Differential expression and phosphorylation of CTCF, a c-*myc* transcriptional regulator, during differentiation of human myeloid cells

M. Dolores Delgado[a], Igor V. Chernukhin[b], Anna Bigas[c], Elena M. Klenova[b], Javier León[a,*]

[a] *Grupo de Biología Molecular del Cáncer, Departamento de Biología Molecular, Facultad de Medicina, Universidad de Cantabria, 39011 Santander, Spain*

[b] *Genetic Unit, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, UK*

[c] *Cell Therapy Department, Institut de Recerca Oncologica, 08907 Hospitalet, Barcelona, Spain*

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Abstract CTCF is a transcriptional repressor of the c-*myc* gene. Although CTCF has been characterized in some detail, there is very little information about the regulation of CTCF activity. Therefore we investigated CTCF expression and phosphorylation during induced differentiation of human myeloid leukemia cells. We found that: (i) both CTCF mRNA and protein are down-regulated during terminal differentiation in most cell lines tested; (ii) CTCF down-regulation is retarded and less pronounced than that of c-*myc*; (iii) CTCF protein is differentially phosphorylated and the phosphorylation profiles depend on the differentiation pathway. We concluded that CTCF expression and activity is controlled at transcriptional and post-transcriptional levels.

Key words: CTCF; c-Myc; Phosphorylation; Differentiation; Myeloid leukemia cell

1. Introduction

c-Myc is a transcription factor of the helix-loop-helix/leucine zipper family involved in the regulation of proliferation, differentiation and apoptosis in many different cell types (reviewed in [1,2]). Myeloid leukemia cell lines have been very useful models in elucidating molecular mechanisms of c-Myc function. Studies on these cells have established that: (i) in cell lines such as HL60, U937, HEL and K562, c-*myc* expression is dramatically down-regulated during the induced differentiation into several phenotypes [3–6], (ii) c-*myc* ectopic expression inhibits erythroid differentiation of murine F-MEL [7] or human K562 cells [8], and monocytic differentiation of U937 cells [4], (iii) c-*myc* inhibition by antisense oligonucleotides in HL60 [9] or overexpression of *myc* inhibitory mutants or *max* in K562 [10] induces myeloid cell differentiation. However, less progress has been made in defining the mechanisms regulating the expression of c-*myc* itself in general, and transcription factors involved in c-*myc* regulation in particular. The extensive and complex regulation of c-Myc expression has led to the identification of a number of factors which regulate its expression upon binding to specific sequences within the 5′ regulatory regions. Most of them are positive transactivators (reviewed in [11]), but transcriptional repressors of c-Myc have also been described [12,13]. We had previously discovered and characterized one of the factors involved in the regulation of c-*myc*, the CTCF protein.

CTCF was first identified as the protein interacting with three repeats of the CCCTC motif in the chicken c-*myc* promoter [14]. The CTCF gene encodes an 11-zinc finger protein, it is ubiquitously expressed and highly conserved [15,16]. Interestingly, different zinc fingers are involved in the binding of chicken or human c-*myc* promoters, thus classifying CTCF as a 'multivalent' transcriptional factor with multiple sequence specificity [16]. CTCF binds to a negative element upstream of the chicken c-*myc* promoter and to several sites in the promoter and coding regions of the mouse and human c-*myc* genes [15,16]. CTCF has been shown to negatively regulate the expression of the c-*myc* gene [16], the chicken lysozyme gene [17] but to positively regulate the amyloid-β protein precursor promoter [18]. It has been found that the human *CTCF* gene is localized at the chromosome 16q22.1 locus, a region commonly deleted in breast and prostate cancers [19]. These data suggest that *CTCF* is a tumor suppressor gene.

Despite the fact that many of the structural and functional properties of CTCF have been described, much less is known about regulation of CTCF expression. This is particularly important since CTCF is a ubiquitous factor, found in all tissues and cell lines analyzed so far [19]. In order to gain insight into how CTCF expression is modulated, we studied CTCF mRNA and protein levels as well as CTCF protein phosphorylation during myeloid cell differentiation. We used several human leukemia cell lines arrested at different stages of differentiation and determined CTCF expression and phosphorylation state during the chemically induced differentiation. We compared *CTCF* and c-*myc* expression and found differential expression and phosphorylation of CTCF, which was dependent on the cells' differentiation pathways.

