TIF1 β functions as a coactivator for C/EBP β and is required for induced differentiation in the myelomonocytic cell line U937

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Representational difference analysis (RDA) cloning has identified transcriptional intermediary factor 1 beta (TIF1 β) as a gene inducibly expressed early during myeloid differentiation of the promyelocytic cell lines HL-60 and U937. To assess the role of TIF1 β , U937 cell lines were made that expressed antisense-hammerhead ribozymes targeted specifically against TIF1 β mRNA. These cells failed to differentiate into macrophages, as determined by several criteria: a nonadherent morphology, a failure to arrest cell cycle, lowered levels of macrophage-specific cell surface markers, resistance to *Legionella pneumophila* infection, a loss of the ability to phagocytose and chemotax, and decreased expression of chemokine mRNAs. One way TIF1 β acts in macrophage differentiation is to augment C/EBP β transcriptional activity. Furthermore, we show by EMSA supershifts and coimmunoprecipitation that C/EBP β and TIF1 β physically interact. Although TIF1 β is necessary for macrophage differentiation of U937 cells, it is not sufficient, based on the inability of ectopically expressed TIF1 β to induce or augment phorbol ester-induced macrophage differentiation. We conclude that TIF1 β plays an important role in the terminal differentiation program of macrophages, which involves the coactivation of C/EBP β and induction of C/EBP β -responsive myeloid genes.

[Key Words: TIF1 β ; C/EBP β ; U937 cells; macrophage; antisense]

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Macrophages play a critical role in development, physiology, innate and adaptive immunity, and in the pathogenesis of many infectious, immunologic, and degenerative disease processes. They possess many specialized cellular functions such as phagocytosis, chemotaxis, antibody-dependent cell cytotoxicity, antigen presentation, and specific expression of a repertoire of cytokines, chemokines, and cell surface markers (Gordon 1995).

Several myelocytic cell lines have been established which, when differentiated with a variety of agents, make the study of these diverse functions tractable. HL-60 is a human myeloid cell line derived from a patient with acute promyelocytic leukemia (Collins et al. 1977). In response to different treatments, HL-60 cells can be induced to differentiate toward mature granulocyte-like cells or monocyte/macrophage-like cells (Collins et al. 1978). HL-60 cells show bilineage differentiation similar to normal granulocyte-macrophage progenitor cells (CFU-GM; Koeffler 1983; Koeffler et al. 1985). The U937

line, established from a patient with histiocytic lymphoma (Sundstrom and Nilsson 1976), has properties consistent with an immature monocyte (Ralph et al. 1976; Sundstrom and Nilsson 1976) and can be induced by phorbol esters to undergo differentiation to a macrophage (Ralph et al. 1982). Thus, both the HL-60 and U937 cell lines provide useful model systems for the study of macrophage differentiation.

Several transcription factors are known to be involved in macrophage differentiation (Clarke and Gordon 1998). The ets-family member PU.1 is critical for myeloid differentiation. Both the granulocyte and monocyte lineages are absent in PU.1-deficient mice (McKercher et al. 1996), and the activation of several macrophage-specific genes requires PU.1 (Pahl et al. 1993; Feinman et al. 1994; Moulton et al. 1994; Perez et al. 1994; Zhang et al. 1994; Rosmarin et al. 1995). The C/EBP family has also been shown to play a direct role in macrophage differentiation. Cotransfection of C/EBP α , β , and δ expression plasmids can activate transcription from macrophage-specific promoters (Ness et al. 1993; Hohaus et al. 1995; Mink et al. 1996; Zhang et al. 1996). C/EBP β , in particular, has been shown to be induced upon macrophage differentiation and to be a potent activator of cytokine gene transcription. The proteins Blimp-1 and ICSBP were re-

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cently shown to be potent regulators of myeloid differentiation. Ectopic expression of Blimp-1 is sufficient to trigger myeloid differentiation (Chang et al. 2000). Mice lacking ICSBP lack macrophages and develop a Chronic myeloid leukemia (CML)-like leukemia (Tamura et al. 2000). *Hoxa9*, when overexpressed, blocks myeloid differentiation (Calvo et al. 2000).

Our objective was to identify early genes expressed during myeloid differentiation, in the hope that such genes might encode regulators of the differentiation process. Toward this end, we used the subtractive cloning technique known as representational difference analysis (RDA) (Hubank and Schatz 1994) to isolate genes expressed during differentiation of the promyelocytic cell line HL-60. One of the cDNAs isolated was that encoding transcriptional intermediary factor 1 beta (TIF1 β). To study the functional role of TIF1 β , antisense-hammerhead ribozymes were designed and used to make stable myelomonocytic U937 cell lines in which endogenous TIF1 β mRNA is ablated. U937 stable clones expressing the antisense-ribozymes were unable to differentiate into macrophages. They have deficiencies in cell cycle arrest, macrophage-specific cell surface marker expression, *Legionella pneumophila* parasitism, phagocytosis, chemotaxis, and chemokine expression. Since C/EBP β has been implicated in the regulation of genes involved in these processes, we investigated the effect of TIF1 β on C/EBP β transcriptional activity. TIF1 β was found to interact with C/EBP β in coimmunoprecipitations and in EMSAs, and functions as a coactivator of transiently transfected and endogenous C/EBP β responsive genes. We propose that TIF1 β is a critical regulator of macrophage differentiation and functions, at least in part, by augmenting the expression of C/EBP β -dependent genes.

