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Research Article

Phorbol-12-myristate-13-acetate-mediated stabilization of leukemia inhibitory factor (*lif*) mRNA: involvement of Nucleolin and PCBP1

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Leukemia inhibitory factor (LIF) is a potent pleiotropic cytokine involved in diverse biological activities, thereby requiring precise spatial and temporal control of its expression. The present study reveals that enhanced expression of LIF in response to PMA (phorbol-12-myristate-13-acetate) in human histiocytic lymphoma cell line U937 largely happens through stabilization of its mRNA. Functional characterization of the long 3′-untranslated region of human *lif* mRNA revealed several conserved sequences with conventional *cis*-acting elements. A 216 nucleotide containing proximal *cis*-element with two AUUUA pentamers and four poly-rC sequences demonstrated significant mRNA destabilizing potential, which, on treatment with PMA, showed stabilizing activity. Affinity chromatography followed by western blot and RNA co-immunoprecipitation of PMA-treated U937 extract identified Nucleolin and PCBP1 as two protein *trans*-factors interacting with *lif* mRNA, specifically to the proximal non-conventional AU-rich region. PMA induced nucleo-cytoplasmic translocation of both Nucleolin and PCBP1. RNA-dependent *in vivo* co-association of both these proteins with *lif* mRNA was demonstrated by decreased co-precipitation in the presence of RNase. Ectopic overexpression of Nucleolin showed stabilization of both intrinsic *lif* mRNA and *gfp* reporter, whereas knockdown of Nucleolin and PCBP1 demonstrated a significant decrease in both *lif* mRNA and protein levels. Collectively, this report establishes the stabilization of *lif* mRNA by PMA, mediated by the interactions of two RNA-binding proteins, Nucleolin and PCBP1 with a proximal *cis*-element.

Introduction

Leukemia inhibitory factor (LIF), a secreted glycoprotein of the IL-6 family cytokines, mediates an extraordinary range of biological events. Some of its activities include the capacity to induce terminal differentiation in leukemic cells, induction of hematopoietic differentiation [1,2], and the development and differentiation of neuronal [3] and nephronal cells [4]. LIF protects myocardium during acute stress of ischemia–reperfusion and contributes to cardiac repair and regeneration post-myocardial infarction [5]. It is indispensable in embryogenesis and implantation [6,7], stem cell maintenance in mouse [8], bone metabolism [9] and inflammation [10]. LIF contributes to the pathogenesis of rheumatoid and osteoarthritis [11,12]. Up-regulation of LIF is also associated with breast cancer progression [13]. Taken together, LIF was found to be a potent poly-functional cytokine capable of acting in a variety of tissues, both in adult and embryo, thus requiring a very precise spatial and temporal control of its expression.

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In mammalian cells especially, the alteration of mRNA stability is one of the most prevailing and rapid means to modulate protein expression. The level of all mRNAs is determined by a complex interplay between '*cis*-elements' of the 3′-untranslated region (3′-UTR) and one or more *trans*-acting RNA-binding proteins or micro-RNAs [14,15]. These *cis–trans* interactions are modulated by a wide variety of physiological conditions like hypoxia, serum starvation or pharmacological agents [16]. Considering the pleiotropic actions of LIF in cells of diverse lineage, its regulation at the level of mRNA stability could be of high significance.

Human *lif* mRNA possesses a long (3199 bases) and fairly conserved 3′-UTR, thereby raising a possibility of this gene to be regulated significantly at the post-transcriptional level. A report showed that when activated human monocytes were stimulated with LPS or phorbol esters, the mRNA levels of HILDA/LIF were regulated post-transcriptionally by mRNA stabilization probably through newly synthesized labile proteins [17]. A few studies have shown that change in *lif* mRNA stability affects conditions like rheumatoid arthritis [18] and inflammation [19]. Though a few preliminary reports exist [17–19], the detailed mechanisms of post-transcriptional regulation of human *lif* remained largely unidentified. Investigation on the regulation of expression of LIF by glucocorticosteroids (GCs) established that its expression was inhibited mainly by increased turnover of its mRNA [18]. A recent study, however, revealed a mechanistic insight into the H_2O_2-redox signaling in *lif* mRNA stabilization in mouse Müller glial cells mediated by ILF3 [19]. Nevertheless, LIF expression is highly context- and system-dependent, and the detailed mechanism of its regulation in humans is yet to be divulged.

PMA is a known inducer of LIF and has been used in several studies for elucidation of the cytokine's functions or for the study of regulation of its expression. Thus, phorbol-12-myristate-13-acetate (PMA) was used as the inducer of LIF expression [20,21]. The PKC-dependent production of LIF in response to PMA is documented in bone marrow stromal cells [20] and in T-lymphocytes [21]. Additionally, PMA is reported to induce terminal differentiation of myeloid leukemia cell line HL60 through the activation of the MAP kinase pathway [22], and LIF is known to induce growth arrest and terminal differentiation of myeloid leukemia M1 cells by STAT3 activation [23] and suppress clonogenicity, thereby inducing differentiation in human leukemic cell lines HL-60 and U937 [2]. All the above reports indicate that LIF contributes significantly to sustain PMA-induced monocytic differentiation of human myeloid leukemia cell lines. Thus, the present study aims at dissecting the molecular mechanisms governing *lif* mRNA turnover by PMA in U937 cells.

The findings of the present study are aimed to identify and characterize the *cis*-acting elements and some of the *trans*-acting factors that could modulate *lif* mRNA stability in U937 cells. They also offer valuable insights into the understanding of the post-transcriptional regulatory mechanisms of this patho-physiologically important cytokine.

Materials and methods

Reagents

The list of reagents is provided in Supplementary Material.

Cell culture

Human histiocytic lymphoma U937 cells (NCCS, Pune, India) were grown in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml amphotericin B. Cells were maintained at 37°C (5% CO_2) in a fully humidified incubator.

Treatment with PMA and actinomycin D

U937 cells (0.3×10^6 cells/ml) were grown in fresh medium for 2 h before the addition of 32 nM PMA. To measure steady-state mRNA levels, cells were grown at 37°C in PMA for 0–24 h and harvested at various time points. The half-life of *lif* mRNA was determined according to ref. [24].

RNA extraction and RT-PCR

Total RNA was extracted using TRIzol according to the manufacturer's instructions. Total RNA (3 µg) was reverse-transcribed to prepare cDNA with MMuLV Reverse transcriptase. Quantitative PCR was performed with specific primers (Supplementary Table S1 in Supplementary Materials) using the DNA thermal cycler (Step One Plus, Applied Biosystems). Relative expression of genes was analyzed by the ΔΔCT method.

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Cloning

lif-B-3′-UTR (216 nt # 1636–1851) fragment of *lif* mRNA (NCBI Reference Sequence: NM_002309.3) was prepared by PCR amplification of cDNA synthesized from the total RNA of U937 cells and cloned in TA cloning vector pTZ57R/T (primer sequences in Supplementary Table S1). The clones were confirmed by sequencing in an automated sequencer (Gene Analyzer 3130, Thermo Scientific). The lif-B-3′UTR (restriction digested fragment from the pTZ57R/T construct) was further cloned downstream of the *gfp* reporter gene of the mammalian expression vector pEGFPC1. The lif-B-3′-UTR fragment downstream 150 base pairs from the 3′-end of the coding region of GFP (green fluorescent protein) from the pEGFP–lif-B construct was PCR-amplified and re-cloned in the pTZ57R/T vector to form the pT-GFP–lif-B construct.

