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Cyclin E enhances P53-mediated transactivation

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Plasmids expressing G1 and G2 cyclins were introduced into the Saos-2 cell system monitoring p53-mediated transactivation [(1993) Oncogene 8, 543]. Cyclin E, but not other cyclins, enhanced the p53-mediated transactivation about 2-fold. Co-transfection of a CDK2 expression plasmid caused a 30% increase in the extent of the p53-mediated transactivation. Moreover, the transfected p53 protein became phosphorylated coordinately with the enhanced transactivation. The close correlation between transactivation and p53 phosphorylation suggests that phosphorylation is involved in positive regulation for the transactivation by p53.

p53; Transactivation; Cell cycle; Cyclin E; CDK2; Phosphorylation

1. INTRODUCTION

Wild-type (wt) p53 has been implicated in the control of cell proliferation and tumor suppression [1]. Like another tumor suppressor, the retinoblastoma susceptible gene, the wt but not the mutant type (mt) p53 is known to down-regulate the activity of various promoters [2]. Recently, Seto et al. [3] reported that the wt p53 can bind directly to the TATA box-binding protein, resulting in repression of transcription from TATA-containing promoters. In contrast, the wt p53 enhances transcription from the promoters containing the 33-base pair p53-binding sequence [4] detected in the ribosome non-transcribed spacer region 3.5 kb upstream of the 45 S rRNA start site [5]. The p53-mediated transactivation is abrogated by DNA tumor virus oncogene products which are able to bind to wt p53, such as SV40 large T antigen [6–8], adenovirus E1B product [9], and human papilloma virus E6 product [7]. In the virus uninfected cells, the *mdm2* gene product is supposed to be a candidate for the modulator of p53 [10], although, in addition to wt, mt p53 can also bind to the *mdm2* product [11].

In this report, we studied the effects of G1 and G2 cyclins and cyclin-dependent kinases on the p53-mediated transactivation in order to exploit the modulator of wt p53 in normal cells. The results showed that cyclin E enhances p53-mediated transactivation and phosphorylation of p53 protein in the transfection cells.

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2. MATERIALS AND METHODS

2.1. *Plasmids*

cDNAs encoding cyclin A [12], cyclin C, D1 and E [13], and cyclin B1 and B2 (Nishimoto, T., unpublished) were subcloned into pcDL-SR 296 [14] at the *Cla*I–*Eco*RI, *Eco*RI–*Kpn*I, or *Cla*I–*Kpn*I site. The CDC2 and CDK2 expression plasmids, pME18S-CDC2 and pME18S-CDK2, respectively, were described previously [15]. pMSVcL and ptk53CAT used for chloramphenicol acetyltransferase (CAT) assays were previously described [8].

2.2. *Transfection and CAT assay*

Saos-2 cells (1×10^6 cells) were transfected with 1 μg of ptk53CAT, 10 μg of cyclin expression plasmids and 4 μg of pRSVβ-gal for 18 h as described previously [8]. Cells were re-fed with new growth medium and incubated for 36 h. Cellular extracts were prepared and subjected to CAT assays as described [8]. Transfection efficiencies were normalized by the β-galactosidase activities from co-transfected pRSVβgal [8]. All CAT assays were repeated more than five times and the reproducibility of results was confirmed.

2.3. *Histone H1 kinase assay*

Saos-2 cells transfected with 10 μg of pMSVcL, 10 μg of cyclin E expression plasmid, with or without 10 μg of CDK2 expression plasmid, were lysed with RIPA buffer containing 1% Triton X-100, 0.1% SDS, 50 mM Tris-HCl (pH 7.5), and 1% trasylol (Sigma). The cell extracts were incubated with anti-cyclin E antibody [16] (2 μl), normal rabbit sera (2 μl), or anti-p53 antibody (1 μl, pAb421, Oncogene Sci. Inc.) at 4°C for 2 h. Immune complexes were collected by adding protein A–Sepharose beads and subjected to histone H1 kinase assay as described [16].

