Alterations in expression of E2F-1 and E2F-responsive genes by RB, p53 and p21$^{Sdi1/WAF1/Cip1}$ expression

Keizou Ookawa[a,*], Shigeki Tsuchida[a], Takashi Kohno[b], Jun Yokota[b]

[a] *Second Department of Biochemistry, Hirosaki University School of Medicine, Zaifu-cho 5, Hirosaki, Aomori 036-8562, Japan*
[b] *Biology Division, National Cancer Center Research Institute, 1-1, Tsukiji 5-chome, Chuo-ku, Tokyo 104-0045, Japan*

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Abstract RB, p53 and p21$^{Sdi1/WAF1/Cip1}$ interact in the induction of G1 arrest. We established osteosarcoma cell lines in which a tetracycline-regulatable promoter controls the induction of RB, p53 and p21. By using these cell lines, we investigated whether RB, p53 or p21 regulates, in the same manner or differently, expression and function of E2F-1 and its responsive genes. E2F-1 gene products and transcripts of the E2F-responsive genes decreased in response to RB. Similar changes occurred to p53 and p21 when RB is present. However, in the absence of RB, some of the E2F-responsive genes decreased in response to p53 but not to p21. Thus, RB is a critical component for regulating the E2F-responsive genes, while p53 alone affects only a subset of these genes.

Key words: E2F-1 expression; RB; p21; p53

1. Introduction

E2F-responsive elements are present in the promoters of cell cycle-related genes, including E2F-1's promoter [1–8]. Unphosphorylated RB protein binds to E2F-1 gene product [9,10], inhibiting E2F-1 activity [11–13] and inducing G1 arrest [14]. The level of unphosphorylated RB gene product is thought to be critical for entering the G1 checkpoint [15–18]. On the other hand, the levels of E2F-1 mRNA and protein fluctuate in the cell cycle [7,10] under complex modulation mechanisms. Transcription of the E2F-1 gene is regulated by E2F DNA-binding sites within its promoter region [7,8], therefore, E2F-1 expression can also be repressed by RB [19,20]. However, by binding E2F-1 gene product, RB also protects E2F-1 protein from degradation [21,22]. Recently, it has been demonstrated that p53 also binds to E2F-1 and inhibits E2F-1 transcriptional activity [23]. In addition, p21, a cyclin-dependent protein kinase inhibitor and a potential mediator for p53-induced growth suppression [24,25], also inhibits the E2F activity in the cells either expressing or lacking functional RB protein [26,27]. These findings suggest that transcription of the E2F-1 gene can also be independently repressed by p53 and p21. However, alterations of E2F-1 transcription and protein expression by RB, p53 and p21 have not been demonstrated.

Defects in the p53 and/or RB signaling pathways are frequently detected in human cancer [28]. To assess the biological significance of RB and p53 inactivation in osteosarcoma, we previously established several osteosarcoma cell lines in which the RB and p53 expressions were placed under tetracycline (Tc) regulatory control [29]. Studies using these derived cell lines demonstrated that RB induced differentiation, p53 caused apoptosis, and either induced cell cycle arrest. The results also indicated the presence of a pathway for p53-induced cell cycle arrest independent of RB. However, it remains unclear whether E2F-1 is an intermediate in the pathway of p53-induced cell cycle arrest. To clarify the mechanisms of E2F-1 regulation by p53 and RB, we analyzed the effects of RB, p53 and p21 expression on E2F-1 and E2F-responsive genes, using a Tc-regulated inducible expression system. Here, we demonstrate that the E2F-1 gene product decreased in response to RB, but not to p53 or p21 in the absence of RB, and that transcripts of the E2F-responsive genes were decreased by RB, while only a subset of responsive genes were reduced by p53 in the absence of RB.

2. Materials and methods

2.1. Plasmids and cDNA probes

Plasmids pTA-Hyg, pT2Xp53neo, pT2p21/neo and pT2p16neo were constructed as described previously [29,30]. pUHD13-3, a Tc-responsive trans-activator (tTA)-responsive luciferase reporter construct [31], was provided by Dr. H. Bujard. E2F-1 cDNA probe was provided by Dr. H. Matsushime. cDNA probes for B-MYB, dihydrofolate reductase (DHFR), thymidylate synthase (TS) and glyceraldehyde-3-phosphate dehydrogenase were generated by reverse transcription-polymerase chain reaction.

