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Retroviral Reporter Systems for Assessing the Activity of Stress-Inducible Signal Transduction Pathways Controlled by the p53, HIF-1, and HSF-1 Transcription Factors

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Abstract

The tumor suppressor p53, hypoxia-inducible factor 1 (HIF-1), and heat-shock factor 1 (HSF-1) are the key transcription factors involved in the stress response to damage of the genetic material, hypoxia, or heat shock, respectively. Since these factors play a considerable role in tumor development and progression, modulation of their activities may be used in cancer therapy. To quantitate the transcriptional activities of p53, HIF-1, and HSF-1, reporter constructs were obtained on the basis of retroviral and lentiviral vectors, allowing generation of reporter cultures from almost all cell types. Induction of the reporter β -galactosidase gene, which reflected the activity of p53 or HIF-1, proved to depend on the concentration of an activating agent and to correlate with induction of the endogenous target genes of the transcription factors. Inhibition of *p53* or *HIF-1 α* expression with specific small interfering RNAs (siRNAs) completely abolished the activating effect of stress conditions. The reporter constructs were proposed for screening chemical compounds or genetic elements (siRNA or cDNA libraries) that modulate the activity of p53, HIF-1, or HSF-1 and for studying the components of the relevant signaling pathways.

Keywords

stress factors; transcription factors; p53; HIF-1; HSF-1; responsive element; reporter gene; β -galactosidase; small interfering RNAs

INTRODUCTION

Stress initiates adaptive responses, which are controlled by transcription factors. Changes in the activity of signal transduction pathways in stress play an important role in some diseases.

The tumor suppressor p53 is activated under conditions threatening heritable alteration of the genetic material and either arrests cell division or induces cell death (apoptosis) [1]. A loss of *p53* leads to uncontrollable accumulation of genetic lesions and malignant transformation of the cell. The hypoxia-inducible factor 1 (HIF-1) is responsible for the maintenance of oxygen homeostasis and plays a considerable role in carcinogenesis and cardiovascular disorders [2]. The heat-shock factor 1 (HSF-1) is involved in the cell response to a loss of native conformations by proteins, e.g., in hyperthermia [3]. Changes in HSF-1 activity determine the apoptosis resistance mediated by HSF-1 target genes in some malignancies [4]. Thus, modulation of the activities of these three factors *in vivo* is of interest from the viewpoint of therapy for cancer and cardiovascular disorders. The major biological role of p53, HIF-1, and HSF-1 is their function as transcription factors [5–7]. Since specific nucleotide sequences (response elements, REs) that are responsible for the binding of these transcription factors are well known [8–10], it is possible to obtain reporter constructs suitable for studying the functional properties of p53, HIF-1, and HSF-1. However, the essential feature of reporter constructs used to assay the activity of stress-inducible signaling pathways is the absence of a stress response to the procedure of construct introduction into cultured cells. We developed reporter constructs on the basis of retroviruses and lentiviruses to prevent artifacts caused by cell transfection with DNA. The constructs proved to be suitable for quantitating the activities of p53, HIF-1, and HSF-1 with sufficient accuracy.

EXPERIMENTAL

Reporter plasmid sensitive to p53 was based on the self-inactivating vector pUSTdS-mCMV-lacZ. After excising the *Bam*–*H*I *Bgl* II fragment adjacent to the H4 promoter and containing a transcriptional silencer, the resulting vector pUSTdSB-mCMV-lacZ was used to clone the p53-RE (WafConA), which was obtained as a *Xho* I–*Spe* I fragment of pSIP-WafConAmHsp70-GFP (A.V. Ivanov, unpublished data).

Reporter plasmid sensitive to HIF-1 α

The oligonucleotides 5'-HRE *Xba*I (5'-ctagaggacgtgacaaacagaagccacacgtcctagggacgtggggagtgctgaggagtacgtgaggacacgtgggta) and 3'-HRE *Spe*I (5'-ctagtaccacgtgtctctcactcactcactccacgtccctaggacgtgtggcttctgtttgtcacgtcct) were annealed and multimerized by ligation. To obtain multimers with the head-to-tail orientation of monomers, the reaction product was digested with *Xba*I and *Spe*I. Multimers of the synthetic DNA fragment were separated in agarose gel, and the fragment corresponding to a 12-mer tandem (HRE12) was isolated from gel and cloned into the *Xba*I site of pGEM-7Zf(+) (Promega, United States). HRE12 with the restriction site order *Xho*I–*Xba*I–*Xba*I/*Spe*I–hybrid site–*Sph*I was cloned into the *Xho*I–*Sph*I sites of pUSTdS-mCMV-lacZ.