Results

A representational difference analysis of early genes in macrophage differentiation isolates the cDNA for TIF1 β

The promyelocytic line HL-60 was used for the RDA. HL-60 cells become fully differentiated in response to the phorbol ester phorbol 12-myristate 13-acetate (PMA) after 2 d (Rovera et al. 1979). To ensure that early genes were isolated, the HL-60 cells used were treated with PMA for only 2 h. RDA subtraction was performed using uninduced HL-60 cDNA as the driver and 2 h PMA-treated HL-60 cDNA as the tester. One of the resulting amplified fragments, when subjected to a BLAST sequence homology search (Altschul et al. 1990), was 100% homologous to the cDNA for TIF1 β . Northern blots of HL-60 RNA (Fig. 1A) showed that TIF1 β mRNA was induced rapidly (by 30 min) and sustained throughout 2 d of PMA-dependent macrophage differentiation (Fig. 1A, lanes 1–6). It was also strongly induced when HL-60 cells underwent granulocytic differentiation in response to treatment with DMSO (Fig. 1A, lanes 7–11). An identical expression pattern of TIF1 β mRNA was seen with PMA-treated U937 cells (Fig. 1A, lanes 12–17).

When human peripheral mononuclear cells (PMNs) were analyzed, TIF1 β mRNA levels were significantly induced upon macrophage differentiation in response to GM-CSF and M-CSF (Fig. 1A, cf. lanes 20 and 21 with U937 levels in lanes 18 and 19). PMA-induced TIF1 β mRNA in U937 cells was unaffected by the addition of the protein inhibitor cycloheximide, thus defining TIF1 β as an immediate-early gene (Fig. 1B). Additionally, TIF1 β protein levels correlated with TIF1 β mRNA expression (Fig. 1C). Thus, TIF1 β is identified as an early response gene for the differentiation of HL-60 cells to either macrophages or granulocytes, for the differentiation of U937 cells to macrophages, and for the differentiation of PMNs to macrophages.

Antisense-hammerhead ribozymes suppress endogenous TIF1 β expression

We sought to determine whether TIF1 β induction was important for macrophage differentiation. Antisense-hammerhead ribozymes were designed to suppress endogenous TIF1 β mRNA. The RNA folding program mfold (Patel et al. 1998; Mathews et al. 1999; Zuker et al. 1999) was used to predict regions of single-strandedness within the TIF1 β RNA molecule, and two antisense constructs were made corresponding to these regions. To further ensure inhibition of the endogenous TIF1 β transcript, catalytic hammerhead ribozyme sequences were incorporated into the antisense constructs (Homann et al. 1993; Tabler et al. 1994; Holmes et al. 1997). As a negative control, a comparable antisense-ribozyme was directed against the β -galactosidase mRNA. Expression plasmids for these three antisense-ribozymes were stably transfected into U937 cells, and four clones expressing each antisense-ribozyme were selected for further study. To monitor expression of the antisense-ribozymes, Northern analyses were performed using as a probe the hammerhead ribozyme sequence (Fig. 1D, top panel, lanes 1–12). To test for the ability of the antisense-ribozymes to degrade endogenous TIF1 β mRNA, the Northern blots were hybridized with a cDNA probe for TIF1 β mRNA (Fig. 1D, middle panel). Whereas the antisense-ribozymes directed against β -gal mRNA had no effect on TIF1 β mRNA levels (Fig. 1D, middle panel, lanes 1–4), each of the four clones with the ribozymes directed against TIF1 β mRNA significantly suppressed expression (Fig. 1D, middle panel, lanes 5–8 and 9–12). Conversely, because the TIF1 β antisense-ribozymes had no effect on GAPDH mRNA levels (Fig. 1D, bottom panel), they appear to be specific for the TIF1 β mRNAs. These data show that the TIF1 β antisense-ribozymes specifically ablate expression of endogenous TIF1 β mRNA.

TIF1 β is required for many aspects of myeloid differentiation

Cell cycle arrest during macrophage differentiation of U937 cells Ribozyme-induced degradation of TIF1 β mRNA in these clones allowed the functional role of

Figure 1. TIF1 β expression during macrophage differentiation. (A) Northern blot of TIF1 β mRNA in differentiating HL-60, U937, and human peripheral blood monocytes (PBMs). HL-60 cells were induced with PMA (lanes 2–6) or DMSO (lanes 7–11). U937 cells were induced with PMA (lanes 12–17). Human PBMs were induced with GM-CSF and M-CSF (lanes 20–21). (B) TIF1 β mRNA is not affected by cycloheximide. Northern analysis was performed on untreated U937 cells untreated (lane 1), treated for 4 h with PMA (lane 2) and treated with PMA and cycloheximide for 4 h (lane 3). (C) Immunoblots of extracts from differentiating U937 cells using antibodies specific for TIF1 β (top) and β -actin (bottom). (D) Expression of TIF1 β antisense-hammerhead ribozymes ablates TIF1 β mRNA levels. U937 cells were stably transfected with plasmids expressing β -gal or TIF1 β antisense-ribozymes and induced with PMA for 24 h. Four hygromycin-resistant cell clones were isolated and analyzed for expression of the mRNAs encoding antisense-ribozymes (top), endogenous TIF1 β (middle), and GAPDH (bottom).

TIF1 β in U937 differentiation to be determined. One distinguishing feature of differentiating macrophages is that they undergo cell cycle arrest. To analyze the state of the cell cycle in the antisense-ribozyme-expressing clones, DNA content was determined by propidium iodine staining, and DNA replication was measured by BrdU incorporation (Fig. 2A). After one day of PMA treatment, the β -gal antisense-ribozyme stable U937 cell lines have undergone cell cycle arrest, as seen by the decrease in number of cells in S phase. In contrast, in the TIF1 β antisense-ribozyme cell lines, no difference was seen in the status of cell cycle progression between untreated and PMA-treated cells. TIF1 β antisense-ribozyme stable U937 cell lines continue to progress through S phase and fail to undergo G₁ arrest. These results demonstrate that TIF1 β is required for G₁ arrest during U937 differentiation into macrophages.

