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# TCR pathway involves ICBP90 gene down-regulation via E2F binding sites

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**Abstract**

Antigen-induced cell death is essential for function, growth and differentiation of T-lymphocytes through legation of the T cell receptor. Since TCR-induced cell death occurs at late G1 checkpoint of the cell cycle and considering that ICBP90 is critical for G1/S transition, we studied the ICBP90 regulation through the TCR pathway in Jurkat cells. ICBP90 expression was strongly decreased after TCR triggering concomitantly to cyclin D3 and topoisomerase IIα expression decreases. Cell stimulation with PMA and/or calcium ionophore A23187 down-regulated ICBP90 expression. The decrease of ICBP90 protein and mRNA expressions was accompanied with cell growth arrest. A luciferase reporter assay demonstrated that activation of TCR pathways inhibit ICBP90 gene promoter activity. Three consensus E2F binding sites (called from E2F-a to E2F-c) were identified in the ICBP90 gene promoter and were subjected to mutations. The E2F-a, located in a highly active promoter fragment, shows a strong positive functional activity in proliferating cells. E2F-a and E2F-c binding sites are involved in the TCR-induced down-regulation of ICBP90 gene transcription. Altogether, our data demonstrate that TCR signaling pathways regulate ICBP90 gene expression through pRb/E2F complex. We propose that ICBP90 down-regulation is a key event in G1 arrest preceding T cell death.


*Keywords:* Apoptosis; Cell cycle; Gene regulation; T cells; Signal transduction; Transcription factors

## 1. Introduction

T cell receptor (TCR) engagement leads to genetic programs that both activate and negatively regulate T cells functions [1]. The T cell outcome is determined by the context in which TCR is engaged. In inflammation for instance, antigen is presented by activated APC in the context of costimulation resulting in T cell activation [2]. Conversely, when antigens are presented by resting APC, T cells are conducted to apoptosis or anergy that are fundamental states in self-tolerance and homeostasis [3]. Induction of apoptosis is the major mechanism controlling the self-reactive T cells [4]. Two major pathways are known in these apoptotic scenari: (i) antigen-induced cell death (AID), (ii) T cell autonomous cell death. Stimulation of the TCR in Jurkat cells, that are actively proliferating, results in proliferation cessation in G1 and subsequent apoptosis [5,6]. It has been proposed that up-regulation of p27 and p21, cell cycle inhibitors, in anergic cells play an important role in maintaining the anergic status [7–9]. In contrast, T cell anergy upon TCR engagement is not only a p27-independent but also a p21-independent mechanism since T cell anergy can be preserved in p27 and p21 knock-out mice [10].

The transcription factors involved in TCR-induced proliferation mechanisms are well-described. Indeed, NF κB, NFAT and AP-1 are the major transcription factors involved in the TCR-induced cell cycle progression [11,12]. In contrast, the transcription factors expression patterns during TCR-induced G1-arrest remain poorly documented. NF κB has been reported to be involved in

*Abbreviations:* AID, antigen-induced cell death; ICBP90, inverted CCAAT box binding protein of 90 kDa; TopoIIα, topoisomerase IIα; NIRF, Np95/ICBP90 ring finger

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the G1-arrested cell cycle in TCR-induced cell death [13]. TCR induced cell death was recently shown to occur from a late G1 checkpoint in a pRb-dependent fashion [14]. Furthermore, it was shown that AID occurs from a late G1 cell cycle checkpoint that is dependent on E2F-1 and p73 (a p53 gene related protein) [15,16]. Immunosuppressive compounds such as sanglifehrin (SFA, a novel cyclophilin-binding ligand) exert their effects through T cell proliferation blocking in G1 [17] and are responsible for p53 induction at the transcription level. Considering that we have recently shown that ICBP90 expression peaks in late G1 [18], that ICBP90 is critical for G1/S transition [19] and that its expression is governed by the p53/p21 pathway, and that apoptosis is dependent upon ICBP90 expression downregulation and that ICBP90 exhibits anti-apoptotic properties and a target for casein kinase 2 [20,21], we believe that down-regulation of ICBP90 is a critical event in G1 arrest preceding AID.

ICBP90 is a human nuclear protein that binds the ICB2 element of the TopoIIα gene promoter [22,23]. It acts as a transcription factor and regulates the expression of the TopoIIα gene during the cell cycle [24,25]. The thymus and the bone marrow are among the richest tissues for ICBP90 mRNA [22] suggesting an important role of ICBP90 in T cell functions. In a recent report, we demonstrated that, in non tumoral cells, overexpression of ICBP90 is associated with an elevation of TopoIIα expression and an increase of cell proliferation rate [24]. ICBP90, characterized by a RING finger domain, shares structural homology with the mouse nuclear phosphoprotein Np95 and the human NIRF, both involved in cell cycle regulation [26–29].

There is increasing evidence that ICBP90 belongs to a family of proteins corresponding to a subset of E3 ligases of the RING finger type. This is supported by recent observations showing that NIRF and Np95 are able to ubiquitinate the PEST-containing nuclear protein and the histone H3, respectively [29,30]. NIRF, Np95 as well as ICBP90 themselves can be ubiquitinated. E3 ligases are important determinants in cell cycle progression, gene transcription and embryonic development [31]. Recent attentions were focused on the diverse roles played by E3 ligase family in T cell responses [32,33]. Consequently, we hypothesized that down regulation of ICBP90 expression occurs in late G1 phase following TCR engagement and that this mechanism is responsible for the hindrance of the cells to enter the S phase with subsequent apoptosis. We have used Jurkat cells for their proliferation capacity independent from TCR activation or co-stimulatory signals. T cell activation can be mimicked by direct activation of one of the downstream signaling pathways of the TCR. For example, activation of protein kinase C by adding PMA, either alone or in combination with calcium ionophore can induce growth inhibition and apoptosis in leukemic T cell lines [5,6].