Transient transfection of plasmids and siRNAs

U937 cells (0.4×10^6 cells/well) were seeded in six-well tissue culture plates 1 day prior to transfection. Cells were transfected using TurboFect Transfection Reagent (Thermo Scientific) according to the manufacturer's protocol. At 44 h after transfection, actinomycin D (5 μg/ml) was added and cells were harvested in TRIzol up to 4 h (at an interval of 1 h). For siRNA, duplex RNA (50 nM each) for PCBP1 (sense 5′-GUCUGGCCCAGUAUCUAAU-3′ and antisense 5′-AUUAGAUACUGGGCCAGAC-3′) or Nucleolin (sense 5′-GGGAUUGCUUAUAUUGAAU-3′ and antisense 5′-AUUCAAUAUAAGCAAUCCC-3′) or control (Eurogentec, Belgium) were transfected using jetPRIME plasmid/siRNA transfection reagent (Polyplus-transfections, Illkirch, France) according to the manufacturer's protocol.

Preparation of total, cytoplasmic and S_{100} extracts

Untreated and PMA-treated U937 cells (5×10^6) were washed twice with PBS and immediately used for extract preparation. Whole-cell extract was prepared by resuspending the cell pellet in 100 μl of cell lysis buffer (BD Pharmingen). After incubating on ice for 30 min, the supernatant was collected by centrifugation at 10 000 rpm for 10 min. The cytoplasmic and S_{100} extracts were prepared according to ref. [24].

Preparations of RNA transcripts

lif-B transcript was synthesized using T7 RNA polymerase and pT-lif-B plasmid. ^{32}P-labeled RNA was synthesized in transcription reactions containing ^{32}P-CTP. To maximize the amount of full-length product, reactions contained unlabeled CTP (250 μM), along with ATP, UTP and GTP (each at 500 μM). The purity of [^{32}P]-RNA was monitored by analysis on 6% polyacrylamide–8 M urea gels, where the amounts of full-length products were generally ≥90%.

RNA gel mobility shift assay, competition and supershift assay

Cytoplasmic extracts (2 μg) were mixed with 20 nM ^{32}P-RNA transcripts in RNA-binding buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.025 mg/ml yeast RNA, 0.25 mg/ml BSA (bovine serum albumin)] and incubated on ice for 10 min. Samples were separated on a 2% agarose/TAE (Tris/acetate/EDTA) gel, which was dried on nitrocellulose paper and analyzed using PhosphorImager (Typhoon Trio+, GE Healthcare). For competition assays, 2× (40 nM) or 5× (100 nM) cold homologous transcript was added to 20 nM ^{32}P-RNA transcript bound to proteins of PMA-treated (24 h) cytoplasmic extracts. For antibody supershift assays, the ^{32}P-*lif-B* transcript was preincubated with cytoplasmic extracts of PMA-treated (24 h) U937 cells using anti-Nucleolin or anti-PCBP1 antibodies, as mentioned previously in ref. [24].

UV-induced *in vitro* RNA–protein cross-linking assays

Assays were performed according to ref. [25] with slight modifications. Briefly, 1 μl of ^{32}P-*lif-B* RNA (1 μM) in binding buffer and PMA-treated cytoplasmic extracts of U937 cells (20 μg) were incubated on ice for 10 min. For competition assays, 5× concentrations of homologous RNA (*lif-B*) or non-homologous RNA (*β-globin*) were added to samples containing ^{32}P-*lif-B* RNA, before the addition of cytoplasmic extracts of cells treated with PMA for 24 h. Reactions were taken in a 96-well microtiter plate and exposed to UV irradiation (254 nm) from a distance of 5 cm for 15 min on ice using a mineral light lamp in a UV cross-linker (Bio-Rad, CA, U.S.A.). Following UV-cross-linking, samples were treated as described previously [25]. RNA-cross-linked protein bands were separated on 10% polyacrylamide gels and visualized by phosphorimaging.

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RNA affinity column chromatography

This was performed similarly to the method in ref. [26] using *in vitro*-transcribed *lif-B* RNA polyadenylated using a poly(A) tailing kit according to the manufacturer's protocol and Oligo (dT)-agarose beads.

RNA–protein co-immunoprecipitation assays

Immunoprecipitation of RNA–protein complexes was performed as described by Niranjanakumari et al. [27] with minor modifications. Briefly, U937 cells (5×10^7) treated with 32 nM PMA were harvested, washed with cold PBS and suspended in 10 ml of PBS followed by cross-linking with formaldehyde [final concentration of 0.1% (v/v)]. Cross-linking was quenched with glycine (pH 7.0, 0.25 M final concentration). The cells were harvested by centrifugation, followed by two washes with ice-cold PBS. Fixed cells were resuspended in 1 ml of RIPA buffer [50 mM Tris–HCl (pH 7.5), 1% NP-40, 0.05% SDS, 1 mM EDTA and 150 mM NaCl] containing protease inhibitors. The cells were lysed by sonication (3×15 s), cell lysate was pre-cleared and supernatant was diluted with RIPA buffer containing RNase inhibitor and protease inhibitors, mixed with 20 µl of protein A/G Sepharose beads preincubated for 1 h with 4 µg of anti-Nucleolin, anti-PCBP1 or normal IgG antibodies and incubated for 2 h with shaking at 4°C. The Sepharose beads were washed five times with RIPA buffer, resuspended and incubated at 70°C for 45 min for reverse cross-linking. RNA was extracted from the precipitates using TRIzol, treated with DNase I, reverse-transcribed and amplified by semi-quantitative PCR for observing *lif* and *β-actin* mRNA levels. Products were viewed on 6% native PAGE by ethidium bromide staining.

In vitro decay assay

For *in vitro* decay assay, *gfp* and *gfp-lif B* transcripts synthesized respectively from Xba1-linearized pT-GFP and pT-GFP-lif-B plasmid and 10µg each of untreated, PMA-treated or PCBP1-depleted cytosolic (S_{100}) extracts of U937 cells were used. The experiment was performed according to [25].

Immunoprecipitations and western blot assays

Immunoprecipitations were performed with cytoplasmic extracts of PMA-treated U937 cells with anti-Nucleolin, anti-PCBP1 and normal mIgG antibodies followed by western blots as described in ref. [26]. Western blots were performed with the precipitate using anti-Nucleolin, anti-PCBP1 and β-actin/GAPDH antibodies to check for successful immunoprecipitation.

Statistical analysis

All graphs were generated in Microsoft Office Excel 2007 (Microsoft Corporation, Washington, U.S.A.). Error bars indicate mean ± SEM. A parametric unpaired *t*-test was used for analysis of statistical significance with KyPlot version 2.0 (KyensLab Incorporated, Tokyo, Japan). *P*-values <0.05 were considered to be statistically significant, whereas values of $P > 0.05$ were considered non-significant (NS).

Results

PMA-induced *lif* mRNA stabilization in U937 cell line

U937 cells, treated with PMA (32 nM) for 24 h, resulted in a time-dependent steady increase in the levels of mature *lif* mRNA with no change in DMSO-treated controls (Figure 1A). To determine whether *lif* mRNA stabilization contributed to the observed increase in *lif* mRNA level, its decay rates [half-life ($t_{1/2}$)] were compared in control or PMA-treated (20 h) cells after inhibiting transcription with actinomycin D. Figure 1B shows the semi-logarithmic plot of relative expression (fold change after normalization with respective *β-actin* mRNA levels) of *lif* mRNA in control or PMA-treated U937 cells. It was observed that the half-life of *lif* mRNA increased from 2.7 h (DMSO-treated control cells) to >4 h due to PMA treatment.

lif 3′-UTR is significantly conserved among different species

Megablast (for highly similar sequences) of *lif* 3′-UTR using NCBI BLASTN2.2.31+ program and *refseq_rna* database (NCBI transcript reference library) revealed its complete homology with apes like *Pan troglodytes*, *Gorilla gorilla*, *Colobus angolenesis* and *Macaca fascicularis* [99% query cover (QC) for all with 99, 98, 94 and 94% identities (I), respectively] and partial homology with *Felis catus* (55% QC; 77% I), *Canis lupus* (42% QC; 76% I) *Bos taurus* (25% QC; 92% I) and *Mus musculus* (12% QC; 89% I) along with many others. Assessing the degree of conservation, five distinct regions of the *lif* 3′ UTR (namely lif-A, lif-B, lif-C, lif-D and lif-E) were

