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A luciferase reporter assay to investigate the differential selenium-dependent stability of selenoprotein mRNAs

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Abstract

The mechanisms regulating the differential selenium (Se)-dependent stability of selenoprotein mRNAs are partially characterized. To further study the Se-dependent regulation of selenoproteins, we developed a novel chemiluminescent reporter to monitor the steady-state mRNA level of an artificial selenoprotein. Our reporter is a fusion of the *Renilla* luciferase gene and of the β-globin gene, but contains features required for incorporation of selenocysteine (SEC), namely, a UGA-SEC codon and a 3′ untranslated region RNA stem loop called a SEC incorporation sequence (SECIS). At various levels of Se, the activity of reporters containing *GPX1* or *GPX4* SECIS elements is proportional to the steady-state mRNA level of the reporter construct and reflects the level of the corresponding endogenous mRNA. In a reporter containing a UGA codon and a functional *GPX1* SECIS, Se-dependent nonsense-mediated decay (NMD) occurred in the cytoplasm, as opposed to the more typical nuclear location. To validate the reporter system, we used genetic and pharmacologic approaches to inhibit or promote NMD. Modulation of UPF1 by siRNA, overexpression, or by inhibition of SMG1 altered NMD in this system. Our reporter is derived from a Renilla luciferase reporter gene fused to an intron containing B-globin gene and is subject to degradation by NMD when a stop codon is inserted before the second intron.

Keywords: Selenium; Selenoprotein; Glutathione peroxidase; Nonsense-mediated decay; Reporter; Luciferase

1. Introduction

Selenoproteins are a small group of proteins that incorporate selenocysteine (SEC), a selenium (Se)-containing amino acid, into their primary structure [1]. The 25 known human selenoproteins include several with important antioxidant properties, including five glutathione peroxidases (GPXs), three thioredoxin reductases and selenoprotein P (a major plasma Se-delivery molecule with GPX-like activity). While the standard 20 amino acids all have codons of their own, SEC appears to be a late addition to the genetic code [2,3]. In this regard, SEC does not have a codon of its own, but rather must recode an in-frame UGA "stop" codon to allow incorporation of SEC. The recoding of the UGA "stop" codon is mediated by a unique stem-loop structure in the 3′ untranslated region (UTR) of the selenoprotein mRNA called a SEC insertion sequence or SECIS [4–6]. SECIS elements are necessary for the insertion of SEC at a UGA codon, but they are not sufficient [4]. A number of protein factors assemble on the SECIS to form an insertion complex [7–9]. Essential factors in the complex include a SECIS binding protein (SBP2), a specific elongation factor (EFSEC) and the $tRNA^{SEC}$ (encoded by *TRSP*). When this complex assembles on the SECIS, SEC is delivered to the ribosome and incorporated into the growing peptide chain when directed to do so by the presence of a UGA codon [7].

An interesting phenomenon first noted in rats and later confirmed in tissue culture experiments is that, under conditions of Se deficiency, the expression of *GPX1* mRNA is reduced [10,11]. In contrast to what is observed with the level of *GPX1* mRNA, the level of *GPX4* mRNA remains relatively stable under conditions of Se deficiency [12]. Like the *GPX1* mRNA, some other selenoprotein mRNAs including *SelH* and *SelW* are also regulated by Se deficiency, and a few mRNAs are resistant to Se-dependent degradation [13]. Based on their structure, some, but not all, selenoprotein mRNAs are predicted to be degraded by a process known as nonsense-mediated decay (NMD) [12,14]. NMD is a surveillance pathway used by the cell to detect and degrade aberrant mRNA transcripts containing premature termination codons (PTCs). In general, termination codons are usually situated within the last exon and typically lack a downstream exon–junction complex, a feature required for the initiation of NMD, and therefore do not generally trigger the degradation of the mRNA [15]. According to the classic rules, in order for an mRNA to be flagged for destruction by NMD, it must have a PTC followed by an appropriately spaced intron, features present in the pre-mRNAs of both *GPX1* and *GPX4* [16]. One hypothesis is that the differential susceptibility of selenoprotein mRNAs to Se-dependent NMD is a regulatory mechanism by which a limited supply of Se may

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be preferentially used to support the production of more essential selenoproteins [13,17].

