGACAAACCCG (h) CAACCATGTCTCTGAGAAACCCG	TGCAGCATCCAAATCCAGTC (m) GCTCTAGATTACGATTGCCAGCTTG (h) GATTGCCTGCTTGTCTCTCC	393 (m) 135 (h) 135
8 Thymosin β4	M34043	↑	↑	→	(h) CAACCATGTCTCTGAGAAACCCG	(h) GATTGCCTGCTTGTCTCTCC	(h) 135
9 EST-MNCb4008 Telomeric repeat binding	BF168890	↑	→	↑	GGAGCAAAGAATGCATAAGC	CAGTGGGAAGAGAGGCCATG	
10 Factor 2	NM_02058	↑	↑	→	CCTTTCCTGCCAACTCTCCAC	GAGACTCTGGTTGGCCAGAG	300
11 EST-MNCb1040	AU035914	↑	↑	N.T.	(m) ATGTGAGGCCGTTTGCGCCGGA	(m) TTTTCTAATGGAATGCTTCCCC	(m) 540
12 mKIAA1039 protein	AK122424	↑	↑	→	AGGTTCCGAGGAGCCCTGG (m) CCGGAATTCGAGAGCCAAGGCCAAAGC (h) CAATGTGGAGAAGCCTGGGG (m) GCGAAGGCTGAAAAGTGG (h) GACTGTGAGGAAGGATGGGC	CTTGATGATGGGGTCTCTG (m) GCTCTAGATTAGTTTGAATGTCATTTT (h) AGATGTGTGATTTAATCG (m) GGGGTAAGTCAAGATGCAGGGTTC (h) GGTCTGTGAGGACATGTTGG	(m) 1085 (h) 312 (m) 908 (h) 330
13 Selenoprotein P	X99807	↓	↑	→	(h) CAATGTGGAGAAGCCTGGGG (m) GCGAAGGCTGAAAAGTGG (h) GACTGTGAGGAAGGATGGGC	(h) AGATGTGTGATTTAATCG (m) GGGGTAAGTCAAGATGCAGGGTTC (h) GGTCTGTGAGGACATGTTGG	(h) 312 (m) 908 (h) 330
14 GARG16 Phosphatidic acid	Q64828	↓	↑	→			
15 Phosphatase type 2B	BC005558 NM_032003	↓	↓	→	CCGAGCCAGCCATGCAAA	GTAATAGATCCGGTAGAATTC	344
16 Ectonucleotide pyrophosphatase		↓	→	→	GATCACAACACAGAGGGCAG	TCTGTGGAGTTCATGGCTTC	684
17 Phosphodiesterase 5/amyloid beta (A4) precursor like protein 2	NM_009691	↓	→	→	GAGGCTCTTGCCAGCCAATGC	CTGGAAC TAGCAGGACATCAC	337
18 Lysosomal membrane glycoprotein	J05287	↓	→	→	TGTCTGTGGCTACCATGGG	CTGCACTGCAGTCTTGAGCTG	418
19 EST-MNCb1423 (RAMP)	XM_14918	↓	↑	↓	CTGGGCAGCGCTGTGAAAATC	GTAATGGTGTCTCCCTTGAC	366

The mRNA expression levels were determined by Northern blot analyses (\*) or by semi-quantitative RT-PCR (†). N.D., not detectable; N.T., not tested; M, mouse; H, human.

growth media at 32.5°C. The fluorescent signals were quantified by ScanArray 4000 (GSI Lumonics, Moorpark, CA) and the data were analyzed by QuantArray software (GSI Lumonics).

### Construction of Human Microdystrophin cDNA and Transfection

Construction of the human microdystrophin cDNA containing four rod repeats and three hinges was performed based on a published report.<sup>23</sup> By using cDNA derived from human skeletal muscle cells (Cambrex, Baltimore, MD) as the template, two polymerase chain reactions (PCRs) were independently performed with two sets of primers, namely, HD1 (5'-CTCGAGATGCTTTGGTGGGAAGAAGT-3') and HD2(5'-TCTTCAAGGGTATCCACAGTAATCTGCCTCTTC-3') or HD3 (5'-ATTACTGTGGATACCCTTGAAAGACTC-CAGGAAC-3') and HD4 (5'-GCGGCCGCTACATTGTGTC-CTCTCTCAT-3'). Subsequently, a mixture of the two PCR products was reamplified with the HD1 and HD4 primers to obtain a long PCR band of 4.6 kb pairs that was subsequently cloned into a pPCR-Script Amp SK(+) vector (Stratagene, La Jolla, CA). A *XhoI/NotI* fragment was inserted into the reverse tet-regulated retrovirus vector pLRT-X<sup>24</sup> (a kind gift from Dr. Hagiwara, Tokyo Medical and Dental University, Tokyo, Japan). A subclone of the *mdx*-derived muscle cell line was transfected with the pLRT-microdystrophin vector by FuGENE 6 (Roche, Mannheim, Germany) and cultured in growth medium

containing blasticidin S (Wako) at 5 μg/ml for 3 weeks to establish stable transfectants.

### Western Blot Analysis

Total cell lysates were separated by electrophoresis on a 5 to 10% sodium dodecyl sulfate-polyacrylamide gel, transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA), and incubated with anti-dystrophin DYS2 monoclonal antibody (1:100 dilution; Novocastra, Newcastle, UK). Immunoreacting bands were visualized by using the ECL-Plus detection reagent (Amersham Pharmacia Biotech, Piscataway, NJ).

