v-Myb suppresses phorbol ester- and modifies retinoic acid-induced differentiation of human promonocytic U937 cells

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The c-*myb* protooncogene as well as its transforming derivate, the v-*myb* oncogene code for transcription factors. They regulate transcription of specific target genes thus controling proliferation, differentiation and apoptosis of hematopoietic cells. Up-regulation of the c-*myb* expression or rearrangement/amplification of the *myb* locus are often involved in leukemogenesis. Enforced *myb* expression blocks differentiation of various leukemic cell lines. Human promonocytes U937 can be induced to differentiate to monocyte/macrophage-like cells using phorbol esters or to granulocytes using retinoic acid. In order to investigate transforming capability of v-*myb*, we expressed the v-*myb* oncogene of avian myeloblastosis virus in U937 cells. We found that v-Myb efficiently suppressed formation of macrophages upon treatment with phorbol ester. Some features of granulocytic differentiation of retinoic acid-treated U937 cells were affected by the v-Myb protein as well. These results document that v-Myb is significantly involved in control of myeloid differentiation.

Key words: v-Myb, U937, differentiation, macrophage, phorbol ester, retinoic acid

The c-*myb* gene plays a critical role in development of hematopoiesis in vertebrates [1]. It is generally expressed in immature, proliferating hematopoietic precursors including erythroid, myeloid, and lymphoid lineages [2, 3], and its expression declines when these cells differentiate to mature forms [4]. Deregulated c-*myb* expression interferes with differentiation into monocyte/macrophage-like cells [5–7], granulocytes [8-10] and erythrocytes [3, 11–13]. Several lines of evidence indicate that c-Myb plays a role in leukemogenesis. Insertional activation of the mouse c-*myb* gene results in myeloid leukemias [14, 15]. Up-regulation of the c-*myb* expression or rearrangement/amplification of the *myb* locus was found nearly in all human leukemias and lymphomas [16–19], and in many other human tumors [20, 21].

There are structurally altered forms of c-*myb*, the v-*myb* oncogenes that have been transduced by two acutely transforming avian retroviruses: avian myeloblastosis virus (AMV), and avian leukemia virus E26. The v-*myb* of AMV causes acute leukemia in animals and transforms hematopoietic cells in culture [22, 23]. The v-*myb*-transformed monoblasts BM2 can be induced to terminal differentiation by various stimuli, including treatment with phorbol ester TPA [24, 25], trichostatin A [26], okadaic acid [27] or by overexpression of certain genes, such as retinoic acid receptors RAR/RXR [28, 29], c-*jun* [30], and *p53* [31]. Irrespectively of the stimulus specifity, the v-*myb* transformed monoblasts followed only the monocyte/macrophage differentiation pathway.

In order to evaluate the effects of v-Myb in regulation of myeloid differentiation, we expressed the AMV v-*myb* in bipotent human promonocytes U937. These cells can differentiate along monocytic pathway in response to TPA or granulocytic pathway upon treatment with retinoic acid [32–36]. We found that v-Myb blocks the TPA-induced differentiation of U937 cells to monocyte/macrophage-like cells, and partially affects the RA-induced differentiation to granulocytes allowing development of some but not all features of granulopoiesis.

Materials and methods

Cell cultivation and transfection. U937 human histiocytic lymphoma cells [37] were cultured in RPMI-1640 medium, HEPES modification (Sigma), supplemented with 10% heat-

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inactivated fetal calf serum (Invitrogen), 2 mM L-glutamine and antibiotics (100 μg/ml streptomycin, 100 U/ml penicilin) (Sigma) in a humidified incubator at 37°C in a controlled 5% CO_2 atmosphere. To prepare U937 cells ectopically expressing v-*myb*, we cotransfected the cells with pMTvMYBCD4 [38] (5.4 μg) and pSV2neo [39] (0.6 μg) plasmids using a Lipofectamine 2000 Transfection reagent (Invitrogen) according to the manufacturer's instructions. The neomycin analog G418 (Gibco) was added to cultivation media at a final concentration of 400 μg/ml 24 h after transfection. Stable G418-resistant transfectants were selected within 3 weeks and cloned by limiting dilution. In order to induce expression of v-*myb* from the metallothionein promoter, we used $ZnCl_2$. 100 μM concentration of $ZnCl_2$ was used in experiments that did not require incubation period longer than 12 hours, otherwise we used 70 μM $ZnCl_2$. Phorbol ester TPA (Sigma) and all-*trans* retinoic acid (RA, Sigma) were used as differentiation inducers. TPA was diluted in dimethyl sulfoxide (DMSO) and used at a final concentration of 50 ng/ml, RA was diluted in ethanol used at final concentration of 1 or 5 μM.

