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CIAPIN1 targets Na^+/H^+ exchanger 1 to mediate K562 chronic myeloid leukemia cells' differentiation via ERK1/2 signaling pathway

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ABSTRACT

CIAPIN1 (cytokine-induced antiapoptotic inhibitor 1) was recently identified as an essential downstream effector of the Ras signaling pathway. However, its potential role in regulating myeloid differentiation remains unclear. In this study, we found depletion of CIAPIN1 by shRNAs led to granulocytic differentiation of K562 cells. Meanwhile, the decrease of NHE1 and up-regulation of phosphorylated ERK1/2 were observed after CIAPIN1 depletion. Interestingly, targeted inhibition of NHE1 further promoted the differentiation of K562 cells with CIAPIN1 silencing. Accordingly, ectopic expression of NHE1 reversed this phenotype. Furthermore, ERK1/2 inhibition with the chemical inhibitor, PD98059, abolished CIAPIN1 silencing-induced differentiation of K562 cells after NHE1 inhibition. Thus, our results revealed important mechanism that CIAPIN1 targeted NHE1 to mediate differentiation of K562 cells via ERK1/2 pathway. Our findings implied CIAPIN1 and NHE1 could be new targets in developing therapeutic strategies against leukemia.

1. Introduction

CIAPIN1, is a recently identified antiapoptotic molecule which has been found without any homology to widely known apoptosis regulatory molecules including members of Bcl-2 family and caspase family. CIAPIN1 has been recognized as a downstream effector of the receptor tyrosine-Ras signaling pathway in mouse Ba/F3 pro-B cell line. It also plays pivotally roles in malignant phenotypes of gastric cancer and renal cancer [1–3]. The expression of CIAPIN1 is dependent on stimulation with growth factors such as interleukin 3, stem cell factor, and thrombopoietin in factor-dependent hematopoietic cell lines. Forced expression of CIAPIN1 confers resistance to apoptosis caused by growth factor deprivation [4]. In addition, CIAPIN1 protects Ba/F3 cells against etoposide, γ radiation and stauroporine. But the underlying mechanisms remains unclear [5], especially in malignant cells. Moreover, enhanced expression of CIAPIN1 in the host induces some solid tumors and leukemia development [6].

NHE1 (Na^+/H^+ exchanger 1) is an electroneutral plasma membrane transporter that catalyzes the extrusion of intracellular proton ions in exchange for extracellular sodium ions, thereby regulating intracellular pH (pH_i) [7,8]. NHE1 is ubiquitously expressed in essentially cell types and multiple roles of NHE1 have been described recently. In addition to its primary roles in pH_i homeostasis and cell volume regulation, a substantial body of evidence indicates that NHE1 also plays essential roles in control of multiple cellular processes, including cell proliferation and differentiation, migration and cell death/survival.

CML (chronic myeloid leukemia), is a clonal disorder of the pluripotent hematopoietic stem cell (resulting in a progressive granulocytosis), in which a reciprocal translocation *t*(9; 22) (q34; q11) forms a Ph (philadelphia) chromosome and thus creates a novel fusion gene, *BCR-ABL* [9,10]. The fusion protein has a constitutively activated tyrosine kinase which is essential to the pathogenesis of CML [11]. Impaired differentiation is a common feature of many hematologic malignancies, including CML. K562 cell line, characterized by *BCR-ABL* positive, was derived from a patient with CML in 1977 [12]. However, the role of CIAPIN1 in CML has not been reported yet. Our study was an attempt to characterize the role of CIAPIN1 in differentiation of K562 CML cells. pH_i (intracellular pH) regulation is extremely important, because virtually every biological process is pH sensitive. Changes in pH_i are important in modulating transepithelial solute transport, in modifying biochemical or endocrinological function, and in controlling cell growth and differentiation [13]. It has been documented that increase of pHi caused by NHE1 provides either a permissive or

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Table 1
The sequences of primers designed for real-time quantitative PCR.