Macrophage-specific cell surface protein expression Another consequence of macrophage differentiation is the expression of specific cell surface proteins. Among these are the β 2-integrin receptor for ICAM-1 and the CR3 complement receptor, CD11b (Mac1), the β 2-integrin receptor for fibrinogen, CD11c, and the LPS receptor, CD14. CD11b and CD11c both mediate macrophage attachment to endothelial cells and subsequent extravasation to areas of inflammation or infection. In addition, CD11b and CD11c both serve as complement receptors, activating an inflammatory response when bound by complement, and facilitating phagocytosis of complement-bound organisms. CD14, the LPS receptor, allows macrophages to recognize, bind to, and phagocytose bacteria. As seen in Figure 2B, CD11b, CD11c, and CD14 levels increased in response to PMA-treatment of U937 clones expressing the β -gal antisense-ribozyme. However, expression did not increase in the TIF1 β antisense-ribozyme stable U937 cell line. Indeed, the levels of CD14 were suppressed to levels below the untreated state. These data show that TIF1 β is required for inducible expression of cell surface molecules of differentiation markers.

L. pneumophila parasitism The cell surface expression of CD11b is required for the infection of macrophages by *L. pneumophila*, a facultative intracellular pathogen which parasitizes human alveolar macrophages (Horwitz and Silverstein 1980). Antibodies specific for CD11b have been shown to block *L. pneumophila* infection of the HL60 cell line (Marra et al. 1990). Since the suppression of TIF1 β blocked CD11b expression, we predicted it would block *L. pneumophila* infection and killing, which requires functional CD11b. To test this prediction, we performed a *L. pneumophila* infection/killing assay. Live *L. pneumophila* were coincubated with PMA-treated U937 antisense-ribozyme stable cell lines for 4 d, and cell survival was assayed for the ability to reduce MTT, an indication of cell viability. As seen in Figure 3A, stable U937 cell lines expressing the antisense-ribozyme against β -gal were effectively killed by *L. pneumophila*, whereas the stable U937 cell lines ex-

pressing the TIF1 β antisense-ribozymes were completely resistant to killing.

We also investigated the ability of PMA-treated U937 cells to phagocytose *L. pneumophila*. U937 cells were assayed for their ability to phagocytose *L. pneumophila* (Fig. 3B) by determining the number of viable intracellular bacteria after a 2 h incubation of *L. pneumophila* and stable U937 cell lines. The stable U937 cell lines expressing the antisense-ribozyme against β -gal effectively phagocytosed bacteria, whereas the stable U937 cell lines expressing the TIF1 β antisense-ribozymes did not.

Phagocytosis of opsonized *S. aureus* Whereas phagocytosis of *L. pneumophila* occurs through the CD11b complement receptor, phagocytosis may also occur via Fc receptors (Aderem and Underhill 1999). To investigate the effect of suppressing TIF1 β expression on Fc receptor-dependent phagocytosis, the stable cell lines were used in phagocytosis assays using opsonized FITC-labeled *S. aureus*, followed by FACS analysis. Whereas the β -gal antisense-ribozyme stable U937 cell lines were able to phagocytose upon differentiation with PMA, both TIF1 β antisense-ribozyme stable cell lines were completely blocked in the ability to phagocytose (Fig. 3C).

Chemotaxis in response to RANTES chemokine Differentiated macrophages are able to undergo chemotaxis towards an area of inflammation or infection. To test for chemotaxis ability, the β -gal and TIF1 β antisense-ribozyme stable cell lines were differentiated with PMA, placed on top of transwell membranes and tested for the ability to migrate to the lower chambers containing the chemoattractant chemokine, RANTES. As seen in Figure 3D, although the β -gal antisense-ribozyme stable cell lines retained the ability to undergo chemotaxis, both of the TIF1 β antisense-ribozyme stable cell lines were completely blocked.

Induction of chemokine mRNAs Activated macrophages exhibit a distinct pattern of cytokine and chemokine expression. These products serve to recruit other members of the immune system to combat infection and inflammation. Chemokine mRNA expression in the β -gal and TIF1 β antisense-ribozyme stable cell lines was assayed using a ribonuclease protection assay (RPA) (Fig. 4A). Whereas IP-10, MIP1 β , MIP1 α , MCP1, and IL8 mRNA levels are induced upon PMA treatment of the β -gal antisense-ribozyme stable U937 cell line, there was a complete failure in the TIF1 β antisense-ribozyme stable U937 cell lines to induce these cytokines.

TIF1 β functions as a coactivator of C/EBP β in U937 cells

C/EBP β is an important transcriptional activator that is induced during PMA treatment of U937 cells and activation of primary macrophages (Akira and Kishimoto 1992; Natsuka et al. 1992). Many of the macrophage functions assayed in this work depend on genes whose

Figure 2. (A) Cell cycle analysis of ribozyme-expressing U937 cell lines. BrdU/PI FACS analysis of representative β -gal and TIF1 β antisense-ribozyme stable cell lines. Untreated (left column) and 24 h PMA-treated (right column) cells of each clone were analyzed. Propidium iodide is on the horizontal axis, and BrdU incorporation is on the vertical axis. (B) Expression of macrophage cell surface proteins in stable U937 cell lines. Individual stable transfected clones expressing β -gal or TIF1 β antisense-ribozymes were analyzed by FACS for expression of Mac1 (CD11b–CD18 heterodimer), CD11c, and CD14. Unstained cells (dashed line), untreated (normal line), and 24 h PMA-treated cells (bold line).