Cytocentrifugation and staining. Cells (2×10^5) were washed with 1 × phosphate buffer saline (PBS), resuspended in 200 μl 1×PBS, and spread onto glass slides by cytocentrifugation for 5 min/500 g. Air-dried samples were fixed with methanol and stained using modified Wright-Giemsa stain Diff-Quik (Dade Behring). Morphology of at least 200 cells was evaluated by light microscopy.

Analysis of nonspecific esterase (NSE) activity. NSE activity was determined using 10 μM α-naphthyl acetate (Sigma) by coupling the reaction product with Fast Blue BB salt (Sigma) at neutral pH for 30 min at 37°C. The activity was measured at 37°C for 30 min. Relative enzyme activity was assesed spectrophotometrically at 492 nm and 620 nm (reference value) after cell lysis.

Analysis of CD11b and CD15 expression by flow cytometry. Expression of cell surface differentiation markers CD11b and CD15 was determined by flow cytometry. The cells (5×10^5) were washed twice with 1× PBS, resuspended in 50 μl 1×PBS containing 1% (w/v) BSA (PBSA) and incubated either with the CD11b-specific monoclonal antibody (C0551, Sigma, 10 μg/ml) or with CD15-specific antibody conjugated with FITC (555401, BD Pharmingen) at 4°C for 30 min. Cells were rinsed twice in ice-cold PBSA. For analysis of CD11b, the cells were incubated with secondary FITC-conjugated antibody at 4°C for 30 min and rinsed twice in PBSA. Next, the cells were resuspended in 600 μl PBSA and analyzed by flow cytometry using FC500 Cytomics (Beckman Coulter). The amount of fluorescence emitted by at least 20.000 cells was analyzed in each sample.

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. SDS-PAGE and immunoblotting of protein lysates were performed as described previousely [25]. Briefly, the cells were lysed by boiling in 2×CSB buffer containing 0.1 M Tris (pH 6.8), 16% v/v glycerol, 3.2% w/v SDS, 10% v/v β–mercaptoethanol, and 0.005% w/v bromphenol blue for 5 min. Denatured proteins were resolved by 10% SDS-PAGE and electroblotted onto the nitrocellulose membrane (BioRad). Blots were blocked with 5% non-fat milk in TBS-Tween (Tris-buffered saline with 0.1% Tween 20) for at least 30 min, washed in TBS-Tween and probed with the Myb-specific monoclonal antibody kindly provided by J. Sleeman [40]. To control for sample loading, the blots were probed with the actin-specific polyclonal antibody (A5060, Sigma). The blots were incubated with either goat anti-mouse or anti-rabbit secondary antibody conjugated to alkaline phosphatase (Promega) and developed by standard procedure using 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium.

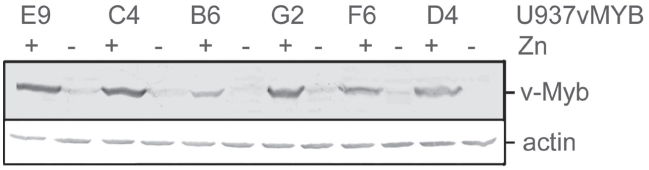

Figure 1. v-Myb is inducibly expressed in U937vMYB cells. Six independent clones of U937vMYB cells were treated with 100 μM $ZnCl_2$ or left untreated for 6 hours. Protein extracts were resolved by SDS-PAGE and analyzed by immunoblotting using the Myb-specific antibody (upper box) or the actin-specific antibody for control of sample loading (lower box).

Results

U937 cells were cotransfected with plasmid pMTvMYBCD4 [38] harbouring the v-*myb* oncogene under control of the human metallothionein II_A promoter, and the pSV2neo plasmid confering G418-resistance to transfected cells [39]. We selected six independent clones of stable transfectants U937vMYB inducibly expressing v-*myb* upon treatment with zinc ions as shown by SDS-PAGE and immunoblotting (Fig. 1). The clones E9 and C4 were used in next experiments.

