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The MHC class II transactivator (CIITA) mRNA stability is critical for the HLA class II gene expression in myelomonocytic cells

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The human promyelocytic U937 cells express detectable levels of MHC class II (MHC-II) molecules. Treatment with 12-o--tetradecanoyl phorbol 13-acetate (TPA), inducing macrophage-like differentiation, produces a dramatic decrease of MHC-II expression as result of down-modulation of the activation of immune response gene 1 (*AIR-1*)-encoded MHC-II transactivator (CIITA). This event is specific, as MHC class I remains unaffected. Similar results are observed with U937 cells expressing an exogenous full-length CIITA. Molecular studies demonstrate that TPA treatment affects the stability of CIITA mRNA rather than CIITA transcription. Importantly, *cis*-acting elements within the distal 650 bp of the 1035-bp 3′ untranslated region (3′UTR, nucleotides 3509–4543) are associated to transcript instability. Transcription inhibitors actinomycin D and 5,6-dichlororibofuranosyl benzimidazole, and the translation inhibitor cycloheximide significantly rescue the accumulation of CIITA mRNA in TPA-treated cells. A similar effect is also observed after treatment with staurosporine and the PKC-specific inhibitor GF109203X. The instability of CIITA mRNA produced by TPA in U937 cells is not seen in B cells. These results demonstrate the presence of an additional level of control of MHC-II expression in the macrophage cell lineage depending upon the control of CIITA mRNA stability, most likely mediated by differentiation-induced, 3′UTR-interacting factors which require kinase activity for their destabilizing function.

Key words: MHC · Gene regulation · Monocytes · Transcription factors · Hematopoiesis

Introduction

Major histocompatibility complex class II (MHC-II) molecules play a key function in the homeostasis of the immune system by presenting antigenic peptides to $CD4^+$ T helper cells, the key cells in the activation of immune effector functions. MHC-II molecules are expressed constitutively in dendritic cells (DC), B cells, and in cells of the monocyte/macrophage lineage, cumulatively referred to as professional antigen-presenting cells (APC). Moreover, cytokines, mainly IFN-γ, may further increase, or *de novo* induce, MHC-II expression in macrophage-like cells or in cells of extrahematopoietic origin [1].

Expression of MHC-II genes is mainly regulated at the transcriptional level [2]. Triggering of MHC-II transcription is mediated by a non-DNA-binding specific coactivator encoded by the activation of immune response gene 1 (*AIR-1*), designated MHC-II transactivator (CIITA) [3, 4]. CIITA acts by integrating the functions of several DNA-binding proteins in the MHC-II

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Abbreviations: TPA: 12-o-tetradecanoyl phorbol 13-acetate · ***AIR-1:*** Activation of immune response gene 1 · **MHC-II:** MHC class II · **CIITA:** MHC-II transactivator · **ActD:** Actinomycin D · **DRB:** 5,6-Dichlororibofuranosyl benzimidazole · **CHX:** Cycloheximide · **3′UTR:** 3′ Untranslated region · **EGFP:** Enhanced green fluorescent protein

transcriptosome, and is required for both constitutive and inducible expression. In addition, post-translational modifications influence the functional activity of CIITA [5–9] and thus the MHC-II transcription. Beside the transcriptional control, MHC-II function depends also on post-transcriptional events. It has been found that MHC-II mRNA stability depends on active protein synthesis [10] and can be increased by bacterial CpG DNA in mouse B cells [11]. Moreover, in interspecies somatic B cell hybrids, impaired expression of HLA-DQ but not of HLA-DR and HLA-DP is observed, notwithstanding the presence of normal amounts of DQα and DQβ transcripts [12]. Lastly, DC express low levels of cell-surface, but high levels of intracellular MHC-II molecules, which are mobilized to the cell surface during functional maturation [13]. Concomitantly, CIITA expression is shut off [14].

U937 is a promyelocytic cell line that constitutively expresses low levels of cell surface MHC-II molecules. Following 12-o-tetradecanoyl phorbol 13-acetate (TPA) treatment, U937 cells acquire a macrophage-like phenotype and MHC-II surface expression is shut off. In this study we have investigated the molecular correlates of this MHC-II down-regulation. We show that the MHC-II down-regulation is due to a post-transcriptional block resulting from a dramatic decrease of the stability of CIITA mRNA. This event is counteracted by treatment with inhibitors of transcription, of protein synthesis, and of protein kinase C (PKC), strongly suggesting the existence of TPA-induced differentiation factors destabilizing CIITA mRNA, which require kinase activity for their functioning. Furthermore *cis*-acting determinants of transcript instability were mapped within the distal 650 bp of the 3′ untranslated region (3′UTR) of CIITA mRNA. These results add a new level of complexity to the regulation of MHC-II expression and may be important to better understand the control of MHC-II-dependent antigen presentation in APC.

Results

TPA down-modulates endogenous MHC-II expression in U937 cells by acting at the level of CIITA expression

The promyelocytic cell line U937 displays a low constitutive MHC-II cell surface expression which cannot be further induced by IFN-γ treatment (data not shown). The treatment of U937 with phorbol ester TPA for 72 h strongly decreases HLA-DR surface expression (Fig. 1A, left panel). The effect of TPA is specific for MHC-II molecules because HLA-A, B, C cell surface expression is unaffected (Fig. 1A, right panel).

