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IDENTIFICATION OF CELLULAR PROTEINS THAT BIND THE CENTRAL CONSERVED REGION OF P53

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The bacterial fusion protein between glutathione S-transferase and the central conserved region of human p53(GST-p53) was purified and fixed on the beads and then used in the binding assay with radiolabeled cell extract from human hepatocarcinoma cell line, Hep3B. The binding asssay disclosed the presence of cellular proteins that interact with GST-p53 but not with GST. SV40 large T antigen abrogated the bindings of two cellular proteins with molecular weights of 50 kda and 40 kda. The binding of the proteins to p53 was observed in a cell cycle-dependent manner. These two proteins are candidate cellular proteins which regulate the function of p53.

p53 was discovered as a cellular protein which co-immunoprecipitated with SV40 large T antigen. Several lines of evidence from the studies during the past several years have highly suggested that wild-type p53 is a tumor suppressor gene product while mutant forms of p53 are oncogenic (1 - 4).

One important character of p53 is its interaction with viral oncogene products. SV40 large T antigen, adenovirus Elb and human papilloma virus E6 proteins form stable complexes with p53 and inactivate the function of this anti-oncogene product. This is an important mechanism by which the oncogenic DNA viruses can transform the normal cells (1, 2). T antigen binds the central conserved regions of p53 and masks this DNA-binding domain of p53. It is not unreasonable to speculate that some cellular proteins interact with this region and regulate the function of p53 as T antigen does.

Herein, we report the presence of cellular proteins which specifically interact with the T antigen-binding region of human p53 and discuss the potential biological functions of these proteins.

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MATERIALS AND METHODS

Cell culture

The hepatocellular carcinoma cell line Hep3B was grown in complete media [Dulbecco's modified Eagle's medium (D-MEM) with 10% fetal calf serum, 100U/ml penicillin (GIBCO), 100μg/ml streptomycin (GIBCO) and 2 mM L-glutamine (GIBCO)]. The cells were grown at 37°C in a 5% CO_2-incubator.

Fusion protein preparation from bacteria

Expression and purification of glutathion S-transferase fusion protein were performed as previously described (8). Details of construction of pGST-Hp53(III-V) are available upon request.

Binding reaction between fusion protein on beads and ^{35}S-labeled cell extracts

[^{35}S]-labeled Hep3B extracts were prepared as described (8). Salt (NaCl) concentration of the [^{35}S]-labeled cell extract was adjusted to 100mM. Then the extract was preabsorbed with approximately 1/10 volume of glutathion Sepharose 4B beads, which prefixed GST, at 4°C for 1 hour. After centrifugation, the supernatant was used in binding reaction, mixed with GST-p53 fusion protein (or GST) on the beads and incubated for 2 hrs. After the binding reaction, the beads were washed 4 times with NETN[20mM Tris-Cl(pH8.0), 100mM NaCl, 1 mM EDTA and 0.5% NP-40] and bound proteins were eluted with protein sample buffer and analyzed with SDS-PAGE followed by fluorography or image analysis. The details of the competition assays are described in figure legends.

Cell cycle synchronization

To order to synchronize in the Go/G1 phase of the cell cycle, Hep3B cells were starved for 48hrs with medum containing 0.5% FCS. After the starvation, the cells were stimulated with complete medum(10% FCS). The cells were incubated in complete medium containing 1.5 mM Hydroxyurea(HU) for 48 hrs to synchronize in the early S phase. After washing with PBS(-) to remove HU, the cells were incubated with complete medium for 4 hrs to synchronize in the middle S phase. The cells were incubatd in complete medium containing 1μg/ml of Nocodazol for 24 hrs to synchronize in the G2/M phase. Cell cycle synchronization was confirmed by FACS analysis.

RESULTS

To identity cellular protein(s) which interact specifically with the SV40 T antigen-binding region of p53, the binding reactions between radio-labeled cell extracts and an affinity-purified recombinant p53 on beads were performed. Bipartite regions of mouse p53, amino acid 165-199 and amino acid 232-285, are required to bind SV40 T antigen (9). Corresponding to the T antigen-binding region of mouse p53, a part of the cDNA of human p53, which spans from the conserved region III (CRIII) through V (CRV), was amplified by PCR and then cloned into a

GST-fusion protein plasmid, pGEX-3X. The resulting new construct, pGST-Hp53(III-V), was used to prepare bacterial fusion protein between glutathione S-transferase(GST) and human p53. The bacterial fusion proteins were purified on glutathione Sepharose 4B beads and used for binding reaction with [^{35}S]-radiolabeled extract of a hepatocellular carcinoma cell line, Hep3B. We used Hep3B cell line as a source of cell extract, as this cell line has no endogeneous p53 expression due to deletion(s) or rearrangement(s) of both alleles of p53 genes(10). The use of this cell line was thought advantageous, since endogenous p53 might hinder the binding reaction by acting as a competitor.

