JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Article

pubs.acs.org/JACS

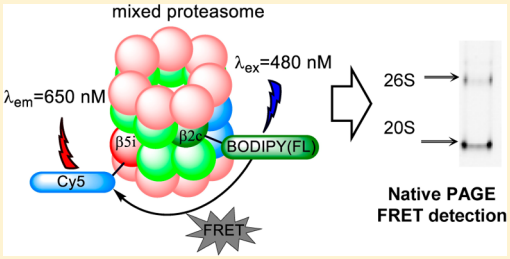
Proteasome Subunit Selective Activity-Based Probes Report on Proteasome Core Particle Composition in a Native Polyacrylamide Gel Electrophoresis Fluorescence-Resonance Energy Transfer Assay

Gerjan de Bruin, Bo-Tao Xin, Bogdan I. Florea, and Herman S. Overkleeft*

Gorlaeus Laboratories, Leiden Institute of Chemistry, Einsteinweg 55, 2333 CC Leiden, The Netherlands

Supporting Information

ABSTRACT: Most mammalian tissues contain a single proteasome species: constitutive proteasomes. Tissues able to express, next to the constitutive proteasome catalytic activities (β1c, β2c, β5c), the three homologous activities, β1i, β2i and β5i, may contain numerous distinct proteasome particles: immunoproteasomes (composed of β1i, β2i and β5i) and mixed proteasomes containing a mix of these activities. This work describes the development of new subunit-selective activity-based probes and their use in an activity-based protein profiling assay that allows the detection of various proteasome particles. Tissue extracts are treated with subunit-specific probes bearing distinct fluorophores and subunit-specific inhibitors. The samples are resolved by native polyacrylamide gel electrophoresis, after which fluorescence-resonance energy transfer (FRET) reports on the nature of proteasomes present.



INTRODUCTION

26S proteasomes are responsible for the degradation of the majority of cytoplasmic and nuclear proteins in eukaryotic cells.[1] Proteins destined for degradation are tagged with poly ubiquitin chains for recognition by 19S (PA700) caps. Subsequently and in an ATP-dependent process, proteasome substrates are unfolded and funneled through the α-rings to the inner side of 20S proteasome core particles (CP), where they are degraded.

CPs are 28-mer multiprotein complexes consisting of four heptameric rings: two outer α-rings onto which 19S caps can dock and two inner β-rings in which the catalytic subunits reside. Each β-ring of the constitutive proteasome (cCP, Figure 1), constitutively expressed in all eukaryotic cells, contains three different active subunits: β1c (caspase-like, cleaving preferentially C-terminal of acidic residues), β2c (trypsin-like, cleaving preferentially after basic residues) and β5c (chymotrypsin-like, cleaving preferentially after hydrophobic residues).

Proteasomes produce oligopeptides varying in length between 3 and 12 amino acid residues.[2] These are further processed by aminopeptidases and in part escape to the ER lumen, where they bind to major histocompatibility complex class I (MHC-I) heterodimers for antigen presentation. Another type of proteasomes, immunoproteasomes (iCP, Figure 1), are constitutively expressed in bone marrow derived cells and can be induced in other tissues by the inflammatory cytokines, interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α).[3] iCPs generate peptide pools containing a comparatively (with respect to cCP-produced oligopeptide pools) higher number of peptides prone to bind to MHC-I complexes.

In tissue expressing both active β-subunit sets (β1c/β2c/β5c and β1i/β2i/β5i)s iCPs and cCPs are not formed exclusively

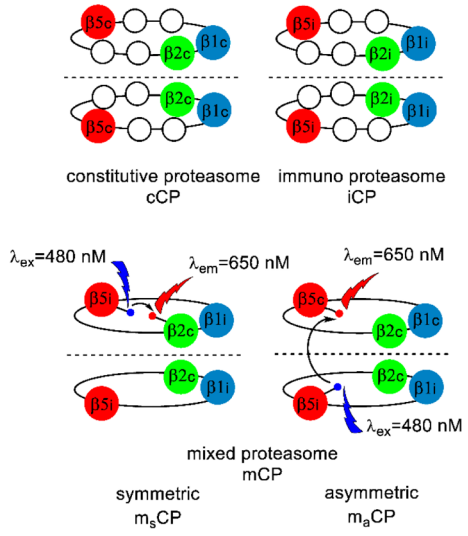


Figure 1. 20S proteasome subtypes and examples of ABP-based FRET. Only β-rings are shown.

but also mixed proteasomes (mCPs) containing cCP and iCP catalytic subunits can be formed (Figure 1). mCPs can either be symmetric (m_sCP, identical β-rings) or asymmetric (m_aCP, different β-rings).[4,5]

Mathematically, 33 different CP particles are possible (26/2 + 1); however, β1i and β2i can only be incorporated together with β5i.[6] Taking this restriction in account, five different β-

Received: April 25, 2016
Published: July 18, 2016

DOI: 10.1021/jacs.6b04207
J. Am. Chem. Soc. 2016, 138, 9874−9880

rings are possible, and thus 15 different proteasome types may exist simultaneously in cells expressing all cCP and iCP subunits (Table 1).