Like many other leukemic cell lines, U937 cells can be induced to differentiate to monocyte/macrophage-like cells by phorbol ester TPA. Upon treatment with 50 ng/ml TPA for 24 hours, the U937 cells aggregated and developed adherency. Large macrophage-like cells developed within 4 days. In contrast, the Zn/TPA-treated U937vMYB cells did not form flat macrophages but rather retained size and spherical shape of immature cells (Fig. 2A). The level of the Myb proteins in the same samples was determined by SDS-PAGE and immunoblotting. We found that endogenous c-Myb protein was produced in similar extent in untreated U937 and U937vMYB cells. This level of expression did not change significantly in U937 cells treated with Zn/TPA for 1 and 2 days, it droped dramatically in day 3 and disappeared in day 4 (Fig. 2B). In contrast, the v-Myb protein was produced in detectable level in U937vMYB cells even in day 4 of treatment with Zn/TPA. Interestingly, expression of endogenous c-*myb* was silenced in

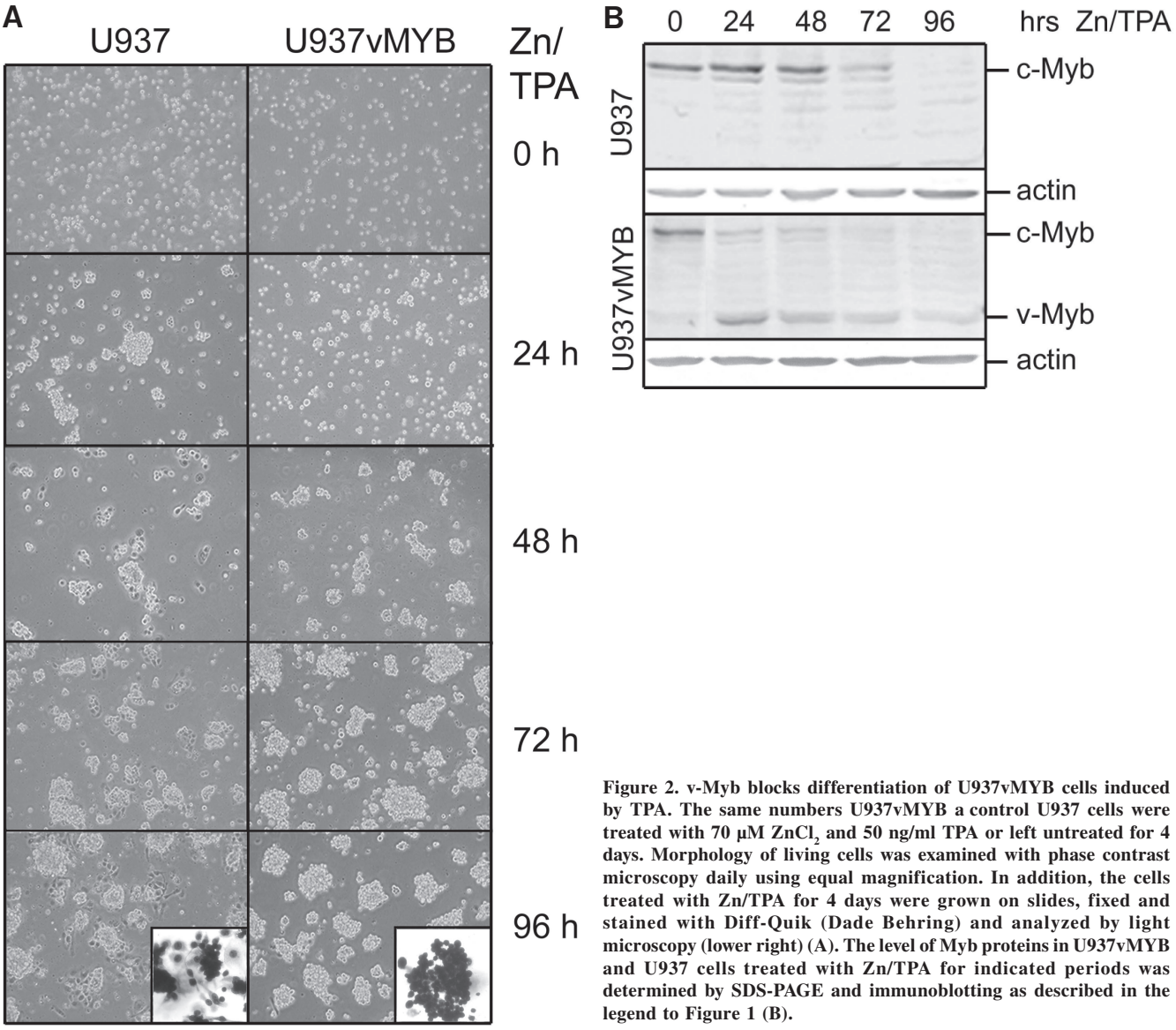

Figure 2. v-Myb blocks differentiation of U937vMYB cells induced by TPA. The same numbers U937vMYB a control U937 cells were treated with 70 μM $ZnCl_2$ and 50 ng/ml TPA or left untreated for 4 days. Morphology of living cells was examined with phase contrast microscopy daily using equal magnification. In addition, the cells treated with Zn/TPA for 4 days were grown on slides, fixed and stained with Diff-Quik (Dade Behring) and analyzed by light microscopy (lower right) (A). The level of Myb proteins in U937vMYB and U937 cells treated with Zn/TPA for indicated periods was determined by SDS-PAGE and immunoblotting as described in the legend to Figure 1 (B).

v-*myb*-expressing U937vMYB cells, suggesting that v-Myb suppresses production of c-Myb (Fig. 2B).