2. Materials and methods

2.1. Cells and induction of differentiation

Human cell lines NB4, Raji, CA46, Manca and Daudi were kindly donated by S. Collins (Fred Hutchinson Cancer Research Center, Seattle, WA, USA). The other leukemia cell lines were obtained from ATCC. Cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics. Exponentially growing K562 cells were induced to differentiate with 1 μM 1-β-D-arabinofuranosylcytosine (ara-C), 10 nM 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) or 100 nM staurosporine (STA). HL60 cells were exposed to 1.2% dimethyl sulfoxide (DMSO), 10^{-5} M all-*trans*-retinoic acid (RA) or 16 nM TPA. U937 and THP1 cells were treated with 20 nM TPA. Cell morphology was determined by Giemsa staining. The erythroid differentiation was assessed with the benzidine cytochemical test and ε-globin gene expression [8]. Monocytic and granulocytic differentiation was assessed by standard morphological criteria. Cell clustering activity, adherence to surface and induction of the vimentin

*Corresponding author. Fax: (34) (942) 201945.
E-mail: leonj@medi.unican.es

Abbreviations: Ara-C, 1-β-D-arabinofuranosylcytosine; IEF, isoelectric focusing; STA, staurosporine; TPA, 12-*O*-tetradecanoyl phorbol-13-acetate

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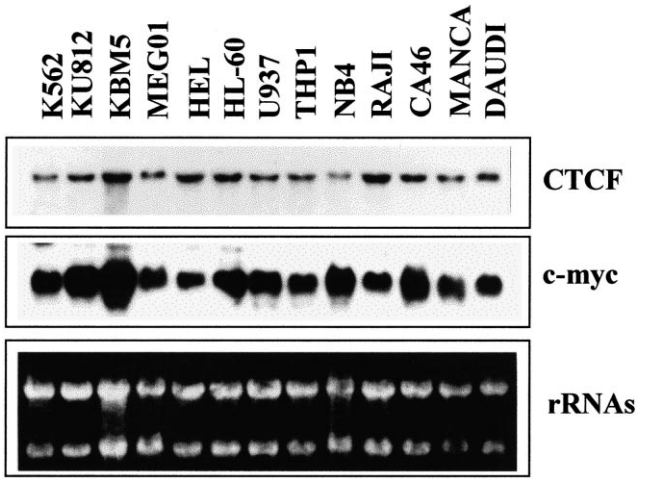

Fig. 1. Expression of *CTCF* and c-*myc* in hematopoietic cell lines. Total RNA was isolated from exponentially growing cells of the indicated cell lines and analyzed by Northern blot. The same membrane was consecutively hybridized to the *CTCF* and c-*myc* probes. A picture of the filter after transfer is shown to assess the loading and integrity of the RNAs.

gene [20] were also used as markers of monocytic differentiation. The differentiation towards monocytic versus granulocytic lineages was assessed by staining for the α-naphthyl acetate esterases (monocytic esterases) and for the chloroacetate esterases (neutrophil esterases).

2.2. RNA preparation and Northern blot analysis

For RNA analysis, total RNAs were prepared by the guanidine-thiocyanate method [21], separated by electrophoresis through agarose-formaldehyde gel and transferred to nitrocellulose. Filters were hybridized sequentially to the different probes labeled with [α-^{32}P]dCTP by random priming. Semiquantitative determination of mRNA levels was carried out by measuring the radioactivity of the filters with a Molecular Imager apparatus (Bio-Rad). Probes for human c-*myc*, ε-globin and vimentin were as described [8]. For human *CTCF*, a 2 kb *Pvu*II cDNA fragment from pCI7.1 CTCF [16] was used.

2.3. Western blot assay

For immunodetection of CTCF, cell pellets were lysed in a buffer containing 100 mM Tris (pH 6.8), 8% β-mercaptoethanol, 4% SDS and 20% glycerine. Protein content was measured using the Bio-Rad Protein Assay. 30 μg of protein per lane was separated in 8% acrylamide gels and transferred to nitrocellulose. Equal protein loading was assessed by Coomassie blue staining of the gels after transfer. CTCF protein was detected by a rabbit anti-CTCF polyclonal antibody kindly donated by V. Lobanenkov (Fred Hutchinson Cancer Research Center, Seattle, WA, USA). Immune complexes were revealed by chemiluminescence (ECL, Amersham, UK).

