Triiodothyronine utilizes phosphatidylinositol 3-kinase pathway to activate anti-apoptotic myeloid cell leukemia-1

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Abstract

Triiodothyronine (T3) regulates apoptosis in cells according to their developmental stage, cell type, and pathophysiological state. The molecular mechanisms of this regulation, however, have been largely unknown. In this work, we show that the expression of the myeloid cell leukemia-1 (MCL-1) protein, an anti-apoptotic member of B-cell lymphoma-2 (BCL-2) family, increases in thyroid hormone receptor-expressing human kidney-2 (HK2) cells upon 6-h incubation in 100 nM T3; we also describe the molecular mechanisms leading to this phenomenon. Transcription regulation assays performed in human embryonic kidney (HEK) 293 cells show that 100 nM T3 increases transcription from the MCL-1 promoter twofold in the presence of thyroid hormone receptor β1, but not of its α1 isoform. However, this increase is not a result of direct activation via the thyroid hormone-response element, TRE-DR4, located at the −998 to −983 position in this promoter; furthermore, the presence of 9-cis-retinoic acid receptor is not required. The promoter’s activation is abolished in the presence of phosphatidylinositol 3-kinase (PI3-K) inhibitor, wortmannin. The −295 to −107 promoter fragment contains all sequences involved in T3-dependent activation of the MCL-1 promoter, and cAMP-responsive element located at the −262 to −255 position is a major mediator in this process. Therefore, MCL-1 expression is activated by T3, which increases its promoter activity by a non-genomic mechanism using the PI3-K signal transduction pathway. We propose that this is another mechanism by which T3 regulates apoptosis.

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Introduction

Triiodothyronine (T3) regulates cellular metabolism, proliferation, differentiation, as well as apoptosis (Puzianowska-Kuznicka et al. 2006, Tata 2007) using a variety of mechanisms. The genomic mechanism is mediated by nuclear receptors (TRs) (Oetting & Yen 2007), a ligand-dependent transcription factor that binds specific DNA sequences (thyroid hormone-responsive elements, TREs) present in the promoters of target genes (Harvey & Williams 2002). Four major TR isoforms TRz1, TRz2, TRβ1, and TRβ2, and a number of other isoforms, are encoded by the TRA and TRB genes (Gosden et al. 1986, Drabkin et al. 1988, Cheng 2000, Weiss & Ramos 2004). Upon binding to TRE, TRs either activate or inhibit the transcription of these genes; whether activation or inhibition occurs depends on the presence or absence of T3 (Eckey et al. 2003, Yen et al. 2006, Oetting & Yen 2007), and on their interaction with coactivators, with corepressors (Chen & Evans 1995, Onate et al. 1995, McKenna et al. 1999, Weiss & Ramos 2004), and with other proteins such as 9-cis-retinoic acid receptors (RXRs) (Rastinjead 2001, Szanto et al. 2004). The non-genomic mode of action of T3 is more complex and, in some cases, is mediated by extra-nuclear fraction of TRs. For example, transcription from mitochondrial DNA is regulated by T3 bound to the truncated form of TRz1—p43 (Casas et al. 1999). The interaction of liganded cytoplasmic fractions of TRs with the p85z subunit of phosphatidylinositol 3-kinase (PI3-K) induces the kinase activity and generation of phosphatidylinositol-3,4,5-triphosphate. Subsequently, Akt kinases are phosphorylated and they activate their downstream targets (Lei et al. 2004, Cao et al. 2005, Kuzman et al. 2005, Moeller et al. 2005, 2006, Kenessey & Ojamaa 2006, Storey et al. 2006, Verga Falzacappa et al. 2006, 2007).