### Northern Blot Analysis and Reverse Transcriptase (RT)-PCR

Poly(A)<sup>+</sup> RNAs (2 μg) from various tissues of adult (12 to 16 weeks old) C57BL/6 mice were separated by electrophoresis on a 1% formaldehyde-agarose gel, transferred to a Hybond-N<sup>+</sup> nylon membrane (Amersham Pharmacia Biotech), and hybridized with [ $\alpha$ -<sup>32</sup>P] dCTP-labeled probes prepared from each cDNA at 42°C overnight. To verify the amount of RNA loaded in each lane, the blot was rehybridized with a β-tubulin probe. After washing under the most stringent of conditions, the membrane was subjected to autoradiography. For RT-PCR analysis, total RNAs were prepared from skeletal muscle tissues and various muscle

cell lines using TRIzol (Invitrogen). Five  $\mu\text{g}$  of the RNA from each sample was reverse-transcribed by using the superscript preamplification system for first strand cDNA synthesis and an oligo(dT) primer (Invitrogen). Part (1/125) of the cDNA mixture was subjected to a PCR reaction using 56°C as an annealing temperature, ExTaq DNA polymerase (Takara, Tokyo, Japan), and the specific primer sets listed in Table 1.

### *Muscle Injury Model and in Situ Hybridization Analysis*

A crush-injury was given by puncturing the gastrocnemius muscle of 8-week-old male C57BL/10 mice with a 23-gauge needle. At different time points (5 hours to 14 days) after the injury, the gastrocnemius muscles were isolated and frozen in liquid nitrogen for RNA extraction. For *in situ* hybridization analysis, the tibialis anterior muscle samples from C57BL/10 and *mdx* mice were dissected on the day 6 after the crush injury or the injection of 100  $\mu\text{l}$  of 10  $\mu\text{mol/L}$  cardiotoxin (Wako), and they were frozen in isopentane precooled in liquid nitrogen. Ten- $\mu\text{m}$  cryostat longitudinal sections were prepared and fixed in 4% paraformaldehyde in PBS (pH 7.4) and treated with 1  $\mu\text{g/ml}$  of proteinase K (Wako) in PBS at room temperature for 7 minutes. After being acetylated with acetic anhydride in triethanolamine, the sections were hybridized with a digoxigenin-labeled anti-sense or sense RNA probe at 65°C for 18 hours and subjected to the colorimetric detection of signals as previously described.<sup>25</sup>

### *Establishment of Myoblastic Cell Lines from Patient Biopsy Samples*

Skeletal muscle biopsy samples were provided from a normal donor (52 years of age), two BMD patients (1 to 6 years of age), and six DMD patients (1 to 12 years of age) after obtaining the informed consent of donors or their parents. The enzymatically dissociated human cells were subjected to primary culture in Dulbecco's modified Eagle's medium supplemented with 20% fetal calf serum and 4% Ultrosor G or 5% of chicken embryo extract. Fibroblastic cells were removed by a 1-hour attachment in the tissue culture plate. Remaining myoblastic cells were subcultured for 1 to 2 weeks at 37°C, and infected with the SV40 tsT retrovirus for 2 days followed by a continuous culture at 32.5°C. The amphotropic retrovirus was produced by using the PLAT-A packaging line (SM and TK, unpublished). The above-described experimental protocols were approved by the ethical committees of the institute and associated universities.

## **Results**

### *Establishment of Skeletal Muscle-Derived Cell Lines from Mdx and B10 Mice*

To assess whether the gene expression program of myogenic cells in skeletal muscle is affected by the *mdx*

mutation, we immortalized the myoblastic cells isolated from the skeletal muscle of *mdx* and B10 mice. The immortalization was achieved by introducing SV40 tsT by retrovirus-mediated gene transfer into the primary myoblast culture. The newly established skeletal muscle cell lines derived from the *mdx* mice were termed *mdx-sm* whereas those from the B10 control mice were termed B10-sm. In the absence of viral challenge, the primary cells ceased growing after the third to fourth passage. In contrast, the *mdx-sm* and B10-sm cell lines have proliferated for more than a year when grown at 32.5°C. Their doubling times are comparable (data not shown). When these cells are cultured at 39.5°C, all stop proliferating and myotube formation is initiated. This indicates that the cells have been successfully immortalized in a temperature-dependent manner. In terms of their morphology, both cell lines are myoblastic in general but some of the *mdx-sm* cells spontaneously differentiate into myotube-like structures in the presence of horse serum even at the permissive temperature of 32.5°C. Concordantly, the frequency of desmin-positive myotubes in the *mdx-sm* cell line is much higher than that in the B10-sm cell line (Figure 1A). This phenotypic difference was also observed when six subclones of the *mdx-sm* cell line (*mdx-sm*1 to *mdx-sm*6) and two subclones of the B10-sm cell line (B10-sm1, B10-sm2) were compared. Thus, it appears that the higher differentiation capacity of *mdx-sm* cells is not because of the mixed cell populations in the founding lines, rather it results from the altered genetic program in the *mdx* myoblasts. For the following studies, we used the *mdx-sm*2 and B10-sm1 subclones as representative cell lines.