2.4. Isoelectric focusing (IEF) assay

Cells (5×10^6) were collected and lysed in a standard 0.5 M salt RIPA buffer. The cell lysates were then spun to remove DNA and the proteins in the supernatants were precipitated with acetone. After drying, the proteins were resolved in the sample buffer containing 6 M urea, 5% CHAPS, 1% ampholyte pH 4–6, 50 mM NaF and 2% β-mercaptoethanol, in a volume which gives the equivalent of 0.5×10^5 cells in 20 μl sample buffer. IEF was performed on a horizontal system (Pharmacia) using precast IEF gel on a glass plate with pH range 4–6. The IEF gel contained 5% acrylamide, 10% v/v sorbitol, 1% CHAPS and 2% ampholyte (Fluka). The electrode buffers were 0.1 M β-alanine and 0.1 M glutamic acid in 0.5 M H_3PO_4 for the cathode and anode, respectively. The electrophoresis was performed at 2000 V and 10 mA at 10°C during 2.5 h. Immediately after the electrophoresis was completed, the gel was soaked in the buffer containing Tris base 10 mM, 0.5% SDS, 20% methanol for 30 min and proteins were transferred onto PVDF membranes using a semidry transfer system. After blocking in 1% BSA and 1% PVP, the membrane was first probed with the anti-CTCF antibodies and then with the secondary anti-rabbit IgG peroxidase-conjugated antibodies. The bands were visualized by ECL.

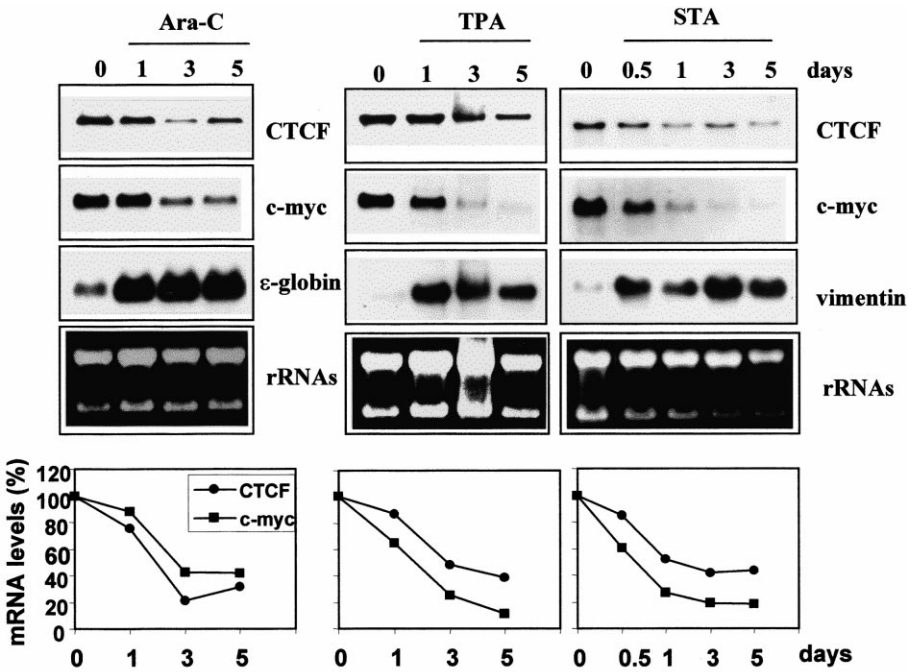

Fig. 2. Expression of *CTCF* and c-*myc* in K562 cells treated with ara-C, TPA or STA. Exponentially growing K562 cells were exposed to 1 μM ara-C, 10 nM TPA or 100 nM STA for up to 5 days. Lane 0 shows control cells prior to drug addition. Total RNAs were isolated and analyzed by Northern blot. The filters were consecutively hybridized to the indicated probes. The experiments were performed at least twice and similar results were obtained. Graphs at the bottom represent the quantification of the Northern blots. Relative expression levels were determined by measuring the *CTCF* and c-*myc* signals, as described in Section 2, and the values normalized with respect to 28S rRNA.

2.5. Two-dimensional IEF

The IEF was performed on precast 2D-IEF gel strips on a plastic film support. The gel was composed of 5% acrylamide, 6 M urea, 5% glycerol, and 2.2% carrier ampholyte pH 3.5–10. 0.5 M NaOH and 0.15 M H_3PO_4 were used for the cathode and the anode, respectively. The electrophoresis was run on the horizontal system (Pharmacia) at a maximum of 1500 V and 10 mA at 10°C during 3 h. After the electrophoresis was complete, the strips were soaked in SDS-PAGE standard sample buffer and further applied to 10% SDS-PAGE. The proteins resolved in SDS-PAGE were transferred onto PVDF membranes by semidry transfer. The filter was probed with the rabbit anti-CTCF antibodies as described above.