In this report, we show that ICBP90 is down-regulated in growth arrested TCR-activated Jurkat cells. Cell growth and ICBP90 expression are also inhibited after PMA stimulation or after $Ca^{2+}$ mobilization induced by ionophore A23187. This down-regulation is a result of the decrease in the ICBP90 gene promoter activity through TCR engagement involving pRb/E2F complex. We propose ICBP90 gene down-regulation as a key event in G1-arrested TCR-activated Jurkat hindering cells to enter S phase.

## 2. Materials and methods

### 2.1. Cell culture and treatment

The human lymphocyte Jurkat cell line was cultured in RPMI-1640 medium (Sigma–Aldrich Chimie, St. Quentin Fallavier, France) supplemented with 100 μg/ml gentamycin, 50 μg/ml penicillin/streptomycin, 2 mM L-glutamine and 10% FCS (Invitrogen, Cergy-Pontoise, France). Jurkat cells ($1 \times 10^6$ cells/well) were synchronized by serum starvation for 48 h with fresh RPMI-1640 medium. Cell treatment was carried out at 37 °C in 5% $CO_2$-humified incubator at the concentration of $4 \times 10^5$ cells/ml in the presence of 10% FCS. Cell activation with anti-CD3 (UCHT-1) or anti-CD28 mAbs (Beckman Coulter France, Villepinte Roissy, France) was established as described elsewhere [6]. Briefly, culture plates were coated overnight at room temperature with mAbs with concentrations indicated in the figures. Stimulation with PMA and/or calcium ionophore A23187 (Sigma) was established in 12-well plates, Jurkat cells were cultured in the presence of these drugs prepared in DMSO as indicated in the legend of figures.

### 2.2. Proliferation and apoptosis assays

After stimulation of Jurkat cells with mAbs, living cell numbers were quantified in 96-well micro-plates by a colorimetric assay by addition of 20 μl per well of XTT reagent mixture as described in the manufacturer's instructions (Roche Diagnostics, Meylan, France). In PMA and/or A23187 stimulated cells, 100 μl of cell suspension were transferred in triplicate into 96-well micro-plates and 20 μl per well of XTT reagent mixture were added. Microplates were then incubated at 37 °C in a 5% $CO_2$-humified incubator for 4 h. Optical densities were measured with a spectrophotometer at 450 nm versus 650 nm.

Apoptosis was analyzed using annexin V-FITC kit (Beckman Coulter France). Jurkat cells were treated as described in the legends of figures. Cells were harvested and washed twice in PBS buffer at room temperature. $5 \times 10^5$ cells were resuspended in 100 μl of annexin V binding buffer (140 mM NaCl, 5 mM $CaCl_2$ and 10 mM HEPES, NaOH, pH 7.4) supplemented with 1 μl of

annexin-V-FITC solution and 5 μl of propidium iodide solution (250 μg/ml). Cells were incubated for 15 min in dark at room temperature. Samples were then diluted with 400 μl of annexin-binding buffer and subjected immediately to fluorescence-activated cell sorter analysis using a FACStar Plus flow cytometer (Becton Dickinson France, Le-Pont-de-Claix, France). The early and late stages of apoptotic cells were stained with annexin V and annexin V plus propidium iodide, respectively, whereas the living cells are double negative. Cytometric experiments were run in our local facilities (service commun de cytométrie de l'Institut Fédératif de Recherche "Gilbert Laustriat") on a FACStar Plus using 400 mW of 488 nm light from an argon ion laser. Sort windows were used on forward and side scatter to eliminate debris. Granulation, size and fluorescence intensities were recorded at a rate of ~800 cells/s.

### 2.3. Cells extracts and Western blotting

Crude cell lysates were prepared by harvesting and washing the Jurkat cells in PBS at 4 °C. After a 5 min centrifugation at 200 × *g*, cell pellets were resuspended in PBS supplemented with protease inhibitor cocktail (Roche Diagnostics) and were sonicated in a final volume of 200 μl. After centrifugation (20,000 × *g* for 10 min at 4 °C), the supernatants were collected and protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad, Marnes-la-Coquette, France). Equal amount of proteins (5 or 10 μg) from each sample were resolved by SDS-PAGE (8%) and transferred onto nitrocellulose membranes by electroblotting. Blots were blocked with 5% non-fatty dried milk in TBST 0.1% (Sigma), probed with the mouse mAbs raised against β-actin (0.8 μg/ml) (Sigma), pRb (1 μg/ml) (Chemicon, Euromedex, Mundolsheim, France), cyclin D3 (1 μg/ml) (Becton Dickinson Pharmingen, BD France), TopoIIα (1 μg/ml) (MBL, Nagoya, Japan) or ICBP90 mAb (1RC1C-10; 0.5 μg/ml) engineered as described elsewhere [22]. HRP-conjugated anti-mouse Ab (Jackson ImmunoResearch Laboratories, West Grove, PA) was used to visualize proteins by the ECL detection kit (Amersham Biosciences Europe, Orsay, France).