Depending on the developmental stage of the cell, its pathophysiological state and its type, T3 either inhibits or activates apoptosis. For example, T3 serves as a survival factor for human pancreatic β cells (Verga Falzacappa et al. 2006), but stimulates apoptosis in rat hepatic cells (Upadhyay et al. 2004), optic lobe cells of the chick embryo (Ghorbel et al. 1997), and muscle cells of Xenopus laevis tadpole tail undergoing metamorphosis (Sachs et al. 1997). Molecular mechanisms of apoptosis regulation by T3 are obscure. In part, the hormone may act via the members of BCL-2 family of proteins, as T3 treatment increases the level of BCL2-associated X protein (Bax) mRNA in the optic lobe of...
the chick embryo (Ghorbel et al. 1997) and in the caudal muscle of Xenopus tadpole tail (Sachs et al. 1997), and results in mitochondrial depolarization, the increase of mitochondrial pro-apoptotic Bax and Bak and in the decrease of anti-apoptotic BCL-2 in Jurkat cells (Yehuda-Shnaidman et al. 2005).

Myeloid cell leukemia (MCL-1) is an anti-apoptotic member of the BCL-2 family (van Delft & Huang 2006). It resides in the outer mitochondrial membrane (Yang et al. 1996) where it binds and prevents pro-apoptotic BCL-2 antagonist killer (BAK) from oligomerization and the formation of the channels necessary for cytochrome C release and for the triggering of apoptosis (Nijhawan et al. 2003, Leu et al. 2004, Willis et al. 2005). MCL-1 interacts with pro-apoptotic BCL-2 homology 3 (BH3)-only BCL-2 family members BCL-2-interacting mediator of cell death (BIM) and truncated BH3 interacting domain death agonist (tBID), blocking their function (Clohessy et al. 2006, Han et al. 2006). Multiple interactions of MCL-1 with other BCL-2 family members, its short half-life time, rapid down-regulation in response to stress signals, and multiple levels of expression control indicate that MCL-1 plays an important role in the regulation of apoptosis (Nijhawan et al. 2003, Michels et al. 2005, Yang-Yen 2006).

In this work, the molecular mechanism by which T3 regulates the expression of one of the members of the BCL-2 family is presented. We provide evidence for the activation by T3 of the MCL-1 promoter via TRβ1-dependent activation of PI3-K signaling pathway, and its influence on the increase of MCL-1 protein.

Materials and methods

MCL-1 promoter cloning and sequence analysis

The 1826 bp human MCL-1 promoter fragment (fragment A) including its transcription start site (Townsend et al. 1999) was cloned from 200 ng human genomic DNA with Taq polymerase and 5'MCL1 5'-AATCCCGGATTGTCTCTAGCGACCTTG-3' and 3'MCL1 5'-ACGAAGCTTACTGGAGGGAACG-3' forward and reverse primers (SmaI and HindIII restriction sites in bold) respectively. After a 3-min initial denaturation at 94°C, 3 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 3 min, and then 37 cycles of 94°C for 30 s, 58°C for 1 min, and 72°C for 3 min were performed, followed by a final extension at 72°C for 10 min. The amplified DNA was cloned into the pGEM-T vector (Promega), restricted with SmaI and HindIII and recloned into the pGL2 luciferase reporter vector (Promega) to make the pGL2-MCL-1 reporter vector. The correctness of the promoter sequence was verified by sequencing. In silico search for putative binding sites for thyroid hormone receptors was performed with Transcription Element Search Software (TESS, Technical Report CBIL-TR-1997-1001-v0.0). The search was performed with a consensus TRE hexamer sequence (AGGTCA), with a maximum of one mismatch allowed.

MCL-1 promoter mutants

Deletion mutant E lacked 1515 bp from the 5' end of the pGL2-MCL-1 promoter (restriction with SacI and SmaI, blunting SacI end with Klenow, and re-ligation) and therefore, contained the −295 to +16 part of this promoter (pGL2-MCL-1E). Fragment EE (−107 to +16, pGL2-MCL-1EE) was generated by PCR with proofreading Platinum Pfx DNA polymerase (Invitrogen), with the pGL2-MCL-1 vector as a template, with 5'MCL1EE 5'-CATCCCGGCCCCTTTTATGG-GAATATTATTTT-3' and 3'MCL1 5'-ACGAAAGCTTACTGGAAGGAACGGAACG-3' forward and reverse primers (incorporated SmaI and HindIII restriction sites in bold) respectively. After a 3-min denaturation at 94°C, 35 cycles of 94°C for 30 s, 56°C for 30 s, and 68°C for 40 s were performed, and followed by a final extension at 68°C for 5 min. The PCR product was cloned into the pGL2-basic vector.

