NGF Inhibits Human Leukemia Proliferation by Downregulating Cyclin A1 Expression through Promoting Acinus/CtBP2 Association

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Abstract

Cyclin A1 is essential for leukemia progression, and its expression is tightly regulated by acinus, a nuclear speckle protein. However, the molecular mechanism of how acinus mediates cyclin A1 expression remains elusive. Here we show that transcription corepressor CtBP2 directly binds acinus, which is regulated by NGF, inhibiting its stimulatory effect on cyclin A1 but not cyclin A2 expression in leukemia. NGF, a cognate ligand for the neurotrophic receptor TrkA, promotes the interaction between CtBP2 and acinus through triggering acinus phosphorylation by Akt. Overexpression of CtBP2 diminishes cyclin A1 transcription, whereas depletion of CtBP2 abolishes NGF’s suppressive effect on cyclin A1 expression. Strikingly, gambogic amide, a newly identified TrkA agonist, potently represses cyclin A1 expression, thus blocking K562 cell proliferation. Moreover, gambogic amide ameliorates the leukemia progression in K562 cells inoculated nude mice. Hence, NGF down-regulates cyclin A1 expression through escalating CtBP2/acinus complex formation, and gambogic amide might be useful for human leukemia treatment.

Keywords

Acinus; CtBP2; Cyclin A1; Gambogic amide; Leukemia; NGF

INTRODUCTION

NGF (nerve growth factor) is a family member of neurotrophins that are essential for neuronal differentiation and survival (Skaper, 2008). Docking of NGF on its cognate receptor TrkA initiates receptor homo-dimerization, autophosphorylation of cytoplasmic...
tyrosine residues on the receptor, and a cascade of cell signaling events including Ras/Raf/ MAPK, PI 3-kinase/Akt and PLC-γ1 (Kaplan and Stephens, 1994). Neurotrophin receptors are also expressed in peripheral tissues. For instance, TrkA is expressed in a variety of leukemia cell lines and primary cells from acute myelogenous leukemia patients (Kaebisch et al., 1996). Nevertheless, NGF signaling and functions in these leukemia cells are not fully understood. TrkA expression in K562 cells is enhanced by differentiation inducer all-trans retinoic acid (RA) (Xie et al., 1997), suggesting NGF might be able to trigger cellular differentiation. Later studies further demonstrate that NGF alone, or in combination with other inducers, actuates K562 megakaryocytic differentiation (Xie et al., 2000). NGF also displays a synergistic effect to cell differentiation in other leukemic cell lines like Mo-CM and KG-1 (Tsuda et al., 1991). Most recently, we showed that gambogic amide (GA-amide) selectively binds to TrkA cytoplasmic juxtamembrane domain and robustly induces its tyrosine phosphorylation and activates downstream signaling including Akt and MAP kinases. By mimicking NGF, GA-amide exhibits a great neuroprotective role in various neural injury animal models (Jang et al., 2007).

There are two mammalian A-type cyclins, cyclin A1 and A2. Cyclin A1 is limited to male germ cells, whereas cyclin A2 is broadly expressed. While Cyclin A2 regulates both G1/S and G2/M transition, cyclin A1 is critical for passage of spermatocytes into meiosis I (Wolgemuth et al., 2004). In addition to expression in male germ cells, cyclin A1 is also found in hematopoietic stem cells and primitive precursors (Yang et al., 1997; Yang et al., 1999). Elevated levels of cyclin A1 have been detected in several leukemic cell lines and in patients with myeloid hematological malignancies (Kramer et al., 1998; Yang et al., 1999). Transgenic mouse model reveals that cyclin A1 overexpression results in abnormal myelopoiesis, supporting an important role of cyclin A1 in hematopoiesis and the etiology of myeloid leukemia (Liao et al., 2001).

Acinus (Apoptosis Chromatin Condensation Inducer in the Nucleus) is a family of nuclear proteins, which trigger chromatin condensation during apoptosis. It has three isoforms (acinus-L, S and -S′), which have different N-terminal structures that probably arise from alternative splicing (Sahara et al., 1999). During apoptosis, acinus proteins are cleaved by caspases, releasing the active form (p17) to induce chromatin condensation (Sahara et al., 1999). We have shown previously that acinus-S is a substrate of Akt, which phosphorylates acinus on S422 and S573 residues and prevents its apoptotic cleavage (Hu et al., 2005). This cleavage could also be inhibited by the tethering of zyxin, a focal adhesion protein that are constantly translocated into the nucleus, where it provokes the anti-apoptotic functions (Chan et al., 2007; Kato et al., 2005). Acinus proteins are also involved in transcription and mRNA splicing. Tange et al. showed that acinus proteins are components of the exon junction complex (EJC), which stabilize the association between RNPS1 and SAP18 (Tange et al., 2005). Moreover, both acinus-L or –S form a trimeric complex with SAP18 and RNPS1, which functions in inhibiting RNA processing (Schwerk et al., 2003). We have shown recently that acinus-S regulates cyclin A1 transcription in leukemia cells (Jang et al., 2008). This transcription enhancing activity could be regulated by SRPK phosphorylation. The role of acinus in transcription is further established by showing a direct interaction with...
retinoic acid receptor (RAR), leading to a repression of RAR-regulated gene (Vucetic et al., 2008).

