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P300 Regulates the Human RLIP76 Promoter Activity and Gene Expression

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Abstract

A 76-kDa Ral-interacting protein (RLIP76) has been implicated in the pathogenesis of cancer and diabetes. It is often overexpressed in human malignant cell lines and human tumor samples and has been associated with metastasis and chemoresistance. RLIP76 homozygous knockout mice exhibit increased insulin sensitivity, hypoglycemia, and hypolipidemia, and resist cancer development. Little is known about the mechanism by which the expression of RLIP76 is regulated. In the present study, we functionally characterized the RLIP76 promoter using deletion mapping and mutational analysis to investigate the regulation of RLIP76 transcription. We have identified the promoter regions important for RLIP76 transcription, including a strong *cis*-activating element in the proximal promoter containing overlapping consensus cMYB and cETS binding sites. Transcription factor cMYB and the coactivator p300 associated with RLIP76 gene promoter as shown by CHIP assay. Knockdown of p300 in HEK293 cells reduced the activity of the promoter fragment containing wild type cMYB/cETS binding site in comparison to that with deleted or mutated cMYB/cETS binding site. Knockdown of p300 also decreased the RLIP76 expression as indicated by immunoblotting, immunocytochemistry and flow cytometry analysis. Thus, we report for the first time that p300 associates with the RLIP76 promoter via an overlapping cMYB and cETS binding site and regulates RLIP76 promoter activity and its expression.

Keywords

RLIP76; Transcription; Promoter Activity; p300; cMYB; cETS

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1. Introduction

RLIP (Ral-interacting protein)-76/RalBP1¹ (Ral-binding protein-1) is a multifunctional protein, implicated in cell proliferation, metastasis and ligand-dependent receptor endocytosis. It is a stress-inducible non-ABC transporter that is overexpressed in most cancer cell lines and many human cancers [1–5]. RLIP76 was identified and cloned as an effector of Ral, a GTPase activated during Ras signaling [6–8]. Later we established the identity of RLIP76 with dinitrophenyl-S-glutathione (DNP-SG)-ATPase that mediates ATP-dependent efflux of glutathione conjugates (GS-E) of electrophilic compounds, and other xenobiotics including chemotherapeutic agents [9, 10]. In our initial studies using biochemical and molecular biological approach, we have established that RLIP76 is a transporter of GS-E as well as multiple drugs. These studies also demonstrate that in the absence of RLIP76, GS-E accumulates in the cells resulting in an increased sensitivity to apoptosis caused by stress, xenobiotics, drugs, and high-energy radiation [11–13]. Subsequent studies using knockout-mouse models show that despite markedly increased tissue oxidative-stress, RLIP76^{−/−} mice are highly resistant to chemical carcinogenesis and are even resistant to the growth of subcutaneously implanted cancer cells [1–3, 14–16]. Moreover, these mice are exquisitely insulin sensitive, hypoglycemic and hypolipidemic indicating that RLIP76 plays an important role in mediating insulin-resistance typical of type II diabetes [17, 18]. Our recent studies show the near absence of clathrin-dependent-endocytosis (CDE) in RLIP76^{−/−} mouse embryonic fibroblasts results in the inhibition of insulin receptor endocytosis, and thus increase in insulin sensitivity [17, 18]. Incremental increase in RLIP76 in RLIP76^{−/−} mouse embryonic fibroblasts decreases insulin/glucose ratio and insulin sensitivity, which are the hallmarks of diabetes [17]. These studies suggest that suppression of RLIP76 can serve as a treatment for cancer and diabetes.

Studies from our group and by others clearly show that the expression levels of RLIP76 play an important role in cancer and diabetes [1, 15, 16, 19–24]. Human embryonic kidney 293 (HEK293) and MCF cells has been chosen as a model as these cells have high transfection efficiency and the expression and activity of RLIP76 has been characterized in each of these cells lines. While the functions of RLIP76 in these pathological conditions have been studied in great details, there are no reports elucidating the mechanisms involved in the regulation of RLIP76 gene expression. In the present study, we have performed deletion and mutational analysis of the RLIP76 gene promoter to investigate its transcription regulation. Our results show that p300, which interacts with a transforming protein E1A and has been implicated in the regulation of gluconeogenesis and lipid metabolism, is a key transcriptional regulator of RLIP76. Thus, this finding is consistent with the role of RLIP76 in both cancer and diabetes.

