Analytical Biochemistry 390 (2009) 85–87

Contents lists available at ScienceDirect

Analytical Biochemistry

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

Notes & Tips

A time-resolved fluorescence resonance energy transfer-based assay for DEN1 peptidase activity

Ingo H. Engels *, Catherine Daguia, Truc Huynh, Hugo Urbina [1], Jörg Buddenkotte [2], Andrew Schumacher, Jeremy S. Caldwell, Achim Brinker

Genomics Institute of the Novartis Research Foundation, San Diego, CA 92121, USA

ARTICLE INFO

Article history:
Received 12 December 2008
Available online 27 March 2009

ABSTRACT

Neural precursor cell expressed, developmentally down-regulated gene 8 (NEDD8) is a recently discovered ubiquitin-like posttranslational modifier. NEDD8 acts predominantly as a regulator of ubiquitin–protein ligases and as a decoy for proteins targeted for proteasomal degradation. It thereby controls key events in cell cycle progression and embryogenesis. Deneddylase-1 (DEN1/NEDP1/SENP8) features a selective peptidase activity converting the proNEDD8 precursor to its mature form and an isopeptidase activity deconjugating NEDD8 from substrates such as cullins and p53. In this study, we describe a high-throughput screening (HTS)-compatible time-resolved fluorescent resonance energy transfer (TR–FRET) assay measuring the peptidase activity of DEN1.

© 2009 Elsevier Inc. All rights reserved.

NEDDylation is a process of reversible posttranslational protein modification and involves an enzymatic cascade for the conjugation and deconjugation of neural precursor cell expressed, developmentally down-regulated gene 8 (NEDD8),[3] very similar to the ubiquitin system [1]. NEDD8 is a 9-kDa polypeptide that shares 80% sequence homology with ubiquitin. NEDD8, like ubiquitin, is synthesized as precursor protein with a C-terminal extension [2]. However, a single amino acid mismatch in the otherwise sequence identical C termini of NEDD8 (Ala72) and ubiquitin (Arg72) underlies the specific recognition of NEDD8 by the heterodimeric E1-activating enzyme APPBP1–UBA3 and the deneddylase DEN1 (deneddylase-1) [3,4]. The binding of NEDD8 to APPBP1–UBA3 and DEN1 is mediated via extensive protein interfaces and induces extensive conformational changes in all binding partners, thereby stimulating the catalytic activity of DEN1.

DEN1 is a cysteine protease that plays a dual role in the NEDD8 system [5]. First, DEN1 features an isopeptidase activity deconjugating NEDD8 from a wide variety of substrates, including cullins and p53 [5–7]. Second, the same catalytic site of DEN1 has a peptidase activity that acts on proNEDD8 precursors to generate the C-terminal conjugation motif LRGG [5]. The main function of NEDD8 conjugation appears to be the regulation of protein stability either indirectly by modulating ubiquitin ligase activities or directly by marking protein substrates for proteasomal degradation [8,9]. Small molecule inhibitors of DEN1 are wanted to facilitate the interrogation of the intracellular NEDD8 network and to investigate the potential of DEN1 as a cancer drug target.

Here we present a time-resolved fluorescent resonance energy transfer (TR–FRET)-based assay to measure the processing of proNEDD8 by DEN1 in a high-throughput screening (HTS)-compatible, high-density, 1536-well plate assay format. By using full-length proNEDD8 as opposed to short oligopeptide substrates mimicking the C-terminal end of NEDD8, this assay takes advantage of the increased catalytic activity of DEN1 toward its protein substrates and is tailored to target the most physiologically relevant and substrate selective enzyme conformation [4]. Relative to assays relying on straight fluorescence intensity measurements and using, for example, C-terminally labeled ubiquitin constructs [10–12], TR–FRET assays are less prone to fluorescence interference and, therefore, are especially well suited for the screening of large diverse compound libraries [13,14]. Moreover, TR–FRET assay formats are generally highly sensitive at low target concentrations in the picomolar to nanomolar range and low assay volumes of around 10 µl and less.

The principle of the featured DEN1 activity assay is illustrated in Fig. 1A. Unprocessed DEN1 substrate His6–Myc–proNEDD8–FLAG is quantified by TR–FRET through interactions with anti-Myc–Eu–cryptate- and allophycocyanin (APC)-conjugated anti-FLAG

* Corresponding author. Fax: +1 858 812 1597.
E-mail address: iengels@gnf.org (I.H. Engels).

[1] Present address: Amylin Pharmaceuticals, San Diego, CA 92121, USA.