To obtain the pGL2-MCL-1TREmut reporter vector with mutated putative TRE, point mutagenesis was performed using the QuikChange multi site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA), strictly following the manufacturer’s instructions. The mutagenic primer TREmut 5'-AGGCCGAGACGG-CAAAAACTTGGAGGCATGAGTTC-3' was used in the reaction with the pGL2-MCL-1 vector as a template. PCR was carried out as follows: 95°C for 1 min, and 30 cycles of 95°C for 1 min, 55°C for 1 min, and 65°C for 15 min. The PCR product was treated with DpnI, and 1·5 μl of the reaction mix was used to transform XL1-Gold ultracompetent Escherichia coli cells. To obtain the pGL2-MCL-1Emut reporter vector containing the −295 to +16 MCL-1 promoter fragment with mutated putative cAMP-responsive element (CRE), another point mutagenesis was performed. The mutagenic primer CREmut 5'-CTCGGAGGACGCGCAAAAAAATCCCGGAGACGCGCACTCAG-3' was used in the reaction with the pGL2-MCL-1E vector as a template. The PCR was carried out as detailed above. The correctness of the mutated promoter fragments was verified by sequencing.

Cell culture and transcription regulation assay

HK2 cells were cultured in a 24-well dish in Dulbecco Modified Eagle’s (DME)/Ham’s medium (Sigma–Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (FBS), according to the protocol of Fraser et al. (2002), in a 37°C humidified incubator...
with 5% CO₂. Just before the experiment, cells were washed with PBS, and 0.5 ml serum-free BIO-MPM-1 multi-purpose serum-free medium for adherent cells (Biological Industries, Kibbutz Beit Haemek, Israel) supplemented with 2 mM glutamine were added. Cells were incubated for 6 h either in the presence or absence of 100 nM T₃. Following the incubation, cells were washed with PBS, and used for the production of whole-cell protein extracts.

HEK 293 cells were cultured in high glucose DMEM (Sigma–Aldrich) containing 10% FBS. Twenty-four hours before transfection, cells were seeded 1:2 onto a 24-well dish and maintained in DMEM supplemented with 10% FBS. Directly before transfection, cells were washed once with PBS and 0.5 ml serum-free BIO-MPM-1 medium supplemented with 2 mM glutamine were added to each well. Cells were transfected with metafectene (Biontex Laboratories Gmbh, Munich, Germany) according to the manufacturer’s directions with 1.5 µl metafectene, 150 ng pGL2-MCL-1 vector containing firefly luciferase reporter gene driven by different MCL-1 promoter fragments, 100 ng pEGFP-C1 expression vector (Clontech Laboratories) or the same vector encoding wild-type TRβ1 or TRα1, 100 ng pcDNA3.1(+) encoding wild-type human RXRα, and 20 ng phRL-CMV internal control vector (Promega). After 24 h incubation without T₃, or in the presence of 100 nM of this hormone, the cells were washed with PBS and lysed with 100 µl passive lysis buffer (Dual-Luciferase Reporter Assay System, Promega). Firefly and Renilla luciferase activities were measured in a microplate luminometer (BMG Labtech, Offenburg, Germany). All experiments were repeated six to nine times. Certain incubations were performed in the presence of the PI3-K inhibitor, wortmannin, according to the protocol of Wang et al. (2003) with the following modifications: after transfection was performed as described above, the cells were incubated for 3 h in BIO-MPM-1 serum-free medium, then wortmannin was added to a final concentration of 100 nM. T₃ was added 30 min later to a final concentration of 100 nM and the incubation lasted for an additional 24 h.

