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Characterization of nuclear factors modulating the apolipoprotein D promoter during growth arrest: Implication of PARP-1, APEX-1 and ERK1/2 catalytic activities

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ABSTRACT

Human Apolipoprotein D (apoD) is upregulated under several stress conditions and pathological situations such as neurodegenerative diseases and cancers. We previously showed that apoD mRNA expression is induced in growth-arrested cells and demonstrated the specific binding of nuclear proteins to the region −514 to −475 of the promoter. Such region contains a pair of Serum Responsive Elements (SRE), an Ets-Binding Site (EBS) and a Glucocorticoid Responsive Element (GRE). In this study, we show that Parp-1, HnRNP-U, CBF-A, BUB-3, Kif4, APEX-1 and Ifi204 bind these regulatory elements of the apoD promoter. Specific binding of HnRNP-U and Parp-1 was confirmed by Electrophoretic Mobility Shift Assay (EMSA). In a biotin pull-down assay, Kif4 and BUB-3 bind preferentially the SRE1 and the EBS-GRE sites, respectively, while APEX-1 seems recruited indirectly to these elements. We found that the mRNA expression of some of these binding factors is upregulated in growth-arrested cells and that these proteins also transactivate the apoD promoter. In agreement with these results, mutants of APEX-1 and of Parp-1 defective for their DNA-binding and catalytic activities could not transactivate the promoter. The knockdown of Parp-1 and HnRNP-U and the use of specific inhibitors of MEK1/2 and of Parp-1 also inhibited the induction of apoD gene expression. Moreover, ERK1/2 was found activated in a biphasic manner post serum-starvation and the inhibition of Parp-1 causes a sustained activation of ERK2 but not ERK1 for up to 2 h. Altogether, these findings demonstrate the importance of Parp-1, APEX-1 and ERK1/2 catalytic activities in the growth arrest-induced apoD gene expression.

1. Introduction

Human apolipoprotein D (apoD) is a 29-kDa secreted glycoprotein and a member of the lipocalin superfamily [1,2]. Since its discovery, which dates for more than 35 years ago [3] and despite the identification of a variety of candidate ligands such as arachidonic acid [4], progesterone and pregnenolone [5,6], bilirubin [7], E-3-methyl-2-hexenoic acid [8] and cholesterol [9], the physiological function of apoD remains unknown. Therefore, apoD is considered as a protein with pleiotropic functions depending on its ligands and its tissues or organs of expression [10].

In human, the apoD gene is expressed at high levels in the adrenal glands, spleen, testes, lungs, pancreas, kidneys and the nervous system [10]. ApoD expression is also found increased in several neurological disorders such as Alzheimer's disease, meningoencephalitis, dementia, motor neuron disease [11], Parkinson's disease [12], schizophrenia [13,14], multiple sclerosis [15] and several animal models of neurodegenerative injuries [10]. Besides neuropathological situations, apoD expression is elevated in several cancers. This overexpression of apoD gene is observed particularly in differentiated tumours with low proliferation rate and several studies suggested the importance of cellular growth arrest as a triggering factor of apoD expression [16–18]. Effectively, in cultured cells, apoD expression is associated with cellular growth arrest, senescence and inflammation. In agreement with these facts, apoD is also upregulated in aging rats [19] and humans [20,21] and this senescence characteristic, which is an irreversible growth-arrested state in contrary to quiescence, is also considered as a physiological mechanism capable of counteracting tumour proliferation [22–26].

Furthermore, apoD expression and proliferation are also inversely correlated in response to cellular stress. Indeed, cellular stresses provoking an extended growth arrest such as high doses of UV light and H_2O_2 increased apoD expression [27].

We have already identified the promoter region comprised between −558 and −179 to be responsible for the induction of apoD following growth arrest [28]. More precisely, we found that the Alternating Purine-Pyrimidine stretch (APP) and two Serum Responsive Elements (SRE-1 and -2) were the major determinants of growth arrest-induced apoD

Abbreviations: apoD, apolipoprotein D; SRE, Serum responsive element; EBS, Ets-Binding site; GRE, Glucocorticoid responsive element; EMSA, Electrophoretic Mobility Shift Assay; HnRNP, Heterogeneous nuclear ribonucleoprotein; CBF-A, CArG-box Binding Factor A (CBF-A); Parp-1, Poly(ADP-ribose) polymerase-1; Ifi204, Interferon-inducible protein 204; APEX-1, Apurinic/Apyrimidinic Endonuclease-1; Kif4, Kinesin family member 4; BUB-3, Budding Uninhibited by Benzimidazole 3; KifC1, Kinesin family member C1; Rbp1, Ribosome-binding protein 1

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gene expression. The SRE is a 23-bp element with an inverted repeat of dyad symmetry [29]. Only the SRE1 of the apoD promoter contains a CArG-box core described as a 10-bp element with the consensus sequence $CC(A/T)_6GG$. In many growth-related or immediate early genes, the CArG-box element is often in the vicinity of an Ets-Binding-Site (EBS) [29–33]. Detailed analysis of the apoD promoter also revealed the presence of an EBS and a Glucocorticoid Responsive Element (GRE) between the two SREs in the region −514 to −475 [28]. The EBS, with its central core sequence 5'-GGA(A/T)-3', is known to cooperate tightly with the SRE through the binding of several nuclear proteins, particularly the Serum Response Factor (SRF) and members of the *E26 transforming specific* (ETS) family which regulate several genes implicated in cell proliferation and differentiation [33,34]. Also, the GRE is known to interact with the Glucocorticoid Receptor (GR), a nuclear receptor ligand-dependant transcription factor which recognizes the consensus sequence 5'-GGTACAnnnTGTTCT-3' [35].

Thus, it appeared important to further study the regulation of the apoD gene, especially in relation with cellular proliferation. As the SREs in the region −558 to −179 were identified as major determinants of growth arrest-induced gene expression, we focused our study on this region of the promoter that contains the SRE1, EBS and GRE sites (Table 1). Here, we present the identification and the characterization of several nuclear proteins that bind the apoD promoter under normal and growth arrest conditions in NIH/3T3 fibroblast cells. Our results show that Parp-1, HnRNP-U, CBF-A, BUB-3, Kif4, APEX-1 and Ifi204 bind elements of the promoter. We also investigated the implication of MEK/ERK pathway in the regulation of the apoD gene expression. We demonstrate the implication of these nuclear factors in the modulation of the apoD promoter and that Parp-1, APEX-1 and ERK1/2 catalytic activities regulate positively the apoD gene expression in growth arrested-cells.

2. Materials and methods

2.1. Cell culture and reagents

Mouse fibroblasts (NIH/3T3) were obtained from American Type Culture Collection (ATCC, Rockville, MD) and maintained in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Carlslab, CA) supplemented with 10% calf serum, penicillin G (100 units/ml) and streptomycin (100 μg/ml). NIH/3T3 cells were maintained at 37 °C in a 5% CO_2 humidified atmosphere and were fed every 2 days with fresh medium. For the analysis of gene expression in sparsely growing cultures (normal conditions), cells were maintained in medium supplemented with 10% calf serum and harvested at 50% of confluence. For the analysis in growth arrest conditions, cells were maintained in 0.5% serum when they reached 80% of confluence. Cells were kept in this medium and harvested at different days post serum starvation. When specific inhibitors of Parp-1 or MEK1/2 were used, the medium was replaced by medium containing 10 mM 3-ABA (Sigma, St. Louis, MO) or 10 μM U0126 (Calbiochem EMD Biosciences, San Diego, CA), respectively.