3. Results

CTCF is present in a wide range of tissues [19]. We have further investigated CTCF expression in a variety of cell lines derived from chronic myeloid leukemia in blastic crisis (K562, KU812, KBM5 and MEG01), acute myeloid leukemia (HEL, HL60, U937, THP1 and NB4) or Burkitt lymphomas (Raji, CA46, Manca and Daudi). As shown in Fig. 1, *CTCF* mRNA with an approximate size of 4 kb is expressed in all cell lines. We did not observe major differences in *CTCF* mRNA expression among cell lines from different leukemic origins. The same blot was re-hybridized to a human c-*myc* probe to compare the expression of *CTCF* with that of its target gene, c-*myc*. There are small differences in the ratio *CTCF*/c-*myc* among the different cell lines (Fig. 1). Thus the NB4 promyelocytic cell line shows the lowest ratio while the Raji Burkitt lymphoma cell line has the highest ratio. To further analyze the regulation of *CTCF* during myeloid differentiation, we chose four of these lines representing different stages of myeloid differentiation: K562, HL60, U937 and THP1.

The K562 cell line represents immature hemopoietic precursors that express erythroid markers. Cells can be differentiated into the erythroid lineage by incubation with ara-C [22,23] or into the monocytic/macrophagic lineage by phorbol esters, i.e. TPA (in these cultures, cells express both megakaryocytic and monocytic markers [23,24]). We have recently described that the PKC inhibitor STA induces megakaryocytic differentiation of K562 cells (Lerga et al., submitted). Exponentially growing K562 cells were exposed to these three agents and cell proliferation and effectiveness of differentiation were monitored for up to 5 days after the addition of the inducing agents. Treatment with ara-C induced cell growth arrest, increased the ε-globin expression (Fig. 2, left) as well as the fraction of benzidine positive, i.e. hemoglobinized, cells (data not shown). Treatment of K562 cells with TPA led to the appearance of cells with specific monocyte features: ability for latex phagocytosis, adherence to the surface, formation of cell clusters (data not shown) and vimentin mRNA up-regulation (Fig. 2, center). On the other hand, the TPA differentiated cells lost the expression of erythroid markers such as ε-globin (not shown). Treatment of K562 cells with STA resulted in the appearance of cells with megakaryocytic features such as increased cell size, appearance of multinucleated cells, loss of ε-globin expression (not shown) and increased vimentin expression (Fig. 2, right).

To investigate expression of *CTCF* and c-*myc*, total RNA was isolated from K562 cells, electrophoretically separated, blotted to filters and hybridized sequentially with ^{32}P-labeled probes specific for human *CTCF* and c-*myc*. We found that the *CTCF* mRNA decreased during the erythroid differentiation, in parallel with the down-regulation of c-*myc* expression (Fig. 2, left). Quantification of *CTCF* and c-*myc* signals showed about three-fold reduction of expression of both genes after 5 days of exposure to ara-C, as shown in the graph (Fig. 2, left). Similar down-regulation of *CTCF* mRNA (2.5-fold) was found in K562 cells after induction of monocytic differentiation by TPA (Fig. 2, center). However, the decrease of c-*myc* expression with TPA was faster and more pronounced (about 10-fold), thus confirming previously published observations [5]. Comparable results were obtained when K562 cells were induced with STA towards the megakaryocytic pathway (Fig. 2, right): a strong and rapid down-regulation of c-*myc* expression (10-fold) and a gradual decrease in *CTCF* expression (three-fold).

As has been previously reported, CTCF migrates as a 130 kDa protein on SDS-PAGE [25]. We found a decrease of the CTCF protein during the erythroid, monocytic and megakar-