Figure 3. (A) Susceptibility of stable U937 cell lines to infection and killing by *L. pneumophila*. PMA-differentiated U937 stable cell clones were coincubated with the indicated number of *L. pneumophila* bacteria for 4 d, and then assayed for cell survival by the ability to reduce MTT. Each point represents three independent results. (B) Measurement of *L. pneumophila* uptake by stable U937 cell lines. The ability of U937 stable cell lines to phagocytose *L. pneumophila* was determined by assaying for the number of viable intracellular *L. pneumophila* bacteria after a 2 h coincubation of PMA-treated U937 stable cell lines with *L. pneumophila*. (C) Phagocytosis of FITC-labeled *S. aureus* bacteria. Cell clones expressing β -gal and TIF1 β antisense-ribosymes were analyzed for the ability to phagocytose FITC-labeled *S. aureus* bacteria. Results using untreated (normal line) and 24 h PMA-treated (bold line) are shown for each clone. (D) Chemotaxis. Shown are the number of cells/mL that have migrated through the transwell membrane towards the chemoattractant chemokine, RANTES, after 6 h.

transcription is known to be regulated by C/EBP β . For example, expression of *MIP1 α* (Matsumoto et al. 1998), *MCPI* (Yamamoto et al. 1999), and *IL-8* (Kunsch et al. 1994) are in part regulated by C/EBP β . C/EBP β also regulates genes encoding cell surface molecules CD14 (Fig. 3; Matsumoto et al. 1998) and CD16 (Fc γ RIII receptor) (Feinman et al. 1994). A previous report showed that TIF1 β and C/EBP β coimmunoprecipitate in the P388D1 macrophage cell line (Chang et al. 1998). In transient transfections of BHK cells, TIF1 β functions as a coactivator with C/EBP β of the alpha-1 acid glycoprotein (*AGP*) gene (Chang et al. 1998). Thus, we suspected that one mechanism by which TIF1 β may function during U937 differentiation is as a coactivator of C/EBP β .

To investigate this hypothesis, we performed cotransfections with C/EBP β and TIF1 β expression plasmids and C/EBP β -dependent luciferase reporters in U937 cells, followed by a 24-h PMA treatment. As seen in Figure 4B (bars 3–5), expression of C/EBP β alone resulted in a modest activation of the p8XC/EBP β -luc promoter (containing eight C/EBP β -responsive elements), and expression of TIF1 β alone had no effect. However, coexpression of TIF1 β with C/EBP β expression synergistically increased promoter activity (Fig. 4B, bars 6–8). Identical results were obtained using a natural C/EBP β -responsive promoter/reporter which contains –205 to +1 bp of the HIV LTR (Henderson et al. 1995). This region contains two C/EBP β binding sites, two NF- κ B sites, and three SP-1 sites. As seen in Figure 4C (bar 2), ectopic expression of C/EBP β in PMA-treated U937 cells alone activated the p–205 HIV LTR-luc promoter. Although TIF1 β alone had no effect (Fig. 4C, bars 3–5) on the p–205 HIV LTR-luc promoter, in combination with C/EBP β , it synergistically activated p–205 HIV LTR-luc promoter activity (Fig. 4C, bars 6–8). This increase was further shown to depend on C/EBP β binding sites, since no augmentation was observed in a promoter harboring mutant C/EBP β sites (Fig. 4D, bars 3–6). The promoter construct with mutated C/EBP β sites also shows that other transcription factors binding to the two intact NF- κ B-response elements and the three intact Sp1-response elements are unaffected by either C/EBP β or TIF1 β cotransfections, and suggests that the coactivator function of TIF1 β is specific to C/EBP β . Further evidence of the specificity of TIF1 β for C/EBP β is seen in the persistent augmentation of the reporter construct with both NF- κ B binding sites mutated (see Fig. 6C, lanes 7–9). Thus, TIF1 β specifically augments C/EBP β -dependent transcription in PMA-differentiated U937 cells in transient cotransfection experiments.

To determine whether endogenous C/EBP β transcriptional activity requires TIF1 β , we transfected the β -gal Rz, TIF1 β Rz1, and TIF1 β Rz2 stable cell lines with p8XC/EBP β -luc and p–205 HIV LTR-luc, followed by treatment with PMA. Transfection efficiency of the different cell clones was normalized using a cotransfected renilla luciferase plasmid. With both reporters, p8XC/EBP β -luc (Fig. 4E) and p–205 HIV LTR-luc (Fig. 4F), maximal activity was seen using 50 μ g of reporter when transfected into the β -gal Rz cell line (Fig. 4E,F, bar 4). In

contrast, little activation of the two reporters was seen in the TIF1 β Rz1 and TIF1 β Rz2 U937 cell clones lacking TIF1 β . Essentially no activation (or repression) was seen when undifferentiated U937 cells were used. Therefore, we conclude that TIF1 β augments the transcriptional activity of endogenous C/EBP β on reporter constructs in differentiating U937 cells.

TIF1 β and C/EBP β associate in vivo and in vitro

TIF1 β and C/EBP β cooperate to induce transcription. To determine whether they physically associate (directly or indirectly), we used total cellular extracts from U937 cells treated with PMA for 24 h in coimmunoprecipitation studies using antibodies against TIF1 β and C/EBP β . As seen in Figure 5A, whole cell extracts were immunoprecipitated with either preimmune (lane 1) or an antibody directed against C/EBP β (lane 2). When separated by PAGE and immunoblotted with an antibody directed against TIF1 β , a band of the size of TIF1 β (109 kD) was found in anti-C/EBP β immunoprecipitated samples. In a reciprocal experiment, anti-TIF1 β immunoprecipitated samples, but not preimmune immunoprecipitated, were shown by Western analysis to have coimmunoprecipitated C/EBP β , which appears as a doublet of ~48 kD (Fig. 5A, lanes 3 and 4).

Another test for the association of TIF1 β and C/EBP β was performed by incubating PMA-stimulated U937 cell extracts with immobilized GST-C/EBP β or GST-TIF1 β . After extensive washes, retained proteins were analyzed by immunoblotting. Endogenous TIF1 β associated with GST-C/EBP β (Fig. 5B, lane 1). Similarly, as seen in lane 2, endogenous C/EBP β associated with GST-TIF1 β . Thus, endogenous C/EBP β and TIF1 β physically associate (directly or indirectly) both in vivo and in vitro.