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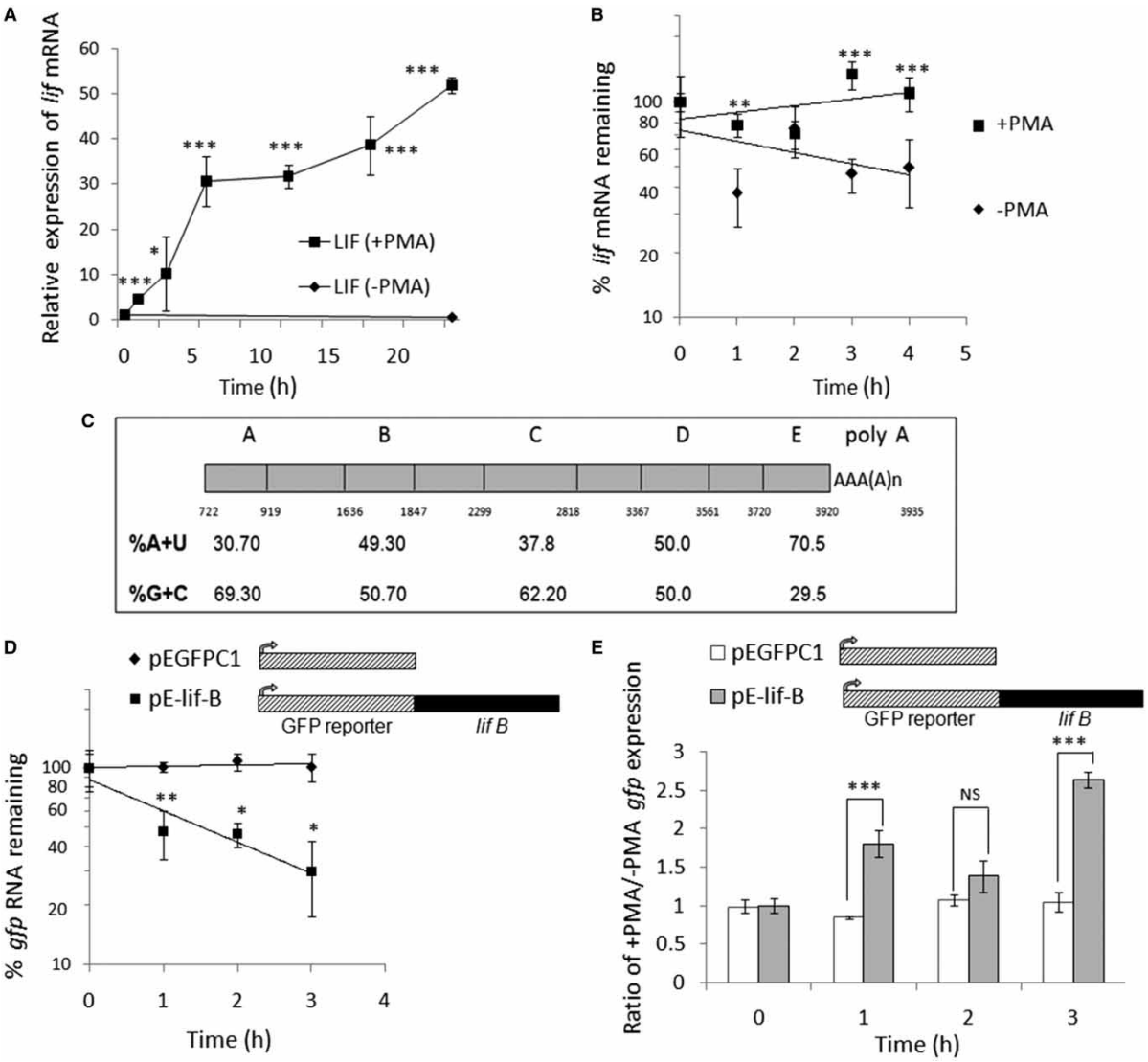

Figure 1. Effect of PMA on steady-state levels and half-life of *lif* mRNA.

(**A**) Levels of mature *lif* mRNAs of U937 cells treated with PMA (32 nM) for time indicated as measured by qRT-PCR. The relative expression was calculated by the ΔΔCT method and normalized with β-actin mRNA. (**B**) Semi-log plot showing decay of *lif* mRNA in the presence of actinomycin D. Control or PMA-treated cells were grown for 20 h followed by treatment with actinomycin D (5 μg/ml) for 0–4 h. The levels of mature *lif* mRNAs were measured by qRT-PCR normalized with β-actin mRNA as mentioned in (**A**). (**C**) *In silico* characterization of *lif* 3′-UTR. Schematic representation showing mapping of the 3′-UTR into regions (A–E) based on sequence conservation. (**D**) pEGFPC1- or pE-lif-B-transfected U937 cells harvested at 0, 1, 2 and 3 h after treatment with 5 μg/ml of actinomycin D. The relative expression of reporter (*gfp*) was quantified using the ΔΔCT method (qRT-PCR) after normalization with neomycin RNA. Data plotted in **A**, **B** and **D** are means of three independent experiments and presented as mean ± SEM. (**E**) pEGFPC1- or pE-lif-B-transfected U937 cells harvested at 0, 1, 2 and 3 h after treatment with 5 μg/ml of actinomycin D. The relative expression of *gfp* was quantified using the ΔΔCT method (qRT-PCR) after normalization with *neomycin* RNA. Columns representative of fold changes in gfp expression between PMA-treated and -untreated U937 cells with 0–3 h of actinomycin D treatment. Data plotted in **E** are means of three independent experiments and presented as mean ± SEM; significance is calculated between corresponding treated and untreated conditions, where NS indicates ($P > 0.05$), * is ($P \leq 0.05$), ** is ($P \leq 0.01$) and *** is ($P \leq 0.001$).

designated to be especially conserved (lying within the QC with other organisms and showing maximum identities) and contain significant *cis*-acting elements. These regions were characterized *in silico* with respect to their A + U, G + C contents (Figure 1C). The distal regions of the *lif* 3′-UTR (lif-D and lif-E) are AU-rich (50 and 70.5%) containing class I, class II and class III AREs [adenylate(A)- and uridylate(U)-rich element].

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The rest of the 3′-UTR was found to be significantly GC-rich (lif-A and lif-C), except a proximal region lif-B (216 nt long with AU content of 49.3%) containing two AUUUA pentamers in a not so AU-rich background. Owing to the presence of four poly(rC) sequences (putative hnRNP E1/E2/E3/E4/K/J binding sites [28]), this region is assumed to be a more non-canonical ARE and thus has been studied for potential mRNA-stabilizing/-destabilizing activity.

lif-B region of *lif* 3′-UTR contains potential *cis*-elements for mRNA stability

Transfection of a chimeric reporter construct containing the pE-*lif*-B region of the *lif*-3′-UTR in U937 cells demonstrated its potential as a *cis*-acting element in the context of mRNA stability. The parent pEGFPC1 and its derivative pE-*lif*-B were transfected in U937 cells as described in the 'Materials and Methods' section. The half-life ($t_{1/2}$) of *gfp* mRNA (normalized with *neomycin* RNA) was found to be more than 4 h in cells transfected with pEGFPC1 only, whereas the half-life of *gfp* mRNA reduced to 1.5 h in cells expressing gfp–*lif*-B, thereby confirming the destabilizing potential of this region (Figure 1D).

To check the effect of PMA on $t_{1/2}$ of *lif*-B, U937 cells transfected with pE-lif-B were treated with PMA (for 20 h) followed by actinomycin D (for 4 h). Figure 1E revealed the fold changes of *gfp* reporter RNA (normalized to *neomycin*) in the transfected cells after and before (in the presence and absence of PMA) PMA treatment, where it was observed that, for *lif*-B, the change was 2.6-fold higher than its untreated counterpart after 3 h of actinomycin D treatment.