The binding reaction revealed that several cellular proteins of the Hep3B cell line bind the GST-p53 fusion protein (Fig. 1). Proteins with a

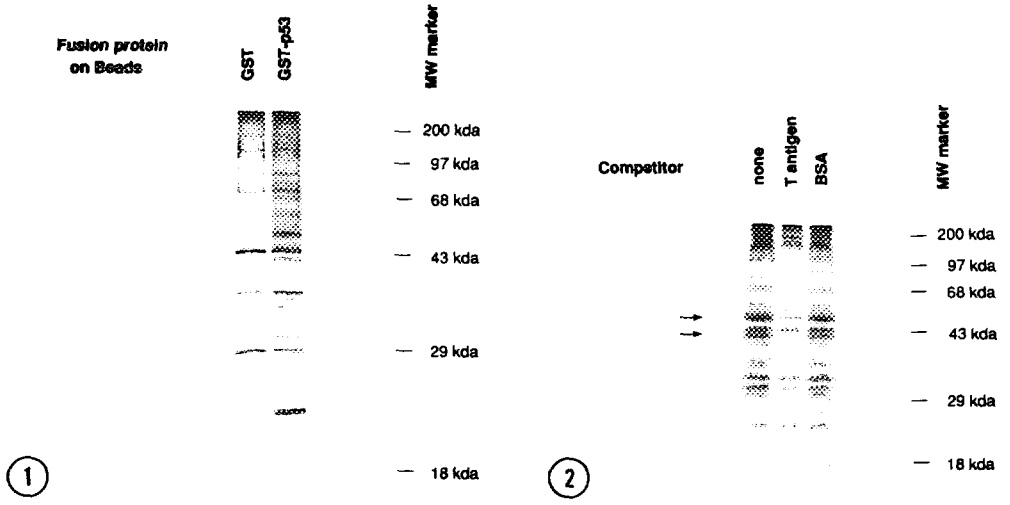

Figure 1. Detection of cellular proteins that bind the central conserved region of human p53.

The conditions of the binding reactions were as described in materials and methods. Two and a half μg of bacterial (fusion) proteins fixed on glutathion Sepharose 4B beads and 1.2 x 10^7 cpm of [^{35}S]-labeled Hep3B cell extracts were used for the binding reactions.

Figure 2. SV40 T antigen specifically inhibits the binding of cellular proteins with molecular weights 50kda and 40kda to GST-p53.

GST-p53 on glutathion Sepharose 4B beads was firstly incubated with immunopurified T antigen or bovine serum albumin(BSA), 25μg each, at 4°C for 1 hr. It was then incubated with about 0.6 x 10^7 cpm of [^{35}S]-labeled Hep3B cell extracts, which had been preabsorbed with GST, for the binding reaction. Arrows indicate p50 and p40 that were significantly inhibited for binding to GST-p53 by T antigen but not by BSA.

MW of 43kda, 35kda and 29kda interact not only with GST-p53 but also with GST, indicating that the bindings of these proteins are non-specific. However, proteins with a MW of 50kda(p50), 40kda(p40) and 22kda(p22), interact with GST-p53 but not with GST. Although we have performed several binding reactions using different preparations of [^{35}S]-radiolabeled Hep3B cell extracts, the binding of these proteins to GST-p53 but not to GST was reproducible, suggesting that cellular proteins, p50, p40 and p22, are candidate proteins which interact specifically with the central conserved region of human p53.

To further confirm the binding specificities of these cellular proteins, competition assays were performed using SV40 T antigen and BSA as a specific and as a non-specific competitor in the binding reactions, respectively. Whereas BSA did not compete with the binding of any of the cellular proteins, the T antigen inhibited significantly the bindings of p50 and p40. Inhibition of binding of p22 by the T antigen was not observed (Fig. 2). These findings indicate that the interactions of the two cellular proteins, p50 and p40, with the T antigen-binding region of human p53 are specific.

