Mechanisms of Ageing and Development 133 (2012) 575–579

Contents lists available at SciVerse ScienceDirect

Mechanisms of Ageing and Development

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

Short communication

Quantitative analysis of WRN exonuclease activity by isotope dilution mass spectrometry

Aswin Mangerich [a,1,*], Sebastian Veith [a,1], Oliver Popp [a], Jörg Fahrer [a,2], Rita Martello [a], Vilhelm A. Bohr [b], Alexander Bürkle [a,*]

[a] Molecular Toxicology Group, Department of Biology, University of Konstanz, 78457 Konstanz, Germany
[b] Laboratory of Molecular Gerontology, Biomedical Research Center, National Institute on Aging, NIH, Baltimore, MD 21224, USA

ARTICLE INFO

Article history:
Received 9 December 2011
Received in revised form 11 May 2012
Accepted 19 June 2012
Available online 2 July 2012

Keywords:
Werner syndrome
WRN
Exonuclease
Mass spectrometry
Aging
PARP1

ABSTRACT

Werner syndrome is a disorder characterized by a premature aging phenotype. The disease is caused by mutations in the *WRN* gene which encodes a DNA helicase/exonuclease which is involved in multiple aspects of DNA metabolism. Current methods mostly rely on radiometric techniques to assess WRN exonuclease activity. Here we present an alternative, quantitative approach based on non-radioactive isotope dilution mass spectrometry (LC–MS/MS). A oligoduplex substrate mimicking the telomeric sequence was used for method development. Released nucleotides, which correlate with the degree of oligoduplex degradation, were dephosphorylated, purified, and quantified by LC–MS/MS. Heavy-isotope-labeled internal standards were used to account for technical variability. The method was validated in terms of reproducibility, time-course and concentration-dependency of the reaction. As shown in this study, the LC–MS/MS method can assess exonuclease activity of WRN mutants, WRN's substrate and strand specificity, and modulatory effects of WRN interaction partners and posttranslational modifications. Moreover, it can be used to analyze the selectivity and processivity of WRN exonuclease and allows the screening of small molecules for WRN exonuclease inhibitors. Importantly, this approach can easily be adapted to study nucleases other than WRN. This is of general interest, because exonucleases are key players in DNA metabolism and aging mechanisms.

Werner syndrome (WS) is a rare autosomal recessive disorder characterized by a segmental premature aging phenotype, including early onset of atherosclerosis, osteoporosis, and a high cancer incidence. The disease is caused by loss-of-function mutations in the *WRN* gene which encodes the WRN protein, a member of the RecQ helicase family. On the cellular level, fibroblasts derived from WS patients display genomic instability and a reduced replicative life span (Kudlow et al., 2007). This phenotype is in accordance with experimental data demonstrating that WRN is involved in multiple aspects of DNA metabolism, such as DNA replication, genomic maintenance, and telomere regulation (Bohr, 2008; Reddy et al., 2010; Rossi et al., 2010). In contrast to the other five members of the human RecQ helicase family, WRN also possesses a unique 3′ → 5′ exonuclease activity (Huang et al., 1998).

The WRN exonuclease cleaves the DNA phosphodiester bond and releases free 5′-dNMPs from the DNA strand (Kamath-Loeb et al., 1998). To elicit its exonuclease activity, WRN requires a 3′ recessed end (5′-overhang) substrate. WRN does not degrade duplex DNA with blunt ends, unless the substrate also contains a junction or alternate DNA structures such as a fork (Brosh et al., 2006; Shen and Loeb, 2000). It is largely inactive on short single-stranded DNA substrates (Kamath-Loeb et al., 1998), but longer ssDNA substrates are efficiently degraded (Machwe et al., 2006). Its activity is regulated by posttranslational modifications and protein interactions. For instance, phosphorylation of WRN by DNA-PK inhibits its exonuclease activity (Karmakar et al., 2002; Yannone et al., 2001). In addition, p53, BLM, and PARP1 cause inhibitory effects (Brosh et al., 2001; Sommers et al., 2005; von Kobbe et al., 2002, 2004), whereas the Ku70/80 complex stimulates exonuclease activity (Cooper et al., 2000; Kudlow et al., 2007; Li and Comai, 2000, 2001).

