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RTP801 is a Novel Retinoic Acid Responsive Gene Associated with Myeloid Differentiation

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Abstract

Objective—Retinoids are crucial in the regulation of fundamental cellular processes including terminal differentiation of both normal and malignant myeloid progenitors. The aim of this study was to identify and characterize retinoic acid (RA) target genes.

Methods and Results—RTP801 is a recently cloned stress response gene that acts as a negative regulator of the mTOR pathway. Here we identified RTP801, as a novel early RA target gene in myeloid cells. RTP801 mRNA levels are induced in acute myeloid leukemia (AML) cell lines during RA-dependent differentiation and are differentially expressed during maturation of normal CD34⁺ cells. The myeloid specific, differentiation related transcription factor C/EBP ϵ also induces RTP801 expression. Overexpression of RTP801 in the U937 leukemic cells leads to growth inhibition and apoptosis. Conversely, silencing of endogenous RTP801 by shRNA reduces RA-induced differentiation of the U937 cells. Downregulation of RTP801 also abrogates hypoxia-induced inhibition of mTOR in those cells.

Conclusion—Taken together, our data suggest that RTP801 is an important RA regulated gene involved in myeloid differentiation, which could represent a therapeutic target in leukemia.

Introduction

One of the main factors contributing to the development of cancer is a block in normal differentiation. Retinoids play an important role in diverse cellular processes, including proliferation and differentiation. They have also been found to have chemopreventive and therapeutic activity in various types of tumor cells (1,2). In hematopoietic progenitors, retinoids are powerful inducers of differentiation, particularly towards the neutrophil lineage (3,4). The biological functions of retinoids are mediated primarily through the retinoic acid receptors (RARs) that regulate the expression of multiple downstream target genes (5,6). RTP801 (also known as REDD1/Dig2/DDIT4) is a recently cloned gene that is induced at the transcriptional

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level in response to various stresses such as hypoxia, glucocorticoids and DNA damage (7–9). One of the recognized functions of RTP801 is its role in cell survival; depending on the cellular context, RTP801 can act as a pro- or anti-apoptotic factor. RTP801 is a transcriptional target of p63 and p53 and is implicated in regulation of reactive oxygen species (ROS) by these two tumor suppressors, as well as in p63-mediated regulation of epithelial differentiation (8). Further studies showed that RTP801 is a critical regulator of the Mammalian target of rapamycin (mTOR) pathway in response to certain stress signals (10–14). Here, we show that RTP801 is an early responsive retinoid target that may contribute to the intricate process of myeloid differentiation.

Materials and Methods

Cell Culture

NB4 cells were a generous gift from M. Lanotte (St. Louis Hospital, Paris, France). The KG-1 cell line was established by our group. Additional cell lines were obtained from the American Type Culture Collection (Rockville, Maryland, USA). Differentiation was assessed by measuring CD11b expressions using phycoerythrin-conjugated monoclonal antibodies and FACS[®]. For hypoxic response studies, cells were incubated at 5% CO₂, 1% O₂.

Northern blot analysis

Total RNA (10 µg) was electrophoresed on a denaturing formaldehyde gel, transferred onto a nylon membrane, and hybridized with [α -³²P]dATP labeled (Strip-EZ DNA, Ambion, Austin, TX) DNA probes.

Plasmids and transfections

RTP801 cDNA was obtained by PCR and cloned into pcDNA3.1 (Invitrogen, Carlsbad, CA). The RTP80 short hairpin RNA (shRNA) plasmid was kindly provided by J. Kaufmann [(Atugen AG, Berlin, Germany, (13)]. The control shRNA (15) and the C/EBP ϵ (16) vectors have been previously described. U937 cells were electroporated using a Gene Pulser electroporation apparatus (Electro Square Porator T820, BTX, San Diego, CA.). NIH 3T3 cells were transfected using Lipofectamine 2000 (Invitrogen).

Western blot analysis

Preparation of whole cell extracts and immunoblot analysis were performed according to standard methods as previously described (15). The following antibodies were used: RTP801 from Chemicon (Temecula, CA); PARP from Santa Cruz Biotechnology (Santa Cruz, CA); phospho-4E-BP1 (Thr37/46) and 4E-BP1 from Cell Signaling Technology (Beverly, MA); β -actin from Sigma-Aldrich (St. Louis, MO).

