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Identification of a 22 bp DNA *cis* Element that Plays a Critical Role in Colony Stimulating Factor 1-Dependent Transcriptional Activation of the SPHK1 Gene

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Abstract

Sphingosine-1-phosphate (S1P) is an anabolic clastokine. Colony Stimulating Factor 1 (CSF1) induces expression of the rate limiting enzyme required for S1P synthesis, sphingosine kinase 1 (SPHK1) in bone in vivo, and in osteoclasts in vitro. To study the mechanism of CSF1-induced SPHK1 gene expression, a 2608 bp fragment of the murine SPHK1 gene (– 2497 to + 111 bp relative to the transcription start site) was cloned and transfected into pZen cells (murine fibroblasts engineered to express c-fms). SPHK1 promoter activity was assessed using a dual-luciferase reporter assay system. By analyzing a series of 5′-deletions, a CSF1-responsive region was identified in the region – 1250 to – 1016 bp. To define putative DNA binding site(s) in this fragment, two biotin-labeled fragments that completely overlapped this region were generated, one 163 bp in length (– 1301 to – 1139) and one 169 bp in length (– 1157 to – 989). EMSAs revealed the 163 bp fragment as the target for protein binding. Using EMSAs, the nuclear protein binding region was further narrowed to an 85 bp fragment, (– 1223 to – 1139). Using a series of unlabeled DNA sequences as “cold competitors” in EMSAs, a 22 bp sequence is identified as the smallest fragment that could successfully compete away protein binding. The same 22 bp sequence also competed DNA binding in EMSAs using nuclear protein isolated from primary murine osteoclasts. A full-length wild-type SPHK1 promoter and an SPHK1 promoter in which the ATGGGGG motif was mutated were subsequently expressed in pZen cells. Mutating this ATGGGGG motif nearly completely abrogated the ability of CSF1 to activate the promoter. Although two transcription factors, KLF6 and Sp1 have been reported to bind to this sequence, supershift EMSAs failed to detect either among the proteins bound to the 85 bp DNA fragment.

Keywords

CSF1; SPHK1; Bone formation; Osteoclasts; Transcription factor

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Conflict of interest Gang Qing Yao, Meiling Zhu, Joanne Walker and Karl Insogna declare no conflict of interest.

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Introduction

Colony Stimulating Factor 1 (CSF1) is a key regulator of osteoclastogenesis, but a role for this cytokine in bone anabolism has not been well studied. However, transgenic mice with targeted overexpression of CSF1 in osteoblasts and mice receiving daily injections of CSF1 have increased bone formation rates [1, 2]. The mechanisms underlying this anabolic effect are not known.

In vivo and in vitro evidence indicate that Sphingosine-1-Phosphate (S1P) is a clastokine that can augment osteoblast function [3, 4]. S1P is a phospholipid with pleotropic effects on growth and differentiation that is generated by metabolism of ceramide to sphingosine [5]. Sphingosine is phosphorylated to S1P by one of two sphingosine kinase isoforms, SPHK1 and SPHK2. SPHK1 acts at the cell membrane to generate S1P for export from the cell as a signaling molecule [5], while SPHK2 is localized to the endoplasmic reticulum, the nucleus, and mitochondria [5]. SPHK1 can be activated either transcriptionally or post-transcriptionally by phosphorylation at S²²⁵. Phosphorylation of S²²⁵ is dependent on the actions of ERK1/2 [5, 6].

S1P stimulates the differentiation and survival of osteoblasts [7, 8] as well as osteoblast function [7, 9]. Importantly, two recent in vivo experiments support the hypothesis that S1P production by osteoclasts acts as an anabolic clastokine. Lotinun et al. found that selective deletion of cathepsin K in osteoclasts resulted in an increase in bone formation rates in these animals [3]. This was due to increased expression of SPHK1 and S1P production by these cells. Media conditioned by cathepsin K^{-/-} osteoclasts induced osteoblast differentiation that could be blocked by interdicting S1P signaling in osteoblasts [3]. Keller et al. reported that selective deletion of the calcitonin receptor in osteoclasts did not increase osteoclast number but did lead to an increase in rates of bone formation in vivo [4]. Expression of Spinster homology 2 (the S1P exporter) was higher in osteoclasts lacking the calcitonin receptor than in wild-type cells, suggesting that this was the mechanism for increased S1P secretion by these cells. Further, they showed that S1P-induced expression of collagen 1 α 1 and bone sialoprotein in osteoblasts, and inhibiting ERK1/2, prevented this effect.