[2] Present address: Department of Dermatology, Ludwig Boltzmann Institute for Cell Biology and Immunobiology of the Skin, University of Münster, 48149 Münster, Germany.

[3] *Abbreviations used:* NEDD8, neural precursor cell expressed, developmentally down-regulated gene 8; DEN1, deneddylase 1; TR–FRET, time-resolved fluorescent resonance energy transfer; HTS, high-throughput screening; APC, allophycocyanin; GST, glutathione *S*-transferase; UV, ultraviolet; Ni–NTA, nickel–nitrilotriacetate; VS, vinylsulfone; DTT, dithiothreitol; S/N, signal/noise; DMSO, dimethyl sulfoxide.

0003-2697/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2009.03.035

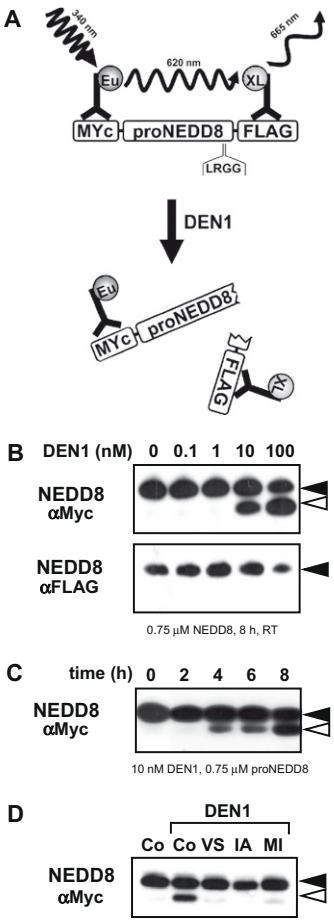

Fig. 1. (A) Principle of a TR–FRET-based DEN1 peptidase assay monitoring the maturation of proNEDD8. (B–D) Validation of DEN1 assay concept by Western blot analysis. Proteolytic processing of NEDD8 is indicated by closed arrows for the unprocessed form and by open arrows for the processed form. (B) proNEDD8 processing in the presence of increasing DEN1 concentrations. His6–Myc–proNEDD8–FLAG was combined with the indicated concentrations of GST–DEN1 for 8 h. RT, room temperature. (C) Kinetics of NEDD8 processing. His6–Myc–proNEDD8–FLAG was incubated with 10 nM GST–DEN1 for the indicated amounts of time. (D) Inhibition of DEN1 activity. His6–Myc–proNEDD8–FLAG was either left untreated (Co) or incubated with 10 nM GST–DEN1 for 8 h after DEN1 had been preincubated with either dimethyl sulfoxide (Co + DEN1), 100 nM NEDD8–vinylsulfone (VS), 30 mM iodoacetamide (IA), or 30 mM maleimide (MI) for 30 min.

antibodies. After excitation with ultraviolet (UV) light at 340 nm, Eu–cryptate is able to transfer energy to the adjacent acceptor (APC), which can emit light at 665 nm. Energy that is not transferred is emitted from Eu at 615 nm. The intensity of light emitted by the APC acceptor at 665 nm, or alternatively the 665/620-nm intensity ratio, is a measure for the relative His6–Myc–proNEDD8–FLAG concentration. DEN1-mediated substrate cleavage decreases the TR–FRET signal.

Materials required for the assays were obtained as follows. Glutathione *S*-transferase (GST)–DEN1 was expressed and purified as described previously [5]. His6–Myc–proNEDD8–FLAG was PIPE-cloned into pSpeedET [15] using the forward primer AACCTGTACTTCCAGGGCATGGAACAAAAACTTATTTCTGAAGAAGATCTG and the reverse primer GAGTTAATTAAGTCGCGTTATCACTTGTCATCGTCGTCTTTGTAGTC, expressed in *Escherichia coli* TOP10, and purified by affinity chromatography using first nickel–nitrilotriacetate (Ni–NTA) and then anti-FLAG M2 sepharose (Sigma, St. Louis, MO, USA) to ensure the presence of both the N- and C-terminal tags necessary for TR–FRET detection. The initial nickel column purification yielded three bands: the full-length His–Myc–NEDD8–FLAG and two bands that migrated slightly lower and appeared to be C-terminal cleavage fragments based on Coomassie and anti-His Western blot analyses. Based on Coomassie blue staining, approximately two-thirds of this material appears to be the full-length protein, whereas the remaining one-third is the truncated material. Therefore, the subsequent anti-FLAG purification was deemed necessary. Anti-c-Myc and anti-FLAG antibodies for Western blot analysis were purchased from MP Biomedicals (Solon, OH, USA) and Stratagene (La Jolla, CA, USA), respectively. The Eu–cryptate-conjugated anti-Myc–Eu and the APC (XL665)-conjugated anti-FLAG antibodies were purchased from Cisbio–US (Bedford, MA, USA). The TR–FRET assays were performed at least in triplicates using white solid-bottom 1536-well plates (Greiner, San Diego, CA, USA) in a final detection volume of 10 μl. NEDD8–vinylsulfone (VS) was purchased from Boston Biochem (Cambridge, MA, USA), and iodoacetamide and maleimide were purchased from Sigma.