### 2.4. Northern blot analysis

The double stranded ICBP90-specific probe was generated from the pSG5-ICBP90 plasmid [24] by PCR amplification of a 680 bp fragment (sense: 5′-GTCGGATCATCTTCGTGGACG-3′ and antisense: 5′-CTGTTCCGCGGTCCTCTTGTT-3′, nucleotides 806–1485 from Genbank accession number AF_129507). GAPDH specific probe was amplified from pGEM-T-GAPDH plasmid [34] leading to a 556-bp PCR product (sense: 5′-GGCT–GCTTTTAACTCTGGTA-3′ and antisense: 5′-GATGTT CTGGAGAGCCCCGC-3′, nucleotides 135–690 from Genbank accession number NM_002046). PCR products were resolved on 1% agarose gel and extracted with Ultrafree-DA (Millipore, St. Quentin, France). Sequence analysis of the amplified products revealed 100% homology with the human ICBP90 and GAPDH cDNA. These probes were quantified and 25 ng was used for $^{32}$P-labeling using RadPrime DNA Labeling System (Amersham). Radio-labeled probes were then purified through a Micro Bio-spin 30 chromatography column (Bio-Rad) and hybridization was performed at a probe concentration of $10^6$ cpm/ml. The probes were heated at 95 °C for 5 min and chilled on ice before adding to the ExpressHyb Hybridization Solution (Becton Dickinson Clontech, BD France), After stimulation with PMA or A23187, cells were collected by centrifugation and washed twice with PBS. Total RNA was isolated from cells by RNeasy® extraction Kit (Qiagen, Courtaboeuf, France) and quantified by measuring the absorbance at 260 nm and 280 nm in a spectrophotometer. Four micrograms of total RNA were resolved by electrophoresis on 1% agarose gel containing formaldehyde (8%) and transferred to Nylon membrane (Hybond-N, Amersham) by capillary action of high salt. Membranes were pre-hybridized with ExpressHyb Hybridization Solution and treated as described by the manufacturer. Briefly, membranes were hybridized with $^{32}$P-labeled ICBP90 or GAPDH specific probes in ExpressHyb Hybridization Solution for 1 h at 68 °C. Post-hybridization washes were performed four times in 2× SSC, 0.1% SDS (10 min per wash at room temperature) and four times in 0.1× SSC, 0.1% SDS (10 min per wash at 50 °C). For radioactivity detection, membranes were then exposed for X-OMAT AR detection Kodak films (Sigma). The same membrane was successively hybridized with the ICBP90 and GAPDH $^{32}$P-labeled probes.

### 2.5. Plasmid constructs

Five fragments of the promoter region of ICBP90 gene (Nucleotides 4849011–4850897 from Genbank accession number NT_011255.14) [35], ICBP-I (position −1887 to −1), ICBP-II (position −1887 to −1369), ICBP-III (position −1387 to −1094), ICBP-IV (position −1113 to −574) and ICBP-V (position −657 to −1), were amplified by PCR performed on human placenta genomic DNA using the primers shown in Table 1. To mutate the E2F binding sites in the fragments ICBP-II, ICBP-III and ICBP-IV, we used a classical PCR method with primers containing the mutated E2F binding sites, E2F-a TTTCGCGGGAAA becomes TTTCGATGGAAA, E2F-b TTTCCCGC becomes TTTCCATC and E2F-c TTTCGCGC becomes TTTCGATC. The PCR-amplified fragments were subsequently cloned into the Mlu I and Bgl II restriction sites of the pGL3-enhancer vector (Promega France, Charbonnières, France) upstream of the firefly luciferase reporter gene to obtain the ICBP (I–V)-pGL3 plasmid constructs as shown in (Fig. 5A). All constructs were confirmed by sequencing analysis.

Table 1
Primer sequences used for PCR amplification of the ICBP90 gene promoter fragments[a]

| Fragement | size (bp) | Primer name | | Primer sequences 5′–3′ | Position from ATG |
|---|---|---|---|---|---|
| ICBP-I (Wt) | 1911 | ICBP-S1 | Sense | TGTGTGACGCGTGCGGGTGGGTAGAGTGGGG | −1899 |
| | | ICBP-AS1 | Antisense | TACTTAAGATCTGGTGTCGGCGCTGAGGGGA | 11 |
| ICBP-II (Wt) | 543 | ICBP-S1 | Sense | TGTGTGACGCGTGCGGGTGGGTAGAGTGGGG | −1899 |
| | | ICBP-AS4 | Antisense | CAGGGTAGATCTGCGCTGCCAGCTGCTCTGA | −1357 |
| ICBP-II (Mut) | 543 | ICBP-S1 | Sense | TGTGTGACGCGTGCGGGTGGGTAGAGTGGGG | −1899 |
| | | ICBP-AS4(m) | Antisense | TTTTTCCatGAAAACTCG | −1357 |
| ICBP-III (Wt) | 318 | ICBP-S2 | Sense | TATGTGACGCGTTCAGAGCAGCTGGCAGCGC | −1399 |
| | | ICBP-AS3 | Antisense | CAGTCGAGATCTAAGGGGAGGCCCTGGATCC | −1082 |
| ICBP-III (Mut) | 318 | ICBP-S2 | Sense | TATGTGACGCGTTCAGAGCAGCTGGCAGCGC | −1399 |
| | | ICBP-AS3(m) | Antisense | GCTGGCCGCGatGGAAAGTTTTGC | −1145 |
| | | ICBP-S2(m) | Sense | AAAACTTTCCatCGCGGCCAGCCC | −1166 |
| | | ICBP-AS3 | Antisense | CAGTCGAGATCTAAGGGGAGGCCCTGGATCC | −1082 |
| ICBP-IV (Wt) | 564 | ICBP-S3 | Sense | TATGTAACGCGTGGGATCCAGGGCCTCCCCT | −1125 |
| | | ICBP-AS2 | Antisense | CACCTGAGATCTGCGGTGGCCATGGTAACCG | −562 |
| ICBP-IV (Mut) | 564 | ICBP-S3 | Sense | TATGTAACGCGTGGGATCCAGGGCCTCCCCT | −1125 |
| | | ICBP-AS2(m) | Antisense | TCAGGGCGatCGAAATCGGGCTCG | −888 |
| | | ICBP-S3(m) | Sense | CGATTTCGatCGCCCTGAGTTCCC | −905 |
| | | ICBP-AS2 | Antisense | CACCTGAGATCTGCGGTGGCCATGGTAACCG | −562 |
| ICBP-V (Wt) | 681 | ICBP-S4 | Sense | GATGATACGCGTCGGGGGTTGGAGGTCGAGG | −669 |
| | | ICBP-AS1 | Antisense | TACTTAAGATCTGGTGTCGGCGCTGAGGGGA | 11 |

[a] Wild type (Wt) and mutants (Mut) fragments of the ICBP90 gene promoter were amplified by PCR using the indicated sense and antisense primers. Putative E2F binding sites are underlined and mutated bases are indicated in lower cases.