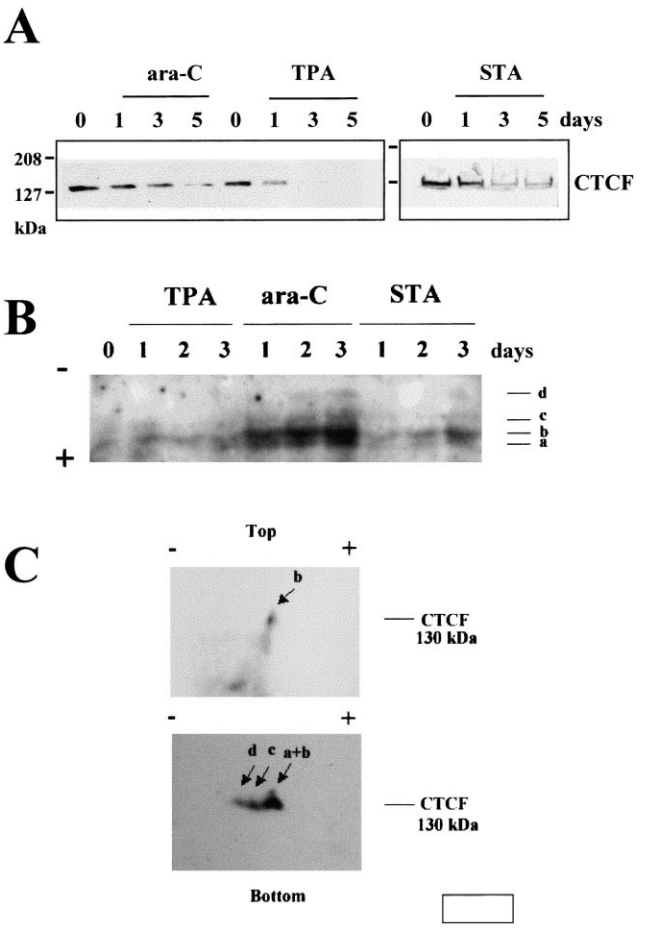

Fig. 3. Analysis of CTCF protein expression and phosphorylation in K562 cells. A: Immunoblot of CTCF protein in cells treated with ara-C, TPA and STA, as described in Fig. 2, for the indicated periods of time. The positions of molecular mass markers are indicated on the left. B: Isoelectric focusing assay of the CTCF protein in K562 cells. Cell extracts were prepared from the cells treated for up to 3 days with the three inducers and the IEF assay was conducted as described in Section 2. The positions of differentially phosphorylated forms of CTCF are shown on the right (from 'a' to 'd'). The positions of the anode ('+') and cathode ('−') are shown on the left. Lane 0 refers to untreated control cells. C: Two-dimensional IEF assays of CTCF in untreated K562 cells (top panel) and cells after 3 days of exposure to ara-C (bottom panel). The position of a CTCF protein of 130 kDa is shown on the right and the differentially phosphorylated forms of CTCF are indicated by arrows.

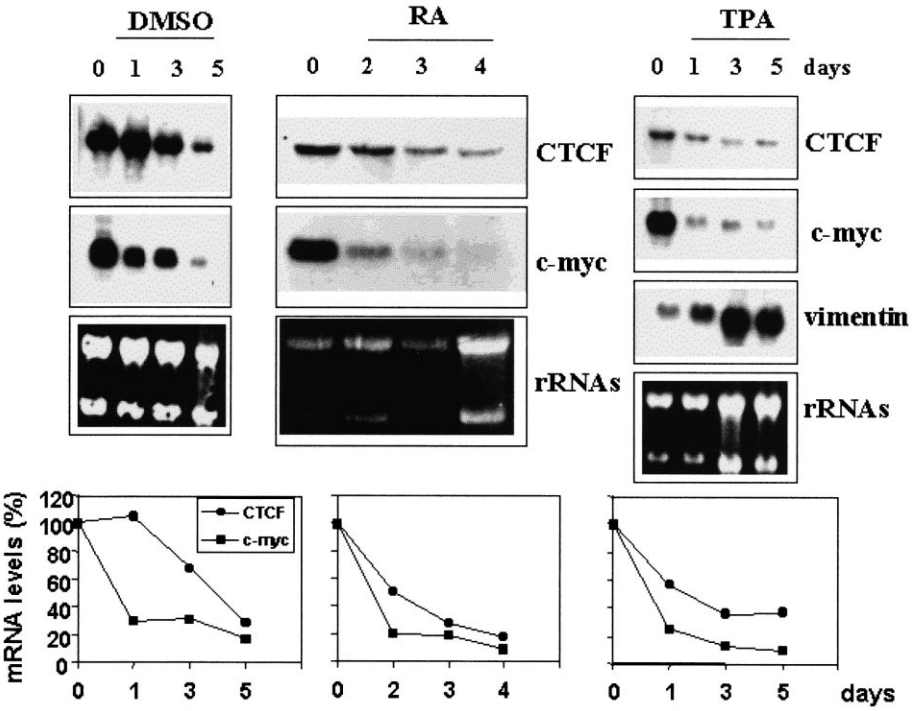

Fig. 4. Expression of *CTCF* and c-*myc* in HL60 cells treated with DMSO, RA or TPA. Exponentially growing HL60 cells were exposed to 1.2% DMSO, 10^{-5} M RA, or 16 nM TPA for the indicated times. Total RNAs were isolated and analyzed by Northern blot as described in Fig. 2. Graphs at the bottom represent the quantification of the Northern blots.