Table 1. Theoretical Possible Proteasome Subtypes[a]

Subtype	β1	β2	β5	β1	β2	β5
cCP	■	■	■	■	■	■
iCP						
m_sCP_1	■	■		■	■	
m_sCP_2		■			■	
m_sCP_3	■			■		
m_aCP_1	■	■	■	■	■	
m_aCP_2	■	■	■		■	
m_aCP_3	■	■	■	■		
m_aCP_4	■	■	■			
m_sCP_5	■	■			■	
m_aCP_6	■	■		■		
m_sCP_7	■	■				
m_aCP_8		■		■		
m_aCP_9		■				
m_aCP_{10}	■					

[a]Given that β5i is required for incorporation of β1i and β2i, 15 subtypes are theoretically allowed. Gray: constitutive proteasome subunit. White: immunoproteasome subunit. m_sCP: mixed symmetric core particle; m_aCP: mixed asymmetric core particle.

mCPs containing either β5i-β1c-β2c or β5i-β1i-β2c β-rings are encountered most often and have been identified in human liver, colon, small intestine and kidney tissues.[7] mCPs produce peptide pools distinct from both those produced by cCPs and iCPs, thus adding to the diversity of MHC-I ligands and thereby to a broad $CD8^+$ T-cell repertoire. Tumor-specific antigenic peptides[7,8] as well as virally encoded antigenic peptides[9] have been identified that appear to be produced uniquely by mCPs.

A rapid and accurate assay to detect mixed proteasomes and that would report on the nature of their composition would be of considerable use to get insight in the contribution of these in protein turnover and MHC-I antigenic peptide pool production. Here, we describe a native-PAGE Fluorescence Resonance Energy Transfer (FRET) assay that reports on proteasome CP composition of crude cell lysates. For this purpose proteasome-subunit selective irreversible inhibitors were equipped with suitable fluorophores to yield a panel of activity-based probes (ABPs) for FRET mediated detection of proteasome compositions.

RESULTS

Development of FRET Donor and Acceptor ABPs. FRET is a physical process in which energy is transferred from a donor fluorophore to an acceptor fluorophore via dipole−dipole coupling. This nonradiative energy transfer depends on whether the fluorophores are in close proximity (>100 Å): whether there is substantial overlap between the donor emission and acceptor excitation spectra and whether the fluorophores are properly oriented (the dipoles of the fluorophores should be approximately parallel).[10] FRET has been widely used to study protein−protein interactions and conformational changes,[11] but its potential to determine the composition of protein complexes has not been fully exploited.[12] The distances between all active site threonine residues fall well within the FRET range (<100 Å).[13] Native-PAGE separation of proteasomes has provided important insights in proteasomal composition, assembly and binding characteristics.[14] On native gel, proteasome complexes separate in three bands, corresponding to doubly capped 30S proteasomes, singly capped 26S-proteasomes and 20S proteasome CPs. These complexes are revealed by either Western blotting or in-gel fluorogenic substrate assays.[14] We reasoned that, in analogy to SDS-PAGE, it should be possible to visualize intact proteasome complexes on native-PAGE using ABPs. Indeed (Figure S1, lane 1−3), clear labeling of both 26S proteasomes and 20S proteasomes was observed in crude cell lysate using either Cy5-NC001 (β1-selective), BODIPY(FL)-LU112 (β2-selective) or BODIPY(TMR)-NC005 (β5-selective) (See Figure S1 for excitation/emission wavelengths and Figure 2 and S1 for structures).[15] In the first instance we investigated whether FRET signals emerge from proteasomes exposed to combinations of these probes and next resolved by native-PAGE. For this purpose, lysates were treated with each of the three combinations of two probes simultaneously. Clear FRET signals were observed for each combination (Figure S1, lane 4−6, Cy2-Cy3, Cy3-Cy5 and Cy2-Cy5 channels). However, due to the spectral overlap with both Cy2 excitation and Cy5 emission, the use of BODIPY(TMR) as either FRET donor or acceptor proved suboptimal (Figure S1). Hardly any background signal was observed in the samples treated with BODIPY(FL)- and Cy5-modified probes (Figure S1, lane 1 and 2, Cy2-Cy5). As well, FRET efficiency between these fluorophores appeared close to 100%, indicating near complete quenching of BODIPY(FL) fluorescence (Figure S1a, Cy2).

Given these results, we decided to develop BODIPY(FL) and Cy5 ABPs for each subunit-pair (β1c/β1i, β2c/β2i and β5c/β5i). Structures of the ABPs used in this study are shown in Figure 2. In keeping with the tradition of naming our compounds, the last digit indicates which subunits/subunit pairs are targeted (β1, β2 or β5) and "c" or "i" indicates respectively cCP or iCP selectivity. BODIPY(FL)-NC001[16] **2** and BODIPY(FL)-LU112[17] **4** have been described previously, whereas Cy5-LU112 **3** was readily synthesized following established procedures (see Supporting Information). Cy5-LU015 **5** and BODIPY(FL)-LU015 **6** were used to selectively label β5c/β5i (see Supporting Information for their synthesis). Furthermore, in order to study m_aCPs, ABPs selective for a single catalytic subunit, namely BODIPY(FL)-LU001c **7** (β1c-selective), Cy5-LU001i **8** (β1i-selective), BODIPY(FL)-LU015c **9** (β5c-selective) and Cy5-LU035i **10** (β5i-selective), were developed (see Supporting Information). The single subunit selective ABPs are based on our previously reported subunit selective inhibitors.[15,18] The selectivity window and concentrations required for complete labeling of the respective subunits by ABPs **1**−**10** was assessed in Raji- and HEK cell lysates (ABP **1**−**6**) (Figure S2, Table S1). β2-selective probes **3** and **4** as well as β1-selective probe **2** are partially cross-reactive toward β5c and β5i at concentrations required for full labeling. To avoid this to happen, the β5 subunits are to be blocked previous to treatment with **2**, **3** or **4** by either a β5-selective inhibitor (NC005 **13**, Table S1) or by β5 probes **5** or **6** (neither of which are cross-reactive). β5c selective probe BODIPY(FL)-LU015c **9** partially labels both β2 subunits (Figure S2m), which however can be prevented by pretreatment with the β2-selective inhibitor, LU102 **12**.

