# Original Article

# Downregulation of *myc* Promoter-Binding Protein 1 (MBP-1) in Growth-Arrested Malignant B cells

(follicular lymphoma / transforming growth factor β1 / *myc*-binding protein 1 / two-dimensional gel electrophoresis / matrix-assisted laser desorption/ionisation time of flight mass spectrometry)

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**Abstract. Normal human B lymphocytes are sensitive to the growth-inhibitory action of TGF-β1 whereas malignant B lymphoma cells are mostly resistant to TGF-β1 effects. We have shown in our previous work that, TGF-β1 treatment resulted in significant growth inhibition of the DoHH2 cell line. In the present study we showed that TGF-β1-induced growth arrest was associated with notable downregulation of the *myc*-binding protein-1 (MBP-1). Moreover, our results indicated that c-Myc overexpression in TGF-β1-arrested malignant B cells is mediated by binding of MBP-1, as a transcription repressor, to the (+118/+153) element of the promoter region of the *myc* gene.**

## Introduction

The c-*myc* protooncogene in particular may have a critical role in the normal control of cell proliferation. The cells in which c-Myc expression is specifically prevented will not divide even in the presence of growth factors. Conversely, cells in which Myc expression is specifically switched on independently of growth factors cannot enter the G0 phase. If the cells are in G0 when the c-Myc protein is induced, they will leave G0 and begin to divide even in the absence of growth factors – a behaviour that ultimately causes them to undergo the programmed cell death (Alberts et al., 1994). Modulation of c-Myc expression is a common event in many malignant cell types (Erikson et al., 1983; Cole, 1986; Malliri et al., 1996) and it is abrogated by viral transforming proteins with pRb binding domains (Pietenpol et al., 1990).

The c-Myc protein contains a basic helix-loop-helix and leucine zipper motif that dimerizes with Max, and binds to 5'-CACGTG-3' or 5'-CATGCG-3' target sequences, known as the E box (Blackwell et al., 1990; Blackwood and Eisenman, 1991). DNA bound to the c-Myc-Max heterodimers activates transcription, whereas Max acts as a transcriptional repressor (Amati et al., 1993; Amin et al., 1993).

The human c-*myc* protooncogene contains two TATA box sequences separated by 165 base pairs, located near the 5' end of the first exon (Blackwell et al., 1990). Four promoters of c-*myc*, named P0, P1, P2, and P3, have been characterized, although in normal and transformed cells the majority of mRNAs initiates at the P2 promoter (Potter and Marcu, 1997), which contains binding sites for many known transcription factors including MBP-1 (Marcu et al., 1992).

*myc*-binding protein-1 (MBP-1) is a 37 kDa protein with 97% sequence homology to the 3' portion of the α-enolase gene (Giallongo et al., 1986). The product of this gene is a 48 kDa protein, which plays a critical role in the glycolytic pathway. The enzyme catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate and it is present in mammalian tissues as three isoforms, α-, β- and γ-enolase, that are encoded by three distinct genes (Giallongo et al., 1990,1993; Oliva et al., 1991). The presence of two single-base insertions in the *mbp-1* sequence results in a shift in the reading frame compared to the α-enolase coding region (Ray and Miller, 1991; Onyango et al., 1998). This reading frame shift causes the shorter form of α-enolase to be alternatively translated from the full-length α-enolase mRNA. Comparison of the amino acid sequence of α-enolase and MBP-1 indicated that the MBP-1 initiator methionine corresponds to a methionine at position 97 in α-enolase (Met-97). This methionine is encoded by the AUG co-

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Abbreviations: bp – base pairs, kDa – kilodaltons, MBP-1 – *myc*-binding protein 1, TGF-β1 – transforming growth factor β1.

don and it is conserved in both α-enolase isoforms isolated from different species, while it is absent in the β- and γ-enolase isoforms, although the three isoforms share more than 90% similarity at the amino acid level in the same species (Lebioda and Stec, 1991).

Recent studies have shown that MBP-1 and TATA-binding protein (TBP) bind simultaneously in the minor groove of the c-*myc* P2 promoter (Chaudhary and Miller, 1995), which suggests that MBP-1 may negatively regulate c-*myc* transcription by preventing the formation of the transcription initiation complex.

TGF-β1 has a profound effect on the growth of various mammalian cell types, including B lymphocytes (Bouchard et al., 1997). Our experimental DoHH2 cell line model was derived from a patient bearing a malignant non-Hodgkin's lymphoma (NHL) of follicular origin (Kluin-Nelemans et al. 1991). As published in our previous works (Djaborkhel et al., 2000; Tvrdík et al., 2002, 2006), the inhibitory action of TGF-β1 is mediated by deactivation of the cell-cycle machinery.

In this study we were interested in the effect of TGF-β1 treatment on the expression of proteins, particularly MBP-1 and c-Myc. The hypothesis (Feo et al., 2000) whether the alternatively translated product of the gene encoding α-enolase could be a potential candidate as a tumour suppressor is discussed.