2. Materials and Methods

2.1. Reagents

The primers and oligonucleotides used in this study were synthesized by Bio Synthesis Inc. (Lewisville, TX) or Invitrogen (Carlsbad, CA). The Firefly-luciferase plasmid, pGL3-basic was kindly provided by Dr. M. Kim, and the Renilla-luciferase plasmid, pRL-CMV, was a gift from Dr. P. Mathew, University of North Texas Health Science Center, Fort Worth, TX. p300 siRNA and non-targeted control siRNA were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Mouse monoclonal antibody against p300 was from

¹RLIP76 is a 76-kDa splice variant protein encoded by the human *RALBP1* gene (18p11.22) (HGNC: 9841). In the present communication, to avoid confusion, we have used “RLIP76” to refer to both the gene and protein. Although the designation RIP or RIP1 has previously been used for the mouse gene or protein, its use in referring to RLIP76/RALBP1 should be avoided because the distinct gene RIP1K (HGNC: 10019) has been referred to previously and currently as either RIP or RIP1.

Millipore (Billerica, MA), and rabbit monoclonal antibody against cMYB was purchased from Abcam (Cambridge, MA). Horse-radish peroxidase-conjugated goat anti-mouse and donkey anti-rabbit antibodies were obtained from Jackson Immuno Research Lab Inc. (West Grove, PA). Mouse polyclonal antibody against RLIP76 and mouse IgG were from Santa Cruz Biotechnology Inc., while rabbit IgG was from Thermo Fisher Scientific (Waltham, MA). QuickChange II Site-Directed Mutagenesis Kit was bought from Agilent Technology (Santa Clara, CA), while Chip-IT® Express Enzymatic kit was from Active Motif (Carlsbad, CA). Dual luciferase assay system was purchased from Promega Corporation (Madison, WI). Thermo-TAQ polymerase, Lipofectamine 2000 and Lipofectamine RNAiMax were from Invitrogen (Carlsbad, CA). All other reagents used were of analytical grade and described elsewhere [25, 26].

2.2. Plasmid constructs

The upstream promoter sequence of the human RLIP76 gene was PCR-amplified using normal human genomic DNA (Promega) as template and was ligated in T-easy vector (Promega). Different 5' deletion fragments of the promoter were PCR amplified using primers with KpnI and HindIII sites and ligated in Firefly-luciferase reporter vector pGL3-basic (Promega) to generate -2965/-1, -2486/-1, -1986/-1, -1582/-1, -966/-1, -512/-1, -245/-1, -199/-1, -167/-1, -152/-1, -101/-1, -72/-1 and -50/-1-pGL3 plasmid constructs. The insert sequences were verified by sequencing. Renilla-luciferase plasmid containing CMV promoter (pRL-CMV) was used as an internal control. The primers used for the PCR amplification of the RLIP76 promoter fragments are listed in Table I.

2.3. Cell culture and transfection

HEK293 cell lines (obtained from ATCC) were maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS). MCF7 cells (ATCC) were cultured in RPMI 1640 medium supplemented with 10% FBS. Cells were kept in a humidified incubator at 37°C with 95% air and 5% CO₂. For luciferase-assays, equal copy numbers of the luciferase plasmids carrying different 5' deletion fragments of the promoter were transfected in the cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Equal amount of pRL-CMV was co-transfected in all the experiments as an internal control. The siRNA was transfected using Lipofectamine RNAiMax (Invitrogen) according to the manufacturer's protocol.

2.4. Site directed mutagenesis

The specific core sequences of the potential transcription factor binding sites (TFBSs) were mutated using Quickchange® site directed mutagenesis kit (Agilent Technology) following the manufacturer's instructions. The primers are listed in Table II.

2.5. Luciferase assay

The Firefly luciferase reporter constructs harboring the RLIP76 promoter fragments were co-transfected into the cells along with Renilla luciferase reporter plasmid containing CMV promoter using Lipofectamine 2000 (Invitrogen) transfection reagent following the manufacturer's protocol. The cells were lysed 24 h later and luciferase assays were performed using a Dual-Luciferase® Reporter Assay System (Promega) on a TD-20/20 luminometer (Turner Designs) following the manufacturer's protocol. Transfection of each construct was performed in triplicate in each assay and three to six independent experiments were carried out.

2.6. Preparation of biotinylated probes

Single stranded oligonucleotides were labeled using biotin 3' end DNA labeling kit (Thermo Scientific) according to the manufacturer's protocol. Equal amount of labeled complementary oligonucleotides were annealed by denaturing at 94 °C for 3 minutes in a thermo cycler followed by gradual cooling to 25 °C over a period of two hours.

2.7. Nuclear extraction

Nuclear extract from HEK293 cells was prepared using nuclear extraction kit NE-PER (Thermo Scientific) following the manufacturer's protocol. Protein concentration in the nuclear extract was calculated using Bradford reagent method [27].