Thus, these results indicated that treatment of U937 cells with PMA induced stabilization of *lif*-B, which was otherwise a potent destabilizing *cis*-element of *lif* mRNA.

PMA-treated U937 cell extracts contain RNA-binding proteins that interact with *lif-B*

To address which *trans*-factor(s) recognize this *cis*-acting element (*lif*-B) and impart stabilization in response to PMA, REMSA were performed using radio-labeled *in vitro*-transcribed *lif-B* and PMA-treated cytoplasmic extracts of U937 cells. As shown in Figure 2A, cytoplasmic extracts of untreated cells produced weak RNA–protein complexes, whereas extracts of cells treated with PMA for 6–24 h resulted in the formation of progressively intense RNA–protein complexes. The transcripts that were not bound by proteins were presumably degraded by cytosolic nucleases present in the extracts and ran off the gel. The specificity of RNA–protein interactions (competition assay) is shown in Supplementary Figure S1.

UV-cross-linking assays in Figure 2B illustrated increased binding of at least five proteins (approximate molecular mass being 110, 90, 70, 38 and 36 kDa) of PMA-treated cytoplasmic extracts to the radio-labelled *lif-B* transcript when compared with untreated cell extract, where the RNA-protein complexes formed were competed out with the addition of homologous (unlabelled *lif-B* RNA) but not with heterologous RNA (β-globin RNA) (Figure 2C). Coomassie blue-stained gels are shown in Supplementary Figure S2, indicating uniform protein loads.

Identification of the proteins that bind *lif-B*

For identification of proteins comprising the RNA–protein complex of *lif-B* as mentioned in Figure 2B, affinity chromatography was performed with oligo(dT) agarose bead-bound polyadenylated *lif-B* transcript and PMA-treated cytoplasmic extracts. Resolving the eluted fractions in a 10% SDS–PAGE, proteins corresponding to molecular masses of 110, 48, 38 and 36 kDa were prominent (Figure 2D). The band corresponding to 48 kDa could be non-specific and presumably that of RNase inhibitor. Western blots for known RNA-binding proteins of similar molecular mass revealed the presence of Nucleolin (55–110 kDa; containing several proteolytic fragments), PCBP1 (38 kDa) and HuR (36 kDa; Figure 2E). The specificities of these interactions were confirmed by the absence of GAPDH or another mRNA-stabilizing protein PCBP2 (38 kDa) in the eluted fractions. These observations were further confirmed through antibody supershift assays where a supershifted complex appeared with the addition of anti-Nucleolin or anti-PCBP1 antibodies, while no such complex was formed with control anti β-actin antibody (Figure 2F). Thus, these results clearly indicate that Nucleolin and PCBP1 are present in the *lif*-B RNA–protein complex.

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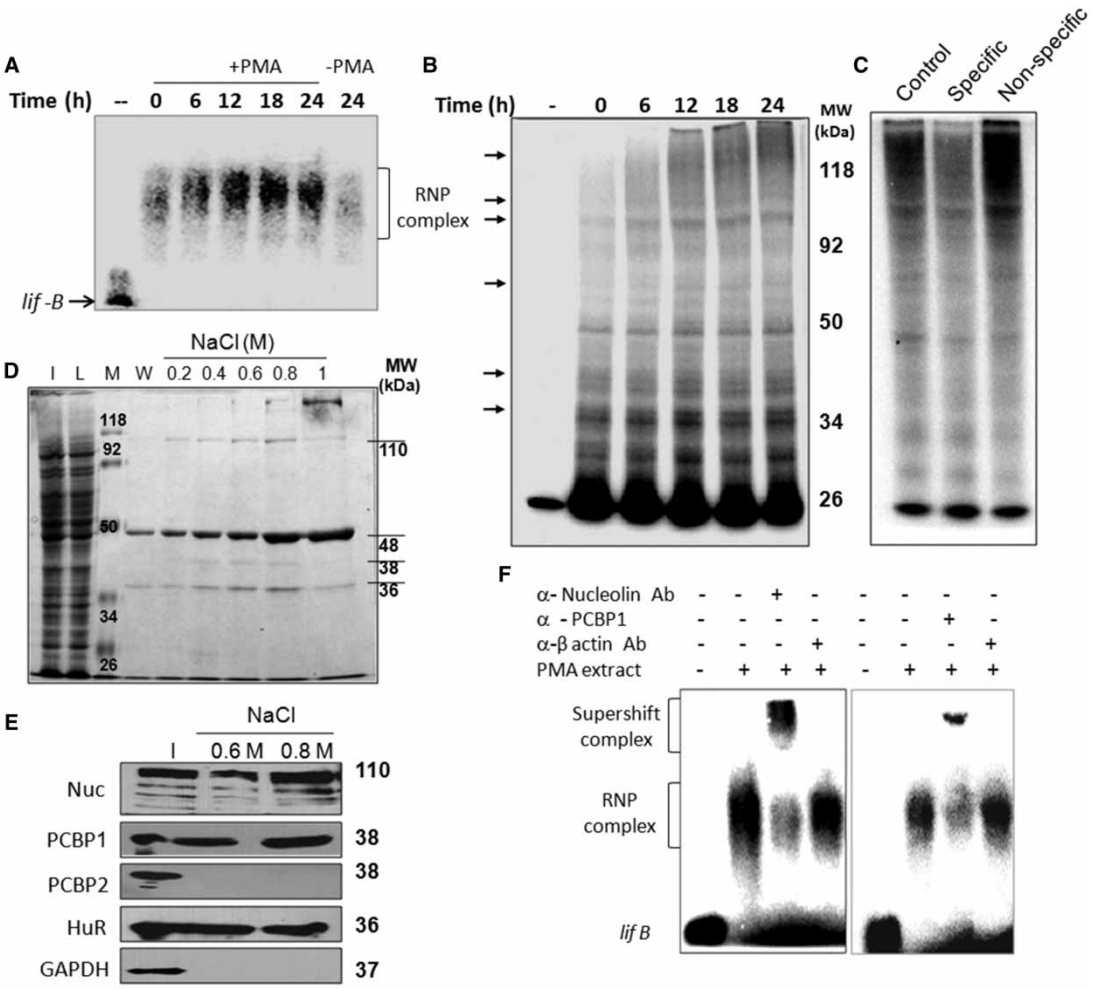

Figure 2. Identification of proteins present in the RNP complex of *lif-B* with U937 cytoplasmic extracts.

(**A**) Phosphorimage of ^{32}P-labeled *lif-B* transcripts (20 nM, ∼20 000 cpm) incubated with cytoplasmic extracts (2 μg) prepared from 0 to 24 h PMA-treated and 24 h untreated U937 cells, electrophoresed on 2% agarose/TAE gels. (**B**) UV-cross-linking assay of untreated or PMA-treated cytoplasmic extracts with ^{32}P-labeled *lif-B* transcript. Complexes were digested with RNase A/T1 and separated in 10% SDS–PAGE, stained, dried and analyzed by phosphorimaging. Arrows indicate proteins in PMA-treated extracts that were cross-linked more efficiently to *lif-B* in comparison with untreated control. (**C**) Phosphorimage of ^{32}P-*lif-B* cross-linked to PMA-treated (24 h) cytoplasmic extract in the absence (control) or presence of 5-fold molar excess of unlabeled homologous *lif-B* (specific) or heterologous *β-globin* (non-specific) competitor RNA transcripts. (**D**) Elution profile of the proteins in PMA-treated U937 cytoplasmic extracts eluted from the RNA affinity (polyadenylated *lif-B*-bound oligo-dT beads) column, where eluted fractions were separated in 10% SDS–PAGE and stained with Coomassie blue. (**E**) Western blot of column fractions eluted with 600 and 800 mM NaCl and blotted with anti-Nucleolin, anti-PCBP1, anti-PCBP2, anti-HuR and anti-GAPDH antibodies. (**F**) Antibody supershift assays where RNP complex of ^{32}P-*lif-B* and PMA-treated cytoplasmic extracts were supershifted on addition of antibodies as indicated. The complexes were separated on 2% agarose/TAE gel, dried and visualized by phosphorimaging (abbreviations: M: molecular mass markers; I: pre-cleared input; L: loading flow through; W: wash flow through; Nuc: Nucleolin; Ab: antibody; Extract: cytoplasmic extract).