Microarray expression analysis

Gene expression analysis was performed as previously described (17).

Semiquantitative RT-PCR

Bone marrow aspirates were obtained from healthy volunteers after their informed consent, and CD34⁺ cells were isolated using a magnetic cell sorting system (MiniMACS; Myltenyi Biotec, Auburn, CA). CD34⁺ cells were differentiated in vitro with 50 ng/mL stem cell factor and PIXY321 [20 ng/mL interleukin-3 (IL-3)/granulocyte macrophage-colony-stimulating factor (GM-CSF) conjugate]. RNA was isolated and semiquantitative RT-PCR for RTP801 and C/EBP ϵ was performed using gene-specific primers as described previously (18).

Cell proliferation, and apoptosis assays

Cell proliferation was determined by MTT assays (Roche Diagnostics) according to the manufacturer's protocol. Apoptosis analysis was performed with Annexin V-FITC Apoptosis Detection Kit I (BD PharMingen) according to the manufacturer's instructions.

Yeast two-hybrid screen

RTP801 cDNA was cloned into the pGBT8 (Clontech) vector in-frame with the GAL4 DNA-binding domain and used as a bait plasmid. A human leukocyte Matchmaker cDNA library (Clontech) was used according to the manufacturer's directions. Selection was performed with synthetic dropout medium lacking Trp, Leu, and His and containing 5 mM 3-aminotriazole. Clones were selected on the basis of β -galactosidase-positivity via a filter assay. The β -galactosidase-positive clones were amplified by PCR using Matchmaker 5' and 3' AD LD-insert screening amplimers (Clontech) and sequenced.

Reporter assay

NIH 3T3 cells were cotransfected with a RTP801 promoter reporter construct [pGL3-RTP801, generous gift from Y. Chen, Indiana University, Indianapolis, IN (19), 0.6 μ g], along with either C/EBP ϵ expression vector or empty vector (0.6 μ g). Lysates were harvested 48 h post-transfection and luciferase activity was measured with the Dual-Luciferase reporter 1000 assay system (Promega, Madison, WI). Transfection efficiency was normalized using pRL-TK vector (0.1 μ g).

Results

RTP801 and Lfng are regulated by RA during granulocytic differentiation

To search for novel RA target genes, we have previously performed a PCR-based screen (18). From this screen, we identified RTP801, a stress response gene (7–9), and Lfng, a gene involved in Notch signaling (20) as potential novel RA-target genes (data not shown). To verify retinoid regulation of these genes, we used the RA-sensitive AML cell lines, NB4 and HL60. Early induction of RTP801 and Lfng during all-trans retinoic acid (ATRA)-induced granulocytic differentiation was demonstrated in both cell lines (Fig. 1). These results suggest that RTP801 and Lfng are new early RA targets induced during granulocytic differentiation.

RTP801 is differentially expressed during normal granulocytic differentiation

Additional experiments focused on RTP801. Northern blot analysis of multiple tissue panels demonstrated that RTP801 is expressed at various levels, in most adult tissues (Fig. 2A). ATRA treatment of cell lines from various tissues led to an increase in RTP801 mRNA levels in most of the cell line tested (Fig. 2B), demonstrating that RA regulation of RTP801 is not specific to AML cells blocked at the myeloblast stage of differentiation.

We next examined if the regulation of RTP801 is specific to ATRA-induced differentiation of AML cells, or whether it also occurs during normal lineage-specific differentiation. Analysis of microarray data showed that during cytokine-induced granulocytic differentiation of CD34⁺ normal bone marrow cells, RTP801 mRNA levels decreased (2.7-fold) by day 1 and subsequently increased slightly above basal levels by day 4 (Table 1). GAPDH expression levels at each time point were essentially unchanged (data not shown). Temporal expression of RTP801 in normal CD34⁺ progenitors induced to differentiate with cytokines was also shown by semiquantitative RT-PCR, confirming the microarray data (Fig. 3A). CCAAT enhancer binding protein β (C/EBP β) is involved in transcriptional control of RTP801 in keratinocytes (19). Overexpression of C/EBP ϵ induces granulocytic differentiation in U937 cells (16). Real time PCR analysis showed that induction of expression of C/EBP ϵ in U937

cells stably transfected with an inducible C/EBP ϵ gene, led to an increase in RTP801 levels (Fig. 3B). Reporter assays showed that C/EBP ϵ expression resulted in a 7-fold stimulation of RTP801 promoter activity (Fig. 3C). In addition, during cytokine-induced differentiation of CD34⁺ bone marrow cells, induction of C/EBP ϵ (by day 2) precedes the induction of RTP801 (at day 5, Fig. 3A).

