Regulation of the Rb gene by normal and mutated RAS, TPA and EGF

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Abstract. Complete inactivation of the human retinoblastoma gene is believed to be an essential step in tumorigenesis of several different cancers. Using the plasmid pRbCAT2 that contains the Rb promoter region was tested for its ability to promote transcription of the bacterial chloramphenicol acetyltransferase (CAT) gene in a transient expression assay. This plasmid was co-transfected in a short term transfections with the plasmids pH06T1 and pH06N1 that contains the mutant and normal H-ras gene respectively, into the human cell line HeLa, by the calcium phosphate technique. It was found that the mutant H-ras gene enhances the activity of the Rb gene promoter in contrast to the normal H-ras gene that inhibits it. The expression of the CAT gene in stable clones of HeLa cells carrying the promoter of Rb gene after treatment with TPA and EGF respectively, was also investigated, whereas TPA enhanced, EGF had no effect on the activity of the Rb gene promoter.

Introduction

The Rb gene was the first human tumor suppressor gene isolated and is located on the long arm of human chromosome 13 (1-3). This gene contains 27 exons dispersed within 200 Kb of genomic DNA (4) and expresses a 4.7 Kb mRNA transcript in all normal tissues (2). Negative regulation of the cell cycle by Rb-1 was implied from the finding that TPA induces transcription of cellular proto-oncogenes, e.g. c-fos (16,17), c-myc (16,18) and c-sis (19). In an attempt to identify the target of transcription regulation by ras, we examined its effect on the promoter of the Rb gene, in expectation of activation of Rb transcription by ras. We also tested whether the Rb promoter could be activated by TPA and EGF. Our results show that mutant H-ras gene enhances the activity of the Rb gene promoter in contrast to the normal H-ras gene that inhibits it. TPA, was able to induce expression of a CAT gene linked to the human Rb promoter region, whereas EGF had no effect on the activity of the Rb gene promoter.

Materials and methods

Cells and plasmids. pRbCAT2 contains human Rb-1 promoter sequence between -1546 and +186 (relative to the major start site of transcription) linked to the chlor-
amphenicol acetyltransferase (CAT) gene (2). Plasmid p+53ΔCAT is the 5' deletion construct of pRbCAT2 (20). Plasmid p201 carry the aph gene and the CAT gene linked to the human mutant T24 H-ras promoter region (21). Plasmids pH06T1 and pH06N1 contains the 6.6 Kb of the mutant and normal H-ras gene, respectively (22). Plasmid pRbCAT2-A carry the human Rb-1 promoter sequence between -1546 and +186 and linked to the CAT gene, the 1.9 Kb Bam HI fragment of the aph gene (Fig 1).

HeLa cells (ATCC CCL 2) were cultured in Dulbecco's F12 medium supplemented with 10% fetal calf serum.

**DNA transfection and CAT assays.** Transfections of HeLa cells with plasmid DNA were performed using a modification (23) of the calcium phosphate technique (24). Stable transfecants were isolated in the presence of 200 μg/ml geneticin. For CAT assays, cells were grown exponentially in absence of geneticin. TPA was purchased from Sigma and 1000x stocks were made in DMSO. EGF was purchased from Collaborative Research Incorporation and 50 ng/ml stock was made in Dulbecco's-F12 medium. Cells in the presence or absence of TPA or EGF were harvested and assayed for CAT activity as previously described (25). HeLa cells were cotransfected by the calcium phosphate precipitation technique with 10 μg of reporter construct and 30 μg of each effector construct (pH06T1, pH06N1 and pHomer 6). All CAT assays were repeated at least twice and the reproducibility of the results was confirmed.

**Results**

**Suppression of Rb transcription by the normal H-ras protein.** In transient-expression assays using HeLa cells (Fig. 2) cotransfection of pRbCAT2 with the normal H-ras construct phO6N1, resulted in less CAT activity than that on its cotransfection with pHomer 6. In contrast, cotransfection of pRbCAT2 with the mutant H-ras construct pH06T1, resulted in enhanced CAT activity. In two independent experiments, we observed an average of 3-fold reduction and 2-fold increase in CAT activity with the normal and mutant H-ras, respectively.

**TPA increased, whereas EGF had no effect on Rb transcription.** It was of interest to examine the responsiveness of Rb-1 promoter to phorbol ester TPA. The possibility that the
human Rb-1 promoter could respond to the TPA was examined by treating stable transfectants carrying a CAT gene linked to the Rb-1 promoter. Transfectant cell line was obtained by transfecting plasmid pRB-CAT-A into recipient HeLa cells and selecting for geneticin resistance. Treatment with TPA was performed at various TPA concentrations and time intervals. At optimum conditions, that is, after 12 h exposure to 50 ng TPA/ml, CAT expression in HeLa Rb-CAT-A transfectants which carried CAT plasmid linked to the Rb-1 promoter was increased (2.3-fold) compared to untreated cells (Fig. 3). On the other hand HeLa cells transfected with the same plasmid did not respond to EGF (Fig. 4).

Discussion

The mechanism by which several transcription factors interact with the Rb promoter and trigger RNA synthesis, remains unknown. However, it is obvious that an important step towards understanding the regulation and function of the Rb promoter is the investigation of such interactions.

In this study we have shown that normal ras suppresses Rb transcription in contrast to mutated ras which enhances it. To our knowledge suppression or activation of the Rb transcription by the normal or the mutated ras gene, respectively has not so far been reported. However, it is known that mutated ras leads to phosphorylation of the p53 protein through activation of MAP-2 kinase and inactivation of the Rb promoter (20). Thus, we can suggest that mutated ras enhances the Rb transcription via phosphorylation and consequently inactivation of the p53 protein. Alternatively, the ras genes can influence the Rb transcription through activation of raf-1 and MAP-2 kinase which can then activate both S6 kinase and DNA synthesis-promoting factor (SPF). SPF contains cyclin, p107, the transcription factor E2F and tumor suppressors such as the retinoblastoma gene product and p53 (26).

It is well known that stimulation of cell growth by TPA occurs through its direct interaction with protein kinase C (27). Also, it has been reported that TPA can induce expression of a CAT gene linked to the human H-ras1 promoter region (21). We found that TPA activates the transcription of Rb gene. This activation may be either a direct effect of the TPA on the Rb promoter, possibly through a TPA-responsive element or an indirect effect through activation of the mutated ras gene and subsequent activation of the Rb gene.

It has been found that many tyrosine kinases including the receptors for epidermal growth factor, nerve growth factor and insulin transmit intracellular signals through ras proteins (28-31). Ligand binding to such receptors stimulates ras guanine-nucleotide exchange activity (32-36) and increases the level of GTP-bound ras (37,38). Whether EGF acts also through inhibition of the Rb gene has not been reported. In order to investigate this possibility we treated stable clones of HeLa cells carrying the Rb promoter with various concentrations of EGF. Our results show that EGF has no effect on the transcription of the Rb gene.

In conclusion, our results indicate that the transcription of the Rb gene may be regulated through normal and mutated ras as well as phorbol esters. Even if the suppression of the Rb transcription by normal ras or the activation by its mutated form seems to be a paradox, it may reflect an additional mechanism through which the cells avoid tumorigenesis.

References