2.8. Electrophoretic mobility shift assay (EMSA)

Biotinylated oligonucleotides were incubated with 3–5 µg of nuclear extract in the presence of 10mM Tris, 50mM KCl, 1mM DTT, 10% glycerol and 1 µg poly (dI.dC) for 20 minutes at roomtemperature. The EMSA reaction mixture was electrophoresed on 4% to 8% gradient native polyacrylamide gel followed by electrophoretic transfer to nylon membrane. The transferred DNA was cross-linked to the membrane by UV exposure and detected using chemiluminescent EMSA kit (Thermo Scientific).

2.9. Chromatin immunoprecipitation assay

The chromatin immunoprecipitation assay was performed using Chip-IT express kit from Active Motif using the manufacturer's protocol. The eluted DNA was subjected to PCR amplification for the RLIP76 gene promoter region, –211/+4, using primers GTAGTTTGCTTCCTTGCGA (forward) and ATGATTGGGACGGAACGGGACG (reverse). PCR amplification conditions were initial 94 °C for 2 min followed by 6 cycles of 94 °C for 30 s, touchdown annealing at 62 °C (–1°C/cycle) for 30 s and 72 °C, and additional 36 cycles with annealing at 56 °C. Hot start PCR was used to amplify RLIP76 promoter region with 5% DMSO in the reaction mix. The primers used to amplify the control promoter sequence from a gene the monoamine oxidase B gene unrelated to RLIP76 were TTGCTGTCTCAGGCCCTTATA (forward), ATGAATGGAGAGGATCTGCTACG (reverse) [28]. PCR amplification conditions were initial 94 °C for 2 min followed by 36 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C.

2.10. Immunoblot analysis

Equivalent amounts of total cellular extracts were electrophoresed by SDS-PAGE and electrophoresed to PVDF membranes. Immunoblot analyses were carried out as described previously [26].

2.11. Flow cytometry analysis

HEK293 cells were harvested and suspended in ice cold PBS (approximately 1×10^6 cells/mL). Cells were treated with 0.1% v/v Tween 20 in PBS and incubated at room temperature for 15 min. Cells were washed twice with PBS containing 0.1% Tween 20 and centrifuged at 2000 rpm for 5 min. Cells were incubated with anti-RLIP76 IgG (1 µg/mL) in 3% BSA/PBS solution at 4 °C for 2 h followed by washing with PBS and incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibody for 30 min at room temperature in dark. Cells were washed with PBS, resuspended in ice-cold PBS containing 3% BSA and 1% sodium azide, and were analyzed by flow cytometry.

2.12. Immunocytochemistry

Immunocytochemistry was performed on HEK293 cells by a method described previously with slight modifications [29]. Briefly, HEK293 cells were transfected with non-targeted control siRNA and P300 siRNA using Lipofectamine RNAiMax following the manufacturer's instructions. After 24 h, cells were trypsinized and plated on sterilized glass cover-slips in 12 well plates ($\sim 0.2 \times 10^6$ cells/well). After 24 h, cells were fixed with cold methanol and acetic acid (3:1). Nonspecific antibody interactions were minimized by pre-treating the cells with 1% BSA in PBS for 60 min at room temperature. Subsequently, rabbit anti-human-RLIP76 IgG and mouse-anti-human P300 IgG were added (at 100 $\mu\text{g/mL}$) and incubated overnight at 4 °C in a humidified chamber. After washing five times with PBS, the cells were subjected to FITC-conjugated rabbit anti-mouse and Rhodamine Red-X-conjugated goat anti-rabbit IgG for 2 h at room temperature in humidified chamber, followed by washing five times with PBS. DAPI (4', 6-Diamidino-2-phenylindole) was used as a nuclear counter-stain. Finally, the cover slips were removed, air-dried and mounted on slides upside down with Vectashield mounting medium. Slides were analyzed by laser-scanning confocal microscope (Zeiss LSM510 META, Germany).

2.13.1. Gene expression correlation analysis using microarray database

Correlations between p300 and RLIP76 mRNA expression levels were calculated using downloaded microarray gene expression data from the City of Hope BRAVO database [30] containing 1376 brain tumor, 2790 breast cancer, 1085 colorectal cancer, 1505 ovarian cancer, and 1442 lung samples. Correlations were calculated separately by probe, gene, and study. P-values were calculated using the correlation test in R [31], and correlations were considered significant if they had a p-value < 0.05. Cohorts with conflicting results (for example, one positive correlation for one probe and one negative correlation for another probe) were considered not significant.