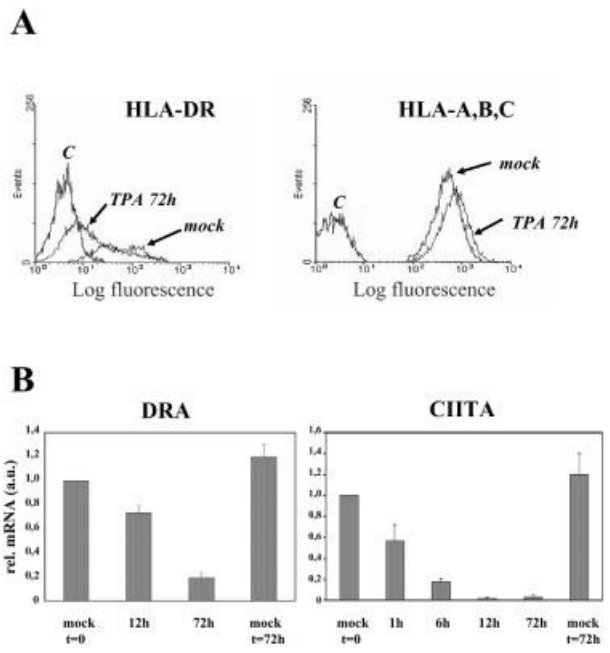

Fig. 1. Expression of HLA class II and CIITA in U937 cells treated with TPA. (A) Cell surface expression of HLA class II (HLA-DR) and class I (HLA-A,B,C) molecules in U937 cells treated with TPA. Cells were cultured for 72 h in absence (mock) or in presence of 80 nM TPA (TPA 72 h) and then analyzed by indirect immunofluorescence and flow cytometry. HLA-DR (left panel) and HLA-A,B,C cell surface molecules were assessed by the use of the D1–12 anti-DR and the B9.12.1 anti-HLA class I mAb, respectively. Cells stained with an isotype-matched antibody were used as negative control (C). Results are expressed in the abscissa as log fluorescence value in arbitrary units. (B) Quantitative analysis of HLA-DRA- and CIITA-specific transcripts in U937 cells treated with phorbol ester. Cells were cultured in presence of 80 nM TPA for the indicated times (in the abscissa) and then analyzed for the presence of HLA-DRA- (left panel) or CIITA- (right panel) specific mRNA by real-time RT-PCR. Two samples of mock-treated cells from cultures at time=0 and at 72 h were included to better estimate possible variations in the amount of specific mRNA at the beginning and at the end of the culture period. The results are expressed in the ordinate as relative amount of transcript in arbitrary units (a.u.). The amount of HLA-DRA and CIITA transcripts detected in untreated U937 cells at the beginning of the culture were arbitrarily set to 1. Bars represent standard deviations of real-time RT-PCR triplicate values. The results shown are from one representative experiment of four with similar results.

Because the total MHC-II protein expression level is also strongly decreased by TPA treatment (see below), we assessed by real-time RT-PCR whether MHC-II-specific transcripts were modified. The results show that HLA-DRA mRNA is reduced to less than 20% of its initial content after 72 h of phorbol ester treatment (Fig. 1B, left panel). Furthermore, CIITA mRNA levels are clearly decreased after 1 h of TPA treatment and are 20% and 3% at 6 h and 12 h, respectively (Fig. 1B, right panel). The steady-state level of CIITA mRNA remained very low throughout the remaining period of the kinetics

analyzed. Thus, TPA-dependent MHC-II down-modulation correlates to reduced transcription of the corresponding genes generated by reduced expression of CIITA.

TPA treatment affects the stability of CIITA mRNA: mapping of the instability regions to the 3′UTR of the transcript

To assess whether the decreased accumulation of CIITA-specific mRNA after phorbol ester treatment was due to transcriptional or post-transcriptional events, a Rous sarcoma virus (RSV) promoter-based vector driving the expression a full-length CIITA cDNA containing 1035 bp of its 3′UTR (CIITA/3509–4543) was transfected into U937 cells. Stable transfectants express four times more CIITA mRNA than untransfected cells (Fig. 2, right panel; compare the second with the first histogram), and this correlates with a four- to sixfold higher expression of HLA-DR cell surface molecules with respect to untransfected U937 cells (compare Fig. 2, left panel, mock, with Fig. 1A, left panel, mock). Interestingly, TPA treatment of U937/CIITA cells results again in a strong reduction of HLA-DR expression (Fig. 2, left panel) as a consequence of a dramatic decrease of exogenous CIITA transcripts (Fig. 2, right panel). It must be noted that exogenous CIITA mRNA is reduced with a kinetics virtually superimposable to the one observed for the mRNA transcribed from the endogenous gene. Therefore, CIITA expressed under the control of a heterologous promoter behaves similarly to CIITA expressed from the endogenous promoter, suggesting that the effect of TPA is exerted on the stability of CIITA-specific mRNA rather than on the rate of transcription.