TIF1 β and C/EBP β associate on C/EBP binding sites

Since TIF1 β augments C/EBP β -dependent transcription, we reasoned that a complex of the two proteins might bind to C/EBP β binding sites. To test this hypothesis, we performed EMSAs using two different C/EBP β binding site oligonucleotides: one from the HIV LTR and another from the *AGP* promoter, together with nuclear extracts from U937 cells untreated or treated with PMA for various times (Fig. 6A). A protein–DNA complex appeared at 2 h and reached a maximum at 24 h. Cold competitions (Fig. 5B,C) established the specificity of this complex. Furthermore, antisera to TIF1 β as well as that to C/EBP β supershifted the complex (Fig. 6, lanes 8 and 9). Since TIF1 β alone cannot bind DNA, this shows that a C/EBP β /TIF1 β complex is bound to the DNA. In contrast, control mouse antisera, or antisera specific to C/EBP α (not present in U937 cells) (Henderson et al. 1995) had no effect on binding (Fig. 6, lanes 6 and 7). Identical results were obtained using nuclear extracts from the promyelocytic cell line HL-60 (data not shown).

(legend on facing page)

Figure 5. TIF1 β and C/EBP β associate. (A) Whole-cell extracts from 24 h PMA-treated U937 cells were immunoprecipitated with preimmune (PI) (lanes 1,3), anti-C/EBP β (lane 2) or anti-TIF1 β (lane 4). Western analysis was then performed using anti-TIF1 β (lane 2) or anti-C/EBP β (lane 4) antibodies. (B) Whole-cell extracts from 24 h PMA-treated U937 cells were incubated with prewashed GST-C/EBP β (lane 1), GST-TIF1 β (lane 2), or GST alone (lanes 3,4). After extensive washing, the protein complexes were subjected to SDS-PAGE and immunoblotted with anti-TIF1 β antibody (lanes 1,3) or anti-C/EBP β antibody (lanes 2,4).

TIF1 β is not sufficient for myeloid differentiation of the myelocytic cell line U937

To determine whether ectopic expression of TIF1 β alone is sufficient to cause differentiation of U937 cells, TIF1 β

was overexpressed in U937 cells using bicistronic retroviruses. Cells were transduced with a retrovirus encoding the cDNAs for TIF1 β and yellow fluorescent protein (YFP) or a control expression only YFP (Fig. 7A). They were untreated, or treated with suboptimal amounts of PMA, and allowed to differentiate for 24 h. TIF1 β alone did not cause myeloid differentiation, based on cell morphology, and FACS staining of surface Mac-1 and CD11c (Fig. 7B). TIF1 β was also unable to augment differentiation of transduced cells treated with suboptimal doses of PMA. We conclude that TIF1 β is necessary, but not sufficient, for the differentiation of the myelocytic cell line U937.

Discussion

Identification of TIF1 β as necessary for myeloid differentiation

Our data provide the first indication that TIF1 β mRNA is induced upon monocyte activation and that it plays a role in macrophage differentiation. Steady-state levels of TIF1 β mRNA are significantly increased when HL-60 cells differentiate in response to PMA or DMSO treatment. Similarly, TIF1 β is expressed when the slightly more mature line, U937, differentiates into macrophages in response to PMA treatment. TIF1 β mRNA is also found in peripheral blood monocytes, and levels increase following treatment with GM-CSF and M-CSF. These data clearly demonstrate that TIF1 β mRNA is induced during activation and differentiation of promyelocytic cells lines and primary monocytes.

Expression of antisense-hammerhead ribozymes was used to ablate TIF1 β mRNA in U937 cells. Cell lines lacking TIF1 β mRNA were unable to differentiate in response to PMA treatment, thus establishing a requirement for TIF1 β in U937 differentiation. However, TIF1 β is apparently not sufficient for U937 differentiation, based on the inability of ectopic TIF1 β expression to induce or augment PMA-dependent differentiation. Due to technical limitations, we have not been able to express TIF1 β and C/EBP β together in U937 cells, so we do not know if this would be sufficient to drive their differentiation. However, based on the results from the U937 system and the expression and induction of TIF1 β mRNA observed in PBMs, it is reasonable to suggest that TIF1 β is required for macrophage differentiation *in vivo*.

Figure 4. (A) Chemokine mRNA levels. Ribonuclease protection assay (RPA) was performed using probes specific for the mRNAs encoding chemokines RANTES, IP10, MIP1 β , MIP1 α , MCP1, IL8, and for the control L32 and GAPDH transcripts. Total RNA samples were used from cells treated with PMA for the indicated times. (B) Cotransfection of C/EBP β and TIF1 β expression plasmids with the 8XC/EBP β -luciferase reporter plasmid into U937 cells followed by a 24 h PMA differentiation. (C) Cotransfection of C/EBP β and TIF1 β expression plasmids with the -205 HIV-LTR-luciferase reporter plasmid in U937 cells followed by a 24 h PMA differentiation. (D) Cotransfection of C/EBP β and TIF1 β expression plasmids with -205 HIV-LTR-luciferase reporter plasmids with mutations in the two C/EBP β binding sites (-205 HIV LTR m2m3) or in the two NF- κ B binding sites (-205 HIV LTR mkB) in U937 cells followed by a 24 h PMA differentiation. The absolute luciferase values were 250,000 light units with C/EBP β alone, and 1.5×10^6 light units with both TIF1 β and C/EBP β cotransfected. (E) Transfections of U937 stable cell lines expressing β -gal and TIF1 β antisense-ribozymes with increasing amounts of the 8XC/EBP β -luciferase reporter plasmid followed by a 24 h PMA differentiation. (F) Transfections of U937 stable cell lines expressing β -gal and TIF1 β antisense-ribozymes with increasing amounts of the -205 HIV-LTR-luciferase reporter plasmid followed by a 24 h PMA differentiation.