C-terminal Binding Proteins (CtBP) are co-repressors that inhibit transcription through interacting with a variety of transcription factors. Human and mouse contain two CtBP genes, which encode two proteins (CtBP1 and CtBP2) of high similarity (Katsanis and Fisher, 1998). Structurally, CtBPs share a significant degree of homology to NAD+-dependent dehydrogenase (Kumar et al., 2002), in which the NAD+ binding domain has an important role in the formation of homo- or heterodimers (Thio et al., 2004). Binding of NAD+/NADH also affects the function of CtBPs, thus making the protein as a switch linking cellular redox status to transcription control (Fjeld et al., 2003). For example, association of CtBP2 and neuron restrictive silencing factor (NRSF) is enhanced when cellular NADH concentration is decreased, which results in a repression of BDNF expression (Garriga-Canut et al., 2006).

In this report, we show that CtBP2 associates with acinus. This interaction is NAD+ dependent and can be enhanced by NGF stimulation. CtBP2 associates with acinus-S and dampens its cyclin A1 transcriptional enhancing activity. The formation of CtBP2/acinus-S complex is essential for mediating NGF-induced cyclin A1 repression in leukemia cell line K562. Functionally, NGF inhibits K562 cell proliferation in vitro and administration of NGF mimetic GA-amide ameliorates leukemia progression in K562 cells inoculated mice. These results provide a molecular mechanism for the functional role of NGF in leukemia cells and suggest the potential therapeutic application of GA-amide to leukemia treatment.

MATERIAL AND METHODS

Animal experiment

Animal experiments were conducted according to the institutional ethical guidelines for animal experiments and approved by the Institutional Animal Care and Use Committee (IACUC) at Emory University. Six-week-old nude mice (athymic nu/nu, Taconic, USA) were irradiated (1 × 550 cGy) and tail vein injected with 1 × 10^6 K562 cells suspended in 0.6 ml Hanks’ Balanced Salt Solution. One week after K562 inoculation, 2mg/kg GA-amide were injected (i.p.) daily for 3 weeks.

Yeast two-hybrid screening

The experiments were executed exactly as described (Ye et al., 1999).

Co-immunoprecipitation and in vitro binding assay

Experimental procedures for co-immunoprecipitation and in vitro binding assays are as described (Ye et al., 1999). Western blotting analysis was performed with a variety of antibodies from Santa Cruz Biotechnology (USA) (Cyclin A2, cyclin D1 and Trk), BD Pharmingen (USA) (CtBP2 and cyclin A1), Cell signaling (USA) (phosphor-Akt S473 and phosphor-TrkY490) and Upstate (USA) (acinus).
Luciferase assay

HEK293 cells were co-transfected with CtBP2 and acinus-S plasmids with the pGL3 plasmid consists of cyclin A1 promoter as previously described (Jang et al., 2008). Luciferase activity of cell lysates was then determined using Dual Luciferase Assay Kit (Promega, USA).

RT-PCR

Total RNA from was prepared by using Trizol Isolation Reagent (Invitrogen, USA). Five micro grams total RNA were extracted and first-strand cDNA was synthesized by using Superscript III reverse transcriptase (Invitrogen, USA) and Oligo-dT17 as primer with recommended procedures. Amplification of cyclin A1 and cyclin A2 were performed using the following primers: cycin A1-RT5 (5′-GCCTGGCAAACTATACTGTG-3′), cyclin A1-RT-3 (5′-CTCCATGAGGGACACACACA-3′), cyclin A2-RT5 (5′-TCCATGTCAGTGCGACACACA-3′), and cyclin A2-RT5 (5′-GAAGGTGCTCGAGGAAAGGC-3′). GAPDH fragment was also amplified as internal standard using primers GAPDH-F (5′-CGCATCTTCTTGCTCGATGCC-3′) and GAPDH-R (5′-GCCCTCTACTTGCCCCCTGAATTT-3′). A kinetic profile of the amount of PCR product generated at different PCR cycles was constructed and the cycle number used for individual gene expression study was chosen within the exponential region of the amplification curve. This is to ensure that the amount of the PCR product reflects the amount of template in the original sample.

Immunohistochemical staining

Immunohistochemical assays were performed on formalin-fixed paraffin-embedded sections. Sections from spleen were cut, deparaffinized in xylene and rehydrated in graded alcohols. The slides were then treated with 0.3% hydrogen peroxide and SuperBlock blocking buffer (Pierce Biotechnology, Inc., USA) and incubated with mAb against human CD45 (BD Biosciences, USA). Finally, HRP activity was detected by Zymed Histostain Kit (Invitrogen, USA) and the cells were counterstained with hematoxylin.

Cell cycle analysis

K562 cells were treated with either PBS or 100nM GA-amide for 48 hr. The cells were then fixed and stained with 1μM propidium iodide for 45 min. Cell cycles status were examined using flow cytometry.