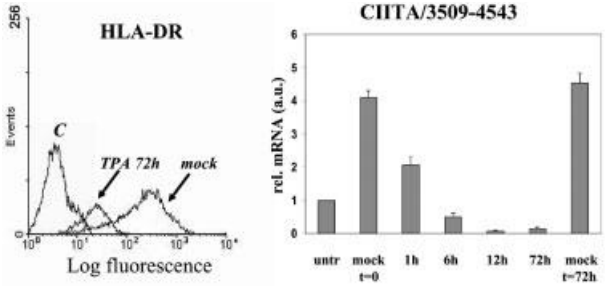

Fig. 2. TPA treatment of U937 cells decreases the stability of CIITA mRNA. Cell surface expression of HLA-DR class II molecules (left panel) and expression of CIITA mRNA (right panel) in U937 cells stably transfected with a full-length CIITA cDNA containing the entire 1035-bp 3′UTR before (mock) and after treatment with 80 nM TPA for the indicated length of time. HLA-DR cell surface expression was assessed by indirect immunofluorescence and flow cytometry using the anti-DR mAb D1–12. Cells stained with an isotype-matched antibody were used as negative control (C). CIITA mRNA content was assessed by real-time RT-PCR. The results are expressed in the ordinate as relative amount of transcript in arbitrary units (a.u.) as compared to the CIITA transcripts detected in untreated and untransfected parental U937 (untr) cells. Bars represent standard deviations of real-time RT-PCR triplicate values. Results are representative of three distinct experiments.

It is well established that the 3′UTR of many mammalian mRNA contain destabilizing signals [15]. Experiments were then performed to assess whether TPA-mediated destabilization of CIITA mRNA could be associated to its 3′UTR. To this purpose, U937 cells were stably transfected with a CIITA cDNA construct bearing an 800-bp deletion in the distal region of its 3′UTR (CIITA/3509–3744). The transfectants express substantial amounts of HLA-DR molecules (Fig. 3A, left panel). Interestingly, TPA treatment does not decrease the expression of MHC-II molecules at all. Moreover CIITA mRNA derived from the transcription of the transfected gene is not substantially down-modulated (Fig. 3A, right panel). These results indicate that the 800-bp 3′UTR of CIITA mRNA contains *cis*-acting destabilizing sequences.

To further delineate the CIITA 3′UTR including the instability region and to assess whether the instability can be transferred to another open reading frame (ORF), two additional deletion mutants encompassing the proximal 385 bp (nucleotides 3509–3893) and the distal 756 bp (nucleotides 3789–4543) of the CIITA 3′UTR, respectively, as well as the entire 3′UTR (nucleotides 3509–4543), were cloned downstream to the d2-enhanced green fluorescent protein (EGFP) cDNA whose expression is driven by an RSV promoter. This ORF codes for a GFP variant containing a PEST domain that targets the protein for rapid degradation; the corresponding mRNA, however, is very stable, as it contains only six nucleotides between the stop codon and the polyadenylation signal [16]. The three different constructs (pEGFP/3509–4543, pEGFP/3509–3893, and pEGFP/3789–4543) were transfected into U937 cells. Stable transfectants were isolated on the basis of their GFP-positive phenotype as assessed by flow cytometric analysis (data not shown) and then treated with TPA.

Results presented in Fig. 3B show that the CIITA 3′UTR carries indeed destabilizing *cis*-acting sequences that can be transferred to another ORF; moreover, most of these sequences are contained within the distal 650 bp (nucleotides 3789–4543) region of the CIITA 3′UTR. Interestingly, the destabilizing effect on the GFP mRNA induced by TPA over time follows similar kinetics as observed for CIITA mRNA. As expected, the GFP mRNA of U937 cells transfected with the d2EGFP cDNA vector alone was not destabilized by TPA treatment (data not shown).