Apoptosis detection and flow cytometric analysis

HK2 cells were seeded onto a 60 mm Petri dish and cultured in DMEM supplemented with 10% heat-inactivated FBS. After 24 h, cells were washed with PBS and 3 ml DMEM without FBS were added. Cells were cultured for 6 h either in the presence of 100 nM T₃ or without this hormone. The detection of apoptotic cells was performed with ApoTarget Annexin-V FITC Apoptosis Kit (BioSource International, Inc., Camarillo, CA, USA) according to the manufacturer’s protocol. Cell viability was determined by propidium iodide exclusion. Cells were analyzed by flow cytometry using FACS Calibur (Becton Dickinson Biosciences, San Jose, CA, USA).

RNA isolation and semi-quantitative RT-PCR

HK2 cells were seeded onto a 60 mm Petri dish and cultured in DMEM supplemented with 10% heat-inactivated FBS. After 24 h, cells were washed with PBS and 3 ml DMEM without FBS were added. Cells were cultured for 6 h either in the presence of 100 nM T₃ or without this hormone, collected, and subjected to RNA isolation with TRIzol Reagent (Invitrogen) following the manufacturer’s protocol. About 250 ng of each total RNA were used as a template in RT-PCRs performed with SuperScript One-Step RT-PCR with Platinum Taq System (Invitrogen). Each reaction was supplemented with 40 U RNasin ribonuclease inhibitor (Promega). Initial RT-PCR was carried out with control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene primers GAPDH-U 5'CGCTGAGTACGCCTGGAGTC-3' and GAPDH-L 5'-GTCGCCATTGAGCATGGAC-3' (product size 278 bp) as follows: one cycle of 30 min at 50 °C and 2 min at 94 °C, 40 cycles of 94 °C for 20 s, 58 °C for 30 s, 68 °C for 1 min. Ten microliters of aliquots were removed from the reaction tube after 20, 25, 30, 35, and 40 cycles, and GAPDH product was analyzed onto a 2% agarose gel to ensure that the main reaction will be stopped while in the exponential growth phase. Next, a main RT-PCR was performed as above with GAPDH and MCL-1 primers (MCL-1F 5'-GCCATAATCCTCTTGCCACTTG-3' and MCL-1R 5'-GCCATAATCCTCTTGCCACTTG-3', product size 344 bp), and stopped after 30 amplification cycles. PCR products were resolved onto a 2% agarose gel.

Whole-cell protein extracts, nuclear protein extracts

To make a whole-cell protein extract, the cells (2 × 10⁶) were resuspended in 70 µl lysis buffer consisting of 75 mM Tris–HCl (pH 8.0), 2% SDS, 15% glycerol, and boiled for 5 min. The extracts were flash-frozen in liquid nitrogen and stored at −80 °C.

To obtain nuclear protein extract, the cells (5 × 10⁶) were resuspended in 500 µl buffer A consisting of 10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM MgCl₂, supplemented with a protease inhibitors mix (Complete Protease Inhibitor Cocktail, Roche Applied Science) and phenylmethylsulphonyl fluoride up to 400 µM, and incubated on ice for 1 h. After incubation, the cells were homogenized in an ice-cold glass–teflon homogenizer. The resulting homogenate was transferred to an Eppendorf tube and centrifuged at 400 g for 5 min at 4 °C. The pellet was resuspended in an equal volume of buffer A, centrifuged as before, and again resuspended...
in two volumes of buffer B consisting of 20 mM HEPES (pH 7-9), 10% glycerol, 420 mM NaCl, 1.5 mM MgCl2, and 0.2 mM EDTA, supplemented with protease inhibitors as above. After a 30-min incubation on ice, the sample was centrifuged at 15 000 g for 20 min at 4 °C, then the supernatant was transferred to a new tube, and supplemented with an equal volume of buffer C consisting of 20 mM HEPES (pH 7-9), 30% glycerol, 1.5 mM MgCl2, 0.2 mM EDTA, and protease inhibitors. The sample containing soluble nuclear proteins was aliquoted into pre-chilled Eppendorf tubes, flash-frozen in liquid nitrogen, and stored at −80 °C.