DOI: 10.1021/jacs.6b04207
J. Am. Chem. Soc. 2016, 138, 9874−9880

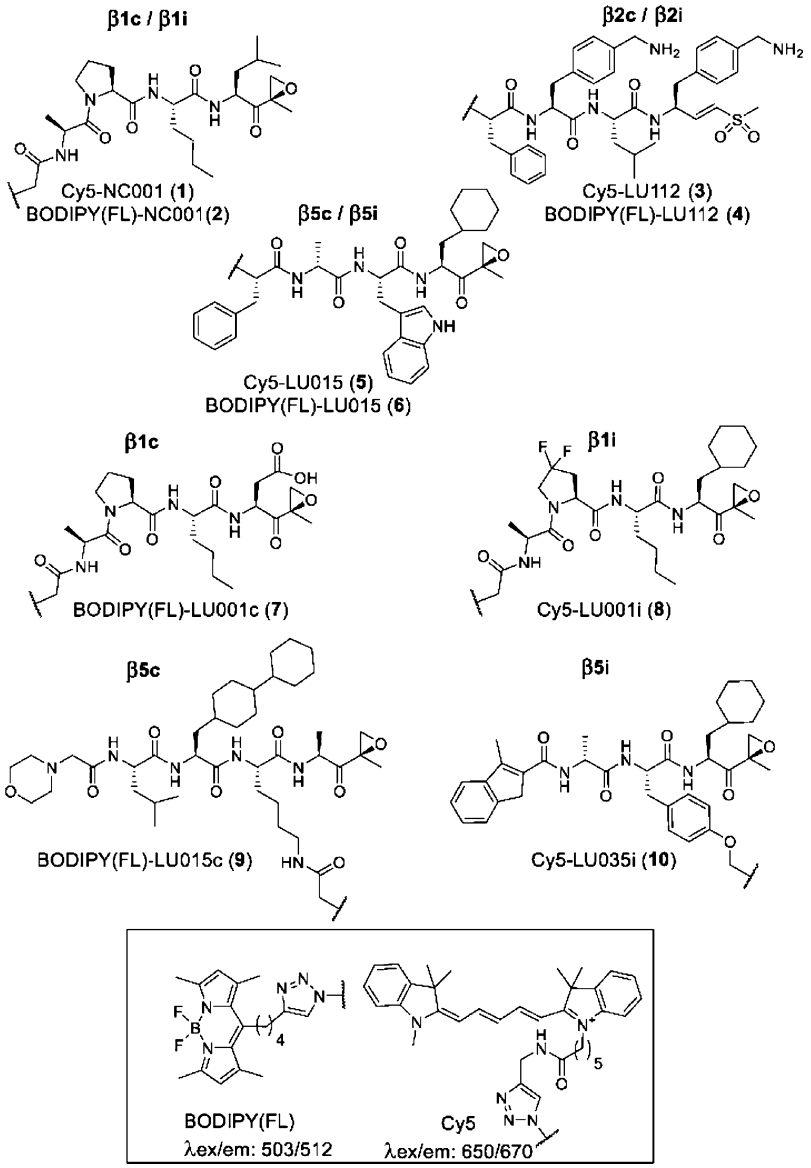


Figure 2. Structures of FRET acceptor (Cy5) and (BODIPY(FL)) activity-based probes used in this study.

Evaluation of ABPs 1−6 as Native-PAGE FRET Proteasome Probes. From the pool of ABPs **1**−**6**, six FRET donor/acceptor pairs can be assembled. We evaluated all these pairs in Raji and HEK-293 lysates on their behavior as FRET couples in a native-PAGE fluorescence readout setting. In the first step, both β5 subunits were either inhibited with NC005 **13** (in case FRET signals emerging from ABP labeling of β1 and β2 were sought for) or labeled with ABPs **6** or **7** (β1-β5 or β2-β5 labeling) for 1 h. Subsequently, β1 and/or β2 targeting probes **1**, **2**, **3** and/or **4** were added and the samples were again incubated for 1 h. One half of each sample was resolved by native-PAGE (Figure 3a) and the other half by SDS-PAGE (Figure 3c). Clear FRET signals and near complete quenching of FRET donor ABPs were observed for each FRET pair. Following quantification of the fluorescence bands of the acceptor ABPs (Cy2 channel), the FRET efficiencies (E) were calculated, and high FRET efficiencies for each FRET pair (E > 0.8, Table S2) were revealed. Swapping the FRET donor (BODIPY-FL) and acceptor (Cy5) on the subunit-selective ABPs did not result in significant differences in FRET efficiency. In order to verify whether true intraproteasomal FRET signals are observed, the native-PAGE slab was transferred to a fixing solution (5:4:1 H_2O/MeOH/AcOH) and heated in a microwave oven. This process results in denaturation of the proteins, and separation of the fluorophores. Indeed, after fixation, FRET signals have disappeared almost entirely (Figure 3b), with concomitant return of fluorescence of the donor ABPs. This result confirms the occurrence of intraproteasomal FRET and the suitability of ABPs **1**−**6** for native-PAGE FRET analysis of proteasome compositions. Remarkably, mutual differences in fluorescence intensity on native-PAGE between samples treated with a single ABP were observed, while on SDS-PAGE the intensities are similar (Figure 3a,c; compare lanes 1−6 in both gels). For instance, BODIPY(FL)-NC001 **2** shows the highest fluorescent signal with the intensities for BODIPY(FL)-LU112 **4** and BODIPY(FL)-LU015 **6** being respectively 1.5 and 4.0 times lower (Figure 3a; compare lane 2, 4 and 6). However, after gel fixation the fluorescence intensity for the three probes became almost equal.

