Musclin, a Novel Skeletal Muscle-derived Secretory Factor*§

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Skeletal muscle is involved in the homeostasis of glucose and lipid metabolism. We hypothesized that the skeletal muscle produces and secretes bioactive factors, similar to adipocytokines secreted by fat tissue. Here, we report the identification of a novel secretory factor, musclin, by signal sequence trap of mouse skeletal muscle cDNA libraries. Musclin cDNA encoded 130 amino acids, including NH₂-terminal 30-amino acid signal sequence. Musclin protein contained a region homologous to natriuretic peptide family, and KKKR, a putative serine protease cleavage site, similar to the natriuretic peptide family. Full-length musclin protein and KKKR-dependent cleaved form were secreted in media of mouse skeletal muscle-transfected mammalian cell cultures. Musclin mRNA was expressed almost exclusively in the skeletal muscle of mice. Musclin mRNA levels in skeletal muscle were markedly low in fasted, increased upon re-feeding, and were low in streptozotocin-treated insulin-deficient mice. Musclin mRNA expression was induced at late stage in the differentiation of C2C12 myocytes. In myocytes, insulin increased, while epinephrine, isoproterenol, and forskolin reduced musclin mRNA, all of which are known to increase the cellular content of cyclic AMP, a counter-regulator to insulin. Pathologically, overexpression of musclin mRNA was noted in the muscles of obese insulin-resistant KKα mice. Functionally, recombinant musclin significantly attenuated insulin-stimulated glucose uptake and glycogen synthesis in myocytes. In conclusion, we identified musclin, a novel skeletal muscle-derived secretory factor. Musclin expression level is tightly regulated by nutritional changes and its physiological role could be linked to glucose metabolism.

Adipose tissue has shown to produce secretory factors conceptualized adipocytokines (1, 2).

Muscle-specific glucose transporter GLUT4 (3) and peroxisome proliferator-activated receptor-γ (4, 5) knock-out mice exhibited the alterations in insulin sensitivity in fat and liver. Muscle-specific insulin receptor knockout mice showed adipocyte hyperplasia (6). These findings suggest that the skeletal muscle may release bioactive factors (myokines), like adipocytokines, to target fat, liver, and potentially skeletal muscle itself.

In the present study, we attempted to identify skeletal muscle-derived secretory factors using an efficient signal sequence trap (SST) method (7). Here, we report a novel skeletal muscle-derived secretory factor, musclin, whose mRNA was dynamically regulated by nutrition and hormonal factors.

EXPERIMENTAL PROCEDURES

Cloning of Mouse Musclin cDNA—Poly(A)+ RNAs were extracted from gastrocnemius muscles of 10-week-old male C57BL/6J mice under ad libitum, 24-h fasting or 24-h refeeding after 24-h fasting condition and ad libitum db/db mice. Equal amounts of poly(A)+ RNA from each group were pooled to synthesize cDNA. To selectively clone the genes that possess signal sequence at the NH₂-terminal end of cDNAs, SST-REX system (signal sequence trap by retrovirus-mediated expression screening system) was introduced as we reported previously (7). Interleukin-3-independent Ba/F3 cells were harvested, and the integrated cDNAs were isolated from the cells by genomic PCR and sequenced.

To identify full-length cDNA sequence of musclin, the original fragment obtained by SST-REX system was subjected to 5'- and 3'-RACE, using SMART™ RACE cDNA amplification kit (BD Biosciences). Rat and human full-length cDNA were cloned, using homology search for rat and human genome (GenBank™), with mouse musclin sequence, RACE, and reverse transcription-PCR.

Animals and Experimental Protocol—Male C57BL/6J mice (10 weeks old) were divided into three groups (n = 5, each): mice fed ad libitum with standard chow, mice fasted for 48 h, and mice re-fed 24 h after 48-h fasting. Male C57BL/6J mice (10 weeks old) received one-shot intraperitoneal injection of streptozotocin (STZ, Sigma) (100 mg/kg body weight) every day for 3 days (total three times). The mice were sacrificed 4 days after the final injection. Musclin mRNA expression in various tissues of mice was analyzed in 10–12-week-old male C57BL/6J mice. Muscin mRNA expression was also analyzed in muscles of 13-week-old female C57BL/6J and KKα mice.

All experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of Osaka University.