Results

3.1. Identification of cis-acting elements in the RLIP76 promoter using deletion analysis

The human RLIP76 gene is located at chromosome 18p11.22. From the reported full-length cDNA [8], the 223 bases long 5' UTR sequence of RLIP76 [8] was used in a BLAST search of the human genome to determine the sequence of the 5' upstream region of the human RLIP76 gene. Primers were designed to amplify 5' upstream promoter from -2965 to -1 position that was subsequently cloned in promoter-less pGL3-basic plasmid vector upstream of the luciferase reporter gene. Luciferase reporter constructs harboring various 5' deletion fragments of the promoter in the same orientation were generated (Figure 1). These plasmid constructs were designated as -2965/-1, -2486/-1, -1986/-1, -1582/-1, -966/-1, -512/-1, -245/-1, -199/-1, -167/-1, -152/-1, -101/-1, -72/-1 and -50/-1-pGL3. The assigned numbers corresponded to the position with respect to the transcription start site taken as +1. The promoter activity of these constructs was measured in HEK293 and MCF7 cells because constitutive RLIP76 mRNA expression is high in these cells. Different promoter luciferase plasmids were each subjected to transient co-transfection assay with plasmid expressing Renilla-luciferase reporter gene under the regulation of CMV promoter (pRL-CMV) that served as an internal control. All promoter constructs including the one with smallest fragment of the promoter showed clearly detectable and measurable activity in the cells confirming that we cloned a functional promoter of the human RLIP76 gene. The comparison of the promoter activities of these constructs is shown as fold change (Figure 1) normalized to the activity of -50/-1 construct. As shown in Figure 1, a comparison of the promoter activities of the -2965/-1 and -512/-1 fragments indicates that the deletion of -2965 to -512 promoter region did not have a significant effect on the promoter activity in HEK293 cells implying that there was no important cis-acting element in -2965/-512 promoter region.

The results from deletion analysis of the promoter as shown in Figure 1 also demonstrated that in HEK293 cells, the most active promoter fragment was -512/-1. Also, the deletion of -2486 to -1986 fragment reduced the promoter activity significantly in MCF-7 cells, while it had no effect in HEK293 cells (Figure 1). The comparison of -512/-1 and -50/-1 promoter activities (Figure 1) indicated that the deletion of -512 to -50 promoter region drastically reduced its activity in HEK293 as well as in MCF7 cells which indicated the presence of cis-activating elements in this region. The search for the specific cis-activating elements was therefore focused on this region. Further deletion analysis of the -512/-50 promoter region revealed specific sequences harboring potential cis activating elements (Figure 1). The deletion of -512 to -167 region reduced the promoter activity by approximately 50% whereas the deletion of the -167 to -152 region had a profound effect on the promoter activity that was reduced by 86% in HEK293 cells and 80% in MCF7 cells (Figure 1). These results strongly indicated the presence of a cis-activating element in -167/-152 promoter region.

The identified cis-activating element -167/-152 was subjected to *in silico* analysis to predict the potential transcription-factor binding sites (TFBSs) in this region. We used online bioinformatics tools PROMO and TRANSFAC [32, 33], which predicted potential TFBSs for leukemia-related factor (LRF), thyroid transcription factor-1 (TTF1), and overlapping binding site for c-ETS, and cMYB (Figure 2A) in -167/-152 cis-activating element. This analysis also revealed that the RLIP76 promoter lacked a typical consensus TATA box, which is typical for GC-rich promoters. The RLIP76 gene promoter scored for a GC rich promoter with 72% GC content in the -250/-1 fragment, as compared to 52% GC content in the -2965/-1 promoter region.

3.2. Determination of the functional cMYB/cETS binding site in the human RLIP76 promoter

Once the potential transcription-factor binding sites (TFBSs) were identified in the -167/-152 promoter cis-activating element (Figure 2A), we used mutational analysis of the promoter coupled with luciferase reporter assay to determine whether these predicted sites played a functional role in the promoter activity. The core sequences of the predicted TFBSs were mutated individually. The promoter sequence with mutated nucleotides and the corresponding potential TFBS disrupted as a result of the mutation is shown in Figure 2B. All mutations were introduced in -199/-1 pGL3 luciferase construct. Mutating LRF (Mu1) and TTF1 (Mu2) binding core sequences did not have any significant effect on the promoter activity (Figure 2C). On the other hand, mutation of two bases that are common in cETS and cMYB binding core sequence (GGGAACT to GGGTCCT) (Mu3) and mutation of a single nucleotide in cMYB binding core sequence (GGGAACT to GGGAATT) (Mu4) remarkably reduced the promoter activity of -199/-1 fragment close to the level of that observed in -152/-1 fragment activity (Figure 2C). These results confirmed that overlapping binding sites for cMYB and cETS played an important role in regulating the RLIP76 promoter activity and suggested that transcription factors (TFs) cMYB or cETS potentially bind to the RLIP76 gene promoter and activate it.