### 2.6. Transfection of human Jurkat cells

Jurkat cells ($4 \times 10^5$ cells/ml) were plated in culture plates and then transiently transfected with 1 μg of pGL3 plasmid reporters together with 0.1 μg of Renilla luciferase pRL-TK vector as an internal standard (Promega) using jetPEI trensfection agent (3 μl) (Polyplus-transfection, Illkirch, France) according to the manufacturer's instructions. Twenty four hours after transfection, cells were diluted to the half with fresh completed RPMI-1640 medium. Cells were treated for further 24 h with the various stimuli as indicated and harvested for luciferase activity measurements with the dual luciferase system (Promega). Results are expressed as a ratio of the control pRL-TK activity.

### 2.7. Bioinformatics

FACS data from annexin staining experiments were analyzed using WinMDI 2.8 freeware (http://facs.scripps.edu/software.html/). Protein and mRNA bands were quantified as arbitrary units on scanned blots with the Scion Image beta 4.0.2 freeware (http://www.scioncorp.com/) and band densities were expressed as percentage of the control in non-treated cells. Sequence homology searches were done with the different BLAST programs available at the National Centre for Biotechnology Informatics web site (http://www3.ncbi.nlm.nih.gov./BLAST/). The ICBP90 gene promoters were analyzed for the presence of transcription factor binding motifs with sequence analysis web site (http://www.genomatix.de/). Multiple sequence alignments and primers prediction were done using the BioEdit 7.0.1 freeware (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). All statistical analyses were performed using Student's *t* test.

## 3. Results

### 3.1. Expression of pRb, cyclin-D3, ICBP90 and TopoIIα proteins in activated Jurkat cells

We first explored the expression patterns of G1/S transition proteins involved in the cell cycle of TCR-triggered Jurkat cells (Fig. 1). Western blot analysis shows TopoIIα, ICBP90, pRb and cyclin-D3 expressions were strongly decreased when Jurkat cells were stimulated with 2 μg/ml of anti-CD3 mAb or with simultaneous activation of cells with PMA and calcium ionophore A23187 at 10 ng/ml and 0.3 μg/ml, respectively, as compared with control untreated cells (Fig. 1). Although, in activated cells, pRb was found in its active hypo-phosphorylated form. Triggering the CD28 receptor of Jurkat cells by anti-CD28 mAb had no effect on the expression pattern of these proteins as observed in the untreated cells. No change has been observed in actin expression in both stimulated and unstimulated cells.

### 3.2. ICBP90 expression is dose-dependently down-regulated in anti-CD3 stimulated Jurkat cells

Fig. 2 shows the effect of TCR stimulation induced by anti-CD3 mAb, on the living cell number and ICBP90

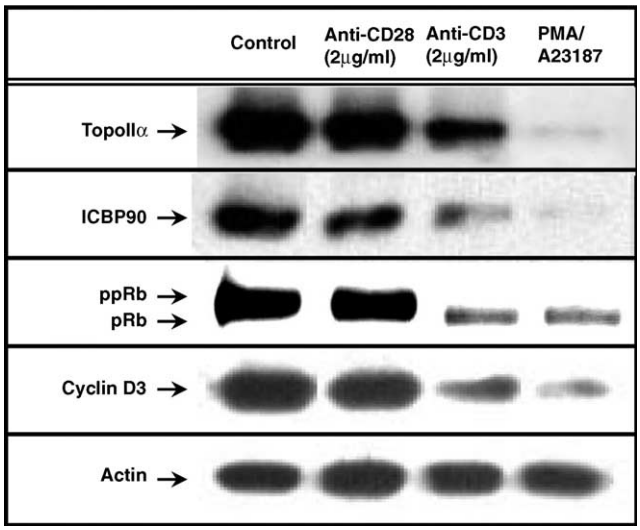


Fig. 1. Expression patterns of cell cycle regulatory proteins TopoIIα, ICBP90, pRb and cyclin-D3 in stimulated Jurkat cells. Cells were stimulated with 2 μg/ml of anti-CD3, anti-CD28 in precoated plates or with PMA (10 ng/ml) and ionophore A23187 (0.3 μg/ml) for 24 h. 10 μg of protein extracts were loaded on SDS-PAGE and transferred onto nitrocellulose membrane for Western blot analysis. Proteins were detected using their corresponding mAbs recognized by HRP-conjugated anti-mouse Ab and revealed by the ECL detection kit.

expression. After 48 h of stimulation with anti-CD3 mAb, a dose-dependent decrease of living cell numbers was observed. The maximum inhibition of about 35% of living cells was reached at 5 μg/ml of anti-CD3 mAb (Fig. 2A). In contrast, activation of Jurkat cells by increased concentrations of anti-CD28 mAb induced a non-significant rise in the living cell numbers (Fig. 2A). By measuring the percentage of annexin-V positive cells, a slight significant increase of cell death (about 8%) was observed in cells activated with anti-CD3 mAb when compared to cells activated with anti-CD28 mAb or to unstimulated control cells (Fig. 2A, inset). ICBP90 mRNA level analyzed by Northern blot in anti-CD3 stimulated cells showed a dose-dependent decrease of ICBP90 mRNA expression from 1 to 5 μg/ml of anti-CD3 mAb after 48 h of treatment (Fig. 2B). In contrast, stimulation by anti-CD28 mAb under the same experimental conditions failed to affect the level of ICBP90 mRNA. We also examined the expression of GAPDH as mRNA loading control, which shows invariable expression. Therefore, ICBP90 mRNA down-regulation appears strongly correlated with the growth inhibition induced by TCR stimulation.

