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Transcription Intermediary Factor 1 γ Decreases Protein Expression of the Transcriptional Cofactor, LIM-Domain-Binding 1

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Abstract

LIM-domain-binding 1 (LDB1) is a cofactor that participates in formation of regulatory complexes involving transcription factors containing LIM domains as well as other factors. We have examined the ability of transcriptional Intermediary factor 1 γ (TIF1 γ) to decrease LDB1 expression. An expression vector for TIF1 γ was found to decrease expression of LDB1. A mutation which disrupts the ubiquitin ligase activity of TIF1 γ was found to block the ability of TIF1 γ to decrease LDB1 expression. Proteasome inhibitors were also able to block TIF1 γ effects on LDB1. Immunoprecipitation studies provided evidence that LDB1 interacts with TIF1 γ in intact cells. Knockdown of TIF1 γ in zebrafish embryos led to increased expression of LDB1 providing evidence for a physiological role of TIF1 γ in regulating LDB1 expression. Reporter gene assays demonstrated that TIF1 γ can alter the activity of LIM-homeodomain transcription factor-responsive promoters. These studies are consistent with a model in which TIF1 γ acts to ubiquitinate LDB1 leading to degradation of LDB1 and changes in transcription of LDB1-dependent promoters.

Keywords

ubiquitin ligase; LIM homeodomain transcription factor; Proteasome

1. Introduction

LIM domain binding protein 1 (LDB1) is a conserved, transcriptional cofactor that functions in transcriptional complexes containing LIM homeodomain transcription factors and other factors [1]. A number of studies in *Drosophila* have provided evidence that an LDB1 dimer interacts with two LIM-homeodomain transcription factors leading to transcriptional activation [2-7]. These studies have shown that the stoichiometry of LDB1 and LIM-homeodomain transcription is important. Either over-expression or decreased expression of either LDB1 or the interacting LIM-homeodomain factor can inhibit transcription. LDB1 regulation of LDB1 expression is likely important for many physiological functions. Indeed, homozygous disruption of the LDB1 gene in mice leads to severe patterning defects during gastrulation [8].

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TIF1 γ is a member of the transcription intermediary factor family of co-factors [9]. While two of the family members, TIF1 α and TIF1 β , have been shown interact with several nuclear receptors and modulate their activity, TIF1 γ does not appear to interact with nuclear receptors [9]. TIF1 γ has been shown to repress transcription when recruited to a promoter [9]. Experiments in zebrafish have shown that TIF1 γ is necessary for differentiation of hematopoietic cells [10]. More recent data suggests that TIF1 γ plays a role in regulating TGF β signaling by interacting with SMAD4 and regulating its levels and/or activity [11-13]. In this report we show that TIF1 γ interacts with LDB1 and decreases expression of LDB1. This activity depends on an intact RING finger domain of TIF1 γ and is inhibited by chemical inhibitors of the proteasome. We also show TIF1 γ can negatively regulate a LIM homeodomain-dependent reporter gene.

2. Materials and methods

2.1 Cell culture, DNA constructs, and transfections

Human embryonic kidney 293 cells (HEK 293), Chinese hamster ovary, and the gonadotrope-derived mouse α T-3 cell line [14] were maintained in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum. A reporter gene containing the mouse glycoprotein hormone alpha subunit promoter linked to the luciferase coding sequence has been described previously [15]. The coding sequence for mouse LDB1 and TIF1 γ was amplified by the polymerase chain reaction using standard protocols. The products were all confirmed by automated DNA sequencing. A mammalian expression vector for human SMAD4 was obtained from Dr. Jan Christian at Oregon Health and Science University. Cells were typically transfected with a total of 2 μ g DNA and 5 μ l of Lipofectamine 2000 (Invitrogen, Inc.) in 35 mm well plates, or 0.8 μ g DNA and 2 μ l Lipofectamine in 22 mm well plates using a protocol provided by the supplier.