2.2. Whole cell and nuclear extracts

For total extracts, 3×10^6 cells were lysed in 100 μl lysis buffer (10 mM HEPES, pH 7.9, 140 mM NaCl, 1.5 mM $MgCl_2$, 0.5% Nonidet P-40, 1 mM PMSF, 1 mM dithiothreitol (DTT), 100 U/μl aprotinin and 200 μM Sodium orthovanadate (Na_3VO_4)). After 5 min of incubation at 4 °C, lysates were cleared by 10 min of centrifugation at 13,000*g* at 4 °C. Nuclear extracts (NE) from growing or from 5-day serum starved NIH/3T3 fibroblasts were prepared as described previously [36]. The protein concentration was determined at 595 nm using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA).

2.3. Electrophoretic mobility shift assays (EMSA)

Sense oligonucleotides (50 ng) were 5'-end-labeled with T4 polynucleotide kinase and (γ-^{32}P) ATP and annealed with 200 ng of the complementary oligonucleotide resulting in a double-stranded sequence with SRE1-EBS-GRE elements found in the apoD promoter and shown in Table **1**. Nuclear extracts were added to 0.8 ng of the labeled double-stranded oligonucleotide and after 20 min incubation at room temperature, the mixture was run on a 6% acrylaminde, non-denaturating gel in 0.5× TBE. The dried gels were autoradiographed on Kodak X-Omat films (Perkin Elmer, Rochester, NY). For competition assays, a 25- and 50-fold excess of cold double-stranded specific and unrelated (UNR) oligonucleotide, shown in Table 1, was incubated with the nuclear extracts before addition of labeled oligonucleotide. For supershift assays, 20 and 50 μg of nuclear extracts were incubated with specific antibodies against HnRNP-U (Santa Cruz Biotechnology, Santa Cruz, CA) and Parp-1 (Chemicon, Temecula, CA).

2.4. Affinity purification of nuclear factors

The biotinylated oligonucleotides corresponded to the region –514 to –475 of the apoD promoter (SRE1-EBS-GRE) or carried mutations on the cis elements; SRE1 and EBS-GRE. As negative control, the IRU5Cas sequence from the Cas-Br-E retrovirus was used (Table 1). Streptavidin beads (500 μg; Dynabeads M-280 Strepavidin, Dynal Biotech, Oslo, Norway) were resuspended and incubated with 1500 pmoles of biotinylated oligonucleotides for 15 min in the buffer W&B (1×) (10 mM of Tris–HCl, pH 7.5, 1 M of NaCl and 1 mM of EDTA) as recommended by the manufacturer. The washing steps were done in the same buffer and the beads linked with the biotinylated oligonucleotides were incubated for 15 min at room temperature with 500 μg of nuclear extracts containing 20 μl of annealing buffer 10× (50 mM of Tris-HCl, pH 7.5, 1 M of NaCl, 100 mM of $MgCl_2$, 0.2 mM of EDTA and 1 mM of DTT), 1 mg of BSA and 20 μg of PolydI:dC in a final volume of 200 μl. The beads were washed with a low salt washing buffer (20 mM Tris–HCl, pH 8.0, 0.05% NP-40, 1 mM EDTA and 75 mM KCl) and eluted with 30 μl of SDS 0.1%. The proteins were loaded on a SDS-PAGE (12%) and migrated at 100 V for 1 h followed by silver nitrate staining or western blot analysis. For the silver nitrate

Table 1
Oligonucleotides used for the binding assay in EMSA and the streptavidin-biotin Technique.

Oligonucleotide	Biotinylated Sequence (sens)
SRE1-EBS-GRE	5′-ATTACCTGACTCCATTAGTGGAGTCAGGAACTCCATGTTCT-3′
Consensus SRE	⟷
Consensus CArG-box	⟷
Consensus EBS	⟷
Consensus GRE	⟷
SRE1-mEBS-mGRE	5′-ATTACCTGACTCCATTAGTGGAGTatcaAgCcCacatgaCT-3′
mSRE1-EBS-GRE	5′-ATTAtCaagCTCacATTAGTttAGTCAGGAACTCCATGTTCT-3′
EBS-GRE	5′-AGTCAGGAACTCCATGTTCT-3′
UNR (unrelated sequence)	5′-CCAAACAGGATATCTGTAATAAGCAG-3′
IRU5Cas	5′-CGCCTCGGGGGTCTTCATT-3′

*Lower case letters, mutated nucleotides.

gel, bands of interest were excised and analyzed by mass spectrometry (LC-MS/MS) at the Genome Québec Innovation Centre Proteomics Platform (Montreal, Qc, Canada) for protein identification.

2.5. Western blot

Protein extracts were eluted from the streptavidin–oligo complex, heated for 10 min at 70 °C and loaded onto a 12% SDS-polyacrylamide gel. The gel was subjected to electrophoresis for 1 h at 100 V and then transferred by electroblotting to a polyvinylidene difluoride (PVDF, Millipore, Bedford, MA) membrane for 45 min at 300 mA. Membranes were blocked for 1 h at room temperature with blocking buffer (PBS containing 0.2% Tween 20 and 5% skim milk powder) and incubated overnight at 4 °C with anti-mouse Parp-1 (1:1000), anti-mouse BUB-3 (1:1000; BD Transduction Laboratories, Mississauga, Ontario), anti-mouse APEX-1 (1:1000; Santa Cruz Biotechnology), anti-rabbit Kif4 (1:500; Imegenex CEDERLANE Laboratories, Hornby, Ontario), anti-rabbit ERK1/2 (1:1000; Cell Signaling Technology, Danver, MA) or anti-rabbit phospho-ERK1/2 (1:1000; Cell Signaling Technology) polyclonal antibodies in the blocking buffer. Subsequently, the blots were incubated at room temperature with a secondary horseradish peroxidase conjugated anti-mouse or anti-rabbit antibody (1:5000; GE Healthcare Bio-Sciences, Little Chalfont, UK) in the blocking buffer. The blots were developed using the enhanced chemiluminescence method with Kodak X-Omat films (Perkin Elmer) with X-ray film.

2.6. Semi-quantitative RT-PCR and siRNA transfections

Total RNA was isolated from cells using RNeasy Mini Kit (Qiagen, Mississauga, Ontario). RNA was reverse-transcribed using Omniscript Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. The reverse transcriptase products (20 ng) were used for amplification of apoD, HnRNP-U, Parp-1, BUB-3, Kif4, Ifi204, APEX-1 or GAPDH genes using specific primers shown in Table 2. The ratio obtained from sparse cultures at day 0 (D0) was given an arbitrary value of one. All reactions were done in triplicate. For the siRNA experiments, only 5 ng of the reverse transcriptase products were used. Two pre-designed siRNA were used per gene: (HRNPU: Mm_Hnrpu_1 HP siRNA or Mm_Hnrpu_5 HP siRNA; Parp-1: Mm_Parp1_3 HP siRNA or Mm_Parp1_4 HP siRNA; Qiagen) and negative controls siRNAs (Allstar negative control, Qiagen) were included in each experiment. All transfections were done twice in triplicate.

