Analytical Biochemistry 482 (2015) 7–15

Contents lists available at ScienceDirect

Analytical Biochemistry

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

The capture proteasome assay: A method to measure proteasome activity in vitro

Nathalie Vigneron *, Joanna Abi Habib, Benoît J. Van den Eynde

Ludwig Institute for Cancer Research, B-1200 Brussels, Belgium
Walloon Excellence in Life Sciences and Biotechnology (WELBIO), B-1200 Brussels, Belgium
de Duve Institute, Université catholique de Louvain, B-1200 Brussels, Belgium

ARTICLE INFO

Article history:
Received 24 February 2015
Received in revised form 4 April 2015
Accepted 8 April 2015
Available online 23 April 2015

Keywords:
Proteasome
Activity assay
Fluorogenic peptides
Proteasome–Glo

ABSTRACT

Because of its crucial role in various cellular processes, the proteasome is the focus of intensive research for the development of proteasome inhibitors to treat cancer and autoimmune diseases. Here, we describe a new and easy assay to measure the different proteasome activities in vitro (chymotrypsin-like, caspase-like, and trypsin-like) based on proteasome capture on antibody-coated plates, namely the capture proteasome assay (CAPA). Applying the CAPA to lysates from cells expressing standard proteasome, immunoproteasome, or intermediate proteasomes β5i or β1i–β5i, we can monitor the activity of the four proteasome subtypes. The CAPA provided similar results as the standard whole-cell proteasome–Glo assay without the problem of contaminating proteases requiring inhibitors. However, the profile of trypsin-like activity differed between the two assays. This could be partly explained by the presence of $MgSO_4$ in the proteasome–Glo buffer, which inhibits the trypsin-like activity of the proteasome. The CAPA does not need $MgSO_4$ and, therefore, provides a more precise measurement of the trypsin-like activity. The CAPA provides a quick and accurate method to measure proteasome activity in vitro in a very specific manner and should be useful for the development of proteasome inhibitors.

The proteasome is a large barrel-shaped particle that is responsible for the bulk of protein degradation in cells. The central core of the complex, called the 20S proteasome, is composed of four stacked heptameric rings that delimit a proteolytic chamber inside which proteins are degraded. The two outer rings of the 20S proteasome are made of α-subunits, and the two inner rings are made of β-subunits, three of which (β1, β2, and β5) are catalytic in the vertebrate proteasome [1]. The N-terminal tails of the α-rings interact with regulatory caps that control substrate access into the proteasome catalytic chamber. Four types of regulator exist: the 19S regulator, the 11S regulators PA28α/β and PA28γ, and the PA200 regulator [2].

Three major catalytic activities are associated with proteasome function: the caspase-like activity, the trypsin-like activity, and the chymotrypsin-like activity, which cleave after acidic, basic, and hydrophobic residues, respectively [3]. Based on the study of yeast proteasome mutants, the caspase-like activity was associated with the β1 subunit, whereas the trypsin-like and chymotrypsin-like activities were linked to the β2 and β5 subunits, respectively [4–9]. However, cleavage specificity is not as clear-cut because overlapping specificities can sometimes be observed for some proteasome subunits [8] and because cleavage efficiency can also be influenced by the sequences surrounding the cleavage site [10,11]. In addition to the standard proteasome, three other subtypes of proteasome exist that contain alternative catalytic subunits. The immunoproteasome, which contains three inducible subunits β1i, β2i, and β5i, is found in immune cells and is induced by pro-inflammatory cytokines such as interferon-γ. Intermediate proteasomes, which are composed of a mixture of standard subunits and immunosubunits, were recently described and contain either one (β5i) or two (β1i–β5i) immunosubunits [12]. These intermediate proteasomes account for approximately 10 to 20% of the total proteasomes found in tumors and 30 to 50% of those found in liver, kidney, small bowel, colon, and dendritic cells [12]. Incorporation of the inducible subunits modifies the catalytic activity of the proteasome complex, with the immunoproteasome showing a greater propensity to cleave after hydrophobic and basic

