Osteoactivin is a type I transmembrane protein upregulated by unloading stresses, including denervation, prolonged bed rest, and space flight, but the regulatory mechanisms of its expression and activation under these conditions remain undefined. Here we report that osteoactivin protein exists in two forms: an intact transmembrane form and a secreted form. The secreted form, the extracellular fragment of osteoactivin, was produced by ectodomain shedding and was released into a culture medium. Amino acid sequence analysis of the carboxy-terminal fragment of osteoactivin (OA-CTF) revealed that cleavage of osteoactivin by proteases occurred both at the cell surface and within the cell membrane. Localization analysis demonstrated translocation of OA-CTF to the nucleus and the endoplasmic reticulum. Moreover, RNA binding proteins, which regulate pre-mRNA splicing, were identified as OA-CTF binding proteins. These results suggest that OA-CTF formed by ectodomain shedding is involved in the regulation of pre-mRNA splicing.

Key words: osteoactivin; ectodomain shedding; translocation; pre-mRNA splicing

Osteoactivin, a type I transmembrane protein, is a rat homolog of the Gpnmb (glycoprotein nonmetastatic melanoma protein B) family, originally reported to be highly expressed in human melanoma cells. Previous studies reported that osteoactivin transcripts increase in various diseases associated with fibrosis, such as osteopetrosis and liver cirrhosis. Furthermore, microarray analysis of the gastrocnemius muscle of space flight rats indicated upregulation of the osteoactivin gene under microgravity conditions. The expression of osteoactivin mRNA is also upregulated in gastrocnemius muscles treated by denervation and tail-suspension, similarly to the findings for space flight, but the regulatory mechanisms of its expression and the physiological function of osteoactivin undefined.

Several type I transmembrane proteins, including Notch and HB-EGF, are activated by proteolysis on the cell surface (ectodomain shedding). Previous studies have established the involvement of metalloprotease disintegrins, ADAM, and certain matrix metalloproteinases (MMPs) in this ectodomain shedding. In addition, ectodomain proteolysis is followed by proteolytic processing by the γ-secretase complex within the transmembrane domain. Furthermore, recent reports indicate that HB-EGF and Notch are cleaved by both ADAMs and γ-secretase and that the released carboxy-terminal fragment of Notch and HB-EGF translocates to the nucleus and acts as a transcriptional regulator. In the present study, we found that ectodomain proteolysis resulted in the formation of intracellular and extracellular osteoactivin fragments. Hence, one can assume that the behavior of the intracellular osteoactivin fragment is similar in Notch and HB-EGF. We were able to identify the site of osteoactivin cleavage using proteases and RNA binding proteins, acting as candidate interacting proteins of the carboxy-terminal osteoactivin fragment (OA-CTF).

Pre-mRNA splicing by RNA binding protein is functionally coupled to transcription, and regulation of it is essential to the survival of cells in biotransformation. Changes in splice site choice are regulated by proteins that bind to the pre-mRNA and affect spliceosome assembly. One of the well-studied protein families of splicing regulatory factors is the SR protein, which plays important roles in both constitutive and alternative splicing and act at various stages of spliceosome assembly. Splicing factor arginine/serine-rich 9 (SFRS9/SRp30c) belongs to the SR family. The heterogeneous nuclear ribonucleoproteins (hnRNPs) are another large group of proteins that bind to nascent pre-mRNAs. HnRNP A1, an hnRNP protein, regulates the transcription, processing of primary transcripts, nuclear export, subcellular localization, and stability of mature mRNA. HnRNPs are also involved in alternative splicing regulation in that they antagonize the activity of the SR family of proteins.

The present study sought to clarify the physiological role of osteoactivin. The results indicate that osteoactivin was activated by shedding and that the released OA-CTF translocated to the nucleus. In addition, OA-CTF interacted with RNA binding proteins. These results are the first to suggest that osteoactivin is a key regulatory protein in pre-mRNA splicing under unloading conditions.
Materials and Methods

Cell lines and culture conditions. HEK293T cells (human embryonic kidney cell line) were purchased from GenHunter (Nashville, TN). COS-7 cells were the kind gift of Dr. Taketani (University of Tokushima). All the cells were maintained and proliferated at 37 °C under 5% CO2/95% air in Dulbecco’s Modified Eagle’s Medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 100 U/mL of penicillin, and 100 μg/mL of streptomycin.

