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Zebrafish p53 protein enhances the translation of its own mRNA in response to UV irradiation and CPT treatment

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ABSTRACT

p53 protein is an important regulatory factor involved in cell growth and development. In our previous study, we demonstrated that recombined zebrafish p53 protein could specifically bind to its own mRNA in vitro. To determine if a similar interaction exists in zebrafish and if this interaction affects zebrafish development, in the present study, we investigated the interaction of p53 protein and its mRNA in zebrafish embryos. Our results revealed that expressed zebrafish p53 protein could bind with its own mRNA in zebrafish embryos. Furthermore, the endogenous activated or ectopically expressed p53 protein could enhance the relative activity of Renilla luciferase fused with p53 3′UTR in response to UV irradiation and CPT treatment, and retarded development of zebrafish embryos was observed.

1. Introduction

p53 protein plays a critical role in controlling the cell cycle, cell growth, and development [1,2]. As a central regulation factor, the expression and stability of p53 protein is regulated at different levels, including transcriptional, translational, and post-translational regulation [3,4]. In unperturbed cells, p53 protein is normally short-lived and maintained at low levels attributing to the rapid proteasomal degradation [2,5,6]. While in response to cellular stresses, such as DNA damage, hypoxia, or nutrient deprivation, intracellular p53 protein is quickly accumulated and leads to growth arrest of cell cycle or apoptosis [1,7]. It has been well established that posttranslational modification, such as phosphorylation, acetylation, and altered intracellular localization, plays an important role in the rapid accumulation of p53 protein in response to cellular stresses. For example, phosphorylation increases the stability of p53 protein, which reduces the degradation of p53 protein by its negative regulation protein mdm2 and hence results in the elevation of p53 protein level in cells [2,5,6]. Increasing documents have suggested that translational control of p53 mRNA is another crucial reason accounting for the elevation of p53 protein in response to stresses [8,9]. For example, HuR and RPL26 protein have been reported to enhance p53 translation by binding with the 3′UTR or 5′UTR of p53 mRNA [4,9]. Thymidylate synthase and nucleolin were reported suppressing p53 translation by binding to the coding sequence and 5′UTR of p53 mRNA respectively [4,8]. Furthermore, p53 protein of murine and human could negatively regulate its own mRNA translation by direct binding with the 5′UTR or 3′UTR of its cognate mRNA [10].

The zebrafish, Danio rerio, is an ideal model organism for studying developmental biology due to its unique characteristics, including high fecundity, short reproductive cycle, external fertilization, and transparency that permits visual assessment of developing cells and organs [11–15]. Studies have shown that p53 plays pivotal roles in the development of zebrafish embryos [16]. Maintaining the appropriate level of p53 protein is important for zebrafish development. Stresses such as a short exposure to UV irradiation or treatment with camptothecin (CPT) can activate p53 protein and result in growth retardation in zebrafish embryos [6]. Knockdown of mdm2, the most important negative regulation factor of p53 protein, results in severe apoptosis and retardation of development [6]. However, little is known about the translation regulation of p53 in zebrafish embryos.

In our previous study, we expressed zebrafish p53 protein using prokaryotic expression system, and have demonstrated that recombined zebrafish p53 protein can specially bind to its own mRNA in vitro as evidenced by immunoprecipitation: RT-PCR analysis and a UV-crosslinking experiment [17]. However, little is known about the function of the interaction between mRNA and proteins in zebrafish, and the effect of this interaction on zebrafish development need to be addressed. In the present study, the interaction of

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p53 protein and its mRNA was studied both in vitro and in vivo, and the effect of this interaction on development of zebrafish embryos was also investigated. Our results revealed that p53 protein could bind with the endogenous p53 mRNA in zebrafish embryos and the interaction between zebrafish p53 protein and its cognate mRNA could enhance the translational efficiency of p53 protein in response to UV irradiation and CPT treatment. Moreover, retarded development of zebrafish embryos was also observed.

2. Materials and methods

2.1. In vitro transcription of full-length p53, p53 5′UTR+ORF and p53 3′UTR mRNA

The full- length p53 gene (including the 5′UTR and 3′UTR), p53 5′UTR+ORF and p53 3′UTR was obtained by PCR amplification using the following specific primers:
full-length p53(forward): 5′-**TAATACGACTCACTATAGGG**GTTTAGT GGAGAGGAGGTCG -3′,
full-length p53(reverse): 5′-AAAGAGTGTATTTTAAAACATTTCTC-3′;
p535′UTR+ORF(forward): 5′-**TAATACGACTCACTATAGGG**GTTTAG T GGAGAGGAGGTCG -3′,
p535′UTR+ORF(reverse): 5′-TTAATGATGATGATGATGATGATCAGA GTCGCTTCTTCCTTC-3′;
p53-3′UTR(forward):5′-**TAATACGACTCACTATAGGG**GCGACTCTGAT TAAGGTGATGGGATG-3′,
p53-3′UTR(reverse):5′-AAAGAGTGTGTATTTTAAAACATTTCTC-3′.

The 5′ end of forward primer contains a T7 promoter (in bold) that permits transcription in vitro by T7 RNA polymerase. The PCR products were resolved on 1% agarose gel, purified using the Gel Recovery Kit (Tiangen, China), and used as templates for the synthesis of corresponding RNAs. Biotin-labeled RNAs were synthesized using the MEGAscript transcription kit (Ambion, CA) [17].