The mechanisms that allow the *GPX4* mRNA to resist being degraded by NMD are partially understood. A distinguishing feature between the *GPX1* and *GPX4* selenoprotein mRNAs is that they have distinct SECIS forms. SECIS elements come in two forms [5]. Form 1 (*GPX1*) SECIS elements have a 10–14-nucleotide adenosine loop. Form 2 (*GPX4*) SECIS elements have an adenosine bulge and an upper stem loop. Most selenoproteins have just one SEC codon and one SECIS element. It is possible that the efficiency of NMD is mediated in part by different proteins that assemble on the various SECIS forms, by the affinity of specific SECIS binding proteins for the SECIS or by the efficiency of SECIS-mediated SEC incorporation. All SECISs differ in sequence and structure; however, only some selenoprotein mRNAs are subjected to degradation under Se deficiency; in addition, some selenoprotein mRNAs are regulated by Se but do not follow the classical rules for NMD [13]. Other cell studies have shown that the GPX4 3′ UTR is as effective as the GPX1 3′ UTR in mediating the incorporation of SEC at UGA codons and that some of the difference in mRNA stability resides in the coding region [18]. Others hypothesize that $tRNA^{SEC}$ isoforms play a role in selenoprotein mRNA stability [19] or that additional stem-loop structures in the coding region might account for differences in stability [20]. Moreover, data from Müller et al. indicate that the stabilizing efficiency of an individual selenoprotein 3′ UTR does not necessarily correlate with the efficiency of read-through at the UGA codon, suggesting that Se-responsive elements may be located in both the translated and untranslated region of the gene [21]. Improved reporter systems will facilitate studies aimed at further characterizing mechanisms regulating the stability of selenoprotein mRNAs and, in the future, could be used to characterize mechanisms regulating the stability of additional members of the selenoprotein family.

Here we describe the development and validation of a chemiluminescence-based selenoprotein reporter system that is susceptible to Se-dependent NMD. To validate our reporter, we confirm that a *GPX1* SECIS dictates in the absence of optimal supplemental Se that a transcript containing both an intron and a UGA-SEC codon is susceptible to NMD. In the presence of optimal supplemental Se, a functional *GPX1* SECIS element inhibits the NMD of the artificial selenoprotein reporter. Our validated reporters are powerful tools that may be useful for rapidly characterizing mechanisms regulating selenoprotein mRNA stability and cytoplasmic NMD.

2. Methods and materials

2.1. Plasmids

A reporter for monitoring the NMD of an artificial selenoprotein mRNA was created by modifying a pCI-*Renilla*/β-globin NMD reporter plasmid developed by Dr. Andreas E. Kulozik [22]. The original reporter is a fusion of the *Renilla* luciferase open reading frame and the human β-globin gene, with or without a nonsense mutation at amino acid position 39 (NS39). The presence of an intron downstream of the PTC renders the NS39 mRNA susceptible to NMD. To allow monitoring of selenoprotein mRNA decay, the β-globin gene was made into an artificial selenoprotein by mutating the premature UAA termination codon located at position 39 to a UGA codon and by inserting a SECIS element into the 3′ UTR of the construct (Fig. 1A). To facilitate the insertion of a SECIS, site-directed mutagenesis was performed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). We sequentially introduced *Xba*I and *Bgl*II restriction sites into the proximal 3′ UTR region of the NS39 plasmid and then introduced a UGA codon at position 39. As the original plasmid contained an additional *Bgl*II restriction site upstream of the reporter, this site was removed by site-directed mutagenesis. The human *GPX1* SECIS element was amplified from human genomic DNA (Coriell sample ID# NA10861; Coriell Institute for Medical Research, Camden, NJ, USA) using polymerase chain reaction (PCR) primers containing *Xba*I and *Bgl*II restriction sites in their tails. Restriction-digested PCR products were ligated into the *Xba*I/*Bgl*II-digested and gel-purified reporter constructs. Control plasmids containing an ACC codon or true stop codon (UAA) were constructed from the parental plasmid using site-directed mutagenesis. Plasmids with mutant SECIS elements were generated by mutating the four-base SECIS core (UGAA) to CCCC or by deleting these four bases. The primers used are reported in Supplemental Table 1.

2.2. Cell culture and plasmid transfection

HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Media Core, Lerner Research Institute) supplemented with 10% fetal calf serum (USA Scientific, Orlando, FL, USA), 100 U/ml penicillin and streptomycin. In six-well plates, cells were transiently transfected using Lipofectamine 200 (Invitrogen, CA, USA) as described in the manufacturer's protocol. Where appropriate, tissue culture medium was supplemented with 1 μM of selenomethionine (SeMet), a concentration of Se empirically determined to be optimal for the incorporation of SEC in a read-through reporter assay (Fig. S1A) and by Western blot (Fig. S1 B). We elected to use SeMet rather than an inorganic form of Se, as SeMet may be less toxic to cells [23] and has been used in human clinical trials [24].