Immunoblot

Forty micrograms of whole-cell protein extract supplemented with β-mercaptoethanol up to 5% and with bromophenol blue up to 0.01% were boiled, and loaded onto a 10% polyacrylamide gel. Proteins were transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Inc.) and processed following the protocol supplied by the ECL kit manufacturer (Amersham Biosciences UK Limited). The membrane was probed with a mouse monoclonal anti-MCL-1 antibody (1:250, Becton Dickinson, Limited), a rabbit monoclonal anti-MCL-1 antibody (1:250, Becton Dickenson, Golden, CO, USA), a rabbit monoclonal anti-TRβ1 antibody (1:5000, Santa Cruz Biotechnology, Inc., Santa Cruz, CO, USA), and a mouse monoclonal anti-β-actin antibody (1:5000, Sigma–Aldrich). Following incubation and washes, a goat anti-rabbit or a goat anti-mouse horseradish peroxidase-conjugated polyclonal secondary antibodies (1:10 000; Calbiochem, San Diego, CA, USA) were applied. Specific bands were visualized by a chemiluminescent reaction performed with an ECL kit. When necessary, the amount of the specific protein was estimated from densitometry measurements after normalization against the β-actin band.

Electrophoretic mobility shift assay (EMSA)

RXRz1, TRz1, and TRβ1 proteins were overexpressed in reticulocyte lysates (T7 Quick Coupled TNT Reticulocyte Lysate System, Promega). One microgram of each pcDNA3.1(+) expression vector (Invitrogen) encoding these receptors were used as templates in 25 μl reactions. The reactions were performed according to the manufacturer’s instructions. EMSA was performed with the mix of all reticulocyte lysates (2 μl each) overexpressing the aforementioned receptors. The probe was made of the two primers that were hybridized to each other to make double-stranded DNA containing putative TRE-DR4 from the MCL-1 promoter: 5’DR4 5’-GAGACAGGCAAGTCACTTGGAGCCATGA3’ and 3’DR4 5’-CGAATCATGCGCCATGACCTGCT-3’. A mutated version of the putative TRE-DR4 was also made with 5’DR4mut 5’-GAGACAGGCCAAAACCTTGAGGCATGA3’ and 3’DR4mut 5’-CGAATCATGCGCCATGACCTGCT-3’. The probes were labeled by fill-in reaction with Klenow enzyme and [α-32P]dCTP. Reticulocyte lysates were incubated for 20 min at RT in a binding buffer containing 20 mM Tris–HCl (pH 7-5), 50 mM KCl, 2 mM DTT, 0.1% Triton X-100, 6% glycerol, in the presence of 250 ng dIdC, and 1 ng probe. To verify binding specificity, a 25-fold excess of the specific or non-specific competitor (5’-CCTGCGATCTATCGACAGATGATC3’) was added to certain samples. In the supershift experiments, 0.5 μg anti-TR antibody recognizing both TRz1 and TRβ1 isoforms (C4, Santa Cruz Biotechnology, Inc.) was added to the binding reaction. In this case, the mix of reticulocyte lysates, binding buffer, dIdC and antibody was incubated on ice for 50 min, then the probe was added and the incubation was extended for an additional 20 min at RT. The samples were then loaded onto a 5% native gel and electrophoresed at 150 V for 2-5 h at RT. The gel was dried and exposed against the film (Biomax MS, Eastman Kodak Company) for 24 h.

