An Essential Role of Small Ubiquitin-like Modifier (SUMO)-specific Protease 2 in Myostatin Expression and Myogenesis*

Received for publication, September 17, 2013, and in revised form, November 14, 2013. Published, JBC Papers in Press, December 16, 2013, DOI 10.1074/jbc.M113.518282

Yitao Qi‡§1, Yong Zuo‡¶1, Edward T. H. Yeh∥1, and Jinke Cheng‡¶2

From the ‡Department of Biochemistry and Molecular Cell Biology, Shanghai Key Laboratory for Tumor Microenvironment and Inflammation, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China, the ¶Texas Heart Institute, St. Luke’s Episcopal Hospital, Houston, Texas 77030, the §State Key Laboratory of Oncogenes and Related Genes, Shanghai Cancer Institute, Shanghai Jiao Tong University School of Medicine, Shanghai 200438, China, and the ¶Department of Cardiology, University of Texas M.D. Anderson Cancer Center, Houston, Texas 77030

Background: It is unknown whether SUMOylation regulates myogenesis in skeletal muscle.

Results: SENP2 de-SUMOylates MEF2A, promoting myostatin expression and suppressing myogenesis in skeletal muscle.

Conclusion: SENP2 plays a critical role in the regulation of myostatin-induced inhibition of myogenesis.

Significance: SENP2 is a potential therapeutic target in skeletal muscle regeneration.

Sentrin/small ubiquitin-like modifier (SUMO)-specific protease 2 (SENP2) has broad de-SUMOylation activities in vitro, which is essential for embryonic heart development. Here, we show that myostatin, a key factor in skeletal muscle development, is markedly reduced in Senp2−/− mouse embryonic fibroblast cells and embryos. SENP2 regulates the transcription of myostatin mainly through de-SUMOylation of MEF2A. Silencing SENP2 can reduce myostatin expression and, therefore, promote myogenesis of skeletal muscle. These results reveal the important role of SENP2 in the regulation of myostatin expression and myogenesis.

The small ubiquitin-like modifier (SUMO)3 covalently modifies a large number of cellular proteins and regulates the localization, function, and protein-protein interaction of proteins (1–3). The transcription factors are the most popular targets for SUMO conjugation, which suggests an important role of SUMOylation in the regulation of gene expression (4, 5). SUMOylation is catalyzed by SUMO-specific E1s, E2s, and E3s, and is reversed by a family of Sentrin/SUMO-specific proteases (SEPNs) (6, 7). Studies have shown that SENPs are important determinants of the SUMO modification status in cells (8–10). Although the biochemical properties of SENPs have been well documented, their targets with involved biological processes are largely unknown.

There are six mammal SENPs with different subcellular locations and substrate specificities (3, 9, 10). SENP2 was originally reported to be associated with the nuclear envelope through the binding to Nup153 (11, 12). We have shown previously that SENP2 contains both nuclear import and export signals that can shuttle between the nucleus and cytoplasm (13). SENP2 is the key regulator of Pc2/CBX4 function through the regulation of its SUMOylation status, which is critical for embryonic heart development (14).

Myostatin (growth and differentiation factor 8, GDF8) is a member of the TGF-β superfamily of secreted growth/differentiation factors (15–17). Myostatin is primarily expressed by skeletal muscle and acts in an autocrine manner to inhibit myoblast proliferation, differentiation, and protein synthesis (18, 19). Disruption of the myostatin gene in mice causes a large and widespread increase in skeletal muscle mass because of cell hyperplasia and hypertrophy (20–22). An inactivating mutation of the myostatin gene in a child has been reported to be hypermuscular (23). However, the overexpression or lack of active myostatin does not affect cardiac systolic function, although it reduces cardiac mass and cardiomyocyte proliferation (24).