2.3. Luminometry

Cells were lysed with Passive Lysis Buffer (Promega, Madison, WI, USA), and luminescence was measured in a luminometer (Victor R^{3v}, Perkin Elmer) using the Dual-Luciferase Reporter Assay System (Promega). *Renilla* luciferase signals were normalized to a cotransfected firefly luciferase control.

2.4. Protein analysis

Immunoblot analysis was performed using 60 μg of total cell extract electrophoresed on 12% sodium dodecyl sulfate–polyacrylamide gels. Subsequently, proteins were transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA) using a semidry electroblotting system. Membranes were blocked with 5% nonfat dry milk (Bio-Rad). Antibodies were used that recognized UPF1, GAPDH, β-actin (dilution, 1:1000; Santa Cruz Biotech Inc., Santa Cruz, CA, USA), GPX1 (dilution, 1:1000; Cell Signalling Technology Inc., Danvers, MA, USA) and GPX4 (dilution, 1:1000; Santa Cruz Biotech Inc.). Reactivity to each antibody was detected using 1:2000 dilution of horseradish-peroxidase-conjugated donkey anti-rabbit (Cell Signaling Technology Inc.) or 1:5000 dilution of anti-mouse antibodies and anti-goat (Santa Cruz Biotech). Reactivity of the secondary antibody was visualized by SuperSignal West Pico solution (Pierce, Thermo Scientific, Rockford, IL, USA).

2.5. Quantitative real-time PCR

Total RNA was purified from cells using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). One microgram of total RNA from transiently transfected cells was subjected to reverse transcription using an i-script cDNA synthesis kit (Bio-Rad). PCR amplification was carried out using iQ-SYBR Green Supermix (Bio-Rad) and an i-Cycler (Bio-Rad). Primers were designed to amplify a 100- to 200-base-pair (bp) product. Before being used in real-time PCR, each primer pair was authenticated by standard reverse transcription-PCR (RT-PCR). The primers used are reported in Supplemental Table 2. For real-time PCR assays, all values were normalized to the ACTB housekeeping gene. The ΔCt method was used for relative quantification of transcripts. Endogenous *GPX1* (Hs 00829989_gh; Applied Biosystems, Carlsbad, CA, USA) and *GPX4* (Hs00157812_ml; Applied Biosystems) levels were determined using Taqman real-time PCR probes and master mix (Applied Biosystems). For relative quantification of the transcripts, we compared the ΔCt value normalized to the transcript level of GAPDH (Hs99999905_ml; Applied Biosystems).

2.6. Nuclear and cytoplasmic separation

Nuclear and cytoplasmic total RNA was isolated from cells grown in 10-cm plates. Cells were transiently transfected with the appropriate plasmid and incubated in the presence or absence of 1 μM SeMet. After 72 h of SeMet supplementation, the total RNA was extracted from both cytoplasmic and nuclear fractions using the Sure Prep Nuclear or Cytoplasmic RNA Purification Kit (Fisher BioReagents, USA). The total RNA was subjected to cDNA synthesis as described above.

2.7. LY294002 treatments

HeLa cells transfected with the NMD reporters were treated for 24 h, and then the medium was replaced by serum-free medium for another 24 h. The cells were preincubated with 20 μM of LY294002 for 2 h in serum-free medium, and then they were incubated for 24 h in serum-containing medium; thereafter, reporter activity was analyzed by luminometry. The treatment of transfected cells with the drug vehicle dimethyl sulfoxide served as a negative control.

2.8. UPF1 knockdown and overexpression

To knock down the expression of *UPF1*, HeLa cells were transiently transfected with a pool of four UPF1 shRNAs (Origene; TR308482) or a GFP-specific control shRNA plasmid (Origene). Twenty-four hours later, cells were subjected to another round of transfection with two plasmids, RenβG-ACC *GPX1* (wild-type control) or RenβG-TGA *GPX1* (UGA at NS 39 position), and a firefly luciferase control plasmid. Western blotting indicated that, relative to GFP-specific shRNA, UPF1 shRNA reduced the level of cellular UPF1 by 95%. *Renilla* luciferase activity was normalized to the firefly luciferase