Statistics analysis

Data are presented as mean ± SEM. Statistical evaluation was carried out by means of Student’s t test or One-way ANOVA. Data were considered statistically significant when P < 0.05. All statistical analysis was performed by the computer program Prism (GraphPad, USA).
RESULTS

CtBP2 interacts with acinus-S

To search for the interaction partners of acinus-S, we performed a yeast two-hybrid analysis using the C-terminal domain (amino acids 228–583) as bait. Out of the 10 independent positive clones isolated, one of them encodes the C-terminus of CtBP2. We observed interactions between the C-terminal portions (CTD) of acinus and CtBP2 regardless of which protein was used as bait or prey. By contrast, the N-terminal portion (NTD) of acinus-S failed to interact with CtBP2 (Table 1). To verify the interaction between these two proteins, we conducted an in vitro binding assay using various recombinant CtBP2 fragments (Fig 1A), and bindings were performed using flag-acinus-S expressed in HEK293 cells. Acinus-S bound independently to the purified NTD of CtBP2 (aa 1–219) and CtBP2 CTD (aa 217–445), fitting with the yeast two-hybrid result. In addition, flag-acinus-S interacted weakly with the CtBP2 mutant lacking the first 48 aa. Surprisingly, intact CtBP2 did not bind to acinus-S in vitro (Fig 1A), indicating the binding motifs in the NTD and CTD were masked in the full-length protein.

Next, we performed mapping assay to pinpoint the CtBP2 interaction site in acinus-S. Immunoprecipitation assay revealed that the full-length protein and the CTD fragments (aa 228–583, a.a. 340–583) of acinus strongly associated with CtBP2; in contrast, the NTD (aa 1–340) of acinus failed, suggesting acinus aa 340–583 is the major binding region for CtBP2 (Fig 1C & D).

NAD$^+$ is essential for acinus-S/CtBP2 association

NAD$^+$/NADH ratio modulates the function of CtBP proteins (Fjeld et al., 2003; Garriga-Canut et al., 2006; Kumar et al., 2002; Thio et al., 2004; Zhang et al., 2002). To test if NAD$^+$ or NADH is necessary for CtBP2/acinus-S interaction, we performed an in vitro binding assay in the presence of 100 μM NAD$^+$ or NADH. CtBP2 associated with acinus-S in the presence of NAD$^+$ but not NADH or vehicle control (Fig 2A, 1st panel). However, neither NAD$^+$ nor NADH promoted acinus-S to bind CtBP1, suggesting that the acinus-S/CtBP association is isoform specific (Fig 2A, 3rd panel). To test whether NAD$^+$ binding to CtBP2 is essential for its interaction with acinus-S, we mutated the NAD$^+$ binding site in CtBP2 (G189 into A) (Thio et al., 2004) and co-transfected with acinus-S into HEK293 cells. Wild-type but not G189A mutant of CtBP2 co-immunoprecipitated with acinus-S, suggesting the CtBP2/acinus-S interaction is impaired when the NAD$^+$ binding ability of CtBP2 is abolished. An irrelevant nuclear protein PIKE-A (Liu et al., 2007) was used in the transfection and immunoprecipitation as a negative control. No association between PIKE-A and acinus-S was detected, indicating the high specificity for CtBP2/acinus-S association (Fig 2B). To further confirm that acinus-S/CtBP2 interaction is regulated by NAD$^+$ level in vivo, we stimulated HEK293 cells overexpressing CtBP2 and acinus-S with chemicals that increase the intracellular NAD$^+$ concentrations ([NAD$^+$]$_i$) (McLure et al., 2004; Ying et al., 2005). As anticipated, NAD and niacinamide were effective in inducing acinus-S/CtBP2 association. Together, these data suggest that NAD$^+$ binding to CtBP2 is essential for its association with acinus-S and changes of [NAD$^+$]$_i$ affect this interaction.
Binding of CtBP2 to acinus-S is regulated by NGF

We have previously reported that the association of acinus-S and zyxin is enhanced by growth factor stimulation (Chan et al., 2007). We tested if growth factor also triggers acinus-S/CtBP2 interaction. PC12 is a phenochromocytoma cell line that expresses TrkA receptor and is NGF responsive. Serum-starved PC12 cells were treated with NGF for various time points and CtBP2 was immunoprecipitated. Association of CtBP2 with acinus-S increased after 6 h stimulation and the interaction was further elevated after 24 h (Fig 3A).

Pre-treatment of PC12 cells with PI3K inhibitor (LY294002) or Akt inhibitor (TCN) abolished NGF-induced CtBP2/acinus-S complex. By contrast, PKC inhibitor GF109203x had no significant effect on the interaction (Fig 3B). These results suggest that NGF-induced Akt activation is able to enhance the CtBP2/acinus-S interaction. To verify that phosphorylation of acinus-S by Akt is required for CtBP2 interaction, we performed a binding assay using different acinus-S mutants (Hu et al., 2005). While the Akt phosphorylation mimetic mutant (S422, 573D) robustly interacted with CtBP2, unphoshorylated mutant acinus-S (S422, 573A) failed (Fig 3C). In vitro phosphorylation assay showed that CtBP2 cannot be phosphorylated by Akt (data not shown).

Immunofluorescent staining further confirmed that CtBP2/acinus-S interaction is phosphorylation dependent. Co-localization of CtBP2 and Acinus-S was evident in the nuclear speckles of HEK293 cells transfected with CtBP2 and wild-type acinus-S or the S422, 573D mutant. In contrast, co-localization of CtBP2 and Acinus-S was significantly diminished, when acinus-S S422, 573A mutant was overexpressed (Fig 3D). Hence, acinus phosphorylation by Akt is critical for its association with CtBP2.