yocytic differentiation of K562 cells, as judged by Western blot analyses of cells treated with the specific inducers for up to 5 days (Fig. 3A). The decrease in CTCF protein levels is consistent with the mRNA down-regulation observed in Fig. 2. We also investigated whether differentiation of K562 provoked changes in the phosphorylation state of CTCF. We performed IEF assays at treatment times where CTCF protein is still present. As shown in Fig. 3B, the IEF reveals that CTCF protein is represented by several forms: the hypophosphorylated closely migrating forms 'a' and 'b', isofocused near the anode, the intermediately phosphorylated form 'c', and the hyperphosphorylated form 'd' isofocused near the cathode. In undifferentiated cells CTCF is in the hypophosphorylated form (Fig. 3B, day 0). No significant changes were observed in the phosphorylated state of CTCF upon induction with TPA and STA, where CTCF remains hypophosphorylated. However, erythroid differentiation of K562 provoked the appearance of two higher phosphorylated forms ('c' and 'd' forms). To confirm that all these forms are specific and correspond to the modified forms of CTCF, we performed a two-dimensional IEF assay. When resolved in the second dimension all proteins detected with the anti-CTCF antibody migrated as 130 kDa proteins (Fig. 3C, top panel, K562 non-induced; Fig. 3C, bottom panel, K562 cells induced with ara-C for 3 days).

Next we studied the CTCF regulation in HL60, a more differentiated myeloid cell line with bipotential capability of differentiation towards granulocytic or monocytic lineages (reviewed in [3]). Treatment of HL60 cell with DMSO or retinoic acid up to 5 days induced granulocytic features as assessed by morphological and cytochemical analysis (see Section 2). As shown in Fig. 4, left, *CTCF* mRNA levels remained elevated 1 day after DMSO treatment, decreasing thereafter (three-fold after 5 days). The RA treatment also led to down-regulation of *CTCF* mRNA after 2 days (Fig. 4, center). To confirm the observed CTCF down-regulation during the granulocytic differentiation process, we used the murine cell line 32-D Cl3 [26]. These cells are non-tumorigenic, IL-3 dependent, and can be induced to granulocytic differentiation with granulocyte colony stimulating factor (G-CSF). We found that the 130 kDa CTCF protein was also down-regulated in this murine cell line after 3 days of treatment with G-CSF (results not shown).

When HL60 cells were exposed to TPA, the monocytic phenotype was clearly induced as assessed by cell morphology, increased adherence to surface and the induction of vimentin gene expression (Fig. 4, right). In this case, a progressive decrease in *CTCF* expression was also observed (2.5-fold after 5 days). A strong down-regulation of c-*myc* (10–15-fold) was detected after differentiation of HL60 cells with all three agents, in agreement with published observations [3,6]. Western blot analysis of the lysates from HL60 cells treated with TPA and DMSO (Fig. 5A) revealed that the levels of CTCF protein progressively decreased after induction of cell differentiation towards the monocytic (with TPA) or granulocytic (with DMSO) pathways, although CTCF suppression was more pronounced after TPA treatment. Analysis of the phosphorylation profile of the CTCF protein in HL60 cells upon induction of monocytic and granulocytic pathways demonstrated that, in contrast to K562, all four CTCF forms ('a', 'b', 'c' and 'd') were present in HL60 non-differentiated cells. After treatment with TPA, only hypophosphorylated forms of CTCF ('a' and 'b') were observed, and form 'c' (intermediate) disappeared after treatment with DMSO (Fig. 5B).

U937 and THP1 are more mature monoblastic cell lines with the capacity to differentiate only towards the mono-