Human gene	Primer sequence (forward)	Primer sequence (reverse)
GAPDH	5′-GAAGGTGAAGGTCGGAGTC-3′	5′-GAAGATGGTGATGGGATTTC-3′
CIAPIN1	5′-AGTGGTCTGGGATAAGTC-3′	5′-CCTGGGACTAAACCTGAC-3′
NCF1	5′-GACACCTTCATCCGTCACATC-3′	5′-CTGCCCGTCAAACCACTT-3′
ORM1	5′-CTCCTGGTCTCAGTATGGC-3′	5′-GTTGCTCCTTGGTCGTCT-3′
HIF1α	5′-CACCTATGACCTGCTTGG-3′	5′-TGTTTGTTGAAGGGAGAA-3′
Elastase 2	5′-GCTAATCCACGGAATTGC-3′	5′-GATGATAGAGTCGATCCAGTT-3′
Cathepsin G	5′-CGCATCTTCGGTTCCTAC-3′	5′-CTTCTCATTGTTGTCCTTATCC-3′
MPO	5′-AGTATGAGGACGGCTTCT-3′	5′-TCGGCTTGGTTCTTGATG-3′
Lactoferrin	5′-CTTACCTGGAACTCTGTGAA-3′	5′-AGTGTAGCCGTAGTATCTCT-3′
NHE1	5′-GCTTCAGTCTGCCGCCGGGGCC-3′	5′-GGCCCCGGCGGCAGCAGACTGAAGC-3′

an obligatory signal for cell proliferation [14,15] and differentiation [16,17]. NHE1 has been proved to be associated with serum deprivation-induced differentiation in immortalized rat proximal tubule cells [13]. It was also shown to play important roles in the process of cardiomyocyte differentiation of embryonic stem cell [18] as well as retinoic acid-induced differentiation of HL60 cells [16]. Our recent findings also demonstrated that decrease of pH_i contributed to human umbilical cord-derived mesenchymal stem cells differentiation [19]. Another study from our group pointed out that NHE1 inhibition facilitated the differentiation of K562 cells [20]. Nevertheless, whether CIAPIN1 has any association with NHE1 remains elusive. Thus, in this study, we initially measured the effect of CIAPIN1 depletion on K562 cells differentiation, and then explored the potential association between CIAPIN1 and NHE1. The potential function of MAPKs signaling pathway during this process was also investigated.

2. Materials and methods

2.1. Reagents and chemicals

We obtained RPMI1640 medium, penicillin and streptomycin from Gibco-BRL Life Technologies, Inc. (Burlington, ON, Canada), FBS (fetal bovine serum) from HyClone (Logan, UT, USA), Cariporide from Santa Cruz Biotechnology (Santa Cruz, CA, USA), MEK1 inhibitor PD98059 from Beyotime (Shanghai, China). For western blotting analysis, antibodies GAPDH, NHE1, anti-phospho-specific ERK1/2, p38, JNK1-2/SAPK, and non-phosphorylated ERK1/2, p38, JNK were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-phospho-specific Akt and non-phosphorylated Akt from Cell Signaling Technology (MA, USA); antibody CIAPIN1 from Abcam (MA, USA). ECL (enhanced chemiluminescence reagent plus) reagents were purchased from BD Transduction Laboratories (CA, USA).

2.2. Cell culture

The K562 CML cell line was preserved by our laboratory. K562 cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humid atmosphere with 5% CO_2.

2.3. Plasmids and transfection

The independent hairpins targeting CIAPIN1 were designed by using software from Ambion and cloned into the eukaryotic vector pGPU6/GFP/Neo (GenePharma, China). The plasmid pGPU6-shCIAPIN1 (CIAPIN1 interfering DNA plasmid) and pGPU6-scramble (unrelated sequence DNA plasmid) were prepared from transformed *E. coli* DH5α cells and transfected into K562 cells with Lipofectamine™2000 (Invitrogen, CA, USA) according to the protocol provided by the company. The transfectants were selected with G418 for at least 2 weeks. When the stable clones were obtained, the GFP positive cells were sorted by flow cytometry.

