Osteoclast-stimulating Factor Interacts with the Spinal Muscular Atrophy Gene Product to Stimulate Osteoclast Formation*

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We have recently identified and cloned an intracellular peptide termed osteoclast-stimulating factor (OSF) that increases osteoclast (OCL) formation and bone resorption through a cellular signal transduction cascade, possibly through its interaction with c-Src or related family members. To further identify participants in the OSF signaling cascade, we used yeast two-hybrid screening with Saccharomyces cerevisiae, and we found that the 40-kDa spinal muscular atrophy disease-determining gene product, survival motor neuron (SMN), interacts with the OSF-Src homology 3 domain. Reverse transcription-polymerase chain reaction analysis of SMN mRNA expression in cells of the OCL lineage demonstrates that expression of the exon 7 splice variant of SMN is restricted to mature OCLs, whereas the unspliced transcript was expressed in OCL precursors as well as mature OCLs. Treatment of murine bone marrow cultures with conditioned media (5% v/v) from 293 cells transiently expressing the SMN cDNA significantly increased OCL formation, compared with treatment with conditioned media from mock-transfected cells. Furthermore, OCL-stimulatory activity by OSF or SMN was abolished by antisense constructs to SMN or OSF, respectively. These data confirm the participation of SMN in the OSF-enhanced expression of an OCL stimulator. OSF-SMN interaction may provide more insights into novel cellular signaling mechanisms that may play an important role in congenital bone fractures associated with type I spinal muscular atrophy disease.

The osteoclast (OCL)† is the primary bone-resorbing cell (1). Recent evidence suggests that factors produced by OCLs themselves play an important role in regulating OCL formation and activity (2). Using an expression cloning approach with a human OCL cDNA library, we previously identified annexin II and OIP-1/hSca as autocrine/paracrine factors that regulate OCL formation and activity (3, 4). Recently, using this approach, we also identified a novel 28-kDa intracellular peptide termed osteoclast-stimulating factor (OSF) that indirectly enhances OCL formation and bone resorption through a cellular signal transduction cascade, possibly through its interaction with c-Src or related family members (5). OSF is highly expressed in OCL and contains structural domains such as SH3 and ankyrin repeats, which are potentially involved in protein-protein interactions. OSF is ubiquitously expressed analogous to other regulators of OCL activity such as c-Src or transforming growth factor β. We have recently assigned the OSF gene (OSTF1) to human chromosome band 12q24.1 by fluorescent in situ hybridization (6).

Local factors produced by stromal cells or osteoblastic cells in the bone microenvironment play an important role in OCL differentiation. Recent evidence supports a central role for tumor necrosis factor gene family members receptor activator of nuclear factor κB (RANK) ligand and RANK receptor in OCL differentiation (7). Transcription factors such as PU.1 and Foxo3a deficiency caused osteopetrosis in mice (8, 9). Recently, an OCL-derived zinc finger protein, OCZF, a possible transcriptional repressor, has been shown to be involved in osteolastogenesis (10). Therefore, intracellular signals in response to external stimuli play an important role in inducing OCL differentiation. Although, Src tyrosine kinase is ubiquitously expressed and has been implicated in a wide variety of signal transduction pathways, Src-deficient mice showed an osteoprotic phenotype (11). It has also been reported that distinct ligand preferences for c-Src/SH3 domains mediate protein-protein interactions through binding of short proline-rich regions in the ligand proteins (12). High affinity binding of OSF with c-Src in vitro suggested a potential role for OSF in the OCL bone resorption process through protein interaction with the cellular signal transduction machinery (5). In addition, the presence of ankyrin repeats in OSF further suggested potential interaction with other cellular proteins. Ankyrin repeats are present in proteins involved in cell cycle and tissue differentiation. These motifs have been shown to be confined to specific membrane domains and bind integral membrane proteins such as Na⁺- and K⁺-ATPase and the voltage-dependent Na⁺ channel in hematopoietic cells (13). Therefore, it is important to identify OSF-interacting proteins in addition to c-Src to unravel the novel signaling cascade that results in the secretion of soluble stimulator(s) of OCL formation and activity. In the present study, we used yeast two-hybrid screening and identified the spinal muscular atrophy (SMA) disease-determining gene product, SMN protein (40 kDa), and its interaction with the OSF-SH3 domain. We further characterized SMN expression in OCL and its participation in OSF-induced OCL formation.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screening