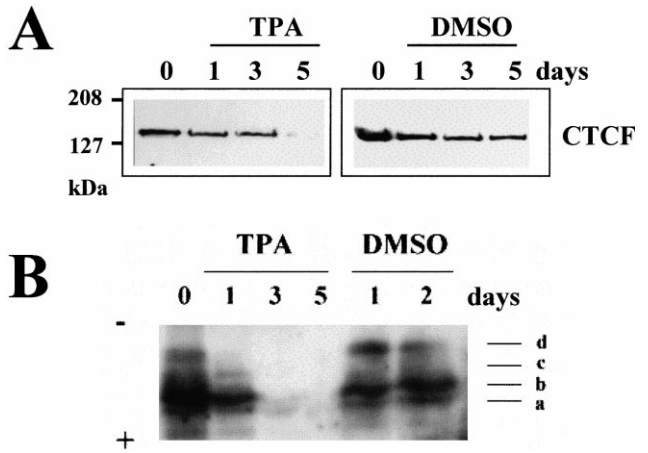

Fig. 5. Analysis of CTCF protein expression and phosphorylation in HL60 cells. A: Immunoblot of CTCF protein in HL60 cells treated with TPA or DMSO, for the indicated periods of time. The positions of molecular mass markers are indicated on the left. B: IEF assay of the CTCF protein in HL60 cells. Cell extracts were prepared from the cells treated with TPA and DMSO (days after treatment are indicated). The positions of differentially phosphorylated forms of CTCF ('a' to 'd') are shown on the right and the anode (+) and cathode (−) positions on the left. Lane 0 refers to untreated cells.

cytic/macrophagic lineage by treatment with TPA [4,6,27]. This phenotype was confirmed by the appearance of several monocytic morphological characteristics described above (data not shown) and also by the increase of vimentin mRNA expression (Fig. 6). Upon cell differentiation of these two cell lines, the *CTCF* mRNA levels were only reduced around 2–2.5-fold, while the c-*myc* expression decreased dramatically. In particular, c-*myc* mRNA levels in U937 cells were almost undetectable 12 h after TPA addition, while the *CTCF* mRNA levels remained elevated. Consistently, the CTCF protein contents were found to be only slightly decreased after 5 days of TPA treatment in both cell lines (Fig. 7A).

Despite the absence of apparent differences between the CTCF protein levels in THP1 and U937 upon cell differentiation, the phosphorylation of CTCF changed dramatically. Thus, the positions of the CTCF forms in these cells differed from HL60 and K562, therefore they were called 'e', 'f', 'g' and 'h'. An intermediate form 'f' is characteristic for non-induced U937 cells, whilst the hypophosphorylated form 'e' in addition to hyperphosphorylated forms 'g' and 'h' was seen in the induced cells (Fig. 7B, lanes 1–3). In non-induced THP1 cells two bands 'g' and 'f' could be detected, which disappeared upon induction with TPA. However, the presence of a strong band corresponding to form 'h' was seen 1 day after TPA addition (Fig. 7B, lanes 4–6).

4. Discussion

In this study we have investigated differential expression and phosphorylation of CTCF during differentiation of four leukemia cell lines into erythroid, megakaryocyte, granulocyte and monocyte-like cells. Three general conclusions could be drawn from this study: (i) both mRNA and protein CTCF are generally down-regulated during differentiation into the different phenotypes, although there are marked differences depending on the cell line and differentiation pathway; (ii) CTCF down-regulation is delayed and less pronounced than that of c-*myc*; (iii) there is a differential phosphorylation of CTCF protein which depends on the cell line and differentiation pathway.

It is noteworthy that the decrease in CTCF protein is detected only once the differentiation process has become irreversible, i.e. the cells are committed to terminal differentiation. In K562 this commitment requires 24, 12 and 2 h of ara-C, staurosporine and TPA treatment, respectively ([5] and Lerga et al., submitted). In most cases, down-regulation of c-Myc precedes that of CTCF, in full agreement with the described negative effect of CTCF on c-*myc* promoter activity [16]. There are noticeable differences between the CTCF and c-Myc expression profiles among the different cell lines induced into the monocytic pathway (K562, HL60, U937 and THP1 treated with TPA). These differences may reflect the different stages of maturation of these cell lines. For example, CTCF protein down-regulation is much smaller and slower in U937 and THP1 cells, which may reflect the fact that both cell lines are unipotent (promonocytic) and more mature than K562 or HL60.

We have recently demonstrated that CTCF is a phosphoprotein and is phosphorylated in vivo in COS6 cells by casein kinase II (Klenova et al., in preparation). It is conceivable that the phosphorylation pattern of CTCF depends on the cell type, particular inducers or cell state (i.e. whether it is differentiated or cycling). Our present study shows that CTCF has a specific phosphorylation profile in different cell types, thus implying that other protein kinases may be involved in CTCF phosphorylation. Furthermore, the phosphorylation pattern of CTCF changes upon terminal differentiation, therefore suggesting a functional importance of CTCF phosphorylation in the cells. In most cases CTCF is under- or de-phosphorylated in proliferating cells, but higher