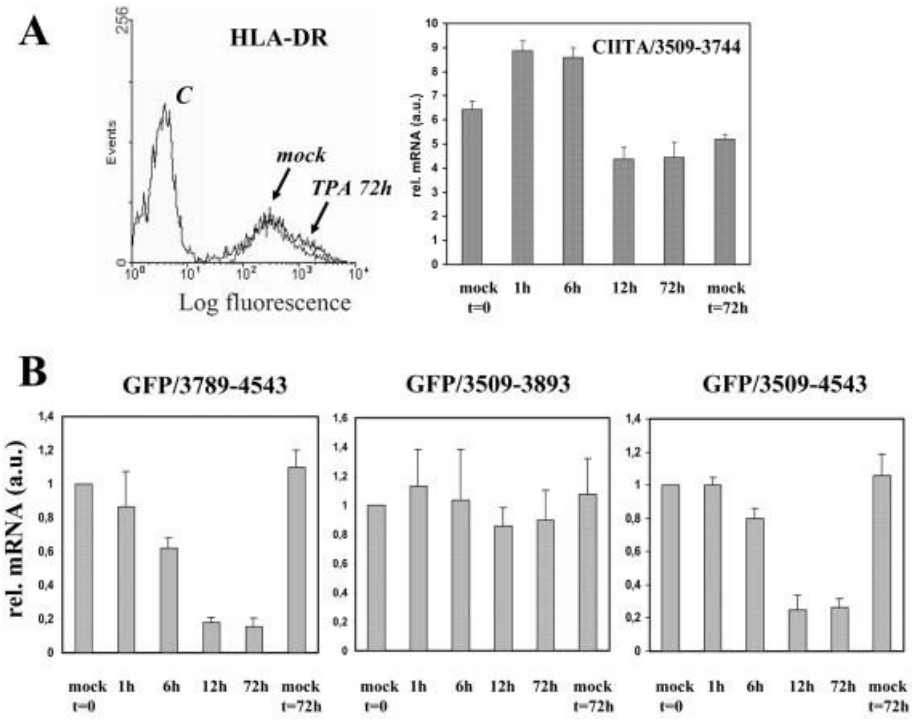

Fig. 3. The 3′UTR of CIITA mRNA is the target of the TPA-mediated CIITA mRNA destabilization. (A) Cell surface expression of HLA-DR class II molecules (left panel) and expression of CIITA mRNA (right panel) in U937 cells stably transfected with a CIITA cDNA bearing a 800-bp deletion in the 3′UTR (CIITA/3509–3744), before (mock) and after treatment with 80 nM TPA for the indicated length of time. HLA-DR cell surface expression was assessed by indirect immunofluorescence and flow cytometry using the anti-DR mAb D1–12. Transfectants stained with an isotype-matched antibody were used as negative control (C). CIITA mRNA content was assessed by real-time RT-PCR. The results are expressed in the ordinate as relative amount of transcript in arbitrary units (a.u.) as compared to the CIITA transcripts detected in untreated, untransfected parental U937 cells as described in the legend to Fig. 1. Bars represent standard deviations of real-time RT-PCR triplicate values. Results are representative of three independent experiments. (B) Real-time RT-PCR of U937 cells stably transfected with d2EGFP cDNA carrying at its 3′ end distinct fragments of CIITA 3′UTR as indicated in the top of each histogram, before and after treatment with 80 nM TPA for the indicated length of time. The results are expressed in the ordinate as relative amount of GFP transcript in arbitrary units (a.u.). The amount of GFP transcript detected in each untreated U937 transfectant at the beginning of the culture was arbitrarily set to 1. Bars represent standard deviations of real-time RT-PCR triplicate values. Results are representative of three independent experiments.

The 3′ untranslated region of CIITA is the target of TPA-induced mRNA-destabilizing factors

Since the function of the 3′UTR sequences are manifested only after treatment with TPA, we reasoned that the phorbol ester may induce and/or activate specific factors which interact with these sequences and destabilize the CIITA mRNA. In order to investigate this point, wild-type U937 cells or U937 cells stably transfected with the CIITA cDNA including the entire destabilizing 3′UTR (the same cells as described in Fig. 2) were incubated for 1 h with TPA alone. At this time, drug inhibitors of either transcription, such as actinomycin D (ActD) and 5,6-dichlororibofuranosyl benzimidazole (DRB), or translation, as cycloheximide (CHX), were added for additional 11 h, always in presence of TPA. At the end of the 12-h culture period, the cells were collected and analyzed for their content of CIITA mRNA. The use of the above inhibitors was adopted because it has been reported that transcriptional or translational inhibitors may super-induce the expression of some genes by preventing the degradation of otherwise labile mRNA [17, 18].

Fig. 4 shows that both transcription inhibitors, ActD and DRB, rescue the accumulation of CIITA mRNA in wild-type (from 2% to 35% and to 28%, respectively) and CIITA-transfected (from 9% to 45% and to 35%, respectively) U937 cells. Moreover, treatment with CHX also counteracts the destabilizing effect of TPA on CIITA mRNA both in wild-type (from 2% to 32%) and CIITA-transfected (from 9% to 40%) U937 cells. The 800 bp-deleted CIITA construct remained unaffected by treatment with the above inhibitors (data not shown). Thus, inhibitors of transcription and protein synthesis have an antagonistic effect on the TPA-induced degradation of CIITA mRNA.

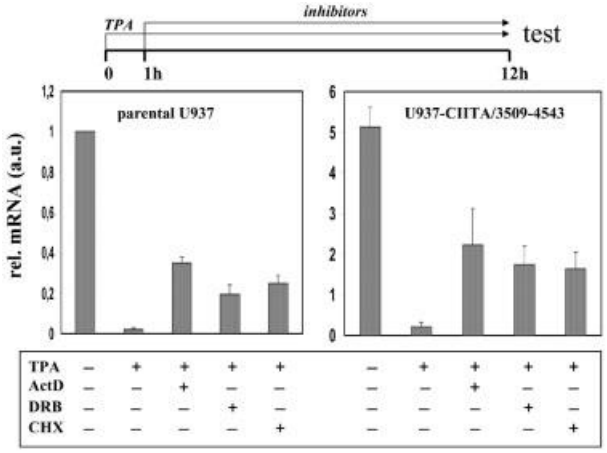

Fig. 4. Inhibition of transcription or of protein synthesis partially protects CIITA mRNA of U937 cells from TPA-induced destabilization. Parental U937 cells or U937 cells stably transfected with a CIITA cDNA containing the 1035-bp 3′UTR (U937-CIITA/3509–4543) were incubated for 1 h with 80 nM TPA before the addition of the indicated transcription (ActD: 5 μg/ml; DRB: 10 μg/ml) or protein synthesis (CHX: 2 μg/ml) inhibitors for additional 11 h of culture. At the end of the 12-h culture period cells were lysed and their CIITA mRNA content was assayed by real-time RT-PCR. The results are expressed in the ordinate as relative amount of transcript in arbitrary units (a.u.) as compared to the CIITA transcripts detected in untreated, untransfected parental U937 cells as described in the legend to Fig. 1. Bars represent standard deviations of real-time RT-PCR triplicate values. Results are representative of three independent experiments.