2.2. Cell culture

The cells from human osteosarcoma cell lines Saos-2 and U-2 OS (American Type Culture Collection, ATCC) were grown in Dulbecco's modified Eagle's medium (Nissui) supplemented with 10% fetal calf serum at 37°C in a humidified 5% CO_2 atmosphere. Saos-2-derived STA-e clone [29] was maintained in media containing 0.3 mg/ml of hygromycin B (Calbiochem) and 1 μg/ml of Tc (Sigma). Saos-2- and U-2 OS-derived subclones described below were maintained in media containing 0.5 mg/ml of G418 (Gibco BRL-Life Technologies), 0.3 mg/ml of hygromycin B and 1 μg/ml of Tc.

2.3. Transfection

Saos-2-derived STA-e cells were transfected with pT2p21/neo using lipofectamine reagent (Gibco BRL-Life Technologies) according to the manufacturer's protocol. Cells were grown in a medium containing hygromycin B, G418 and Tc, and were cloned and expanded for further analyses.

U-2 OS cells were first transfected with pTA-Hyg. Cells grown in a

*Corresponding author. Fax: (81)-172-39 5205.
E-mail: kookawa@cc.hirosaki-u.ac.jp

Abbreviations: Tc, tetracycline; DHFR, dihydrofolate reductase; TS, thymidylate synthase; Ab, antibody; mAb, monoclonal antibody

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medium containing hygromycin B were cloned and tested for expression of tTA by transient transfection of pUHD13-3 in the presence or absence of 1 μg/ml Tc and subsequent luciferase assay. A clone with tTA expression was then transfected with pT2Xp53neo, pT2p21/neo or pT2p16neo. Cells grown in a medium containing G418, hygromycin B and Tc were cloned and expanded for further analyses.

2.4. Western blot analysis

Cells were lysed with lysis buffer as described elsewhere [29]. 50 μg of cellular protein was separated in an 8% or 12.5% SDS–PAGE and electroblotted to Hybond-ECL nitrocellulose membrane (Amersham). Equal loading of protein was confirmed by staining the membrane after detection. After blocking with 4% non-fat dry milk in Tris-buffered saline, the membranes were incubated for 2 h with appropriate antibodies (Abs). They were anti-p53 monoclonal Ab (mAb) PAb1801 (Oncogene Science), anti-RB mAb PMG3.245 (Pharmingen), anti-E2F-1 mAb KH20 (a gift from Dr. J.A. Lees), anti-p21 Ab C-19 (Santa Cruz Biotechnology) and anti-p16 Ab (Pharmingen). The blots were then probed on an ECL Western blotting detection system (Amersham). The signal intensity was measured on an image scanner (Agfa) with NIH Image (1.47) software or on a GS-525 Molecular Imager system (Bio-Rad) using a chemiluminescent substrate Super-Signal ULTRA (Pierce).

2.5. Northern blot analysis

Total RNA was isolated from the cells using a RNeasy™ Total RNA Kit (Qiagen). 12 μg of total RNA was electrophoresed and transferred to nitrocellulose filters. Equal loading of RNA was confirmed by comparing the intensities of stained bands for ribosomal RNA. The filters were hybridized with ^{32}P-labeled cDNA probes and exposed to Kodak XAR-5 or XLS-1 film at −80°C. The signal intensity was analyzed as described above.

2.6. Cell cycle analysis

The cells were cultured using growth media with or without fetal calf serum for 24 h in the presence of 1 μg/ml of Tc, and subjected for the further culture with or without Tc for 12 or 24 h. At each time point, cells were harvested. A part of the cells were stored for Western and Northern blot analyses and the remaining cells were fixed with 70% ethanol. Cells were treated with 1 mg/ml of RNase A (Qiagen), stained with 50 μg/ml of propidium iodide (Molecular Probes), and analyzed for cell cycle status by flow cytometry using a flow cytometer (FACScan; Becton-Dickinson) and Cell Quest and ModFit software.