DOI: 10.1021/jacs.6b04207
J. Am. Chem. Soc. 2016, 138, 9874−9880

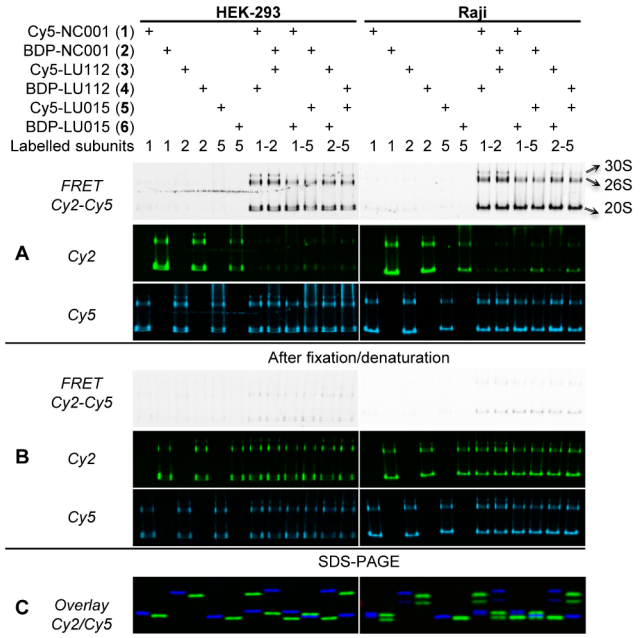


Figure 3. Evaluation of six FRET donor/acceptor pairs in HEK-293 and Raji lysates. (A) Native-PAGE analysis. (B) Native-PAGE analysis after gel fixation. (C) SDS-PAGE analysis to verify correct labeling. Gels were imaged using Cy2, Cy5 or Cy2-Cy5 settings, see Figure S1. See Table S2 for calculated FRET efficiencies.

These differences may be caused by either self-quenching[19] or homo-FRET,[20] processes that can take place when fluorophores have sufficient overlap in their excitation and emission spectra. Since proteasomes encompass two copies of each subunit (pair), two fluorophores are brought in close proximity, which may allow self-quenching or homo-FRET processes to occur. Due to differences in the mutual orientation of- and distances between the two fluorophores, the efficiency of self-quenching of the ABPs may vary, resulting in different fluorescence intensities.

Native-PAGE FRET Allows the Detection of Mixed Proteasomes. In order to detect mixed proteasomes by native-PAGE FRET, each donor or acceptor ABP should bind to a single cCP or iCP subunit. The presence of mixed proteasome core particles in a given sample is revealed by FRET when, for instance, a donor ABP is bound to a cCP subunit and an acceptor ABP to an iCP subunit assembled in the same CP. Selective binding of ABPs **1**–**6** to a single subunit can be attained by making use of our recently published panel of subunit-selective inhibitors (Table S1).[15]

With the exception of LU-002c (targeting β2c) the selectivity windows for all inhibitors are sufficiently large to allow selective and complete blocking of their target subunits. With the panel of five inhibitors selective for β1c, β1i, β2i, β5c, or β5i (Table S1), eight combinations of two inhibitors can be made. With these and together with ABPs **1**–**6** eight different proteasome subunit combinations can in theory be detected. Each possible inhibitor combination was assessed in Raji cell lysates using two FRET ABP pairs (Figure 4). Since both inter- and intra-β-ring FRET can take place, the observed FRET signal is a sum of several possible FRET pathways that emerge from, either, two, three or four ABPs present in a proteasome particle. Interestingly, however, clear FRET signals were observed for each combination, which implies that, next to cCP, iCP also mCPs are present. The various ABP couples yield FRET signals

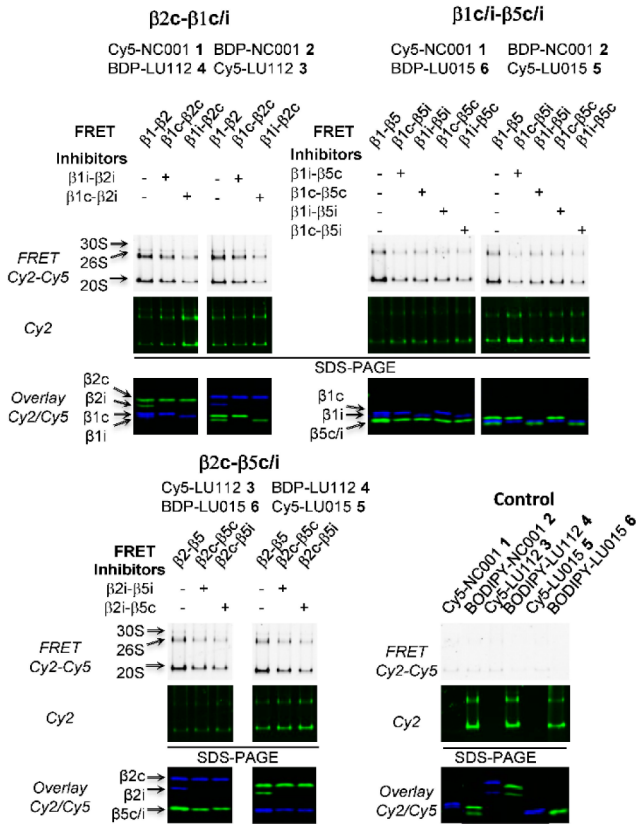


Figure 4. Detection of mixed proteasomes in Raji cell lysates. Samples were preincubated with indicated inhibitors, followed by labeling of residual proteasome activity by indicated ABPs. The samples were analyzed by native-PAGE and SDS-PAGE.

of similar intensities, but subtle differences are observed (see also Table 2).