It has recently been reported that deleting the receptor for CSF1, c-fms, in mature osteoclasts significantly attenuated the anabolic response to PTH [10], suggesting a role for CSF1 in this process. One pathway by which CSF1 could participate in the anabolic response to PTH might be via a paracrine loop in which single daily doses of PTH induce osteoblasts to release CSF1 that binds c-fms in osteoclasts, inducing production of the anabolic clastokine S1P by those cells. The fact that CSF1 signals in part through ERK1/2 [11], a known regulator of SPHK1 expression, is consistent with that hypothesis. In this manuscript, we describe a *cis* element in the SPHK1 promoter that is required for CSF1-induced transcription of that gene.

Materials and methods

Cells

The pZen cell line [12] was a generous gift from Dr. Larry Rohrschneider. pZen cells were routinely grown in α -MEM (Sigma-Aldrich, St. Louis, MO) supplemented with 10% FBS (Atlanta Biologicals, Lawrenceville, GA) and 1% penicillin/streptomycin (GIBCO, Grand Island, NY). To prepare osteoclasts, bone marrow was harvested from C57 BL/6J mice and cultured overnight in α -MEM containing 10% FBS. Non-adherent cells were harvested, layered onto an equivalent volume of Phycol-Hypaque and centrifuged at $700\times g$ for 20 min. Cells at the interface were collected, washed twice in PBS, and plated at a final concentration of 4×10^5 cells/well in 6-well plates in α -MEM containing 10% FBS, 50 ng/mL CSF1 and 100 ng/mL RANKL for 5 days to induce osteoclasts. At day 5, osteoclasts were washed twice in PBS, then treated with 200 ng/mL CSF1 and 100 ng/mL RANKL or, as a control, 50 ng/mL CSF1 and 100 ng/mL RANKL in fresh α -MEM containing 10% FBS for 24 h.

CSF1 Treatment In Vivo

Two groups of C57/BL6J male mice (12 weeks old, 6 animals per group) received intraperitoneal injections of either 100 ng of CSF1 or PBS as a control. Twenty-four hours later, mice were sacrificed, femurs were stripped of soft tissue, and bone marrow was removed by spinning in an Eppendorf microfuge. The isolated femurs were placed in prechilled microtubes (1.5 mL Eppendorf Safe-Lock) containing 1 mL of Tri Reagent (Sigma-Aldrich, St Louis, MO) and two stainless steel beads (5 mm, Qiagen, Valencia CA). The bones were disrupted using the Tissue Lyser II (Qiagen) at an oscillation frequency of 30/s for 1.50 min. The pulverized bone material was placed on ice and 200 μ L of chloroform was added to each sample. Samples were then mixed and microfuged for 15 min. at 13,800 g. Following centrifugation, RNA was isolated from the resulting bone lysate using an RNeasy Plus Mini Kit (Qiagen, Valencia CA) following the company's instructions.

qPCR

cDNA was synthesized from osteoclasts and bone RNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Taqman probes and primer sets for mouse SPHK1(Mm00473044), SPHK2(Mm01195726), and β -glucuronidase (Mm00446953) were purchased from Applied Biosystems (Foster City, CA). qPCR was performed using a Bio-Rad MyiQ2 detection system. All qPCR reactions were performed in duplicate. Cycling conditions were 95 °C for 20 s and 60 °C for 1 min for 40 cycles. The expression level of each transcript was determined using the comparative C^T method for relative quantitation using GusB as an endogenous reference.