Myostatin promoter activity is upregulated by differentiation. Both the myogenic regulatory factor and the myocyte enhancer factor 2 family of muscle transcription factors increase myostatin promoter activity (25, 26). Myostatin transcription is also upregulated by dexamethasone treatment (27). However, it is not currently known whether SUMOylation regulates myostatin expression and myogenesis. In this study, we found that the deletion of the Senp2 gene in mice diminished myostatin expression. We further confirmed that MEF2A is a target for SENP2 de-SUMOylation activity and that SENP2 promotes myostatin expression and inhibits myogenesis through MEF2A. These results reveal the critical role of SENP2 in muscle development through the MEF2A-myostatin pathway.

EXPERIMENTAL PROCEDURES

Cell Culture—The generation of MEF cells has been described previously (14). The C2C12 cell line was purchased...
from the ATCC. Satellite stem cells were isolated from the hind limb muscles of 6-week-old C57BL/6 mice. All cell lines were cultured in DMEM (Invitrogen) supplemented with 1% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Plasmids and Antibodies—Plasmids HA-SUMO-1 and FLAG-SENP2 and the FLAG-SENP2 mutation have been described previously (28, 29). The MEF2A, MEF2B, MEF2C, and MEF2D plasmids were gifts from Dr. Eric Olson (Southwestern Medical Center). siRNA against SENP2, myostatin, MEF2A, MEF2B, MEF2C, MEF2D, and nonspecific siRNA (NS-siRNA) were purchased from Sigma. Probes for SENP2 and myostatin were purchased from Applied Biosystems. Anti-MEF2A, anti-myosin heavy chain, and anti-PAX7 antibodies were purchased from Abcam. Anti-FLAG and anti-HA antibodies were purchased from Sigma. Anti-SUMO-1 antibody was from Zymed Laboratories Inc.

Real-time Quantitative PCR—Total RNA was isolated by an RNeasy kit (Qiagen) and treated with DNase (Promega). Complementary DNA was synthesized using the cDNA synthesis kit (Clontech) according to the instructions of the manufacturer. Fluorescence real-time PCR was performed with the TaqMan probe using the ABI Prism 7300 system (PerkinElmer Life Sciences). PCR was done in triplicate, and standard deviations representing experimental errors were calculated. All data were analyzed using ABI Prism SDS 2.0 software (PerkinElmer Life Sciences).

Myostatin Promoter–Luciferase Plasmids—A myostatin bacterial artificial chromosome (BAC) clone (BACPAC Resources Center) was used to amplify the myostatin promoter (Fig. 2A) with specific primers (the sequences of primers will be provided upon request). The primers included a cleavage recognition site for either KpnI (forward primer) or XhoI (reverse primer) to facilitate subcloning into the multiple cloning sites of the pGL3-basic vector.

Skeletal Myogenesis—C2C12 cells were cultured in growth medium (DMEM supplemented with 10% FBS). When cells reached about 80–90% confluence, the growth medium was aspirated, and cells were switched to differentiation medium (DMEM supplemented with 2% horse serum). Refeeding with fresh differentiation medium was done every 48 h. After 6 days of differentiation, the cells were stained with myosin heavy chain antibody to show myotube formation. DAPI was used to stain the nuclei.

Satellite Stem Cell Differentiation—Satellite stem cells were isolated from C57BL/6 mouse skeletal muscle. Cells with round morphological characteristics were selected and defined as primitive muscle satellite stem cells. The genetic marker PAX7 was used to identify satellite stem cells. To induce satellite stem cell differentiation, the cells were grown to 80–90% confluence, growth medium was aspirated, and cells were switched to differentiation medium (DMEM supplied with 2% horse serum). Refeeding with fresh differentiation medium was done every 48 h. After 6 days of differentiation, the cells were stained with myosin heavy chain antibody to show myotube formation. DAPI was used to stain the nuclei.

Cachexia Mouse Model—10⁶ LLC cells in 0.1 ml PBS and PBS control were injected into the tail veins of 8-week-old C57BL/6 mice. Six weeks after LLC cell injection, when tumors appeared in the lungs, body weight was measured, and the skeletal muscle was harvested. The SENP2 and myostatin mRNA expression levels were measured via real-time PCR.

Statistical Analysis—Results were presented as mean ± S.D. Student’s t test was used to compare the differences between two groups.