An OSF cDNA encoding the 57 amino acids that span the SH3 domain was PCR-amplified with the EcoRI and NcoI restriction enzyme sites flanking the 5′ and 3′ end, respectively. A bait construct was then

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The abbreviations used are: OCL, osteoclast; OSF, osteoclast-stimulating factor; SMA, spinal muscular atrophy; SMN, survival motor neuron; MNC, multinucleated cell; TRAP, tartrate-resistant acid phosphatase; 1,25-(OH)₂D₃, 1,25-dihydroxy vitamin D₃; PCR, polymerase chain reaction; GST, glutathione S-transferase; SH, Src homology; RANK, receptor activator of nuclear factor κB.
prepared by subcloning the OSF-SH3 domain in frame at the carboxyl terminus of the GAL4 DNA-binding domain in the pGBT8 vector (PharMingen, San Diego, CA). The resulting bait plasmid (OSF Hyb SH3#3) was subjected to yeast two-hybrid screening using a HeLa cell cDNA library generated by directional cloning into the EcoRI/XhoI sites located at the carboxyl terminus of the GAL4 activation domain or open reading frame of the pGAD-GH prey vector. Both the bait and prey plasmids were cotransformed by electroporation into the Y166 yeast strain containing a lacZ reporter gene whose expression was controlled by upstream binding sites of the GAL1 promoter (14). The yeast transformants were grown on agar plates containing Trp, His, and Leu dropout nutritional selection media in the presence of 50 μg/ml 3-amino-1,2,4-triazole and assayed for lacZ activity. The prey plasmid containing HeLa cell cDNA, which interacted with the OSF bait plasmid in the lacZ-positive yeast clones, was segregated by transforming it into the MH4 Esherichia coli strain and selected on Leu dropout culture plates. The OSF-SH3 domain-interacting prey plasmid was then isolated and subjected to DNA sequence analysis.

In Vitro Protein Affinity Binding Assay

Glutathione-Sepharose beads were coupled with purified GST or E. coli-derived GST-SMN fusion proteins. GST or GST-SMN fusion proteins (2 μg) were incubated with total cell lysates (500 μg of protein) of 293 cells that were transiently expressed with OSF cDNA at 4 °C for 2 h in binding buffer (1% Triton X-100, 20 mM Tris, pH 7.4, 10% glycerol, 100 mM NaCl, 1 mM EDTA, and 0.1% bovine serum albumin). The samples were spun down briefly, washed four times with the same buffer, and subjected to SDS-polyacrylamide gel electrophoresis (15% gel). Western blot analysis was then performed using rabbit antisera raised against recombinant OSF at a 1:5000 dilution as described previously (5).

Immunoprecipitation of OSF with an Anti-SMN Antibody

The OSF cDNA was transiently expressed in 293 cells, and total cell lysates were prepared using lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1.5 mM MgCl2, 1 mM EDTA, 200 μM DTT, 200 μM phenylmethylsulfonyl fluoride, and 1 mg/ml aprotinin). The cell extract (500 μg of protein) was incubated with 1 μg of an anti-SMN antibody for 1 h, and the immune complex was separated using a protein G-agarose conjugate. A control immunoprecipitation using mouse IgG was run in parallel. The immune complex was washed four times with buffer containing 1% Triton X-100, 20 mM Tris, pH 7.4, 150 mM NaCl, and 1 mM EGTA and subjected to SDS-polyacrylamide gel electrophoresis (15% gel). Western blot analysis was performed using OSF antisera as described previously (5).