SPHK1 Promoter Constructs

A 2608 bp DNA fragment of the SPHK1 gene from 2497 bp upstream of the transcription start site (TSS) to 111 bp downstream of the TSS (– 2497/ + 111) was generated by PCR and cloned into the pGL3 basic vector (Promega, Madison, WI) using Kpn I and Xho I restriction enzymes (New England Biolabs, Ipswich, MA). Two deletion constructs (– 1481/

+ 111; – 992/ + 111) were also generated. The construct – 1540/– 1016 was created by removing the – 1540/– 1016 region of the – 2497/ + 111 construct using the Sph I and Sbf I restriction enzymes (New England Biolabs). Two additional constructs, – 1540/– 1316 and – 1250/– 1016, were cloned into – 1540/– 1016 using an infusion cloning kit (Clontech, Mountain View, CA). All constructs were confirmed by sequencing.

Luciferase Assay

pZen cells were plated 1 day prior to transfection in 24-well plates at a density of 6×10^4 cells/well. The SPHK1 luciferase constructs were transfected using Extreme Gene HP reagent (Roche Applied Science, Indianapolis, IN) at a ratio of 1:3. Twenty-four hours after transfection, cells were stimulated with or without 100 ng/mL CSF1 in α -MEM containing 1% FBS for another 24 h before harvesting. Luciferase assays were performed using the Dual-Luciferase reporter assay system (Promega) with the pGL4.73 vector system (Promega) as the internal control. Data are presented as fold increase over basal (i.e., cells transfected only with the pGL3 basic vector).

Nuclear Extracts and Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts from pZen cells and osteoclasts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Rockford, IL). Protein concentration was measured using the BCA protein assay kit (Thermo Fisher Scientific) and an equal amount of nuclear protein was used in each binding reaction. The probes derived from the SPHK1 promoter were 5'-end biotin-labeled. The sequences for the probes and cold competing fragments used in EMSA are listed in Fig. 3. EMSA was performed using the LightShift Chemiluminescent EMSA Kit (Thermo Fisher Scientific) following the manufacturer's instructions. Binding reactions were performed in a 20 μ L volume with 6 μ g of nuclear extract, 200 ng poly dI-dC. Labeled probe (2 ng) was then added and the reaction mixture is incubated at room temperature for 30 min before loading on to the gel. In competition assays, a 100-fold excess of unlabeled probe was added to the reaction. For supershift assays, 2 μ g of antibody (200 μ g/0.1 mL, Santa Cruz Biotechnologies, Santa Cruz, CA) was added in each reaction and pre-incubated without labeled probe for 30 min on ice. Labeled probe was then added and the reaction mixture incubated at room temperature for an additional 30 min before loading onto the gel.

Site-Directed Mutagenesis

Site-directed mutagenesis was performed using the Quick-Change site-directed mutagenesis kit (Agilent Technologies, Englewood, CO). A mutant binding site was generated in the – 2497/ + 111 construct by introducing 2 bp substitutions into the wild-type sequence as follows: ATGGGGG to mutant ATGATGG. The changes were confirmed by sequence analysis.

Statistical Methods

A two-tailed test was used for all statistical analyses.

Results

CSF1 Induces Expression of SPHK1 in Osteoclasts In Vitro and in Bone In Vivo

C-fms is highly expressed on mature osteoclasts but not on osteoblasts [13]. To determine if CSF1 could directly induce SPHK1 expression in osteoclasts, RNA was isolated from osteoclasts treated either with vehicle or CSF1 for 24 h, as detailed above. CSF1 induced a 3.19 ± 0.86 fold increase in SPHK1 expression, while there was no change in the expression of SPHK2 (1.04 ± 0.16 fold) (Fig. 1a). To determine if CSF1 could also induce expression of SPHK1 in bone, C57/BL6 male mice were given 100 ng/mL of CSF1 by intraperitoneal injection. After 24 h, femur RNA was isolated and expression of SPHK1 was quantified by qPCR. CSF1 induced SPHK1 expression 2.47 ± 0.51 fold and SPHK2 expression 2.31 ± 0.81 in bone. (Fig. 1b).