In order to evaluate differentiation-inducing effect of TPA on U937 and U937vMYB cells in more detail, we assessed expression of CD11b and activity of nonspecific esterases. The CD11b cell surface protein marks myeloid differentiation [41]. The cells treated with Zn/TPA for 4 days were stained with the CD11b-specific antibody and analyzed by flow cytometry. Expression of CD11b increased in both U937 and U937vMYB cells upon Zn/TPA treatment (Fig. 3). Interestingly, frequency of the CD11b-positive U937vMYB cells treated with Zn/TPA (24%) was lower than frequency of the CD11b-positive control U937 cells (62%). This difference was statistically significant ($P<0.05$).

Increased activity of nonspecific esterases also marks maturing monocytes [42]. We estimated activity of the nonspecific esterases in U937 and U937vMYB cells upon treatment Zn/TPA for 4 days by spectrometry. Activity of nonspecific esterases in Zn/TPA-treated U937 cells was almost two-fold higher than in untreated cells (Fig. 4). In contrast to U937, the nonspecific esterase activity in U937vMYB cells did not increased significantly upon treatment with Zn/TPA ($P<0.05$). These results indicate that v-Myb interferes with TPA-induced differentiation of U937 cells.

Maturation of RA-treated U937 cells along granulocytic pathway is characterized by nuclear lobulation and reduction of cell size [35,43]. We exposed U937 and U937vMYB cells

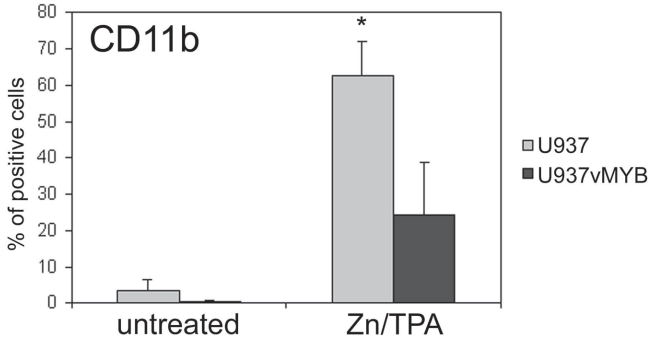

Figure 3. v-Myb blocks expression of CD11b in TPA-treated U937vMYB cells. U937vMYB and U937 cells were treated with 70 μM $ZnCl_2$ and 50 ng/ml TPA or left untreated for 4 days. Expression of CD11b was analysed by flow cytometry. The graph summarizes results of three independent experiments. The columns represent frequency of CD11b-expressing cells. Error bars indicate standard deviations, * represents statistically significant difference of U937vMYB from control U937 cells (P<0.05).