2.4. *Phosphorylation of p53*

Saos-2 cells were transfected with p53, cyclin E and/or CDK2 expression plasmids. At 36 h after transfection, cells were labeled with [^{32}P]orthophosphate (1.0 mCi/ml; ICN) for 4 h. Cellular extracts prepared as described above were subjected to immunoprecipitation with

anti-p53 antibody (pAb421), followed by SDS-PAGE. Phosphorylated p53 was detected by autoradiography.

3. RESULTS

3.1. *Modulation of p53-mediated transactivation by cyclin expression plasmids*

The wt p53 plays a crucial role in G1 arrest [17], enabling us to study the effect of cell cycle-specific cyclins on the modulation of p53-mediated transactivation. The plasmids encoding cyclins A, B1, B2, C, D1, and E, were placed under the control of SRα promoter [14]. These plasmids were transfected to p53-null Saos-2 cells with the p53 expression plasmid, pMSVcL, and the reporter plasmid, ptk53CAT or ptkCAT, as described previously [8]. The CAT activities detected in the presence of cyclin expression plasmids were compared with those in the absence of the plasmids. The CAT activity of the cyclin E-transfected extract was reproducibly increased about 2-fold, when ptk53CAT, but not ptkCAT, was used as a reporter plasmid (Fig. 1). Co-transfection with the plasmid encoding CDK2, which is known to be associated with cyclin E in vivo [16], caused a 30% increase in the extent of the p53-mediated transactivation, as compared with that in the cells transfected with cyclin E only (Fig. 1). Transfection with only CDK2 expression plasmid did not enhance CAT activity. In contrast, the introduction of cyclin C decreased CAT activity by about 50%. However, the CAT activity was recovered by co-transfection with CDC2 or CDK2 expression plasmid. Upon transfection with other cyclins, there were no changes in CAT activities.

To study the expression of cyclin E in the transfected cells, cellular extracts were immunoprecipitated with anti-cyclin E antibody or control normal rabbit sera, followed by histone H1 kinase assay (Fig. 2). The histone H1 kinase activity associated with cyclin E, if any, was faintly detected in the cyclin E-untransfected cells. By co-transfection with the cyclin E expression plasmid, however, the extent of histone H1 phosphorylation was clearly increased. Moreover, the introduction of CDK2 expression plasmid further enhanced phosphorylation of histone H1 by 4 times. No phosphorylated bands were detected in the immunoprecipitated extracts with normal rabbit sera (Fig. 2B). These results indicate that cyclin E is definitely expressed in the transfected cells.

3.2. *Phosphorylation of p53 in the transfected cells*

We then examined the phosphorylation of p53 under conditions that the enhanced p53-mediated transactivation and histone H1 kinase activity were detected. The transfected cells were labeled with [^{32}P]phosphate and immunoprecipitated with anti-p53 antibody. The band of phosphorylated p53 was faintly detected in the cells transfected with p53 expression plasmid only (Fig. 3, lane 3). By co-transfection with cyclin E (lane 2), or cyclin E plus CDK2 expression plasmids (lane 1), the extent of phosphorylation was dramatically increased. These results indicate that p53 is more heavily phosphorylated in cells in which enhanced transactivation was observed.