Before attempting the development of a microtiter plate assay, we first confirmed the activity and selectivity of our GST–DEN1 enzyme preparation by Western blot analysis, looking at both the N-terminal Myc tag and the C-terminal FLAG tag of the His6–Myc–proNEDD8–FLAG substrate construct (Fig. 1B). The peptidase reaction was performed at room temperature using 750 nM substrate in 50 mM Tris–HCl (pH 7.4), 100 mM NaCl, 10 mM dithiothreitol (DTT), and 0.05% Chaps (buffer A). Both DEN1 concentration and time-dependent C-terminal proNEDD8 processing could be observed and followed through the occurrence of a distinct truncated NEDD8 species that maintained its N-terminal Myc tag (Fig. 1B and C). At higher DEN1 concentrations or longer incubation times, the His6–Myc–proNEDD8–FLAG turnover could also be registered in the anti-FLAG blots as a weakening of the unprocessed precursor substrate band (Fig. 1B and data not shown). Protease inhibitors were tested to further validate the specificity of this assay (Fig. 1D). We found that not only the pan-cysteine protease inhibitors iodoacetamide and maleimide but also the DEN1-specific inhibitor NEDD8–VS abrogated the proteolytic processing of proNEDD8. This result demonstrates the dependence of the proteolysis reaction on the specific DEN1 peptidase activity and eliminated possible contaminants in GST–DEN1 preparation as major contributors to the reaction.

Next, the peptidase activity of DEN1 was measured in a 1536-well plate TR–FRET assay (Fig. 1A) in a final reaction volume of 10 μl per well. The enzyme reaction was performed in a 4-μl volume in buffer A and was followed by the addition of an anti-Myc–Eu/anti-FLAG–APC antibody mix diluted in reconstitution buffer (Cisbio–US). Relative anti-Myc–Eu and anti-FLAG–APC (data not shown) and His6–Myc–proNEDD8–FLAG concentrations were optimized for maximal TR–FRET signal/noise (S/N) detection. Fig. 2A shows titrations of His6–Myc–proNEDD8–FLAG at various incubation times post- antibody addition. Ternary antibody–substrate complex formation equilibrated after 2 h of room temperature incubation. At fixed concentrations of 2 nM anti-Myc–Eu and 10 nM anti-FLAG–APC, increasing His6–Myc–proNEDD8–FLAG concentrations between 3 and 100 nM resulted in correspondingly increased TR–FRET signals. At higher His6–Myc–proNEDD8–FLAG concentrations, a hook effect set in (Fig. 2A).

Based on the time-dependent decline of the TR–FRET signal at concentrations of 100 pM GST–DEN1 and 75 nM His6–Myc–proNEDD8–FLAG (Fig. 2B), the effects of a number of DEN1 inhibitors on the DEN1 peptidase activity were tested at a 6 h endpoint (Fig. 2C). DEN1 activity was completely inhibited by 100 pM NEDD8–VS as well as by 1 mM iodoacetamide and 1 mM maleimide. The HTS compatibility of the TR–FRET-based DEN1 peptidase assay was then assessed in a "pilot screen" by testing a randomized 1536-well compound plate chosen from the 2.5 million compound library of the Genomics Institute of the Novartis Research Foundation (GNF, San Diego, CA, USA). In the final protocol, 2 μl of GST–