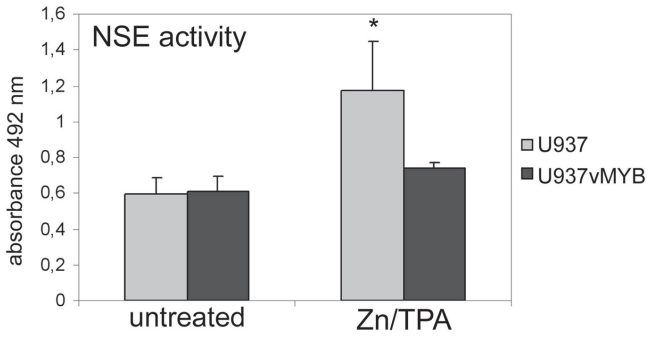

Figure 4. v-Myb suppresses activity of nonspecific esterases (NSE) in TPA-treated U937vMYB cells. Cells were treated as described in the legend to Figure 3. The NSE activity was measured by spectrophotometry. The columns represent the average absorbance (492 nm) from three independent experiments. Error bars indicate standard deviations, * represents statistically significant difference of U937vMYB from control U937 cells (P<0.05).

to 1 μM RA in the presence of $ZnCl_2$ (70 μM) for 7 days and evaluated frequency of lobulated nuclei by visual inspection of cytocentrifuged and stained samples by light microscopy. However, we did not find any significant difference in cell size and frequency of nuclear lobulation in U937vMYB and U937 controls (Fig. 5).

In order to address the effect of v-Myb on RA-induced granulocytic differentiation, we analyzed expression of the cell surface proteins CD11b and CD15. Expression of these antigens increases as cells mature along granulocytic pathway. U937vMYB and control U937 cells were treated with Zn/RA for 4 days, stained with either CD11b- or CD15-specific antibody and analyzed by flow cytometry. Indeed, frequency of the CD11b positivity increased from 2% in untreated U937 cells to more than 28% upon treatment with Zn/RA (Fig. 6). However, this increase of the CD11b-positive cell fraction was efficiently suppressed in similarly treated U937vMYB cells. Frequency of the CD11b-positive U937vMYB cells treated with Zn/RA did not exceed 3%. This result suggests that v-Myb suppresses RA-induced granulocytic differentiation of U937 cells.

However, analysis of the CD15 protein, which is also widely used as a marker granulocytic differentiation, provided different results. Frequency of U937 cells expressing CD15 slightly increased from 30% to about 38% upon treatment with Zn/RA (Fig. 7). In contrast, frequency of CD15-positive U937vMYB cells increased from 28% in the absence of Zn/RA to about 56% in the presence of Zn/RA (Fig. 7). The difference between Zn/RA-treated U937vMYB and control U937 cells was statistically significant (P<0.05). Interestingly, endogenous c-Myb protein was produced constitutively in U937 and U937vMYB cells both in the presence and absence of Zn/RA as documented by SDS-PAGE and immunoblotting (Fig. 8). The level of the c-Myb protein in

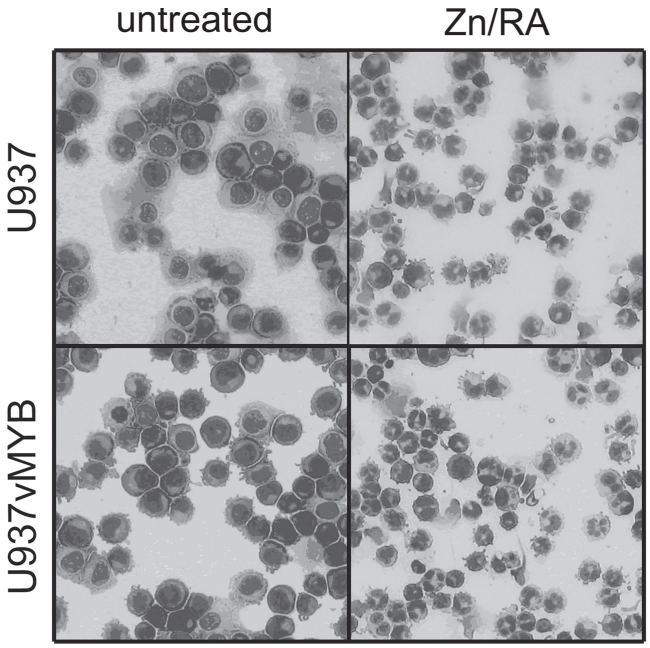

Figure 5. v-Myb does not affect morphology of U937vMYB cells treated with retinoic acid (RA). The same number of U937vMYB and U937 cells was treated with 70 μM $ZnCl_2$ and 1 μM RA or left untreated for 7 days. The cells were cytocentrifuged, fixed and stained with Diff-Quik (Dade Behring) and analyzed by light microscopy. Representative fields taken under equal magnification are shown.