* Corresponding author at: Ludwig Institute for Cancer Research and de Duve Institute, 74 Avenue Hippocrate UCL 74.59, B-1200 Brussels, Belgium. Fax: +32 2 7647590.
E-mail address: nathalie.vigneron@bru.licr.org (N. Vigneron).

http://dx.doi.org/10.1016/j.ab.2015.04.019
0003-2697/

amino acids, whereas the intermediate proteasomes β5i (hereafter called SIP for single intermediate proteasome)[1] and β1i–β5i (called DIP for double intermediate proteasome) display chymotrypsin-like and trypsin-like activities that are intermediate between those of the standard proteasome and the immunoproteasome [12]. The SIP displays a caspase-like activity (cleavage after acid amino acids) similar to that of the standard proteasome, whereas this activity is low in the DIP and the immunoproteasome. Because of their particular cleavage properties, each type of proteasome produces a specific peptide repertoire [12–14].

Proteasome-dependent proteolysis is essential to maintain cellular homeostasis and regulate a number of essential cellular processes such as cell differentiation, cell cycle progression, and apoptosis. Therefore, the development of efficient proteasome inhibitors has become one of the focuses of anticancer therapies. The proteasome inhibitor bortezomib (PS-341 or Velcade), which was the first proteasome inhibitor approved by the U.S. Food and Drug Administration (FDA) for the treatment of multiple myeloma [15] and mantle cell lymphoma [16], reversibly targets the chymotrypsin and caspase-like activities of the proteasome. Recently, the proteasome inhibitor carfilzomib (PR-171 or Kyprolis) was also FDA approved, and many other β5/β5i-targeting inhibitors are under clinical evaluation. More recently, the proteasome inhibitor PR-957, which specifically targets proteasome immunosubunit β5i, was shown to decrease, in animal models, the immune signaling cascade and the cytokine secretion associated with autoimmune inflammation and, therefore, might be useful for the treatment of autoimmune disorders such as rheumatoid arthritis [17].

The measurement of proteasome catalytic activities is often based on the use of fluorogenic peptides composed of a small stretch of amino acids bound to a fluorescent probe. Proteasome assays typically require either high-performance liquid chromatography (HPLC)-purified 20S or 26S proteasomes or proteasomes contained in whole-cell extracts. HPLC purification of proteasomes is a time-consuming procedure that requires the expansion of a large number of cells and is technically very demanding. On the other hand, proteasomes obtained from total cell extracts are not pure and, therefore, assay specificity is often questionable despite the inclusion of specific proteasome inhibitors to exclude background activities related to nonproteasomal proteases. Recently, a high-sensitivity bioluminescent assay, Proteasome–Glo (Promega), was developed that enables the measurement of proteasome activities directly from cultured cells [18]. In this assay, cell lysis is achieved directly at the step of substrate addition with the detergent-containing substrate buffer. The high sensitivity of this assay lies in the nature of the substrates, which consist in peptides bound to aminoluciferin. Cleavage of these substrates by the proteasome leads to liberation of free aminoluciferin, which is the substrate of the buffer-contained luciferase. One of the drawbacks of this assay is the occurrence of background cleavages due to nonproteasomal proteases, which makes the addition of proteasome and protease inhibitors essential to monitor proteasome-specific activity. This is especially true when measuring the trypsin-like activity [18,19]. In addition, the bovine serum found in the cell culture medium can also influence the specificity of the assay [20]. Here, we describe a new type of proteasome assay based on the specific capture of proteasomes on 96-well plates. It enables the quick monitoring of proteasome activity from any cell type, using a very limited number of cells per sample. It bypasses the need for biochemical proteasome purification while retaining the concept of "pure" proteasome assay, thereby limiting problems related to contaminating proteases. When used with a set of four different cell lines, each expressing a distinct proteasome subtype, it allows individual monitoring of the specific activity of these proteasome subtypes, namely the standard proteasome, immunoproteasome, SIP, and DIP. This should help the development of proteasome inhibitors specific for a given catalytic subunit.