Figure 6. Detection of C/EBP β - and TIF1 β -containing complexes in EMSA analyses. (A) Nuclear extracts were prepared from untreated and PMA-treated U937 cells and used in EMSA analyses with oligonucleotides containing C/EBP β binding sites from the α 1-acid glycoprotein (*AGP*) promoter and the HIV LTR. (B) The shifted complex is sequence-specific and contains C/EBP β , TIF1 β , but not C/EBP α . The protein/DNA complex is competed with excess unlabeled wild-type (lanes 2,3), but not mutated (lanes 3,4), *AGP* oligonucleotide. EMSAs were intentionally overexposed to reveal the supershifts. Supershifted complexes are seen using antibodies against C/EBP β (lane 8) and TIF1 β (lane 9), but not with preimmune (lanes 6,10) or an antibody against C/EBP α (lane 7). (C) The experiment was identical to that shown in (B), but used the HIV LTR oligonucleotide.

Formal proof of this awaits analysis of mice lacking TIF1 β in their monocytes.

Myeloid differentiation is accompanied by cell cycle arrest, and other screens for myeloid-specific genes have identified such cell cycle genes as the cdk inhibitors p21 (Liu et al. 1996) and p27 (Freedman 1999). However, of

the 50 cDNAs cloned, only one that encoded a protein associated with cell cycle arrest, CLN3, was cloned. We also isolated two cDNAs that encode proteins associated with cell cycle progression: CYCLIN1 and CDC2/CDK1. This apparent discrepancy is probably due to differences in the times when the cloning was performed. Unlike

Figure 7. Ectopic expression of TIF1 β in U937 cells. (A) Retroviral constructs used to transduce U937 cells. An IRES (internal ribosomal entry site) ensures that TIF1 β and YFP are coexpressed from a bicistronic mRNA message. (B) Effect of ectopic expression of TIF1 β on myeloid cell surface markers Mac1 and CD11c. U937 cells were transduced with pGC-IRES-YFP (*top* table) or pGC-TIF1 β -IRES-YFP (*bottom* table) induced for 24 h with optimal and suboptimal amounts of PMA. YFP-positive cells (expressing TIF1 β) were assayed for levels of Mac1 and CD11c by FACS using PE-conjugated antibodies.

other studies where cDNAs were isolated toward the end of differentiation, our study isolated cDNAs expressed very early. It is known that U937 cells (and possibly HL-60 cells) undergo an early burst in proliferation before the onset of cell cycle arrest (Rots et al. 1999).

Functional aspects of TIF1 β during myeloid development

We have shown that TIF1 β functions as a coactivator for C/EBP β . Expression of C/EBP β -responsive promoters was augmented by TIF1 β . Either mutation of the C/EBP β

binding sites or ablation of TIF1 β by antisense-ribosomes eliminated this augmentation. Further, we showed by two methods, coimmunoprecipitation and GST-pull downs, that endogenous TIF1 β and C/EBP β physically associate (directly or indirectly). EMSA experiments showed that an inducible complex that binds to C/EBP β response elements can be supershifted with antibodies to TIF1 β and C/EBP β . We conclude that TIF1 β associates with (directly or indirectly) and functions as a transcriptional coactivator of C/EBP β .

However, this does not rule out other possible mechanisms by which TIF1 β may function in myeloid cells.

TIF1 β binds to and acts as a corepressor for Kruppel-associated box (KRAB) domain-containing proteins (Friedman et al. 1996; Moosmann et al. 1996; Agata et al. 1999). The KRAB domain is a conserved motif at the N-termini of proteins that contain multiple Kruppel-class (C₂H₂) zinc fingers in their carboxyl termini (Bellefroid et al. 1991). Approximately 700 human genes encode C₂H₂ zinc finger proteins (Klug and Schwabe 1995), and one-third of these contain KRAB domains (Bellefroid et al. 1991). Because consensus binding sequences are not currently known for any of the KRAB domain proteins, their putative targets are unknown. However, the differential expression of several KRAB domain-containing zinc finger proteins during myeloid differentiation suggests they could be important in this developmental process. If so, another function for TIF1 β might be to modulate their function (Bellefroid et al. 1991).

Additionally, TIF1 β may also be involved in remodeling chromatin structure to alter gene expression during macrophage differentiation. It was originally isolated based on its ability to associate with the mouse homologue of the *Drosophila* heterochromatinic protein 1 alpha (HP1 α) (Le Douarin et al. 1996). HP1 α is a nonhistone chromosomal protein that exerts dose-dependent effects on heterochromatin-mediated gene silencing (Eisenberg et al. 1995; Elgin 1996) and shares a conserved N-terminal domain with *Drosophila* Pc, a repressor of homeotic gene expression (Koonin et al. 1995). TIF1 β colocalizes with HP1 α in heterochromatin, and also with HP1 γ , present in euchromatin (Ryan et al. 1999). TIF1 β contains a bromo domain, and in yeast bromo domain-containing proteins associate with chromatin remodeling complexes (Aasland et al. 1995; Jeanmougin et al. 1997 and references therein), consistent with a role for TIF1 β in determining chromatin structure. Also, TIF1 β activity can be blocked by trichostatin A, an inhibitor of histone deacetylases (Nielsen et al. 1999).