RESULTS

SENP2 Modulates Myostatin Expression—Because SENP2 is highly expressed in the dermomyotome in mouse embryos (data not shown), we reasoned that SENP2 might be an important regulator in myogenesis. Microarray profiles from Senp2+/+ and Senp2−/− embryos at embryonic day 10.5 showed that myostatin mRNA in Senp2−/− embryos was reduced significantly compared with that in Senp2+/+ embryos. We evaluated this observation in Senp2−/− MEF cells and embryos by using real-time PCR, and the evaluation showed that in both MEFs and embryos, myostatin mRNA was decreased in comparison with that in the Senp2+/+ controls (Fig. 1A). To further determine the role of SENP2 in myostatin expression, we overexpressed SENP2 in murine myoblast C2C12 cells, which showed that the overexpression of SENP2 increased myostatin expression (Fig. 1B). Interestingly, the SENP2 catalytic mutant did not have such an effect (Fig. 1B), suggesting that the de-SUMOylation activity is essential for SENP2 to regulate myostatin expression. We also confirmed the role of SENP2 in the regulation of myostatin expression by using the SENP2 siRNA approach. As shown in Fig. 1C, silencing SENP2 significantly reduced myostatin expression. Collectively, these results show a crucial role of SENP2 in myostatin expression.

MEF2A Mediates the Effect of SENP2 on Myostatin Expression—To explore how SENP2 regulates myostatin expression, we generated a myostatin promoter (~2858 to +152 bp of myostatin genomic DNA)-luciferase report gene (Fig. 2A, pMSTN6). Cotransfection of SENP2 significantly
induced the transcription activity of the myostatin promoter (Fig. 2B). To further map the SENP2 response element of the myostatin promoter, we tested a series of truncated forms of myostatin promoter-luciferase plasmids and found that the SENP2-responsive element was located between -1938 to -1358 bp of the myostatin promoter (Fig. 2A). Consistent with the observation in Fig. 1B, we found that SENP2 catalytic activity was also required to activate myostatin promoter transcription (Fig. 2B). The TESS program predicted a MEF2 binding site located in this area (−1336 to −1326 bp of the myostatin promoter) (Fig. 2A). We tested whether this MEF2 binding site might mediate SENP2 action on myostatin promoter activity by a mutation approach. As expected, we found that the SENP2 activation on the myostatin promoter was almost abolished when the MEF2 binding site was mutated (Fig. 2C).

The MEF2 family includes four members, denoted as MEF2A, MEF2B, MEF2C, and MEF2D. Coexpression of MEF2A, but not of other MEF2 members, could promote the transactivation

**FIGURE 2.** **MEF2A mediates SENP2 activity on the myostatin promoter.** A, schematic of the various deletion fragments generated to identify the minimal promoter region required for myostatin activity. There is one MEF2 binding site (MBS) in pMSTN4 but not in pMSTN3. C2C12 cells were transfected with various deletion fragments and SENP2, and luciferase activity was measured 24 h after transfection. The data are presented as mean ± S.D. of three independent experiments. B, C2C12 cells were transfected with FLAG-tagged SENP2 or SENP2m plasmid, and relative luciferase activity was measured 24 h after transfection. The data are presented as mean ± S.D. of three independent experiments. Differences between SENP2 and SENP2m or control cells were significant (p < 0.05, Student’s t-test). C, C2C12 cells were transfected with pMSTN4 or pMSTN4m plasmid, and relative luciferase activity was measured 24 h after transfection. The data are presented as mean ± S.D. of three independent experiments. Differences between SENP2 and SENP2m or control cells were significant (p < 0.05, Student’s t-test). D, C2C12 cells were transfected with MEF2A, MEF2B, MEF2C, or MEF2D plasmid, and luciferase activity was measured 24 h after transfection. The data are presented as mean ± S.D. of three independent experiments. Differences between MEF2A and MEF2B, MEF2C, MEF2D, or the control were significant (p < 0.05, Student’s t test). E, C2C12 cells were transfected with NS-siRNA (si-NS), MEF2A siRNA (si-MEF2A), MEF2B siRNA (si-MEF2B), MEF2C siRNA (si-MEF2C), or MEF2D siRNA (si-MEF2D), and the expression of MEF2 proteins was analyzed by Western blot analysis (right panel). Luciferase activity was measured 24 h after transfection. The data are presented as mean ± S.D. of three independent experiments.
We next determined whether SENP2 de-SUMOylated MEF2A in vivo and in vitro. In the in vitro assay, MEF2A SUMOylation was detected in C2C12 cells transfected with MEF2A and HA-SUMO-1. The overexpression of SENP2, not the SENP2 mutant, deconjugated SUMO-MEF2A (Fig. 3B). The role of SENP2 in de-SUMOylation of MEF2A was also confirmed in Senp2+/+ and Senp2−/− MEF cells. SUMO-MEF2A was detected easily detected and accumulated in Senp2−/− MEF cells (Fig. 3C). These data indicate that SENP2 can de-SUMOylate MEF2A and enhance MEF2A transactivation on the myostatin promoter.