### 3.3. Downstream signaling pathways of the TCR induce ICBP90 down-regulation

TCR pathways involve protein kinase C activation and calcium mobilization that can be mimicked by PMA and ionophore A23187, respectively [5,36]. Fig. 3 shows that ICBP90 mRNA level is down-regulated in Jurkat cells stimulated with PMA to 60% and 32% at 1 or 10 ng/ml, respectively (Fig. 3A). Although, ICBP90 mRNA level

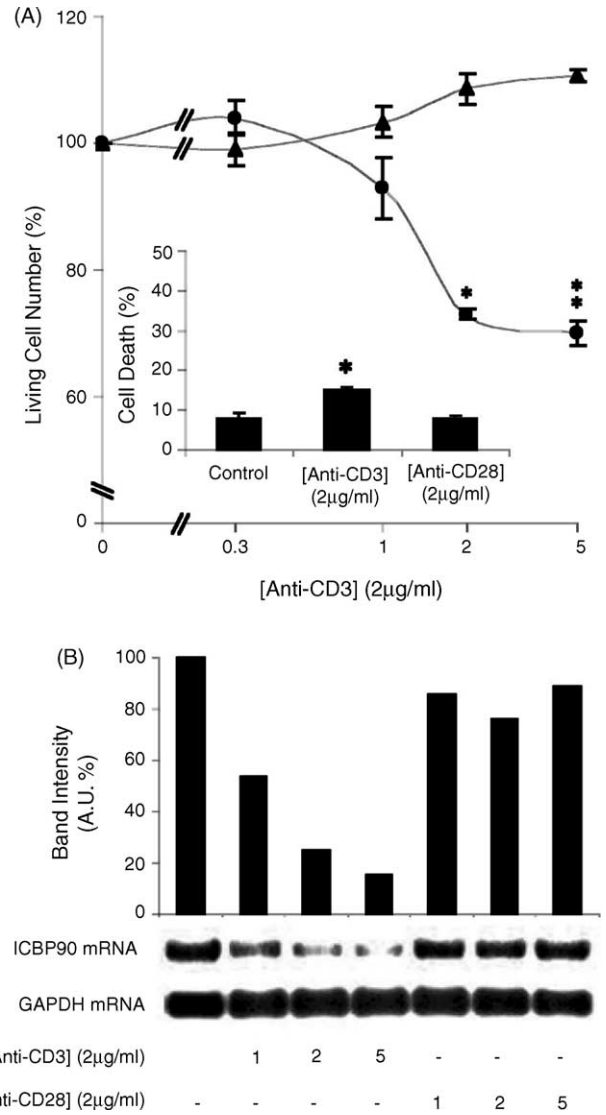


Fig. 2. Inhibition of ICBP90 expression in anti-CD3 stimulated Jurkat cells. (A) Cells were cultured in precoted plates with indicated concentration of anti-CD3 (●) or anti-CD28 (▲) mAbs for 48 h and living cell numbers were expressed as percentage of control (i.e., in the absence of mAbs). The percentage of cell death was determined using annexin-V and propidium iodide staining (inset). Data are means ± S.E.M. of three separate experiments performed in triplicate. Significantly different from control, $^{*}P < 0.05$; $^{**}P < 0.01$. (B) ICBP90 and GAPDH mRNAs expressions were analyzed after 24 h of cell treatment with various concentrations of immobilized anti-CD3 or anti-CD28 mAbs by Northen blot. Band densities are expressed as relative percentages of the untreated control cells.

was reduced to 76% and 40% in cells stimulated with 0.3 and 1 μg/ml of A23187, respectively, when compared with the untreated control cells. ICBP90 mRNA level was reduced to only 9% when ionophore A23187 (0.3 ng/ml) and PMA (10 ng/ml) were added simultaneously. We also studied cell viability and cell death in Jurkat cells after 48 h of incubation with 10 ng/ml of PMA and/or 0.3 μg/ml of A23187 (Fig. 3B). A pronounced decrease in living cell number reached about 96% was obtained in cell populations incubated with both stimuli. This decrease in living cell numbers was more important than that in cells treated

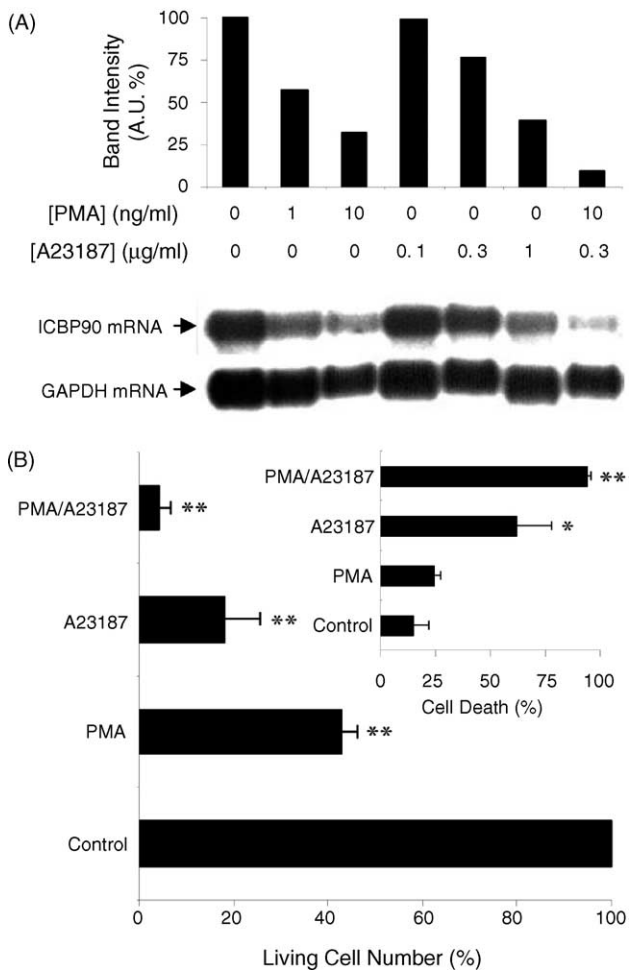


Fig. 3. Effect of PMA and ionophore A23187 on ICBP90 expression in Jurkat cells. (A) ICBP90 mRNA expression was analyzed after 24 h of cell treatment with the indicated concentrations of PMA and/or ionophore A23187 by Northen blot. Band densities are expressed as relative percentages of the untreated control cells. (B) Cells were cultured in the presence of 10 ng/ml of PMA and/or 0.3 μg/ml of ionophore A23187 for 48 h and living cell numbers were determined and expressed as described above. The percentage of cell death was determined using annexin-V and propidium iodide staining (inset). Data are means ± S.E.M. of three separate experiments performed in triplicate. Significantly different from control, $^{*}P < 0.05$; $^{**}P < 0.01$.

with either PMA alone (57%) or with ionophore A23187 alone (82%) (Fig. 3B). On the other hand, cell death percentage was increased from 15% in untreated control cell population to 94% in those stimulated with both drugs. This apoptotic effect was lower in cells stimulated with A23187 alone whereas a low but non significant cell death was observed with PMA alone (Fig. 3B, inset). Altogether, these results show that ICBP90 down-regulation may be induced via an increase in cytosolic calcium and/or protein kinase C activation.