Reporter plasmid sensitive to HSF-1, pUSTdSHSE18-mCMV-lacZ, was obtained using the same procedure as for the HIF-1 α -sensitive plasmid. The difference was that we used the oligonucleotides 5' HSE *Xba*I (5'-ctagagaacattcctcagagagaggaaggttctggaggagacctggaatattcctga) and 3'-HSE *Spe*I (5'-ctagtcaggaatattccaggtctctccagaacctctctctctgaggaatgttct) and cloned a tandem of 18 monomers of the synthetic DNA fragment (HSE18).

To obtain p53- and HIF-1 α -sensitive reporter constructs in a self-inactivating lentiviral vector (pLVWafConA-mCMV-lacZ and pLV-HRE12-mCMVlacZ), the expression cassettes WafConA-mCMVlacZH4- Puro R and HRE12-mCMV-lacZH4- Puro R were cloned into the *Xho* I–*Sal* I sites of pLV-CMV-H4 vector.

Plasmids expressing siRNA

To obtain the construct pLSLG-HIF-1 α -siRNA, the oligonucleotides siRNA-HIF-1 α -Bam HI (5'gatccgtatggttctcacagatgatggcttctgtcaccatcatctgtgagaacctattttg) and siRNAHIF-1 α -Eco RI (5'aattcaaaaatatggttctcacagatgatgggtacaggaag-ccatcatctgtgagaacctatcg, regions of homology to human HIF-1 α cDNA, GenBank NM 001530, are underlined) were annealed and cloned into the Bam HI–Eco RI sites of the self-inactivating lentiviral vector pLSLG (P.M. Chumakov, unpublished data). The construct pLSLG-p53-siRNA was obtained using the oligonucleotides siRNA-p53- Bam HI (5' gatccggactccagtggttaactctctctgtcagtagattaccactggagctcttttg) and siRNA-p53- Eco RI (5' aattcaaaaagtagattaccactggagctgtgacaggaaggtagattaccactggagtcgcg, regions of homology to human p53 cDNA are underlined [11]). The construct pLSLG-E6-siRNA was obtained using the oligonucleotides siRNA-E6- Bam HI (5' gatccgctaactgggttatacaactctctgtcattgtataaccagtggttagttttg) and siRNAE6- EcoRI (5' aattcaaaaactaactgggttatacaatgacaggaaggtgtataaccagtggttagcg, underlined are regions of homology to the human papilloma virus 18 E6 gene, GenBank X04354). The siRNA specifically inhibiting E6 expression was used as a negative control. Nucleotide sequences corresponding to E6 were absent from the genome of cell cultures used in our experiments.

Virion preparations of retroviral and lentiviral vectors, infection of target cells, and selection of infected cells

To obtain retroviral vector virions, retroviral constructs were transduced into PhoenixAmpho cells [12] by lipofection with Lipofectamine-Plus (Invitrogen, United States). In the case of lentiviral vectors, we used 293T cells and the helper plasmids pCMV Δ R8.2 and pVSV-G, which code for lentiviral packaging proteins. Infected cells were selected on the standard culture medium supplemented with 1 μ g/ml puromycin.

β -Galactosidase activity assays

In the case of X-Gal staining, cells were fixed with a cold 0.5% glutaraldehyde and 1 mM MgCl₂ solution in PBS and stained with a 1 mM MgCl₂, 3.3 mM K₄Fe(CN)₆, 3.3 mM K₃Fe(CN)₆, 0.02% NP-40, and 0.2% X-Gal (Sigma, United States) solution in PBS at 37°C for several hours. In the case of ONPG staining, cells were incubated in a 1 mM MgCl₂, 250 mM Tris-HCl (pH 7.4), 0.02% NP-40, and 2 mg/ml ONPG (Sigma, United States) solution in PBS at 37°C. Staining intensity was quantitated at A 405 on a Wallac Victor 2 1420 Multilabel counter (Perkin Elmer Life Sciences, United States).