Results

**T3 increases the amount of MCL-1 protein**

T3 either inhibits or activates apoptosis depending on the type of cell. Therefore, the initial experiment was aimed at establishing whether T3 had an impact on the rate of apoptosis of HK2 cells (renal tubular epithelial cells) expressing endogenous TR proteins. The cells were incubated in a serum-free medium in the presence of 100 nM T3 or without this hormone. After the incubation, the percentage of apoptotic cells was determined using flow cytometry analysis. We found that in HK2 cells T3 acted as apoptosis-protective factor: after 48-h incubation in the presence of this hormone the frequency of apoptosis of cells was only 1%, while in the absence of this hormone it was 7.5% (Fig. 1A).

Next, to check if apoptosis-protective function of T3 in HK2 cells could be related to the increased amount of MCL-1 mRNA and protein, cells were incubated in a serum-free medium, in the presence of 100 nM T3 or in the absence of this hormone. Semi-quantitative RT-PCR revealed that 6 h of T3 treatment resulted in an approximately twofold increase of the amount of the MCL-1 mRNA compared with cells incubated without T3 (Fig. 1B). This was paralleled by twofold increase of the MCL-1 protein, while the amounts of TRz1 and of TRβ1 remained unaltered (Fig. 1C).
T₃ activates transcription from the MCL-1 promoter in the presence of TRβ₁, but not TRα₁

Having shown that T₃ treatment resulted in an increase of the MCL-1 protein amount, it became pertinent to determine the mechanism underlying this phenomenon. To establish whether this was a result of increased transcription from the MCL-1 promoter, transcription regulation assays were performed in HEK 293 cells containing biologically insignificant amounts of endogenous TRs (Turowska et al. 2007). The cells were transfected with the pGL2-MCL-1 reporter vector containing firefly luciferase reporter gene driven by the 1.82 kb MCL-1 promoter fragment (−1810 to +16 respective to the transcription start site (Townsend et al. 1999)), with expression vectors encoding wild-type TRα₁ or TRβ₁ fused to EGFP tag by their N-terminal ends, and with the expression vector encoding their heterodimerization partner, RXRa. Initially, it was demonstrated that EGFP-TR proteins were efficiently expressed in HEK 293 cells (Fig. 2A), that EGFP did not change their nuclear localization (Fig. 2B), and that the activity of the MCL-1 promoter did not change in the presence of 100 nM T₃ and pEGFP-C1 vector encoding EGFP protein (Fig. 2C). Transcription regulation assays performed in the presence of 100 nM T₃ showed that the activation of the MCL-1 promoter required the presence of TRβ₁, but not of TRα₁ (Fig. 2D). Under
our experimental conditions the MCL-1 promoter was activated twofold. This indicates that TRβ1, but not TRα1, is crucial for MCL-1 activation by T3.

MCL-1 promoter activation by T3 does not require binding to TRE

In silico analysis of the MCL-1 promoter sequence revealed the presence of putative TRE-DR4 (AGGTCActtgAGGCCA, the only mismatch in bold). To establish whether this sequence might mediate T3-dependent activation of the MCL-1 promoter, EMSA was performed with a probe identical to this putative TRE, and with reticulocyte lysate containing overexpressed TRα1, TRβ1, and RXRα. It revealed that the overexpressed receptors formed complexes with the probe. The complexes were specific, as demonstrated by the results of control experiments. The intensities of the specific bands decreased in the presence of the specific competitor and remained unaltered in the presence of non-specific competitor, the supershifted band was present in lanes containing samples supplemented with antibody recognizing both TRα1 and TRβ1, and there was no specific binding in the samples containing mutated MCL-1 TRE-DR4 probe (Fig. 3A).
Next, transcription regulation assays were performed with the reporter vectors containing either the wild-type 1.82 kb MCL-1 promoter fragment or the same promoter fragment with the first hexamer of the putative TRE-DR4 replaced by AAAAAA sequence (MCL-1TREmut). In the absence of T3, the activity of the wild-type 1.82 kb MCL-1 promoter fragment was the same as the activity of the wild-type 1.82 kb MCL-1 promoter fragment. In addition, in the presence of TRβ1 both promoters were activated twofold by 100 nM T3 (Fig. 3B). These data show that the MCL-1 promoter is activated by T3 in a mechanism independent of this TRE-DR4. No other TRE-like sequences were identified within the MCL-1 promoter.