The high β2c over β2i ratio (see Table 2) is reflected in the relatively high FRET intensities emerging from ABPs bound to β2c-β1c/β5c compared to β2c-β1i/β5i, indicating that β2c is preferentially incorporated together with β1c and β5c. The low FRET signal between β2c-β1i reflects the preferential incorporation of β2i together with β1i. The FRET intensities for β1c-β5c and β1i-β5i are higher than those for β1i-β5c and β1c-β5i, indicating preferential formation of β1c-β5c and β1i-β5i containing β-rings.

Asymmetric Mixed Proteasomes (m_aCPs) Can Be Detected Using Native-PAGE FRET. When applied at appropriate concentrations, ABPs **7**, **8**, **9** and **10** selectively and completely block a single proteasome subunit, namely β1c, β1i, β5c and β5i, respectively. Inclusion of these compounds in the native-PAGE FRET experiments allows labeling of for instance β1c with a FRET donor and β1i with a FRET acceptor ABP. FRET signals emerging from samples treated in this way and resolved on native-PAGE, can only be caused by m_aCPs. Clear FRET signals were observed for both FRET pairs **7**/**8** and **9**/**10**, confirming the presence of one or more m_aCPs (see Figure 5). Although relative amounts of m_aCPs could not be established due to lack of appropriate reference samples, this method demonstrates the presence of m_aCPs in a given sample in an unambiguous fashion.

Assessment of Proteasome Composition after Induction of iCPs by IFN-γ. The expression of iCP subunits can be induced by exposure to the inflammatory cytokine

DOI: 10.1021/jacs.6b04207
J. Am. Chem. Soc. 2016, 138, 9874–9880

Table 2. Relative FRET Intensities in Raji and HeLa Lysates

		relative FRET intensity (% of FRET no inhibitor)[a]						ratio subunits[b]
			HeLa			HeLa		
subunits		Raji	−IFN-γ	+IFN-γ	Raji	−IFN-γ	+IFN-γ	Raji
ABPs		Cy5-NC001 − BDP-LU112 (**1**−**4**)			BDP-NC001 − Cy5-LU112 (**2**−**3**)			β1c/β1i = 1.24 ± 0.03 (55/45)
β1c	β2c	53	89	57	50	102	58	β2c/β2i = 2.28 ± 0.07 (70/30)
β1i	β2c	18	22	20	17	20	19	β5c/β5i = 1.04 ± 0.03 (51/49)
ABPs		Cy5-NC001 − BDP-LU015 (**1**−**6**)			BDP-NC001 − Cy5-LU015 (**2**−**5**)			Hela − IFN-γ
β1c	β5i	26	29	21	23	32	22	β1c/β1i = 5.52 ± 0.04 (85/15)
β1i	β5i	28	14	41	35	13	40	β2c/β2i = 1.00 ± 0.00 (100/0)
β1c	β5c	31	54	33	44	67	40	β5c/β5i = 2.47 ± 0.12 (72/28)
β1i	β5c	23	17	7	20	15	10	Hela + IFN-γ
ABPs		Cy5-LU112 − BDP-LU015 (**3**−**6**)			BDP-LU112 − Cy5-LU015 (**3**−**5**)			β1c/β1i = 1.44 ± 0.07 (85/59)
β2c	β5c	39	72	36	50	74	47	β2c/β2i = 2.72 ± 0.01 (100/37)
β2c	β5i	27	30	30	29	19	31	β5c/β5i = 0.98 ± 0.04 (72/72)

[a]Determined by quantification of FRET signals from Figure 4 (Raji) or Figure 5 (HeLa). [b]Determined by quantification of SDS-PAGE.

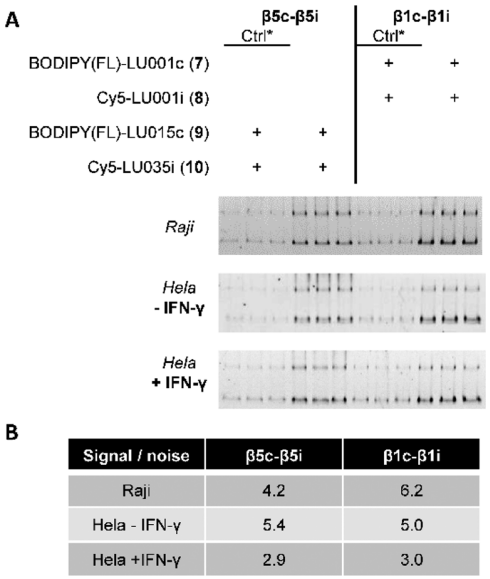


Figure 5. Asymmetric mixed proteasomes in Raji lysates and in HeLa cell lysates (before and after exposure to IFN-γ for 24 h). Samples were incubated with a β2-selective inhibitor (LU102, in case of ABPs **9**, **10**) or a β5-selective inhibitor (NC-005, in case of ABPs 7, **8**) followed by treatment with ABPs. *Ctrl: control. Control samples contained twice the amount of protein compared to normal samples were incubated with LU-102 or NC-005 as for normal samples, followed by incubation with one of the ABPs. Next, remaining proteasome activities were blocked by a mixture of NC001, LU102 and NC005 and the samples treated with ABP **7**/**8** and **9**/**10**.