3. Results

3.1. Effect of RB on expression of E2F-1 and E2F-responsive genes

To clarify the effect of RB on E2F-1 mRNA and protein levels, we utilized an osteosarcoma cell (Saos-2)-derived cell line, SRB-1, in which RB expression is controlled by a Tc-repressable promoter [29]. RB induction caused by removal of Tc resulted in G1 arrest as described previously [29]. The amounts of both E2F-1 mRNA and protein decreased markedly at 24 and 48 h post RB induction (Fig. 1A,B). Since E2F-1 protein induces the expression of the E2F-responsive genes B-MYB, DHFR and TS [2–6], the effect of RB induction on E2F-1 activity can be measured by examining alterations in these responsive gene expressions. Induction of RB biosynthesis substantially reduced the expression of all of these E2F-responsive genes at 24 and 48 h (Fig. 1B). It should be noted that the decrease of E2F-1 and TS transcripts began at 12 h, although the level of E2F-1 protein did not decrease at this time. Thus, RB down-regulated E2F-1 activity, which was accompanied by the reduction of E2F-1 protein levels.

3.2. Effect of p53 and p21 on expression of E2F-1 and E2F-responsive genes in RB-retained cell lines

p53 and p21 could, in the presence of RB, down-regulate E2F-1 bilaterally, since either might affect E2F-1 through RB-dependent or RB-independent pathways. To test this possibility, clones that expressed p53 and p21 under the control of the Tc-regulatable promoter were derived from an osteosarcoma cell line U-2 OS. In these clones wild-type RB and p53 genes are expressed, but p16 gene is homozygously deleted [32–34]. Clones that express p16, also under the control of a Tc-regulatable promoter, were also established to examine a RB-dependent pathway. The cells were arrested at G1 by induction of each gene and were also arrested at G2/M by p53 and p21, while apoptosis occurred only by p53 induction (data not shown). Fig. 2 shows the results of Western and Northern blot analyses of the clones, Up53-1, Up21-1 and Up16-2, at 48 h after induction of p53, p21 and p16, respectively. Upon induction of p53 protein expression in Up53-1, endogenous p21 protein was also induced, although its amount was less than that of exogenously induced p21 in Up21-1 cells. In Up16-2 cells, p16 protein was induced in a significant amount by depletion of Tc, although it was also slightly detected in the presence of Tc. With the induction of p53, Western blot analysis of RB electrophoretic mobility demonstrated the appearance of more rapidly moving minor bands below the diffuse major bands of RB, suggesting the appearance of hypophosphorylated RB [15–18]. The induction of p21 or p16

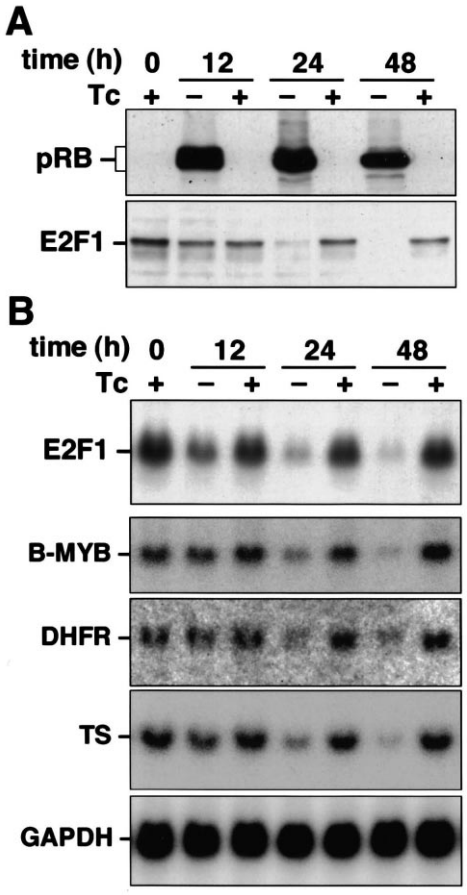

Fig. 1. Time course of RB induction and expression of E2F-1 and the E2F-responsive genes in Saos-2-derived SRB-1 cells analyzed by Western blot (A) and Northern blot (B). Cells were harvested at 0, 12, 24 and 48 h after seeding with or without 1 μg/ml of Tc. The blots were probed with anti-RB or anti-E2F-1 mAbs (A), and with E2F-1, B-MYB, DHFR, TS or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes (B).