RTP801 is required for ATRA-induced differentiation in U937 cells

To investigate further the role of endogenous RTP801 in myeloid cell differentiation, we examined the effect of silencing RTP801 expression. U937 cells transfected with either RTP801 shRNA or control shRNA were selected with puromycin and then treated with ATRA. Suppression of ATRA-induced RTP801 expression was verified by Western blot analysis (Fig. 4). MTT assays, measuring cell growth, showed that while ATRA inhibited the proliferation of the control cells, this inhibition was impaired in the RTP801 shRNA transfected cells. Similarly, the control cells showed an increase in the levels of the granulocytic differentiation marker, CD11b, and this increase was inhibited in the cells transfected with the RTP801 shRNA. These results show that RTP801 induction is required for ATRA-induced differentiation of myeloid cells.

Expression of RTP801 leads to reduced growth and apoptosis in U937 cells

Depending on the cellular context, RTP801 was shown to function as either a pro- or anti-survival factor. To establish the consequences of RTP801 expression on myeloid cell proliferation, U937 cells were transfected either with an RTP801 (pcDNA3.1-RTP801) expression vector, or an empty vector (pcDNA3.1) as control. Expression of RTP801 was verified by Western analysis (Fig. 5A). After a short period of antibiotic selection, equal numbers of polyclonal populations were cultured in fresh media and their growth rate was measured by MTT assays. Forced expression of RTP801 led to a decrease in proliferation (Fig. 5B). Furthermore, an increase in the apoptosis rate, measured by Annexin V, was observed in the RTP801 transfected cells, compared with the control-transfected cells (Fig. 5C). The onset of apoptosis was confirmed by cleavage of PARP, a marker of activated caspases (Fig. 5D).

RTP801 regulates mTOR-dependent phosphorylation in U937 cells in response to hypoxia

In several cell types, RTP801 functions as a negative regulator of mTOR in response to various stresses, including hypoxia. Northern blot analysis showed that RTP801 levels were significantly upregulated in a time-dependent manner in U937 cells cultured under hypoxic conditions (Fig. 6A), while mTOR activity, as evidenced by the phosphorylation levels of its substrate, eukaryotic initiation factor 4E-binding protein 1 (4E-BP1), decreased (Fig. 6B). To determine whether RTP801 is involved in the downregulation of mTOR by hypoxia in AML cells, mTOR activity was measured in U937 cells in which RTP801 expression was inhibited by shRNA (Fig. 6C–D). In cells transfected with control shRNA, hypoxia led to dephosphorylation of 4E-BP1 (Fig. 6E). However, this dephosphorylation was impaired in the shRNA-RTP801 transfected cells, showing that RTP801 is necessary for hypoxia-induced downregulation of mTOR signaling in AML cells.

Identification of RTP801 potential binding partners

RTP801 belongs to a family of highly conserved proteins that possess little detectable homology to known protein domains (7,8). To begin elucidating the molecular mechanisms through which RTP801 functions, we performed a yeast two-hybrid screen in order to identify its binding partners. Full-length RTP801 was fused to the GAL4 DNA binding domain and used as a bait to screen a human leukocyte cDNA library. Several novel proteins, as well as, known proteins were identified as potential interacting partners (Table 2). Among the known

proteins identified, neutrophil elastase (NE) is especially interesting because it is a neutrophil specific gene that has been implicated in myeloid differentiation (21).