Stabilization of *lif*-B mRNA by Nucleolin and PCBP1

To establish the role of these two proteins in *lif* mRNA stabilization, Nucleolin and/or PCBP1 was partially knocked down by respective siRNAs. The effect of knockdown of Nucleolin was more pronounced (∼80% decrease) compared with that of PCBP-1 (∼32% decrease), while knockdown of both showed ∼88% decrease

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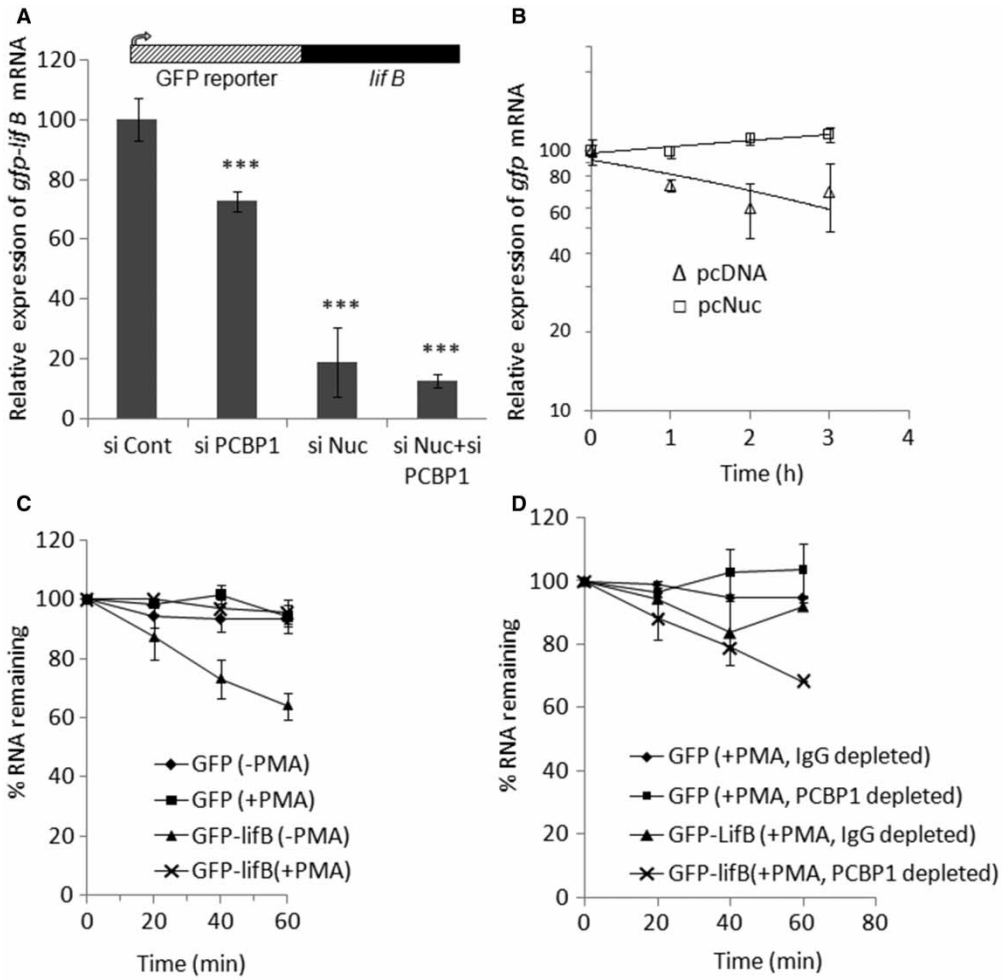

Figure 3. Effects of Nucleolin and PCBP1 on the stability of *lif-B* mRNA.

(**A**) Relative expression of *gfp* normalized against *neomycin* mRNA by the ΔΔCT method from U937 cells co-transfected with pE-lif-B (2 μg) and 50 nM siRNAs (control or PCBP1 or Nucleolin or PCBP1 + Nucleolin) for 48 h, followed by PMA treatment for 24 h. Results are means of three independent experiments and presented as mean ± SEM, where *** is ($P \leq 0.001$). (**B**) Relative expression of *gfp* normalized against *neomycin* RNA from U937 cells co-transfected with pE-lif-B (2 μg) and pcDNA or pcNuc, where cells were harvested at 0, 1, 2 and 3 h after treatment with 5 μg/ml of actinomycin D and *gfp* expression was quantified by qRT-PCR. Results are means of three independent experiments and presented as mean ± SEM. (**C**) Plot of percentage RNA remaining versus time quantitated from the phosphorimages of *in vitro* decay assay using polyadenylated ^{32}P-*gfp* or ^{32}P-*gfp–lif-B* transcripts and S_{100} extracts of untreated or PMA-treated U937 cells and (**D**) ^{32}P-*gfp* or ^{32}P-*gfp–lif-B* transcripts with PMA-treated or PMA-treated S_{100} extracts of U937 cells depleted with anti-PCBP1 antibody. Percentage RNA remaining indicates the amount of full-length RNA recovered as a function of incubation time.

(Figure 3A). Western blots in Figure 5B,C show that transfection of U937 cells by respective siRNAs reduced the protein levels of PCBP1 and Nucleolin.

As the above knockdown experiment indicated that Nucleolin has more prominent effects in the stabilization of PMA-induced *lif-B* mRNA in U937 cells, Nucleolin was ectopically expressed by co-transfection of pcDNA 3.1 and pC-Nuc plasmids (control) in U937 cells for 44 h followed by actinomycin D chase for another 3 h. The results in Figure 3B showed stabilization of GFP reporter mRNA ($t_{1/2}$ increased from 3.3 h to >>4 h), indicating that Nucleolin is a potent stabilizing *trans*-acting factor for *lif*-B mRNA.

From *in vitro* decay assays with *gfp* transcripts (Figure 3C), no appreciable decay was observed for the *gfp* transcript with both untreated and PMA-treated S_{100} extracts of U937 cells, but when *lif*-B was incorporated at

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the 3'-end of this fairly stable gene (*gfp*), *gfp–lif-B* decayed significantly (36.2%) in the presence of untreated S100 extracts. However, in the presence of PMA-treated S_{100} extracts, *gfp–lif-B* decay was much slower (4.7% decay). On depletion of PCBP1 with anti-PCBP1 antibody from PMA-treated S_{100} extract, *gfp–lif-B* decayed similarly as the untreated condition (35% decay; Figure 3D). The images of representative denaturing PAGE and western blots, showing depletion of PCBP1, are available in Supplementary Figures S3A,B and S4. This result demonstrated that along with Nucleolin, PCBP1 is also a key player in mediating the PMA-induced stability of *lif-B* RNA.

Nucleolin and PCBP1 remain associated with *lif* mRNA *in vivo*

To further validate the association of Nucleolin and PCBP1 *in vivo*, co-immunoprecipitations were performed. Semi-quantitative RT-PCR shown in Figure 4A,B indicated the presence of *lif* mRNA in the cytosolic extract co-immunoprecipitated specifically with Nucleolin and PCBP1 antibodies from PMA-treated U937 cells. The presence of *β-actin* RNA only in the inputs and its absence in the immunoprecipitates indicated specificity of immunoprecipitation. The efficiency and specificity of immunoprecipitation were further demonstrated by western blots (Supplementary Figure S6A and C, respectively). Semi-quantitative RT-PCR shown in Supplementary Figure S5A,B indicated the absence of *lif* mRNA in the cytosolic extract co-immunoprecipitated with Nucleolin and PCBP1 antibodies from untreated U937 cells, whereas the efficiency and specificity of immunoprecipitation in this case was demonstrated by western blots (Supplementary Figure S6B and D, respectively). The result clearly indicated exclusive binding of Nucleolin and PCBP1 to *lif* mRNA *in vivo* in PMA-treated U937 cells.