**MCL-1 promoter activation by T3 does not require the presence of RXR**

RXRs are most important heterodimerization partners of TRs, enhancing their affinity for DNA. Since DNA binding by TRs is not necessary for the MCL-1 promoter activation by T3, we decided to determine if RXR molecule is needed for this activation. We performed transcription regulation assays with or without overexpressed RXRα. The sole overexpression of TRβ1 resulted in the activation of the MCL-1 promoter by T3 to the same extent, as in the presence of both TRβ1 and RXRα, i.e. twofold (Fig. 4A). This shows that RXR is not necessary for T3-dependent activation of the MCL-1 promoter.
promoter. Maximal, 2.2-fold activation of the MCL-1 promoter was achieved at 500 nM T3. The increase of T3 concentration to 1000 nM did not further change the activity of the MCL-1 promoter (Fig. 4B).

MCL-1 promoter is activated by T3 via PI3-K signaling pathway

As it had been previously shown by other authors that T3 treatment results in the induction of PI3-K activity (Cao et al. 2005, Kuzman et al. 2005, Moeller et al. 2005, 2006, Kenessey & Ojamaa 2006, Storey et al. 2006, Verga Falzacappa et al. 2007), we decided to establish whether T3-dependent activation of the MCL-1 promoter might be mediated by the PI3-K pathway. To do so, transcription regulation assays were performed as described above, in the presence of 100 nM of PI3-K inhibitor, wortmannin. They revealed that wortmannin completely abolished T3-dependent activation of the MCL-1 promoter observed in the presence of TRβ1 (Fig. 5). This indicated that PI3-K pathway is clearly involved in the regulation of the MCL-1 promoter by this hormone.

The −295 to −108 fragment contains all sequences involved in T3-dependent activation of the MCL-1 promoter

We then attempted to find the promoter fragment that mediates T3-dependent activation. Transcription regulation assays were performed with the reporter vectors containing the 1.82 kb MCL-1 promoter fragment, the −295 to +16 deletion mutant E, and the −107 to +16 deletion mutant EE. The assays revealed that in the presence of 100 nM T3, the 1.82 kb and E fragments of the MCL-1 promoter were similarly activated, while fragment EE was resistant to T3 action (Fig. 6). This shows that T3-dependent activation of the MCL-1 promoter is mediated by the sequences located in the −295 to −108 part of this promoter.

Putative CRE is a major mediator of the MCL-1 promoter activation by T3 and PI3-K signaling pathway

Deletion mutant E, but not EE, contains, among others, a sequence similar to that of the consensus CRE, located at the −262 to −255 position. To determine

![Figure 5](Image)

**Figure 5** T3-dependent activation of the MCL-1 promoter is abolished in the presence of wortmannin, a phosphatidylinositol 3-kinase inhibitor. HEK 293 cells were transfected with the pGL2-MCL-1 reporter vector containing the 1.82 kb MCL-1 promoter fragment, with the pEGFP-TRβ1 expression vector, and with the phRL-CMV internal control vector. The cells were incubated for 24 h with 100 nM T3 or without this hormone. The figure represents the mean results of six experiments, ± s.d.

![Figure 6](Image)

**Figure 6** Deletion of the −295 to −108 fragment abolishes T3-dependent activation of the MCL-1 promoter. HEK 293 cells were transfected with the pGL2-MCL-1 or pGL2-MCL-1E or pGL2-MCL-1EE reporter vector containing the −1810 to +16 or −295 to +16 or −107 to +16 fragments of the MCL-1 promoter respectively, with the pEGFP-TRβ1 expression vector, and with the phRL-CMV internal control vector. The cells were incubated for 24 h with 100 nM T3 or without this hormone. The figure represents the mean results of nine experiments, performed with two different DNA preparations, ± s.d.