Localizing the CSF1-Responsive Element in the SPHK1 Promoter

Since regulatory elements mediating CSF1 induction of the SPHK1 gene have not been described, a series of 5' end deletion constructs of the SPHK1 gene were created and luciferase activity assayed in the presence and absence of CSF1. A fragment of the SPHK1 gene (– 2497 to + 111; relative to the transcription start site) demonstrated 2.2-fold greater activity compared to the activity in cells transfected with the same vector and treated with vehicle (Fig. 2). The response to CSF1 was also analyzed in the – 1481 to + 111 fragment. CSF1 increased the activity of this promoter fragment 2.2-fold compared to transfected cells treated with vehicle. In contrast, the – 992 to + 111 bp fragment was unresponsive to CSF1. These data indicated that CSF1-responsive element was located between bp – 1481 and – 992. Three additional deletion constructs were also studied, – 1540/– 1016, – 1540/– 1316, and – 1250/– 1016. Of these, only the fragment – 1540/– 1316 responded to CSF1 with an increase in luciferase activity. These data further narrowed the CSF1-responsive region in the SPHK1 promoter to bp – 1250/– 1016 (Fig. 2).

Identification of a *cis* Element in the SPHK1 Promoter Required for CSF1 Induction

To identify possible CSF1-responsive *cis* elements in the – 1250/– 1016 sequence, two biotin-labeled fragments, one 163 bp in length (– 1301/– 1139) and one 169 bp in length (– 1157/– 989) that completely encompass the 234 bp sequence – 1250/– 1016 were used as probes in EMSAs using nuclear extracts prepared from pZen cells treated with or without CSF1. Shifted bands were observed with the 163 bp probe but not 169 bp probe (data not shown). The 163 bp fragment was therefore divided into 2 probes: – 1301/– 1219 and – 1223/– 1139. Two shifted bands were identified in EMSAs using the – 1223/– 1139 probe (Fig. 3a middle panel) and the intensity of the shifted bands was increased by treatment with CSF1. Based on these data, it appeared that 85 bp fragment (– 1223 to – 1139) harbored the CSF1 responsive *cis* element. To confirm that the findings in pZen cells were relevant to authentic osteoclasts, the experiment shown in Fig. 3a middle panel was repeated using osteoclast nuclear protein. As shown in Fig. 3a right panel, the result obtained with osteoclast nuclear protein in an EMSA was identical to the findings using pZen nuclear extract. This sequence was interrogated for putative transcription factor binding sites using the online program, Genomatix (<https://www.genomatix.de>) [14] and a *cis* element consensus sequence for the transcription factor Kruppel-like factor 6 (KLF6/CBPB

ATGGGGG) identified. Oligos were generated that contained the KLF6 binding site (W: – 1220/– 1177; F: – 1210/– 1177; and R: – 1220/– 1187) or mutant variants (M1: – 1220/– 1177, in which bp – 1203/– 1994 was changed to a random sequence, M2: – 1220/– 1177, in which bp – 1999/– 1998 was changed to AT) and used as unlabeled probes in a competition assay (Fig. 3b). One shifted band in EMSAs was completely competed using cold probes containing the ATGGGGG motif (W, F and R) while mutations in the ATGGGGG motif in the W fragment (M1 and M2) completely abrogated nuclear protein binding, which indicated the ATGGGGG motif is critical and that a 24 bp sequence (– 1210/– 1187, TGCATGGAATGGGGGAGGTGAACA) is required for DNA binding in the SPHK1 promoter (Fig. 3b, left). To establish the minimum sequence required for DNA binding, EMSAs were repeated using 19 to 24 bp “cold” competitor sequences and either pZen nuclear protein (Fig. 3b, middle panel) or nuclear protein from osteoclasts (Fig. 3b, right panel). These studies identified that the minimum sequence required for DNA binding was 22 bp in length. Supershift assays were next undertaken using the 85 bp probe and antibodies to KLF6 and Sp1. Prior work has established that both KLF6 and Sp1 can sometimes be found in the same DNA binding protein complex [15]. Surprisingly neither antibodies to KLF6 nor Sp1 caused a supershift of the bound protein/DNA complex (Fig. 3c).