CtBP2 represses acinus-S-mediated cyclin A1 expression

Since acinus-S is essential for cyclin A1 expression (Jang et al., 2008) and CtBP2 is a co-repressor, we hypothesized that CtBP2 might down-regulate the transcriptional enhancing activity of acinus-S. To test this possibility, we cotransfected acinus-S and cyclin A1 promoter-linked luciferase reporter into HEK293 cells together with increasing amount of CtBP2. Luciferase activity was escalated, when acinus-S was overexpressed (Fig 4A, lane 2), indicating acinus-S enhances cyclin A1 expression by acting through its promoter.

CtBP2, however, diminished the stimulatory effect in a dose-dependent manner (Fig 4A, lanes 3–6), suggesting that CtBP2 acts as a repressor to acinus-S-mediated transcription. Depletion of CtBP2 using specific its siRNA that substantially increased cyclin A1 promoter activity (Fig 4B). Nonetheless, overexpression of CtBP2 or the NAD⁺ binding deficient mutant (G189A) did not change the promoter activity (Fig 4C, lanes 3 and 4). This is probably due to the high endogenous CtBP2 expression level in HEK293 cells. Fitting with our previous report (Jang et al., 2008), overexpression of wild-type but not the S422, 573A mutant (mGST-Acinus-S AA) of acinus-S enhanced the luciferase activity (Fig 4C, lanes 5 and 6). This augmentation was abolished when wild-type CtBP2 was co-expressed; in contrast, G189A mutant had no effect (Fig 4C, lane 5, 8 and 11). The luciferase activity was further increased, when acinus-S S455, 573D mutant (mGST-Acinus-S DD) was overexpressed (Fig 4C, lanes 5 and 7). Similarly, luciferase activity induced by acinus-S S422, 573D (mGST-Acinus-S DD) was reduced in the presence of wild-type but not G189A mutant of CtBP2 (Fig 4D, lanes 7, 10 and 13). Collectively, these findings support that
CtBP2 is a repressor that down-regulates acinus-S-mediated cyclin A1 expression in a NAD\(^+\) dependent manner.

**CtBP2 is essential for NGF to inhibit cyclin A1 expression**

Our luciferase assay results suggest that CtBP2 inhibits cyclin A1 expression by downregulating the transcriptional stimulatory activity of acinus-S. To further demonstrate the functional consequence of CtBP2/acinus-S interaction in human leukemia, we stimulated K562 cells, a cell line that specifically expresses cyclin A1 (Shankar *et al.*, 2005) and TrkA receptor (Chevalier *et al.*, 1994), with NGF (100 ng/ml) and monitored the CtBP2/Acinus-S association and cyclin A1 expression. The amount of cyclin A1 was significantly decreased after 6 h NGF stimulation. Further reduction of cyclin A1 expression occurred at 24 h (Fig 5A, 6\(^{th}\) panel). By contrast, cyclin A2 and cyclin D1 remained constant throughout the experimentation, suggesting that NGF specifically down-regulated cyclin A1 but not other cyclin proteins (Fig 5A, 7\(^{th}\) and 8\(^{th}\) panels). The association between CtBP2 and acinus-S was increased by NGF. The strongest binding was detected at 24 h (Fig 5A, 1\(^{st}\) panel), which inversely correlated with cyclin A1 expression. This result also fits with our observation in PC12 cells that NGF enhances acinus-S/CtBP2 association (Fig 3A). Akt phosphorylation peaked at 30 min. The level of Akt phosphorylation decreased afterward but remained substantially higher than the basal level at 24 h (Fig 5A, 5\(^{th}\) panel). In agreement with the immunoblotting analysis, RT-PCR revealed that transcription of cyclin A1 but not cyclin A2 was significantly reduced after NGF stimulation for 24 h (Fig 5B).

To further demonstrate the effect of CtBP2 and acinus-S on cyclin A1 expression, we transfected K562 cells with myc-CtBP2 and stimulated the cells with NGF. Compared with control, the amount of the endogenous cyclin A1 was markedly reduced in CtBP2 over-expressed cells. NGF treatment further diminished cyclin A1 level (Fig 5C).

NGF significantly blocked cyclin A1 expression after 24 h, and this inhibition was abolished when CtBP2 was depleted by its siRNA. Moreover, the expression of cyclin A1 was markedly enhanced in CtBP2-deficient cells (Fig 5D, top panel), suggesting that CtBP2 is a major factor in repressing cyclin A1 expression in both basal and growth factor-stimulated fashions. On the other hand, knocking down of acinus-S abolished cyclin A1 expression, which was slightly reduced upon NGF stimulation (Fig 5D, top panel), indicating that acinus is a critical effector to regulate cyclin A1 expression by NGF. The modest reduction effect by NGF in acinus-depleted cells might be due to the remnant acinus that was not completely eradicated by its siRNA (Figure 5D, bottom panel). Similarly, transcription of cyclin A1 but not the cyclin A2 was reduced by NGF in control but not CtBP2-depleted K562 cells (Fig 5E). Depletion of acinus-S also diminished the expression of cyclin A1 but not A2 (Fig 5E).

Since depletion of cyclin A1 significantly reduce proliferation in leukemia cells (Ji *et al.*, 2005), treatment of K562 cells with NGF would therefore trigger similar effect. Titration assay revealed that NGF prevented K562 cell proliferation in a dose-dependent manner. NGF at low concentration of 5, 10 or 50 ng/ml did not affect the cell proliferation (Fig 5F). However, when NGF concentration was increased to 100 and 500 ng/ml, cell proliferation was inhibited to approximately 75% of control. No significant amount of cell death was observed in all NGF-treated cells, indicating the reduction of cell number was not a result of
extensive cell death (data not shown). These results suggested that the down-regulation of cyclin A1 by NGF leads to a reduction of cell proliferation.

