Smad-interacting Protein 1 Is a Repressor of Liver/Bone/Kidney Alkaline Phosphatase Transcription in Bone Morphogenetic Protein-induced Osteogenic Differentiation of C2C12 Cells*

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Przemko Tylzanowski‡§§, Kristin Verschueren¶, Danny Huylebroeck¶¶, and Frank P. Luyten‡‡
From the ‡Laboratory of Skeletal Development and Joint Disorders, University of Leuven, Herestraat 49, 3000 Leuven, Belgium and the §Department of Cell Growth, Differentiation and Development (VIB-07), the Flanders Interuniversity Institute for Biotechnology (VIB) and the Laboratory of Molecular Biology, University of Leuven, Herestraat 49, B-3000 Leuven, Belgium

Up-regulation of liver/bone/kidney alkaline phosphatase (LBK-ALP) has been associated with the onset of osteogenesis in vitro. Its transcription can be up-regulated by bone morphogenetic proteins (BMPs), constitutively active forms of their cognate receptors, or appropriate Smads. The promoter of LBK-ALP has been characterized partially, but not much is known about its transcriptional modulation by BMPs. A few Smad-interacting transcriptional factors have been isolated to date. One of them, Smad-interacting protein 1 (SIP1), belongs to the family of two-handed zinc finger proteins binding to E2-box sequences present, among others, in the promoter of mouse LBK-ALP. In the present study we investigated whether SIP1 could be a candidate regulator of LBK-ALP transcription in C2C12 cells. We demonstrate that SIP1 can repress LBK-ALP promoter activity induced by constitutively active Alk2-Smad1/Smad5 and that this repression depends on the binding of SIP1 to the CACCT/CACCTG cluster present in this promoter. Interestingly, SIP1 and alkaline phosphatase expression domains in developing mouse limb are mutually exclusive, suggesting the possibility that SIP1 could also be involved in the transcriptional regulation of LBK-ALP in vivo. Taken together, these results offer an intriguing possibility that ALP up-regulation at the onset of BMP-induced osteogenesis could involve Smad/SIP1 interactions, resulting in the derepression of that gene.

The molecular aspects of signaling by the TGF-β family of ligands are well characterized and have been reviewed extensively (6, 7). Briefly, dimeric ligands bind to a cognate type II receptor, allowing it to associate with type I receptor into a tetrameric complex. After the formation of signaling receptor complexes, the type I receptor phosphorylates various intracellular proteins such as the receptor-activated Smads (R-Smads). Following the phosphorylation, the activated R-Smads heterodimerize with Smad4 and translocate to the nucleus of the cell. There they directly bind to DNA and/or interact with transcription factors/cofactors, affecting gene expression. The issue of the signaling specificity is not yet completely resolved, because ligands display a certain degree of promiscuity towards different combinations of receptors. Based on experiments in vitro, it is generally acknowledged that the R-Smads 1, 5, and 8 are involved in transducing BMP, whereas R-Smads 2 and 3 signal TGF-β activity.

Because activated R-Smads function predominantly in the cell nucleus, the investigation of their mechanism of action has resulted in the isolation and characterization of various nuclear Smad-interacting proteins. One such protein is Smad-interacting protein 1 (8). SIP1 is one of a few novel proteins isolated by the virtue of its interaction with activated, but not latent, R-Smads. It is a member of an emerging family of cellular proteins such as the receptor-activated Smads (R-Smads). It is generally acknowledged that the R-Smads

Major advances have been made toward the understanding of molecular pathways involved in the progression and termination of the osteo/chondrogenic differentiation. The identity of the molecular, cell-autonomous players involved in the onset of those processes remains elusive. Significant progress came with the discovery of Cbfa1 (1–4) and Sox9 (5) and the characterization of their role in the onset of osteo- and chondrogenic differentiation, respectively. Nonetheless, some of the issues remain unresolved. One of the most important ones is that although, unquestionably, various members of the TGF-β superfamily are involved in these differentiation processes, their precise role and the gene transcription programs they modulate are not clear.