2.7. Luciferase assays

The cDNA of Parp-1, HnRNP-U, Ifi204, BUB-3 and APEX-1 were cloned in pcDNA3.1A expression vector (Invitrogen, Burlington, Ontario). Mutants of APEX-1 and Parp-1 were created by PCR directed mutagenesis using specific primers listed in Table 2. The mutants of APEX-1 were named *C64A/C92A, K5R/K7R, D218A* and Parp-1 lacking its catalytic domain: *del-Parp-1*. The mutated cDNA were cloned in pcDNA 3.1A in fusion with myc tag. Each plasmid was transiently co-transfected in NIH/3T3 cells in the presence of Polyfect (Qiagen) as recommended by the manufacturer, with a vector containing the apoD promoter upstream of the luciferase reporter gene (−558/−4-apoD-Luc). Twenty-four hours later, cells were rinsed twice with phosphate-buffered saline and maintained in a medium supplemented with 10 or 0.5% serum. Transfected cells were lysed 48 h later and luciferase activity was measured with the Dynex MLX microplate luminometer (MLX Dynex Technologies, Chantilly, VA) as already described [28]. Each sample was co-transfected with the β-gal-expressing vector for normalization (pRSVβ-GAL). The β-galactosidase activity was measured using the Galacto-Light™ kit (Applied Biosystems, Bedford, MS) according to manufacturer's suggestions. Luciferase activity is presented in Relative Light Units (RLU) and represents the calculated mean ± SD of nine transfected samples normalized by the measured β-galactosidase activity.

2.8. Statistical analysis

Statistical significance of the experiments was evaluated using an unpaired Student's *t*-test. Results were considered statistically significant at $P<0.05$.

3. Results

3.1. Purification and identification of nuclear factors binding the apoD promoter

To isolate the nuclear factors that bind to the apoD promoter, nuclear extracts from NIH/3T3 cells cultured under normal (10% serum; NE+) and serum-starved (0.5% serum; NE−) conditions were incubated with a biotinylated oligonucleotide linked to streptavidin beads. This oligonucleotide contained the SRE1 with a CArG-Box core, as well as the EBS and the GRE sites of the apoD promoter, shown in Table 1 and named SRE1-EBS-GRE herein. The proteins were eluted from the stretpavidin–oligo complex and visualized by SDS-PAGE and

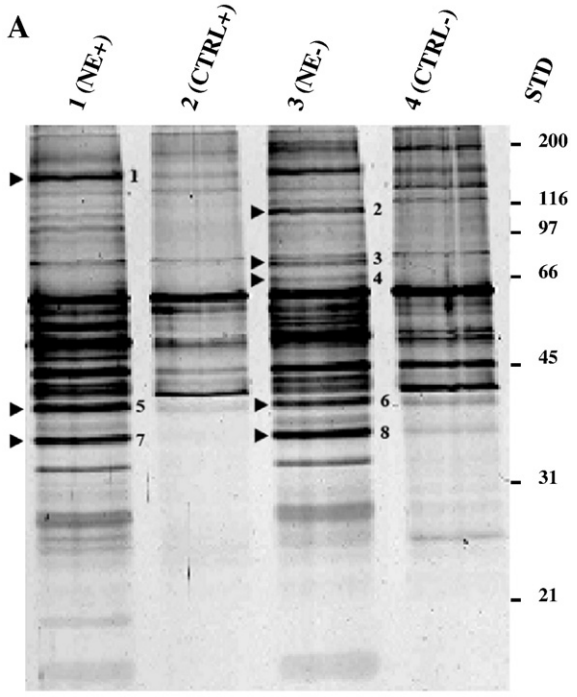
Table 2
Primers used for gene expression analysis by RT-PCR and mutagenesis.

Gene	Primers sequence forward	Primers sequence reverse
Primers for RT-PCR		
ApoD	5'-CCACCGGCACCCTACTGGATC-3'	5'-CGGGCAGTTCGCTTGTTGATCTGT-3'
HnRNP-U	5'-GAAGAAGAAGACGAACACTTCGA-3'	5'-AAAATTTCGCTTCTTACGGGCAG-3'
Parp-1	5'-ATGGCGGAGGCCTCGGAGAG-3'	5'-TCTGGATCCACCATCTTCTTGGACAG-3'
BUB-3	5'-ATGACCGGTTCGAACGAATTCAA-3'	5'-TTACGTGGACTTGGGCTTTGTTT-3'
Kif-4	5'-ATGAAAGAAGAGGTGAAGGGGA-3'	5'-GTAGCTTTCTCACGAGATGAAC-3'
Ifi204	5'-ATGGTGAATGAATACAAGAGAATTG-3'	5'-TCCAGTGATGTTTCTCCTCTTA-3'
APEX-1	5'-ATGCCAAAGCGGGGAAAGAAAG-3'	5'-CAACGCTGTCGGTATTCCAG-3'
GAPDH	5'-GGTGAAGGTCGGTGTGAACGG-3'	5'-ATGGGGGCATCGGCAGAAGG-3'
Primers for mutagenesis		
Del-Parp-1	5'-GTT**AAGCTT**GCAGCACGAGAAGGAGGATGGC-3' (forward)	
Del-Parp-1	5'-CAGCAGCCTCCGG**TCTAGA**AAGCTGCA-3' (reverse)	
APEX-1	5'-GTT**AAGCTT**GGGCTTTCGTCACAGCGATGCC-3'(forward)	
APEX-1	5'-GCTT**TCTAGA**CAGTGCTAGGTAAAGGGTGATG-3' (reverse)	
*C64A	5'-CCACACTCAAGATA**gc**CTCCTGGAATGTGG-3'	
*C92A	5'-ACCAGATATCTTG**gc**CCTCCAACACACCAA-3'	
*K6R/K7R	5'-GTT**AAGCTT**GGGCTTTCGTCACAGCGATGCCAAAGCGGGGAAgGAgA-3'	
*D218A	5'-ATGAAGAAATTG**c**CCTCCGTAACCCCAAAG-3'	

*, amino acid mutated in APEX-1 gene; lower case letters bold, mutated nucleotide; upper case bold, restriction endonuclease site (forward, *Hin*dIII; reverse, *Xba*I).

silver staining (Fig. 1A). Bands were selected for mass spectrometry analysis based on their stronger intensity in NE (NE+, NE−) compared to those obtained from streptavidin beads without biotinylated oligonucleotide used as negative controls (CTRL+, CTRL−). The corresponding bands in the negative controls were also analyzed in order to eliminate false-negatives. Several nuclear proteins were identified by correlating the mass of their tryptic peptides with the theoric values found in NCBInr database through the Mascot server (www.matrixscience.com) and listed in Fig. 1B.