Reverse transcription-PCR Analysis

Approximately 2 μg of total RNA isolated from highly purified OCLs and OCL precursor cells using RNAzol reagent (Biotex, Houston, TX) (15) was reverse transcribed with random hexamers and avian myeloblastosis virus reverse transcriptase. The resulting cDNAs were then amplified by PCR using 0.15 μM concentrations of forward (5′-CTCCATATGTCGACATCTCCTTAT-3′) and reverse (5′-ACTGCTTCAACCACCGTGCTGCTGG-3′) SMN gene-specific primers in a final volume of 100 μl containing 2 units of Taq DNA polymerase (PerkinElmer Life Sciences), 2 mM MgCl2, 50 mM KCl, and 10 mM Tris-HCl (pH 8.3). The PCR reaction was carried out by incubating the samples at 94 °C for 1 min, followed by 35 cycles of 94 °C for 1 min and 60 °C for 1 min, with a final extension for 5 min at 60 °C. The amplified products were electrophoresed on a 1.2% agarose gel with a 123-bp base pair DNA ladder (Life Technologies, Inc.) as a size marker. The bands were visualized by ethidium bromide staining.

Osteoclast Cultures

Osteoclast Precursors—Human bone marrow-derived mononuclear cells were cultured in 0.8% methyl cellulose in the presence of recombinant human granulocyte macrophage colony-stimulating factor (1 ng/ml) for a 1-week period to form early OCL precursors. These cultures were then overlaid with 10−9 M 1,25-(OH)2D3 and cultured for an additional week to induce the formation of more differentiated or late OCL precursors. Late OCL precursors were purified from these cultures using the 23E6 monoclonal antibody, which recognizes the OCL vitronectin receptor (16).

Osteoclast Formation: Human Bone Marrow Culture Assay—Human bone marrow-derived mononuclear cells (105 cells/ml) were cultured in a 96-well plate for 3 weeks in the presence of 10−9 M 1,25-(OH)2D3 to form OCL-like cells. At the end of the culture period, OCL-like cells formed in these long-term marrow cultures were isolated using the 23E6 anti-vitronectin antibody as described previously (17).

Mouse Bone Marrow Culture Assay

Bone marrow from C57 black mouse tibiae that had been aseptically removed was obtained by flushing the tibiae with 1 ml of a-minimum Eagle's medium using a tuberculin syringe fitted with a 27.5-gauge needle. The bone marrow-derived cells were washed twice, resuspended in a-minimum Eagle's medium-10% fetal calf serum, and depleted of plastic-adherent cells by incubating the marrow cell suspension in sterile 10-cm tissue culture dishes for 2 h. The nonadherent marrow cells were collected and cultured in quadruplicate for 8 days in 48-well plates at a density of 5 × 104 cells/well in a-minimum Eagle's medium-10% fetal calf serum supplemented with 10−9 M 1,25-(OH)2D3 as described by Takahashi et al. (18). The cultures were fixed with 4.5% formaldehyde, and acetone and washed twice in distilled water. The cultures were then stained for tartrate-resistant acid phosphatase activity using an acid phosphatase staining kit (Sigma). The tartrate-resistant acid phosphatase-positive MNCs containing three or more nuclei were scored with an inverted microscope.

Immunostaining of SMN

Human bone marrow-derived OCL-like multinucleated cells were cultured as described above. At the end of culture period, the cells were fixed in Bouin's solution for 12 h. Cells were permeabilized by adding cold phosphate-buffered saline containing 0.5% bovine serum albumin and 0.1% Triton X-100 for 15 min. After preincubation in 1% bovine serum albumin, monoclonal antibody 2B11, which recognizes human SMN (19), was added (diluted 1:200 in phosphate-buffered saline and 1% bovine serum albumin) overnight at 4 °C. The cells were washed and then incubated with peroxidase-conjugated rabbit anti-mouse IgG1 (1:200) for 1 h. Peroxidase activity was detected by 3,3′-diaminobenzidine in the presence of 0.1% hydrogen peroxide as described previously (17).