Northern Blot and Quantitative Reverse Transcription-PCR Analysis—RNA extraction and Northern blotting were conducted as described previously (8). First strand cDNA was synthesized using Ther.

The abbreviations used are: GLUT, glucose transporter; 2-DG, 2-deoxy-D-glucose; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; CNP, c-type natriuretic peptide; HEK, human embryonic kidney; IGF, insulin like growth factor; SST-REX, signal sequence trap by retrovirus-mediated expression screening; STZ, streptozotocin; RACE, rapid amplification of cDNA ends; RT, reverse transcription.
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The cDNA encoding mouse musclin protein fused to COOH-terminal FLAG epitope was transiently transfected in HEK293 cells, and the culture medium was subjected to immunoprecipitation with anti-FLAG antibody followed by Western blotting using anti-FLAG M2 monoclonal antibody (A8592, Sigma).

**Preparation of Recombinant Musclin Protein**—Recombinant musclin protein was purified from culture medium of stable C2C12 myocytes expressing FLAG-tagged musclin using agarose beads column conjugated with anti-FLAG antibody. Differentiated C2C12 myocytes synthezing wild-type or mutant musclin (76KKKR79) was fused to FLAG at the COOH terminus was transiently transfected in HEK293 cells, and the culture medium was subjected to immunoprecipitation and Western blotting with FLAG antibody (Fig. 1D). The mutated musclin did not produce the cleaved form, suggesting that some amount of wild-type musclin protein was cleaved at the 76KKKR79 site as described above. To test this, expression plasmid carrying wild-type or mutant musclin (76KKKR79→76A) fused to FLAG at the COOH terminus was transiently transfected in HEK293 cells, and the culture medium was subjected to immunoprecipitation and Western blotting with FLAG antibody (Fig. 1D).

When various tissues of C57BL/6J mice were subjected to Northern blotting, musclin mRNA was almost exclusively expressed in skeletal muscles (Fig. 2A), and the mRNA length was about 1.4 kb. By real-time RT-PCR analysis, musclin mRNA was also expressed in brown adipose tissue, spleen, testis, and bone to a much lesser extent than skeletal muscle (Fig. 2B). Rat musclin mRNA was also expressed almost exclusively in skeletal muscle (data not shown).

We examined regulation of musclin mRNA expression in vivo (Fig. 2, C–E). Musclin mRNA expression in gastrocnemius muscle was almost eliminated by 48-h fasting and reversed by 24-h refeeding (Fig. 2C). This nutritional regulation of musclin mRNA was much more dramatic than those of GLUT4 and lipoprotein lipase mRNAs (Supplemental Fig. 1). To determine the effect of insulin on musclin mRNA expression, mice were treated with STZ. Musclin mRNA expression was significantly decreased in the gastrocnemius muscles of STZ-treated insulin-deficient mice (Fig. 2D). With regard to pathophysiological significance, musclin mRNA expression was augmented in gastrocnemius muscles of obese KKAy mice, at both diabetic (13-week-old) (Fig. 2E) and non-diabetic (7-week-old) stages (data not shown). A similar increase of musclin mRNA was observed in the skeletal muscles of obese db/db mice (data not shown).

Next, we analyzed musclin mRNA expression in mouse myoblast cell line, C2C12. Muscle mRNA was not detected before induction (day 0) and markedly induced after differentiation of C2C12 myotubes, reaching a peak level at a later stage of differentiation (Fig. 3A), compared with mRNAs of other myogenic proteins, such as myogenin, GLUT4, PGC-1α, and UCPT3 (Supplemental Fig. 2A). Recombinant musclin and retrovirus-mediated expression of musclin protein had no effect on proliferation and differentiation of C2C12 myoblasts, suggesting musclin had no significant effect on myogenesis (data not shown).
shown). These results suggested that the physiological significance of musclin is related to some yet unknown function of fully differentiated myocytes.