Discussion

We have identified RTP801 and Lfng as novel early RA responsive genes, in myeloid cells. In this study we focused on RTP801. We found that RTP801 is induced during RA-dependent granulocytic differentiation in AML cell lines and is also temporally expressed during cytokine-dependent differentiation of primary CD34⁺ cells, as well as in U937 cells induced to differentiate by C/EBP ϵ . Furthermore, silencing RTP801 inhibits RA-induced differentiation of U937 cells. These results suggest that RTP801 plays an important role in myeloid cell differentiation.

Although the molecular mechanisms underlying RTP801 functions are largely unknown, one of its demonstrated roles is to act as a negative regulator of mTOR in response to certain stress signals, such as hypoxia. The mTOR pathway and the hypoxia signaling have both been extensively studied in solid tumors (22–24). Recently, however, these networks have also been recognized as important therapeutic targets in leukemias (25–27). We found that hypoxia inhibits mTOR activity in U937 cells (and several other AML cell lines, data not shown). RTP801 mRNA levels are upregulated in U937 cells cultured under hypoxic conditions and inhibition of endogenous RTP801 in those cells abrogates hypoxia-induced down regulation of mTOR. These results suggest that RTP801 could play a role in leukemic transformation by modulating the mTOR pathway and that targeting RTP801 may sensitize AML cells to hypoxia, ATRA and other apoptotic signals.

RTP801 has been shown to regulate cell survival in a number of systems. We found that in U937 cells cultured in a low serum concentration, overexpression of RTP801 inhibited growth and induced apoptosis while these effects were not observed in cells grown at high a serum concentration (data not shown). These results are consistent with earlier studies showing that RTP801 can function as a pro- or anti-apoptotic factor depending on the cellular context (7, 13).

To begin addressing the question of how RTP801 mechanistically exerts its diverse effects on cells, we used the yeast two-hybrid system and identified NE as a potential binding partner for RTP801. Preliminary studies confirmed the interaction between RTP801 and NE (data not shown). NE, a serine protease predominantly expressed by neutrophils, is implicated in myeloid differentiation and in development of APL (28,29). Thus, the RTP801-NE interaction further supports our hypothesis that RTP801 is involved in myeloid differentiation.

In summary, we identified RTP801 as a novel RA inducible gene. Our data support a model wherein RTP801 regulates cellular pathways involved in both normal and malignant myelopoiesis. Finding novel RA-target genes, like RTP801, and identifying pathways that intersect with the retinoids signaling is important for our understanding of the physiological functions of retinoid and may also contribute to the development of new, specific strategies for cancer therapy.

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Figure 1. RA-dependent expression of RTP801 and Lfng

Northern blot analysis of time-dependent expression of RTP801 and Lfng in NB4 and HL60 cells treated with ATRA (5×10^{-7} M) for various durations.

Figure 2. Detection of RTP801 mRNA in human tissues and RA-dependent expression of RTP801 in various cancer cell lines

(A) Human multiple-tissue Northern blots (Clontech) were hybridized with RTP801. Blots were stripped and hybridized with GAPDH. Bar graph represents quantification analysis of RTP801 expression using densitometry and normalized against GAPDH expression levels.

(B) Northern analysis of RTP801 expression in cell lines untreated (–) or treated (+) with ATRA (10⁻⁶ M) for 2 h. Blots were stripped and hybridized with GAPDH. Cell lines from the following cancers were examined: prostate (LNCaP), breast (MCF-7, MDA-MB-231), osteosarcoma (SAOS), cervical carcinoma (C33A), monoblastic leukemia (U937), acute promyelocytic leukemia (NB4), acute myeloblastic leukemia (HL60), CD34⁺ leukemia (KG1), erythroid CML blast crisis (K562) and T-cell leukemia (Jurkat).