## Material and Methods

### *Cell line*

The B-cell line DoHH2 derived from malignant centrocytic/centroblastic lymphoma (Kluin-Nelemans et al., 1991) was purchased from DSMZ (Braunschweig, Germany). The cells were cultured in RPMI 1640 (Sigma, St. Louis, MO) supplemented with 10% foetal bovine serum (Sigma), 80 μg/ml of gentamycin (Léčiva, Prague, Czech Republic) in a humidified incubator at 37°C with 5% $CO_2$. The cells were passaged every third day. At time zero, the cells ($0.5 \times 10^6$/ml) were reseeded into a fresh medium either in the presence of or in the absence of TGF-β1 (10 ng/ml, Sigma).

### *Protein isolation and sample preparation*

The cell pellets were resuspended in 350 μl of lysis/rehydration buffer containing 7 M urea, 2 M thiourea, 40 mM Tris-base, 1% (w/v) ASB-14 detergent, 2 mM tributylphosphine (TBP), 50 U DNase (Fermentas, Ontario, Canada) and 1x protease-inhibitor cocktail (Sigma). The cell extracts were incubated for 5 min at room temperature and protein concentration was measured by the Bradford method (Aushubel et al., 1992). The protein extracts were then purified by using ReadyPrep 2D Cleanup Kit (Bio-Rad, Hercules, CA), and the protein pellets were resuspended in 350 μl of rehydration buffer (8 M urea, 2% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulphonic acid), 50 mM dithiothreitol (DTT), 0.2% (w/v) Bio-Lyte 3/10 ampholyte (Bio-Rad) and 0.001% (w/v) bromphenol blue.

### *Two-dimensional gel electrophoresis*

Three hundred and fifty μl of lysis/rehydration buffer (see above) containing 100 μg of each protein sample were used for overnight rehydration of a 17-cm ReadyStrip IPG Strip (pH 3-10, Bio-Rad) at room temperature. Isoelectric focusing (IEF) was performed using the following voltage gradient: 0–250 V, 20 min; 250–10 000 V, 2.5 h; 10 000 V, 40 kVh (total 41.56 kVh). After IEF, the IPG strips were stored at -80°C until their use in the second dimension.

Prior to the second dimension the strips were equilibrated in a buffer containing 6 M urea, 2% (w/v) sodium dodecyl sulphate (SDS), 20% v/v glycerol, 375 mM Tris-HCl pH 8.8, reduced with 2% (w/v) DTT, and subsequently alkylated with 2.5% (w/v) iodoacetamide. After the equilibration, the proteins were separated on a 10% SDS-PAGE gel, 20 cm × 20 cm, at constant 30 mA. The run was completed when the bromphenol blue reached the bottom of the gel.

Following the electrophoresis, the gels were fixed, silver-stained, dried and scanned (Aushubel et al., 1992).

### *In-gel protein digestion*

Silver-stained protein spots of interest were excised from the 2D gels, cut into small pieces, and washed several times with a fresh 1 : 1 solution of 30 mM potassium ferricyanide and 100 mM sodium thiosulphate. Upon complete destaining, the gel pieces were washed with water, dehydrated in ACN (acetonitrile) and rehydrated in water again. The supernatant was removed and the gel was dried in a vacuum concentrator. The gel pieces were then reconstituted in a cleavage buffer containing 0.01% 2-mercaptoethanol, 0.1 M 4-ethylmorpholine acetate, 1 mM $CaCl_2$, 10% ACN and sequencing grade trypsin (50 ng/μl, Promega, Madison, WI). After overnight digestion, the resulting peptides were extracted with 40% ACN/0.5% TFA (trifluoracetic acid). For the low-abundance proteins, the peptides were purified and concentrated using C18 ZipTips (Millipore, Billerica, MA) prior to mass spectrometry (MS) analysis.

### *Matrix-assisted laser desorption/ionisation mass spectrometry (MALDI MS) and protein identification*

A solution of CHCA (α-cyano-4-hydroxy-trans-cinnamic acid – Bruker Daltonik, Billerica, MA) at 10 mg/ml in aqueous 30% ACN/30% MeOH/0.2% TFA was used as a MALDI matrix. A sample (1 μl) was deposited on a polished steel MALDI target plate, allowed to air-dry at room temperature, and 1 μl of the matrix solution was added. Positive ion mass spectra were measured in a MALDI-TOF/TOF mass spectrometer (Autoflex II, Bruker Daltonik) equipped with a 337 nm nitrogen la-

ser. The spectra were acquired in the mass range of 700–4500 Da in the reflectron mode and calibrated externally with a seven-point calibration using the Peptide calibration standard I (Bruker Daltonik). Mass spectrometric sequencing of the selected peptides was done by MALDI TOF (MS/MS) analysis on the same prepared samples with the same instrument operated in the "LIFT" mode.