Five of these proteins were known to interact directly or indirectly with regulatory elements in the promoter of several genes and to modulate their expression, notably, *Heterogeneous nuclear ribonucleoproteins* (HnRNP)-U [37] and HnRNP-A/B also known as *CArG-box Binding Factor A* (CBF-A) [38,39], *Poly(ADP-ribose) polymerase-1* (Parp-1) [40], *Interferon-inducible protein 204* (Ifi204) [41] and *Apurinic/Apyrimidinic Endonuclease-1* (APEX-1) [42,43]. *Kinesin family member 4* (Kif4) and *Budding Uninhibited by Benzimidazole* (BUB)-3 were also considered because of their role in cellular proliferation and their interaction with Parp-1 [44,45]. As shown in Fig. 1A, CBF-A, BUB-3 and APEX-1 were purified in both normal and growth arrest conditions. In growth conditions, the two known isoforms of CBF-A (p37 & p42) [46] were co-purified (lane 1; bands 5 and 7) in contrast to growth arrest conditions where only the p42 isoform was purified (lane 3; band 6). HnRNP-U, Parp-1 (band 2), Ifi204 (band 3), Septin 9 and *Kinesin family member C1* (KifC1) (band 4) were exclusively present in growth arrest conditions while Kif4 (band 1) only in normal growth conditions. Several other band differences were observed but the protein concentration was under the mass spectrometry limit of detection.



B

Band No. on the gel	Protein identification	Score	Tryptic peptides	Accession No. (NCBInr)
1 (NE+)	Kif4	68	1	gi|6680568
	HnRNP U	244	5	gi|7949051
2 (NE-)	Parp-1	153	3	gi|15080598
	Ribosome-binding protein 1	195	3	gi|23822106
3 (NE-)	Ifi204	205	3	gi|109501
4 (NE-)	Septin 9 protein	248	3	gi|28204888
	Kinesin family member C1	151	2	gi|4050097
5 (NE+)	HnRNP A/B or CBF-A (p42)	243	4	gi|6754222
	BUB-3	97	1	gi|5020213
6 (NE-)	HnRNP A/B or CBF-A (p42)	199	3	gi|6754222
	BUB-3	271	5	gi|5020213
7 (NE+)	APEX-1	201	1	gi|6753086
	HnRNP A/B or CBF-A (p37)	104	2	gi|6754222
8 (NE-)	APEX-1	200	2	gi|6753086

Fig. 1. Purification of nuclear proteins binding to the SRE1-EBS-GRE elements of the human apoD promoter. (A) Nuclear extracts from normal growth (10% serum: +) or growth arrest (0.5% serum: −) conditions were incubated with either the biotinylated oligonucleotide bound to streptavidin beads (NE) or streptavidin beads alone (CTRL). The bound proteins were eluted and analyzed by SDS-PAGE and silver staining. The numbers and arrows on the gel indicate the excised bands. (B) Mass-spectrometry analysis of the eluted bands. Nuclear factors were identified by mass spectrometry (LC-MSMS) after tryptic digestion. STD: Standard molecular weight in kDa; this experiment was done in duplicate.

3.2. PARP-1, HnRNP-U, Kif4, BUB-3 and APEX1 bind to the apoD promoter in vitro

In order to confirm the binding specificity of the selected proteins on the apoD promoter, we performed an electrophoretic mobility supershift assay (EMSA) using commercially available antibodies against these proteins. The same oligonucleotide as that used above for the biotin–streptavidin purification (Table 1) was radiolabeled and incubated with nuclear extracts from serum-starved NIH/3T3 cells. As shown in Fig. 2A, we observed several protein-DNA complexes (CTRL+) compared to the negative control (CTRL−). In the presence of an antibody directed against HnRNP-U, the complex corresponding to band II disappeared (indicated with an asterisk). Competition assay using cold (SRE1-EBS-GRE) and unrelated (UNR) oligonucleotides in 25 and 50 molar excess demonstrated the specificity of formed complexes. In contrast, for Parp-1, 50 μg of nuclear extract were required in order to detect the supershift. Four bands could be detected but the two major (bands II and III) disappeared in favor of a supershifted band (Fig. 2A, arrow). Although, the binding specificity of these complexes was not verified in this experiment with cold oligonucleotide excess, the intensity of band IV was increased in the presence of 0.25 μg of Parp-1 specific antibodies and reduced progressively with increasing amounts of antibodies (0.5 to 2 μg).

The antibodies directed against Kif4, BUB-3 and APEX-1 failed to supershift the DNA-complexes observed (data not shown). However, in a pull-down assay using biotinylated oligonucleotides containing regulatory consensus sequences that were either intact or mutated or deleted (Table 1), we observed by western blot that Kif4 was preferentially bound to the SRE1 in growth conditions (Fig. 2B). In growth arrest conditions, BUB-3 did not bind the SRE1-mEBS-mGRE but was specifically bound to the mSRE1-EBS-GRE and the EBS-GRE where the SRE1 had been mutated or deleted, respectively. APEX-1 could bind both the SRE1-mEBS-mGRE and the mSRE1-EBS-GRE but not the EBS-GRE.

3.3. mRNA expression of nuclear factors in growth-arrested NIH/3T3 cells

We next analyzed the mRNA expression of these nuclear factors in NIH/3T3 fibroblasts to determine if they were also modulated by growth arrest as the apoD gene. Cells were collected in growth conditions and at different times after serum deprivation. Using specific primers for the different genes (Table 2), their expression level was analyzed by semi-quantitative RT-PCR and normalized to that of GAPDH gene expression. As expected, apoD mRNA expression was significantly induced in serum starvation conditions (Fig. 3). Expression levels of Parp-1 were also induced by 2.1-fold after 1 day of serum starvation and reached a peak at day 2 with a 3-fold induction to finally decrease until day 5. This induction was even stronger by Northern analysis (up to 9 fold at day 2). Ifi204 and APEX-1 were also induced after 2 days of serum starvation by respectively 2.8 and 1.8 fold. In contrast, Kif4 mRNA expression was down-regulated after 2 days of serum starvation which is consistent with the