Although all three known activities of TIF1 β may be involved in macrophage differentiation, we have provided evidence of its ability to function as a coactivator of C/EBP β . C/EBP β is known to be a key transcriptional activator that is induced during macrophage activation and differentiation (Natsui et al. 1992; Akira and Kishimoto 1996; Akira 1997). Indeed, mice lacking C/EBP β have defective macrophage function (Tanaka et al. 1995; Poli 1998), showing the critical importance of C/EBP β in macrophage biology. Our data show that TIF1 β augments C/EBP β -dependent transcription in U937 cells from both natural and artificial promoters. Furthermore, we have shown that induction of endogenous chemokine mRNAs known to depend on C/EBP β , such as MIP1 α (Matsumoto et al. 1998), MCP1 (Yamamoto et al. 1999), and IL8 (Kunsch et al. 1994) is ablated in U937 lines lacking TIF1 β . An exception is the chemokine RANTES, which has been shown to be regulated by C/EBP β in dengue-2-virus-infected human liver cells (Lin et al. 2000) and by respiratory syncytial virus (RSV)-infected airway epithelial cells (Casola et al. 2001), but whose expression in our hands is unaffected by the loss of TIF1 β . This discrepancy may be due to tissue-specific

differences in RANTES expression levels. We have shown that expression of CD14, also known to depend on C/EBP β (Matsumoto et al. 1998), is inhibited in cells lacking TIF1 β . Finally, antisera specific for C/EBP β and TIF1 β produce supershifts in EMSA experiments using oligonucleotides containing C/EBP β binding sites and nuclear extracts from differentiated U937 cells. Thus, our data provide strong evidence that one function of TIF1 β during U937 differentiation is to be a coactivator for C/EBP β , augmenting C/EBP β -dependent transcription.

Materials and methods

Representational difference analysis

RDA was performed as described previously (Hubank and Schatz 1994). HL-60 cells, untreated or treated with PMA for 2 h, were used to prepare total RNA by the Guanidinium/CsCl method (Ausubel et al. 1994). Poly A⁺ mRNA was selected using Oligotex beads (Qiagen), and cDNA was synthesized using Superscript RT (GIBCO BRL). Two rounds of RDA were performed.

Plasmids

-20 $\times$ Δ LTR-luciferase, m κ B LTR-luciferase, and mC2,3 LTR-luciferase have been described previously (Henderson et al. 1995). pEVA10E4 (called p8XCARE luciferase in the study) was described previously (Artandi et al. 1994). pMSCV-NF-IL6-IRES-GFP was a gift from A.J. Henderson (Veterinary Science, Pennsylvania State University, University Park).

Antisense-hammerhead ribozymes and construction of stable U937 cell lines

Modeling of TIF1 β mRNA secondary structure was performed using mfold v.3.0 at the Washington University server address (<http://mfold1.wustl.edu/~mfold/rna/form11.cgi>). For TIF1 β Rz1, the antisense region corresponding from 907 to 1002 (relative to the transcriptional start site) was amplified, incorporating a three-nucleotide helix I hammerhead ribozyme (Tabler et al. 1994), using the primers 5'-GCTCTAGAGCGGGTGAAGTACACC-3' and 5'-CCCCCAAGCTTATTCCTGATGAGGCCTCGAGGCCGAATGCTTGTGTACGTTG-3'. For TIF1 β Rz2, the antisense region corresponding from 1442 to 1537 was amplified using the primers 5'-GCTCTAGACTTTTGCTTTC TAAGA-3' and 5'-CCCCCAAGCTTAGGACTGATGAGGCCTAGAGGCCGAAGTGAAGCTTCATCTC-3'. For β -gal Rz, the antisense region corresponding from 1220 to 1315 was amplified using the primers 5'-GCTCTAGAATGAAGCCAATAT TGA-3' and 5'-CCCCCAAGCTTAGCTCTGATGAGGCCTCGAGGCCGAATTCGCGTTACGCGTT-3'.

PCR reactions were carried out in an MJ thermocycler using as the template a plasmid containing full-length TIF1 β cDNA (a kind gift from W. Schaffner, Institute für Molekularbiologie II der Universität Zürich, Zürich, Switzerland) or β -gal (pcDNAHygro(+)/lacZ) (Invitrogen). Amplified products were digested with *Xba*I and *Hind*III and ligated into the pcDNAHygro(+)(Invitrogen) driven by the CMV promoter. Polymerase fidelity was verified by DNA sequencing.

Stable, Hygromycin^r U937 cell lines containing the pcDNA-antisense-ribozyme constructs were produced by electroporation and cloned by limiting dilution. HL-60 and U937 cells (ob-

tained from ATCC) were cultured in RPMI-1640 media supplemented with 10% fetal calf serum and 50 μ g/mL gentamicin. Cells were induced using 25 nM PMA or 1.25% DMSO.

Isolation of human PMNs

Blood from normal healthy human volunteers was collected and pooled and used to isolate PMNs as described (Denholm and Wolber 1991). Cell viability (>95%) was assessed by trypan blue exclusion. The purity of the monocytes (80%) was determined by differential counts of Wright's stained cytocentrifuge preparations. PMNs were treated for 24 h with (20 ng/mL) GM-CSF and (50 ng/mL) M-CSF (both from R&D Systems).

Northern analysis

Northern analysis was performed as described previously (Ausubel et al. 1994). Total RNA (20 μ g) was blotted onto Hybond-X Nylon membranes (Amersham), and hybridizations were performed using random primed probes (>10⁸ cpm/ μ g) according to the manufacturer's instructions. Northern analysis for antisense-ribozyme expression employed a riboprobe corresponding to the hammerhead ribozyme.

Flow cytometry

Cells were washed with ice-cold PBS containing 0.5% BSA, preincubated for 60 min with mouse whole Ig to block Fc receptors, washed, and incubated with fluorochrome-conjugated monoclonal antibody for 60 min. Cells were stained with 7-AAD to facilitate selection of the viable cell population and analyzed on a Becton-Dickinson FACScan using CellQuest software. The monoclonal antibodies used were R-phycoerythrin (PE)-conjugated anti-CD11c, R-phycoerythrin (PE)-conjugated anti-CD14 (both from Pharmingen), and R-phycoerythrin (PE)-conjugated anti-Mac1 (Boehringer Mannheim). BrdU/PI staining was done using an in situ cell proliferation labeling kit (Boehringer Mannheim).