For PMF database searching, peak lists in XML data format were created using the flexAnalysis 2.4 program (Bruker Daltonik) with the SNAP peak detection algorithm. Statistical calibration was included in the program and no smoothing was applied; the maximum number of assigned peaks was set at 50. After peak labelling, all known contaminant signals were manually removed. The peak lists were searched using the MASCOT search engine (Matrix Science, Boston, MA) against either the Swiss-Prot (Swiss Institute of Bioinformatics, Basel, Switzerland) or the NCBInr (National Center for Biotechnology Information, Bethesda, MD) database subsets of human proteins with the following search settings: peptide tolerance of 100 ppm, missed cleavage site value set to one, fixed carbamidomethylation of cysteine, and variable oxidation of methionine. No restrictions were applied on protein molecular weight (MW) and pI value. If the probability-based Mowse score was only slightly higher than the threshold value calculated for the set parameters or the sequence coverage was too low, identity of the protein candidate was confirmed by MS/MS peptide sequencing.

### *Western blotting*

The whole-cell protein extracts were prepared from $1 \times 10^7$ of DoHH2 cells. Cells were lysed in a lysis buffer (50 mM Tris-HCl pH 7.5, 250 mM NaCl, 0.1% Triton X-100, 5 mM EDTA, 50 mM NaF, 0.1 mM sodium orthovanadate, 0.1 mM PMSF (phenylmethylsulphonyl fluoride), 1 μg/ml leupeptin, 1 μg/ml aprotinin ) for 1 h at 4°C, then denatured with a 2×SDS sample buffer (Aushubel et al., 1992) at 100°C for 5 min and fractionated (50 μg of total protein per well) by electrophoresis in 12% SDS-polyacrylamide gel. The proteins were transferred onto nitrocellulose membrane (Bio-Rad) under semi-dry conditions. To block unspecific binding, the membranes were incubated in a blocking buffer (PBS containing 5% non-fat dry milk) supplemented with 0.1% Tween-20 at 4°C overnight, and incubated with primary antibody.

The mouse monoclonal antibody against c-Myc (dilution 1 : 25, Santa Cruz, CA) and the mouse monoclonal antibody against β-actin (dilution 1 : 2500, Sigma) were diluted in the blocking buffer and incubated for 1 h at room temperature. Finally, the membranes were incubated with anti-mouse IgG horseradish peroxidase-conjugated secondary antibody (dilution 1 : 2000 in the blocking buffer; Santa Cruz, CA) for 1 h at room temperature. After washing, the immune complexes were visualized using the Enhanced Chemiluminescence System (Amersham, Buckinghamshire, UK).

### *RNA isolation and one-step RT-PCR analysis*

The isolation of RNA and one-step RT-PCR analysis were performed by standard procedures described in our previous work (Tvrdík et al., 2006). The PCR conditions were as follows: one initial cycle of cDNA synthesis at 55°C for 30 min and denaturation at 94°C for 2 min, followed by 40 cycles of denaturation at 94°C for 15 s, annealing at 50 or 70°C (see Table I) for 30 s, and extension at 68°C for 1 min. The complete list of primer sets including their sequences, annealing temperatures, and PCR product sizes are presented in Table I.

The image analysis of the PCR product in each band was automatically integrated, adjusted in respect to background noise and expressed as pixel density units (PDU) by the Molecular Analyst Software (Bio-Rad). The semi-quantification was then performed by comparison of single PCR products with the amplification efficacy of glyceraldehyde-3-phosphate-dehydrogenase (G3PDH) mRNA as we published in our previous work (Djaborkhel et al., 2000). The results were expressed as the equation: PDU of specific PCR product in TGF-β1-treated cells / PDU of specific PCR product in control unstimulated cells. The equation was further multiplied by index 1.06 (PDU of the G3PDH signal in TGF-β1-treated cells / control unstimulated cells).

### *Gel mobility shift assay*

The nuclear extracts from the cells were prepared as described (Aushubel et al., 1992). Protein concentration in extracts was measured by the colorimetric method (Aushubel et al., 1992).

Sense and anti-sense oligonucleotides (see Table 1) were annealed to generate a double-stranded oligonucleotide probe, which was radiolabelled as described (Aushubel et al., 1992). The binding reaction mixture