SDS–PAGE and immunoblotting. SDS-8, 12%-polyacrylamide gel electrophoresis (PAGE) was performed under reducing conditions by the method of Laemmli.20) For immunoblotting analysis, proteins in the gels were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 3.5% skim milk in 50 mM Tris–HCl, 150 mM NaCl, pH 7.4 (TBS), and then incubated with primary antibodies for 1 h at 25 °C. The primary antibodies used were as follows: anti-osteoadhesive (R&D Systems, Minneapolis, MN), anti-V5 (Invitrogen, Carlsbad, CA), and anti-FLAG (Sigma, St. Louis, MO) antibody. The bound antibodies were detected using an appropriate secondary antibody and the enhanced chemiluminescence system (ECL) (GE Healthcare UK, Buckinghamshire, UK). Protein concentrations were determined by the BCA method, with bovine serum albumin as standard.

Detection of osteoadhesive cleavage sites. HEK293T cells were transfected with V5-tagged osteoadhesive and cultured for 24 h. The cell lysates were incubated overnight with anti-V5 antibody-conjugated agaroase (Sigma, St. Louis, MO) at 4 °C. After washing, OA-CTF was eluted with 0.1 M glycine-HCl (pH 3.5). The eluant was applied to SDS–PAGE and was detected by staining with silver nitrate by standard procedures. The peptide mixtures were applied to high-performance liquid chromatography (HPLC) on an Atlantis dC18 column (0.075 × 150 mm) (Waters, Milford, MA). Analytical separation was done using a gradient elution with 5% acetonitrile as mobile phase A and 95% acetonitrile as mobile phase B, both containing 0.1% formic acid. After an isotropic step of 5% B for 3 min, this was gradually increased to 95% B in 44 min, and held for 5 min. The flow rate was maintained at 0.2 mL/min−1. HPLC was connected to an ion-trap mass spectrometer equipped with an orthogonal electrospray ionization interface (NanoFlow-LC ESI, positive). MS control, data acquisition, and data analysis were done using Q-ToF Ultima API (Waters/Micromass UK, Manchester, UK). The MS conditions were capillary voltage 2.8 kV, and collision energy 13–65 eV. Mass spectra were registered in m/z 300–1950, and mass/neutral mass in m/z 50–1950. The data obtained were analyzed by Mascot Search (MS/MS Ion Search).

Expression and purification of recombinant proteins. For the expression of GST-fusion proteins, the SFRS9 gene was amplified using a specific primer pair, 5'-TTGAATTCCAGTCGAGCTGCT-3' and 5'-GGCTCGACAGTAGGACC-3', and a specific primer pair for HisRNP A1 gene, 5'-CCGAATTCATGTCGGGCTG-3' and 5'-GGCTCGAGGATTCACTCCT-3'. Both the PCR products were cloned into the XhoI and BglII sites of pGEX 6p-1 vector (GE Healthcare UK). GST fusion proteins were expressed in BL21 cells. After induction with 0.5 mM isopropyl-thiogalactopyranoside (IPTG) (12 h, 20 °C), the cells were sonicated in PBS containing 1% Triton X-100. Purification of the fusion proteins was performed as described using GSTrap FF (GE Healthcare UK). For the expression of His- and Myc-tagged OA-CTF, the OA-CTF gene was amplified using a specific primer pair, 5'-CCGGATTCTGACTCAGAGACG-3' and 5'-GGCTCGAGGATTCACTCCT-3'. The PCR product was cloned into the XhoI/EcoRI site of the pBAD vector (Invitrogen). OA-CTF-myc/His were expressed in Top10 cells (0.002% arabinose, 4 h, 30 °C). For purification of the expressed protein, Ni-NTA affinity chromatography (Invitrogen) was done following the instructions supplied by the manufacturer.