The Identified *cis* Element is Responsible for CSF1-Induced SPHK1 Promoter Activation

To examine the biological relevance of CSF1-induced binding to the identified *cis* element in the SPHK1 promoter, a 2 bp substitution was made in the wild-type sequence (ATGGGGG to ATGATGG). This mutation was introduced into the – 2497/ + 111 promoter construct and analyzed in a luciferase assay. The mutated construct had no residual luciferase activity (Fig. 4). These data indicate that the *cis* element identified in the SPHK1 promoter is required for CSF1 transcriptional activation and that the ATGGGGG motif is the critical sequence.

Discussion

The process of bone remodeling involves a still-as-yet-to-be-fully-clarified process in which new bone formation occurs precisely at sites of previous bone resorption [16]. A host of putative mechanisms and mediators have been suggested to participate in this coupling event [17]. Recent interest has focused on the possibility that osteoclasts themselves secrete cytokines, so-called clastokines, that induce bone formation via paracrine effects on cells in the osteoblast lineage [18]. This putative mechanism is distinct from resorption-dependent release of growth factors from bone matrix, such as IGF1, which have also been postulated to participate in coupling [19]. It also differs from the direct cell–cell contact thought, for example, to be required for ephrin-mediated coupling [20].

Among the putative paracrine-acting anabolic clastokines, S1P has drawn recent attention because the two *in vivo* experiments, summarized in the Introduction, highlight the potential importance of that phospholipid in mediating coupling [3, 4]. As also noted in the Introduction, SPHK1 is thought to generate S1P at the cell membrane for export as a paracrine factor. Understanding factors that regulate S1P production via SPHK1 is of

considerable clinical interest since strategies to enhance bone anabolism provide therapeutic opportunities to correct severely reduced bone mass in patients.

To date, no studies have reported hormonal or cytokine regulation of SPHK1 in bone. The two studies summarized in the Introduction have characterized two mechanisms that result in increased SPHK1 production in bone. In one, deletion of cathepsin K led not only to an increase in numbers of osteoclasts in bone, but also to greater production of S1P [3]. In the second in vivo model, deletion of the calcitonin receptor led to an increase in the export mechanism for SPHK1 from osteoclasts [4].

The final common pathway for bone resorption is through the RANKL/RANK and CSF1/c-fms signaling cascades [21]. Bone cells in the osteoblast lineage express RANKL and CSF1, which engage their respective receptors, RANK and c-fms, on osteoclast precursors and mature osteoclasts to stimulate osteoclastogenesis and regulate mature osteoclast function [21]. PTH is known to induce RANKL and CSF1 production in osteoblasts. CSF1 is the principal colony stimulating activity released by osteoblasts, constitutively, and in response to PTH [13, 22]. The receptor for CSF1, c-fms, is expressed on osteoclast precursors but is most highly expressed in mature osteoclasts [13, 22]. A putative anabolic paracrine pathway in bone is one in which PTH-induced CSF1 production by osteoblasts binds c-fms on osteoclasts to transcriptionally induce SPHK1, thereby increasing S1P production and bone formation. The observation that the deletion of c-fms in mature osteoclasts impairs the anabolic response to PTH provides support for this putative paracrine pathway [10]. The results of the current study are also consistent with this model. SPHK1 is induced directly by CSF1 in mature osteoclasts and also in bone in vivo. Further, the data provide a molecular basis for CSF1-induced transcriptional activation of the SPHK1 gene. An analysis of the 2.6 kb SPHK1 promoter demonstrated significant endogenous promoter activity that was further increased by treatment with CSF1. Deletional analysis identified a *cis* element responsible for CSF1-induced activation of the promoter in the region – 1250/– 1016 from the TSS.

EMSA showed that nuclear extracts from CSF1-treated cells demonstrated increased binding to the SPHK1 promoter at position – 1223/– 1139 bp, relative to the TSS that was completely competed using a nucleotide designed to match the minimum 22 bp consensus sequence at position – 1210/– 1191. Two mutated oligos failed to block nuclear protein binding. To further support the conclusion that this *cis* element was required for CSF1-mediated transcriptional activation of the SPHK1 gene, the relevant sequence was mutated in the 2.6 kb SPHK1 promoter, and introduced into the pGL3 expression vector. A 2 bp mutation in this site completely abrogated CSF1-induced transcriptional activation of the SPHK1 gene.