*Table 1. List of primers used for RT-PCR analysis*

*Each primer was chosen to span exons. Specific annealing temperature ($T_a$) of each primer and the size of expected PCR products are listed below.*

| Gene | Primer | Sequence (5' to 3') | $T_a$ (°C) | PCR product (in bp) |
|---|---|---|---|---|
| *G3PDH* | sense | AGG GGT CTA CAT GGC AAC TG | 55 | 228 |
| *G3PDH* | antisense | CGA CCA CTT TGT CAA GCT CA | 55 | 228 |
| c-*myc* | sense | AGT GCA TCG ACC CCT CGG TGG TCT TCC CCT A | 70 | 551 |
| c-*myc* | antisense | CAG CTC GTT CTT CCT CTG GCG CTC CAA GAC GTT | 70 | 551 |

contained 2 µl of 5× binding buffer (20% glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 0.25 mg/ml poly(dI-dC)poly(dI-dC), 50 mM Tris-HCl pH 7.5), 2 µl of nuclear extract, and 1 µl of $^{32}$P-labelled oligonucleotide probe (20 000 cpm) in a final volume of 10 µl. The reaction mixture was incubated at room temperature for 20 min and analysed by 4% native polyacrylamide gel electrophoresis in 0.5× TBE buffer (10× : 890 mM Tris-HCl, 890 mM boric acid, 20 mM EDTA pH 8.0) and visualized by autoradiography. To determine specific DNA-binding proteins, we performed a supershift analysis. The nuclear extracts were incubated with 2 µl antibody either against α-enolase or against TBP (both Santa Cruz, CA) overnight at 4°C before the addition of the labelled probe. The α-enolase antibody recognizes both α-enolase and MBP-1 proteins (Subramanian and Miller, 2000).

### *Image analysis*

The gel images were processed using the open source GelScape software (Young et al., 2004) under the Linux platform. The X-ray films (FOMA Bohemia, Hradec Králové, Czech Republic) were scanned using an HP ScanJet 3800 (Hewlett-Packard, Palo Alto, CA), and processed using the Molecular Analyst Software (Bio-Rad) and GelScape software.

## Results

### *Two-dimensional protein maps of the control and TGF-β1 treated cells*

As we described in our previous studies (Djaborkhel et al., 2000; Tvrdik et al. 2006), the treatment with TGF-β1 (10 ng/ml) for 48 h led to a significant increase in the number of DoHH2 cells arrested in the G0/G1 phase. Therefore, we were keen to examine the expression pattern of proteins isolated from the control unstimulated and TGF-β1-treated cells.

In order to determine the plausible mechanism of TGF-β1-induced growth arrest in TGF-β1-sensitive malignant follicular lymphoma cells, we performed two-dimensional gel electrophoresis on IPG strips of the 3–10 pH range. After the comparative analysis of the silver-stained gels, we found 102 differences in expressions of proteins due to the treatment (Fig. 1A, B).