**TrkA agonist gambogic amide inhibits leukemia cell proliferation**

NGF has poor pharmacokinetic properties. It has a very short distribution half-life of about 5.4 min in circulation (Tria et al., 1994). We therefore tested the anti-proliferative function of NGF using its mimetic GA-amide (Jang et al., 2007) in leukemic mice model. As shown in Fig 6A, stimulation of K562 cells with GA-amide for 30 min activated the TrkA phosphorylation in a concentration as low as 40 nM. The expression of cyclin A1, but not cyclin A2, was inhibited when the cells were challenged by GA-amide for 24 hr. Significant reduction of cyclin A1 was observed when 80 nM or 100 nM of GA-amide was used (Fig 6B). Similar to NGF, GA-amide enhanced the formation of CtBP2/acinus-S complex (Fig 6C) and prolonged treatment for 48 h significantly inhibited cells proliferation (Fig 6D). Titration assay revealed that cell proliferation was reduced to about 50% at 60 nM GA-amide stimulation. Moreover, GA-amide altered the cell-cycle status of K562 cells (Fig 6E). The G1/S transition was significantly reduced in GA-treated K562 cells (G1: 45.8 ± 0.3 vs 36.5 ± 0.1; S: 20.7 ± 0.2 vs 16.6 ± 0.2). In contrast, the number of K562 cells in G2/M phase after GA-amide stimulation was augmented (14.7 ± 0.5 vs 21.9 ± 0.1), indicating GA-amide induces a G2/M arrest in K562 cells. This cell-cycle pattern resembles to previous finding that downregulation of cyclin A1 in ML1 leukemic cells by siRNA challenge slowed S phase entry with G2/M phase accumulation (Ji et al., 2005). Intraperitoneal (i.p.) injection of normal mice with GA-amide (2 mg/kg) induced TrkA phosphorylation in brain 1 h after injection and peaked at 4 h, and the phosphorylation sustained till 8 h (Fig 6F), suggesting the GA-amide is effective in triggering TrkA activation in mice.

Next, we investigated the effect of GA-amide in leukemia progression in nude mice inoculated with K562 cells. All K562 inoculated mice remained alive throughout the experimentation. However, the spleen weight of both K562 inoculated nude mice was significantly reduced when GA-amide was administrated (Fig 6G). We further assessed the leukemia progression in the nude mice by examining the presence of K562 cells in the spleen of the mice by immunohistochemical staining. Infiltrations of K562 cells were detected in both white and red pulp of the spleen of saline injected mice. However, no K562 cells were found in the white pulp and only a small number of K562 were detected in the red pulp of GA-amide administrated mice (Fig 6H). Collectively, these results suggested that GA-amide administration could reduce the in vitro leukemia cell proliferation and ameliorate the leukemia progression in vivo.

**DISCUSSION**

CtBP1 is a D2-hydroxyacid dehydrogenase using NAD⁺ as the cofactor (Kumar et al., 2002). Binding of NAD⁺ or NADH to CtBPs not only triggers their enzymatic action but also affects their co-repressor activity. It has been reported that NADH is critical for CtBP1 and 2 to interact with HIC1 and diminish its repression on SIR1 expression (Hong et al., 2007). Similarly, mutation of the NAD⁺ binding site in CtBP2 abolishes its homodimerization as well as repression activity in the Gal4 tethering assay (Thio et al., 2004). Our results
provide further evidence for the critical role of NAD\textsuperscript{+} to CtBP2 function. First, the presence of NAD\textsuperscript{+} but not NADH is essential for the in vitro interaction between acinus-S and CtBP2 (Fig 1A). Second, chemicals that increase cellular NAD level like niacinamide are able to enhance their interaction \textit{in vivo} (Fig 2C). Furthermore, mutation of the NAD\textsuperscript{+} binding site in CtBP2 abolishes the interaction in intact cells (Fig 2B), thus inhibiting the repressive activity of CtBP2 on cyclin A1 promoter activation by acinus-S (Fig 4C). It is noteworthy that the changes of cellular NAD\textsuperscript{+} level also contribute to NGF-induced CtBP2/acinus-S complex formation, as NGF increases NAD\textsuperscript{+} concentration in PC12 cells (Jackson \textit{et al.}, 1992). Presumably, NGF triggers the phosphorylation of acinus-S by Akt on one hand; it also increases the cellular NAD\textsuperscript{+} level to enhance CtBP2 binding activity on the other hand, which synergistically increases the association between acinus-S and CtBP2.

The identification of CtBP as the interaction partner of viral protein E1a suggests that the co-repressor favorably associates with protein containing a short motif with aa PLDLS (Boyd \textit{et al.}, 1993). Later studies reveal that similar motif is conserved in a lot of CtBP binding proteins like FOG-2 (Fox \textit{et al.}, 1999) and BKLF (Turner and Crossley, 1998), and mutation of the motif abolishes the interaction. Surprisingly, there is no similar motif in acinus-S, indicating that a non-classical interaction occurs between CtBP2 and acinus-S. Indeed, our in vitro binding assay suggests that an overall tertiary structure rather than a specific motif of CtBP2 is necessary for CtBP2/acinus-S complex formation (Fig 1B).