We also examined the regulation of musclin mRNA in fully differentiated C2C12 myocytes (day 7). Insulin markedly and dose-dependently augmented musclin mRNA expression (Fig. 3B), but not that of myogenin, a myocyte differentiation marker gene (Supplemental Fig. 2B), suggesting that insulin-stimulated musclin mRNA expression in myocytes was not due to overdifferentiation of myocytes but rather to direct induction of musclin mRNA. IGF-1 also induced musclin mRNA expression (Fig. 3B). On the other hand, epinephrine and /H9252-adrenergic receptor agonist, isoproterenol, counter-regulators to insulin in glucose disposal in skeletal muscles, markedly decreased musclin mRNA expression (Fig. 3C) but not GLUT4 and myogenin (Supplemental Fig. 2C). Forskolin, which increases the intracellular cAMP contents similarly to epinephrine and isoproterenol, markedly and dose-dependently suppressed musclin mRNA expression (Fig. 3D) but not those of GLUT4 and myogenin (data not shown).

Finally, we assessed the biological role of musclin on insulin-stimulated glucose uptake and glycogen synthesis in differentiated C2C12 myocytes using recombinant musclin protein purified from media of stable C2C12 expressing FLAG-tagged musclin (Fig. 3, E and F). The cells were pretreated with 0.5 μg/ml recombinant musclin or FLAG peptide for 5 h and stimulated with phosphate-buffered saline or 100 nM insulin for 30 min (for 2-DG uptake) or 60 min (for glycogen synthesis). Treatment with insulin significantly increased 2-DG uptake and glycogen synthesis (Fig. 3, E and F). Musclin significantly attenuated insulin-stimulated 2-DG uptake (Fig. 3E) and glycogen synthesis at both basal and insulin-stimulated status (Fig. 3F). This 5-h treatment of musclin did not alter the expression levels of GLUT4, GLUT1, and hexokinase II mRNAs (data not shown).

**DISCUSSION**

Based on the hypothesis that skeletal muscles produce nutritionally regulated secretory factors, we identified musclin cDNA using an efficient SST technique. Although the precise physiological significance of musclin has yet to be determined, several important features were defined in the present study. 1) Musclin protein is actively secreted; 2) musclin mRNA is almost exclusively expressed in skeletal muscle; 3) musclin mRNA expression is dynamically regulated by nutritional changes and hormonal factors, especially insulin; 4) musclin mRNA expression is augmented in the skeletal muscle of obese insulin-resistant mice; and 5) recombinant musclin inhibited insulin-stimulated glucose uptake and glycogen synthesis in myocytes. These observations suggest that musclin could func-
tion as an autocrine and paracrine factor in skeletal muscles and that increased production of musclin might be related to insulin resistance in the skeletal muscle of obese mice.

Musclin protein contained a homologous region to the functional 17 amino acids of the NP family, and basic KKKR sequence, a putative serine protease cleavage site. Proteins of the NP family are cleaved at this site and produce COOH-terminal active forms (12). Indeed, in cultures of mammalian cells transfected with muscle cDNA, full-length musclin protein and a trace amount of76KKKR79-dependent cleaved form were secreted from skeletal muscles of 13-week-old KKAy mice and age-matched C57BL/6J mice (n = 8, each) were used for real-time RT-PCR. The values were normalized to the level of cyclophilin mRNA. Data are mean ± S.E. values. *, p < 0.05; **, p < 0.01.

We still do not know whether musclin is secreted into blood as an endocrine factor. Bruning et al. (6) reported that muscle-specific insulin receptor knock-out mice exhibited adipose hyperplasia and increased mass, suggesting insulin-mediated production of muscle-derived secretory factor that decreases adiposity. In preliminary experiments, we noted that adenovirus-mediated production of musclin protein in plasma significantly decreased fat mass in mice. Musclin could be the missing link from skeletal muscles to fat, receiving intense insulin regulation and controlling adiposity, although musclin mRNA expression was low in skinny STZ-treated mice and high in obese KKAy mice.

Conceivably, musclin may transmit important signal(s) to the skeletal muscle itself or to remote organs. Thus, establishment of a reliable method to measure musclin protein level in blood and knockout mice should enhance our understanding of the physiological significance of this muscle-derived secretory factor.

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Addendum—Thomas et al. (15) recently reported an osteoblast-derived factor, osteocrin, which was expressed in embryonic bone but disappeared after birth (15). The protein sequence of musclin seems to be identical to that of osteocrin. In our study, the expression level of musclin mRNA in skeletal muscle was more than 15-fold higher in obese KKAy mice than in 10–12-week-old mice (Fig. 2B) and more than 10-fold higher than in bones of 6-week-old rats (data not shown).

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