Zn/RA-treated cells remained the same as in untreated cells and did not drop even after 10 days of treatment with 5 μM RA (data not shown).

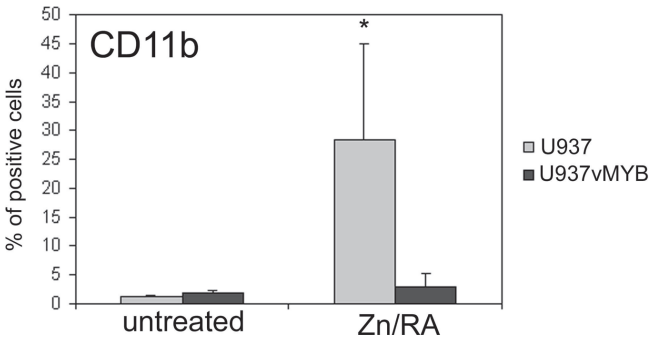

Figure 6. v-Myb blocks expression of CD11b in U937vMYB cells treated with RA. U937vMYB and U937 cells were treated with 70 µM $ZnCl_2$ and 1 µM RA or left untreated for 4 days. Frequency of cells expressing CD11b was determined by flow cytometry. The graph summarizes results of three independent experiments. Error bars represent standard deviations, * represents statistically significant difference of U937vMYB from control U937 cells (P<0.05).

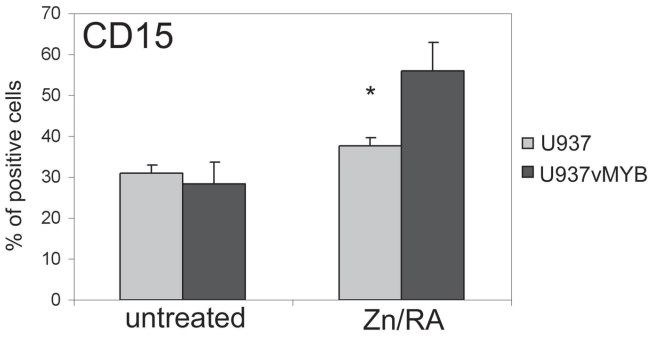

Figure 7. v-Myb stimulates expression of CD15 in U937vMYB cells treated with RA. The cells were cultured and treated similarly as described in the legend to Figure 6. Frequency of CD 15-positive cells was determined by flow cytometry. The graph summarizes results of three independent experiments. Error bars represent standard deviations, * represents statistically significant difference of U937vMYB from control U937 cells (P<0.05).

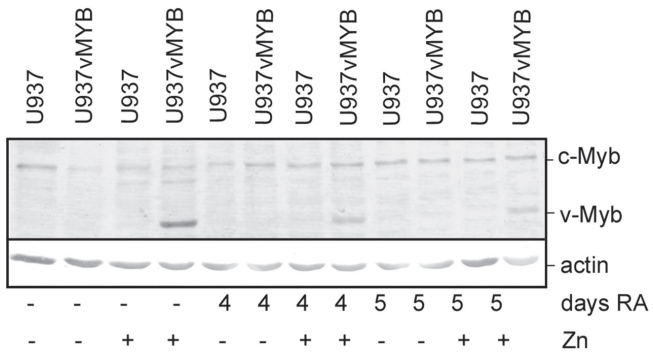

Figure 8. c-Myb expression in U937 and U937vMYB cells is not affected by RA. The cells were treated with 1 µM RA in the presence or absence of 70 µM $ZnCl_2$ or left untreated for 4 or 5 days. The level of Myb proteins was determined by SDS-PAGE and immunoblotting using the Myb-specific antibody. The same blots were probed with the actin-specific antibody to control for sample loading.