SENP2 Inhibits Skeletal Myogenesis—Because SENP2 promotes myostatin expression, we speculated that SENP2 would repress myogenesis as myostatin does. We tested this hypothesis in two cell models. We first used C2C12 cells transfected with SENP2 siRNA, MEF2A, or myostatin siRNA (Fig. 4). Myostatin expression was reduced significantly in these cells compared with si-NS control cells (Fig. 4, A and B). These cells
were induced into myogenesis by 2% horse serum in culture. Myotube formation, as a marker for myogenesis, was checked in these cells by using immunofluorescence staining with anti-myosin heavy chain antibody. As shown in Fig. 4C, silencing of MEF2A or SENP2 enhanced myotube formation in C2C12 cells as myostatin siRNA did. Another cell model used was satellite stem cells isolated from C57BL/6 mouse skeletal muscle. The isolated satellite stem cells were confirmed by PAX7 immunostaining (data not shown). Satellite stem cells transfected with MEF2A, SENP2, or myostatin siRNA were induced into differentiation by addition of 2% horse serum. As observed in the C2C12 cell model, myostatin expression was decreased significantly after MEF2A or SENP2 knockdown in satellite stem cells (Fig. 4B). Differentiation was shown by immunostaining with anti-myosin heavy chain antibody in myostatin siRNA- or SENP2 siRNA-transfected satellite stem cells (Fig. 4C). Like myostatin, SENP2 is a negative regulator in myogenesis.

**DISCUSSION**

In this study, we found that SENP2 contributes to the regulation of myostatin expression in muscle cells. We also observed SENP2 acting as a negative regulator in myogenesis, like myostatin. We further observed that MEF2A, a specific transcription factor of myostatin, mediates SENP2 promotion for myostatin expression through de-SUMOylation. We detected the increase of both the expression of SENP2 and myostatin mRNA showed a remarkable increase in the skeletal muscle of the cachexia mice (Fig. 5, B and C), suggesting that SENP2 might promote cachexia through the regulation of myostatin expression in cancer-bearing mice.

**The Correlation of Senp2 and Myostatin Expression in Cachexia**—Because myostatin expression is increased and related to weight loss and muscle atrophy during cachexia, we reasoned that SENP2 might be involved in cachexia through the regulation of myostatin expression. To test this possibility, we created a cancer cachexia mouse model by injecting LLC cells via the tail vein. Six weeks after LLC cell injection, the mice not only grew lung tumors (data not shown) but also showed a body weight loss of 45%, which is a typical cachexia condition (Fig. 5A). Interestingly, the expression of both Senp2 and myostatin mRNA showed a remarkable increase in the skeletal muscle of the cachexia mice (Fig. 5, B and C), suggesting that SENP2 might promote cachexia through the regulation of myostatin expression in cancer-bearing mice.
heart formation in mouse embryonic hearts (14). Interestingly, here we found that silencing SENP2 would promote the differentiation and myogenesis in skeletal muscle progenitor cells. The two phenotypes in Senp2 deficiency cells look contradictory. However, SENP2 acts with different mechanisms in these two different processes. In cardiomyocytes, SENP2 regulates Gata4/6 expression through the PcG complex, which is essential for heart development (14). However, the function of SENP2 in skeletal muscle is through the induction of myostatin, which suppresses myogenesis. These observations suggest that SENP2 has multiple functions in regulating biological processes involving different targets. In a future study, it will be interesting to identify by which mechanism SENP2 targets specific proteins in different signaling pathways.