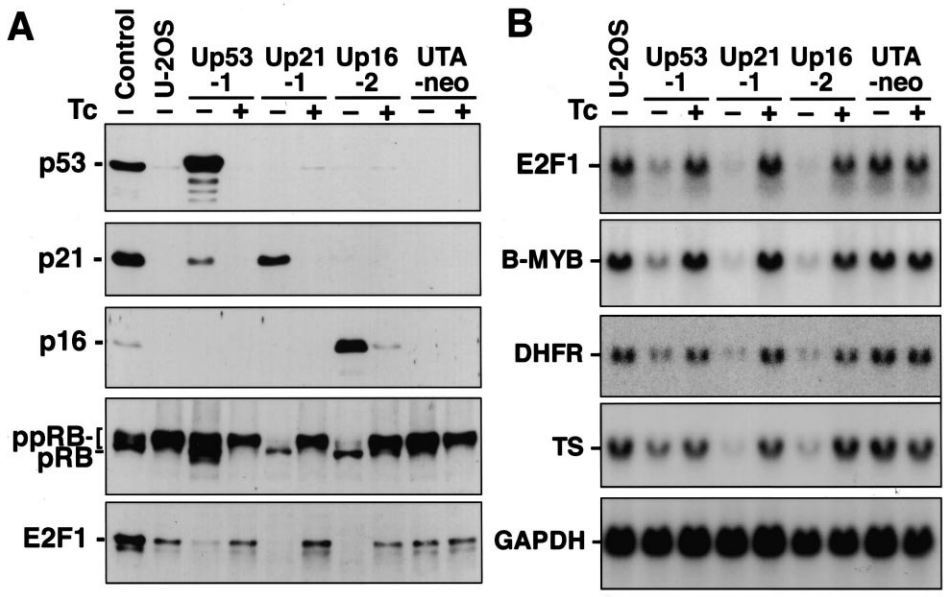

Fig. 2. Western blot analysis of p53, p21, p16, RB and E2F-1 (A) and Northern blot analysis of E2F-1, B-MYB, DHFR, TS and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (B) in U-2 OS-derived subclones. Up53-1, Up21-1 and Up16-2 are subclones that express p53, p21 and p16, respectively, under the control of a Tc-regulatable promoter. UTA-neo is a control clone, which was isolated following transfection of pT2p21/neo and negative for exogenous p21. Samples in control lanes were small cell lung cancer cell line Lu135 in p53 [29] and E2F-1, p21-induced Sp21-2 cells in p21, Saos-2 cells in p16, and lung adenocarcinoma cell line A427 in RB [29]. Cells were harvested at 48 h after seeding with or without 1 μg/ml of Tc. The blots were probed with Abs against p53, p21, p16, RB or E2F-1 (A), and with cDNA probes (B).

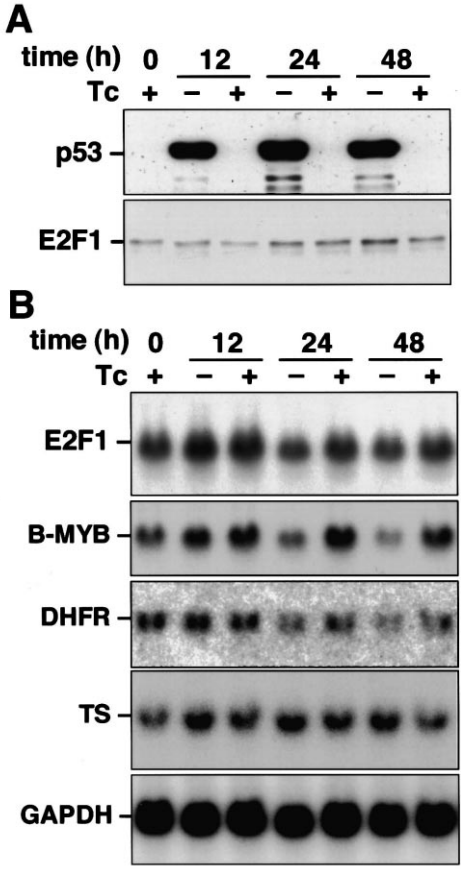

Fig. 3. Time course of p53 induction and expression of E2F-1 and the E2F-responsive genes in Saos-2-derived Sp53-3 cells analyzed by Western blotting (A) and Northern blotting (B). Cells were harvested at 0, 12, 24 and 48 h after seeding with or without 1 μg/ml of Tc. The blots were probed with anti-p53 or anti-E2F-1 mAbs (A), and with the indicated cDNA probes (B).