Western blotting

Cells were lysed with RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% sodium deoxycholate, 1% NP-40, 0.1% SDS) supplemented with Complete mini protease inhibitors (Roche, United States). Protein extracts (20 μ g of total protein) were analyzed by PAGE in 4–20% gradient gel (Invitrogen, United States). We used antibodies against p53 (DO-1, Santa Cruz, United States), p21 (F-5, Santa Cruz, United States), or β -actin (A1978, Sigma, United States) for detecting the corresponding proteins.

Reverse transcription–polymerase chain reaction (RT–PCR)

The first cDNA strand was synthesized with SuperScript II reverse transcriptase (Invitrogen, United States) on 5 μ g of total RNA. PCR was carried out with primers directed to HIF-1 α (HIF-1 α -direct, 5'-tgagcttgctcatcagttgc and HIF-1 α -reverse, ttctaacaagctgacgctg and PGK1-reverse, 5'-ttcttctccacatgaaagc), and the cyclophilin gene (PPIA-direct, 5'-cttcacacgccataatggc and PPIA-reverse, 5'-gtgatcttctgtgctgttctg).

RESULTS

Generation of Retroviral Reporter Constructs for Assessing the Transcriptional Activities of p53, HIF-1, and HSF-1

We obtained several reporter constructs in order to estimate the transcriptional activities of p53, HIF-1, and HSF-1. The reporter plasmids were based on the self-inactivating retroviral vector pUSTdS-mCMVlacZ, which contains a functional promoter in the 5' long terminal repeat (LTR) and a defective promoter in the 3'-LTR (Fig. 1a). Upon infection of target cells, the nonfunctional 3'-LTR replaces the functional 5'-LTR promoter due to the process of reverse transcription. As a result, virus DNA integrated into the cell genome lacks functional promoters and has no effect on the expression of the *lacZ* reporter gene. Using this vector, we obtained the reporter constructs in which the *lacZ* gene expression was under the strict control of the cytomegalovirus minimal promoter (mCMV) and a RE specifically binding the transcription factor p53 (WafConA), HIF-1 (HRE12), or HSF-1 (HSE18).

The p53-sensitive reporter plasmid was based on the retroviral vector pUSTdS-mCMV-lacZ, in which the silencer affecting the *lacZ* expression was deleted. The plasmid was designated as pUSTdSB-WafConAmCMV-lacZ. The plasmids sensitive to HIF-1 or HSF-1 were named pUSTdS-HRE12-mCMV-lacZ and pUSTdS-HSE18-mCMV-lacZ, respectively. Schemes of their REs are shown in Figs. 1b–1d. The p53-RE (WafConA) consists of a tandem of six p53-binding sites from the *p21/Waf1* gene promoter (Waf) [13], a consensus binding site (Con), and fragment A of the ribosomal gene cluster [14]. The HIF-1-RE is a tandem of short fragments originating from the *PGK1*, *ENO1*, and *LDHA* regulatory regions and containing the consensus binding sites for HIF-1 [9, 15]. Knowing that one turn of the DNA double helix corresponds to approximately 11 bp, we arranged the HIF-1-binding sites so that they became all exposed on one side of the double helix. Since the sensitivity of a reporter system is higher when several copies of the HIF-1-binding site are used [16], 12 copies of the RE (HRE12) were cloned to enhance the binding capacity toward HIF-1. The HSF-1-sensitive reporter construct was generated using the same approach with fragments of the *MDR1*, *Hsp40*, and *Hsp70* promoter regions; the RE was cloned in 18 copies (HSE18). In addition to the above retroviral constructs, we obtained the p53- and HIF-1 α -sensitive constructs pLV-WafConAmCMV-lacZ and pLV-HRE12-mCMV-lacZ on the basis of a self-inactivating lentiviral vector.