1 The abbreviations used are: TGF-β, transforming growth factor β; R-Smad, receptor-activated Smad; SIP1, smad-interacting protein 1; bp, base pairs; LBK, liver/bone/kidney; ALP, alkaline phosphatase; BMP, bone morphogenetic protein; CA, constitutively active; Alk, type I receptor; PCR, polymerase chain reaction; EMSA, electric mobility shift assay; dpc, days post-coitus.

2 R. Verschueren and D. Huylebroeck, unpublished data.

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‡ To whom correspondence should be addressed. E-mail: przemko@med.kuleuven.ac.be.
associated with the onset of osteogenic differentiation. The gene is using at least two promoters; one of them, located upstream of exon 1A, is responsible for the expression of LBK-ALP in multiple tissues including bone. The second promoter, located upstream from exon 1B, is activated only in heart muscle. Few regulatory sequences were identified in the first promoter, but none of them has been linked directly to the previously documented induction of endogenous LBK-ALP gene transcription by BMP (15–17).

We decided to reanalyze the regulatory region of the LBK-ALP gene upstream of the exon IA using the well characterized system of BMP-induced osteogenic differentiation of C2C12 cells. This mouse skeletal muscle progenitor cell line was used initially to study myogenesis induced by serum starvation (18). It has been subsequently discovered that the differentiation process could be inhibited by exposure of cells to ligands of the TGF-β family. Interestingly, TGF-β and BMP2 have different effects on C2C12 differentiation in vitro. Both ligands are able to inhibit myogenesis, but only BMP2 can redirect C2C12 cells into the osteogenic differentiation pathway (19, 20). The reason for this striking difference is not quite clear.

In the present study we investigated whether SIP1 could be a candidate regulator of LBK-ALP transcription in C2C12 cells. We demonstrate here that SIP1 can repress LBK-ALP promoter activity. Moreover, we show that this repression is related to the binding of SIP1 to the CACCT/CACCGT sites in this promoter. Interestingly, SIP1 and alkaline phosphatase expression domains in developing mouse limb are mutually exclusive, suggesting a possibility that SIP1 might also be involved in the transcriptional regulation of LBK-ALP in vivo.

EXPERIMENTAL PROCEDURES

Cell Culture—All culture media, sera, and supplements were purchased from Life Technologies, Inc. unless stated otherwise. The C2C12 cell line was grown in Dulbecco’s modified Eagle’s medium high glucose (4.5 g/liter) with 10% fetal bovine serum supplemented with 100 Units/ml ampicillin and 100 μg/ml streptomycin. The cells were passaged at 90% confluence and split 1:10 or 1:20, depending on the desired density. To induce differentiation the cells were placed in starvation medium consisting of Dulbecco’s modified Eagle’s medium high glucose, 2% horse serum supplemented with 10 μg of bovine insulin/ml, 10 μg of transferrin/ml, and 3 x 10^-8 M selenium. All cells were grown in a 95% air/5% CO2 atmosphere, in 95% humidity, at 37 °C.

Cos-1 cells used for eukaryotic expression were maintained in the same medium and supplements as described above. The cells were split 1:10 upon reaching 80% confluence. For transfections, the cells were seeded in 24-well plates (NUNC) 24 h prior to the transfection.

Plasmids—The fragments of the first and the second LBK-ALP promoters were provided kindly by E. Garattini (Laboratory of Molecular Biology, Istituto di Ricerche Farmacologiche Mario Negri, Milan, Italy), and constitutively active type I receptors (CA Alks) were obtained from P. ten Dijke (Netherlands Cancer Institute, Amsterdam, The Netherlands). All other constructs were generated in our laboratories using PCR. PCR fragments used in reporter assays were cloned into the pGL3 reporter plasmid (Promega, Madison, WI) containing thymidine kinase minimal promoter cloned upstream of the luciferase gene.