Cellular fractionation. COS-7 cells were transfected with and without plasmids containing carboxyl-terminal-V5-tagged OA-PL, and then cultured for 24 h. They were pre-treated with 50 μg/mL of cycloheximide for 2 h. Then they were fractionated with a ProteoExtract® Subcellular Proteome Extraction Kit, Mini (Calbiochem, San Diego, CA). The extracted proteins (15 μg/lane) were subjected to Western blotting. Protein concentrations were determined by the BCA method with bovine serum albumin as standard.

Osteoadhesive is released by proteolytic cleavage at both the cell surface and the intramembrane

A number of type I transmembrane proteins have an active form after ectodomain shedding on the cell surface.6,14) First, we examined to determine whether the osteoadhesive protein, a type I transmembrane protein, can also shed due to proteases. As shown in Fig. 1A and B, intact transmembrane forms of osteoadhesive with molecular masses of 97 and 116 kDa were detected in the cell lysates, and secreted forms with molecular masses of 90 and 100 kDa were detected in the

Detection of proteins co-immunoprecipitated with OA-CTF. The nuclear proteins obtained from FLAG-tagged OA-CTF-expressing cells were incubated overnight with anti-FLAG antibody-conjugated agaroase (Sigma) at 4 °C. After washing, OA-CTF was eluted with 0.1 M glycine-HCl (pH 3.5). The eluant was applied to SDS–PAGE, and the target proteins were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS).

Liquid chromatography–tandem mass spectrometry (LC-MS/MS). The purified FLAG-tagged OA-CTF and OA-CTF-binding proteins were subjected to SDS–PAGE and were detected by staining with silver nitrate by standard procedures. The peptide mixtures were applied to high-performance liquid chromatography (HPLC) on an Atlantis dC18 column (0.075 × 150 mm) (Waters, Milford, MA). Analytical separation was done using a gradient elution with 5% acetonitrile as mobile phase A and 95% acetonitrile as mobile phase B, both containing 0.1% formic acid. After an isotropic step of 5% B for 3 min, this was gradually increased to 95% B in 44 min, and held for 5 min. The flow rate was maintained at 0.2 mL/min−1. HPLC was connected to an ion-trap mass spectrometer equipped with an orthogonal electrospray ionization interface (NanoFlow-LC ESI, positive). MS control, data acquisition, and data analysis were done using Q-ToF Ultima API (Waters/Micromass UK, Manchester, UK). The MS conditions were capillary voltage 2.8 kV, and collision energy 13–65 eV. Mass spectra were registered in m/z 300–1950, and mass/neutral mass in m/z 50–1950. The data obtained were analyzed by Mascot Search (MS/MS Ion Search).
was the position between Arg points were identified as candidate cleavage sites. One phenylthiohydantoin (PTH)-amino acid, two major were identified. In particular, based on the yield of OA-CTF. As shown in Fig. 1C, three shedding points of osteoactivin by amino acid sequence analysis of OA-CTF. As described in “Materials and Methods.” The amino acid sequences of OA (Ala481-Lys525) are lined up at the top and the results of amino acid sequences are presented at the bottom. Identical sequences are aligned by asterisks. Bold letters of the amino acid sequences in the top panel (Ala481-Leu522) show the putative trans-membrane region. Cleavage sites are indicated by arrows. The two bold arrows at cleavage site indicate higher amino acid yields than the thin arrows. WB, Western blotting; CL, cell lysate; SFCM, serum-free culture medium; MMSTD, molecular mass standards.

Fig. 1. Osteoactivin Is Released by Proteolytic Cleavage Both at the Cell Surface and within the Cell Membrane.