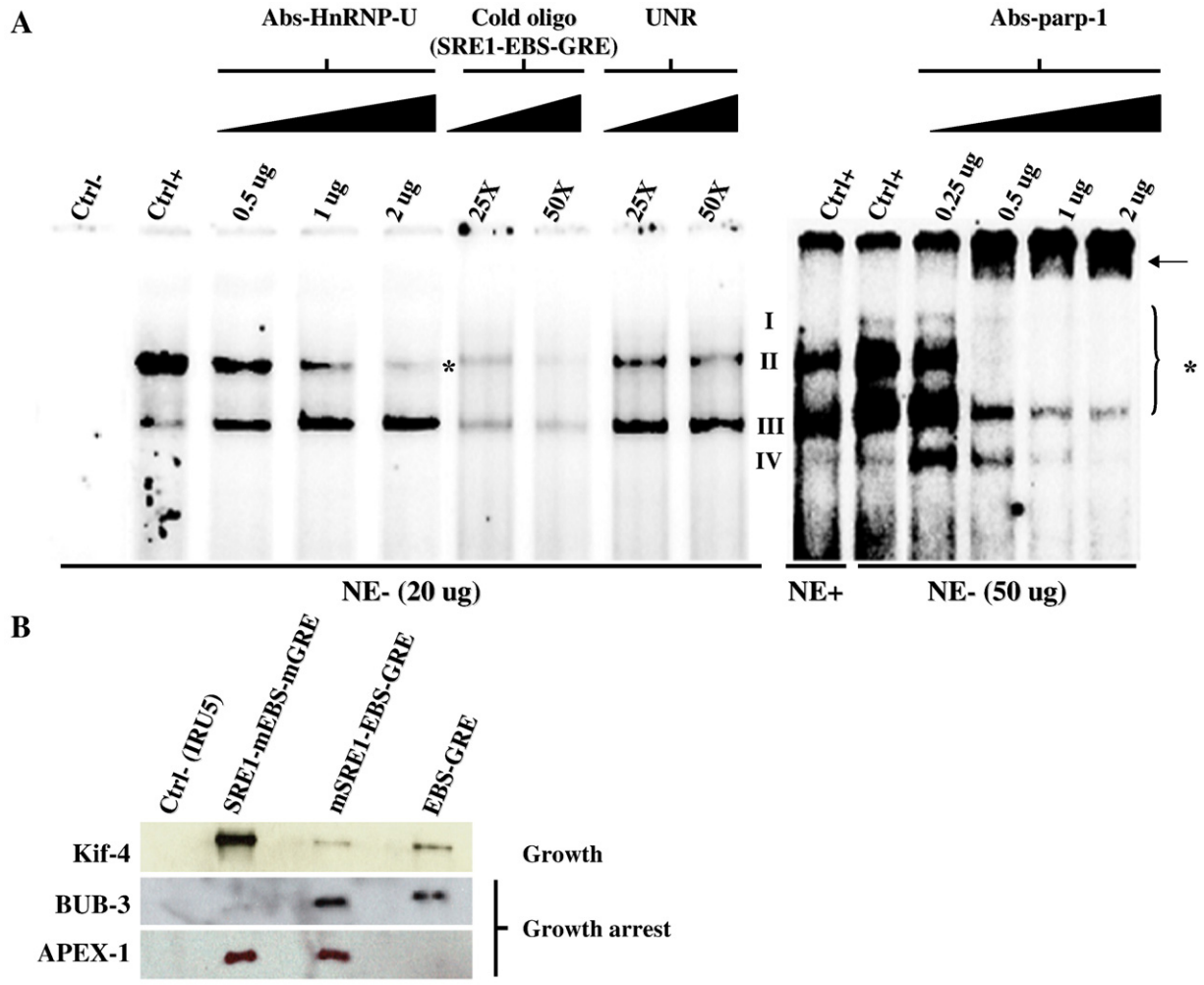


Fig. 2. Binding of HnRNP-U, PARP-1, Kif4, BUB-3 and APEX-1 to the apoD promoter. (A) Nuclear extracts from 5-day serum-starved NIH/3T3 mouse fibroblasts were used in electrophoretic mobility assay with double stranded oligonucleotides containing the SRE1-EBS-GRE of the apoD promoter. 20 µg (HnRNP-U) and 50 µg (Parp-1) of nuclear extracts were incubated in the presence of 0.8 ng of radiolabeled probe and different amounts of specific antibodies. Unlabeled competitor nucleotides were added at 25- and 50-fold molar excess. UNR: (unrelated) non-specific competitor; NE: nuclear extracts; (+): growth condition; (−): growth arrest condition; CTRL: negative control (probe only); Abs: specific antibodies. The asterisks represent the disappearance of the DNA-complex and the arrow the supershifted DNA–protein complex. Representative of three similar experiments. (B) Biotin pull-down assay using different probes in which sequence of specific regulatory element was mutated or deleted as indicated (Table 1). The eluted proteins were then analyzed by western blot using specific antibodies against Kif4, BUB-3 and APEX-1. Sequence from the IRU5Cas LTR of Cas-Br-E retrovirus was used as negative control.

fact that Kif4 was only detected on the apoD promoter in growth conditions (Fig. 1A). In addition, HnRNP-U and BUB-3 were not modulated under serum starvation and seem ubiquitous.

3.4. Transactivation of the apoD promoter

To determine if these nuclear proteins are directly involved in the modulation of apoD gene expression, the corresponding cDNAs cloned in expression vectors were transiently co-transfected in NIH/3T3 cells with a construct containing the apoD promoter upstream of the luciferase reporter gene (−558/−4-apoD-Luc) and with pRSVβGAL vector. The luciferase activity was analyzed in normal and serum-starved conditions. In growth conditions (10% serum; Fig. 4A: white bars), none of the proteins tested had an effect on apoD promoter activity compared to the control (empty vector). However, after 2 days of serum starvation (0.5% serum; grey bars), Parp-1, HnRNP-U, APEX-1 and Ifi204 significantly activated the apoD promoter. In contrast, BUB-3 did not transactivate the apoD promoter.

To confirm these results, we created specific mutations on APEX-1 gene on cysteines 64 and 92 which were replaced by alanine (*C64A*/*C92A*). These cysteines are known to be implicated in the redox activity of APEX-1 which modifies and modulates the activity of several transcription factors [47]. Lysines 6 and 7, located in the nuclear localization signal motif (NLS) and known to be acetylated by p300 were replaced by arginine (*K6R*/*K7R*). This double mutation abrogates the redox activity of APEX-1 without affecting its nuclear localization [48]. Finally, aspartic acid 218 which is known to be important for the endouclease and DNA-binding activities [49] was replaced by alanine (*D218A*). As shown in Fig. 4A, all these mutants were unable to transactivate the apoD promoter in growth arrest conditions in contrast with the wild type APEX-1. Interestingly, mutants *C64A*/*C92A* and *K6R*/*K7R* induced the proliferation of NIH/3T3 cells cultured in condition of serum starvation compare to the wild-type APEX-1 or the control (CTRL−; empty vector) where the cells were maintained in a growth arrest state (Supplemental Fig.1). In addition, the Parp-1 deletion mutant lacking its catalytic domain (del-Parp-1) could no longer transactivate the apoD promoter indicating the importance of the catalytic function in the apoD promoter regulation. Furthermore, we tested the role of Parp-1 and HnRNP-U on the apoD expression using a siRNA approach. The pre-designed siRNAs used to block either HnRNP-U or Parp-1 inhibited their target gene expression by more than 80% up to 4 days under growth arrest (grey bars; Fig. 4B) and efficiently and specifically blocked apoD expression (grey bars; Fig. 4C).