RESULTS

Identification of OSF-SMN Protein Interaction by Yeast Two-hybrid Screening—To identify proteins that interact with the OSF-SH3 domain, we used the yeast two-hybrid screening of a HeLa cell cDNA library in Saccharomyces cerevisiae. The basic scheme of yeast two-hybrid assay using a bait plasmid containing the OSF-SH3 domain and prey plasmid containing SMN cDNA isolated from a HeLa cell cDNA library and the GAL1 promoter region driving lacZ reporter gene expression in Y166 yeast strain used in two-hybrid assay. GAL1 promoter upstream activating sequence (GAL-1 UAS) drives expression of the lacZ reporter plasmid in Y166 yeast strain. The bait plasmid construct contains OSF-SH3 domain in frame with the GAL4 DNA-binding domain (GAL 4 DBD). The prey plasmid containing SMN fusion in the GALA activation domain (GAL 4 AD) was isolated by yeast two-hybrid screening of a HeLa cell cDNA library. The bait and prey interaction was performed in yeast Y166 strain as described under “Experimental Procedures.” B, isolation and identification of SMN clone. Y166 yeast transformants with the OSF SH3 bait plasmid and HeLa cell-derived prey plasmid library were cultured on agar plates containing Trp, His, and Leu dropout nutritional selection media in the presence of 50 μM 3-amino-1,2,4-triazole. A putative candidate clone was replica plate-lifted onto nitrocellulose filter paper and stained for lacZ activity. The prey plasmid was segregated in MH4 E. coli strain and identified as the SMA disease-determining gene product SMN.
yeast strain is schematically represented in Fig. 1A. Two-hybrid screening in the Y166 yeast strain resulted in the identification of a putative candidate prey plasmid from a HeLa cell cDNA library that interacts with the OSF-SH3 bait, resulting in expression of lacZ activity (Fig. 1B). The prey plasmid isolated from the lacZ-positive yeast clone upon sequence analysis further identified the cDNA (1 kb) encoding the SMA disease-determining gene product, SMN protein, which contains 294 amino acids with an apparent molecular mass of 40 kDa.

In Vitro Affinity Binding of OSF with SMN—The interaction between OSF and SMN was further confirmed by in vitro binding of OSF with E. coli-derived GST-SMN fusion protein. As evident from Fig. 2A, OSF demonstrated binding to the GST-SMN fusion protein. However, GST alone did not bind OSF in vitro. We then tested the in vitro affinity binding of OSF to GST-SMN exon deletion mutant proteins to identify the functional domains of SMN involved in OSF interaction. OSF demonstrated affinity binding with GST-SMN mutant proteins containing exons 6 and 7 alone. In contrast, GST-SMN mutant protein containing the exon 1–4 region did not demonstrate affinity binding with the OSF in vitro.

We also confirmed the OSF-SMN interaction by communoprecipitation using an anti-SMN antibody. OSF was communoprecipitated from 293 cell lysates with an anti-SMN antibody. In contrast, a control IgG did not demonstrate communoprecipitation of OSF (Fig. 2B). We previously reported OSF affinity binding to c-Src SH3-SH2 domain (5). However, anti-SMN antibody did not demonstrate communoprecipitation of c-Src from 293 cell lysates (data not shown). These data further confirm the specificity of SMN interaction with OSF.