resulted in a significant decrease in the major bands, and increase in the intensity of more rapidly moving bands. In these three clones, the induction of respective p53, p21 or p16 substantially decreased both E2F-1 mRNA and protein levels (Fig. 2A,B), as well as expressions of the B-MYB, DHFR and TS genes (Fig. 2B). Although down-regulation of E2F-1 activity by p53 was not as intense as that by p21 or p16, the overall effects of p53, p21 and p16 in U-2 OS-derived cells were thus very similar to those of RB in Saos-2-derived SRB-1 cells.

3.3. *Effect of p53 on expression of E2F-1 and E2F-responsive genes in a RB-deficient cell line*

To evaluate the possibility of a RB-independent pathway mediated by p53 repression of E2F-1 activity, we analyzed alterations in expression of E2F-1 and the E2F-responsive genes in a Saos-2-derived subclone, Sp53-3, in which expression of p53 was placed under Tc regulatory control [29]. In Sp53-3, apoptosis and G1 and G2/M arrest were induced by p53 as described previously [29]. With p53 induction, the E2F-1 protein level was slightly increased at 48 h after induction (Fig. 3A), while its mRNA level showed a slight decrease at 24 and 48 h (Fig. 3B). On the other hand, expression of the B-MYB and DHFR genes decreased significantly at 24 and 48 h, similar to the case of RB induction. However, TS gene expression did not decrease (Fig. 3B). Thus, in the absence of RB, p53 repressed only a subset of E2F-responsive genes without reducing E2F-1 protein levels.

3.4. *Effect of p21 on expression of E2F-1 and E2F-responsive genes in RB-deficient cell lines*

To investigate whether p21 is a mediator of p53 for repression of E2F-1 activity, we established Saos-2-derived subclones, Sp21-2, Sp21-5 and Sp21-7, in which expression of p21 was under Tc regulatory control (Fig. 4). Sp21-N was