2.2. UV-crosslinking

Biotin-labeled full-length p53, p53 5′UTR+ORF and p53 3′UTR mRNA were obtained through transcription in vitro as described above. recombinant p53 protein was prepared as described in our previous report[17]. RNA–protein binding reaction mixtures (20 μl), containing 100 ng of biotin-labeled full- length p53, p53 5′UTR+ORF or p53 3′UTR mRNA and 1 μg of recombinant renatured p53 protein, were incubated at room temperature for 30 min and then cross-linked by UV irradiation for 15 min at 254 nm [18]. The mixtures were digested with RNaseA1 (1 mg/ml) at 37 °C for 30 min and resolved on 12% SDS–PAGE. The gel was electroblotted onto a Plus Positively Charged Nylon Membrane (Ambion, CA), and then the membrane was irradiated by UV irradiation to immobilize the RNA probe onto the membrane. The BrightStar BioDetect™ Kit (Ambion, CA) was used to detect the binding complex following the manufacturer's protocol. In brief, the membrane was washed two times for 5 min each time using washing buffer and then incubated in blocking buffer for 30 min. Streptavidin–alkaline phosphatase buffer (1:10,000) was added and the membrane was allowed to incubate for 30 min, followed by washing three times with washing buffer. CDP-Star was used as chemiluminescent substrate for detection of alkaline phosphatase to visualize the protein–RNA complex.

2.3. Synthesis of zebrafish p53 expression constructs and luciferase report vectors

The isolated total RNA from Zebrafish embryos was reverse transcribed into cDNA using M-MLV reverse transcriptase (Takara, Japan) [11]. The open reading frame (ORF) and the 3′ untranslated region (UTR) of zebrafish p53 were amplified by PCR using the following primers respectively:
p53-ORF(forward):5′-GGATCCATGGCGCAAAACGACAGCCAAG-3′,
p53-ORF(reverse):5′-GAATTCTTA*ATGATGATGATGATGATGA*TCAG AGTCGCTTCTTCCTTC-3′
p53-3′UTR(forward): 5′- CTCGAGGCGACTCTGATTAAGGTGATGG-GATG-3′,
p53-3′UTR(reverse):5′-GCGGCCGCAAAGAGTGTGTATTTTAAAA-CATTTCTC-3′

For the convenience of detection, a His-tag sequence, which is indicated by italic in the p53-ORF reverse primer, was introduced into the C- terminus of recombined p53 protein just before the stop codon.

The PCR products of p53 ORF and 3′UTR were inserted into pMD-18T cloning vectors (Takara, Japan). After sequencing, p53 ORF was sub-cloned into the corresponding sites of BamHI and EcoRI on the eukaryotic expression pcDNA3.1 vector (Fig. 1A). psiCHECK-2(Promega, WI)), which contains both Renilla and firefly luciferase reporter genes, enable the monitoring of changes in expression of a target gene fused to the reporter gene (Renilla luciferase). The firefly reporter cassette has been specifically designed to be an intraplasmid transfection normalization reporter. The 3′UTR of p53 was ligated into the psiCHECK-2 vector, between the XhoI and NotI sites, immediately downstream of the Renilla luciferase gene (Fig 1C). The construct of pcDNA3.1/his-TS (thymidylate synthase) was constructed previously by our laboratory (Fig 1D) [11] .

2.4. Culture of zebrafish embryos and manipulation of microinjection and UV irradiation

Zebrafish were maintained on a 14–10 h light dark cycle at 28 °C according to standard laboratory conditions of Kimmel [19]. The embryos at 1–2 cells stage were obtained by natural crossing. The linearized plasmids (100 pg) of Luciferase report constructs psiCHECK/p53 3′UTR were microinjected alone or co-microinjected with pcDNA3.1/his-p53 into embryos at 1–2 cell stage, and then the embryos were transferred to a 6-well plate and raised in embryo medium at 28 °C [20,21]. The UV irradiation experiment was performed as described by Mazan-Mamczarz [9]. Briefly, plasmids were microinjected into the embryos at 1–2 cell stage. After cultured for 12 h, the medium was removed, and embryo cells were washed with PBS and irradiated with 15 J/m2 UVC for 4 h and fresh medium was supplemented.

2.5. Western immunoblot analysis

Zebrafish embryos were microinjected without or with pcDNA3.1/his-p53 plasmids. After cultured for 48 h, the zebrafish embryos were harvested by centrifugation at 1000×*g*. Zebrafish embryos homogenate were prepared using ice-cold RIPA buffer (50 mM Tris–HCl, 1% NP40, 0.25% Na-doxycholate. 150 mM NaCl, 1 mM EDTA) which contains PMSF at a final concentration of 1 nm/mL. Protein concentrations were determined using BCA protein assay kit (Thermo Scientific, FL). Immunoblot analysis was performed as previously described [11]. Briefly, whole-cell lysates were resolved by 12% SDS–PAGE and then electroblotted onto a nitrocellulose membrane (Bio-Rad, CA). The membrane then was incubated in blocking buffer (1× PBS, 0.1% Tween-20, and 5% non-fat dry milk powder) for 2 h. Membrane was incubated for 1 h with primary antibodies anti-his monoclonal antibody (1:200, Santa Cruz, CA). After three 10 min washes, membranes were incubated with a dilution of 1:2000 of horseradish peroxidase-conjugated secondary antibody (Goat anti-mouse, BioRed) for 1 h at room temperature. chemiluminescence method was employed to