SMN Expression in OCL Lineage—Expression of SMN protein in OCL-like cells formed in long-term human bone marrow cultures was examined by immunocytochemistry. Immunostaining using the 2B1 mouse anti-human SMN monoclonal antibody confirmed the expression of SMN protein in OCL-like cells and mononuclear cells (Fig. 3). The control IgG did not show a positive reaction in these cells, confirming the specificity of SMN expression in OCL. It has been reported that the SMN mRNA demonstrates exon 7 splicing and that the relative levels of native and splice variant forms correlated with the severity of the SMA disease. Therefore, we used reverse transcription-PCR analysis to examine SMN mRNA expression in cells of the OCL lineage using sense and antisense primers flanking exons 6 and 8, respectively (20). As shown in Fig. 4, total RNA isolated from highly purified early OCL precursors (CFU-GM), more differentiated OCL precursors, and mature OCLs demonstrated expression of the SMN mRNA containing exons 6, 7, and 8. However, only mature OCLs expressed the exon 7 splice variant of SMN mRNA. The native SMN mRNA appeared to be more abundant compared with the exon 7 splice variant. This suggests that the exon 7 splice variant of SMN may have a functional role in mature OCLs.

Participation of SMN in OSF-enhanced OCL Formation—To determine whether SMN could participate in the production of an OCL stimulator, 293 cells were transiently expressed with SMN cDNA. Conditioned media collected from these cells were tested at 0–10% concentrations for their effects on OCL formation in mouse bone marrow cultures. Treatment of mouse marrow cultures with SMN-conditioned media at a 5% concentration significantly increased tartrate-resistant acid phosphatase-positive MNCs and immunocytochemical staining was performed using rabbit antisera raised against recombinant OSF at a 1:5000 dilution. Western blotting using rabbit antisera raised against recombinant OSF at a 1:5000 dilution.
Recently, using an expression cloning approach, we have identified and cloned OSF, a novel intracellular protein produced by OCL that indirectly enhances OCL formation and bone resorption (5). It is a 28-kDa peptide that contains a c-Src homology 3 domain and ankyrin repeats, suggesting that it is involved in intracellular signaling. Furthermore, recombinant OSF demonstrated high affinity binding to c-Src, an important regulator of OCL activity (21). This interaction of OSF with c-Src results in secretion of soluble stimulator(s) of OCL formation and activity.

**DISCUSSION**

OSF interacts with SMN, a protein that binds to the SMN protein carboxyl terminus (amino acids 240–267). Although SMN itself had only weak antiapoptotic activity (25). In addition, SMN has been shown to interact with Bcl-2, and coexpression of SMN with Bcl-2 prevented Bax-induced or Fas-mediated apoptosis, although SMN itself had only weak antiapoptotic activity (25). In addition, SMN has been shown to interact with the SIP-1 protein complex (26). Although the exact mechanisms for SMN-SIP-1 nuclear translocation are unclear, it is evident that the SMN-SIP-1 complex is directly involved in the biogenesis of spliceosomal small nuclear ribonucleoproteins. It is also unclear whether SMN could directly participate in gene transcription upon translocation to nucleus.

**OSF-\(\text{SH}3\) domain. In vitro affinity binding studies further mapped OSF binding to the SMN exon 6 region. Liu et al. (22) have previously identified that the P2 peptide region of the SMN protein carboxyl terminus (amino acids 240–267) was involved in the interaction with the Sm proteins (small nuclear ribonucleoprotein core protein). Coimmunoprecipitation of OSF with an anti-SMN antibody further confirmed the specificity of OSF-SMN interaction. We had previously identified OSF interaction with the c-Src SH3-SH2 domain (5). However, c-Src does not immunoprecipitate with an anti-SMN antibody (data not shown), further suggesting that OSF may play a central role in the cellular signal transduction cascade leading to the release of soluble factor(s) that stimulates osteoclast formation and activity.

SMA disease is the second most common fatal autosomal recessive disorder (23). Although SMA disease is characterized primarily by the degeneration of lower motor neurons, it also involves congenital bone fractures (24). Immunocytochemical staining confirmed the expression of SMN in OCL. Therefore, the OSF interaction with SMN may have a potential role in OCL formation and bone resorption. Recently, SMN has been shown to interact with Bel-2, and coexpression of SMN with Bel-2 prevented Bax-induced or Fas-mediated apoptosis, although SMN itself had only weak antiapoptotic activity (25). In addition, SMN has been shown to interact with the SIP-1 protein complex (26). Although the exact mechanisms for SMN-SIP-1 nuclear translocation are unclear, it is evident that the SMN-SIP-1 complex is directly involved in the biogenesis of spliceosomal small nuclear ribonucleoproteins. It is also unclear whether SMN could directly participate in gene transcription upon translocation to nucleus.