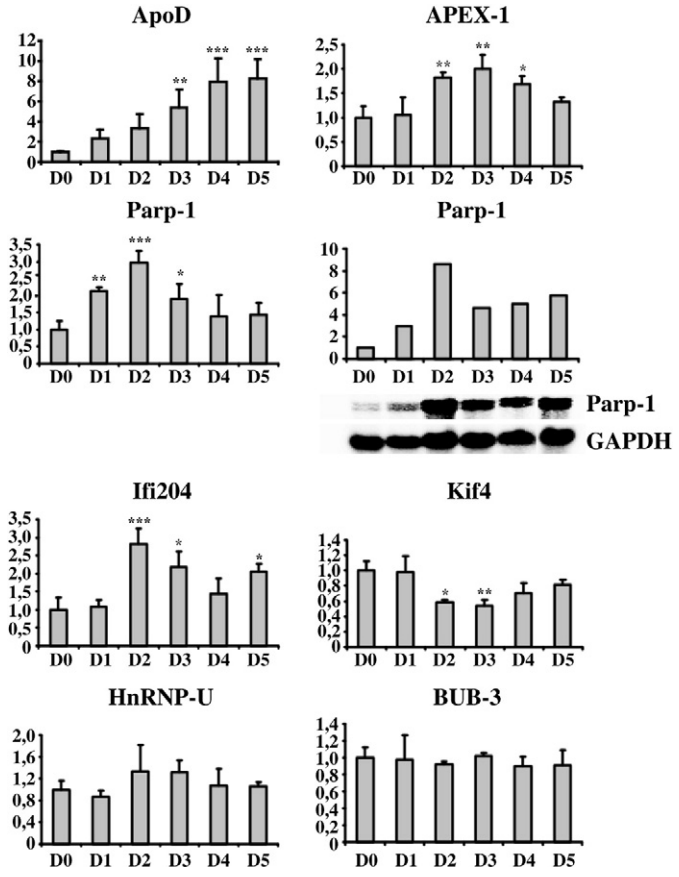


Fig. 3. Analysis of mRNA expression of nuclear factors in NIH/3T3 growth arrested cells. Semi-quantitative RT-PCR using specific primers for the indicated genes (Table 2). The expression of each gene tested was normalized by GAPDH expression. ($N=3$, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$). An example of one Northern analysis is presented for Parp-1.

3.5. Effect of Parp-1 and MEK1/2 inhibitors on the apoD gene expression

To further investigate the role played by the catalytic activity of Parp-1 in the regulation of the apoD promoter during growth arrest, we used 3-Amino-Benzamide (3-ABA), a specific inhibitor of Parp-1. This inhibitor blocks the binding of NAD^+ to the catalytic domain of Parp-1. This binding is crucial for the transfer of ADP-ribose unit on nuclear proteins mediated by Parp-1 [50]. As expected, the inhibition of Parp-1 catalytic activity with 3-ABA at 10 mM completely abolished the stimulating effect of Parp-1 on the apoD gene expression in growth-arrested cells (Fig. 5A). This result was consistent with those obtained with the deletional mutant del-Parp-1 transfection experiment and the siRNA assay (Fig. 4A and C).

Furthermore, considering the important role played by the MEK/ERK pathway in cellular proliferation and differentiation as well as the interplay between ERK and Parp-1, which modulates their respective catalytic activity [51,52], we also analyzed the effect of a MEK1/2 inhibitor (U0126 at 10 μM) on the apoD gene regulation. Interestingly, the inhibition of MEK1/2, which phosphorylate ERK1/2, significantly reduced the apoD gene expression under growth arrest conditions (Fig. 5B). Complete inhibition of ERK1/2 phosphorylation in presence of U0126 was verified by western blot analysis (results not shown). Consistent with this result, upregulation of phosphorylated ERK1/2 was detected as early as 5 min after serum starvation without inhibitor, reached a peak at 15 min and decreased after 30 min (Fig. 5C). Surprisingly, at 24 h post serum starvation, ERK1/2 were again phosphorylated. Moreover, in the presence of Parp-1 inhibitor, activation of ERK2, but not ERK1, is maintained after 15 min for up to 2 h (Fig. 5D). Subsequently, ERK2 phosphorylation was completely inhibited after 2 h in the presence of Parp-1 inhibitor which is consistent with the apoD gene downregulation observed in Fig. 5A and B.

4. Discussion

The apoD promoter was previously characterized and its complexity is reflected by the numerous cis elements located within its promoter [28,53]. Using a biotinylated oligonucleotide containing the SRE1, EBS and GRE elements of the apoD promoter, we were able to purify and identify several nuclear proteins by mass spectrometry analysis. Among these proteins, HnRNP-U and CBF-A (Fig.1A), which belong to the Heterogeneous nuclear ribonucleo-protein family, are also known to interact with elements in the promoter of several genes and to regulate their expression. Indeed, HnRNP-U is known as a transcriptional activator of Kruppel-like factor 2 gene [54] and of Osteopontine gene expression, which is also modulated by CBF-A [37]. CBF-A is also involved in the regulation of the Ha-ras gene expression [39]. The mRNA binding ability of these two proteins led us to speculate that they could be recruited at the promoter level and play a role in both the apoD mRNA expression and processing, in this context of growth arrest. All these different crucial roles of HnRNP-U may explain its ubiquitous gene expression profile in growth-arrested NIH/3T3 cells (Fig. 3). Binding of HnRNP-U to the promoter was further demonstrated by EMSA (Fig. 2A). The addition of HnRNP-U antibodies specifically reduced the complex intensity. This interaction with the SRE1-EBS-GRE, whether direct or indirect, is in agreement with previous reports showing that HnRNP-U can play a role in gene regulation via its interaction with the GRE site located in the promoter of the tryptophan oxygenase gene [55] and therefore suggests that HnRNP-U might interact with the apoD promoter through the GRE binding site.

Parp-1 is an abundant nuclear protein with a poly(ADP-ribosyl)-ation catalytic activity which uses NAD^+ as substrate to modify several nuclear proteins, such as transcription factors thus affecting their functions [40,56]. Although, several studies have shown its implications in DNA repair pathways, its DNA-binding and catalytic activities are also implicated in transcription regulation [40,56]. Despite the fact that Parp-1 is reported as a ubiquitous protein, we found that its gene expression is up-regulated in growth-arrested NIH/3T3 cells and we clearly demonstrated its binding to the apoD promoter, highlighting its importance in this stress condition. We were able to obtain a supershift by EMSA upon addition of a specific antibody coupled with the disappearance with the major bands II and III. Bands I and IV were very faint and detectable only when 50 μg of extracts were used. Both bands appeared more abundant in growth arrest conditions and seem specific since they disappeared in the presence of Parp-1 antibodies (Fig. 2A).

In agreement with our results, Parp-1 can be activated by its binding to conformational or non-B DNA structures such as cruciforms or hairpins [57,58]. More importantly, cruciforms can be formed within a sequence that contains inverted repeats with the appearance of duplex arms. The SRE was already reported to be able to form cruciforms which resemble the Holliday junction [59]. Moreover, several studies demonstrated the interaction of Parp-1 with DNA in a sequence-specific manner. Tapia-Paez and collaborators [60] reported the binding of Parp-1 to a SRE-CArG-like element. Parp-1 was also shown to bind a recently named Parp-1-binding element (PBE) [61] whose consensus sequence resembles that of the GRE.