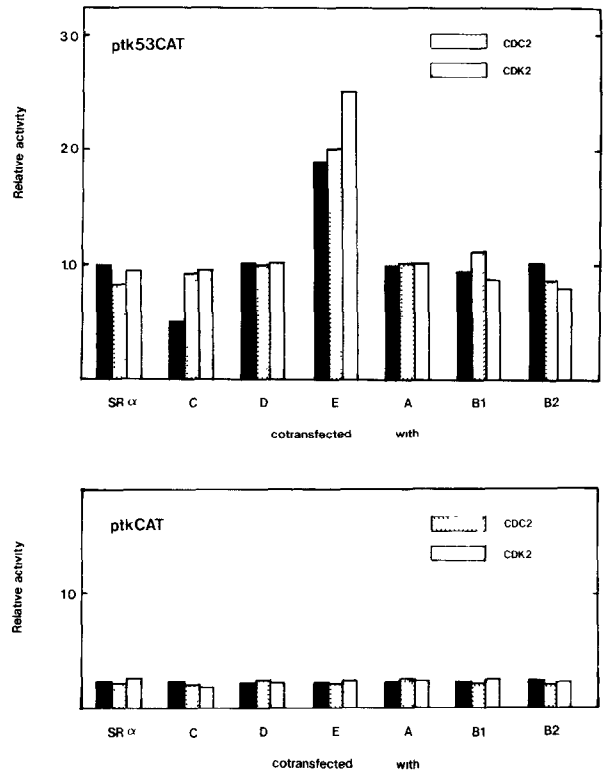

Fig. 1. Transactivation by p53 in the presence of cyclin and/or cell cycle control kinase genes. Saos-2 cells were transfected with ptk53CAT or ptkCAT (1 μg), pMSVcL (5 μg), cyclin expression plasmid (10 μg) shown as a capital letter below the figure, and/or cell cycle control kinase expression plasmids (CDC2 and CDK2). The CAT activities in the extracts from the transfected cells were measured as described [8]. The relative CAT activities compared with the control value obtained from the cell extract transfected with ptk53CAT, pMSVcL, and backbone plasmid pcDL-SRα296 are shown as closed bars. Hatched and open bars represent the values obtained from CDK2 or CDC2 expression plasmid-transfected cells, respectively

4. DISCUSSION

Various G1 and G2 cyclin expression plasmids were transfected in the p53-mediated transactivation monitoring system reported previously [8]. Only cyclin E, but not the other G1 and G2 cyclins, reproducibly enhanced transcription from the promoter containing the p53-binding sequence (Fig. 1). Moreover, the co-transfection of the plasmid encoding CDK2, which is known to be associated with cyclin E in vivo [16], further enhanced CAT activity (Fig. 1). In parallel with the elevated transactivation, the extent of p53 phosphorylation in the transfected cells was also increased (Fig. 3). The close correlation between transactivation and p53 phosphorylation suggests that phosphorylation is in-

volved in the positive regulation of the transactivation by p53. Recently, Hupp et al. [18] reported that phosphorylation at the C-terminal site of p53 by casein kinase (CK) II is responsible for the activation of the DNA-binding ability of p53. Thus, phosphorylation of p53 may allow very tight binding to the p53-binding sequence or may stabilize the complex of p53 and DNA, resulting in transcriptional activation, as in the case of the c-Fos/c-Jun heterodimer [19]. Alternatively, phosphorylation might affect the conformation of the N-terminus transactivation domain in p53, as reported in the case of CREB [20]. It is important to study whether p53 is directly phosphorylated by cyclin E–CDK2 or by some other kinase(s) controlled via the cyclin E–CDK2 system. To search for certain kinases for p53, in vitro phosphorylation experiments using purified p53 and CDC2, CDK2 and CKII are in progress.

A number of results support the hypothesis that G1 cyclins and cyclin-dependent kinases play crucial roles to control G1 progression [21]. Ohtsubo and Roberts [22] have recently published that the over-expression of cyclin E, but not other G1 cyclins, shortens the duration of G1, decreases cell size, and diminishes the serum requirement for the transition from G1 to S. Therefore, the enhanced p53-mediated transactivation by cyclin E might be one of the important events controlling the transition from G1 to S. In this context, it should be noted that the synthesis of p53 is increased in parallel with the transition from G1 to S [23], and the amount of cyclin E is maximal near the G1–S boundary [16]. However, this assumption is irrelevant to the previously published findings that wt p53 stops cells at G1 [17] and transactivates *gaad45* gene, which is responsible for growth arrest [24]. The discrepancy needs to be clarified by identifying cellular genes transactivated by wt p53 and by analyzing their functions.