Although the structure of CtBP2 has not been reported, crystal structure of CtBP1 shows that it is a dumbbell-shaped protein contained a large and a small domain separated by the hinge region (Kumar \textit{et al.}, 2002). The small domain (or referred as substrate binding domain) composes of the residues from both N- and C-termini. Since both N- and C-termini of CtBP2 interact with acinus-S, it is thus reasonable to infer that the substrate binding domain is responsible for the CtBP2/acinus-S association. It has been suggested that the C-terminus of CtBPs maintains an unstructured conformation which might be instrumental for its recognition and binding to diverse molecular partners (Nardini \textit{et al.}, 2006). This observation might explain the unsuccessful interaction between full-length CtBP2 and acinus-S in vitro (Fig 1A), which could be rescued in the presence of NAD\textsuperscript{+} (Fig 2A). Since conformational change upon NAD\textsuperscript{+} binding is a key feature of NAD\textsuperscript{+}-dependent dehydrogenase (De Weck \textit{et al.}, 1987), binding of NAD\textsuperscript{+} to CtBP2 would somehow stabilize the C-terminal structure of CtBP2, which favors its binding to acinus-S.

Acinus proteins are a part of splicing machinery (Schwerk \textit{et al.}, 2003), however, their roles in mRNA transcription have not been well studied. We have reported that acinus-S is essential for cyclin A1 expression, which provides the first evidence that these proteins regulate gene transcription (Jang \textit{et al.}, 2008). Recently, Vucetic and colleagues reported that acinus-S\textsuperscript{′}, another isoform of acinus-S, interacted with retinoic acid receptor (RAR) and repressed RAR-regulated gene expression like CYP26 (Vucetic \textit{et al.}, 2008). These results suggest that acinus proteins could function as a transcription repressor or activator in a gene-specific manner. Yet the detailed mechanism of how acinus proteins control gene transcription remains to be studied, our findings that CtBP2 inhibits the transcriptional enhancer activity of acinus-S provide further insight into this process. Conceivably, the
transcriptional activity of acinus proteins could be modulated by co-operating with various nuclear proteins to achieve a particular physiological function.

Since cell-cycle arrest is critical for cell differentiation (Miller et al., 2007), our results that NGF inhibits proliferation via CtBP2/acinus-S/cyclin A1 pathway might provide a prerequisite mechanism for K562 differentiation. It is noteworthy that NGF also enhances proliferation of TF1 erythroleukemia cells (Chevalier et al., 1994). Moreover, a recent study reported that neutrophin receptors (Trk) are constitutively activated and induced leukemogenesis in blasts from patients with acute myeloid leukemia (Li et al., 2009). One of the possible explanations to this discrepant role of TrkA lies on the differential signaling cascade activation. It has been reported that PI3K and mTOR, but not Akt, is the decisive transformation pathway for mutated TrkA-mediated leukemogenesis (Meyer et al., 2007). Activation of Akt, however, is necessary for CtBP2/acinus-S interaction and its subsequent cyclin-A1 suppression. Conceivably, NGF might have dual functions in the hematopoiesis that it triggers either proliferation or differentiation in a cell-type specific and signaling cascade-specific manner.

GA-amide is a TrkA agonist, which mimics the physiological functions of NGF in protecting the neurons from apoptotic stress (Jang et al., 2007). In the present report, we have demonstrated that GA inhibits K562 cells proliferation and reduces leukemia progression in mice (Fig 6). These results suggest that GA-amide can be an effective agent for inhibiting cancer cell proliferation in vivo and highlights its therapeutic potential in leukemia treatment. Indeed, gambogenic acid and its derivatives have been reported to inhibit proliferation of tumor cells in a variety of tissues (Chen et al., 2008; Guo et al., 2004; Liu et al., 2005; Qiang et al., 2008; Shu et al., 2008; Tao et al., 2007; Xu et al., 2009). Interestingly, our finding that NGF or GA-amide inhibits cell growth is contradictory to the well-recognized anti-apoptotic function of NGF. The anti-tumorigenic activity of NGF, however, has been reported in pituitary adenomas, small cell lung cancer, prostate cancer and thyroid tumor (Missale et al., 1993; Missale et al., 1998; Paez Pereda et al., 2000; Sigala et al., 1999), which further supports the notion that NGF can act as an inhibitor to cell proliferation.

In summary, we have provided evidence that NGF enhances the interaction of CtBP2 and acinus-S, which reduces the expression of cyclin A1, leading to inhibiting proliferation of leukemia cells (Fig 6I). By using the NGF mimetic compound GA-amide, we demonstrate that systemic administration of GA-amide effectively inhibits the leukemia progression. Therefore, GA-amide might be a potential therapeutic agent against leukemia.

Acknowledgments

This work is supported by Grant from NIH (RO1 NS060680) to K.Ye. We would like to thank Dr. G. Chinnadurai (Saint Louis University Health Sciences Center) for GST-CtBP1 plasmid.