2.2 Real time polymerase chain reaction

RNA and then first strand cDNA was prepared from transfected HEK293 cells using TRIZOL and SuperScript II reverse transcriptase according to the manufacturer's instructions (Invitrogen). Real time PCR was performed using a SYBR green containing reagent mix from Applied Biosystems with the ABI Taqman 7900HT real time PCR machine. Primers used for mouse GAPDH were: 5'-CTCTGCCACCCAGAAGACTGT and 5'-GGAAGGCCATGCCAGTGA. Primers used for FLAG-tagged mouse LDB1 were: 5'-AGCCAAGAGAGCAGATCGGAGAAT and 5'-TGCCTTGTCATCGTCGTCCTTGTA.

2.3 Preparation of cell extracts

For immunoblotting or immunoprecipitations, cells were scraped from the culture dishes in phosphate buffered saline. The cells were pelleted in a microfuge and resuspended in 100 mM sodium phosphate with 0.1% NP-40. The cells were disrupted by 4 cycles of freeze thaw using dry ice/ethanol and 37 $^{\circ}$ C water baths. After centrifugation at 10,000 $\times$ g for 5 min at 4 $^{\circ}$ C, the supernatant was saved as a whole cell extract. For preparation of cell extracts for luciferase assays cell monolayers were rocked for 15 minutes in 100 mM sodium phosphate, pH 7.8, 1 % Triton X-100. Cell debris was removed by transferring the extract to microfuge tubes and centrifuging for 2 min.

2.4 Antiserum, immunoprecipitations, and immunoblotting

A monoclonal antibody to the FLAG epitope was obtained from Sigma, Inc. Rabbit polyclonal antiserum to LDB1 was obtained from Biovintage. For immunoprecipitations, cell extracts were adjusted to contain 0.1% Tween-20. Aliquots containing equal amounts of total protein were combined with 15 μ l of a 50% slurry of anti-FLAG agarose or rabbit anti-

AU1 serum and protein A/G agarose. The immunoprecipitation mixtures were rotated for 2 hours at 4° C and the agarose bound antibodies were collected by centrifugation. The agarose beads were then washed 3 times with 1 ml each of 10 mM Tris, pH 7.4, 150 mM NaCl, 0.1 % Tween-20, 0.1% Triton X-100. Proteins bound to the agarose beads were then analyzed by electrophoresis on a denaturing, polyacrylamide gel. For immunoblotting, proteins were transferred to polyvinylidene difluoride membranes (Millipore). Blocking reactions, incubation with a 1:5,000 dilution of antiserum to FLAG or LDB1, incubation with a 1:10,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse antibody (Santa Cruz) and incubation with chemiluminescent reagent (Amersham Renaissance) were all performed as suggested by the suppliers.

2.5 Morpholino knockdown of TIF1 γ in zebrafish embryos

A morpholino oligonucleotide (Gene Tools, Philomath, OR) with sequence, CCTTTCCGAACCTTACCGATTTCGAGT, was directed against the first exon splice-donor site of zebrafish TIF1 γ . A standard control morpholino with fluorescein (5'-CCTCTTACCTCAGTTACAATTTATA-3') was also used to determine that the phenotype observed was not due to toxicity. Morpholinos were diluted to 1 mM in 1 $\times$ Danieau solution (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO₄, 0.6 mM Ca(NO₃)₂, 5.0 mM HEPES pH 7.6), and injected into 1-2 cell wild-type embryos with 0.05% phenol red as a marker.