### 3.4. Involvement of pRb/E2F pathway in TCR-induced ICBP90 gene down-regulation

In order to understand the regulation mechanisms of ICBP90 gene expression, we studied its promoter activity

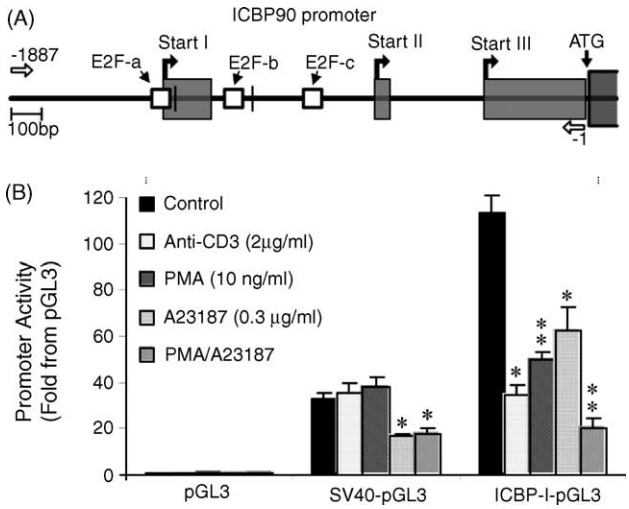


Fig. 4. ICBP90 gene promoter regulation by TCR activation. (A) Structural organization of the ICBP90 gene promoter, grey boxes indicate three non-coding exons with three putative transcription start sites (broken arrows). Three E2F binding sites (a–c) are indicated by white boxes. White arrows indicate the position of the primers used to amplify the ICBP-I fragment (see Table 1 for primer sequences). (B) Cells were transiently transfected with pGL3, SV40-pGL3 or ICBP-I-pGL3, stimulated for 24 h with the indicated concentrations of anti-CD3 mAb, PMA and/or ionophore A23187 and prepared for luciferase activities. Promoter activities are presented as over fold from basal activity of pGL3 vector. Data are means ± S.E.M. of three separate experiments performed in triplicate. Significantly different from control, $^{*}P < 0.05$; $^{**}P < 0.01$.

in activated Jurkat cells under various conditions (Fig. 4). A large fragment ICBP-I (from −1887 to −1) of ICBP90 gene promoter, was constructed by PCR from human placenta genomic DNA and cloned upstream of a luciferase reporter gene in the pGL3 vector (ICBP-I-pGL3). ICBP-I fragment contains three potential E2F binding sites that were homologue with the consensus E2F binding site TTTSSCGS (S = C or G). The E2F-a (TTTCGCGGGAAA) is from −1403 to −1392, the E2F-b (TTTCCCGC) is from −1161 to −1154 and the E2F-c (TTTCGCGC) is from −902 to −895. The E2F-c binding site manifests 100% of homology with the consensus E2F binding site (TTTCGCGC) (Fig. 4A). We studied the promoter activity of ICBP-I fragment in Jurkat cells. In unstimulated Jurkat cells, the ICBP-I-pGL3 shows 110-fold promoter activity higher than the pGL3 vector (basal activity) whereas SV40-pGL3 (positive control) shows only a 30-fold promoter activity (Fig. 4B). TCR triggering by anti-CD3 mAb induced a strong and significant decrease in the ICBP-I promoter activity that reached 69.5 ± 10.1% inhibition when compared to unstimulated control cells. Stimulation by PMA or ionophore A23187 resulted in 56.3 ± 9.9% and 45.2 ± 15.9% inhibition, respectively, with a strong inhibition of 82.28 ± 10.25% observed when both stimuli PMA and ionophore A23187 were used. In SV40-pGL3 transfected cells, no effect of anti-CD3 mAb or PMA was observed on the promoter activity in contrast to the ionophore A23187 which induced a strong inhibition alone or in combination with PMA (Fig. 4B).