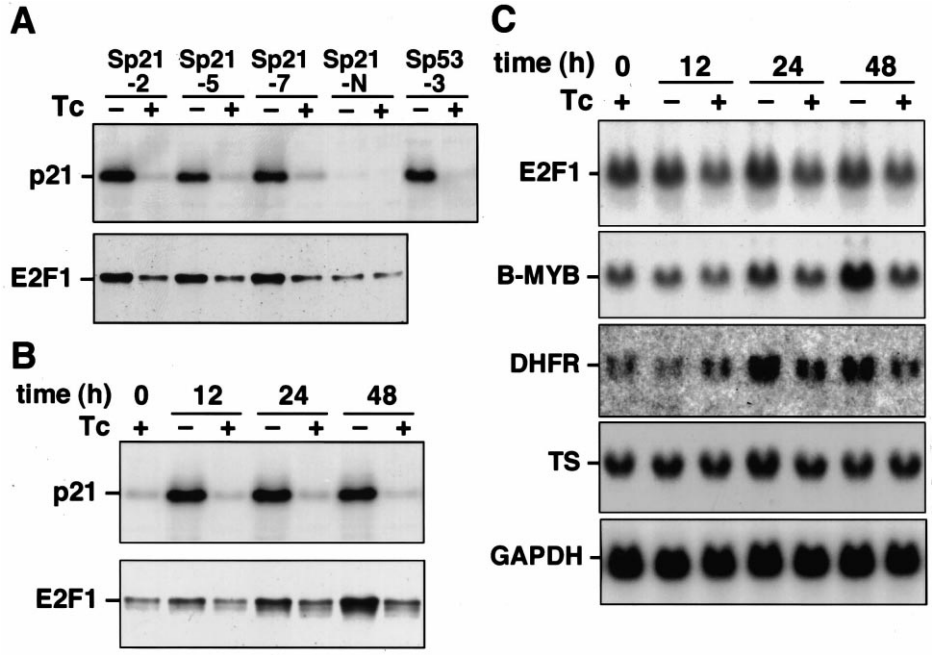

Fig. 4. Western blot analysis of p21 and E2F-1 proteins (A,B) and Northern blot analysis of the E2F-1 and E2F-responsive gene mRNAs (C) in Saos-2-derived subclones, in which p21 expression is controlled by a Tc-regulatable promoter. Cells were harvested at 48 h (A), and at 0, 12, 24 and 48 h (B,C; Sp21-2 was used) after seeding with or without 1 μg/ml of Tc. The blots were probed with Abs against p21 or E2F-1 (A,B), and with the cDNA probes (C).

made as a negative control for p21 induction. Induction of p21 in these cells was examined using the clone Sp53-3, which also induces p21, as a positive control. At 48 h after seeding without Tc, significant amounts of p21 proteins were detected in the p21-inducible clones as in the case of the clone Sp53-3 (Fig. 4A). These subclones showed G1 and G2/M arrest as a result of p21 induction (data not shown). However, p21 induction significantly increased the E2F-1 protein levels in these clones (Fig. 4A). In the time course experiment with Sp21-2, the induced p21 protein level reached a maximum at 12 h post induction and remained at this level for up to 48 h (Fig. 4B). This was accompanied by a gradual increase in E2F-1 protein levels that reached 5.0 times at 48 h (Fig. 4B), although its mRNA level was up by only 30% even at earlier time points (Fig. 4C). p21 induction also increased transcripts of B-MYB and DHFR at 24 and 48 h (Fig. 4C). Thus, p21 alone was unable to repress the activity of E2F-1, directly or indirectly, in Saos-2 cells.

3.5. *Alterations in the expression level of E2F-1 and E2F-responsive genes by RB and p53 expression in a serum-starved state*

Some of the changes in E2F-1 and E2F-responsive genes could be the result of more indirect effects of the general cell cycle alteration. In order to rule out the possible role of indirect effects, we evaluated the effects of RB and p53 induction in SRB-1 and Sp53-3 cells, respectively, in a serum-starved state, in which the alterations in the expression of induced cell cycle genes were minimized (Table 1). By the serum depletion, the E2F-1 protein levels decreased in both clones, but were not further altered by RB or p53 induction (Fig. 5A). Serum starvation also resulted in the decrease of

Table 1
Effect of RB and p53 expression on the cell cycle distribution in Saos-2-derived clones in a serum-starved state

Clone	Serum	Tc	Time after induction (h)	Cell cycle distribution (%)		
				G0/G1	S	G2/M
SRB-1	+	+	0	49.2 ± 3.5	36.3 ± 2.5	14.6 ± 1.1
	+	−	12	54.3 ± 2.3	20.5 ± 2.0	25.3 ± 0.3
	+	+	12	46.7 ± 0.5	29.6 ± 1.1	23.7 ± 0.6
Sp53-3	+	+	0	42.2 ± 1.5	39.7 ± 0.3	18.2 ± 1.9
	+	−	24	51.1 ± 1.7	19.8 ± 0.2	29.2 ± 1.5
	+	+	24	51.7 ± 2.1	29.3 ± 1.6	19.0 ± 0.5
SRB-1	−	+	0	75.3 ± 2.7	6.8 ± 1.5	18.1 ± 1.3
	−	−	12	85.1 ± 1.6	4.9 ± 0.2	10.1 ± 1.5
	−	+	12	84.6 ± 1.6	5.1 ± 0.2	10.4 ± 1.7
Sp53-3	−	+	0	73.1 ± 0.8	9.9 ± 0.5	17.1 ± 0.2
	−	−	24	71.4 ± 2.6	13.1 ± 1.1	15.6 ± 1.6
	−	+	24	70.9 ± 2.6	15.8 ± 1.6	13.4 ± 0.9

Each value is expressed as the mean ± S.D. of three independent experiments.