We were not able to confirm binding by EMSA for the other selected proteins for which specific antibodies were commercially available most likely because the recognized epitope is hidden in the protein native form. However, using the biotin pull-down approach followed by western blot analysis, we confirmed that these proteins effectively bind the region of the promoter containing the SRE1, EBS and GRE sites (Fig. 2B). Obviously, BUB-3 does not recognize the SRE1 since the protein still binds an oligo lacking the SRE1 or an oligo in

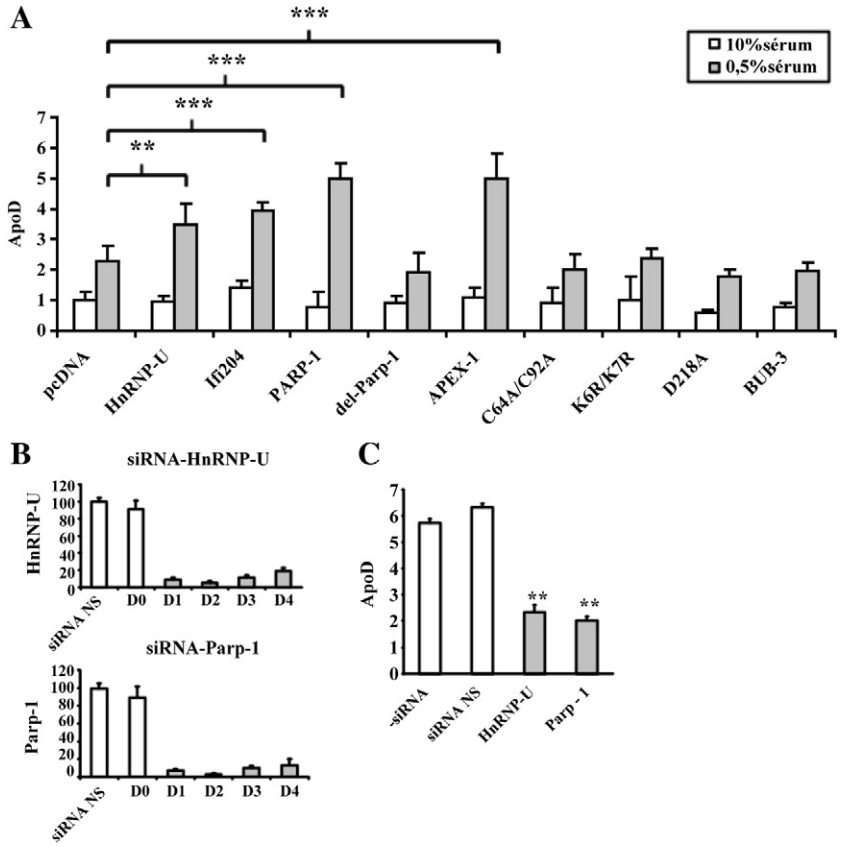


Fig. 4. ApoD mRNA induction in growth-arrested cells and siRNA analysis. (A) NIH/3T3 cells were co-transfected with vectors containing a luciferase reporter construct under the 5'-flanking region (−558/−4) of the apoD promoter gene, the cDNAs corresponding to the identified proteins or APEX-1 cDNA carrying mutations (C64A/C92A, K5R/K7R and D218A) and the plasmid pRSVβGAL. After transfection, cells were cultured in media with (10%; white bars) or without serum (0.5%; grey bars) for 2 days. The luciferase activity was analyzed and normalized by the β-Galactosidase activity. The empty pcDNA 3.1 expression vector was used as control. The asterisks indicate statistically significant results ($N=9$, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$). Del-Parp-1 corresponds to the protein lacking its C-terminal end and its enzymatic activity. (B) NIH-3T3 cells were transiently transfected with siRNA directed against HnRNP-U and Parp-1 and exposed to serum-starved conditions for 4 days. Total RNA was extracted and analyzed by RT-PCR using specific primers for the HnRNP-U, Parp-1 gene and HPRT as control. (C) ApoD mRNA expression analyzed by RT-PCR in NIH-3T3 cells exposed to serum starved conditions for 4 days after siRNA suppression of HnRNP-U and Parp-1 genes. As negative controls, transfections with non-specific siRNA (siRNA NS) or without siRNA (−siRNA) were used.

which the SRE1 was mutated. It is worth mentioning that, by mutating the EBS, we also mutated the GRE as well as the SRE1 because these sites are superimposed (see Table 1). Moreover, BUB-3 did not modulate the apoD promoter (Fig. 4A) and the gene was not activated under growth arrest (Fig. 3).

Based on our results, Kif4 binds the SRE1 most probably through its CArG-box core since it only binds the intact SRE1 (Fig. 2B) and as already mentioned above, most of the mutated EBS consensus sequence is included within the inverted repeat of the SRE1. Kif4 is a member of the kinesin superfamily of proteins known to transport cargos of macromolecules along the microtubules of cells, such as membrane organelles, protein complexes and mRNAs [44,62]. The Kif4 nuclear localization was shown to be regulated by its interaction with Parp-1 in juvenile neurons. In addition, Kif4 can repress Parp-1 enzymatic activity in the nucleus of NIH/3T3 cells in normal culture conditions [44]. Thus, the apoD gene expression could be modulated by the interaction of these two proteins. In our study, Parp-1 could not be purified in normal growth conditions suggesting that the possible interaction of Parp-1 and Kif4 in NIH/3T3 cells, reported by Midorikawa and collaborators [44], may inhibit Parp-1 binding on the apoD promoter. Therefore, the presence of Parp-1 on the apoD promoter in growth arrest conditions may be favored by both the down- and upregulation in gene expression of Kif4 and Parp-1, respectively (Fig. 3).

In contrast, APEX-1 binds the promoter but binding persists despite the mutations of each consensus sequence (Fig. 2B). However, the deletion of the SRE1 abolished the binding. This implies that APEX-1 probably binds the apoD promoter through a greater affinity for the mutated sequences or through the interaction with other proteins, such as Parp-1, that bind either the SRE1 or the EBS-GRE sites. APEX-1 is a multifunctional protein implicated in DNA repair through the base-excision repair (BER) pathway, where Parp-1 was also shown to be involved but more importantly, APEX-1 is well known for its role in transcription regulation through a redox activity. Indeed, the redox activity of APE1 was shown to modify and activate several stress-inducible transcription factors such as AP-1, NF-κB, p53, Egr-1, CREB, ATF and HIF-1α [47,63]. The fact that APEX-1 gene expression was found induced in growth arrest conditions may also indicate its importance in such stress conditions (Fig. 1B). Also, APEX-1 was shown to be induced in several stresses such as oxidative conditions, cancers [47] and several neurodegenerative disorders [63]. It is interesting to note that such stresses including H_2O_2 and UV induce apoD gene expression as well as the catalytic activity of Parp-1 [27,64,65]. Therefore, it would be of interest to investigate if Parp-1 and APEX-1 also modulate the apoD gene expression under these conditions and will definitely clarify the mechanism of regulation of the gene. Indeed, apoD is considered as a stress protein, and we demonstrated that it plays a protective effect for neurons of mice treated with paraquat [65]. A similar protection against oxidative stress was observed in Drosophila and plants [65,66]. Protection was also observed in mice infected with OC43, a coronavirus that induces brain inflammation and neurodegeneration [64].