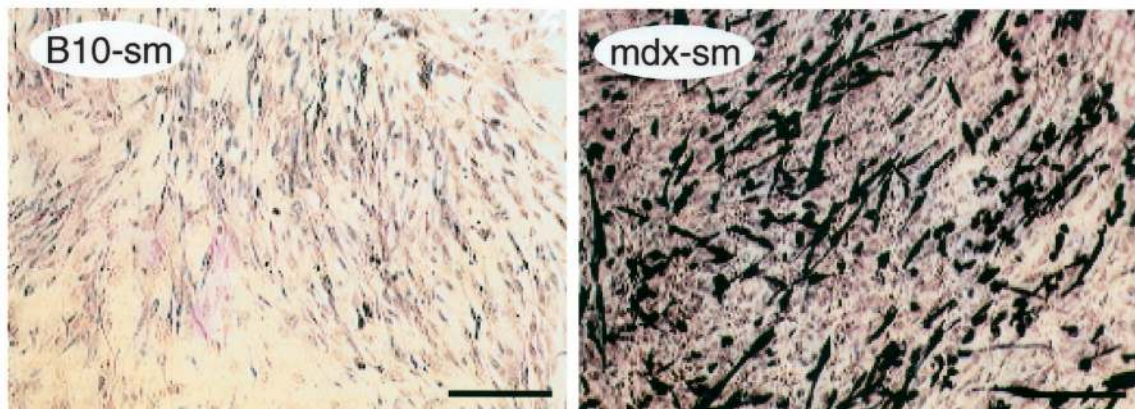
### *Identification of Differentially Expressed Genes in Mdx-sm Cells by cDNA Microarray Analysis*

DNA chip analysis has been shown to be a powerful way to compare the overall gene expression profiles of test cells with those of control cells. We used mouse cDNA microarrays holding ~4000 distinct mouse cDNA fragments (>0.5 kb) originating from a mouse fetus and an adult mouse brain. After hybridization with fluorescently labeled cDNAs from the *mdx-sm* and B10-sm cells, we selected those genes in which the ratio of *mdx-sm* fluorescence to that of B10-sm was either higher than 1.5 or lower than 0.62 when both fluorescent color combinations were used (data not shown). In this way, we identified 20 up-regulated and 21 down-regulated genes in the *mdx-sm* cells.

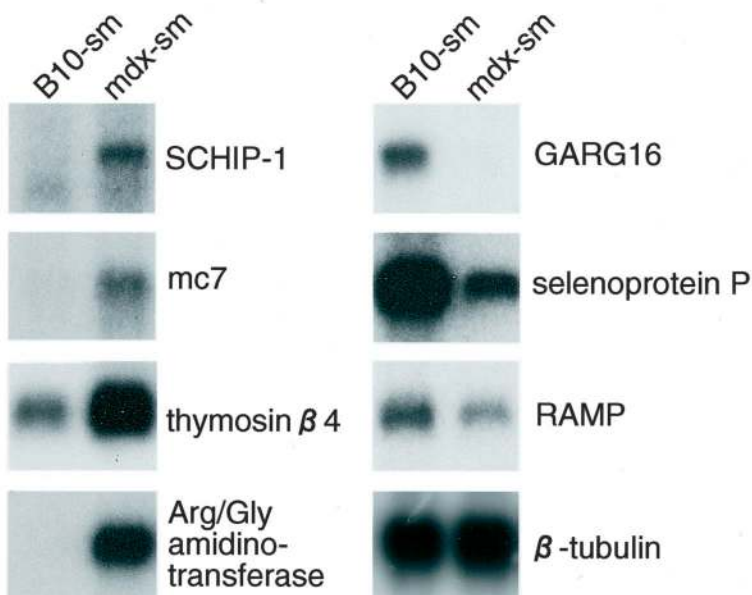
To confirm the differential expression of these candidate genes, we performed Northern blot analyses with mRNAs from *mdx-sm* and B10-sm cells. As shown in Figure 1B, mRNAs for schwannomin-interacting protein 1 (SCHIP-1),<sup>26</sup> mc7 (dystrophin-interacting protein),<sup>27</sup> thymosin  $\beta$ 4 (G-actin sequestering protein),<sup>28</sup> and L-arginine:glycine (Arg/Gly) amidinotransferase<sup>29</sup> are more abundantly expressed in *mdx-sm* cells than in B10-sm cells, whereas the mRNA levels of GARG16 (glucocorticoid-attenuated response gene),<sup>30</sup> selenoprotein P (anti-