Both Nucleolin and PCBP1 are predominantly localized in the nucleus (reviewed in refs [29,30]), yet in the present study they were found to be associated with *lif* mRNA in the cytoplasm. This demanded their presence in the cytosol of PMA-treated U937 cells. Recently, Saha et al. [24] have reported that Nucleolin is translocated from the nucleus to the cytoplasm on PMA treatment of U937 cells. Figure 4C shows the similar nuclear to cytoplasmic translocation of Nucleolin protein by PMA treatment, where β-actin was used as a loading control, and the absence of histone 2A indicated no nuclear contamination in the preparation of cytosolic extract. Western blot (Figure 4D) revealed that both the cellular and nuclear levels of PCBP1 remained almost the same, whereas the levels are elevated in the cytoplasm of U937 cells on PMA treatment, where β-actin served as the loading control, confirming their cytoplasmic translocation.

To further confirm this observation, experiments were done where the cells were treated with different doses of PMA for 24 h and *lif* mRNA levels were checked by qPCR, and the levels of cytosolic Nucleolin and PCBP1 proteins in U937 cells treated with different doses of PMA were measured by western blot. The result (Figure 4E,F) indicated that the cytosolic level of Nucleolin gets elevated with the increasing concentrations of PMA. From Figure 4D, it is clear that the total amount of PCBP1 stays the same when compared with that of the untreated control (comparing the level of PCBP1 at 0 and 24 h in the whole-cell extract), and Saha et al. [24] have reported that by PMA treatment, the nucleolin protein gets translocated to the cytosol inspite of its unchanged cellular level, which in turn is responsible for the binding and stabilization of the *Oncostatin-M* mRNA. Thus, it is clear that, in this case, the cytoplasmic shuttling of these nuclear proteins is significantly responsible for *lif* mRNA stabilization.

Nucleolin and PCBP1 are concurrently bound to *lif* mRNA

Immunoprecipitation with anti-Nucleolin or anti-PCBP1 antibodies followed by western blots with both the antibodies demonstrated that Nucleolin and PCBP1 associate with each other in the cytoplasm of PMA-treated U937 cells, where anti-GAPDH antibody acted as a negative control (Figure 4G). This association was, however, perturbed when the cytoplasmic extract was treated with RNase A prior to antibody-mediated pull-down (Figure 4H), indicating an RNA-dependent interaction between the two proteins in the cytoplasm of PMA-treated U937 cells.

Nucleolin and PCBP1 lead to stabilization of *lif* mRNA and elevated LIF protein expression

To determine the functional role of Nucleolin and/or PCBP1 in expressional control of LIF, siRNA-mediated partial knockdown of these two proteins was done and intrinsic levels of *lif* mRNA and LIF protein were determined in untreated and post-PMA-treated U937 cells. Partial knockdown of Nucleolin and PCBP1 does not

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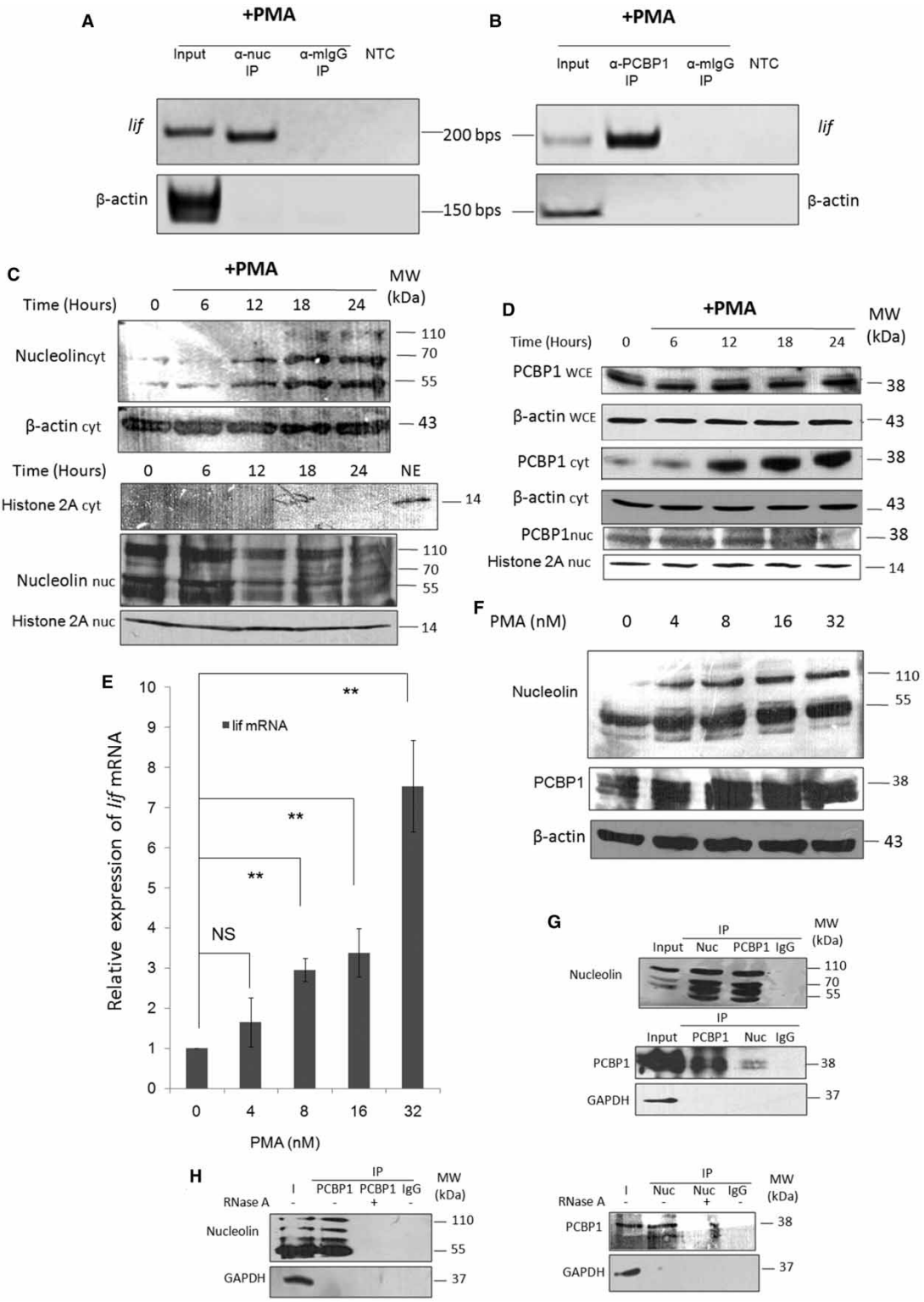

Figure 4. Elucidation of the *in vivo* interactions among intrinsic *lif* mRNA, Nucleolin and PCBP1. Part 1 of 2

PCR products obtained by semi-quantitative RT-PCR using *lif* and *β-actin*-specific primers from chemically cross-linked RNA–protein complex IP with (**A**) Nucleolin or IgG antibodies (**B**) PCBP1 or IgG, from PMA-treated U937 cells separated on 6% native PAGE. Western blots of Nucleolin (**C**) and PCBP1 (**D**) with untreated and PMA-treated U937 cells extracts (whole cell, cytoplasmic and nuclear), where β-actin and histone 2A were used as respective loading controls. (**E**) Relative expression of