Satellite stem cells are quiescent muscle stem cells (31–33). How to activate the differentiation process of satellite stem cells into new myofibers is still a critical issue for skeletal muscle regeneration. It seems that SENP2 functions in maintaining the quiescent status of satellite stem cells. When SENP2 expression is silenced or SENP2 activity is inactivated, satellite stem cells can differentiate into myocytes. Thus, SENP2 is a potential therapeutic target in skeletal muscle regeneration.

REFERENCES

1. Geiss-Friedlander, R., and Melchior, F. (2007) Concepts in sumoylation. A decade on. *Nat. Rev. Mol. Cell Biol.* 8, 947–956
2. Hay, R. T. (2005) SUMO: A history of modification. *Mol. Cell* 18, 1–12
3. Yeh, E. T. (2009) SUMOylation and de-SUMOylation. Wrestling with life's processes. *J. Biol. Chem.* 284, 8223–8227
4. Gill, G. (2003) Post-translational modification by the small ubiquitin-related modifier SUMO has big effects on transcription factor activity. *Curr. Opin. Genet. Dev.* 13, 108–113
5. Ouyang, J., Valin, A., and Gill, G. (2009) Regulation of transcription factor activity by SUMO modification. *Methods Mol. Biol.* 497, 141–152
6. Yeh, E. T., Gong, L., and Kamitani, T. (2000) Ubiquitin-like proteins. New wines in new bottles. *Gene* 248, 1–14
7. Schwenhorst, I., Johnson, E. S., and Dohmen, R. J. (2000) SUMO conjugation and deconjugation. *Mol. Gen. Genet.* 263, 771–786
8. Cheng, J., Kang, X., Zhang, S., and Yeh, E. T. (2007) SUMO-specific protease 1 is essential for stabilization of HIF1α during hypoxia. *Cell* 131, 584–595
9. Hay, R. T. (2007) SUMO-specific proteases. A twist in the tail. *Trends Cell Biol.* 17, 370–376
10. Mulkhopadhyay, D., and Dasso, M. (2007) Modification in reverse. The SUMO proteases. *Trends Biochem. Sci.* 32, 286–295
11. Chow, K. H., Elgort, S., Dasso, M., and Ullman, K. S. (2012) Two distinct sites in Nup153 mediate interaction with the SUMO proteases SENP1 and SENP2. *Nucleus* 3, 349–358
12. Hang, J., and Dasso, M. (2002) Association of the human SUMO-1 protease SENP2 with the nuclear pore. *J. Biol. Chem.* 277, 19961–19966
13. Ithana, Y., Yeh, E. T., and Zhang, Y. (2006) Nucleocytoplasmic shuttling modulates activity and ubiquitination-dependent turnover of SUMO-specific protease 2. *Mol. Cell. Biol.* 26, 4675–4689
14. Kang, X., Qi, Y., Zuo, Y., Wang, Q., Zou, Y., Schwartz, R. J., Cheng, J., and Yeh, E. T. (2010) SUMO-specific protease 2 is essential for suppression of polycomb group protein-mediated gene silencing during embryonic development. *Mol. Cell* 38, 191–201
15. McPherron, A. C., and Lee, S. J. (1997) Double muscling in cattle due to mutations in the myostatin gene. *Proc. Natl. Acad. Sci. U.S.A.* 94, 12457–12461
16. Lee, S. J., and McPherron, A. C. (1999) Myostatin and the control of skeletal muscle mass. *Curr. Opin. Genet. Dev.* 9, 604–607
17. Lee, S. J., and McPherron, A. C. (2001) Regulation of myostatin activity and muscle growth. *Proc. Natl. Acad. Sci. U.S.A.* 98, 9306–9311