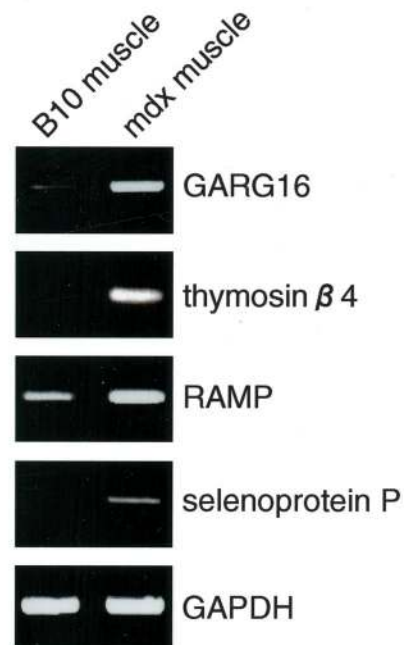
A



B



C

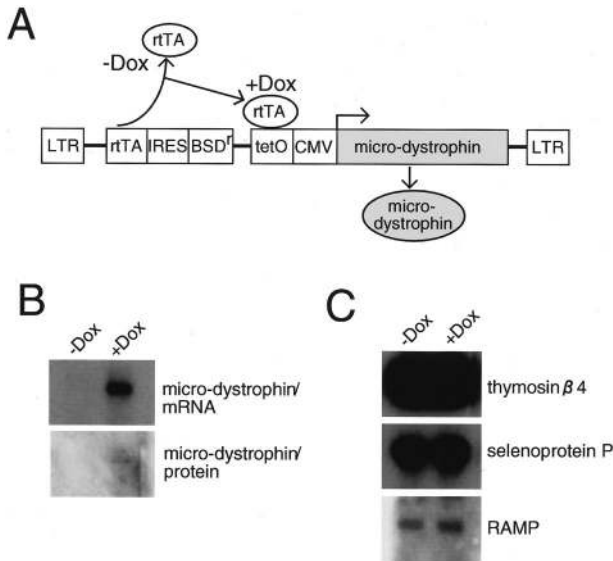


**Figure 1.** Identification of up- and down-regulated genes in the *mdx*-derived skeletal muscle cell line. **A:** Extent of spontaneous myogenic differentiation in the B10-sm and *mdx*-sm myoblastic cell lines derived from B10 and *mdx* mice, respectively. Cells cultured in differentiation-inducing conditions were immunostained with anti-desmin antibody. **B:** Northern blot analysis of various *mdx* up- and down-regulated genes in *mdx*-sm and B10-sm cell lines. The integrity and amount of loaded RNAs were assessed by probing with  $\beta$ -tubulin cDNA. **C:** RT-PCR analysis of various *mdx* up- and down-regulated genes in the intact skeletal muscle of B10 and *mdx* mice. Total RNAs were converted to cDNA by random hexamers and subjected to 20 cycles of amplification with specific primer sets for each gene as indicated. DNA bands after ethidium bromide staining are shown with GAPDH, which acts as the control that ensures equal amount of template cDNA were used.

-oxidant protein),<sup>31</sup> and EST-MNCb1423 (accession number, XM 149185; designated as RAMP in this study) are lower in *mdx*-sm cells. In addition, eight other genes including myogenic transcription factor MEFC<sup>32</sup> were found to be up-regulated in *mdx*-sm cells while four other genes were down-regulated (data not shown, Table 1). The expression patterns of these genes were similar in all of the *mdx*-sm subclones (data not shown).

Of the 19 differentially expressed genes, 15 encode proteins with known function and 4 are registered in the public database only as expressed sequenced tags (Table

1). When we examined the mRNA expression of these genes in the intact skeletal muscle of *mdx* and B10 mice by RT-PCR, five genes, including thymosin  $\beta$ 4 and Arg/Gly amidinotransferase showed the same patterns revealed by the cell lines (Figure 1C, Table 1). In contrast, the mRNAs for GARG16, selenoprotein P, and RAMP were increased in the intact muscle of *mdx* mice (Figure 1C, Table 1). Although the exact reason for these discrepancies remains to be determined, our results demonstrate that the genetic programs of the growing myoblastic cells and the intact muscle fibers in *mdx* mice are not identical.



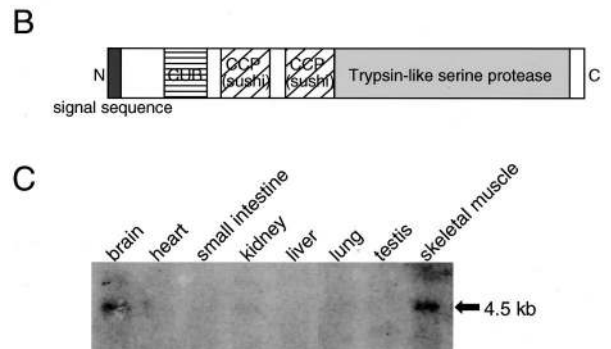
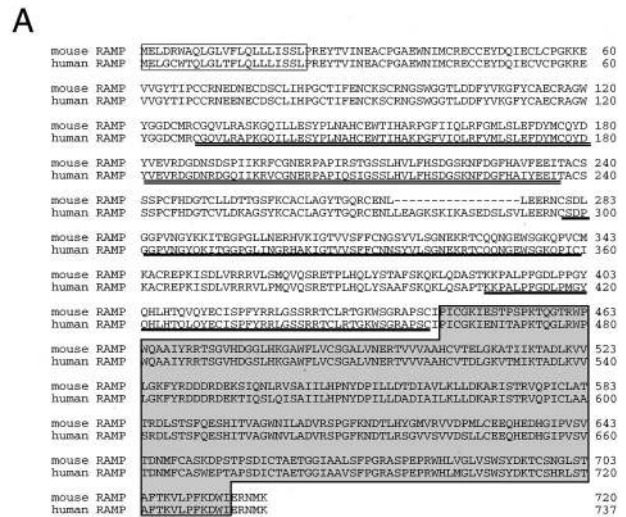
**Figure 2.** Effect of enforced expression of microdystrophin protein in mdx-sm cell line. **A:** Structure of the pLRT vector carrying a mouse microdystrophin gene under the Dox-inducible promoter. **B:** Dox-inducible expression of microdystrophin mRNA and protein in mdx-sm cells transfected with pLRT-microdystrophin. **C:** Effect of introducing microdystrophin into mdx-sm cells on the expression of some of the *mdx* up- and down-regulated genes. The levels of the mRNAs specific for thymosin  $\beta$ 4, selenoprotein P, and RAMP before and after adding Dox were verified by Northern blot analyses.