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Figure 4. Elucidation of the *in vivo* interactions among intrinsic *lif* mRNA, Nucleolin and PCBP1. Part 2 of 2

intrinsic *lif* mRNA normalized to β-actin mRNA by the ΔΔCT method measured on treatment with different doses of PMA (0–32 nM). (**F**) Western blots of Nucleolin and PCBP1 with cytoplasmic extracts of U937 cells treated with PMA (0–32 nM) for 24 h, where β-actin was used as loading control. (**G**) Western blots with Nucleolin, PCBP1 or GAPDH antibodies using immunoprcipitates of PMA-treated U937 cytoplasmic extracts with antibodies against Nucleolin, PCBP1 or normal IgG. (**H**) Western blots for proteins as indicated from immunoprecipitates of PMA-treated U937 cytoplasmic extracts preincubated with or without RNase A with antibodies as indicated. All experiments were repeated three times, producing the same results (abbreviations: IP: immunoprecipitate; NTC: no template control; cyt: cytosolic extract; WCE: whole-cell extract; nuc: nuclear; NE: nuclear extract (positive control for blot with histone).

significantly reduce the levels of *lif* mRNA in non-PMA-treated cells (Supplementary Figure S7); however, in the PMA-treated condition, *lif* mRNA levels were decreased by ∼81 and ∼42% by partial knockdown of Nucleolin and PCBP1, respectively, when compared with the control, while knocking down of both decreased that to 10% (Figure 5A, *left panel*). Additionally, Nucleolin was overexpressed with or without partial knockdown of PCBP1, and the corresponding *lif* mRNA levels were checked. Results from this experiment, demonstrated in Figure 5A (*right panel*), indicate that partial knockdown of PCBP1 decreased *lif* mRNA levels to 69% when compared with control (taken as 100%), which by overexpression of Nucleolin reverts back to 93%.

Western blots illustrated decreased levels of LIF protein resulting from partial knockdown of Nucleolin (Figure 5B) and PCPB1 (Figure 5C), where reduced levels of these proteins due to their transient knockdown demonstrate the efficiency of the knockdown of Nucleolin (Figure 5B) and PCPB1 (Figure 5C). The increase in *lif* mRNA and LIF protein levels post-PMA treatment in U937 cells is reversed upon depletion of PCBP1 and Nucleolin, signifying Nucleolin and PCBP1 as the two major regulators of PMA-induced *lif* mRNA stability in U937 cells.

It was also observed that ectopic overexpression of Nucleolin by transfecting U937 cells with pC-Nuc plasmids (pcDNA 3.1 was used as a control) stabilized intrinsic *lif* mRNA (Figure 5D) as indicated by the increased half-life of its mRNA (from 2.5 h to much higher than 4 h) when the cells were chased for 4 h with actinomycin D after 44 h of transfection.

The results therefore clearly indicate that knockdown of Nucleolin and PCBP-1 proteins decreased *lif* expression, and ectopic overexpression of Nucleolin increased its level, altogether demonstrating that Nucleolin and PCBP-1 are potent stabilizing *trans*-acting factors for *lif* mRNA.

Discussion

Human LIF is an important cytokine that plays key roles in myriads of cellular processes and its production is highly inducible with a wide range of inducing agents (physiological or pharmacological like LPS (lipopolysaccharide), TNF (tumor necrosis factor), GM-CSF (granulocyte-monocyte colony stimulating factor), PMA and retinoic acid) depending on the cell type involved [31]. LIF is a potent and pleiotropic ligand secreted from a vast variety of tissues like fibroblasts, activated T-cells, spleen or macrophage cells, chondrocytes, bone marrow stromal cells, mesenchymal stem cells, endothelial cells and astrocyte tumor cells, and is responsible for regulating numerous physiological phenomena and is also important in several pathological conditions. The constitutive levels of *lif* mRNA are low in several hematopoietic as well as leukemic cells, which upon treatment with different inducers showed higher levels of its expression promoting cell differentiation [1,2,17,32]. Interestingly, elevated expression of LIF has varied roles in health and disease; therefore, a tight but subtle control of LIF expression is necessary. The detailed mechanisms of regulation of LIF expression, however, are yet to be divulged. The present findings render an insight into the molecular mechanisms of post-transcriptional regulation of human *lif* expression when induced with PMA in human histiocytic lymphoma cell line U937.

First, the study has revealed that PMA induces *lif* expression by stabilization of its mRNA in U937 cells. In mammalian cells, a change in the half-life of a particular mRNA can result in significant difference in its abundance without any change in the rate of mRNA synthesis. Moreover, a small change in the mRNA level can have significant effects on the abundance of its encoded protein, which, in turn, may profoundly affect cellular physiology [14]. The half-life of an mRNA is determined by its sequence and the structural elements present in it. The interactions of these *cis*-elements with *trans*-acting factors can either shorten or lengthen the half-life of

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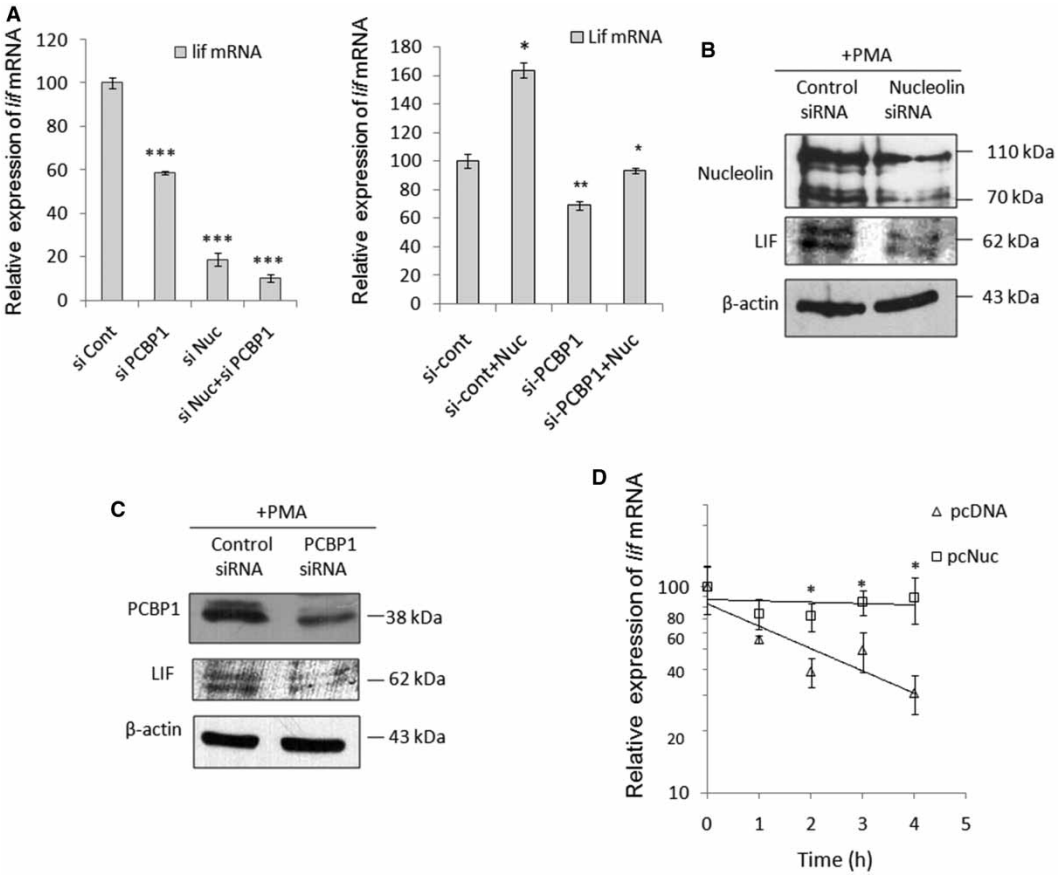

Figure 5. Effects of Nucleolin and PCBP1 on the stability of intrinsic *lif* mRNA.