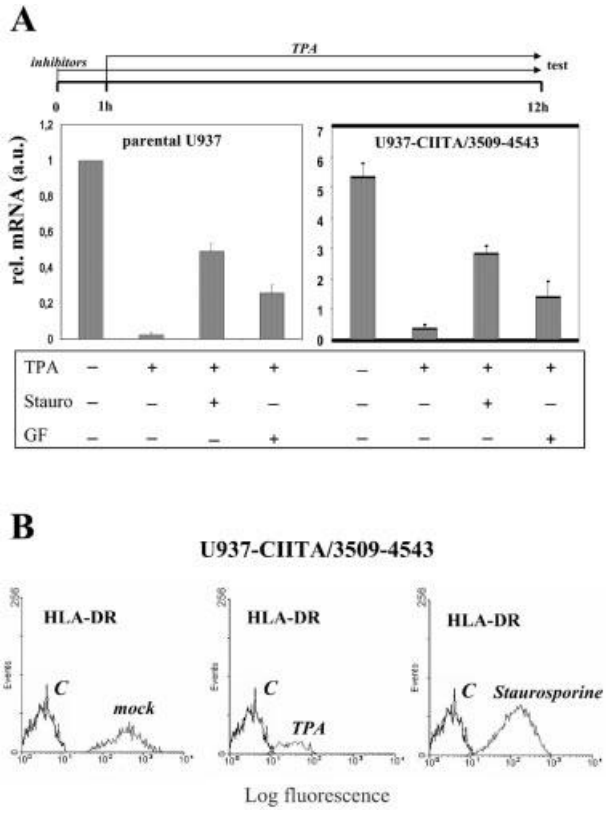

Fig. 5. Inhibition of protein kinase activity partially protects CIITA mRNA of U937 cells from TPA-induced destabilization and rescues HLA class II expression. (A) Parental U937 cells or U937 cells stably transfected with a CIITA cDNA containing the 1035-bp 3′UTR (U937-CIITA/3509–4543) were incubated for 1 h with protein kinase inhibitors (staurosporine: 150 nM; GF109203X: 500 nM) before the addition of 80 nM TPA for additional 11 h of culture. At the end of the 12-h culture period cells were lysed and their CIITA mRNA content assayed by real-time RT-PCR. The results are expressed in the ordinate as relative amount of transcript in arbitrary units (a.u.) as compared to the CIITA transcripts detected in untreated, untransfected parental U937 cells as described in the legend to Fig. 1. Bars represent standard deviations of real-time RT-PCR triplicate values. Results are representative of three independent experiments. (B) Flow cytometric analysis of CIITA/U937 transfectants. Cells were incubated with staurosporine for 1 h, then TPA was added. After 16 h an equal volume of fresh medium was added to the culture. Cells were collected at 72 h and analyzed. HLA-DR cell surface expression was assessed using the anti-DR mAb D1–12. Cells stained with an isotype-matched antibody were used as negative control (C). Mock, untreated cells; TPA, cells treated with TPA only; Staurosporine, cells treated with staurosporine and TPA.

The function of CIITA mRNA-destabilizing factors requires kinase activity

Phorbol esters act as agonists of diacylglycerol in the PKC pathway [19]. To further investigate the functional requirements of TPA-mediated destabilization of CIITA mRNA, experiments were performed to assess the role of protein kinases. Parental and CIITA-transfected U937 cells were pre-incubated for 1 h with the broad kinase inhibitor staurosporine or with the selective PKC inhibitor bis-indolylmaleimide GF109203X. TPA was then added and CIITA mRNA content was assessed 12 h after the beginning of the culture.

Staurosporine potently rescued CIITA mRNA stability in both cell types from 2% and 7%, respectively, up to 50% of the level of control untreated cells (Fig. 5A). Similarly, GF109203X significantly rescued, although to lesser extent than staurosporine, CIITA mRNA stability in wild-type (from 2% to 25%) and CIITA-transfected (from 7% to 22%) U937 cells. Interestingly, the strong antagonistic effect of staurosporine on the TPA-mediated destabilization of CIITA mRNA resulted in an increased biological effect of the CIITA protein, since MHC-II transcription and corresponding protein cell surface expression was also appreciably increased (Fig. 5B). Taken together, these results strongly suggest that CIITA mRNA-destabilizing factors induced by TPA require protein kinase activity, including PKC activity, to become functionally competent.

Cell type specificity of the CIITA mRNA-destabilizing effect by TPA

To assess whether the effect of TPA was specific for cells of the myelomonocytic lineage, MHC-II and CIITA expression were studied in THP-1, another myelomonocytic cell line, and in the B lymphoblastoid cell line Raji. Treatment with TPA for 72 h decreased the total MHC-II protein expression level in THP-1 cells whereas it did not affect at all the MHC-II protein expression level in Raji cells, as assessed by cell surface cytofluorimetric analysis (data not shown) and by Western blotting of cell extracts (Fig. 6A).