Transfections—Transfections were carried out using the Fugene 6 reagent (Roche) according to manufacturer instructions. We found that in our case the optimal Fugene/DNA ratio was 2 μl of Fugene to 1 μg of DNA; therefore, when necessary, the amount of DNA was adjusted to 1 μg/well with the empty expression vector DNA. In all the assays the results were normalized to β-galactosidase activity values obtained from the cotransfected RSVlacZ expression plasmid (1 ng/well).

Reporter Assays—Transfected cells were washed once with PBS at room temperature, and then 50 μl of PBS/0.05% Triton X-100 (UltraTune, Pierce) was added to the wells. After two freeze-thaw cycles at −80 °C the lysates were transferred to an ice-cold-bottomed 96-well plate, and the cell debris was removed by centrifugation at 2000 x g. For all subsequent assays 5 μl of the lysates was used, and the remaining lysates were stored at −80 °C for future use. For the luciferase reporter assays we used a luciferase kit from Promega, β-galactosidase expression was measured with the TropiX Kit (PerkinElmer Life Sciences), and the endogenous ALP was measured with the ALP kit (KPL Labo-
as expected, was unable to compete for binding to SIP1, but single-site mutants competed in a distinct fashion. The right-site mutant (lane 2) was not able to abrogate the SIP1 binding to the radiolabeled probe, whereas the left-site mutant (lane 3) did compete partially for the binding. This indicated that the right site (the E2 site CACCTG) alone was still able to bind SIP1, although with apparently lower affinity, whereas the left site alone could not.

Taken together, the above results indicate that SIP1 can bind to the LBK-ALP promoter fragment in vitro and that this binding depends on the presence of an intact bipartite CACCT/CACCTG binding site.

2B, lane 4), as expected, was unable to compete for binding to SIP1, but single-site mutants competed in a distinct fashion. The right-site mutant (lane 2) was not able to abrogate the SIP1 binding to the radiolabeled probe, whereas the left-site mutant (lane 3) did compete partially for the binding. This indicated that the right site (the E2 site CACCTG) alone was still able to bind SIP1, although with apparently lower affinity, whereas the left site alone could not.

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SIP1 Interferes with the Induction of Endogenous ALP—BMP signaling induces LBK-ALP activity in vitro in a number of cellular systems. In addition, previous studies of the regulation of the Xbra gene (8, 12, 22) have indicated that SIP1 could act in that case as a transcriptional repressor. Therefore we decided to test whether SIP1 could repress BMP-induced LBK-ALP activity in vitro. To investigate the response only in cells overexpressing SIP1, instead of using ligand, we cotransfected

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FIG. 1. A schematic representation of the promoter fragment of LBK-ALP upstream of the exon 1A. A, a comparison of the distribution of CACCT/CACCTG clusters in the mouse (upper panel) and human (lower panel) promoters of the LBK-ALP gene. The downward arrows indicate clusters that contain the CACCT/CACCTG cluster separated by less than 60 bp. The first exon in both cases is indicated with a black arrow. The horizontal bar represents 100 bp. B, a part of the mouse LBK-ALP promoter with indicated positions of the primers used to generate DNA fragments for EMSAs and reporter construction (black arrows PT133 and PT135).