3. Results

There is evidence that ubiquitin-mediated degradation of the transcriptional cofactor, LDB1, plays a role in modulating gene expression [16-17]. In the present study we have tested the possibility that transcriptional intermediary factor, TIF1 γ , can play a role in regulating LDB1 expression. TIF1 γ is a widely expressed member of a family of transcriptional regulators [9]. TIF1 γ contains a RING finger domain and it has been shown that TIF1 γ can function as an E3 ubiquitin ligase leading to ubiquitination of SMAD4 [11]. As an initial test we examined the ability of TIF1 γ to alter LDB1 protein levels in transiently transfected HEK293 cells. TIF1 γ co-expression with LDB1 resulted in a reduction in LDB1 expression (Fig. 1A lanes 1 and 2). The ability of TIF1 γ to reduce expression of LDB1 was dependent on the RING finger of TIF1 γ as a mutation that abolishes ubiquitin ligase activity [11] greatly reduced the ability of TIF1 γ to decrease LDB1 expression (Fig. 1A, lane 3). The effects of TIF1 γ are relatively specific for LDB1 as co-expression of TIF1 γ with Ets-1 had little or no effect on expression levels (Fig. 1A, lane 5) and there was no effect on ERK1, used as a loading control. The effect of TIF1 γ to reduce LDB1 expression appears to be at the post-transcriptional level as TIF1 γ did not significantly alter the amount of LDB1 RNA in transfected HEK293 cells (Fig.1B). These findings are consistent with the possibility that TIF1 γ may act as an E3 ubiquitin ligase leading to ubiquitination and subsequent degradation of LDB1. It has been reported that TIF1 γ serves as an E3 ubiquitin ligase leading to ubiquitination of SMAD4 [11] although there are also conflicting findings [13]. In view of the possibility that TIF1 γ may play a role in regulating SMAD4 expression, we compared TIF1 γ effects on SMAD4 and LDB1 in HEK293 cells. TIF1 γ modestly reduced the expression of SMAD4 (Fig. 3) and in the same experiment TIF1 γ had a somewhat greater ability to reduce expression of LDB1. To further explore the possibility that TIF1 γ functions as an E3 ubiquitin ligase leading to proteasomal degradation of LDB1, we utilized two different cell permeable chemical inhibitors of the proteasome. A twelve hour treatment of transfected HEK293 cells with either epoxomicin [18] or MG-132 [19] greatly increased expression of LDB1, suggesting that LDB1 is normally degraded via the proteasome (Fig 1D, lanes 15-18). These inhibitors were both able to substantially block the ability of TIF1 γ to reduce LDB1 levels (Fig. 1D, lanes 19-21). These findings provide evidence that TIF1 γ -mediated reduction in LDB1 requires a functional proteasome.

We have also obtained evidence that TIF1 γ interacts with LDB1 in intact cells. FLAG tagged-TIF1 γ efficiently co-immunoprecipitates with LDB1 even though TIF1 γ substantially reduces LDB1 protein levels in HEK293 cells (Fig. 2A, lane 7). The ubiquitin ligase inactive TIF1 γ mutant also efficiently co-immunoprecipitates LDB1 (Fig 2A, lane 8). The same interaction is observed in CHO cells that express transfected LDB1 at a higher level than 293 cells (Fig 2B). This finding is consistent with previous studies showing that the RING finger of TRIM family ubiquitin ligases bind to an E2-conjugating enzyme while other domains of the protein interact with the substrate [20].

The preceding observations are consistent with a model in which TIF1 γ serves as a ubiquitin ligase leading to LDB1 ubiquitination and proteasomal degradation. However, although we have explored a variety of approaches including use of proteasome inhibitors, we have been unable to detect TIF1 γ -stimulated ubiquitination of LDB1 (data not shown). It is possible that TIF1 γ effects on LDB1 are indirect, mediated by ubiquitination of another protein. We cannot rule this possibility out, but the direct binding of TIF1 γ to LDB1 suggests a direct mechanism.

Our finding in tissue culture cell lines suggests that TIF1 γ can regulate LDB1 protein levels. To further explore the physiological relevance of this observation we used morpholino knockdown technology to reduce the level of TIF1 γ expression in developing zebrafish. In zebrafish, the TIF1 γ loss of function mutant is designated *moonshine* and TIF1 γ is an essential regulator of both embryonic and adult hematopoiesis [10]. The results of the studies in HEK293 cells predict that reducing the levels of TIF1 γ in developing zebrafish would result in increased levels of LDB1. To test this, a morpholino was designed spanning the splice donor site of exon 1 of zebrafish TIF1 γ . Injection of this morpholino into zebrafish embryos causes a phenotype identical to severe *moonshine* mutants (Fig. 3A and 3B). All of the embryos injected with the morpholino targeting TIF1 γ were completely bloodless (n=150). To examine LDB1 expression after TIF1 γ knockdown, whole embryo extract was prepared 48 hours after morpholino injection and equal amounts of protein were analyzed by immunoblot using a rabbit polyclonal antiserum raised against a carboxy-terminal region of human LDB1. Human and zebrafish LDB1 are 94% identical and our preliminary blotting experiment confirmed that this antiserum recognizes zebrafish LDB (data not shown). Knockdown of TIF1 γ in zebrafish causes an increase in the amount of immunoreactive LDB1 visible at 48 hours of development (Fig. 3C). This observation provides evidence that in a whole organism, TIF1 γ modulates LDB1 expression levels. Presumably, some tissues may have higher levels of TIF1 γ and therefore greater effects of TIF1 γ on LDB1 expression than the average response obtained with the whole embryo extract.