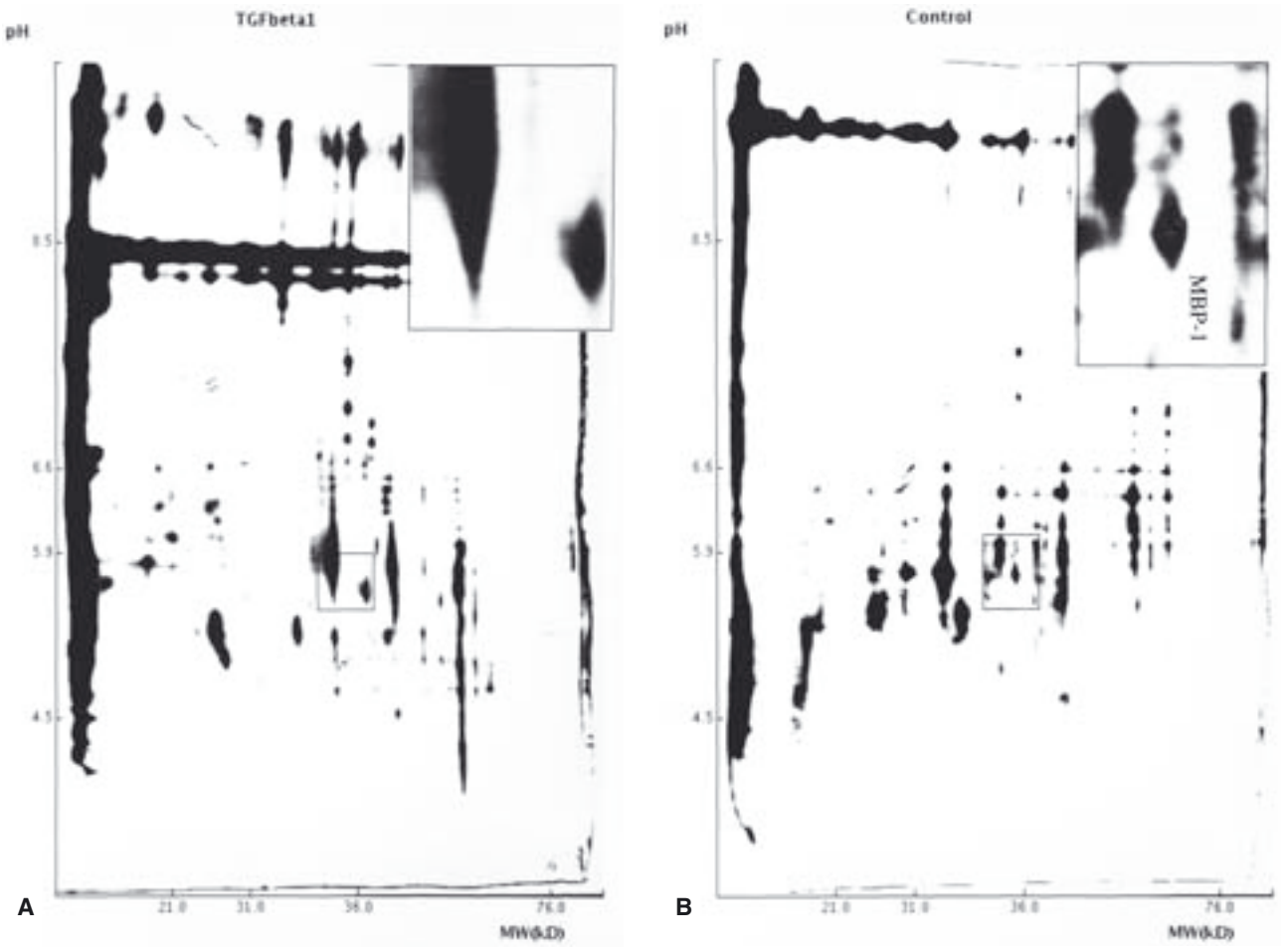


*Fig. 1.* Two-dimensional gel electrophoresis. DoHH2 cells were cultured in the presence (A) or absence (B) of TGF-β1. The 2D patterns were obtained after 2DE separation of a 100 mg protein sample. The protein spot labeled as MBP-1 was found only in control unstimulated cells.

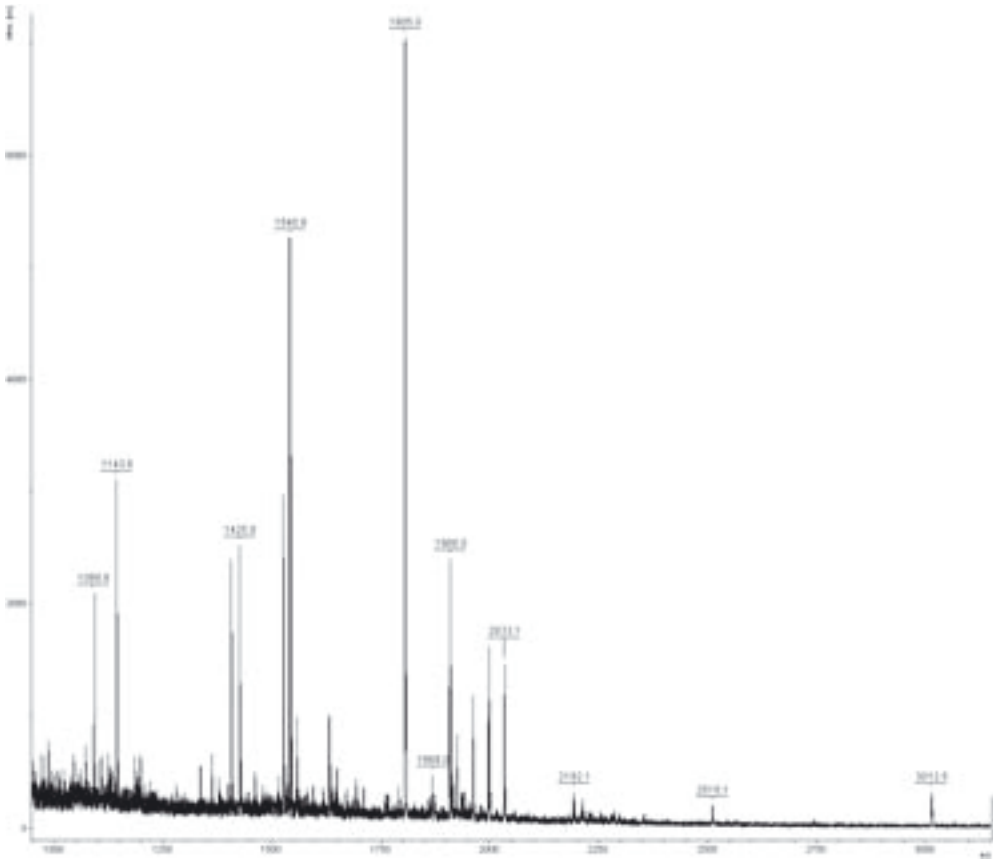

*Fig. 2.* MALDI-TOF mass spectrum of MBP-1. The protein spot was isolated from the silver-stained 2D gel and in-gel digested with trypsin. The mass of the resulting fragments was determined by MALDI-TOF spectrometry.