FIG. 2. SIP1 binds to the LBK-ALP promoter fragment. A, the end-labeled PCR product of 92 bp containing SIP1 binding sites separated by 54 bases was incubated with extracts from Cos-1 cells transfected with an expression construct encoding Myc-tagged SIP1. Lane 1, mock-transfected cells; lane 2, extracts from cells expressing Myc-tagged SIP1; lane 3, the same extract as described in lane 2 but incubated with the anti-Myc antibody. The arrows indicate the shift (lane 2) and supershift (lane 3) of SIP1-DNA complexes. B, schematic representation of the location of primers on the LBK-ALP promoter. The primers PT133 and PT135 containing wild-type CACCT and CACCTG sequences were used to generate a wild-type probe. Primers PT153 and PT154 containing mutation CACCT(G)–CATCT(G) were used to generate mutated probes. C, results of a competition assay between the labeled wild-type probe and a 30-fold molar excess of different, competing PCR fragments. Lane 1 represents competition with the wild-type probe, lane 2 with the right mutant, lane 3 with the left mutant, and lane 4 with the double mutant. The arrow indicates the SIP1-shifted band.
cells with constitutively active forms of BMP type I receptors. We first determined the conditions leading to the highest up-regulation of LBK-ALP by transiently transfecting C2C12 cells with CA BMP receptors in conjunction with various wild-type R-Smads. Neither CA Alk4 nor CA Alk5 induced measurable LBK-ALP activity (data not shown and Ref. 23). Constitutively active Alk1, Alk2, Alk3, or Alk6 did induce the endogenous LBK-ALP activity (Fig. 3). Smads 1 or 5, separately or combined, also led to increased levels of endogenous ALP, but that induction always remained low (Fig. 3, pCS2 lane, and data not shown), whereas the cotransfection of CA Alks with R-Smads induced the endogenous Alp in a synergistic manner (Fig. 3, gray columns). Because CA Alk2 in combination with Smad1/Smad5 consistently gave the strongest induction of the endogenous ALP, we chose these conditions for all subsequent experiments.

We then transiently transfected C2C12 cells with CA Alk2/Smad1/Smad5 combinations and cotransfected with expression constructs encoding SIP1. The induction of endogenous ALP activity by CA Alk2/Smad1/Smad5 was repressed strongly by cotransfection with SIP1 (Fig. 4). To test whether the repression was related to SIP1 binding to DNA, we transfected a mutant of SIP1 that could not bind to the DNA target because both zinc finger clusters had been mutated (SIP1NZF3CZF3) (12). As can be seen in Fig. 4, this mutant failed to repress efficiently the endogenous LBK-ALP activity. The lack of repression was not related to the levels of produced SIP1 (data not shown).

**SIP1 Can Interfere with Induction of LBK-ALP Promoter Plasmids**—Next, we determined whether the repressive activity of SIP1 could be assigned to the DNA fragment containing the SIP1 bipartite binding site. First, we cloned the available promoter fragment located upstream of the exon 1A of LBK-ALP into pGL3. Subsequently, we transfected this plasmid into C2C12 cells and cotransfected with CA-Alk2 and Smad1/Smad5. As demonstrated in Fig. 5A, this reporter was, similar to the endogenous gene, induced by CA-Alk2/Smad1/Smad5 and repressed by SIP1. Thus, the 1.9-kilobase promoter fragment of LBK-ALP contains the regulatory sequences directing the response of the reporter gene to CA ALK2/Smad1/Smad5 induction and SIP1 repression.

Because EMSA demonstrated that a 92-bp fragment of this promoter could specifically bind SIP1, we repeated the reporter assays using this promoter fragment. As shown in Fig. 5B, shaded bars, the reporter containing the wild-type sequences was induced in a similar way by CA Alk2/Smad1/Smad5 and again repressed by SIP1 as we have shown for the endogenous gene or the 1.9-kilobase promoter fragment (Figs. 4 and 5A, respectively). To confirm that the reporter response was related directly to SIP1 binding to DNA, we used a mutant reporter in which the right binding site (CACCTG) was mu-
tated to CA\textsuperscript{ACTG}. This mutant reporter could still be induced by CA Alk2/Smad1/Smad5 but failed to be repressed by SIP1 (Fig. 5B).

To demonstrate that the above effect was related directly to SIP1 binding to DNA, we cotransfected the cells with the SIP1\textsubscript{NZF3CZF3} expression plasmid. This SIP1 mutant protein, which binds to DNA with very low affinity (12), failed to interfere with the induction of the wild-type reporter, although its synthesis levels were comparable with the wild-type SIP1 (data not shown).