TIF1 γ is structurally related to the transcriptional co-factors TIF1 α and TIF1 β [9]. These three TIF family members share the same domain structure and have a high degree of identity and similarity to each other. We co-expressed TIF1 α and TIF1 β with LDB1 to test if the effects of TIF1 γ on LDB1 are specific to TIF1 γ or are shared by all members of the family. Under conditions where TIF1 γ again causes a large decrease in LDB1 expression levels neither TIF1 α nor TIF1 β have any significant effect on LDB1 (Fig. 4A). As we have found that TIF1 γ expression leads to substantial decreases in LDB1 expression, it seems likely that these changes in expression would influence the ability of LDB1 to function as transcriptional cofactor. The stoichiometry of LDB1 to interacting transcription factors is very important to its normal function [2-7]. Over-expression of LDB1 as compared to LIM homeodomain transcription factors can inhibit transcription, presumably by disrupting the formation of tetramer complexes involving LDB1 dimers interacting with the LIM homeodomain factors. Likewise, reducing the amount of LDB1 also disrupts tetramer complexes. To test this we used a reporter gene containing the glycoprotein hormone α -subunit promoter. The glycoprotein hormone α -subunit promoter has been shown to be

activated by LIM homeodomain transcription factors and the binding site for these factors is essential for ras activation of this promoter [21-22]. Mouse α T3-1 pituitary gonadotroph cells were transfected with a glycoprotein hormone α -subunit promoter luciferase reporter gene either alone or with an expression vector for activated ras (Fig 4B). As expected, ras led to robust activation of this reporter gene. Co-expression of TIF1 α or TIF1 β had little effect on the basal or ras activated activity of the reporter gene. However, co-expression of TIF1 γ inhibited both the basal level of reporter gene activity and the response to activated ras. Immunoblot analysis of transfected α T3-1 cells confirms that Tif1 γ reduced LDB1 levels (Fig. 4C, compare lanes 8 and 11). However, the reduction of LDB1 is not as great as seen in HEK293 cells and does not appear to be great as the reduction seen in reporter gene activity. These observations suggest that although TIF1 γ can alter LDB1 levels it may also modify LDB1 activity. Reduced transcriptional activity could reflect LDB1-mediated recruitment of TIF1 γ to the glycoprotein hormone α -subunit promoter. A report describing TIF1 γ activity demonstrated that a GAL4 DNA binding domain fusion to full length TIF1 γ results in transcriptional repression of reporter genes containing GAL4 binding sites [9].

4. Discussion

The present studies provide new information concerning the ability of TIF1 γ to decrease expression of LDB1. We find that an expression vector for TIF1 γ leads to decreases in LDB1 expression at a post-transcriptional level and that mutations that disrupt the ubiquitin ligase activity of TIF1 γ block this activity. The ability of TIF1 γ to decrease LDB1 expression is also blocked by proteasome inhibitors. Knockdown of TIF1 γ in zebrafish embryos leads to increases in LDB1 expression in a whole organism, suggesting that TIF1 γ plays a physiological role in modulating LDB1 expression. Overall, the present findings are consistent with a model in which TIF1 γ serves as an E3 ubiquitin ligase leading to LDB1 ubiquitination and degradation by the proteasome. This role for TIF1 γ in regulating LDB1 expression is similar to a suggested role for TIF1 γ in regulating SMAD4 through ubiquitination [11-13]. While several different findings support a role for TIF γ functioning as a ubiquitin ligase to regulate LDB1 expression, it is surprising that we have been unable to detect TIF1 γ -stimulated ubiquitination of LDB1. It is possible that TIF1 γ effects on LDB1 are indirect, mediated by ubiquitination of another protein. We cannot rule this possibility out, but the direct binding of TIF1 γ to LDB1 suggests a direct mechanism. Perhaps under some conditions, rapid de-ubiquitination of LDB1 prevents the accumulation of TIF1 γ -stimulated increases in ubiquitinated LDB1.