Real-time RT-PCR analysis of CIITA mRNA at different times after TPA treatment shows the progressive and dramatic decrease of CIITA mRNA content in THP-1 cells, with similar kinetics as observed in U937 cells. On the contrary, the steady-state level of CIITA mRNA in Raji cells is only marginally reduced, being 86% of that of untreated cells (Fig. 6B). Then we tested whether treatment with the transcription inhibitor ActD was able to modify the pattern of CIITA mRNA expression in the two cell lines incubated with TPA. As observed for U937 cells, ActD strongly rescues the stability of mRNA in THP-1 macrophage-like cells (from 6% to 40%), whereas it does not further increase the already relatively stable CIITA mRNA in Raji cells. Taken together, these results indicate that within the myelomonocytic cell lineage represented by U937 and THP-1, phorbol ester treatment produces similar destabilizing effects on the CIITA mRNA, whereas these effects are not observed in cells of the B lymphocyte lineage.

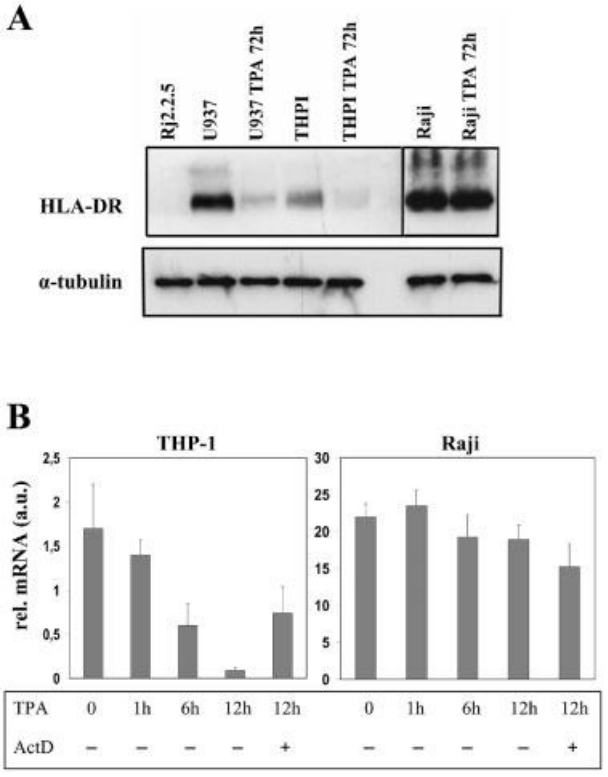

Fig. 6. Effect of phorbol treatment on the HLA-DR and CIITA expression in the promyelocytic THP-1 and the B lymphoblastoid Raji cell line. (A) Western blot analysis of total HLA-DR molecular pool was performed on cell extracts of the various cell lines listed on the top, before and after treatment with TPA for 72 h. In non-denaturing conditions [29], the anti-DR mAb D1–12 recognizes the class II α-β heterodimer as a single band of 64 kDa. As control for a constitutively expressed protein, the Western blot of α-tubulin as detected by the DM1A antibody is shown. All filters were exposed to films for 120 s, with the exception of the filter containing samples of Raji cells and Raji cells treated with TPA, which were exposed for 20 s. Samples were: RJ2.2.5, an HLA-II-negative mutant originated from Raji; U937, untreated parental U937 cells; U937 treated with TPA; untreated promyelocytic THP-1 cells; THP-1 cells treated with TPA; Raji, untreated Raji cells; Raji cells treated with TPA. (B) THP-1 and Raji cells were incubated with TPA for the indicated times and then analyzed for their CIITA mRNA content by real-time RT-PCR. The effect of transcription inhibitor ActD was assessed by adding the drug 1 h after the initiation of the culture in presence of TPA, and measuring CIITA mRNA at the end of the 12-h culture period, as described in the legend to Fig. 4. The results are expressed in the ordinate as relative amount of transcript in arbitrary units (a.u.) as compared to the CIITA transcripts detected in untreated and untransfected parental U937 cells as described in the legend to Fig. 1. Bars represent standard deviations of real-time RT-PCR triplicate values. Results are representative of three independent experiments.

Discussion

The results presented in this report establish for the first time the existence of a novel level of regulation of MHC-II gene expression based on the control of mRNA stability of the *AIR-1* locus product CIITA, the major activator of MHC-II transcription. Most of the available studies on the physiology of CIITA have been concentrated on its transcriptional regulation [2]. Some studies have investigated the protein turnover and the post-synthetic modifications of CIITA [5–9]. Little attention has been given to other possible levels of regulation during CIITA biosynthesis. Although down-modulating effects on MHC-II expression, possibly mediated by a specific targeting of CIITA mRNA, have previously been reported by Han and co-workers [20], the *cis*-acting elements of this destabilization could not be assigned precisely even though they were likely located in the coding region of CIITA mRNA