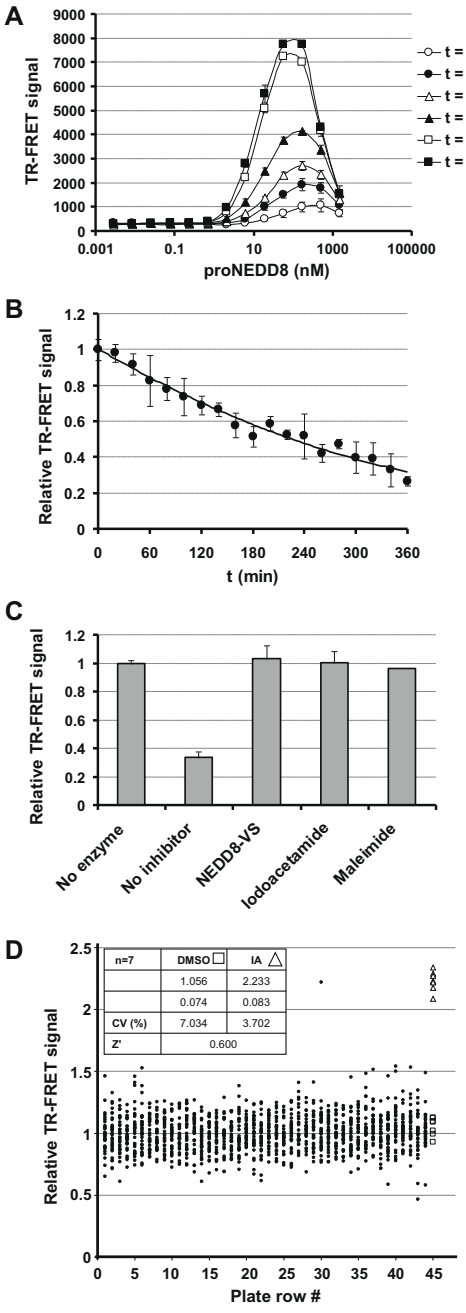

Fig. 2. Development of a TR–FRET-based assay for inhibitors of DEN1 activity. All assays were performed in white solid 1536-well plates. Here, 6 μl of anti-Myc–Eu and anti-FLAG–APC were added to 4 μl enzyme reactions at final concentrations of 2 and 10 nM, respectively. (A) Kinetics of the formation of anti-Myc–Eu/His6–Myc–proNEDD8–FLAG/anti-FLAG–APC tertiary complexes at different concentrations of proNEDD8. (B) Kinetics of DEN1 activity. Here, 100 pM GST–DEN1 was incubated with 75 nM His6–Myc–proNEDD8–FLAG for up to 6 h in a time-dependent manner. Resulting TR–FRET signals were normalized relative to the His6–Myc–proNEDD8–FLAG alone control. (C) Inhibition of DEN1 activity. Here, 100 pM GST–DEN1 was preincubated with either dimethyl sulfoxide (control), 100 pM NEDD8–VS, 1 mM iodoacetamide, or 1 mM maleimide and was reacted with 75 nM His6–Myc–proNEDD8–FLAG. Resulting TR–FRET signals were normalized relative to the His6–Myc–proNEDD8–FLAG alone control. (D) Scatterplot of a 1536-well assay plate. A diverse set of compounds was tested at a concentration of 12.5 μM. Test compounds are shown in black. Controls are indicated by open squares (dimethyl sulfoxide [DMSO]) and open triangles (1 mM iodoacetamide [IA]). TR–FRET signals were normalized relative to the median signals obtained from the 1408 test compound wells. Inset: assay statistics. CV, coefficient of variation.

DEN1 was first added to the 1536-well assay plate using GNF's proprietary liquid dispenser. Subsequently, 50 nl of test compounds (1-mM dimethyl sulfoxide [DMSO] stocks) were pintool-transferred (V&P Scientific, San Diego, CA, USA) from a 1536-well compound plate. Then 50 nl of iodoacetamide (10 mM stock in DMSO) was added from a second control compound plate into 7 reference wells on the same assay plate (position H45–N45). The assay plate was then preincubated at room temperature for 30 min, followed by the addition of 2 μl of His6–Myc–proNEDD8–FLAG. At the end of a 6 h reaction time, 6 μl of detection reagent at final concentrations of 2 nM anti-Myc–Eu, 10 nM anti-FLAG–APC, and 1 mM maleimide in reconstitution buffer were added and incubated at room temperature for another 2 h before reading the TR–FRET signals on an Envision reader (PerkinElmer, Fremont, CA, USA). Fig. 2D shows the resulting data and assay statistic; the Z' factor ($Z' = 1 - [(3\sigma_{Iodo} + 3\sigma_{DMSO})/(\mu_{Iodo} - \mu_{DMSO})]$) [16] for this representative plate was 0.6.

In conclusion, we have developed a robust and HTS-compatible TR–FRET assay for the peptidase activity of DEN1 in a miniaturized 1536-well plate format that is tailored to the screening of large diverse compound libraries. We believe that this assay will facilitate the discovery of DEN1 targeted small molecule inhibitors and valuable probes for the dissection of DEN1 and NEDD8 biology through the screening of targeted protease inhibitor collections and comprehensive diverse molecular libraries.

