Comparative Biochemistry and Physiology, Part C 154 (2011) 326–332

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

journal homepage: www.elsevier.com/locate/cbpc

Characterization of p53 expression in rainbow trout

Michelle Liu [a], Catherine Tee [a], Fanxing Zeng [a], James P. Sherry [b], Brian Dixon [a], Niels C. Bols [a], Bernard P. Duncker [a,*]

[a] Department of Biology, University of Waterloo, Waterloo, Ontario, N2L 3G1, Canada
[b] Aquatic Ecosystem Protection Research Division, Environment Canada, Burlington, Ontario, L7R 4A6, Canada

ARTICLE INFO

Article history:
Received 21 May 2011
Received in revised form 29 June 2011
Accepted 30 June 2011
Available online 13 July 2011

Keywords:
p53
Checkpoint
DNA damage
Rainbow Trout
Biomarker

ABSTRACT

The tumour suppressor protein p53 is a critical component of cell cycle checkpoint responses. It upregulates the expression of cyclin-dependent kinase inhibitors in response to DNA damage and other cellular perturbations, and promotes apoptosis when DNA repair pathways are overwhelmed. Given the high incidence of p53 mutations in human cancers, it has been extensively studied, though only a small fraction of these investigations have been in non-mammalian systems. For the present study, an anti-rainbow trout p53 polyclonal antibody was generated. A variety of rainbow trout (*Oncorhynchus mykiss*) tissues and cell lines were examined through western blot analysis of cellular protein extracts, which revealed relatively high p53 levels in brain and gills. To evaluate the checkpoint response of rainbow trout p53, RTbrain-W1 and RTgill-W1 cell lines were exposed to varying concentrations of the DNA damaging agent bleomycin and ribonucleotide reductase inhibitor hydroxyurea. In contrast to mammals, these checkpoint-inducing agents provoked no apparent increase in rainbow trout p53 levels. These results infer the presence of alternate DNA damage checkpoint mechanisms in rainbow trout cells.

© 2011 Elsevier Inc. All rights reserved.

1. Introduction

To ensure proper DNA replication, surveillance mechanisms known as checkpoints are in place at various stages of the cell cycle. Specific checkpoints can be activated in the presence of genotoxic stress, including double strand breaks (DSBs), single strand breaks (SSBs), and stalled replication forks. Such perturbations typically activate several groups of proteins through a kinase cascade. The initial DNA damage or replication stress is detected by sensor proteins that proceed to stimulate the activity of transducer proteins, which in turn lead to the activation of downstream effector proteins, resulting in cell cycle arrest, DNA repair, or apoptosis (reviewed in Warmerdam and Kanaar, 2010).

The tumor suppressor protein p53 is a key factor in the mammalian DNA damage checkpoint pathway. It is a transcription factor which stimulates the expression of target genes encoding proteins that can help lead the cells to a resolution of the checkpoint (reviewed in Brady and Attardi, 2010). As one of the main effector proteins in the checkpoint pathway, mutations in its functional domains can lead to tumorigenesis and cancer. Indeed, p53 mutations have been found in over 50% of human tumors (reviewed in Olivier et al., 2010). Due to its importance in cancer research, p53 has been extensively characterized in mammalian models; however, its regulation and mechanisms of action remain poorly understood for lower vertebrate species.

Functional conservation relative to mammalian p53 has been shown in studies with zebrafish (reviewed in Storer and Zon, 2010). For example, it has been reported that p53 is necessary for apoptosis of cells in response to DNA damaging agents in developing zebrafish (Langheinrich et al., 2002; Berghmans et al., 2005). As in mammals, zebrafish p53 is negatively regulated by MDM2, and embryos that are depleted of MDM2 display developmental defects and an increased incidence of apoptosis (Langheinrich et al., 2002). Furthermore, zebrafish p53 has been found to regulate the transcription of several of the same genes as in mammals (Langheinrich et al., 2002; Lee et al., 2008), and homozygous p53 mutants develop tumors (Berghmans et al., 2005).