Standard methods to assess WRN exonuclease activity utilize radioactively or fluorescently 5′ end-labeled DNA substrates to detect the degradation of the full-length DNA molecules (Boubriak et al., 2009; Brosh et al., 2006). Here we present an alternative approach to assess WRN exonuclease activity based on isotope dilution mass spectrometry (LC–MS/MS). This method may be

* Corresponding authors at: Molecular Toxicology Group, Department of Biology, University of Konstanz, D-78457 Konstanz, Germany.
E-mail addresses: aswin.mangerich@uni-konstanz.de (A. Mangerich), alexander.buerkle@uni-konstanz.de (A. Bürkle).
[1] Both authors contributed equally.
[2] Present address: Institute of Toxicology, University Medical Center Mainz, D-55131 Mainz, Germany.

0047-6374/$ – see front matter © 2012 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.mad.2012.06.005

particularly useful in two situations: Firstly, for laboratories that wish to replace the common radioactive assays with a non-radioactive one and, secondly, the method can be incorporated into high throughput screening approaches for small molecules that affect exonuclease activity.

We have validated our newly developed method and compared it to a modified version of an established protocol that uses a 5′-biotin-end-labeled DNA substrate to detect activity of recombinant WRN exonuclease (Brosh et al., 2006) (Suppl. Fig. 1). Importantly, using a telomeric substrate mimics one of the key functions of WRN which is to operate at the telomere (Bohr, 2008). To assess if this oligoduplex indeed serves as a suitable substrate for WRN in our hands, an exonuclease reaction was carried out as published previously (Brosh et al., 2006). The reaction mixture contained 75 fmol of the oligoduplex and 0.1–1 pmol of recombinant WRN. Subsequently, digestion products were resolved by denaturing polyacrylamide gel electrophoresis (PAGE) and biotin was detected by streptavidin-POD (Suppl. Fig. 2). In this method, loss of signal intensity of the full-length end-labeled DNA substrate was used as readout to assess exonuclease activity. As is evident from Suppl. Fig. 2, WRN efficiently catalyzes the degradation of this oligoduplex to truncated DNA molecules of various lengths in a concentration dependent manner. Initial degradation of the substrate is visible at an enzyme to substrate ratio (E/S) of ~1 (10 nM WRN) and reached saturation at an E/S of ~8 (60 nM WRN) with a maximum efficiency of ~80%.

Instead of detecting the shortened DNA substrate, the rationale of the LC–MS/MS-based method is to detect degradation end-products, *i.e.*, free nucleosides, to assess WRN exonuclease activity. To allow comparability to the biotin-end-labeling technique, the same substrate and identical reaction conditions were chosen to develop the LC–MS/MS-based method. Since the oligoduplex contains a repetitive telomeric sequence $(TTAGGG)_4$, LC–MS/MS quantification of free 2′-deoxyguanosine (dG) was expected to be a suitable readout to assess WRN exonuclease activity. Fig. 1 shows a flow chart of the experimental procedure; a detailed protocol is available in the supplementary information section. Briefly, after the exonuclease reaction was carried out, samples were placed on ice and ^{15}N-labeled dG was added as an internal standard to the reaction mixture to account for technical variability during sample work-up and mass spectrometric measurement. Thereafter, recombinant WRN was removed by spin column filtration, followed by dephosphorylation of the nucleotides to nucleosides using alkaline phosphatase (AP), removal of the phosphatase by spin column filtration, and subsequent LC–MS/MS analysis. The recovery rate of the internal standard was usually ~70%. Typical LC–MS/MS chromatograms of unlabeled and ^{15}N-labeled dG as well as a calibration curve are shown in Suppl. Fig. 3.