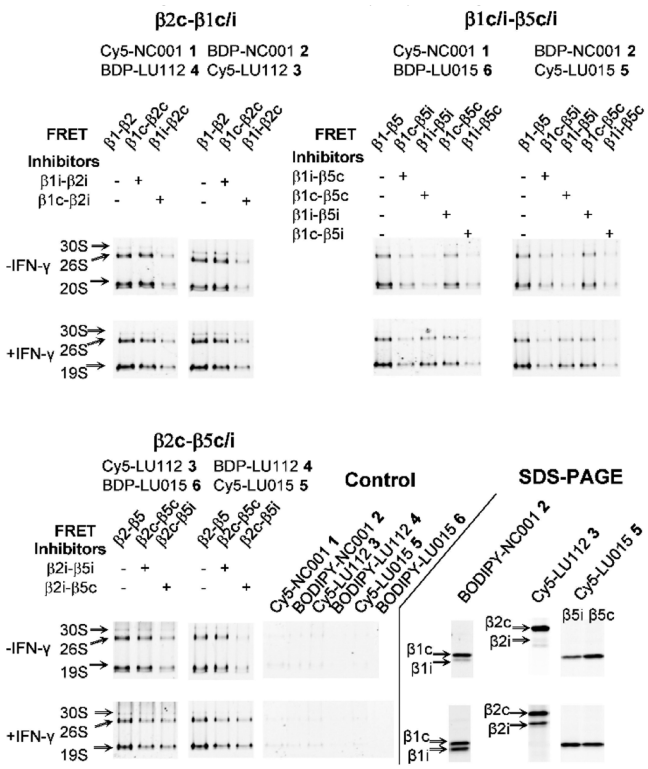


Figure 6. Mixed proteasomes in HeLa cell lysates, with or without exposure to IFN-γ for 24 h. Samples were preincubated with indicated inhibitors, followed by labeling of residual proteasome activity by indicated ABPs. SDS-PAGE analysis shows the relative amounts of iCP and cCP subunit before and after exposure to IFN-γ. To label β5c or β5i specifically, either β5i was blocked by LU-035i or β5c by LU-005c.

interferon-γ (IFN-γ). Dahlmann and co-workers reported that HeLa cells exposed to IFN-γ express both m_sCPs and m_aCPs.[5] These observations were re-evaluated using the above-described native-PAGE FRET assay. For this, HeLa cells were either exposed to IFN-γ for 24 h or left untreated. Next, both samples were subjected to various inhibitor/ABP combinations and evaluated by native-PAGE FRET as described above. When not exposed to IFN-γ, HeLa cells express only small amounts of β1i and β5i while β2i could not be detected. Following exposure to IFN-γ, a substantial increase of the amount of all iCP subunits was found (Figure 6, Table 2). As expected, nonexposed HeLa cells show high inter-cCP subunit FRET signals. Since the FRET signals emerging from β5i-β1c are higher than those observed for β5i-β1i, it is likely that the majority of the β5i subunits in these nonexposed cells are present in proteasomes that also contain at least one β1c subunit.

As well, substantial β5i-β5c and β1i-β1c FRET signals were observed (Figure 5), indicating the presence of m_aCPs. Most likely, the majority of these m_aCPs contain, a single β5i (m_aCP_1), or both one β5i and one β1i in the same β-ring (m_aCP_2), as witnessed by the observed FRET signal between β5c and β1i. After exposure to IFN-γ, a strong increase in β5i-β1i FRET signal is observed, while β5i-β1c, β5c-β1i and β5c-β1c combinations are slightly decreased. This decrease does not

DOI: 10.1021/jacs.6b04207
J. Am. Chem. Soc. 2016, 138, 9874−9880

necessarily reflect a decrease of the absolute amounts of these subunit-pairs, but is probably caused by the increase in expression and incorporation into proteasome particles of both β5i-subunits and β1i-subunits, with an increased total FRET intensity as the result. Given the long half-life of cCPs (up to 5 days),[21] IFN-γ induced expression of iCP subunits presumably leads to a net increase of the total proteasome amount. When for convenience it is assumed that limited proteasome degradation takes place during the 24 h exposure with IFN-γ, the slight decrease in β5i-β1c and β5c-β1i-derived FRET signals indicates that no new proteasomes containing these subunit-pairs are formed, that newly expressed β5i is mainly incorporated together with β1i and that newly formed CPs are symmetric with respect to their β1/β5 subunit composition. Interestingly, the β2c-β5i FRET signals significantly increase (Figure 6) and their intensities become closer to the β2c-β5c FRET signals. The same applies to β1i-β2c, indicating that β5i is to some extend incorporated with β2c, which is also reflected by the lower net increased amount of β2i (+37%) compared to β1i and β5i (both +44%).

Altogether it can be concluded that, following exposure to IFN-γ, HeLa cells predominantly produce two distinct proteasome types: mCPs featuring β-rings composed of either β1i-β2i-β5i or β1i-β2c-β5i. This observation is further confirmed by the lower relative amount of m_aCPs found after exposure to IFN-γ (lower signal/noise ratio compared to no IFN-γ, Figure 5), indicating that the newly formed proteasome are symmetric with respect to their β1 and β5 subunit composition.