*Effect of Introducing Dystrophin on the Gene Expression in Mdx-sm Cells*

Dystrophin is known to be expressed in myotubes and mature muscle fibers but not in proliferating myoblasts. To exclude the possibility that the altered gene expression in mdx-sm cells is directly linked to the mutation in the dystrophin gene or to the lack of dystrophin mRNA or protein, we established a stable transfectant of mdx-sm cells that expresses the microdystrophin cDNA under the doxycyclin (Dox)-inducible promoter (Figure 2A). As shown in Figure 2B, 48 hours after the addition of Dox to the culture medium, microdystrophin mRNA and protein were produced in the mdx-sm transfectant cells. However, the expression levels of the genes that show disparate expression patterns in *mdx* mice, including thymosin  $\beta$ 4, selenoprotein P, and RAMP, were not altered by the addition of Dox (Figure 2C, data not shown). These results suggest that the differential gene expression between mdx-sm and B10-sm cells is independent of dystrophin levels.

*Primary Sequence and Expression of RAMP*

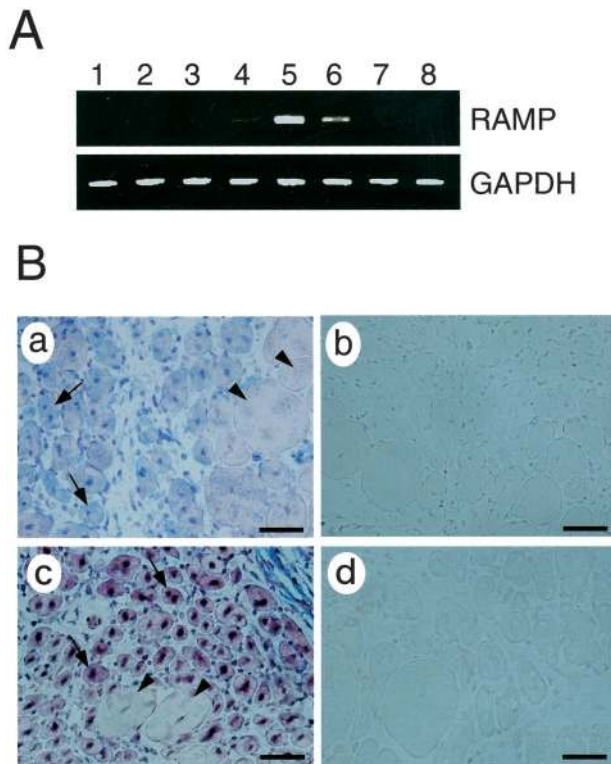
Because myoblasts play a central role in muscle regeneration, we hypothesized that the 13 differentially expressed genes in mdx-sm cells might play a role in the progression of muscle wasting seen in DMD patients and/or the milder myopathy in *mdx* mice. We especially focused on the RAMP gene because we found its mRNA expression was enhanced in regenerating muscle fibers (see Figure 4). Mouse RAMP cDNA in the database is 3085 bp in length and its predicted open reading frame codes for 720 amino acid



**Figure 3.** Sequence and tissue distribution of RAMP. **A:** Sequences of human and mouse RAMP proteins. Predicted signal sequences are **boxed**. CUB and CCP/Sushi domains are indicated by a **double line** and a **solid line**, respectively. The trypsin-like serine protease domain is shown by a **light gray box**. **B:** Schematic representation of the structural motifs in RAMP. **C:** Predominant expression of RAMP mRNA in skeletal muscle and brain. Two  $\mu$ g of poly(A)<sup>+</sup> RNA prepared from various organs of adult C57BL/6 mouse were electrophoresed, blotted, and hybridized with <sup>32</sup>P-labeled mouse RAMP cDNA. The band size of the detected transcript is shown.

residues (Figure 3A). A BLAST search of the database revealed that mouse RAMP protein shows 88% identity at the amino acid level with the uncharacterized human protein DKFZP586H2131 (accession number, MN 015430), which we refer to as human RAMP in this study. As shown schematically in Figure 3B, mouse RAMP protein contains a putative signal peptide at its N-terminal region (1 to 22),<sup>33</sup> the CUB domain (122 to 236),<sup>34</sup> two complement control protein (CCP) modules (also known as Sushi domains) (280 to 342 and 389 to 442),<sup>35</sup> and a trypsin-like serine protease domain (444 to 715).<sup>36</sup> CUB and CCP/Sushi domains are often found in developmentally regulated proteins and cell adhesion molecules, respectively. These subdomain structures are conserved in human RAMP as well. The calculated molecular mass of mouse RAMP was 82.1 kd with an isoelectric point of 7.3. In the normal adult mouse, RAMP mRNA is only detectable in the brain and skeletal muscle as a single band at the position of 4.5 kb (Figure 3C). Thus, it appears that RAMP is a novel secreted protease that potentially plays some functions in skeletal muscle.