3.3. Confirmation of transcription factor binding by EMSA

The above results were verified using an *in vitro* DNA-protein binding electrophoretic mobility shift assay (EMSA) to determine whether this region did indeed bind transcription factors. The sequences of the oligonucleotides of RLIP76 promoter used in these experiments are listed (Table III). A competitive-binding EMSA was performed to demonstrate whether unlabeled oligonucleotides could compete with transcription-factor binding to the region of interest. For these studies, we used the biotinylated -199/-150 oligonucleotide and used unlabeled smaller fragments for competition to determine the

region where the transcription factors bind. The results from these experiments demonstrated that TFs bind to the promoter region -177/-150 (Figure 3A). Biotinylated oligonucleotides corresponding to -165/-135 promoter sequence with wild-type cMYB/cETS binding site, -165/-135(WT) and those with the mutation in cMYB/cETS - binding site, -165/-135 (C-152T), were then used in the EMSA assay with equal amounts of nuclear extract to confirm that the transcription factor/s specifically interacted with the RLIP76 promoter through cMYB/cETS binding site. These studies show specific shifts with the wild-type -167/-135 but not C152T mutated oligonucleotide (Figure 3B). Thus, these findings corroborate the results with the luciferase assay.

3.4. p300 and cMYB associate with the human *RLIP76* promoter

Next, we investigated *in vivo* binding of these transcription factors to the RLIP76 promoter. As the mutation in the core sequence of cMYB binding site -199/-1Mu4 was sufficient to reduce the promoter activity, we determined the binding to cMYB transcription factor to the RLIP76 promoter. Both cMYB and cETS are known to act through the same transcriptional co-activator p300, which activates transcription by associating with the target gene promoter via these transcription factors [34, 35]. To determine if p300 binds to the RLIP76 gene promoter via cMYB/cETS binding site, we performed CHIP assay to monitor protein-DNA binding *in vivo*. The cross-linked DNA-protein complexes were immunoprecipitated with monoclonal antibody to either cMYB or p300. The DNA in the complexes was analyzed by PCR for the enrichment of the RLIP76 promoter sequence. The results in Figure 4 show that while the RLIP76 promoter sequence (-211/+4) flanking the cMYB/cETS site was enriched in cMYB or p300 containing DNA-protein immune complexes, the promoter sequences from monoamine oxidase B gene (that is unrelated to RLIP76 and does not contain cMYB/p300 elements) did not. These data demonstrate that p300 and cMYB associate with the RLIP76 gene promoter and suggest a role for these factors in the regulation of RLIP76 gene transcription.

3.5. Knockdown of p300 reduced RLIP76 promoter activity

Since our results showed that p300 associates with the RLIP76 promoter *in vivo*, we determined the effect of p300 knockdown on the RLIP76 gene promoter activity. HEK293 cells were transfected with either non-targeted control (N. C.) siRNA or p300 siRNA and after 48 hours, cells were separately transfected with luciferase constructs containing RLIP76 promoter with different deletions (-152/-1 and -199/-1) or with mutations (-199/-1 Mu3 and -199/-1 Mu4). Results of these studies demonstrated that knockdown of p300 attenuated the increase in the activity of the -199/-1 promoter containing cMYB/p300 site over -152/-1 promoter, whereas control non-targeting siRNA had no effect. The knockdown of p300 also did not influence the activity of -199/-1(C-152T) promoter with a mutation in the cMYB/p300 site (Figure 5). These results indicate that p300 specifically acts through the element, -157GGGAATA-150, in the RLIP76 promoter and activates it.

3.6. Knockdown of p300 suppressed RLIP76 expression in HEK293 cells

Since p300 regulates the RLIP76 promoter activity, we examined if RLIP76 protein expression is affected by p300. Silencing of p300 in HEK293 cells by siRNA decreased RLIP76 protein level as determined by Western-blot analysis (Figure 6A), flow cytometry (Figure 6B) and immunofluorescence (Figure 6C). Flow-cytometry study revealed a decrease in RLIP76 expression from 18% to 8.5 % ($p < 0.01$) in RLIP76 siRNA-transfected cells compared to control non-targeting siRNA-transfected cells (Figure 6B). Knockdown of p300 decreased both membrane and cytoplasmic staining for RLIP76 (Figure 6C). Taken together, these results provide strong evidence that p300 is an essential transcriptional co-activator for RLIP76 expression.

3.7. p300 expression displays a significant positive correlation with RLIP76 expression in solid tumors

Next, we investigated the correlation of p300 and RLIP76 expression in patient tumor samples using microarray gene expression data from the City of Hope BRAVO database. Analysis of the downloaded microarray data from different studies across different solid tumors including brain cancer, breast cancer, colorectal cancer, ovarian cancer and lung cancer, revealed a significant positive correlation between p300 and RLIP76 mRNA expression levels (Table IV).