Induction of Reporter Gene Expression in Response to Cell Treatment with Transcription Factor Activators

The reporter constructs were transduced into several cell lines by means of retroviral infection. The p53 activity was assayed in primary human embryonic fibroblasts (HEF) and in H1299 human lung cancer cells, which lacked p53 expression and served as a negative control. The HIF-1 activity was assayed in H1299 and 786-0 human renal adenocarcinoma cells (a negative control due to disturbed HIF-1 function [17]). The HSF-1 activity was assayed in H1299 cells. After introducing the constructs, positive cells were selected in the presence of puromycin. Transcriptional activity of p53 was induced by cell treatment with 50 μ g/ml 5-fluorouracil (5-FU). To stabilize HIF-1 α , hypoxia was simulated by treating cells with ironchelating desferrioxamine (DFO). To induce HSF-1-dependent transcription, cells were exposed to heat shock at 44°C for 1 h. Cell cultures were stained with X-Gal to estimate the β -galactosidase activity 12 h after exposure to the above stress factors. Expression of *lacZ* increased in cells with intact transcription factor activation pathways: these cells were intensely stained with X-Gal (Fig. 2).

Induction of the Reporter Gene Is Dependent on the Activator Concentration

The reporter cell cultures HEF/pUSTdSB-Waf-ConA-mCMV-lacZ and H1299/pUSTdS-HRE12-mCMV-lacZ were treated with various concentrations of 5-FU or DFO for 12 h. The

β -galactosidase activity was measured spectrophotometrically at 405 nm in the reaction of colorless ONPG conversion into a colored derivative (Fig. 3). Since β -galactosidase has a short half-life (about 2 h), the dependence of its activity on dosage was distinct (Fig. 3). In studying the dependence of the reporter gene induction on the activity of a transcription factor, expression of the transcription factor gene was suppressed with the corresponding siRNA and a lack of induction in this case served as a control. Reporter cultures were infected with the lentiviral construct pLSLG-p53siRNA or pLSLG-HIF-1 α siRNA and treated with an activator 48 h after infection. When expression of the transcription factor was suppressed, the concentration dependence was undetectable (Fig. 3), and the basal expression of the reporter gene was lower (Fig. 3, zero point).

Correlation between Induction of the Reporter Gene and Endogenous Target Genes in Response to Activating Treatment

Induction of *p21/Waf1*, an endogenous target of p53, in response to 5-FU (50 μ g/ml, 12 h) was assayed by Western blotting. A lack of *p21* expression upon *p53* inhibition with the *p53* siRNA served as a control in studying the *p21* induction as dependent on the *p53* activity. The *E6* siRNA virus was used as a control siRNA. As Fig. 4a shows, both *p53* and *p21* expression was induced by 5-FU and was abolished in the presence of the *p53* siRNA.

Induction of *PGK1*, a target of HIF-1, in response to DFO (225 μ M, 12 h) was assayed by RT-PCR with primers specific to *HIF-1 α* , *PGK1* or the cyclophilin gene (a control HIF-1-independent gene). The *HIF-1 α* and *E6* siRNAs were also used in these experiments. DFO induced *PGK1* expression, and the *HIF-1 α* siRNA inhibited expression of *HIF-1 α* and abolished induction of *PGK1* (Fig. 4b).

Thus, induction of reporter gene expression correlated with an increase in transcription of endogenous target genes of p53 and HIF-1.

DISCUSSION

Earlier studies have already employed reporter constructs containing the p53-RE [14,18–20], HIF-1-RE [21–30], or HSF-1-RE [31–34]. However, these constructs are based on plasmid vectors and have some limitations characteristic of reporters that are delivered into target cells by means of plasmid transfection. First, transfection with exogenous DNA is known to activate p53 in several cell lines [35]. Second, induction of a reporter protein is weaker in cells with stable integrated constructs than in temporarily transfected cells with several nonintegrated copies of the reporter gene. Third, selection of a cell line for experiments with reporters depends to a certain extent on the DNA transfection efficiency, which varies considerably from line to line. To overcome these limitations, we constructed alternative reporter cassettes in self-inactivating virus vectors. The efficiency of DNA transfer into target cells reaches 100% with retroviral or lentiviral vectors, allowing transduction of almost all cell lines. Taking advantage of the puromycin resistance gene, which was present in all reporter constructs, we obtained several cell lines that carried stable integrated constructs and showed inducible expression of the reporter gene even after as many as 10–15 passages (data not shown). The first-generation p53-responsive reporter constructs were previously obtained in our laboratory on the basis of self-inactivating retroviral vectors [36]. In this work, we developed reporter constructs of the second (pUSTdSBWafConA-mCMV-lacZ) and third (pLV-WafConA-mCMV-lacZ, a similar construct based on a lentiviral vector) generations. These reporter constructs are more sensitive to p53 activation as compared with the first-generation constructs, because their improved reporter cassette contains six p53-binding sites from the *p21/Waf1* promoter in addition to the ConA p53-RE, a stronger minimal promoter (mCMV in place of mHsp70), and an optimized vector part. With a higher sensitivity of the constructs, it became problematic to use the green fluorescent protein (EGFP) gene as a reporter of p53 activity (data not shown). The EGFP half-