The above results identify in the promoter of the mouse \textit{LBK-ALP} gene a region (between \textsuperscript{H}11002\textsuperscript{326} and \textsuperscript{H}11002\textsuperscript{381}) necessary and sufficient for SIP1-mediated repression.

\section*{DISCUSSION}

\textbf{SIP1 and ALP Are Expressed in Distinct Nonoverlapping Domains in Developing Mouse Limbs—}The results obtained during our \textit{in vitro} studies indicated that, at least mechanistically, SIP1 could be involved in the transcriptional regulation of the mouse \textit{LBK-ALP} gene. To begin addressing the biological significance of this observation we compared the expression of SIP1 mRNA and ALP in developing mouse limbs. As can be seen in Fig. 6, the expression patterns of both genes are quite distinct and not overlapping. At 12.5 dpc, ALP was not yet detectable (Fig. 6A, left panel and Ref. 24). SIP1 mRNA, on the other hand, was expressed in a discrete pattern as seen in Fig. 6A, right panel.

One day later, at 13.5 dpc, the ALP could be detected around the cartilaginous core of the phalanges. SIP1 mRNA expression was excluded from that area but was present in a broad area around the putative tendon. Detailed expression analysis of the midgestation mouse embryo did not reveal any tissues in the developing mouse limb that would show a clear overlap of the expression domains of both genes (data not shown).

\section*{DISCUSSION}

In this paper we provide evidence that SIP1 can bind \textit{in vitro} to a CACCT/CACCTG sequence cluster in the promoter of the mouse \textit{LBK-ALP} gene. Not only is this binding specific, but it is also responsible for the repression of reporter constructs carrying that promoter fragment. These data suggest that SIP1 could be a candidate repressor protein for the \textit{LBK-ALP} gene.

The promoter of the \textit{LBK-ALP} gene has been analyzed partially in mouse as well as in rat. The initial characterization of
the mouse gene (17) led to the discovery of two promoters of that gene. The first promoter, located upstream of the exon 1A, is responsible for the expression of LBK-ALP in a number of tissues including bone. The second promoter, located upstream from the exon 1B is activated uniquely in heart. Subsequent studies focused on the identification of cis-regulatory elements in the promoter of the gene. They resulted in the identification of promoter elements directly involved in the up-regulation of LBK-ALP by retinoic acid (25) or by a combination of vitamin D3 and TGF-β (26). Interestingly, despite the fact that BMPs are known to be very potent inducers of the endogenous LBK-ALP in vitro, no cis-acting promoter elements in that gene could be linked directly to this effect (27).

In this report we delineate a promoter region from position −326 to −381 that is sufficient to mediate BMP-dependent induction and SIP1-dependent repression through the bipartite CACCT/CACCTG cluster. Similar domain distribution has been found in the human LBK-ALP promoter, suggesting evolutionary conservation of the regulatory elements between mouse and human.

How might SIP1 be involved in the regulation of LBK-ALP transcription? One possible explanation could involve the induction of LBK-ALP transcription through a derepression mechanism whereby the activity of SIP1 is extinguished. A similar mode of action was reported for Drosophila brinker, a transcriptional repressor of dpp-responsive genes. In that case, dpp (a Drosophila homologue of BMP2) down-regulated brinker transcription and consequently up-regulated a number of downstream target genes (28, 29).