Discussion

The results of the present studies demonstrate that p300, a histone acetyltransferase and a transcriptional co-activator, associates with the RLIP76 gene promoter via cMYB/cETS binding site -157GGGAATA-150 and activates its transcription. Furthermore, the depletion of p300 using siRNA results in concomitant decrease in the RLIP76 promoter activity and protein levels in the cells.

It has been previously reported that overlapping binding sites for cMYB and cETS in the gene promoters play an important role in the regulation of the transcription via binding of either transcription factor [36, 37]. There are several reports of functional overlapping TFBSs where the TFs bind and regulate the promoter competitively or in a compensatory manner [36–39]. In our DNA protein binding assays, we demonstrate that although cMYB binds to RLIP76 promoter (Figure 4b), the knockdown of cMYB does not result in any decrease in RLIP76 expression (data not shown). This can be explained by possible compensatory role of cETS factors in the absence of cMYB. Since cETS factors comprise of nearly twenty-seven factors in humans with highly conserved DNA binding domain [40], it will be interesting to investigate which of these cETS factors play a role in RLIP76 gene transcription.

Our studies suggest that other promoter fragments may be important for RLIP76 gene transcription. The deletion of the promoter fragment -2486/-1986 had no effect on the promoter activity in HEK 293 cells, but reduced the activity by 75% in MCF7 cells (Figure 2), indicating that the -2486/-1986 promoter fragment displays cell type specific activity. Further investigation of this fragment in breast cancer cells may identify additional important transcription factor/s regulating RLIP76 gene expression.

Transcriptional co-activator p300 has been linked to the pathogenesis of diabetes [41–45] and p300 inhibitors have a great potential in the treatment of this disease [42, 43, 46]. The molecular mechanisms involving the function of p300 in the context of diabetes have been investigated, which involve different downstream targets of p300. Liver specific p300 conditional knockout exhibited significant lower blood glucose levels and increased insulin sensitivity by activating gluconeogenesis genes [47], and liver specific overexpression of p300 resulted in hepatic steatosis and insulin resistance [41]. High glucose levels increased p300 mRNA and protein expression and p300 nuclear localization accompanied by increased binding of p300 to the target promoters, enhanced histone acetylation, and increased mRNA expression of vasoactive factors and extracellular matrix proteins that contributing to the symptoms of chronic diabetes [41]. Moreover, suppression or inhibition of p300 prevented such glucose-induced changes [44]. We have previously shown that RLIP76 knockout mice are hypoglycemic and exhibit improved insulin sensitivity [17]; here we provide direct experimental evidence using a cell culture system that p300 may exert its insulin-resistance through transcriptional regulation of RLIP76. It will be interesting to elucidate the crosstalk between p300 and RLIP76 mediated pathways in diabetes.

In addition to diabetes the expression of both p300 and RLIP76 is relevant to cancer [1, 15, 17, 18, 24, 41–43, 45–49]. RLIP76 is overexpressed in most of the cancer cells [1–3]. Moreover, p300 and RLIP76 expression levels show a strong positive correlation in solid tumors (Table IV) corroborating our observation that p300 positively regulate RLIP76 expression. p300 has been reported to function as a tumor suppressor as well as a tumor promoter depending on the cellular context [50–53]. It is well established that p300 activates the transcription of many important tumor-promoting genes by interacting with known oncogenic transcription factors such as cMYB and cETS at the promoters of the target gene [35, 54]. In case of the RLIP76 promoter, p300 acts through cMYB/cETS binding site and may be responsible for the reported overexpression of RLIP76 in cancers where the oncogenes cETS or cMYB are overexpressed [55–58]. Thus, our novel observation that RLIP76 expression is regulated by p300 is consistent with its established role in cancer and diabetes.

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Abbreviations

RalBP1	Ral-binding protein-1
RLIP76	76 KDa Ral-interacting protein
p300	E1A binding protein
TFBS	transcription factor binding site
DNP-SG	dinitrophenyl glutathione
CHIP	chromatin immunoprecipitation
LRF	leukemia-related factor
TTF1	thyroid transcription factor-1
EMSA	electrophoretic mobility shift assay

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Figure 1.

Deletion analysis of the human RLIP76 promoter. HEK293 and MCF7 cells were co-transfected with the equal copy number of Firefly luciferase reporter plasmids containing one of the indicated fragments of the human RLIP76 promoter and equal amount of Renilla luciferase reporter. Promoter activity was measured by dual luciferase assay. Firefly luciferase activity has been normalized with Renilla luciferase (internal control). The promoter activities of different deletion fragments are relative to the normalized activity of the smallest promoter fragment -50/-1pGL3. The promoter activity values are the average of minimum three independent experiments.

Figure 2.