DISCUSSION

We have reported here an in-depth analysis on the use of ABPs to determine the composition of large protein complexes using a native-PAGE FRET assay. Proteasome subunit-pair selective ABPs equipped with suitable FRET donor and acceptor fluorophores were selected, which target β1c/β1i, β2c/β2i or β5c/β5i. Crude cell extracts were treated with combinations of these FRET donor/acceptor ABPs and resolved on native-PAGE, after which FRET signals can be measured by fluorescence imaging of the gel. In HEK-293 cells, expressing exclusively cCPs, all FRET pairs gave clear FRET signals with high FRET efficiencies ($E > 0.8$), confirming that β1c; β2c and β5c are present in stoichiometric amounts.

Though our assay does not enable the detection of each specific (mixed) proteasome particle, many of the possible particles in fact show up in our Native PAGE FRET assays. In lysates of Raji cells, which express all six proteasome subunits, FRET signals were observed for iCP-iCP and cCP-cCP subunit-pairs. Importantly, also FRET signals of all possible iCP-cCP subunit-pairs were detected, demonstrating the presence of mCPs. Remarkably, a substantial FRET signal was observed for β1i-β5c (Figure 4, Table 2).

As β5i is required for incorporation of β1i, β5c cannot be incorporated in the same β-ring together with β1i, and therefore this result shows that either m_aCP_2 or m_aCP_4, or both, are present. Moreover, using selective β1c-β1i and β5c-β5i targeting FRET donor−acceptor pairs, m_aCPs were visualized that are asymmetric in their β1 and β5 subunit composition (m_aCP_{1-4}: asymmetric in β5 composition; $m_aCP_{2,4,5,7,8,10}$: asymmetric in β1 composition) (see Figure 5).

Altogether, these results reveal a complex mixture of proteasome subtypes to exist in Raji cells. In Raji cells, iCP-iCP and cCP-cCP subunit-pairs show higher relative FRET intensities compared to iCP-cCP subunit-pairs for β1-β5, indicating preferential formation of β1i-β5i and β1c-β5c containing β-rings. Compared to Raji cells, IFN-γ exposed HeLa cells do show much lower relative FRET intensities of β1-β5 iCP-cCP subunit-pairs, and the relative FRET intensities indicate preferential formation of proteasomes containing β-rings composed of β5i-β1i-β2i and β5i-β1i-β2c. Interestingly, both Raji- and IFN-γ exposed HeLa cells express similar amounts of all subunits, indicating that more mCPs are formed when all subunits are constitutively expressed compared to induction of iCP subunits in otherwise low iCP expressing cells.

Dahlmann and co-workers identified CPs asymmetric in their β1 subunit composition in IFN-γ exposed HeLa cells, while the nonexposed HeLa in their hands did not express detectable amount of immunoproteasome subunits.[5] However, in this study it was found that after IFN-γ exposure HeLa cells predominantly express proteasome-containing β-rings symmetric in their β1 subunit composition.

Dahlmann and co-workers used β1c-ZZ transfected HeLa cells to allow specific precipitation of β1-ZZ by binding to IgG and subsequent analysis of subunit composition of precipitated proteasomes. This may result in higher β1c-ZZ than normal β1c expression, causing higher incorporation of β1c-ZZ in newly formed proteasomes resulting in asymmetric proteasome formation.

Current methods to identify proteasome CP composition are based on either chromatographic separation of proteasome subtypes,[5,22] isoelectric focusing electro-phoresis,[23] or antibody mediated depletion of a β-subunit,[7] followed by determination of CP composition by either immunostaining, substrate hydrolysis assays or mass spectrometry analysis of purified proteasomes.[24] Compared to these methods, the method described here has several advantages. For instance, van den Eynde and co-workers used subunit depletion and subsequent immunoblotting to determine proteasome composition.[7] Alternatively, they calculated the quantity of mCPs based on the assumption that β5i can be incorporated as the only iCP subunit or together with β1i, but that β2i is always incorporated together with β1i and β5i. However, in these approaches asymmetric proteasomes are not taken into account and therefore several proteasome subtypes are possibly overlooked. Our FRET-based approach, besides being more sensitive, is also much faster, straightforward and less time-consuming than the methods relying on chromatographic separation of proteasomes.

The measurement of FRET signals in-native PAGE also represents, in our opinion, a major improvement to the methodology developed by Kim and co-workers.[12] In this method, FRET signals were measured in a plate reader, which required removal of unbound probes by filtration. Moreover, whereas β5c-β1i FRET signals were detected, the detection of other subunit-pairs was hampered by the lack of truly subunit selective probes and inhibitors. We feel that their method to prove β5c-β1i containing proteasomes may be compromised, since a β5i selective probe (LKSCy5)[25] was previously claimed to selectively label β5c. As well, a β1c/β1i targeting probe (UKPCy3) was used to selectively label β1i in RPMI-8226 cells and no selective inhibitors were applied to inhibit β5i and β1c.

In conclusion, the native-PAGE FRET assay we report here adds to existing methods for the assessment of proteasome core particle composition. The method provides semiquantitative insights in the abundance of ten different proteasome subunit-pairs and can do so in any crude cell extract.

DOI: 10.1021/jacs.6b04207
J. Am. Chem. Soc. 2016, 138, 9874−9880

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b04207.

Figures, tables, biochemical procedures, synthetic procedures, ^{1}H and ^{13}C NMR spectra and LC−MS data. (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*h.s.overkleeft@chem.leidenuniv.nl

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank The Netherlands Organization for Scientific Research (NWO−CW, TOP-PUNT Grant to HSO), the China Scholarship Council (CSC, PhD fellowship to B.-T. X) and the European Research Council (ErC-2011-AdG-290836, to HSO) for financial support.