(**A**) Relative expression of intrinsic *lif* mRNA normalized to *β-actin* mRNA by the ΔΔCT method measured after 48 h of transfection of U937 with 50 nM siRNA (control or PCBP1 or Nucleolin or PCBP1 + Nucleolin) followed by PMA treatment for 24 h (left panel), and with control or PCBP1 siRNA (50 nM), with or without overexpression of Nucleolin by co-transfecting with pcNuc (right panel), where NS indicates ($P > 0.05$), * is ($P \leq 0.05$) ** is ($P \leq 0.01$) and *** is ($P \leq 0.001$). (**B**) Western blot of Nucleolin, LIF and β-actin with whole-cell extracts of siRNA (control or Nucleolin) transfected PMA-treated U937 cells. (**C**) Western blot of PCBP1, LIF and β-actin with whole-cell extracts of siRNA (control or PCBP1) transfected PMA-treated U937 cells. (**D**) Intrinsic *lif* mRNA levels in U937 cells transfected with pcDNA or pcNuc, where cells were harvested at 0, 1, 2, 3 and 4 h after treatment with 5 μg/ml actinomycin D. The relative expression of mature *lif* mRNA was measured by qRT-PCR and normalized to the levels of intrinsic β-actin mRNA. Results are means of three independent experiments and presented as mean ± SEM. * is ($P \leq 0.05$).

the transcript [14]. In this context, numerous distinct proteins have been identified that bind *cis*-acting elements present in the 3′-UTRs of different mRNAs [15].

The *lif* mRNA has a long 3′-UTR (3199 nucleotides) with a repertoire of known *cis*-acting elements, as predicted by our *in silico* studies, which renders it an interesting template to investigate the mechanisms related to modulation of its mRNA expression under particular physiological or pathological conditions. The sequence analysis of *lif*-3′-UTR revealed that the 3′-end of the 3′-UTR contains canonical AREs (lif-E). The role of this region in *lif* mRNA stability has been addressed elsewhere [unpublished work by Chakraborty, A and Sengupta (Bandyopadhyay), S] and is beyond the scope of this manuscript. The proximal region of *lif*-3′-UTR (1636–1851 nt) was found to have two class I AREs and four poly(rC)-containing regions, and showed efficient destabilization. Pyrimidine (C/CU)-rich *cis*-elements were first identified in the α2-globin mRNA [33] and later in many mRNAs like α (I)-collagen, tyrosine hydroxylase (TH) and 15-lipoxygenase (15-LOX) mRNA [28]. In addition to a consensus pyrimidine-rich sequence shared by all these mRNAs, they were also bound by

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identical *trans*-factors that formed a stabilizing RNA–protein complex, initially termed the α-complex comprising PCBP1, PCBP2 and PABP-C [28].

Binding of RBPs to different regions of the 3′-UTR of mRNAs predominantly modulate their expressions at the post-transcriptional level. Especially upon PMA treatment, association of Nucleolin, HuR and PCBP1 (Figure 2D,E) was documented by our study. Nucleolin is a multifunctional phosphoprotein distributed ubiquitously and is imperative for cellular growth and proliferation [29,34]. Among many other functions, it plays a significant role in post-transcriptional regulation of many mRNAs containing AU-rich [35] and GC-rich sequences [24]. PCBP1, a member of the hnRNP E family of proteins, contains three KH domains for specific DNA or RNA binding and is predominantly localized in the nucleus [36]. It was reported to be present in the RNP complex of more than 160 mRNA species [30] and regulates gene expression via a broad spectrum of regulatory mechanisms that include transcription, mRNA splicing, mRNA stability and translation [37].

It is evident from the co-immunoprecipitation studies that functionality of Nucleolin and PCBP1 with respect to *lif* mRNA stabilization directly correlated with their binding to the RNA. PMA treatment induced nuclear to cytoplasmic transport of both Nucleolin [24] and PCBP1 (Figure 4E), where they associate with *lif* mRNA, becoming constituents of the same mRNP complex in an RNA-dependent manner. A similar study by Lee *et al.* [38] showed that, for gastrin mRNA stabilization by Nucleolin, the binding of Nucleolin to mRNA was facilitated by interaction with the hnRNPK1/PCBP1 complex bound to C-rich regions in the mRNA, which strengthened our finding.

The interplay of different RBPs with the same RNA is capable of fostering diverse effects on the RNA; for example, in the case of *bcl-2* mRNA, Nucleolin and HuR were shown to promote mRNA stability and AUF1 enhanced degradation [35,39,40]. Similarly, in the case of GADD45A mRNA, while Nucleolin stabilizes the mRNA [41], AUF1 antagonizes its effect by enhancing degradation, where TIAR suppresses GADD45A translation [42]. To elucidate the interplay between Nucleolin and PCBP1 in determining the fate of *lif* mRNA, knockdown experiments (Figure 5) were performed, which revealed that Nucleolin is more important for the maintenance of PMA-induced *lif* mRNA abundance in comparison with PCBP1. The effects of these proteins on *lif* mRNA stabilization are likely to be independent of each other since knockdown of both proteins does not show any significant synergistic decrease in *lif* mRNA or *gfp* reporter levels over that observed for Nucleolin knockdown alone. Therefore, Nucleolin binding to *lif* mRNA might not be additionally facilitated by PCBP1. The correlation of these RNA–protein interactions with functionality will be further investigated in terms of the exact sequences on *lif* mRNA to which these proteins bind. Moreover, other proteins that are enriched specifically in the mRNP complex of *lif*-B and PMA-treated U937 cytoplasmic extracts may also influence Nucleolin-mediated stabilization of *lif* mRNA or act independently, leaving ample scope for future investigations.

The overall regulation of *lif* mRNA stability is a very complicated process involving several effectors (*trans*-acting factors), whose co-ordinated behavior in response to a particular stimulus (PMA treatment in this case) influences the fate of mature *lif* mRNA in the cytoplasm. The *lif* mRNA level is extensively up-regulated by PMA treatment, where Nucleolin and PCBP1 are the two *trans*-factors that play important roles in the post-transcriptional stabilization of this mRNA in U937 cells.

Taking together all these results, it can be concluded that elucidation of the mechanisms of PMA-mediated *lif* mRNA stabilization will lead to a better understanding and control of LIF expression not only in differentiation of myeloid leukemia cells but also in several other physiological and pathological conditions where *lif* mRNA stability may play a significant role. Future studies on interactions of *lif* mRNA and *lif* mRNA-binding proteins in regulating the fate of *lif* mRNA are expected to provide insights into the mechanism of regulation of LIF expression in different physiological and pathological conditions.

Abbreviations

3′-UTR, 3′-untranslated region; ARE, adenylate(A)- and uridylate(U)-rich element; GFP, green fluorescent protein; HILDA, Human interleukin for dopamine agonist, alias: Leukemia inhibitory factor; hnRNP, heteronuclear ribonucleoprotein; IL-6, interleukin-6; KH, domain, K homology domain; LIF, leukemia inhibitory factor; LPS, lipo-polysaccharide; PCBP1, poly (rC) binding protein 1; pcDNA3.1, Mammalian expression vector with CMV promoter; pcNuc, pcDNA construct containing Nucleolin gene for Nucleolin ectopic expression in mammalian cells; ^{32}P-CTP, ^{32}phosphorus labeled cytidine triphosphate; PMA, phorbol-12-myristate-13-acetate; Poly(rC), poly cytidine; REMSA, RNA electrophoretic mobility shift assay; TIAR, (T-cell restricted Intracellular Antigen-1

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related protein) RNA binding protein; TAE, Tris-acetate EDTA buffer; q-PCR, quantitative Polymerase Chain Reaction or Real-time PCR; RT-PCR, Reverse Transcription-Polymerase Chain Reaction.

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Competing Interests

The Authors declare that there are no competing interests associated with the informations provided in the manuscript.

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