As p53 stops the cell cycle in the G1 phase, we examined whether the bindings of p50 and p40 with p53 are cell cycle-dependent. The bindings of both proteins are barely detectable in the G_0/G_1 phase. The binding of p50 was observed in the S and G_2/M phases (In the G_2/M phase, proteins larger than 50 kda were detected. Although these proteins could be modified forms of p50, we have no evidence at this point). The binding of p40 was not observed in the early S phase but became slightly detectable in the middle S phase. Significant binding of p40 was observed in the G_2/M phase (Fig. 3).

DISCUSSION

We have found the presence of two cellular proteins which specifically interact with the T antigen-binding region of human p53 by in vitro-binding assay.

Oncogene products of DNA viruses form complexes with p53 and inactivate the function of p53. Thus adenovirus E1B protein binds the N-terminal region of p53 which overlaps with the acidic transactivational domain of p53 (11), and human papiloma virus E6 protein binds p53, facilitating the degradation of p53 (12). SV40 T antigen binds the central conserved region of p53, causing its inactivation. Recently, the T antigen-binding region of p53 was shown to be bound with zinc metal ions, and suggested to form secondary structure(s) and the secondary structure is required to bind DNA in a

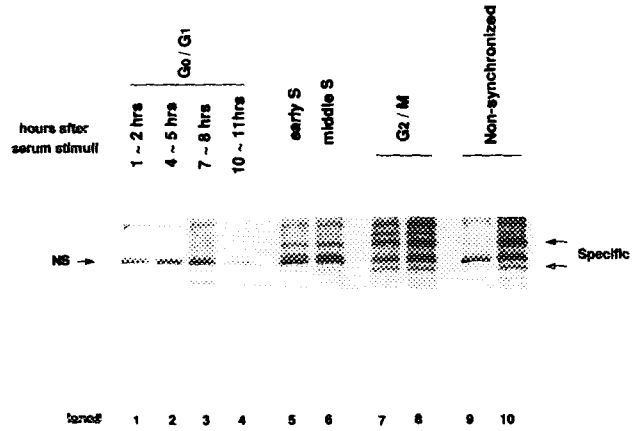

Figure 3. The cell cycle-dependent binding of p50 and p40 to the central conserved region of p53.

Hep3B cells were synchronized at each phase as described in *Materials and Methods*. After the synchronization, the cells were washed with PBS(-) three times to remove synchronizing reagents. Nocodazol was kept treated during radiolabeling in lane 7, whereas it was washed out before radiolabeling in lane 8. Fusion proteins (GST-p53) were fixed on beads in lanes 1-8 and 10, whereas GST was fixed in lane 9. Left and right arrows indicate specific and non-specific (NS) band(s), respectively.

sequence specific manner (5). The T antigen inhibits the function of p53 by direct binding and masking this DNA-binding region of p53.

Several cellular proteins, such as the mdm2 gene product, have been shown to interact with p53 and regulate the function of p53 (3, 13-16). As yet, no cellular protein has been discovered which binds the central conserved region of p53, except for Hsp70 which binds only the mutated form of p53. Recently, Maxwell et al. identified three cellular proteins in the cells of non-small lung carcinomas with MW of 70kda, 56 kda and 45kda which bind the central conserved region(17). The proteins described here, p50 and p40, could be distinct proteins from them.

At this point there is no evidence that p50 and p40 interact with p53 in vivo. However, the specific interaction of these proteins with p53 in a cell cycle-dependent manner lead us to speculate their potential functions. One of the possible functions of p50 and of p40 is their inhibitory effect on the DNA-binding activity of p53, as the central conserved region of p53 is recently shown to be the DNA-binding domain where these p50 and p40 bind (5). The bindings of p50 and p40 were not observed in the G1 phase, in which p53 acts as a transcription factor, but in the S and G2/M phases, in which p53 does

not exert its effect. These observations are consistent with the idea that p50 and p40 bind and mask the DNA-binding domain of p53. Although a number of cellular proteins have been reported to bind transcription factors, few of them have been proved to bind the DNA-binding domains, except for Calreticulin that was shown to bind and mask the DNA-binding domain of steroid hormone receptors (18, 19).

As the roles of p53 in DNA repair and replication have been suggested(6, 7, 14, 15), involvement of p50 and of p40 in these process might be other potential functions for them.

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