References

[1] M. Hochstrasser, All in the ubiquitin family, Science 289 (2000) 563–564.
[2] T. Kamitani, K. Kito, H.P. Nguyen, E.T. Yeh, Characterization of NEDD8, a developmentally down-regulated ubiquitin-like protein, J. Biol. Chem. 272 (1997) 28557–28562.
[3] H. Walden, M.S. Podgorski, D.T. Huang, D.W. Miller, R.J. Howard, D.L. Minor, J.M. Holton, B.A. Schulman, The structure of the APPBP1–UBA3–NEDD8–ATP complex reveals the basis for selective ubiquitin-like protein activation by an E1, Mol. Cell 12 (2003) 1427–1437.
[4] L. Shen, H. Liu, C. Dong, D. Xirodimas, J.H. Naismith, R.T. Hay, Structural basis of NEDD8 ubiquitin discrimination by the deNEDDylating enzyme NEDP1, EMBO J. 24 (2005) 1341–1351.
[5] K. Wu, K. Yamoah, G. Dolios, T. Gan-Erdene, P. Tan, A. Chen, C. Lee, N. Wei, K.D. Wilkinson, R. Wang, Z-Q. Pan, DEN1 is a dual function protease capable of processing the C terminus of NEDD8 and deconjugating hyper-neddylated CUL1, J. Biol. Chem. 278 (2003) 28882–28891.
[6] H.M. Mendoza, L. Shen, C. Botting, A. Lewis, J. Chen, B. Ink, R.T. Hay, NEDP1, a highly conserved cysteine protease that deNEDDylates cullins, J. Biol. Chem. 278 (2003) 25637–25643.
[7] D.P. Xirodimas, M.K. Saville, J.C. Bourdon, R.T. Hay, D.P. Lane, Mdm2-mediated NEDD8 conjugation of p53 inhibits its transcriptional activity, Cell 118 (2004) 83–97.
[8] J. Liu, T. Furukawa, T. Matsumoto, Y. Xiong, NEDD8 modification of CUL1 dissociates p120(CAND1), an inhibitor of CUL–SKP1 binding and SCF ligases, Mol. Cell 10 (2002) 1511–1518.
[9] T. Kamitani, K. Ktio, T. Fukuda-Kamitani, E.T. Yeh, Targeting of NEDD8 and its conjugates for proteasomal degradation by NUB1, J. Biol. Chem. 276 (2001) 46655–46660.
[10] T. Gan-Erdene, K. Nagamalleswari, L. Yin, K. Wu, Z-Q. Pan, K.D. Wilkinson, Identification and characterization of DEN1, a deneddylase of the ULP family, J. Biol. Chem. 278 (2003) 28892–28900.
[11] J. Hemelaar, A. Borodovsky, B.M. Kessler, D. Reverter, J. Cook, N. Kolli, T. Gan-Erdene, K.D. Wilkinson, G. Gill, C.D. Lima, H.L. Ploegh, H. Ovaa, Specific and covalent targeting of conjugating and deconjugating enzymes of ubiquitin-like proteins, Mol. Cell. Biol. 24 (2004) 84–95.
[12] A. Tirat, A. Schilb, V. Riou, L. Leder, B. Gerhartz, J. Zimmermann, S. Worpenberg, U. Eidhoff, F. Freuler, T. Stettler, L. Mayr, J. Ottl, B. Leuenberger, I. Filipuzzi, Synthesis and characterization of fluorescent ubiquitin derivatives as highly sensitive substrates for the deubiquitinating enzymes UCH-L3 and USP-2, Anal. Biochem. 343 (2005) 244–255.
[13] G. Mathis, HTRF technology, J. Biomol. Screen. 4 (1999) 309–314.
[14] R.A. Horton, E.A. Strachan, K.W. Vogel, S.M. Riddle, A substrate for deubiquitinating enzymes based on time-resolved fluorescence resonance energy transfer between terbium and yellow fluorescent protein, Anal. Biochem. 360 (2007) 138–143.
[15] H.E. Klock, E.J. Koesema, M.W. Knuth, S.A. Lesley, Combining the polymerase incomplete primer extension method for cloning and mutagenesis with microscreening to accelerate structural genomics efforts, Proteins 71 (2008) 982–994.
[16] J.H. Zhang, T.D. Chung, K.R. Oldenburg, A simple statistical parameter for use in evaluation and validation of high throughput screening assays, J. Biomol. Screen. 4 (1999) 67–73.