18. Artaza, J. N., Bhasin, S., Magee, T. R., Reisz-Porszasz, S., Shen, B., Groome, N. P., Meerasahib, M. F., Fareez, M. M., and Gonzalez-Cadavid, N. F. (2005) Myostatin inhibits myogenesis and promotes adipogenesis in C3H 10T(1/2) mesenchymal multipotent cells. *Endocrinology* 146, 3547–3557
19. McCroskery, S., Thomas, M., Maxwell, L., Sharma, M., and Kambadur, R. (2003) Myostatin negatively regulates satellite cell activation and self-renewal. *J. Cell Biol.* 162, 1135–1147
20. Walsh, F. S., and Celeste, A. J. (2005) Myostatin. A modulator of skeletal muscle stem cells. *Biochem. Soc. Trans.* 33, 1513–1517
21. Bogdanovich, S., Krag, T. O., Barton, E. R., Morris, L. D., Whitemore, L. A., Ahima, R. S., and Khurana, T. S. (2002) Functional improvement of dystrophic muscle by myostatin blockade. *Nature* 420, 418–421
22. Zimmers, T. A., Davies, M. V., Koniaris, L. G., Haynes, P., Esquela, A. F., Tomkinson, K. N., McPherron, A. C., Wolfman, N. M., and Lee, S. J. (2002) Induction of cachexia in mice by systemically administered myostatin. *Science* 296, 1486–1488
23. Schulke, M., Wagner, K. R., Stolz, L. E., Hübnner, C., Riebel, T., Könen, W., Braun, T., Tobin, J. F., and Lee, S. I. (2004) Myostatin mutation associated with gross muscle hypertrophy in a child. *N. Engl. J. Med.* 350, 2682–2688
24. Artaza, J. N., Reisz-Porszasz, S., Dow, J. S., Kloner, R. A., Tsao, J., Bhasin, S., and Gonzalez-Cadavid, N. F. (2007) Alterations in myostatin expression are associated with changes in cardiac left ventricular mass but not ejection fraction in the mouse. *J. Endocrinol.* 194, 63–76
25. Deng, B., Wen, J., Ding, Y., Gao, Q., Huang, H., Ran, Z., Qian, Y., Peng, J., and Jiang, S. (2012) Functional analysis of pig myostatin gene promoter with some adigoninesis- and myogenesis-related factors. *Mol. Cell. Biochem.* 363, 291–299
26. Du, R., An, X. R., Chen, Y. F., and Qin, J. (2007) Some motifs were important for myostatin transcriptional regulation in sheep (Ovis aries). *J. Biochem. Mol. Biol.* 40, 547–553
27. Qin, J., Du, R., Yang, Y. Q., Zhang, H. Q., Li, Q., Liu, L., Guan, H., Hou, J., and An, X. R. (2013) Dexamethasone-induced skeletal muscle atrophy was associated with upregulation of myostatin promoter activity. *Res. Vet. Sci.* 94, 84–89
28. Gong, L., Millas, S., Maul, G. G., and Yeh, E. T. (2000) Differential regulation of sentrinized proteins by a novel sentrin-specific protease. *J. Biol. Chem.* 275, 3355–3359
29. Kamitani, T., Nguyen, H. P., Kito, K., Fukuda-Kamitani, T., and Yeh, E. T. (1998) Covalent modification of PML by the sentrin family of ubiquitin-like proteins. *J. Biol. Chem.* 273, 3117–3120
30. Chiu, S. Y., Asai, N., Costantini, F., and Hsu, W. (2008) SUMO-specific protease 2 is essential for modulating p53-Mdm2 in development of trophoblast stem cell niches and lineages. *PLoS Biol.* 6, e310
31. Yin, H., Price, F., and Rudnicki, M. A. (2013) Satellite cells and the muscle stem cell niche. *Physiol. Rev.* 93, 23–67
32. Brack, A. S., and Rando, T. A. (2012) Tissue-specific stem cells. Lessons from the skeletal muscle satellite cell. *Cell Stem Cell* 10, 504–514
33. Zammit, P. S., Partridge, T. A., and Yablonka-Reuveni, Z. (2006) The skeletal muscle satellite cell. The stem cell that came in from the cold.