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Fig 1. CtBP2 binds acinus-S

(A) Schematic constructs of CtBP2 truncation mutants. Various fragments of GST-tagged CtBP2 were expressed and purified from E. Coli. (B) In vitro mapping of the CtBP2 domains that associate with acinus-S. Purified GST-tagged CtBP2 proteins were incubated with lysates of HEK293 cells transfected with flag-tagged acinus-S. The associated acinus-S was pulled-down and detected using anti-flag antibody (upper panel). The GST-tagged CtBP2 fragments (asterisked) used in the in vitro binding were detected by Coomassie blue staining (lower panel). (C) Diagram of different deletion mutants of acinus-S. (D) Mapping of acinus-S domains that associate with CtBP2. The C-terminus of acinus-S (aa 340–583) was sufficient to bind full-length CtBP2 in transfected cells. The expression of myc-CtBP2 (middle panel) and GST-acinus-S fragments (bottom panel) were verified.
**Fig 2. The association of CtBP2 and acinus-S is NAD⁺ dependent**

(A) In vitro binding of CtBP2 and acinus-S. Flag-acinus-S associated with purified GST-CtBP2 in the presence of NAD⁺ but not NADH (1st panel). However, neither NAD⁺ nor NADH enhanced the association of flag-acinus-S and GST-CtBP1 (3rd panel). The GST-fused CtBP2 and CtBP1 used in the *in vitro* binding were detected by Coomassie blue staining (2nd and 4th panels).

(B) Mutation of NAD⁺ binding domain of CtBP2 diminishes the CtBP2/acinus-S association. Myc-tagged wild-type CtBP2, G189A mutant or PIKE-A were cotransfected with flag-acinus-S into HEK293 cells. The proteins were immunoprecipitated using anti-myc antibody and the associated acinus was detected using anti-flag antibody (top panel). No additional NAD⁺ was included during the immunoprecipitation. The expression of flag-acinus (middle panel) and myc-tagged proteins (lower panel) were also verified.

(C) HEK293 cells were co-transfected with GST-CtBP2
and flag-acinus-S followed by a stimulation of vehicle, 10 mM NAD$^+$ or 12.5 mM niacinamide for 24 h. The CtBP2 were then pulled-down and the associated acinus-S was detected using anti-flag antibody (top panel). The expression of GST-CtBP2 and flag-acinus-S were also verified (middle and lower panels).
Fig 3. NGF regulates the interaction of CtBP2 and acinus-S
(A) Endogenous association of acinus-S and CtBP2 is enhanced upon NGF stimulation. PC12 cells were treated with NGF (50 ng/ml) at the indicated times, CtBP2 was immunoprecipitated. The co-precipitated proteins were analyzed by anti-acinus antibody (1st panel). The amount of acinus-S (2nd panel), phosphorylated-acinus-S (3rd panel), and CtBP2 were also examined. (B) Blockage of PI3K/Akt pathway abolishes the acinus-S/CtBP2 interaction by NGF. PC12 cells were pretreated with GF109203x (10μM), LY294002 (10 μM) and TCN (10 μM) for 45 min, followed by NGF treatment for 6 h. The endogenous CtBP2 was then immunoprecipitated and the associated acinus-S was detected (upper panel). The expression of CtBP2 (middle panel) and acinus-S (lower panel) in the whole cell lysates were shown. (C) Akt phosphorylation of acinus-S is essential for CtBP2 binding. HEK293 cells were co-transfected with myc-tagged CtBP2 and GST-tagged wild-type acinus, Akt unphosphorylate mutant (S422, 573A) or phosphorylation mimetic (S422, 573D). The transfected CtBP2 was immunoprecipitated and the associated acinus-S was
detected using anti-GST antibody (top panel). The expression of myc-CtBP2 (middle panel) and GST-acinus-S (bottom panel) were also examined. (D) Nuclear co-localization of CtBP2 and acinus-S. HEK293 cells were co-transfected with myc-CtBP2 and various GST-acinus-S mutants. Less co-localization were found when CtBP2 was co-transfected with GST-acinus-S S422, 573A (AA) mutant but not the wild-type (WT) or acinus-S S422, 573D (DD).
Fig 4. CtBP2 down-regulates acinus-S-provoked cyclin A1 promoter activity

(A) Luciferase assay by cyclin A1 promoter. Different amount of myc-CtBP2 was co-transfected with GST-acinus-S and luciferase-linked cyclin-A1 promoter in HEK293 cells. Empty vector was added to normalize the amount of DNA used in the transfection. The presence of CtBP2 diminished the promoter activity enhanced by acinus-S as indicated by the luciferase activity (top panel). The expression of CtBP2 and acinus-S were examined by immunoblotting (middle and bottom panels) (***: P<0.001 vs transfection with cyclin A1 promoter alone, one-way ANOVA, n=3).

(B) HEK293 cells were transfected with scramble RNA or siRNA against CtBP2, followed by co-transfection with cyclin A1-promoter and GST-acinus-S. Depletion of CtBP2 enhances the acinus-S mediated cyclin A1 promoter activity (1st panel) (***: P<0.001, Student’s t-test, n=3). The endogenous CtBP2 (2nd panel) and GST-acinus-S (3rd panel) were also examined.