Checkpoint proteins are attractive candidates as biomarkers for detecting the presence of genotoxic compounds in polluted waters, as they are often upregulated and/or post-translationally modified as part of the response to DNA damage. For example, we have previously shown that levels of rainbow trout CHK2 increase dramatically in a rainbow trout brain cell line, following exposure to the radiomimetic drug bleomycin (Steinmoeller et al., 2009). Given the conserved checkpoint role of fish p53, and the fact that mammalian p53 is known to be stabilized in response to genotoxic stress (reviewed in Kruse and Gu, 2009), it is important to assess the potential of p53 as a biomarker for genomic damage in aquatic ecosystems. To date, studies of p53 in fish have resulted in widely differing findings, with some reporting induction following exposure of cells to stressors (Lee et al., 2008;

* Corresponding author. Tel.: +1 519 888 4567x33957; fax: +1 519 746 0614.
E-mail address: bduncker@sciborg.uwaterloo.ca (B.P. Duncker).

1532-0456/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.cbpc.2011.06.018

Brzuzan et al., 2009; Mai et al., 2010) and others observing no changes in response to the agents that normally upregulate mammalian p53 (Chen et al., 2001; Rau Embry et al., 2006).

In reconciling differing outcomes for p53 induction trials, an important consideration is that p53 distribution and activity can vary greatly between different tissue types. Immunoblot analysis of total protein extracts from zebrafish embryos showed no change in global p53 levels following treatment with either R-roscovotine (a negative regulator of MDM2, found to stabilize p53 in human cells) and/or γ-irradiation. In contrast, the same study reported that immunohistochemical staining of p53 in embryos revealed an accumulation in gut epithelium, liver, pancreas and brain tissues following such treatments (Lee et al., 2008). Variability in p53 expression levels between different tissue types has also been observed during normal mouse and human development (Schmid et al., 1991; Lichnovsky et al., 1998).

In the present study, we report the levels of p53 in rainbow trout tissues and cell lines, and assess its potential utility as a biomarker for genotoxic stress.

2. Materials and methods

2.1. Rainbow trout polyclonal p53 antibody production

Rainbow trout (*Oncorhynchus mykiss*, Salmonidae) p53 coding sequence was cloned using RTgill-W1 cDNA as template, along with forward (5'-GACTTCTCGAGCTGGCGGAGAACGTGTCTCTTC-3') and reverse (5'-GGACTTAAGCTTCACTCCGAAGTCCCGTTTGGC-3') primers, including XhoI and HindIII restriction enzyme sites, respectively. These primers amplify a 1125 bp sequence, encoding residues 4–378 of the 396 aa rainbow trout p53 protein (Caron de Fromentel et al., 1992; Danilova et al., 2008). Following PCR amplification, the fragment was gel-purified and inserted into a pRSET A expression vector (Invitrogen) through digestion with XhoI and HindIII restriction enzymes. Ligation was then performed using T4 DNA ligase (Promega), resulting in a construct, pRSETA-RTp53, expressing rainbow trout p53 as a fusion protein with a polyhistindine tag and an Xpress epitope at the N-terminus. The construct was sequenced (Robarts Research Institute DNA Sequencing Facility), and found to contain a single point mutation, resulting in an amino acid substitution of cysteine to arginine in a non-conserved region of the protein (position 365). pRSETA-RTp53 was then transformed into competent (DE3)pLysS *E. coli* cells (Promega) for inducible expression of recombinant rainbow trout p53 according to the manufacturer's suggested protocol. Transformed cells were grown in SOB medium supplemented with 100 μg/mL ampicillin, shaken at 37 °C, 250 rpm to an optical density of 0.4–0.6, before a 4 h induction of recombinant protein expression with 1 mM IPTG. Cells were harvested by centrifugation (5000 *g*, 10 min) and lysed overnight at room temperature with 8 M urea denaturing buffer (100 mM NaH_2PO_4, 10 mM Tris–HCl, 8 M urea, pH 8). The lysate was then centrifuged (10,000 *g*, 6 min) at 4 °C before saving the supernatant and discarding the pellet. To purify recombinant p53, affinity chromatography was performed in an econo-column (Bio-Rad) with Ni–NTA resin (Qiagen). Binding of resin to the polyhistidine tag at the N-terminus of the recombinant protein was carried out through incubation with the lysate on a rotator at 4 °C for 2 h. After incubation, the flowthrough was discarded. The protein was then refolded on the column using a decreasing gradient of urea (6 M–0 M) before elution in its native form with 250 mM imidazole in 8 fractions of 1 mL each. 10 μL of each fraction was then run on a 12% SDS polyacrylamide gel and analyzed by Coomassie blue staining to determine recombinant protein concentration. Elution fractions were dialyzed overnight at 4 °C in 200 mL of 1× PBS. Following dialysis, recombinant protein samples were again analyzed by SDS-PAGE and Coomassie blue staining, and a Bradford assay was then performed to determine protein yield. The dialyzed recombinant protein sample fractions were then stored at −20 °C.