A, HEK293T cells were transfected with plasmids containing V5-tagged OA-FL and cultured for 24 h under serum-free conditions. Cell lysates (lane 1) and conditioned media (lanes 2 and 3) were subjected to Western blotting using anti-V5 (lanes 1 and 2) and anti-OA antibodies (lane 3). The secreted form and OA-CTF are indicated by the arrows. B, Silver staining image of the OA-CTF. The intact full-length form (OA-FL) and OA-CTF are marked by the arrows. C, Amino acid sequence analysis of OA-CTF as described in “Materials and Methods.” The amino acid sequences of OA (Ala481-Lys525) are lined up at the top and the results of amino acid sequences are presented at the bottom. Identical sequences are aligned by asterisks. Bold letters of the amino acid sequences in the top panel (Ala481-Leu522) show the putative trans-membrane region. Cleavage sites are indicated by arrows. The two bold arrows at cleavage site indicate higher amino acid yields than the thin arrows. WB, Western blotting; CL, cell lysate; SFCM, serum-free culture medium; MMSTD, molecular mass standards.

Discussion

Osteoactivin transcripts increase in various diseases associated with fibrosis, including osteopetrosis and liver cirrhosis, but the role of osteoactivin is poorly understood. In the present study, first we determined the cleavage of osteoactivin at both the cell surface and in
Although several proteases may be involved in this proteolysis, MMP or a disintegrin and metalloproteases (ADAM) mainly mediate the process.\(^{8,9}\) In addition, \(\gamma\)-secretase complex, an intramembrane protease, also mediates the shedding process.\(^{13}\) Recent studies have reported that ADAM10 is a candidate sheddase that releases osteoactivin ectodomain from the cell surface,\(^{21}\) but as shown in
Characteristics of an Osteoactivin Carboxyl-Terminal Fragment

Fig. 1C, the cleavage site of osteoactivin is located at both the cell surface and within the membrane. Therefore, not only ADAM10 but also γ-secretase may be involved in the ectodomain shedding of osteoactivin.

In the present study, we also found that OA-CTF appeared to internalize and translocate to the nucleus or involved in the ectodomain shedding of osteoactivin. These reports suggest that OA–CTF as well as Notch and HB-EGF also interact with certain proteins and have certain roles in the nucleus. Unlike Notch and HB-EGF, osteoactivin interacted with RNA binding proteins, which are involved in pre-mRNA splicing. Orvain et al. have reported that transcription factor c-Myb influences alternative pre-mRNA splicing via interactions with splicing factor U2AF and hnRNP A1. The present study suggests that OA-CTF affect alternative pre-mRNA splicing by interacting with prerNA splicing factor SFRS9 and hnRNP A1 based on the finding of interactions between OA-CTF and both SFRS9 and hnRNP A1 (Fig. 4). In contrast to the interaction between OA-CTF and SFRS9, the interaction between OA-CTF and hnRNP A1 appeared to be weak. HnRNP A1 can increase binding affinity to partner proteins in the presence of mediating proteins. Therefore, some mediating protein might exist for hnRNP A1 to bind more firmly to OA-CTF.

Previous studies have documented the functions of the SR protein and hnRNPs A1 in muscles. Chalfant et al. reported that phosphorylated SR protein acts through the phosphatidylinositol 3-kinase pathway via insulin-regulated alternative splicing of PKCβII mRNA in the skeletal muscle. In another study, phosphorylation of hnRNP A1 led to translocation of mRNA-bound hnRNP A1 from the nucleus to the cytoplasmic stress granules (SGs) during stress. The SGs have cytoplasmic domains that harbor translationally arrested mRNAs that accumulate in cells exposed to a broad range of stresses, including oxidative, genotoxic, hyperosmotic, and heat shock. These reports suggest that phosphorylation of RNA binding proteins is required for them to function. Hence, the function of OA-CTF appears to be regulated by phosphorylated SR proteins or hnRNPs.

In summary, here we report three major findings concerning osteoactivin expression and function. First, osteoactivin upregulated by unloading stresses was cleaved by proteases both at the cell surface and within the cell membrane. Secondly, proteolytic release of OA-CTF resulted in translocation to the nucleus. Thirdly, OA-CTF interacted with SR proteins or hnRNPs that belong to pre-mRNA splicing factors. Although further studies are required, OA-CTF appears to regulate pre-mRNA splicing under microgravity conditions.

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