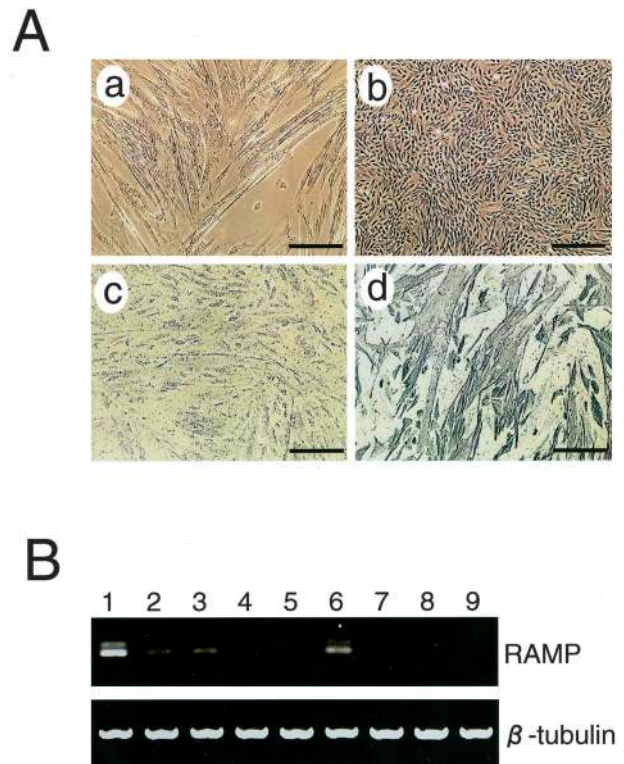




**Figure 4.** Up-regulation of RAMP mRNA expression in skeletal muscle after injury. **A:** Gastrocnemius muscle of mice was dissected 5 hours (lane 1) or 1 (lane 2), 2 (lane 3), 3 (lane 4), 4 (lane 5), 7 (lane 6), 10 (lane 7), or 14 (lane 8) days after injuring the muscle with a needle. Total RNA from each sample was extracted, reverse-transcribed, and amplified by 30 cycles of PCR using RAMP-specific primers. RT-PCR for GAPDH cDNA was performed to verify that equal amounts of template cDNA were used. **B:** Detection of RAMP mRNA in regenerating muscle fibers by *in situ* hybridization. Transverse cryosections of the tibialis anterior muscle of C57BL/10 mice harvested 6 days after crush injury were hybridized with digoxigenin-labeled anti-sense (a) or sense (b) cRNA probes for RAMP. Tibialis anterior muscle sections prepared from *mdx* mice after cardiotoxin injection were also hybridized with digoxigenin-labeled anti-sense (c) or sense (d) cRNA probes for RAMP. Specific signals for RAMP mRNA were detected as blue (a) or brown paints (c) in the centrally nucleated muscle fibers (arrows in a and c), but not in mature fibers (arrowheads in a and c). Scale bars, 50  $\mu$ m.

### Induction of RAMP mRNA in Regenerating Skeletal Muscle Fiber

We next examined whether the expression of RAMP mRNA is changed in regenerating skeletal muscle fibers. A crush injury of gastrocnemius muscle was induced in normal mice and the expression of RAMP mRNA was measured. RAMP expression gradually increased throughout the days after the injury, reaching the highest levels 4 days after the injury, and then being reduced to the baseline within 1 to 2 weeks (Figure 4A). Consistent with the transient up-regulation of RAMP mRNA in the muscle injury model, *in situ* hybridization assays revealed that RAMP mRNA was specifically detected in centrally nucleated regenerating muscle fibers (shown by arrows), but not in unaffected fibers (arrowheads) (Figure 4B, a). In the tibialis anterior muscle of *mdx* mice after cardiotoxin injection, expression of RAMP was again specifically observed in regenerating muscle fibers (arrows) (Figure 4B, c). Thus, the RAMP gene is induced by the injury of skeletal muscle.



**Figure 5.** Expression of RAMP mRNA in cell lines derived from human BMD and DMD patients. **A:** Myotube differentiation capacity of BMD-sm1, a representative cell line derived from a BMD patient's skeletal muscle. The morphology is shown by phase contrast microscopy of the BMD-sm1 cell line cultured at 39.5°C (a) or 32.5°C (b). BMD-sm1 cells cultured at 39.5°C were immunostained with (d) or without (c) anti-desmin antibody. **B:** RT-PCR analysis of RAMP mRNA expression in nine human cell lines derived from skeletal muscle biopsies of patients. Total RNAs were extracted from the control (lane 1), two BMD (lanes 2 and 3), and six DMD (lanes 4 to 9) human muscle cell lines, reverse-transcribed, and amplified by 30 cycles of PCR using specific primers for human RAMP cDNA. Ethidium bromide-staining patterns of DNA bands are shown with  $\beta$ -tubulin, which shows that equal amounts of template cDNA were loaded. Scale bars, 150  $\mu$ m.

### Expression of RAMP mRNA in Skeletal Muscle Cell Lines Derived from BMD and DMD Patients

To evaluate the importance of RAMP in DMD pathology, we established skeletal muscle cell lines derived from biopsies of an unaffected donor and BMD/DMD patients. The strategy used to achieve this was the same as that used to establish the mouse muscle cell lines except that an amphotropic retrovirus was used and the cells were exposed to the virus for a longer period. Continuously growing cells at 32.5°C were obtained from one normal, two BMD, and six DMD biopsies within 1 to 2 months after introducing SV40 tsT. In contrast, uninfected parallel control cultures exhibited senescence. Similar to the *mdx*-sm cell line, the human BMD/DMD-derived muscle cell lines differentiated into myotubes when cultured at 39.5°C. Figure 5 shows a representative BMD cell line, BMD-sm1, during its undifferentiated growth (b) and after it has been placed in differentiation-inducing conditions (a). Immunohistochemical analysis demonstrated that there was a high frequency of desmin-positive myotubes in the differentiated BMD-sm1 cell line (Figure 5A, d). The other eight human patient-derived cell lines proliferated similarly and

also possessed myogenic properties (data not shown), although their growth rates were very low compared to the mouse cell line. By designing specific sets of primers for human orthologs to the *mdx*-up and -down genes, we examined the mRNA expression of these genes in the nine human muscle cell lines by RT-PCR. We found that relative to the unaffected donor-derived cell line, mRNAs for Arg/Gly amidinotransferase and EST-MNCb4008 were always increased in the BMD/DMD patient-derived cell lines (data not shown, Table 1). In contrast, all of the BMD/DMD patient-derived cell lines showed lower RAMP mRNA expression levels relative to the control line (Figure 5B). That both the *mdx*-sm- and the BMD/DMD-derived cell lines show an attenuation of RAMP expression suggests that RAMP could be involved in the pathogenesis of DMD.