### *Identification of differentially expressed proteins*

We focused on identifying proteins that were present either only in control or only in TGF-β1-treated cells. Unfortunately, we were unable to identify many spots because of the fact that some higher intensity spots contained more than one protein and lower intensity spots were under the resolution of mass spectrometry, respectively. For this reason, we performed 2D electrophoresis using smaller or non-linear pH ranges and consequently, tryptic peptide mass fingerprint analysis by MALDI-TOF was used on Coomassie-stained gels (manuscript in preparation). However, we identified a spot of about 37 kDa and pI 6 which presented only in untreated control cells (Fig. 1A, B). The spot was cut off and the tryptic digested products were analysed by MALDI-TOF MS using peptide mass fingerprinting and database search (Fig. 2). The analysis identified a 37 kDa protein as the *myc*-binding protein-1 (MBP-1).

In conclusion, our results indicated that TGF-β1-induced growth arrest was associated with notable down-regulation of MBP-1.

### *Expression of c-Myc*

The *myc*-binding protein-1 is known as a negative regulator of c-*myc* transcription. To determine the plausible role of MBP-1 in the TGF-β1-induced growth arrest, we analysed the expression of c-Myc at both the protein and mRNA levels.

The immunoblot assay showed that the expression of c-Myc was significantly up-regulated at the protein level after TGF-β1 treatment (Fig. 3A). In contrast, the control western blot showed that the expression of β-actin was not modulated after the treatment (Fig. 3A).

Specific oligonucleotide primers that span exons were chosen for the amplification of the c-Myc transcript (see Table 1). In each case, G3PDH was used as an internal control for RT-PCR. Negative controls included reactions lacking RNA and reactions lacking reverse transcriptase (not shown).

In order to determine the correct quality of PCR amplification, we briefly analysed the presence of G3PDH in both TGF-β1-treated and control untreated cells. We found the presence of 228-base pair PCR product, corresponding to spliced mRNA of G3PDH, in equal quantities in both samples (Fig. 3B). Then we analysed the presence of the c-Myc transcript. Similar to the protein level, the mRNA expression of c-Myc was increased due to the TGF-β1 treatment (Fig. 3B).

The evaluation was performed by densitometric measurement of the signal intensity of the bands (Fig. 3B). The signal of the band corresponding to c-Myc mRNA in TGF-β1-treated cells was increased to ap-

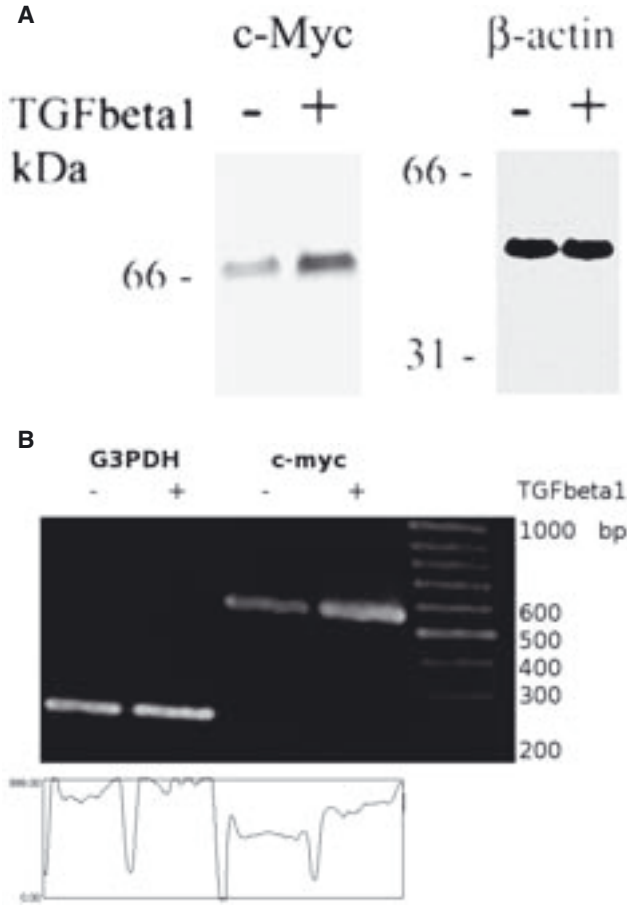


*Fig. 3.* The expression of the c-*myc* oncogene. The cells were cultured in the presence (+) or in the absence (–) of TGF-β1. The expression of c-Myc related to the TGF-β1 treatment was analysed by both immunobloting (A) and RT-PCR (B). Molecular weights of the markers are given in kilodaltons and base pairs, respectively. The semi-quantification was performed by densitometric measurement of the signal intensity of the bands (B).