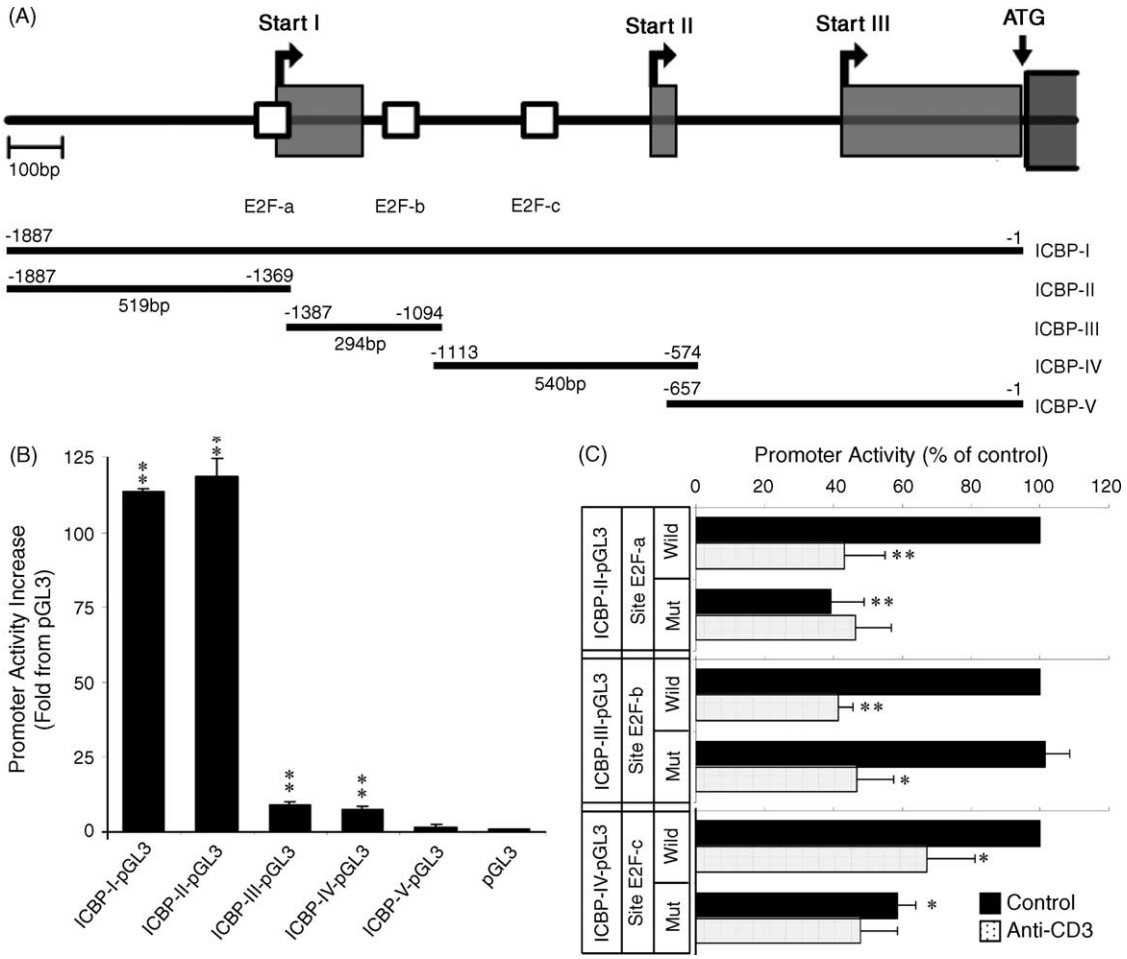


Fig. 5. Role of E2F binding sites in the ICBP90 gene regulation. (A) Schematic representation of the ICBP90 gene promoter with its five designed fragments for luciferase assay. The three E2F binding sites (a–c) are indicated by white boxes. Primer positions used to amplify the different fragments of the ICBP90 gene promoter and fragment sizes are indicated. (B) Cells were transfected with each plasmid construct and lysed 48 h later for luciferas activity quantification. Results are expressed as fold from pGL3 basal activity. Data are means ± S.E.M. of three separate experiments performed in triplicate and were significantly different from control, $^{*}P < 0.05$; $^{**}P < 0.01$. (C) Cells were transfected with wild or mutated constructs (ICBP-II-pGL3, ICBP-III-pGL3 or ICBP-IV-pGL3) or with pGL3 plasmid for 24 h, stimulated or not with anti-CD3 mAb for further 24 h and assayed for luciferase activities. Values are expressed as percentage of unstimulated control cells transfected with the corresponding wild type construct. Data are means ± S.E.M. of three separate experiments performed in triplicate and were significantly different from corresponding control, $^{*}P < 0.05$; $^{**}P < 0.01$.

To study the putative role of E2F binding sites in the regulation of ICBP90 gene, we constructed four other plasmids from the ICBP-I-pGL3 construct (Fig. 5A). The ICBP-II (from −1887 to −1369) includes E2F-a, the ICBP-III (from −1387 to −1094) includes E2F-b and the ICBP-IV (from −1113 to −574) includes E2F-c. These wild type fragments were subjected to mutation in the E2F binding sites. The ICBP-V (from −657 to −1) does not contain any E2F binding site. We compared the promoter activity of each fragment with that of ICBP-I (Fig. 5B). Interestingly, the ICBP-II fragment which includes only the E2F-a binding site showed a very high promoter activity (116-fold) similar to that observed with the ICBP-I fragment (110-fold) when compared with the basal activity of pGL3 vector. The two other fragments, ICBP-III and ICBP-IV showed low but significant promoter activities of nine-fold and seven-fold over basal activity of pGL3 vector, respectively. Since, no promoter activity was found in the ICBP-V fragment, the present data suggest that ICBP90 gene promoter activity is located between −657 and −1887 with a very active region of 519 bp size in ICBP-II fragment located upstream the start I transcription site (Fig. 5A). We further analyzed the effect of TCR stimulation on the promoter activity of each construct. This was performed using the wild type and E2F binding site mutated constructs (Fig. 5C). TCR stimulation of Jurkat cells with anti-CD3 mAb resulted in significant inhibition of 56.9 ± 9.5% in the promoter activity of the ICBP-II fragment. Interestingly, when the E2F-a site was mutated, 60.9 ± 9.9% of promoter activity was lost in proliferating cells but no additional effect of anti-CD3 mAb was observed in stimulated cells (Fig. 5C). A similar effect was observed with ICBP-IV fragment. In fact, 41.5 ± 6.9% of promoter activity was lost in the E2F-c site mutated fragment in proliferating cells. Although, stimulation of cells with anti-CD3 mAb induced

32.8 ± 13.8% inhibition in the promoter activity of the wild type ICBP-IV fragment. This inhibitory effect was abolished in the mutated ICBP-IV fragment (Fig. 5C). Finally, no change in the promoter activity of the ICBP-III fragment containing E2F-b between wild or mutated types was observed. Surprisingly, cell stimulation with anti-CD3 mAb induced similar inhibitions of 58.6 ± 4.3% and 53.3 ± 10.9% in wild or mutated types, respectively. These results suggest that the E2F-a site has an important functional role in the ICBP90 gene promoter and plays a positive role in the regulation of ICBP90 gene transcription in unstimulated Jurkat cells but together with E2F-c site might play a negative role in the regulation of ICBP90 gene in TCR-stimulated Jurkat cells.