## Discussion

In this study, we identified 19 genes that are differentially expressed by newly established skeletal muscle cell lines from *mdx* and control B10 mice. We also showed that the expression of the novel gene RAMP, which is down-regulated in the *mdx* line, was frequently impaired in the muscle cell lines derived from six DMD and two BMD patients.

Gene expression studies using *mdx* mice or DMD biopsies have been published before<sup>10–13,16,17</sup> but unlike these studies, we used immortalized skeletal muscle cell lines as the initial materials for the cDNA microarray and Northern blot analyses. The immortalization was achieved by conditional transformation of the primary cells by infecting them with the SV40 tsT-bearing retrovirus vector. This method has been used successfully to expand target cell types before.<sup>19</sup> It has been reported that myogenic cell lines established by the SV40 large T antigen retain their differentiation capacity *in vitro*.<sup>37,38</sup> Supporting this is that the myoblastic cell lines established in this study possess myogenic differentiation activity. It is also known that rodent cells can be fully immortalized by the SV40 T antigen.<sup>39</sup> However, as the life span of human primary cells *in vitro* is dependent on their telomerase activity, human cells carrying the SV40 large T antigen will eventually undergo senescence and crisis because of telomere shortening. Nevertheless, our approach yielded sufficient numbers of progeny human myoblastic cells from single biopsies from DMD or BMD patients.

The skeletal muscle cell line that was established from *mdx* mice (*mdx*-sm) differentiated more frequently into a myotube-like structure *in vitro* than the line derived from the control C57BL/10 mice (B10-sm) (Figure 1A). That MEF2C expression in *mdx*-sm cells is enhanced (Table 1, data not shown) confirms that the myogenic differentiation program in these cells is engaged. This property could reflect the status of the myoblastic precursor cells in the *mdx* mouse muscle at the time of virus infection. In adult mice, skeletal muscle stem cells (satellite cells) that reside beneath the basal lamina of muscle fibers are mitotically quiescent and are only activated in response to mechanical stimuli such as exercise and injury.<sup>40</sup> In the process of muscle regeneration, satellite cells are believed to generate myoblasts that proliferate and differ-

entiate to make myotubes before fusing with existing myofibers. In *mdx* mice, the destruction of muscle fibers caused by the lack of dystrophin and compensatory muscle regeneration are thought to occur continuously.<sup>9</sup> As is consistent with a previous report, the injury-response signaling pathway mediated by JNK1 MAP kinase may thus be activated in the majority of myoblasts in *mdx* mice.<sup>40</sup> All of the subclonal cell lines from the original bulk culture of *mdx*-sm produced desmin<sup>+</sup> myotube-like structures at a higher rate (data not shown). Because retrovirus infection occurs only in dividing cells in culture, the *mdx*-sm line may represent a proliferating subpopulation of myogenic precursor cells in *mdx* skeletal muscle. This may also be true for the human patient-derived muscle cell lines. Thus, the genetic programs in these cell lines are likely to represent those that are present in most, if not all, myoblasts in intact skeletal muscle.

The comparison of the *mdx*-sm and B10-sm cell lines revealed that 12 genes are up-regulated and 7 genes are down-regulated in the *mdx*-sm line (Figure 1B, Table 1). Dystrophin is expressed in muscle fibers and mature myotubes but not in proliferating myoblasts. To verify that the differential gene expression in *mdx*-sm cells is not because of the absence of dystrophin, we introduced a microdystrophin gene lacking the 4th to 23rd rod domains into *mdx*-sm cells and induced these cells to express the truncated dystrophin protein. Microdystrophin has been proven to be fully functional in restoring myopathy in *mdx* mice.<sup>23,41</sup> However, the presence of this protein in *mdx*-sm cells did not affect the expression of the 19 genes whose expression patterns are altered in *mdx*-sm cells (Figure 2B, data not shown). Thus, the altered gene expression pattern in *mdx*-sm cells is the result of a dystrophin-independent event in the myoblasts of *mdx* mice.

Among the 12 up-regulated genes in the *mdx*-sm cell line, Arg/Gly amidinotransferase is of particular interest because it is a first and rate-limiting enzyme for the creatine biosynthesis that is a major energy source in skeletal muscle.<sup>42</sup> A previous study has shown that Arg/Gly amidinotransferase mRNA is rapidly induced in kidney *in vivo* by adding creatine and growth hormone.<sup>29</sup> It is reasonable to suppose that skeletal muscle cells of *mdx* mice also produce this enzyme more abundantly in response to the creatine and creatine kinase that is released by disrupted muscle fibers. We found that all DMD- and BMD-derived muscle cell lines also produce a higher level of Arg/Gly amidinotransferase (Table 1, data not shown). This suggests that the measurement of mRNA for this enzyme in muscle cells as well as detection of higher creatine kinase levels in serum may be useful in the diagnosis of DMD and other myopathies.