The present study demonstrates that the *cis*-acting elements of CIITA mRNA instability map in the 3′UTR of the transcripts and more precisely within the sequence 3894–4543. Moreover, our findings assume a peculiar relevance because they indicate that CIITA mRNA instability may be involved in the tissue-specific

control of MHC-II gene expression of the granulocyte-macrophage hematopoietic cell lineage. In fact, phorbol ester treatment strongly affects CIITA mRNA stability in both U937 and THP-1, two distinct myelomonocytic cell lines, whereas it does not affect at all the CIITA mRNA stability of Raji cells, representative of the hematopoietic B cell lineage. To this regard our results are similar to those reported by Setterblad and co-workers [21], who showed that phorbol esters do not reduce, and may actually increase, the transcription of the DRA promoter in B cells. In a recent study [7] it was found that the MHC-II expression of CIITA-transfected U937 cells did not decrease and actually was increased by treatment with PMA. Since in that study the CIITA cDNA insert used for transfection carried the 800-bp 3′UTR deletion that eliminates the instability region, the results of Tosi et al. [7] are not in contrast but, indeed, are mostly in line with those presented here.

The presence of destabilizing signals in the 3′UTR of mRNA has extensively been reported in other systems [15]. To date the best-defined *cis*-acting targets of mRNA instability are the AU-rich element (ARE) sequences [22]. Interestingly, the human CIITA transcript reported by Steimle et al. [4] and used in this study (CIITA 1–4543) contains between position 3891–3910 and position 4054–4068 two U-rich, non-AUUUA sequences designated type-III ARE sequences [23–25]. These sequences are included in the 3′UTR whose deletion reverts CIITA mRNA from an unstable to a relatively stable molecule in TPA-treated U937 cells. Thus it is tempting to speculate that the above ARE sequences may be part of the destabilizing signals observed in the present investigation.

ARE sequences are present in several transcripts of genes involved in innate or acquired immune response [15]. However, in most cases the use of phorbol esters to mimic cell activation or an inflammatory status leads to stabilization of transcripts. Steady-state levels of GM-CSF mRNA increase tenfold in mouse T cells treated with TPA as result of mRNA stabilization [26]. In the same cells PMA super-induces ICAM-1 expression by stabilizing its mRNA [27]. For cytokines it is believed that stabilization of their mRNA usually contributes to the strong and rapid induction of immune response genes, particularly during the inflammatory response [28]. Within this frame the apparently opposite behavior of the CIITA mRNA upon inflammatory stimuli in cells of the myelomonocytic lineage may actually play an important role. In fact, the myelomonocytic cell lineage includes classical APC involved in the initiation of the immune response, such as macrophages and DC. In contrast to B cells, which express high amounts of CIITA in a constitutive fashion, macrophages and DC greatly modulate their CIITA expression during functional activation [14, 29]. Thus, in DC and macrophages, CIITA may be regarded as an inflammation-related molecule that is needed for triggering the immune response during the first phases of inflammation, whereas cytokines would play a more important role in the amplification and maintenance of the inflammatory process.

Degradation of the transcripts is not a default process but may be mediated by tightly regulated, transiently expressed factors (the so-called *trans*-acting elements in mRNA instability) [15]. We have observed that in U937 cells the treatment with inhibitors of transcription (ActD and DRB) and of protein synthesis (CHX) strongly counteracts the degradation of CIITA mRNA induced by TPA. Inhibitors of transcription and translation such as those used in this study have been reported to super-induce the expression of many genes by preventing the degradation of otherwise labile mRNA [17, 18]. The molecular mechanisms underlying this effect are still incompletely defined. The most likely hypothesis is that the drug inhibitors block the transcription (ActD and DRB) or translation (CHX) of short-lived repressor proteins which destabilize the mRNA [18, 30], although in certain cases a block in ribosome movement along the mRNA (CHX) required for the rapid decay of the transcript [17, 31] cannot be excluded.

Taken together our results indicate that upon TPA induction of myelomonocytic cells, transient, short-lived trans-acting factors are newly synthesized and/or become functionally competent via a kinase-dependent process which likely requires PKC, and subsequently interact with the 3′UTR of the CIITA mRNA, greatly accelerating the degradation of the transcript.

Materials and methods

Cell cultures and treatments

Two human promyelocytic, macrophage-like cell lines, U937 and THP-1, two human B lymphoblastoid cell lines, Raji and its HLA class II-negative derivative mutant RJ.2.2.5, as well as the human HLA class II-negative T cell line Molt-4, were used in this study. Cells were treated with TPA (Sigma, Milan, Italy) at the following concentrations: U937 and Raji, 80 nM; THP-1, 1 nM. Differentiation toward a macrophage-like cell under TPA treatment was assessed by de novo expression of CD14 and increased phagocytic activity as described [32].

Inhibitors of transcription ActD (Calbiochem, Milan, Italy) and DRB (Sigma) were added to cell cultures at 5 μg/ml (ActD) and 10 μg/ ml (DRB). CHX (Sigma) was used at 2 μg/ ml final concentration. The protein kinase inhibitors staurosporine and GF109203X (Calbiochem) were added to cells at 150 nM and 500 nM final concentration, respectively.

Cell surface phenotyping

HLA-DR and HLA-A, B, C cell surface phenotype was assessed by indirect immunofluorescence and flow cytometry [29]. An irrelevant isotype-matched mAb was used as negative control. EGFP signal was detected by flow cytometry on the FL1 green fluorescent channel.