Materials and methods

Cell lines

Here, 293 cells expressing the standard proteasome (β1–β2–β5), the immunoproteasome (β1i–β2i–β5i), and the intermediate proteasome β5i (SIP) or β1i–β5i (DIP) [12] were grown in Iscove's modified Dulbecco's medium (IMDM, Thermo Scientific, Waltham, MA, USA) containing 10% fetal calf serum (Thermo Scientific) and supplemented with Puromycin (5 μg/ml, Sigma, St Louis, MA, USA) and/or Hygromycin (600 μg/ml, InvivoGen, San Diego, CA, USA). LG2–EBV B cells were cultured in IMDM supplemented with 10% fetal calf serum (FCS). All culture media were supplemented with L-arginine (116 mg/L), L-asparagine (36 mg/L), L-glutamine (216 mg/L), penicillin (100 U/ml), and streptomycin (100 mg/ml) (Thermo Scientific). The MCP21 hybridoma [21] was obtained from European Collection of Cell Cultures (ECACC), and antibody was purified from hybridoma supernatant using HiTrap columns prepacked with Protein G Sepharose.

Cell lysates for Western blots and captured proteasome assay

Pellets washed in phosphate-buffered saline (PBS) were lysed on ice at a cell density of 10^7 cells/ml in 50 mM Tris and 0.1% NP40 (pH 7.5). Postnuclear supernatant was then collected and used for the capture proteasome assay (CAPA), Western blot, or immunoprecipitation. A protease inhibitor cocktail (halt protease inhibitors, Thermo Scientific) was added to the lysis buffer when the lysate needed to be further used for immunoprecipitation and Western blot analysis. No protease inhibitor cocktail was used when the lysate was used for the CAPA.

Capture proteasome assay

Black 96-well MaxiSorp plates (VWR, Radnor, PA, USA) were coated using 5 μg/ml MCP21 antibody and then further blocked for 1 h in PBS containing 2% bovine serum albumin (BSA) before the addition of the fresh postnuclear lysate. The protein content of the lysate was analyzed using the BCA (bicinchoninic acid) protein assay kit (Thermo Scientific) and adjusted to a concentration of 200 μg/ml in lysis buffer (50 mM Tris and 0.1% NP40, pH 7.5). Then, 50 μl of cell lysate was added in each well, and the plates were incubated under rotation (300 RPM) for 2 h at 4 °C. The amount of proteasome captured in these conditions was estimated to be 200 ng/well using quantitative enzyme-linked immunosorbent assay (ELISA) [12]. After proteasome capture, plates were carefully washed in 20 mM Tris and 0.1% NP40 (pH 7.5) and then in 20 mM Tris (pH 7.5) to remove traces of detergent. Proteasome inhibitors diluted in 20 mM Tris (pH 7.5) were then added to the wells and incubated under rotation (300 RPM) for 10 min at room temperature. Finally, the fluorogenic substrates Suc-LLVY-AMC, Z-LLE-AMC, and Boc-LRR-AMC (Enzo, Farmingdale, NY, USA) were added to the wells at a final concentration of 100 μM in 20 mM Tris (pH 7.5). Plates were then sealed with a

[1] *Abbreviations used:* SIP, single intermediate proteasome; DIP, double intermediate proteasome; FDA, U.S. Food and Drug Administration; HPLC, high-performance liquid chromatography; IMDM, Iscove's modified Dulbecco's medium; PBS, phosphate-buffered saline; CAPA, capture proteasome assay; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SP, standard proteasome; IP, immunoproteasome; EDTA, ethylenediaminetetraacetic acid.