TIF1 γ -mediated changes in LDB1 expression likely lead to subsequent changes in transcription of LDB1 target genes. LDB1 was initially identified as a LIM domain-interacting protein [23]. A large body of subsequent research has identified LDB1 as a critical co-factor influencing the developmental roles of LIM homeodomain and other transcription factors [2-7]. An important aspect of LDB1 function concerns the importance of the correct stoichiometry of LDB1 relative to interacting partners. Experimental manipulation of the relative amounts of LDB1 and its interacting partners has profound effects on the function of these complexes [2-7]. Thus our finding that TIF1 γ can decrease expression of LDB1 predicts that TIF1 γ should alter expression of LDB1-dependent promoters. Indeed we find that TIF1 γ has substantial effects in a reporter gene assay. In addition to regulating LDB1 protein levels, LDB1-mediated recruitment of TIF1 γ may lead to transcriptional repression. Recruitment of TIF1 γ to a promoter has been shown to cause silencing activity [9]. This silencing activity is dependent on a relatively small domain unique to the TIF family called the TIF1 signature sequence. Overall our findings provide evidence that TIF1 γ plays a role in regulating LDB1 expression and altering the activity of LDB1-containing transcriptional complexes.

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Abbreviations

LDB1	LIM-domain binding 1
TIF1γ	transcription intermediary factor-1 γ
PCR	polymerase chain reaction
GAPDH	glyceraldehyde-3-phosphate dehydrogenase

Fig. 1.

TIF1 γ reduces the expression of LDB1 in a RING domain dependant manner at a post-transcriptional level. (A) HEK293 cells were transfected with an expression vector for FLAG-tagged LDB1 or ETS-1 and either an empty expression vector (-), or vectors coding for wild type TIF1 γ (WT) or TIF1 γ with a mutation in the RING domain that inactivates ubiquitin ligase activity (Mut). Whole cell extracts were prepared 20 hours after transfection and equal amounts of protein for each sample were resolved by denaturing polyacrylamide gel electrophoresis, transferred to a membrane, incubated with FLAG antiserum and then horseradish peroxidase labeled anti-rabbit secondary antibody was used with a chemiluminescent detection reagent to visualize the immunoreactive proteins. The immunoblots were stripped and then probed with antibody to ERK1 as a loading control. (B) HEK293 cells were transfected with expression vectors for FLAG-LDB1, TIF1 γ , or mutant TIF1 γ and empty expression vectors as indicated. At 20 hours after transfection, RNA was prepared from cells. *Ldb1* and GAPDH RNA were quantitated by real-time PCR. *Ldb1* RNA expression in each sample was normalized to GAPDH in each sample and then relative RNA levels were normalized so that the wild type average value for the *Ldb1* group was set to 1.0. Values are means $\pm$ SEM for three separate transfections. (C) TIF1 γ effects on SMAD4 compared to LDB1. HEK293 cells were transfected with expression vectors for either FLAG-tagged human SMAD4, FLAG-tagged LDB1 or an empty expression vector and a vector coding for TIF1 γ as indicated. Whole cell extracts were prepared 20 hours after transfection and protein resolved by denaturing gel electrophoresis, transferred to a membrane and then incubated with a monoclonal FLAG antibody to detect FLAG-tagged proteins and an antibody to ERK1 (loading control). (D) Proteasome inhibitors increase LDB1 expression and block the ability of TIF1 γ to reduce LDB1 expression. HEK293 cells were transfected with an expression vector for FLAG-tagged LDB1 and either an empty expression vector (-), or vectors coding for wild type TIF1 γ (WT) or TIF1 γ with a mutation that inactivates ubiquitin ligase activity (Mut). At 20 hours after transfection cells received no treatment (-), 50 nM epoxomicin, or 10 mM MG-132 for 5 hours. Whole cell extracts were prepared, equal amounts of protein were resolved by denaturing polyacrylamide gel

electrophoresis, transferred to a membrane and then incubated with FLAG antiserum to detect FLAG-tagged LDB1. ERK1 was also immunoblotted as a loading control.