Data from the current study, coupled with the findings on S1P's role in bone anabolism, suggest that CSF1 may be an important regulator of SPHK1 in vivo. CSF1 is known to act through ERK1 and ERK2 and to activate PI-3 kinase [11, 23]. Interestingly, these molecular mediators also play important roles in TNF- and PMA-induced post transcriptional activation of SPHK1, as well as transcriptional activation of the SPHK1 gene [6, 24].

In summary, the current study supports the conclusion that CSF1 induces transcriptional activation of SPHK1 in osteoclasts. Therapeutic manipulation of this signaling cascade may present an opportunity to augment bone formation.

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Fig. 1.

a To determine the effect of CSF1 on SPHK1 and SPHK2 transcript expression in osteoclasts, RNA was isolated from mature osteoclasts treated with 200 ng/mL CSF1 for 24 h. and analyzed by qRT-PCR. **b** qRT-PCR was used to quantify expression of SPHK1 and SPHK2 transcripts in RNA isolated from bone tissue taken from C57/Bl6 mice treated with 100 ng CSF1 for 24 h. Data are fold-induction in transcript expression compared to untreated osteoclasts or bone RNA from untreated animals. The data summarize the results from 3 of the experiments and are presented as $M \pm SEM$. (* = $p < 0.05$)

Fig. 2.

Basal and CSF1-induced (100 ng/mL) promoter activity of a 2608 bp fragment of the SPHK1 gene (– 2497 to + 111; relative to the transcription start site). A 5′-deletion of that promoter fragment (– 1481/ + 111) was still responsive to CSF1, but a further deletion (– 992/ + 111) lost activity. Selectively deleting the overlapping region of these two fragments (– 1540/– 1016) from the full-length promoter resulted in a loss of activity. Subdividing this region (– 1250/– 1016 and – 1540/– 1316) localized the CSF1 responsive sequence to the region between bp – 1250 and – 1016. The data are fold induction of luciferase activity compared to the pGL3 basic vector and summarize results from 3 of the experiments ($M \pm SEM$; * = $p < 0.05$)

using pZen cell nuclear extracts from cells treated with 200 ng/mL CSF1 for 24 h. The 85 bp sequence was labeled probe and five fragments from that sequence served as cold competitors [Wild type (W)= – 1220/– 1177; M1=– 1220/– 1177, in which a random sequence was introduced between – 1203/– 1994; M2=– 1220/– 1177, in which the bp – 1199/– 1198 were changed from “GG” to “AT”; F=– 1210/– 1177, in which the 5′ ten bp were deleted; R=– 1220/– 1187, in which the 3′ ten bp were deleted; None=no cold competitors added]. The wild-type fragment showed modest competition for the lower band and complete competition for the upper band. The mutated cold oligos M1 and M2 showed no competition for binding. Mutated bases are shown in bold type. Cold oligos F and R also showed competitive inhibition of binding, indicating the nuclear protein binding site could be restricted to a 24 bp sequence (– 1210/– 1187) (left panel). To establish the minimum sequence required for the DNA binding protein, EMSAs were repeated using different size cold oligos as competitors with either pZen nuclear protein (middle panel) or nuclear protein from osteoclasts (right panel). These data indicated that the minimum sequence was 22 bp in length. **c** Supershift EMSA using osteoclast nuclear extract and antibodies specific for KLF6, Sp1, and Tbx3. The 85 bp was used as labeled probe. All antibodies failed to supershift the complex. Tbx 3 was used as a negative control

Fig. 4. Luciferase promoter assays were performed using pZen cells and the indicated SPHK1 promoter constructs. The SPHK1 promoter containing the 2 bp mutation (ATGGGGG to ATGATGG) was unresponsive to CSF1. The data summarize the results from three experiments. Data are presented as $M \pm SEM$ (* = $p < 0.05$)