■ REFERENCES

(1) Hershko, A.; Ciechanover, A. *Annu. Rev. Biochem.* **1998**, *67*, 425.
(2) Kisselev, A. F.; Akopian, T. N.; Woo, K. M.; Goldberg, A. L. *J. Biol. Chem.* **1999**, *274*, 3363.
(3) Ferrington, D. A.; Gregerson, D. S. *Prog. Mol. Biol. Transl. Sci.* **2012**, *109*, 75.
(4) Dahlmann, B.; Ruppert, T.; Kuehn, L.; Merforth, S.; Kloetzel, P.-M. *J. Mol. Biol.* **2000**, *303*, 643.
(5) Klare, N.; Seeger, M.; Janek, K.; Jungblut, P. R.; Dahlmann, B. *J. Mol. Biol.* **2007**, *373*, 1.
(6) Griffin, T. A.; Nandi, D.; Cruz, M.; Fehling, H. J.; Kaer, L. V.; Monaco, J. J.; Colbert, R. A. *J. Exp. Med.* **1998**, *187*, 97.
(7) Guillaume, B.; Chapiro, J.; Stroobant, V.; Colau, D.; Van Holle, B.; Parvizi, G.; Bousquet-Dubouch, M.-P.; Théate, I.; Parmentier, N.; Van den Eynde, B. J. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107*, 18599.
(8) Guillaume, B.; Stroobant, V.; Bousquet-Dubouch, M.-P.; Colau, D.; Chapiro, J.; Parmentier, N.; Dalet, A.; Van den Eynde, B. J. *J. Immunol.* **2012**, *189*, 3538.
(9) Zanker, D.; Waithman, J.; Yewdell, J. W.; Chen, W. *J. Immunol.* **2013**, *191*, 52.
(10) Chirio-Lebrun, M.-C.; Prats, M. *Biochem. Educ.* **1998**, *26*, 320.
(11) Piston, D. W.; Kremers, G.-J. *Trends Biochem. Sci.* **2007**, *32*, 407.
(12) Park, J. E.; Wu, Y.; Carmony, K. C.; Miller, Z.; Sharma, L. K.; Lee, D.-M.; Kim, D.-Y.; Lee, W.; Kim, K.-B. *Mol. BioSyst.* **2014**, *10*, 196.
(13) Borissenko, L.; Groll, M. *Chem. Rev.* **2007**, *107*, 687.
(14) Elsasser, S.; Schmidt, M.; Finley, D. In *Methods Enzymol.*; Academic Press, 2005; Vol. *398*, p 353.
(15) de Bruin, G.; Xin, B. T.; Kraus, M.; van der Stelt, M.; van der Marel, G. A.; Kisselev, A. F.; Driessen, C.; Florea, B. I.; Overkleeft, H. S. *Angew. Chem., Int. Ed.* **2016**, *55*, 4199.
(16) Verdoes, M.; Willems, L. I.; van der Linden, W. A.; Duivenvoorden, B. A.; van der Marel, G. A.; Florea, B. I.; Kisselev, A. F.; Overkleeft, H. S. *Org. Biomol. Chem.* **2010**, *8*, 2719.
(17) Geurink, P. P.; van der Linden, W. A.; Mirabella, A. C.; Gallastegui, N.; de Bruin, G.; Blom, A. E.; Voges, M. J.; Mock, E. D.; Florea, B. I.; van der Marel, G. A.; Driessen, C.; van der Stelt, M.; Groll, M.; Overkleeft, H. S.; Kisselev, A. F. *J. Med. Chem.* **2013**, *56*, 1262.
(18) de Bruin, G.; Huber, E. M.; Xin, B.-T.; van Rooden, E. J.; Al-Ayed, K.; Kim, K.-B.; Kisselev, A. F.; Driessen, C.; van der Stelt, M.; van der Marel, G. A.; Groll, M.; Overkleeft, H. S. *J. Med. Chem.* **2014**, *57*, 6197.
(19) van der Meer, B. W.; Coker, G., III; Chen, S.-Y. *Resonance Energy Transfer: Theory and Data*; VCH Publishers: New York, 1994; Vol. *5*.
(20) Bader, A. N.; Hoetzl, S.; Hofman, E. G.; Voortman, J.; van Bergen en Henegouwen, P. M. P.; van Meer, G.; Gerritsen, H. C. *ChemPhysChem* **2011**, *12*, 475.
(21) Heink, S.; Ludwig, D.; Kloetzel, P.-M.; Krüger, E. *Proc. Natl. Acad. Sci. U. S. A.* **2005**, *102*, 9241.
(22) Dahlmann, B.; Ruppert, T.; Kloetzel, P. M.; Kuehn, L. *Biochimie* **2001**, *83*, 295.
(23) Drews, O.; Wildgruber, R.; Zong, C.; Sukop, U.; Nissum, M.; Weber, G.; Gomes, A. V.; Ping, P. *Mol. Cell. Proteomics* **2007**, *6*, 2021.
(24) Pelletier, S.; Schuurman, K. G.; Berkers, C. R.; Ovaa, H.; Heck, A. J. R.; Raijmakers, R. *Mol. BioSyst.* **2010**, *6*, 1450.
(25) Sharma, L. K.; Lee, N.-R.; Jang, E. R.; Lei, B.; Zhan, C.-G.; Lee, W.; Kim, K.-B. *ChemBioChem* **2012**, *13*, 1899.

DOI: 10.1021/jacs.6b04207
J. Am. Chem. Soc. 2016, 138, 9874−9880