Construction of plasmid vectors and stable transfections

The full-length, 4543-bp B cell form of CIITA cDNA [4], cloned in the *XhoI* multiple cloning site of pREP10 (pREP10-AIR1), has previously been described [33]. It includes 1035 nucleotides of the 3′UTR downstream the stop codon (region 3509–4543) [4]. The 3744-bp CIITA cDNA, contained in the plasmid pcFG3CIITA8 [34], kindly provided by J. Ting, was excised by restriction enzyme digestion and cloned into the *NheI/XhoI* sites of pREP9 (Invitrogen, Milan, Italy). By sequencing and by digestion with restriction enzymes we verified that this cDNA retained the proximal 235 nucleotides (3509–3744) of the 3′UTR while the remaining 800 nucleotides were lost.

Three regions in the 3′UTR of CIITA were obtained by preparative proofreading PCR (Phusion Taq; Finnzymes, Helsinki, Finland). The template DNA for subcloning the CIITA 3′UTR was from pREP10-AIR1. The entire 3′UTR (nucleotides 3509–4543) of CIITA and the distal region of the 3′UTR from nucleotides 3789 to 4543 were amplified using the forward primers 5′-TGAGATGATCCCAGCTGTGCTCT-3′ and 5′-TTCTCTGAAGGACATTGCGGACA-3′, respectively. For both inserts the reverse primer 5′-AGCTGAAGCTTGCTAGAGGCCGCTCG-3′ was chosen in the multiple cloning site of pREP10-AIR1 downstream of the CIITA cDNA. The third CIITA 3′UTR encompassed the proximal 385 nucleotides immediately downstream the stop codon (3509–3893) and the corresponding amplifying primers were: forward 5′-TGAGATGATCCCAGCTGTGCTCT-3′, reverse 5′-GGATCCAGAAGTACTTTCTCCCCAGGGTG-3′.

Forward primers bear an *Xho-I* linker tail and reverse primers bear a *BamHI* linker tail. The destabilized d2EGFP cDNA was excised from pd2EGFP-1 vector (Clontech, Milan, Italy) and cloned in *KpnI/NotI* sites of pREP9 (Invitrogen). Each CIITA 3′UTR fragment was then cloned in *Xho-I/BamHI* sites of pREP9 downstream of the d2EGFP cDNA. The resulting constructs were designated pEGFP/3509–4543, pEGFP/3789–4543, and pEGFP/3509–3893. The correct sequences of the various amplified inserts were assessed by sequencing analysis.

For transfection experiments, 5 μg of each plasmid was transfected in U937 cells by electroporation at 320 V and 350 μF using the Gene Pulser II apparatus (Bio-Rad, Milan, Italy). Cells transfected with pREP10CIITA or with pREP9-based vectors were selected with 250 μg/ml hygromycin (Celbio, Milan, Italy) or 500 μg/ml G418, respectively. A qualitative RT-PCR analysis was set up in stable transfectants of d2EGFP/3′UTR CIITA reporter vectors to ascertain the presence of the predicted GFP/CIITA chimeric transcripts (not shown).

Western blot analysis

The analysis of total HLA-DR protein was performed as previously reported [29]. To verify that equal amounts of proteins were loaded for the various samples, filters were stripped and re-probed with anti-α-tubulin mAb DM1A (Sigma), and developed as described [29].

Real-time RT-PCR

Total RNA was prepared using RNA-clean (Hybaid, Ashford, UK) following manufacturer's instructions. Synthesis of cDNA was performed using murine leukemia virus reverse transcriptase (Promega, Milan, Italy). The PCR was performed on iCycler iQ Real-Time PCR Detection System (Bio-Rad) and the amplification product was detected using the fluorescence reporter dye SYBR Green I. All reagents in the PCR reaction were provided in the iQ SYBR Green Supermix (Bio-Rad). The cDNA was amplified using the following primers: CIITA, forward 5′-CCTGCTGTTCGGGACCTAAA-3′, reverse 5′-GGATCCGCACCAGTTTGG-3′. The PrimerExpress software was used to design DRA and GAPDH primers: DRA, forward 5′-CTCTTCTCAAGCACTGGGAGTTT-3′, reverse 5′-ATGAAGATGGTCCCAATAATGATG-3′; GAPDH, forward 5′-GAAGGTGAAGGTCGGAGTC-3′, reverse 5′-CATGGGTGGAATCATATTGGAA-3′. The d2EGFP primers were: forward 5′-TGAGCAAAGACCCCAACGAGAA-3′, reverse 5′-GCGGCGGTCACGAACTC-3′. Amplifications on total RNA purified from stable CIITA or d2EGFP transfectants were performed after treatment with RNase-free RQ1 DNase (Promega), following manufacturer's instructions.

For each transcript under investigation, the procedure of quantification was validated by analysis of a standard curve consisting in a serial dilution of a positive-control Raji cDNA. For each sample PCR data value (threshold cycle) was normalized to the corresponding value obtained for the GAPDH, the housekeeping transcript chosen as control. The normalized PCR data value obtained in the untreated U937 cells was defined as the arbitrary unit (value 1). All other normalized PCR data values were then expressed as fraction or multiple of the arbitrary unit. As negative control (zero value), the RT-PCR data from the HLA-DR-negative, CIITA-negative Molt-4 T cell line was used (not shown).

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