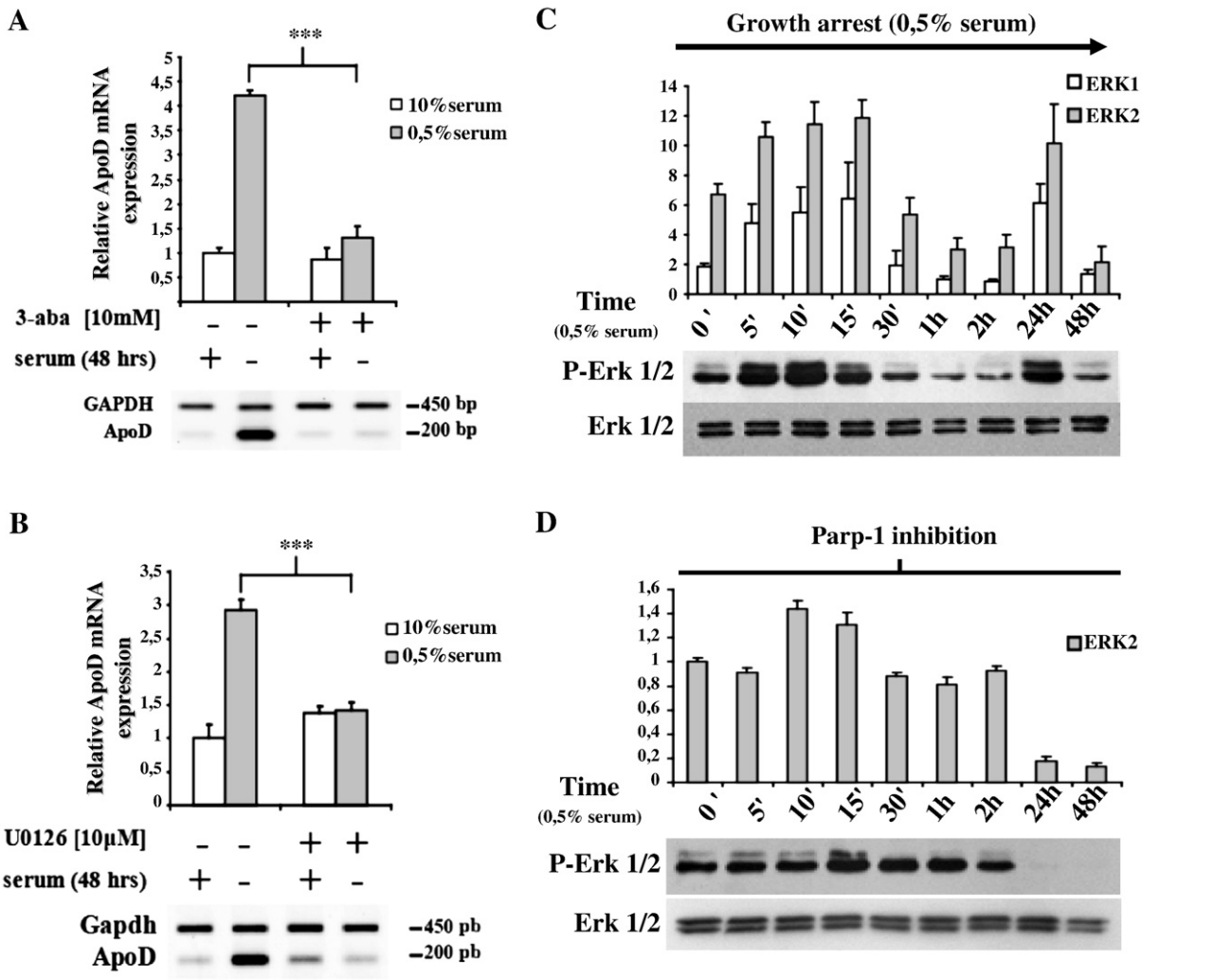


Fig. 5. Analysis of apoD mRNA expression in presence of Parp-1 and MEK1/2 inhibitors. NIH-3T3 cells were cultured under normal (10% serum, +) and serum starvation (0.5% serum, −) conditions for 48 h with or without Parp-1 specific inhibitor: 3-Amino-Benzamide (3-ABA, 10 mM) (A) or MEK inhibitor: U0126 (10 µM) (B). Total RNA was extracted and the expression of apoD gene was analyzed by RT-PCR. The GAPDH gene was used as internal control. The asterisk indicates statistically significant results ($N=4$, $^{***}P<0.001$). Western blots of the phosphorylation levels of ERK1/2 in NIH/3T3 cells under serum starvation at different time points (C) and in the presence of Parp-1 inhibitor (D) (representative of three similar experiments).

Ifi204 is a 72-kDa phosphoprotein and a member of the interferon-inducible p200 (Ifi-200) family of proteins induced by interferons (IFNs) [67]. Due to the lack of specific antibodies against Ifi204, it was not possible to confirm its binding to the apoD promoter by EMSA. However, the protein was clearly identified with a high score in the streptavidine beads technique in extracts from growth arrest conditions (Fig. 1B). More importantly, Ifi204 expression was induced under serum starvation (Fig. 3). These results are consistent with the known interaction of Ifi204 and the Retinoblastoma protein (pRb) which both inhibit proliferation. Also, mutations in Ifi204 confer malignant transformation properties in NIH/3T3 cells [67,68].

In the transactivation assay, we showed that Parp-1, HnRNP-U, APEX-1 and Ifi204 had a positive effect on the apoD promoter in growth arrest conditions only (Fig. 4A). The positive effect of Parp-1 and HnRNP-U was also confirmed by the knock down of their expression with specific siRNA. Their inhibition totally abolished the apoD gene induction under serum starvation conditions (Fig. 4C). Moreover, we further demonstrated the importance of Parp-1 and APEX-1catalytic activities in the induction of apoD gene expression in growth-arrested cells. These results were observed either by deleting the C-terminal portion containing the catalytic domain of Parp-1 (Fig. 4A) or by inhibiting its catalytic activity with a specific inhibitor 3-ABA (Fig. 5A). For APEX-1, both its redox and DNA-binding activities were necessary to transactivate the apoD promoter and we demonstrate a link between APEX-1 redox activity and the cell cycle regulation since inhibition of APEX-1 activity promotes cell proliferation under serum starvation (Supplemental Fig. 1). This is consistent with the strong relation between the redox cycle and cell cycle regulation [69,70].

We also found that the inhibition of MEK1/2 blocks apoD gene induction by growth-arrest after 2 days showing the importance of this pathway in the regulation of apoD gene expression. This is surprising since ERK1/2 activation is essential for cell cycle progression from G1- to S-phase by downregulating several antiproliferative genes [71,72]. However, ERK1, but not ERK2, expression in fibroblasts inhibits oncogenic Ras-mediated proliferation, colony formation as well as tumor production in nude mice [73]. More importantly, the B-Raf isoforms can induce cell-cycle arrest in NIH/3T3 cells [74]. In this study, we showed biphasic ERK1/2 activation in time in growth-arrested NIH/3T3 cells, reaching a peak at 15 min, decreasing after 30 min and activated again 24 h later (Fig 5C). In addition, ERK2 remains phosphorylated for up to 2 h in the presence of Parp-1 inhibitor which blocks apoD expression (Fig. 5D). This result implies that ERK1/2 activation is necessary for apoD gene induction. This regulation of ERK1/2 phosphorylation in growth arrest conditions could be mediated by Parp-1 activity. Further investigations are necessary to verify if ERK1/2 are effectively ADP-(ribosyl)ated in

stress conditions and also to determine if ERK1/2 kinase activity is directly involved in the regulation of the nuclear factors identified herein.

In conclusion, we identified several proteins that bind the apoD promoter through the SRE1, EBS and GRE elements and regulate its gene expression in growth arrest conditions. Our results also highlight the implication of the MEK/ERK pathway in the apoD regulation, itself modulated by Parp-1 catalytic activity. They demonstrate a central role played by Parp-1 and APEX-1, both through their binding to the apoD promoter and their catalytic activity during this process.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamcr.2010.04.011.

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