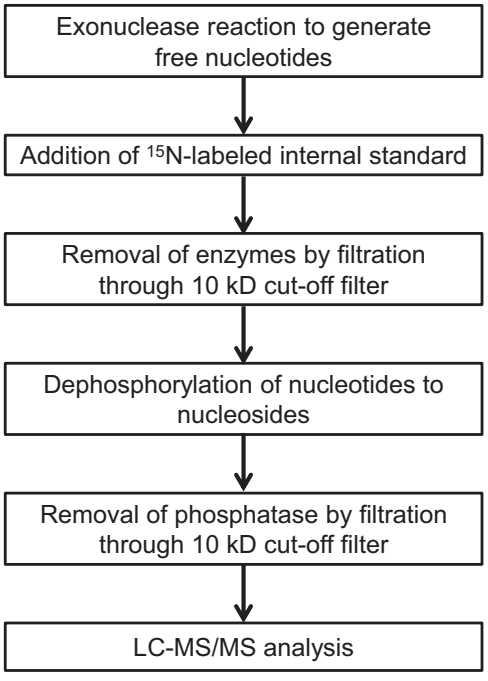

Fig. 1. Flow chart for the LC–MS/MS quantification of WRN exonuclease activity.

The method shows adequate assay-to-assay variability (Suppl. Fig. 4A) and can be performed in 1–2 days dependent on the time chosen for AP digestion (Suppl. Fig. 4B; NB: no significant differences in the quantities of dG were observed between 4-h and overnight AP digestion, indicating that an AP digestion time of 4 h is sufficient for complete dephosphorylation of dGMP).

As shown in Fig. 2, free dG was detected in a time and concentration dependent manner. Since the reaction is still in its dynamic range after 15 min in terms of release of free dGMP (Fig. 2A), a reaction time of 45 min was chosen for the following experiments to achieve maximum oligoduplex degradation. In agreement with results obtained from biotin-end-labeling technique, WRN activity was already detected at an E/S of ~1 (5–10 nM WRN) and reached saturation at an E/S of ~8 (60 nM WRN) (Fig. 2B). A maximum of 32% of the expected total amount of dG was detected in WRN-digested samples. The lower efficiency of WRN compared to phosphodiesterase (PDE) may be related to incomplete annealing of the DNA strands and therefore to incomplete digestion by WRN. A WRN mutant with a mutation in the exonuclease domain (WRN-E84A, X-WRN) (Huang et al., 1998; Machwe et al., 2000) showed strongly diminished exonuclease activity compared to WT-WRN (Fig. 2C). The residual exonuclease activity observed in the X-WRN sample may be related to the sensitivity of the LC–MS/MS method, to some contamination with Zn^{2+} ions, which can trigger minimal exonuclease activity of X-WRN (Choudhary et al., 2004), or to contamination of the X-WRN protein with an unknown nuclease.

Conceivable applications of the LC–MS/MS-based method include studying effects of factors that modulate WRN exonuclease activity, such as posttranslational modifications and protein interaction partners. For example, PARP1, which catalyzes the synthesis of the biopolymer poly(ADP-ribose) upon genotoxic stress, is an established interaction partner of WRN (Rossi et al., 2010; Rouleau et al., 2010). Previously, it was shown that PARP1 inhibits both WRN's helicase and exonuclease activities (von Kobbe et al., 2004). In agreement with these results, PARP1 led to an inhibition of WRN exonuclease activity by up to 80% as detected by LC–MS/MS analysis (Fig. 2D).

Furthermore, the method can be applied to study WRN's substrate and strand specificity. To this end, we tested several newly designed WRN oligoduplex substrates which are compatible with our assay, *i.e.* an optimized forked oligoduplex, a blunt-ended oligoduplex, and oligoduplexes containing a 4-way junction and a 5′-overhang (Suppl. Table 1 for full sequence details). All substrates comprised a strand that contains only dG, dA, dT ('dG' strand) within a repetitive sequence element $(XXXGGG)_6$ (X = A,T in alternating sequence to assure annealing specificity) and a complementary strand that only contains dC, dA, dT ('dC' strand). Except for the blunt-ended substrate, the dG-strand was expected to be accessible for WRN exonuclease activity. In agreement with previous reports (Brosh et al., 2006; Kamath-Loeb et al., 1998), oligoduplexes which contained the fork and 4-way junction served as efficient substrates for WRN exonuclease, whereas the blunt ended oligoduplex showed almost no release of free dG (Fig. 3A). Moreover, significant WRN exonuclease activity was detected with the 5′overhang substrate, however to a lesser extent than with the fork and 4-way junction oligoduplexes. As expected, WRN was not