Effect of mutating potential transcription-factor binding sites (TFBS) on the RLIP76 promoter activity. **(A)** DNA sequence of the -251 to -1 bp 5'-flanking region of the human RLIP76 gene is shown. Arrows indicate the positions corresponding to the transcription start sites taken as +1. The RLIP76 gene promoter sequence was scanned with bioinformatics tools, PROMO and TRANSFAC for the presence of potential transcription factor binding sites (TFBSs). The predicted TFBSs in the human RLIP76 promoter are underlined. **(B)** The core sequences of the potential TFBSs were mutated using site directed mutagenesis with mutations shown in red, and the TFBS/s disrupted as a result of the mutation are indicated in right column. **(C)** The Firefly luciferase reporter plasmids containing mutated and wild type promoter sequences were transiently co-transfected in HEK293 cells with pRL-CMV, and the promoter activity was measured using dual luciferase assay.

Figure 3.

Binding of HEK293 nuclear proteins to the promoter region by EMSA. **(A)** A biotinylated double-stranded oligonucleotide covering the promoter region -199/-150 was incubated with 3 µg of HEK293 nuclear extract (lane 2–6). Lane 1 contains biotinylated double-stranded oligonucleotide without nuclear extract for the control. A 100-fold molar excess of competing unlabeled oligonucleotide were used in lane 3 (-199/-150), lane 4 (-199/-177), lane 5 (-188/-165) and lane 6 (176/-150). **(B)** Biotinylated double-stranded oligonucleotides covering the promoter region -165/-135 containing either wild type (-165/-135 (WT)) or mutated (-165/-135 (C-152T)) cMYB/cETS binding site, were incubated with 3 µg of HEK293 nuclear extract. The mixtures containing DNA and nuclear protein were electrophoresed on 4% to 10% native gradient polyacrylamide gel, transferred to the nylon membrane. The biotinylated oligonucleotide was detected using chemiluminescent EMSA

kit. Arrows are used to indicate the specific band-shifts that indicate nuclear protein binding to the promoter.

*Monoamine oxidase B gene promoter sequence

Figure 4.

cMYB and p300 binds to the RLIP76 promoter in vivo by CHIP Assay. **(A)** RLIP76 promoter sequence from -211 to +4 was chosen for the RT-PCR end-point of the assay. HEK293 cells were cross-linked with 1% formaldehyde for 10 min. Chromatin was isolated, sheared enzymatically and incubated with magnetic beads and immunoprecipitated with either mouse monoclonal anti-p300 antibody **(B)** or rabbit monoclonal anti-cMYB antibody **(C)**. Corresponding mouse pre-immune IgG (mouse IgG) or rabbit pre-immune IgG (rabbit IgG) served as controls for the antibodies. The beads were washed 3 to 4 times, the chromatin was eluted, reverse cross-linked, and the eluted DNA was analyzed by PCR. In both panels, an irrelevant promoter sequence from the monoamine oxidase B gene was used as negative control.

Figure 5.

Effect of p300 knockdown on the RLIP76 promoter activity. HEK293 cells were transfected with either p300 or non-targeting control (N. C.) siRNA. Twenty-four hours later, these cells were trypsinized and equal numbers of the cells were plated in each well of twelve well plates for further experiment. After forty-two hours of siRNA transfections, the cells were transfected with equal copy number of -199/-1pGL3, -152/-1pGL3, -199/-1(Mu4) pGL3 or -199/-1(Mu5) pGL3 luciferase reporter plasmids separately. Equal amount of Renilla luciferase plasmid was cotransfected in all the wells. After twenty-four hours of the plasmid transfections, the cells were processed and the promoter activities were measured using dual luciferase assay.

Figure 6.

Effect of p300 knockdown on RLIP76 expression. **(A)** The effect of p300 knockdown by siRNA was examined by comparing p300 and RLIP76 protein by Western-blot analysis 72 h after transfection with non-targeting (N. C.) siRNA or p300 siRNA, and analyzing total cell lysate using indicated antibodies. Actin was used as a loading control. **(B)** In separate experiments, the cells transfected with N. C. or p300 siRNA were examined by flow-cytometry at 24 h after siRNA treatment using antibodies against RLIP76. **(C)** The simultaneous depletion of p300 and RLIP76 at 48 h after p300 siRNA treatment was also demonstrated by immunocytochemistry using antibodies as indicated.