**The present study begins to identify other signaling molecules that might interact with OSF. Because OSF expression is ubiquitous, and a yeast two-hybrid OCL cDNA library is not available at present, we have performed yeast two-hybrid screening using a HeLa cell cDNA library to identify proteins that interact with OSF. We thus identified the SMN disease-determining gene product SMN as a protein that binds to the SMN gene is located on chromosome 5q13, and the mRNA analysis demonstrated splicing of exon 7, which contains 16 amino acids (20). However, a role for the exon 7 splice variant of SMN is not yet known. Although SMN is ubiquitously expressed, differential expression of the exon 7 splice variant of SMN in mature OCLs may have a potential role in OCL activity. It has been reported that the SMN oligomerization defect that results from increased expression of the SMN exon 7 splice variant correlates with the severity of SMA disease (25). Furthermore, exon 7 C to T transition at codon 280, a translationally silent variation, is sufficient to dictate exon 7 alternative splicing (27). Mouse SMN is 82% identical to the human pro-
tein. The mouse genome contains a single copy of SMN, in contrast to human genome, which contains two copies. SMN does not appear to be alternatively spliced in mouse tissues (28). Furthermore, mice with homozygous SMN disruption display massive cell death during early embryonic development, indicating that the SMN is necessary for cellular survival and function (29). More recently Monani et al. (30) have shown that SMN2 gene expression in SMN(−/−) mice rescued embryonic lethality and that the rescued mice had spinal muscular atrophy. Our data further suggest SMN participation in OSF-induced OCL formation. SMN-conditioned media stimulate OCL formation analogous to OSF, and OCL-stimulatory activity can be blocked by antisense constructs to OSF or SMN, respectively. These data confirm the participation of SMN in the expression of an OCL stimulator through an interaction with the OSF. At present, the identity of downstream targets and soluble factor(s) released in response to OSF-SMN signaling is unknown. However, SMN may directly influence the basic transcription machinery through interaction with specific transcription factors that would induce preferential osteotropic factor(s) gene expression. Recently, it has been shown that the profilins bind to and colocalize with SMN in nuclear gems and that this interaction may be involved in the neuron-specific effects associated with SMA disease (31). Other proteins reported to interact with SMN include transcription factor E2 (nuclear transcription activator of papilloma virus) and for upstream (FUSE) binding protein (32, 33). However, there are no published data showing that these protein interactions and functions are affected by the low SMN levels that exist in SMA patients or in the SMA mouse. It is possible that the deletions or point mutations that occur in many SMA patients and encompass exons 6 and 7 of the SMN protein may influence the OSF-SMN signaling mechanism. In a previous study, neutralizing antibodies to known osteotropic factors such as interleukin 1, interleukin 6, and tumor necrosis factor α did not block OSF-induced osteoclast-stimulatory activity (5). However, we cannot exclude the possibility that other osteotropic factors such as parathyroid hormone, macrophage colony-stimulating factor, and RANK ligand may be responsive to OSF-SMN cellular signaling. Although our data indicate expression of SMN2 in osteoclasts, the interaction of SMN2 with OSF and its functional role in associated signaling are unknown at present. Therefore, SMN2-rescued SMN(−/−) mice would be a potential model to study the role of OSF and SMN in osteoclast development and bone resorption.

Taken together, our data suggest that OSF interaction with SMN may play an important role in a novel signaling cascade that induces stimulus(s) of OCL formation. Therefore, OSF-SMN interaction may provide more insights into cellular signaling mechanisms that lead to congenital bone fractures associated with type I SMA disease.

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