## 4. Discussion

T cell activation program involves induction of genes mediating AID through TCR activation, that is a crucial phenomenon in T cell anergy [1]. AID controls the expansion of antigen-activated T cells after an immune response and deletes self-reactive T cells by negative selection. AID occurs from a late G1 cell cycle checkpoint that is dependent on E2F-1 and p73 [14–16]. TCR-induced growth inhibition via apoptosis was recently shown to occur from a late G1 checkpoint in a pRb-dependent fashion [6,14,37][personal observations]. Recent studies showed that pRb/E2F complexes control at least, 10% of all the genes expressed during cell cycle. The products of these genes are those of DNA repair, replication and checkpoints [38,39]. This pathway was found to be directly implicated in T cells proliferation, anergy or programmed cell death [14,40]. Cell cycle progression is a pre-requisite for AID suggesting that genes involved in proliferation must be down-regulated during apoptotic process.

An emerging family of proteins involved in cell cycle progression, particularly in the G1/S transition, is the ICBP90 family including NIRF (Np95/ICBP90 Ring Finger) and Np95, a murine homologue of ICBP90 [18]. Indeed, down-regulation of ICBP90 blocks cell entry into the S-phase [19]. Interestingly, the members of this family interact with pRb [28]. Also, Bonapace and collaborators have shown the dependence of G1/S transition upon Np95 expression [27]. In contrast, over-expression of NIRF blocks cell entry into the S-phase [28]. Therefore, our hypothesis was that AID, induced by TCR activation, requires ICBP90 down-regulation as a pre-requisite for apoptosis. We show here that TCR engagement in Jurkat T cells, using anti-CD3 mAb, induced down-regulation of ICBP90 accompanied by a decrease in cyclin D3 and TopoIIα. Mimicking TCR activation by using ionophore A23187 and PMA, we show that down-regulation of ICBP90 is caused by protein kinase C activation and cytosolic free calcium increase. The mechanism by which calcium and protein kinase C induces ICBP90 down-regulation is not yet determined but might for instance involve pRb/E2F pathway. Indeed, it was shown in human umbilical vein endothelial cells that protein kinase C has bimodally regulating effects on E2F-1 mRNA [41]. Considering that E2F-1 regulates ICBP90 expression [18], this pathway is likely to occur in Jurkat cells in terms of ICBP90 regulation by TCR.

Cyclin D3 can form an active complex with the cdk4 or cdk6 leading to phosphorylation of pRb [39]. This phosphorylation is considered as a key event in that it hinders pRb to interact with E2F-1 allowing activation of E2F-1 related genes. Cyclin D3 down regulation is the key event in TCR-induced G1 arrest since its ectopic expression can abrogate this arrest and prevent the cell death in actively proliferating T cells [6,9,42]. Although, in stimulated cells, pRb was in its active hypo-phosphorylated form [6,present data]. We suggest that down-regulation of cyclin D3 participate in the TCR-induced ICBP90 decreased expression. Furthermore, we believe that TCR engagement involves the following events starting by cyclin D3 down regulation, prevention of pRb phosphorylation, formation of pRb/E2F-1 inhibitory complex with subsequent ICBP90 down-regulation, all these events hindering cells to enter the S-phase explaining the decrease in cell number. We have previously shown that ICBP90 down-regulation is under the control of p53 [19]. In accordance with this, E2F-1 was shown to mediate apoptosis via p53 up-regulation after TCR stimulation [37]. Nevertheless, TCR-AID appears to be a p53-independent mechanism but dependent on p73, a p53 related-gene [43] and thus we do not exclude that beside p53, ICBP90 regulation may be under the p73 control in TCR activation.

Investigating, the mechanism of ICBP90 down-regulation induced by TCR-AID, we found that ICBP90 mRNA was decreased in TCR-stimulated Jurkat cells. Similar results were obtained when Jurkat cells were stimulated simultaneously with PMA and ionophore A23187 but not with PMA alone. There was a good correlation between cell number decreases and ICBP90 mRNA decreases suggesting that decrease in ICBP90 mRNA is responsible for cell number decrease. We also found that the promoter activity of the ICBP90 gene decreased in TCR-stimulated Jurkat cells as well as in Jurkat cells stimulated by PMA and/or ionophore A23187. This shows that the decrease in the ICBP90 gene promoter activity is participating in the decrease of ICBP90 mRNA. We propose that the ICBP90 mRNA decrease is a fundamental mechanism in AID.

Three potential sites for the transcription factor E2F (a–c) were found in the ICBP90 promoter region with manifestly high homology scores with the consensus E2F binding sequence. Luciferase assays with the ICBP90 gene promoter allowed us to determine the functional promoter located upstream of the first transcription start site from −1887 to −1369. In this active promoter that responds negatively to TCR signals, the mutation of E2F-a was enough not only to reduce the promoter activity to the half,

but also to fully prevent the decrease in the ICBP90 gene promoter activity from upon TCR stimulation. Interestingly, the same E2F sites were identified and at the same positions in Np95 and NIRF gene promoters arguing that pRb/E2F pathway is involved in the regulation of this recently described family of proteins.

The consequences of ICBP90 down-regulation is a block in the G1 phase by preventing probably chromatin remodeling processes. Indeed, NIRF has ubiquitin ligase activity as well as Np95 [29,30]. For this latter, the in vitro substrate was identified as being the histone H3 [30]. Post-translational histone H3 modifications is involved in the histone code and thus is involved in the gene expression regulation governed by the chromatin structure [44]. Furthermore, it was shown that ICBP90, NIRF and Np95, by means of their SRA domains, are able to recruit HDAC to methylated promoter regions of various tumor suppressor genes [45]. Therefore, we propose that in TCR-induced growth arrested cells, ICBP90 down-regulation prevents H3 modifications that would be required for cell cycle progression.

In conclusion, TCR triggering induces ICBP90 down-regulation which prevents the expression of genes involved in the G1/S transition through chromatin remodeling processes and therefore conducting the cells to an apoptotic process.

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