Increased expression of thymosin  $\beta$ 4 in the *mdx* mouse muscle has been reported in previous studies.<sup>12</sup> We found that its expression was up-regulated in *mdx*-sm cells and *mdx* muscle tissue, but not in the DMD patient-derived cell lines. Thymosin  $\beta$ 4 is a bifunctional protein that sequesters G-actin in the cytoplasm and stimulates the migration of endothelial cells and monocytes once secreted outside of cells.<sup>43,44</sup> The acetylated tetrapeptide acSDKP that is proteolytically released from the N-



terminus of thymosin  $\beta$ 4 inhibits proliferation of hematopoietic progenitor cells and enhances angiogenesis.<sup>45</sup> Presumably, thymosin  $\beta$ 4 may play some roles in the regenerating muscle area by acting on inflammation-associated cells. A study is underway to determine whether higher amounts of thymosin  $\beta$ 4 could ameliorate DMD pathogenesis.

The gene products of SCHIP-1 and mc7 are structural proteins in the muscle and brain. It has been suggested that SCHIP-1 links membrane proteins to the cytoskeleton<sup>26</sup> and that mc7 directly binds to the C-terminal region of dystrophin (GenBank data base), although clear evidence for these functions is not yet available. The relevance of the up-regulation of these two genes in the mdx-sm cell line remains uncertain. However, it is tempting to speculate that the expression of sarcolemmal membrane-associated proteins is induced in the absence of dystrophin by a compensatory mechanism. The expression of utrophin in mdx mice is the most famous example of this. Recent gene profiling studies of mdx mice and DMD biopsies also revealed such tendencies.<sup>10,17</sup>

The selenoprotein P gene is also down-regulated in the mdx-sm cell line. This down-regulation could affect the anti-oxidant defense of cells. Selenoproteins possess thioredoxin reductase activity that neutralizes the cytotoxic response of cells to the oxidant stress mediated by thioredoxin.<sup>46</sup> Recently, 10 families with congenital muscular dystrophy were shown to carry mutations in the selenoprotein N gene.<sup>46</sup> Moreover, there are reports showing that selenium deficiency is associated with muscular dystrophy in animals and cardiomyopathy in humans.<sup>47,48</sup> However, we found that the overall mRNA level of selenoprotein P in mdx mice was higher than that in control mice, as has also been shown in a previous report.<sup>10</sup> The presence of increased amounts of selenoprotein P as well as RAMP in adult mdx mice may be related to the milder skeletal myopathic phenotype observed in this model.

Of the seven down-regulated genes in mdx-sm cells, RAMP was the only one whose expression was always lower in muscle cell lines from six DMD and two BMD patients compared to the expression in a line from an unaffected control. Although the normal human myoblastic cell line used in this study was derived from a relatively aged donor, this difference appears to be significant because most of the other genes that showed differential expression in the mdx-sm line had a similar pattern of expression between the BMD/DMD patient-derived cell lines and the unaffected control cell line (Table 1). RAMP is a novel secreted protease that carries three major molecular signatures (Figure 3B). One of these is the CUB domain, which is often found in the extracellular domain of developmentally regulated proteins. Examples of these are bone morphogenic protein 1 (a metalloendopeptidase that induces cartilage and bone formation) and neuropillin (a calcium-independent cell adhesion molecule that functions during the formation of neuronal and vascular circuits). Another molecular signature is the CCP modules/Sushi domain. These exist in a wide variety of complement and adhesion proteins. The third significant molecular motif of RAMP is its serine protease do-

main belonging to the trypsin family at its C-terminus. In normal unchallenged mice, RAMP mRNA is only detectable in the skeletal muscle and brain. Previous studies showed that genes encoding cathepsin S and cathepsin H proteases are up-regulated in mdx muscle,<sup>11,12</sup> but RAMP-related proteases have not been described previously. The reason why the RAMP gene has not been identified in the microarray studies in the past by using mdx mice is probably because its expression level is low and the amount of mRNA in the intact skeletal muscle of mdx and normal mice do not differ significantly. A unique finding of this study is that RAMP mRNA is specifically induced in regenerating muscle fibers found after skeletal muscle injury. As described above, during muscle regeneration, myoblasts differentiate into myotubes and the cells then fuse with existing muscle fiber. Accumulating evidence from previous publications suggests that an elevated calcium concentration in regenerating muscle activates calcineurin, which dephosphorylates nuclear factors of activated T cells (NF-ATs).<sup>49,50</sup> Specific NF-AT isoforms in skeletal muscle play crucial roles in the myogenic differentiation and myoblast fusion events. As with the interleukin-4 induction that triggers myotube fusion in regenerating muscle areas,<sup>51</sup> RAMP mRNA is induced in small centrally nucleated myofibers (Figure 4B). Thus, RAMP could be an important regulator of the muscle regeneration process. Further analysis of its enzymatic targets and biological activities in skeletal muscle would shed light on this novel protease and enhance our understanding of the processes involved in muscle regeneration and the malignant progression of DMD.

### Acknowledgments

We thank Drs. Yuko Miyagoe-Suzuki and Shin-ichi Takeda (National Institute of Neuroscience) for valuable advice; and Drs. Norman Drinkwater (University of Wisconsin Medical School) and Masatoshi Hagiwara (Tokyo Medical and Dental University) for providing us with the pMESVts and pLRT-X retrovirus vectors, respectively.

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