![Figure 7](Image)

**Figure 7** Mutation of the putative CRE located at the −262 to −255 position decreases the level of T3-dependent activation of the MCL-1 promoter from 2- to 1.3-fold. HEK 293 cells were transfected with the pGL2-MCL-1 or with the pGL2-MCL-1Emut reporter vector containing the −295 to −107 fragment of the MCL-1 promoter with wild-type or mutated putative CRE respectively, with the pEGFP-TRβ1 expression vector, and with the phRL-CMV internal control vector. The cells were incubated for 24 h with 100 nM T3 or without this hormone. The figure represents the mean results of nine experiments, ± s.d.
the role of this putative CRE in the activation of the human MCL-1 promoter by T3, transcription regulation assays were performed with promoter deletion mutant E containing either wild-type or mutant CRE. While 100 nM T3 activated the wild-type fragment E of the MCL-1 promoter twofold, the mutation of putative CRE resulted in the decrease of this activation to 1.3-fold (Fig. 7). This is interpreted as indicating that CRE is a major mediator of T3-dependent activation of the MCL-1 promoter.

Discussion

In this work, we showed that MCL-1, an anti-apoptotic member of the BCL-2 family, is activated by T3 using a non-genomic mechanism that involves the PI3-K signal transduction pathway. To our knowledge, this is the first description of a molecular mechanism of T3-dependent expression regulation of a member of the BCL-2 family.

A putative TRE that differed from the consensus TRE-DR4 only by a single nucleotide has been found in the MCL-1 promoter at the −998 to −983 position. Transcription regulation assays showed, however, that it is not involved in promoter’s regulation by T3. The −295 to −107 fragment, mediating T3-dependent activation, did not contain other TRE-like sequences, making direct activation of the MCL-1 promoter by T3 unlikely.

It had been previously shown by other authors that the up-regulation of the human MCL-1 protein in basal cell carcinoma cells (Jee et al. 2002) and in Hep3B hepatic cells (Kuo et al. 2001) in response to interleukin-6 administration was due to PI3-K/Akt pathway activation, and resulted in the inhibition of apoptosis. As mentioned in the introduction, the cytoplasmic fraction of TRs, when bound to T3, also activates the PI3-K pathway (Lei et al. 2004, Cao et al. 2005, Kuzman et al. 2005, Moeller et al. 2005, 2006, Kenessey & Ojamaa 2006, Storey et al. 2006, Verga Falzacappa et al. 2006, 2007). Therefore, we examined a new hypothesis stating that the MCL-1 promoter is activated by T3 in a non-genomic mechanism involving this pathway. We indeed demonstrated that this is so, as the activation of this promoter by T3 was completely abolished by wortmannin, a PI3-K inhibitor. We also showed that the induction of MCL-1 expression required the presence of TRβ1, but not TRα1. This is in agreement with the results of other authors who showed that T3-dependent activation of Akt kinases and certain target genes by PI3-K was specifically mediated by different TR isoforms, with TRβ1 usually indicated as a go-between molecule (Moeller et al. 2006, Storey et al. 2006, Verga Fal Zacappa et al. 2007).

While analyzing the details of murine Mcl-1 promoter activation by interleukin-3 and by PI3-K pathway, Wang et al. (1999) showed that the CRE-2 sequence played a role in this process. Similarly, the −295 to −108 fragment of the human MCL-1 promoter, mediating the T3-dependent activation, has been shown to contain putative CRE. Upon its mutation, T3-dependent activation of this promoter decreased from 2- to 1.3-fold. This demonstrates that CRE is a major mediator in this process.

To sum up, the activation of the human MCL-1 by T3 is a result of the non-genomic action of TRβ1 that activates the PI3-K signaling pathway. We propose that this is one of the mechanisms by which T3 regulates apoptosis.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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