A. Analysis of co-immunoprecipitation in HEK293 cells

B. Analysis of co-immunoprecipitation in CHO cells

Fig. 2.

TIF1 γ and LDB1 interact in HEK293 and CHO cells. (A) HEK293 cells were transfected with vectors for LDB1, FLAG-tagged wild type (WT) or mutant (Mut) TIF1 γ or empty expression vector (-) as indicated. At 20 hours after transfection, whole cell extracts were prepared and samples analyzed by denaturing gel electrophoresis and immunoblotting with polyclonal antisera to LDB1 to detect the input expression of the LDB1 (5% Input, lanes 1-4). FLAG-tagged-TIF1 γ was isolated from the cell extract by immunoprecipitation with FLAG monoclonal antibody and the immunoprecipitate was analyzed by gel electrophoresis and immunoblotting with LDB1 rabbit polyclonal antibody to detect co-immunoprecipitated LDB1 (Immunoprecipitate, lanes 5-8). (B) As in panel A but in CHO cells. Immunoblotting detected proteins with the appropriate migration for full-length LDB1 (closed arrow).

Fig. 3.

A morpholino oligonucleotide to zebrafish TIF1 γ increases LDB1 protein levels. Analysis of the phenotype of embryos injected with a morpholino targeted to the first splice donor site of zebrafish TIF1 γ . Images of live embryos 48 hours post fertilization, heads to the left. (A) Wild-type embryos have approximately 3000 red blood cells in circulation (arrow) that collect near the atrium. (B) Embryos injected with the morpholino targeted to TIF1 γ are bloodless (arrow) and have jagged fins (not shown) but are otherwise normal. (C) Immunoblot analysis of LDB1 expression in zebrafish embryos. At 48 h post fertilization, whole embryo extracts were prepared and equal amounts of protein were examined LDB1 by denaturing gel electrophoresis and immunoblotting with rabbit antiserum to LDB1.

A. Immunoblot analysis of LDB1 in HEK 293

B. Reporter gene analysis of TIF1 effects in α T3-1 cells C. Immunoblot analysis of LDB1 in α T3-1 cells **Fig. 4.**

Selective effects of TIF1 isoforms on LDB expression levels or LIM homeodomain-dependant reporter gene activity. (A) HEK293 cells were transfected with vectors for LDB1 and TIF1 α (α), TIF1 β (β), TIF1 γ WT (γ) or mutant (γ^{mut}) as indicated. At 20 hours after transfection, whole cell extracts were prepared and equal amounts of protein for each sample were resolved by denaturing gel electrophoresis and LDB1 expression assessed by immunoblotting with rabbit antiserum to LDB1. (B) α T3-1 cells were transfected with a reporter gene containing the glycoprotein hormone α -subunit promoter linked to the luciferase coding sequence and expression vectors for TIF1 α , TIF1 β , TIF1 γ , activated ras, or empty vector as indicated as well as an expression vector for β -galactosidase as an internal

standard for transfection efficiency. Cell extracts were prepared 20 hours later and assayed for luciferase and β -galactosidase activity. Luciferase activity was corrected for β -galactosidase activity and the results are presented as means $\pm$ SEM obtained from three separate transfections. (C) α T3-1 cells were transfected with vectors for LDB1 and TIF1a (α), TIF1b (β), TIF1g WT (γ) or mutant (γ^{mut}) as indicated. At 20 hours after transfection cell extracts were prepared and equal amounts of protein for each sample were analyzed as in panel (A).