Polyclonal antiserum production was performed essentially as described previously (Kales et al., 2006). Subcutaneous rabbit immunization was carried out with 200 μL recombinant p53 (0.7 mg/mL) mixed with 200 μL Freund's complete adjuvant (Sigma), at four different injection sites. This was followed with booster shots of a similar emulsion, but with Freund's incomplete adjuvant, at three week intervals. Blood samples were obtained before each boost through the marginal ear vein. To separate the serum from the blood, samples were left at room temperature for 2 h and then overnight at 4 °C to allow the blood to clot. Samples were then centrifuged at 5000 *g* for 10 min at 4 °C to pellet the blood cells, and the serum was collected and assessed for antibody titre through ELISA analysis with purified recombinant p53. At the end of the twelfth week, exsanguination by carotid cannulation was carried out for final collection of total blood.

Antibodies specific to recombinant rainbow trout p53 were purified from crude serum with a SulfoLink Immobilization Kit for Proteins (Pierce). The SulfoLink column was generated by binding recombinant rainbow trout p53 to the resin according to the manufacturer's suggested protocol with minor changes. To prepare the protein for coupling, 1 mL of recombinant p53 (0.7 mg/mL) was reduced with 2-mercaptoethylamine (2-MEA) and applied to the provided desalting column to remove remaining 2-MEA. For affinity purification, 2 mL of pure serum was used for each run. Each wash step was performed the maximum number of suggested times. Bound antibodies were eluted in 4 aliquots of 1 mL each, and concentration was determined through a Bradford assay.

2.2. Whole fish

Rainbow trout (*Oncorhynchus mykiss*) were obtained from the Alma Aquaculture Research Station (University of Guelph) and acclimated for at least two weeks in 200 L aerated tanks with constant water flow at 13 °C and a 12 h light:12 h dark photoperiod, prior to experimentation. Organs were harvested and frozen on dry ice, then stored at −80 °C. Lysates were prepared through homogenization and sonication of frozen tissues in 50 mM Tris–HCl buffer (pH 7.5) supplemented with protease inhibitor cocktail (Roche). Lysates were centrifuged at 16,000 *g* for 10 min to remove cell debris and insoluble proteins. The supernatant was retained and protein concentrations were determined through Bradford protein assay (Bio-Rad) according to the manufacturer's suggested protocol.

2.3. Fish cell lines

Rainbow trout cell lines from the brain (RTbrain-W1), gastrointestinal tract (RTgut-GC), gill (RTgill-W1), gonad (RTG-2), liver (RTL-W1) and spleen (RTS11) were used. Comprehensive descriptions for the development and characterization of RTgutGC, RTL-W1, and RTS11 have been presented respectively by Kawano et al. (2011), Lee et al. (1993), and Ganassin and Bols (1998). RTbrain-W1 was developed by the general methods outlined by Ganassin and Bols (1997) and was used by Steinmoeller et al. (2009). Development of RTgill-W1 is described in Bols et al (1994), and this cell line has been submitted to the American Type Culture Collection (ATCC) (Manassas, VA) from where it can be obtained as CRL-2532 . RTG-2 was obtained from ATCC as CCL-55 and its origins are described by Wolf and Quimby (1962). The routine growth of the cell lines was done at room temperature as described in detail previously (Bols and Lee, 1994).

2.4. Cell culture treatment regimes

Cells were seeded in 25 cm^2 flasks at either 1.5×10^6 (RTbrain-W1) or 3×10^6 (RTgill-W1) cells per flask, 24 h prior to treatment.

Download English Version:

https://daneshyari.com/en/article/10821693

Download Persian Version:

https://daneshyari.com/article/10821693

Daneshyari.com