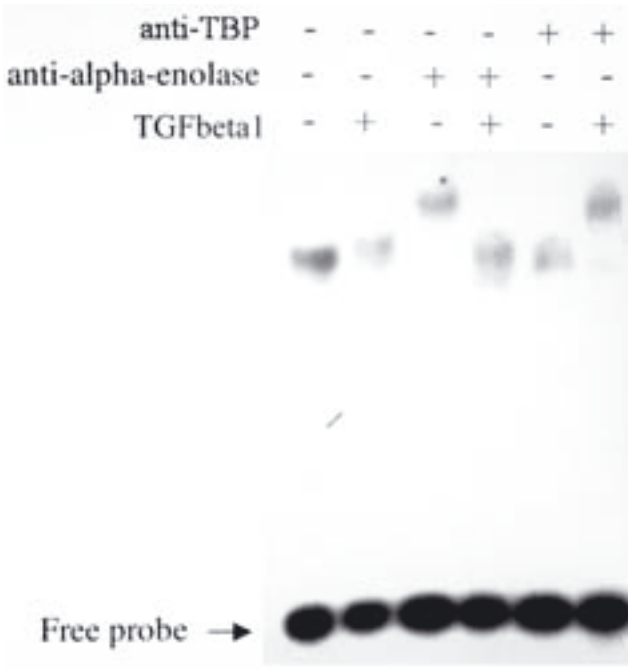


*Fig. 4.* The binding of MBP-1 and TBP to the c-*myc* promoter. The gel mobility shift assay was performed using the nuclear extract from cells cultured in the presence (+) or in the absence (-) of TGF-β1. The oligonucleotide probe which covers the (+118/+153) region of the c-*myc* promoter was used. The supershift experiments were performed by incubation with antibodies against either α-enolase or TBP before addition of the labelled probe. After addition of the antibody against α-enolase, the band was shifted up in unstimulated control cells, whereas the antibody against TBP supershifted the protein-DNA complex in TGF-β1-treated cells only.

proximately 52% of control values in control unstimulated cells: $0.801 \times 10^3$ and $0.520 \times 10^3$ PDU, respectively.

Our results showed that TGF-β1-induced growth arrest was associated with the overexpression of c-Myc.

## *The binding activity of MBP-1 to the promoter of the myc gene*

We further examined the binding activity of MBP-1 using a gel mobility shift assay (GMSA) in order to elucidate its role in c-*myc* up-regulation. For this purpose, an oligonucleotide probe was designed and synthetized covering the (+118/+153) region of the *myc* promoter. This MBP-1 binding region includes the TBP binding site. Sequences of the probe used for GMSA are summarized in Table 2.

We found a single band in both the control unstimulated cells and the TGF-β1-treated cells (Fig. 4). In order to identify the relevant protein corresponding to the shifted bands, we performed a supershift analysis. The nuclear extracts were incubated with antibodies against either α-enolase or TBP before addition of the labelled probe. After addition of the antibody against α-enolase, the band was shifted up in unstimulated control cells,

*Table 2. List of probes used for GMSA*

*The oligonucleotide probe, used for the gel mobility shift assay (GMSA), covers the (+118/+153) region of the myc P2 promoter.*

| Oligonucleotide name | Probe | Sequence (5' to 3') |
|---|---|---|
| Myc P2 | sense | AGG GAT CGC GCT GAG TAT AAA AGC CGG TTT TCG GGG |
| Myc P2 | antisense | CCC CGA AAA CCG GCT TTT ATA CTC AGC GCG ATC CCT |

whereas the antibody against TBP supershifted the protein-DNA complex in the TGF-β1-treated cells (Fig. 4).

In conclusion, we assume that the c-Myc overexpression in TGF-β1-arrested malignant B cells is mediated by the binding of MBP-1 as a negative regulator and TBP as a positive regulator to the (+118/+153) elements of the promoter region of the *myc* gene.

## Discussion

Transforming growth factor β 1 (TGF-β1), a multifunctional regulator of cell growth and differentiation, acts by interacting with specific cell surface receptors through a complex network of intracellular signalling cascades, which ultimately regulate gene transcription and the assembly and activation of the cell-cycle control system.

TGF-β1 has been shown to be stimulatory to the growth of mesenchymal cells in culture (Shipley et al., 1985), while it is a potent growth inhibitor of lymphoid, endothelial, and epithelial cells. In most epithelial cell types, TGF-β1 blocks the cell-cycle progression during the G1 phase in some of the cells due to the inhibition of cyclin D1 mRNA expression or cyclin A and cyclin E mRNA expression (Slingerland et al., 1994; Ko et al., 1995). TGF-β1-induced upregulation of $p21^{WAF1}$ was reported in human pancreatic carcinoma cell lines (Grau et al., 1997), while overexpression of $p27^{KIP1}$ was found in cervical squamous carcinoma cell lines during TGF-β1 treatment (Kim et al., 1998). In humans, TGF-β1 induces growth arrest and apoptosis in normal B cells and in Epstein-Barr virus (EBV)-negative Burkitt lymphoma cell lines (Chaouchi et al., 1995). Although the intracellular mechanisms leading to these pleiotropic effects of TGF-β1 remain poorly understood, there is evidence suggesting that the product of the c-*myc* protooncogene plays an important role in both the inhibitory and stimulatory responses to TGF-β1 (Alexandrow et al., 1995).