Discussion

The v-Myb oncoprotein of avian myeloblastosis virus induces monoblastic leukemia in chickens and transforms myelomonocytic cells *in vitro* [22, 23]. v-Myb is derived from c-Myb, the important regulator of hematopoiesis. There are several lines of evidence indicating the importance of the Myb proteins for development of hematopoietic system. First, the level of c-*myb* expression is high in immature hematopoietic cells of the lymphoid, erythroid and myeloid lineages and is down-regulated as these cells differentiate into mature forms [44–46]. Second, constitutive expression of c-*myb* can block differentiation of hematopoietic cells [3, 5, 8, 9, 11, 25]. Third, mice bearing a homozygous disruption of c-*myb* die *in utero* from failure of embryonal hematopoiesis [1]. Fourth, an amplification or rearrangement of the c-*myb* locus occurs frequently in human leukemias [19,47-49], thus defining new leukemia subtypes in some cases [50]. Interestingly, cells transformed by v-Myb do not contain the c-Myb protein. This raises a question of whether expression of c-Myb is incompatible with transformation by v-Myb. However, we showed earlier that enforced c-*myb* expression in v-*myb*-transformed BM2 monoblasts is compatible with cell proliferation, even increasing the resistance of cells to differentiation inducer TPA [25]. In this study, we used alternative strategy to study outcomes of simultaneous expression of v-*myb* and c-*myb* in leukemic cells. We ectopically expressed v-*myb* in human promonocytic U937 cells that produce endogenous c-Myb. In contrast to BM2, the U937 cells can be induced to differentiate not only to macrophage-like cells using phorbol ester but they can also follow granulocytic differentiation pathway upon treatment with RA. RA has been widely used in differentiation therapy of acute promyelocytic leukemia [51, 52].

Our study shows that v-Myb blocks TPA-induced differentiation of U937 cells to macrophage-like cells. Several differentiation markers, such as cell adherency, CD11b expression and non-specific esterase activity failed to fully develop in TPA-treated U937vMYB cells. This confirms our previous results documenting that co-expression of c-*myb* and v-*myb* in promonocytic cells inhibits TPA-controlled differentiation pathway [25]. While c-Myb was efficiently produced within 2 days, and still detectable in day 3 of treatment with Zn/TPA in U937 cells, the v-Myb protein was detectable in U937vMYB cells treated with Zn/TPA for 4 days. This indicates that ectopic v-*myb* expression possesses similar differentiation suppressing capabilities as constitutive expression of c-*myb* described by others [3, 5, 8, 9, 11].

Next, we wished to evaluate the effect of v-Myb on granulocytic differentiation of U937 cells induced by RA. However, we did not receive conclusive results. The v-*myb*-expression

did not interfere with formation of granulocyte-like shape of nuclei in U937vMYB cells. However, monitoring of differentiation-specific markers provided contradictory results: v-Myb blocked RA-induced increase of CD11b but at the same time it stimulated expression of CD15. The proliferation rate of U937 cells was higher in the presence of v-Myb than in the absence of this protein (data not shown). Similarly, c-Myb was found to stimulate proliferation of several myeloid cell lines [7, 8, 9, 53]. In contrast to TPA, RA did not affect expression of endogenous c-*myb* in U937 and U937vMYB cells suggesting that differentiation to granulocyte-like cells does not require silencing of c-*myb*. There are several reports documenting inhibitory effect of Myb proteins on granulocytic differentiation of 32Dcl3 cells [9, 10, 54]. On the other hand, the Myb protein was also found to at least partially stimulate granulocytic differentiation as documented for RA- and DMSO-treated HL-60 and G-CSF-treated 32Dcl3 cells [8, 9, 55]. Inhibition of c-Myb using antisense oligonucleotides eliminates granulocytic and allows only monocytic differentiation of HL-60 cells [55]. Expression of early markers of granulocytic differentiation is activated in 32Dcl3 cells constitutively expressing c-Myb [8,9] or the B-Myb, the other member of the Myb protein family [10].

We can conclude that v-Myb oncoprotein is significantly involved in control of both pathways of myeloid differentiation of U937 cells. It inhibits TPA-induced differentiation of these cells to monocyte/macrophage-like cells and partially affects RA-induced differentiation to granulocytes allowing development of some but not all features of granulopoiesis. c-Myb and v-Myb proteins can activate expression of different genes [56]. Actual balance of c-Myb and v-Myb proteins in individual U937vMYB cells can be the point that determines the expression level of granulocytic markers.

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