Table I

Primers for the PCR amplification of the RLIP76 promoter fragments

-2965 (F)	GCAGTGGTTGCACTCAAACACCTTGA
-1 (R)	ATGATTGGGACGGAACGGGACGAG
-2965 (F) KpnI	GCAGT GGTACCA CTCAAACACCTTGA
-1 R HindIII	TTACA AAGCTT GGGACGGAACGGGACGAG
-2486 (F) KpnI	TGACCAC CGGTACC ACCTTGAGTCCATC
-1966 (F) KpnI	TCAAAC AGGTACCG TTTGATACTGCAG
-1486 (F) KpnI	CTCCAAAT GGTACC ACGGTCTACATTCTG
-966 (F) KpnI	CAGCGCCAAT GGTACCGG CAACGAAG
-512 (F) KpnI	CAGTCT GGTACC ACTGGTTGGAACGAC
-245 (F) KpnI	CAG GGTAC CTGGGGACGAAGGGGGCGC
-199 (F) KpnI	GTAGTTT GGTAC CTTGGCGAGAGGCCT
-167 (F) KpnI	GCTGGACGCCGGGCCTTGGGAACACTAC
-152 (F) KpnI	CCTT GGTAC CTACGCCTCCCAGCGCGC
-101 (F) KpnI	CTGCC GGTACCGG CTCAGCCGTCTGAG
-72 (F) KpnI	CGGCT GGTACCG TCTGAGCGGAGAG
-50 (F) KpnI	CTGC GGTACC AGGAGGAATGCGCGTG

Table II

Primers used to introduce point mutations in the RLIP76 promoter

199 (CC-161, -160AA) (Mu1) F	GCTGGACGCCGGGGGAATTGGGAACTACGCCTCCCAG
199 (CC-161, -160AA) (Mu1) R	CTGGGAGGCGTAGTTCCCAATTCCCCGGCGTCCAGC
199 (TG-159, -157GA) (Mu2) F	GCTGGACGCCGGGGGCCGTAGGAACTACGCCTCCCAG
199 (TG-159, -157GA) (Mu2) R	CTGGGAGGCGTAGTTCCATCGGCCCGGCGTCCAGC
199 (AA-154, -153TC) (Mu3) F	GCTGGACGCCGGGGCCTTGGTCACTACGCCTCCCAG
199 (AA-154, -153TC) (Mu3) R	CTGGGAGGCGTAGTGACCAAGGCCCGGCGTCCAGC
199 (C-152T) (Mu4) F	GGACGCCGGGGCCTTGGGAATTACGCCTCCCAGCGC
199 (C-152T) (Mu4) R	GCGCTGGGAGGCGTAATTCCTCAAGGCCCGGCGTCC

Table III

Oligonucleotides used in EMSA

199–150	CCTTGGCGAGAGGCCTTCGGGCTGGCTGGACGCCGGGGCCTTGGGA ACTA
	GGAACCGCTCTCCGGAAGCCCGACCGACCTGCGGCCCCGGAACCCTTGAT
199–177	CCTTGGCGAGAGGCCTTCGGGCT
	AGCCCGAAGGCTCTCGCCAAGG
188–165	GGCCTTCGGGCTGGCTGGACGCCG
	CGGCGTCCAGCCAGCCCGAAGGCC
176–150	GGCTGGACGCCGGGGCCTTGGGA ACTA
	TTCCCAAGGCCCCGGCGTCCAGCCGAT
-165/-135(WT)	GGCCTTCGGGCTGGCTGGACGCCG
	CGGCGTCCAGCCAGCCCGAAGGCC
-165/-135 (C-152T)	GGCTGGACGCCGGGGCCTTGGGA ACTAC
	TTCCCAAGGCCCCGGCGTCCAGCCGATG

Table IVCorrelations between RLIP76 and p300 mRNA expression levels in human cancer tissues^{*}

Study		r [#]	p-value
Breast cancer	Huang	0.22	0.041
	TCGA	0.24	1.13×10^{-12}
	expO	0.15	0.0051
	Wang	0.21	0.00060
	Ivshina	0.48	0.00078
	Pawitan	0.21	0.0080
	Finak	0.29	0.024
	Desmedt	0.22	0.0080
Ovarian cancer	Hendrix	0.26	0.0093
	Anglesio	-0.21	0.046
	expO	0.13	0.025
	Bild	-0.23	0.0052
	Tothill	0.13	0.030

Study		r [#]	p-value
Brain cancer	TCGA	0.16	0.0032
	Lee	0.22	2.79×10^{-5}
	Phillips	0.29	0.0037
	Petalidis	0.26	0.0387
Colorectal cancer	Smith	0.27	0.0055
	Expo	0.18	0.011
	Kaiser	0.29	0.0024
	Jorissen	0.076	0.00041
Lung cancer	DCC	0.25	1.59×10^{-6}
	Bhattacharjee	0.20	0.027
	Raponi	0.21	0.014
	Takeuchi	0.16	0.049

^{*} Calculated using microarray gene expression data from the City of Hope BRAVO database.[#] Correlation coefficient; ranges from -1 to 1; 0 means no correlation, positive values mean the positive correlation, negative values mean the negative correlation.