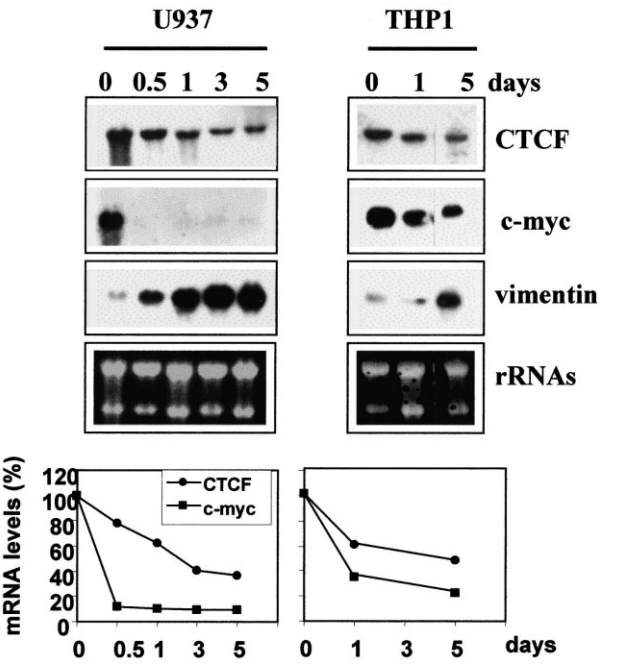

Fig. 6. Expression of *CTCF* and c-*myc* in U937 and THP1 cells treated with TPA. Exponentially growing U937 and THP1 cells were exposed to 20 nM TPA for the indicated times. Total RNAs were isolated and analyzed by Northern blot as described in Fig. 2.

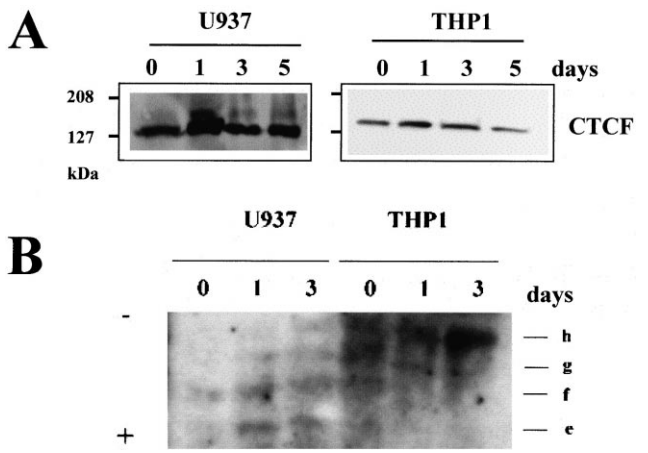

Fig. 7. Analysis of CTCF protein expression and phosphorylation in U937 and THP1 cells. A: Immunoblot of CTCF protein in cells treated with 20 nM TPA for up to 5 days. The positions of molecular mass markers are indicated on the left. B: IEF assay of CTCF protein in U937 and THP1 cells treated with 20 nM TPA for up to 3 days. The positions of differently phosphorylated CTCF protein ('e' to 'h') are shown on the right and the anode (+) and cathode (−) positions on the left. Lane 0 refers to untreated cells.

phosphorylated forms can be observed when cells are induced to differentiate. Since the latter is accompanied by c-*myc* and, in most cases, *CTCF* down-regulation, it is tempting to speculate that external signals triggering the differentiation program also lead to differential phosphorylation of CTCF. Thus, a particular combination of differently phosphorylated forms of the CTCF protein may be responsible for regulating the expression of lineage specific genes, leading to the switch to different differentiation programs. We have previously demonstrated that CTCF acts as a repressor of c-*myc* [16], however, our recent studies revealed that CTCF can also negatively auto-regulate its own promoter (Filippova et al., submitted). Therefore, it is possible that differently modified forms of CTCF could specifically regulate the expression of c-*myc* and *CTCF* genes after induction of differentiation. However, one cannot rule out that other genes, in addition to *CTCF* and c-*myc*, may be targets for regulation by CTCF in those cells. This possibility is particularly suitable to differentiation of U937 and THP1 cells, where there is no correlation between the c-Myc down-regulation and the sustained levels of the CTCF.

In summary, here we present data showing that during the differentiation of human myeloid cell lines, the expression of *CTCF* and also its phosphorylation profile are differentially regulated depending on the specific differentiation pathway. In future studies we will attempt to map the phosphorylation sites in these cells and investigate their importance in the regulation of CTCF at the c-*myc* and *CTCF* promoters.

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