Our experimental model DoHH2 cell line was derived from a patient bearing a malignant non-Hodgkin's lymphoma (NHL) of the follicular subtype (Kluin-Nelemans et al., 1991). Similarly to normal B cells (Arvanitakis et al., 1995), the malignant clone retains its sensitivity to the TGF-β1 inhibitory action. As we have shown in our previous works (Djaborkhel et al., 2000; Tvrdík et al., 2006), TGF-β1 treatment resulted in significant growth inhibition of the DoHH2 cell line. Interestingly, TGF-β1-induced growth arrest is associated with a notably increased expression of the kinase inhibitor $p21^{WAF1}$ at both protein and mRNA levels. This downstream event possibly enhanced the stop before DoHH2 cells enter the S phase. The plausible mechanism of growth inhibition via activation of cyclin-dependent kinase (Cdk) inhibitors was described earlier (Tvrdík et al., 2002, 2006). We assumed that both early and late G1 phase kinase (Cdk4/6 and Cdk2) deactivation was primarily mediated by the induction of the CIP/KIP family of Cdk inhibitors and their binding to relevant Cdk complexes. The binding resulted in the dissociation of cyclin D-Cdk4/6 catalytic complexes due to its targeting by $p21^{WAF1}$ and in the deactivation of the cyclin E-Cdk2 complex due to the action of $p27^{KIP1}$.

In the present work, we were interested in studying the differences between the expression of proteins due to the TGF-β1 treatment. Surprisingly, the expression of c-Myc was slightly increased after the treatment. Although some authors (Munger et al., 1992) reported that TGF-β1 treatment resulted in a decrease of c-Myc RNA and protein levels coincident with G1 growth arrest in keratinocytes, another group of authors (Ushmorov et al., 2005) showed that c-*myc* activation did not accelerate the cell growth in mouse neuroblastoma but conversely decreased growth and led to a cell-cycle arrest in G2/M. p53-dependent G2 arrest by c-*myc* activation without induction of apoptosis has been described in fibroblasts (Felsher et al., 2000), and keratinocytes can also respond to c-Myc overexpression by arresting in G2 (Gandarillas et al., 2000). Moreover, c-Myc overexpression decreased clonogenic growth of NXS2 cells (Ushmorov et al., 2005). These findings may reflect the cell-cycle block induced by c-Myc.

In fact, the c-*myc* protooncogene is one of the most frequently activated oncogenes and it is estimated to be involved in 20% of all human cancers. It regulates transcription through several mechanisms, including recruitment of histone acetylases, chromatin-modulating proteins, basal transcriptional factors, and DNA methyltransferase (Dang et al., 2006). Although thousands of c-Myc responsive genes have been identified and a common view of the c-Myc function is regulating the cell-cycle progression, metabolism, ribosome biogenesis, and cell adhesion, the c-Myc target genes that distinguish the physiologic c-Myc function from the pathologic are currently not completely understood.

Four promoters of c-*myc*, named P0, P1, P2, and P3, have been characterized, although in normal and transformed cells about 80% of c-Myc mRNA is initiated from the P2 promoter (Potter and Marcu, 1997).

A cDNA clone from human cervical carcinoma (HeLa) cells was identified as a *myc*-promoter binding protein 1 (MBP-1) by screening an expression library (Ray and Miller, 1991). MBP-1 is a 37 kDa protein, alternatively translated from the full-length α-enolase mRNA (Feo et al., 2000). α-enolase is the glycolytic enzyme that catalyzes the formation of phosphoenolpyruvate from 2-phosphoglycerate, the second of the two high-energy intermediates that generate ATP in glycolysis (Subramanian and Miller, 2000). The shorter form of α-enolase is able to bind the MBP-1 consensus sequence and to downregulate expression of the luciferase reporter gene under the control of the c-*myc* P2 promoter (Feo et al., 2000).

In our model system, TGF-β1 treatment notably reduced the expression of MBP-1 at the protein level.

Moreover, our data correlated well with the results of other authors (Chaudhary and Miller, 1995), who reported that MBP-1 and TBP bind simultaneously in the minor groove of the c-*myc* P2 promoter, suggesting that MBP-1 negatively regulates c-*myc* transcription by preventing the formation of a transcription initiation complex.

The gene encoding α-enolase maps a region of human chromosome 1 (1p35-p36) that is often deleted in several human malignancies, including neuroblastoma, melanoma, pheochromocytoma, and carcinomas of the breast, liver and colon (Weith et al., 1996). However, exogenous expression of MBP-1 in murine fibroblasts results in rapid cell death and DNA fragmentation (Ray and Steele, 1997) and, in addition to overexpression of MBP-1 in human breast carcinoma cells, results in reduced invasive property, loss of anchorage-independent growth, and suppression of tumour formation in athymic nude mice (Ray et al., 1989, 1995). All of these findings supported the hypothesis that MBP-1, transcriptional repressor of the c-*myc* oncogene, may be a candidate for a tumour suppressor.

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