2.4. Real-time quantitative PCR

Trizol kit (Invitrogen, CA, USA) was utilized to extract total RNA from cultured cells as suggested by the manufacturer. 2 μg RNA was used for reverse transcription in a 20 μl reaction with EasyScript RT (TransGen Biotech, China). Primer premier software 5 was used to design the primers for real-time quantitative PCR. The sequences of primers used in our study were shown in Table 1. Real-time quantitative PCR was performed with ABI 7500 system Instrument by using SYBR Green PCR kit (Takara, Japan). Thermal cycling conditions were 95 °C for 30 s, followed by 40 cycles of 5 s at 95 °C and 34 s at 60 °C. PCR reactions were performed in a total volume of 20 μl, containing 2 μl of sample cDNA, 0.2 μM of each primer, and the SYBR Green PCR kit following the manufacturer's instructions.

2.5. Western blotting

Proteins isolated from cells were resolved by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were blocked for 1 h and then incubated with primary antibodies overnight and then horseradish peroxidase-conjugated secondary antibodies for 1 h, respectively. Specific proteins were visualized with enhanced chemiluminescence detection reagent and determined by densitometric analysis with a Lynx video densitometer (Biological Vision).

2.6. Flow cytometry

K562 cells were harvested when they were 70–80% confluent. After washed with ice-PBS two times, cells were suspended in 100 μl PBS at the density of 1×10^6 cells/ml. Cells were incubated with 20 μl of Human Fc Receptor Binding Inhibitor Purified per 100 μl for 15 min at room temperature prior to staining to block the nonspecific binding. 2 μl anti human CD11b-PE (eBioscience, CA, USA) was added to cells suspension and incubated for 30 min at 4 °C in the dark. Cells were then washed three times and analyzed by LSR II flow cytometry (BD, USA).

2.7. Immunofluorescence

K562 cells with indicated treatments were fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.5% triton for 15 min. Cells were washed with ice-cold PBS, blocked with 0.5% BSA in PBS for 30 min and then incubated with a mouse monoclonal antibody against CIAPIN1, followed by TRITC-conjugated goat anti-mouse IgG antibody. Cells were imaged on Leica TCS SP2 confocal laser microscope (PerkinElmer, USA).

2.8. Statistical analysis

Each experiment was independently repeated at least three times. Statistical analyses were performed by analysis of variance and unpaired two-tailed Student's test. Values were presented as mean ± SD. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Validation of CIAPIN1 knock-down in K562 cells

To explore the potential function of CIAPIN1 on K562 cells differentiation, we constructed two CIAPIN1 shRNA plasmids and transfected into K562 cells to test their RNA interference efficiency. After selected with G418 for 2 weeks, high ratio of GFP^+ cells were obtained. The percentage of GFP^+ cells for scramble, CIAPIN1shRNA1 and CIAPIN1shRNA2 transfected cells were 97.7%, 80.3% and 92.4%, respectively (Fig. 1A). Real-time quantitative PCR and western blotting measurement showed that specific sequence shRNA1 could efficiently deplete the expression of CIAPIN1 ($P < 0.05$), while shRNA2 failed to inhibit CIAPIN1 expression (Fig. 1B). Immunofluorescence staining was also performed to confirm the high-efficient interference of CIAPIN1 (Fig. 1C). Thus, CIAPIN1shRNA1 K562 cells were selected for further study.

3.2. CIAPIN1 knock-down induced granulocytic differentiation of K562 cells

Morphological change is the hallmark of malignant tumor cells differentiation. To explore the potential effects after CIAPIN1 depletion with shRNA, the morphological and ultramicrostructural