Infection of U937 cells with *L. pneumophila*

The *L. pneumophila* strain used in this study was the wild-type Philadelphia-1 strain. Growth conditions were as described previously (Horwitz and Silvers 1980). U937 cells were used as host cells as described (Marra et al. 1990). Dose [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT] reduction assays to quantitate cytotoxicity of bacteria were performed as described (Marra et al. 1990). Uptake of *L. pneumophila* was done as described (Moore and Lambert 1986).

Phagocytosis

Heat-killed *Staphylococcus aureus* (ATCC) were labeled with 0.01% fluorescein isothiocyanate (FITC) isomer I (Sigma), then sonicated, and opsonized in an equal volume of human serum with mixing for 30 min at 37°C. Next, 10⁸ FITC-labeled *S. aureus* were added to 10⁶ U937 cells in 10 mL RPMI-1640 supplemented with 10% human serum and allowed to incubate at 37°C for 3 h. Cells were washed three times in PBS with 0.5% BSA. Fluorescence by extracellular bacteria was quenched by adding trypan blue (final concentration of 30 μ g/mL) for 5 min, and then analyzed on a Becton-Dickinson FACScan.

Chemotaxis assay

Cell migration assays were performed as previously described (Bleul et al. 1996). Briefly, 15 $\times$ 10⁴ U937 cells in 150 μ L of

RPMI-1640 medium containing 0.25% human serum transmigrated through 5 μ m pore-size bare filter Transwell inserts (Costar) for 6 h. Migrated cells were counted using a hemacytometer. Chemotaxis was performed in the presence of optimized ligand concentrations of RANTES (75 ng/mL; R&D Systems) present in the lower chamber.

Ribonuclease protection assay

The ribonuclease protection assay was performed using a kit and templates from Pharmingen and carried out following the instructions of the manufacturer using 20 μ g of total RNA. Protected probe fragments were analyzed by 5% denaturing PAGE.

Transient transfections and reporter assays

U937 transient transfections and luciferase assays were performed as described (Hemperson et al. 1996). In all experiments, luciferase activity was normalized to the amount of protein used in the assay. In transfections for ribozyme-expressing stable cell lines, luciferase activity was normalized to renilla luciferase activity (1 μ g pTK-*renilla*/transfection).

Preparation of whole-cell extracts, immunoprecipitations, and Western blotting

Whole-cell extracts from U937 cells, coimmunoprecipitations, and Westerns were performed as described (Chang et al. 1998). GST pull-down experiments using GST-TIF1 β , GST-C/EBP β , and GST with whole-cell extracts were done as described (Chang et al. 1998).

Retroviral transduction of U937 cells

The full-length TIF1 β cDNA was subcloned into the pGC-IRES-YFP retroviral plasmid (Costa et al. 2000). Infection retroviral supernatant was generated as described previously (Chang et al. 2000). Concentrated VSV-G pseudotyped virus containing pGC-IRES-YFP or pGC-TIF1 β -IRES-YFP was produced and used to infect U937 cells using protocols from G. Nolan et al. (<http://www.stanford.edu/group/nolan/NL-phnrx.html>).

EMSA

Preparation of nuclear extracts and EMSAs were performed as described (Rooney et al. 1994). Antibodies against C/EBP α were from Santa Cruz Biotechnology. Antisera against TIF1 β and C/EBP β was a kind gift from Sheng-Chung Lee (Academia Sinica, College of Medicine, National Taiwan University, Taipei, Taiwan). Five microliter binding reactions were preincubated for 30 min at room temperature with 1 μ L (2 μ g) of antibody.

Oligonucleotides used: HIV (-178 to -159) C/EBPwt top, 5'-GATCGCTAGCATTTTCATCACACGT-3'; HIV (-178 to -159) C/EBPwt consensus bottom, 3'-CGGATCGTAAAGTAGTGTGCACTAG-5'; HIV (-178 to -159) C/EBPmt consensus top, 5'-GATCGCTAGCtgcaggggACACGT-3'; HIV (-178 to -159) C/EBPmt consensus bottom, 3'-CGGATCGacgtccccTGTGCACTAG-5'; AGP wt top (-92 to -70), 5'-GATCGCTGGTGA GATTGTGCCACAGCT-3'; AGP wt bottom (-92 to -70), 3'-CGACCACTCTAACACGGTGTGCTGACTAG-5'; AGP mt top (-92 to -70), 5'-GATCGCTGGTGAacagctgGCCACAGCT-3'; AGP mt bottom (-92 to -70), 3'-CGACCACTgtcgacCGGTGTGCTGACTAG-5'.

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RETRACTED

Retraction

Genes & Development 15: 3023–3038 (2001).

TIF1 β functions as a coactivator for C/EBP β and is required for induced differentiation in the myelomonocytic cell line U937

John W. Rooney and Kathryn L. Calame

“Following publication of this paper, I was informed by a colleague at another institution that experiments performed by Dr. Rooney, which are illustrated in Figure 1, could not be reproduced. Researchers in my laboratory performed the experiments again and found that TIF1 β mRNA is not induced upon treatment of U937 cells with PMA or upon treatment of HL-60 cells with PMA or DMSO. TIF1 β mRNA is expressed at similar levels before and after treatment in both lines. Given the central importance of this observation, I wish to retract the paper and Dr. Rooney agrees to this retraction.”

Kathryn Calame

TIF1 β functions as a coactivator for C/EBP β and is required for induced differentiation in the myelomonocytic cell line U937

John W. Rooney and Kathryn L. Calame

Genes Dev. 2001, **15**:

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[Genes Dev. August , 2002 16: 2170](#)

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