(C) Disruption of CtBP2 and acinus-S
interaction abolishes the CtBP2-repressive activity on cyclin A1 promoter. HEK293 cells were transfected with luciferase-linked cyclin A1 promoter and various combinations of CtBP2 and acinus-S mutants. The cyclin A1 promoter activation was determined by the luciferase activity (top panel). The expression of various mutants was also examined (middle and bottom panels) (***: P<0.001, one-way ANOVA, n=3).
Fig 5. NGF down-regulates cyclin A1 expression by promoting CtBP2 and acinus-S interaction

(A) K562 cells were treated with NGF (100 ng/ml) for various time intervals as indicated. The interaction of endogenous CtBP2 and acinus-S was determined by immunoprecipitation (1st panel). Equal amount of acinus-S (2nd panel) and CtBP2 (4th panel) was used in the immunoprecipitation. Phosphorylation of acinus-S (3rd panel) and Akt (5th panel) were examined to testify the action of NGF. Level of cyclin A1 was reduced after NGF stimulation (6th panel). Cyclin D1 (8th panel) and cyclin A2 were not affected (7th and 8th panels).

(B) NGF down-regulates cyclin A1 transcription. K562 cells were treated with NGF (100 ng/ml) for various time intervals as indicated and the expression of cyclin A1 (top panel) and cyclin A2 (middle panel) were examined by RT-PCR. Equal expression of house-
keeping GAPDH (bottom panel) was confirmed. (C) Over-expression of CtBP2 in K562 cells reduces cyclin A1 expression. K562 cells were transfected with myc-tagged CtBP2 or empty vector. 24 h after transfection, the cells were stimulated with NGF (100 ng/ml) for another 24 h. The expression of endogenous cyclin A1 (upper panel) and the transfected CtBP2 (lower panel) were examined by immunoblotting. (D) Effect of CtBP2 or acinus-S depletion on cyclin A1 expression in K562 cells. K562 cells were transfected with either scramble RNA, CtBP2 or Acinus-S siRNA. The cyclin A1 protein (upper panel) was examined by immunoblotting. The knockdown efficiency of CtBP2 (middle panel) and acinus-S (bottom panel) were verified. (E) Depletion of CtBP2 abolishes NGF-suppressed cyclin A1 transcription. siRNA transfected K562 cells were treated with NGF (50 ng/ml) for 24 hr and the expression of cyclin A1 (upper panel) and cyclin A2 (lower panel) were examined by RT-PCR. (F) NGF inhibits K562 cell proliferation. After treatment by NGF with indicated concentration for 48 hr, the total number of cells was determined by trypan blue exclusion assay. Results were normalized and expressed as the percentage of the control group (*: P<0.05, one-way ANOVA, n=3).
Fig 6. TrkA agonist gambogenic amide (GA-amide) inhibits K562 cells proliferation and ameliorates leukemia progression

(A) GA-amide triggers TrkA phosphorylation in vitro. K562 cells were stimulated with different concentrations of GA-amide as indicated for 30 min. The phosphorylation of TrkA and total TrkA amount were examined by Western blot analysis. (B) GA-amide reduces cyclin A1 but not cyclin A2 expression. K562 cells were stimulated with different concentration of GA-amide as indicated for 24 hr. (C) GA-amide enhances CtBP2/acinus-S interaction. K562 cells were stimulated with 200 nM GA-amide for 24 hr. The CtBP2 was immunoprecipitated and the associated acinus-S was tested using specific antibody (top panel). The expressions of CtBP2 (middle panel) and acinus-S (bottom panel) were also examined. (D) GA-amide inhibits K562 cells proliferation. Various concentrations of GA-amide were applied to K562 cell culture for 48 hr. The number of living K562 cells were examined by Trypan blue exclusion assay. Results were mean ± SEM from three independent experiments (*: P<0.05, one-way ANOVA). (E) Cell cycle analysis of K562 cells treated with 100 nM GA-amide for 48 hr. (**: P<0.001, Student’s t-test, n=3). (F) GA-amide triggers TrkA phosphorylation in vivo. Three-month-old C57BL6 mice were injected with 2 mg/kg GA-amide intraperitoneally and scarified at different time intervals as indicated. The phosphorylation and total expression of TrkA in brain was examined by Western blot analysis. (G) Reduced splenomegaly in GA-amide injected leukemic mice. After three weeks injection of GA-amide (2 mg/kg, daily i.p. injection), the K562 cell inoculated nude mice were scarified and the spleen weight was measured. Data were expressed mean ± SEM from 6 animals in each treatment group (*: P<0.05, Student’s t-test).
(H) Immunohistochemical staining of spleen tissues collected from K562 inoculated nude mice treated with saline or 2 mg/kg GA-amide. The tissues were staining with antibody against the K562 marker (human CD45). Magnified view shown in the right panel of each pulp was marked with dash rectangle. Representative result of 3 mice from each treatment was shown. (I) Proposed mechanistic model of NGF-repressed cyclin A1 expression.
Table 1

Yeast hybrid screening of acinus-S interacting partner. The C-terminal of CtBP2 was identified as the interaction partner of acinus-S.

| Library                      | # Transformants | # his + | # His + and β-gal + |
|------------------------------|-----------------|---------|---------------------|
| Human Fetal Brain            | 1.5 million     | 23      | 10                  |

| BD fusion protein | AD fusion protein | Histone protography | β-gal Filter assay |
|-------------------|-------------------|---------------------|-------------------|
| Acinus (a.a.228–583) | CtBP2 (a.a. 239–445) | +                   | +                 |
| CtBP2 (a.a. 239–445) | Acinus (a.a. 228–583) | +                   | +                 |
| Acinus (a.a. 1–228) | CtBP2 (a.a. 239–445) | −                   | −                 |