A recent analysis of the regulation of the collagen type II promoter by δEF1 (30) provides an interesting context for our observations. SIP1 and δEF1 are two distinct members of the same family of two-handed zinc finger proteins. Their domain structure is very similar, with the highest degree of amino acid sequence conservation in the areas encoding the N- and C-terminal zinc finger clusters (8). The DNA binding specificity of these zinc finger clusters is identical in vitro (12), and the in vivo mRNA expression data suggest only a limited overlap between Sip1 and δEF1 expression in developing mouse limbs (31). It is thus likely that both genes might act as transcriptional repressors in different tissues and that SIP1-mediated repression of LBK-ALP transcription prior to osteogenic differentiation is akin to δEF1-mediated repression of collagen type II prior to the chondrogenic one (30). Interestingly, targeted inactivation of δEF1 in mouse yielded a specific albeit complex skeletal phenotype (31). Some aspects of that phenotype such as hypoplasia of Meckel’s cartilage and intervertebral disks as well as shortening and broadening of the long bones and joint fusions resemble phenotypes arising from inactivation of other genes known to be involved in chondrogenesis, e.g. Indian hedgehog, PTH/PTHrP, noggin (www.jax.org), or from GDF5/CDMP1 overexpression (32). Thus it will be interesting to see if the targeted inactivation of SIP1 would result in skeletal abnormalities.

Finally, a limited analysis of the gene expression pattern in the developing mouse revealed that SIP1 and ALP have distinct nonoverlapping areas of expression. ALP expression in the developing limb was detected from 13.5 dpc onward around the developing cartilage anlage demarcating the cells actively undergoing osteogenic differentiation. SIP1, on the other hand, was detected earlier (12.5 dpc), initially in the central part of the limb. Subsequently, at 13.5 dpc, the expression was seen ventrally to the cartilage anlage and around putative tendon but excluded from the alkaline phosphatase-positive areas. This mutual exclusivity of expression patterns resembles that of δEF1 and collagen type II (30, 31). Indeed, if the extinguishing of δEF1 expression is a prerequisite for the induction of collagen type II, one could envision a similar situation for SIP1 in the case of LBK-ALP. Here, only tissues not expressing SIP1 would be competent to express LBK-ALP. Obviously, in vivo, the situation is most probably more complicated, and a number of other transcription factors may contribute to the regulated expression of alkaline phosphatase. On the other hand, the
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The absence of SIP1 could be a prerequisite for the activation of LBK-ALP transcription. It is noteworthy that SIP1 is expressed around developing tendons. Not much is known about the molecular players participating in tendon formation, although expression of a few genes has been associated with that tissue. The Six1 and Six2 genes, both encoding homeodomain proteins, have been reported to be expressed in tendon and muscle during mouse development (33). The Eph-related receptor tyrosine kinase gene Cek-8 was detected in developing chick tendons (34) and Eya1 and Eya2 transcriptional activators (35) have been associated with patterning of tendons during mouse development. Finally, GDF5 and GDF7 have been implicated in the induction of tendons (36). The broad expression pattern of SIP1 around tendons might suggest that only the cells that are at early stages of differentiation would be expressing this gene. Indeed, perhaps down-regulation of SIP1 expression is one of the prerequisites for terminal differentiation also in this tissue.

The biological function of SIP1 and other family members of that group of transcription factor factors remains an open question. Some indication came from experiments in transgenic frog embryos. In this case, a mutation of 1 bp in the promoter of Xbra abolishing SIP1 binding caused ectopic expression of Xbra mRNA in the gastrula (22). Interestingly, this ectopic expression was suppressed in later stages of frog development clearly indicating that there are other players involved in the regulation of Xbra. Thus, the SIP1/EF1 group of transcriptional repressors might be required to control spatio-temporal expression of target genes by repression rather than activation and thus be involved in the maintenance of a pool of undifferentiated cells required for later stages of development and/or tissue repair.

In summary, we have shown that a CACCT/CACCTG DNA cluster in the promoter of mouse LBK-ALP can bind SIP1 in vitro and that this binding depended on the integrity of those sites. Moreover, CA Alk2/Smad1/Smad5 could up-regulate a reporter plasmid carrying this construct and SIP1 could repress it. SIP1 had the same effect on the activity of the endogenous ALP indicating that the intact CACCT/CACCTG DNA cluster in the promoter of mouse LBK-ALP was necessary and sufficient for the SIP1-mediated repression of its activity.

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Przemko Tylzanowski, Kristin Verschueren, Danny Huylebroeck and Frank P. Luyten

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