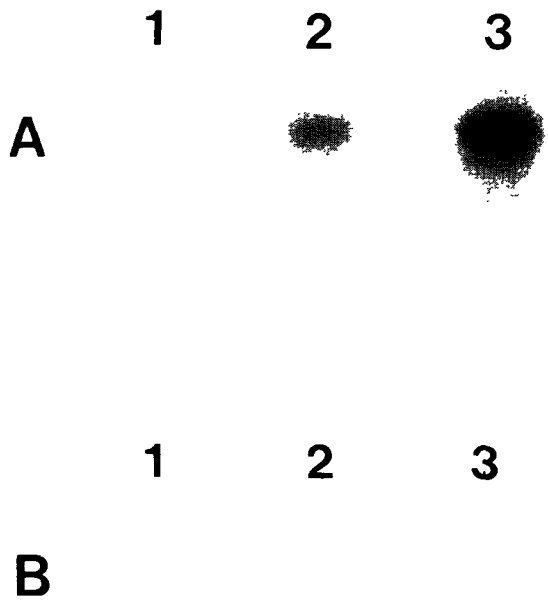

Fig. 2. Histone H1 kinase activity in cyclin E-transfected cells. Saos-2 cells were transfected with 1 μg of ptk53CAT, 5 μg of pMSVcL in the presence of 20 μg of pcDL-SRα296 (lane 1), 10 μg of pSRαcyclin E and 10 μg of pcDL-SRα296 (lane 2), or 10 μg of pSRαcyclin E and 10 μg of pME18S-CDK2 (lane 3). The cellular extracts were immunoprecipitated with rabbit sera containing anti-cyclin E antibody [16] (A), or normal rabbit sera (B), followed by histone H1 kinase assay as described [16]. Only the regions exhibiting phosphorylated histone H1 bands discernible by autoradiography after analyzing by SDS-PAGE are shown.

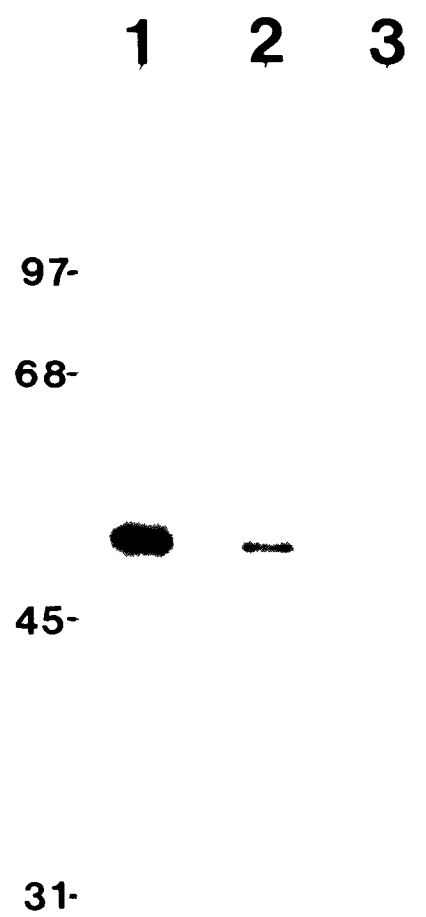

Fig. 3. Phosphorylation of p53 in transfected cells. Saos-2 cells were transfected with 1 μg of ptk53CAT, 5 μg of pMSVcL in the presence of 10 μg of pSRαcyclin E and 10 μg of pME18SCDK2 (lane 1), 10 μg of pSRαcyclin E and 10 μg of pcDL-SRα-296 (lane 2), or 20 μg of pcDL-SRα296 (lane 3). At 36 h post-transfection, cells were labeled with [^{32}P]phosphate for 4 h and lysed with RIPA buffer. The extracts were subjected to immunoprecipitation with the anti-p53 antibody, pAb 421. The phosphorylated p53 bands were discerned by autoradiography after analyzing by SDS-PAGE. The molecular weight markers used which are shown in numbers at the left side of the figure are (in kDa) phosphorylase *b* (97), bovine serum albumin (68), ovalbumin (45), and carbonic anhydrase (31).

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