Figure 3. Expression of RTP801 during granulocytic differentiation of normal human CD34⁺ cells (A) Semiquantitative RT-PCR analysis of RTP801 and C/EBP ϵ expression in human CD34⁺ hematopoietic stem cells induced to differentiate towards granulocytes by culturing in appropriate cytokines (stem cell factor, IL-3, and GM-CSF). GAPDH expression was measured as control. PCR products were electrophoresed on agarose gel, transferred to a nylon membrane and hybridized with internal oligonucleotide probes to confirm specificity of the PCR products. Ethidium bromide staining of agarose gel of PCR products of CD34 and CD11b are shown as differentiation controls (as they were in previously published work by Gery et al, 18). (B) U937 cells stably transfected with either zinc inducible C/EBP ϵ (pMT ϵ) or control empty vector (pMT), were incubated either with or without zinc (100 μ M, 48 h). RTP801 and C/EBP ϵ expression levels were analyzed by real time PCR with specific primers. 18S levels were measured as internal control. The results are expressed in arbitrary units as a ratio of either RTP801 or C/EBP ϵ transcripts/18S transcripts (each value represents the mean of three measurements of the sample). (C) Reporter assays were performed with NIH 3T3 cells cotransfected with RTP801 reporter vector (pGL3-RTP801) and either empty vector (EV) or C/EBP ϵ expression vector. Control transfections indicated that the C/EBP ϵ had little effect on the pGL3 empty reporter vector (data not shown). Results represent the mean of triplicate transfections.

Figure 4. Silencing of RTP801 inhibits ATRA-induced differentiation of U937 cells

U937 cells were transfected with a vector containing the puromycin-resistance gene and either RTP801 shRNA (sh-RTP) or control shRNA (sh-C). Following a 3 day selection with puromycin, cells were incubated in fresh media either without or with ATRA (10^{-6} M) for 4 days. Silencing of RTP801 expression was analyzed by Western blot. β -actin was used to control for equal loading. Cell proliferation was measured by MTT assays. Data represent the mean $\pm$ SD of quadruplicate samples. The experiment was repeated 3 times. Cell surface expression of CD11b was determined by FACS.

Figure 5. RTP801 inhibits U937 cell proliferation

U937 cells were transfected either with RTP801 expression vector (RTP801) or an empty vector (EV), selected with G418 for 5 days and used in subsequent assays. (A and D) Western analysis with RTP801 (A) and PARP (D) antibodies. β-actin was used to control for equal loading. (B) MTT assays. Cells were incubated in media containing 1% serum and cell proliferation was measured by MTT assays. Wild type, non-transfected U937 cells (wt) were included as control. Data represent the mean ± SD of quadruplicate samples. The experiment was repeated 3 times. (C) Apoptosis analysis. Transfected cells were stained with annexin-V-FITC and propidium iodide (PI). Late and early apoptotic populations are in the upper right quadrant and lower right quadrant, respectively.

Figure 6. RTP801 downregulates mTOR in U937 cells

(**A–B**) U937 cells were treated with hypoxia for the indicated time points. (**C–E**) U937 cells were transfected with a vector containing the puromycin-resistance gene and either RTP801 shRNA (sh-RTP) or control shRNA (sh-C). Following a 3 day selection with puromycin, cells were incubated in fresh media and treated with hypoxia for 16 h. RTP801 expression was analyzed by Northern blot (**A** and **C**) and Western blot (**D**), 4E-BP1 phosphorylation status was measured by Western analysis with a phospho-4E-BP1 antibody (**B** and **E**). For loading controls, Northern blots were stripped and hybridized with GAPDH, Western blots were stripped and probed with a 4E-BP1 antibody.

Table 1

Microarray data analysis of RTP801 expression levels during cytokine-induced granulocytic differentiation of CD34⁺ normal bone marrow cells.

Day	RTP801 Expression
0	1
1	0.36
4	1.51
7	1.43

Each of the time points was divided by the expression levels at day 0 (value=1) to generate the final relative expression levels.

Table 2

Potential RTP801 binding proteins identified by yeast two-hybrid screen of a human leucocyte library.

Gene	# of clones
<i>PRPF3</i> : PRP3 pre-mRNA processing factor 3 homolog PRPF3	3
<i>ELA2</i> : elastase 2, neutrophil	4
<i>NDUFB7</i> : NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 7, 18kD	5
<i>TNNT1</i> : troponin T1, skeletal, slow	10
<i>CIQA</i> : complement component 1, q subcomponent	2
<i>GABARAPL2</i> : GABA(A) receptor-associated protein-like 2	4
<i>ATP5O</i> : ATP synthase, H ⁺ transporting, mitochondrial F1 complex, O subunit	3

Shown are clones that were identified in more than a single yeast clone. The table does not include ribosomal proteins and unknown proteins.