life is far longer than that of β -galactosidase, and its accumulation in the cell masks the effect of p53 stimulation. On the other hand, reporter constructs with *lacZ* proved to be adequate to study the dynamics of p53 activity.

Our reporter constructs may be used to convert almost every cell line into a reporter culture suitable for quantitating the changes in the activity of transcription factors in response to various signals transmitted through stress-related pathways. Such reporter cell lines are applicable to screening chemical compounds or genetic elements (siRNA or cDNA libraries) that modulate the activity of transcription factors and to studying the components of the relevant signaling pathways. With a tumor suppressor or toxic (e.g., triggering the apoptosis cascade) gene substituted for the reporter gene, the HIF-1-dependent constructs may be employed in gene therapy of cancer as described for other constructs [37]. Introduced as a component of such constructs into the cells of a solid tumor, the HIF-1-dependent gene would be expressed only in hypoxia to suppress tumor growth or to induce apoptosis.

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Fig. 1. Schemes of (a) the reporter constructs based on the retroviral vector pUSTdS-mCMV-lacZ and the REs for (b) p53 (Waf- ConA), (c) HIF-1 (HRE12), and (d) HSF-1 (HSE18). Restriction sites used in cloning procedures are indicated. H4, the promoter of the histone H4 gene; Puro^R, the puromycin resistance gene; Ap^R, the ampicillin resistance gene

Fig. 2.

Induction of the *lacZ* reporter gene upon cell exposure under transcription factor-activating conditions. (a) The H1299 and HEF reporter cultures carrying pUSTdSB-WafConA-mCMV-lacZ were treated with 50 $\mu\text{g/ml}$ 5-FU for 12 h. (b) The 786-0 and H1299 reporter cultures carrying pUSTdS-HER12-mCMV-lacZ were treated with 225 μM DFO for 12 h. (c) The reporter culture H1299/pUSTdS-HSE18-mHsp70-lacZ was exposed to heat shock for 1 h and then incubated at 37°C for 12 h. Cells were stained with X-Gal.

Fig. 3.

β -Galactosidase activity as dependent on the concentration of (a) 5-FU used to activate p53 in the reporter culture HEF/pUSTdSB-WafConA-mCMV-lacZ or (b) DFO used to activate HIF-1 in the reporter culture H1299/pUSTdS-HRE12-mCMVlacZ. Cell cultures carrying a reporter construct were grown in the absence or presence of 5-FU or DFO for 12 h and stained with ONPG. Optical density (A_{405}) reflects the level of the colored ONPG derivative produced due to β -galactosidase activity. Control experiments were performed with the same reporter cell lines after inhibiting the *p53* or *HIF-1 α* expression with the corresponding siRNA. Standard deviation was estimated with three replications of the experiments.

Fig. 4. Induction of endogenous target genes of (a) p53 (*p21/Waf1*) and (b) HIF-1 (*PGK1*) in response to 12-h exposure to 50 μ g/ml 5-FU or 225 μ M DFO, respectively. (a) Western blotting of the p53 (the DO-1 antibody), p21 (F-5), and β -actin (A1978) protein expression. (b) Agarose gel electrophoresis of the RT-PCR products obtained with primers specific for the *HIF-1 α* , *PGK1*, or cyclophilin genes. In control cell cultures, expression of p53 or *HIF-1 α* was suppressed with the specific siRNA. The E6 siRNA was used as a control for nonspecific effects of siRNA expression.