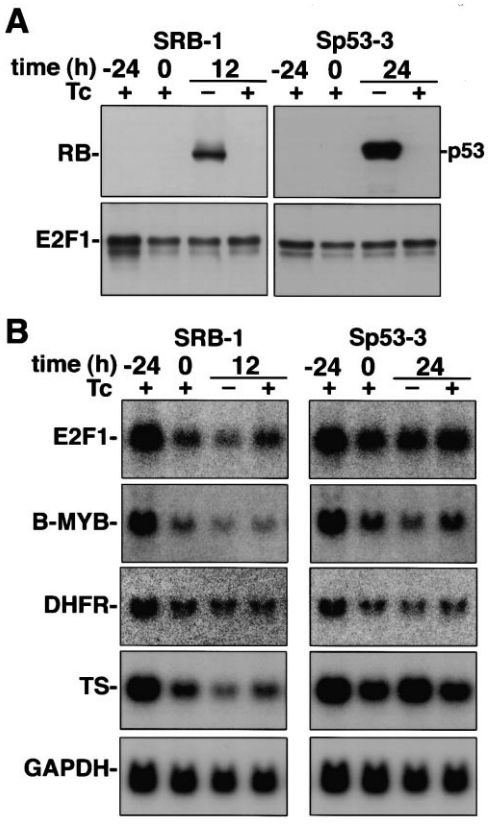

Fig. 5. Effect of RB and p53 expression on E2F-1 and E2F-responsive gene expression in a serum-starved state. Western blot (A) and Northern blot (B) analyses. SRB-1 and Sp53-3 cells were serum starved for 24 h in the presence of Tc, then further cultured for 12 or 24 h with or without Tc. Cells were harvested before the serum starvation (−24 h) and at the indicated time points (0, 12 or 24 h) after the initiation of the second culture. The blots were probed with Abs against RB, p53 or E2F-1 (A), and with the indicated cDNA probes (B).

E2F-1, B-MYB, DHFR and TS gene expressions. However, induction of RB in SRB-1 further decreased E2F-1 and TS gene expressions (Fig. 5B). On the other hand, p53 induction further reduced B-MYB and DHFR gene expressions in Sp53-3 cells (Fig. 5B). These effects of RB and p53 were not accompanied by cell cycle alterations (Table 1), but identical with the case of non-starved cells (Figs. 1B and 3B). Therefore, it was suggested that the effects on E2F-1 and TS gene expression by RB and those on the B-MYB and DHFR genes by p53 were not only the result of alterations in the cell cycle.

4. Discussion

To clarify the effects of p53 and RB on expression and transcriptional function of E2F-1, we investigated the effect of induced RB, p53 and p21 expression on the expression of E2F-1 and the E2F-responsive genes using a Tc-regulated inducible expression system. In the presence of RB, expression of E2F-1 and the E2F-responsive genes decreased either by p53 or by p21, in a similar pattern with induced RB expression. This result supports the concept that RB is a critical component for regulation of E2F-1 in the p53-p21 signaling pathway for induction of G1 arrest. However, in the absence of RB, E2F-1 gene product did not decrease with induction of p53 expression, although G1 arrest accompanied by B-MYB and DHFR repression was observed. This result indicates the existence of another pathway for p53 to induce G1 arrest in addition to the p21-RB-mediated pathway. p21 is improbably a component of such a pathway, since p21 did not have an effect to repress B-MYB and DHFR expression without RB. It is possible that other RB family proteins, as well as other E2F family proteins, are involved in such a pathway, and identification of these regulatory genes is important, because such genes should also play an important role in cell cycle regulation in cancer cells. For this purpose, we are extending our search for genes regulated by the p53 gene using the inducible approach demonstrated in this study.

Expression of E2F-1 itself was reduced by RB, which was accompanied by reduced expression of other E2F-1-responsive genes. The time course experiment showed that E2F-1 and TS transcripts decreased at 12 h after depletion of Tc without reduction of the E2F-1 protein. This would be explained by repression of E2F-1 activity due to RB binding to E2F-1 [12,13]. On the other hand, expression of the B-MYB and DHFR genes was not significantly affected at 12 h post induction. The decrease in B-MYB and DHFR expression only occurred later and was accompanied by reduction of the E2F-1 protein level. Thus, the level of E2F-1 protein expression is also an important factor for regulation of the E2F-responsive genes in addition to its RB binding function. Down-regulation of E2F-1 protein amount by RB has not been demonstrated [35,36]. Therefore, such a reduction should be taken into account when interaction of RB with E2F-1 is considered.

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