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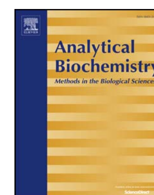

A coupled fluorescence-based assay for the detection of protein arginine N-methyltransferase 6 (PRMT6) enzymatic activity

Jan Kramer[a], Veronika Désor[a], Steffen Brunst[a], Sandra K. Wittmann[a], Jörn Lausen[b], Jan Heering[c], Anna Proschak[a], Ewgenij Proschak[a,*]

[a] Institute of Pharmaceutical Chemistry, Goethe University, Max-von-Laue-Straße 9, D-60438, Frankfurt am Main, Germany
[b] Institut für Transfusionsmedizin und Immunhämatologie, Sandhofstraße 1, D-60528, Frankfurt am Main, Germany
[c] Fraunhofer Institute for Molecular Biology and Applied Ecology IME, Project Group Translational Medicine and Pharmacology TMP, Theodor-Stern-Kai 7, D-60596, Frankfurt am Main, Germany

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ABSTRACT

The protein arginine N-methyltransferase 6 (PRMT6) is overexpressed in a variety of different cancer types and plays a role in human immunodeficiency virus (HIV) infections. Furthermore, the PRMT6 activity might also influence the pathogenesis of neurodegenerative, inflammatory, and cardiovascular diseases, whereby it becomes an interesting target for drug development. Previously reported activity assays for PRMT6 activity are either expensive, time-consuming or use radioactive substrates. To overcome these challenges, we developed a coupled fluorescence-based activity assay using recombinant PRMT6 expressed in *E. coli*. In the first step of the assay, the fluorogenic substrate Nα-Benzoyl-L-arginine-7-amido-4-methylcoumarin (Bz-Arg-AMC) is methylated by PRMT6, while in a second step the remaining un-methylated substrate is cleaved by trypsin, producing the fluorescent 7-amino-4-methylcoumarin.

Introduction

Over the last two decades, a number of cellular processes have been identified where protein arginine methyltransferases (PRMTs) play an important role: e.g. signal transduction, RNA processing, activation of transcription [1] and DNA repair [2]. Furthermore, protein arginine methylation plays a role as part of the histone code [2–5] and is necessary for the modulation of factors interacting with the histones [6]. Dysregulation of these proteins influences the pathogenesis of neurodegenerative, inflammatory, and cardiovascular diseases [7–9]. Until now at least 11 members of the PRMT family have been characterized [10,11]. These proteins catalyze the transfer of a methyl group from their cofactor S-adenosyl-L-methionine (SAM or AdoMet) to the terminal guanidino nitrogens of an arginine sidechain in the target protein [12]. The modified arginine residues are mainly located in glycine- and arginine-rich patches (GAR motifs) [6]. In eukaryotes, three different methylation patterns of arginine residues have been identified, ω-NG-monomethylarginines (MMA), ω-N^G,N^G-asymmetric dimethylarginines (ADMA) or ω-N^G,N'^G-symmetric dimethylarginines (SDMA) [2,12]. The protein family of PRMTs can be further subdivided into three different classes that differ by the methylation pattern they create. Type I and II PRMTs can dimethylate their targets in two consecutive steps. In the first step both protein classes produce MMA intermediates, while in the second methylation step type I PRMTs (1, 2, 3, 4, 6 and 8) create ADMAs and type II enzymes (PRMT 5 and 9) SDMAs [8]. Typ III enzymes only create MMAs (PRMT7) [10,13]. In this work we focus on PRMT6, which is 375 amino acids long and has a molecular mass of 41.9 kDa [1]. This type I enzyme is able to form homodimers. The stability of this homodimer is increased in presence of the cofactor AdoMet and is unchanged or reduced in presence of S-adenosyl-L-homocysteine (SAH or AdoHcy) [14]. The stability of PRMT6 is further increased by an automethylation at position R35 that is important for the restriction of human immunodeficiency virus 1 (HIV-1) replication [10]. Furthermore, Thomas et al. could demonstrate the importance of the PRMT homodimerization for their methyltransferase activity [14].

Up to this point, various assays have been established to determine methyltransferase activity. These assays are used to identify new methylation targets of the PRMT family as well as to develop novel inhibitors and most of them utilize the fact that methyltransferase use AdoMet as cosubstrate while producing AdoHc regardless of the target they methylate [15]. A number of comprehensive reviews and manuscrips give a detailed overview of the available assay systems as well as their application fields [8,16,17]. In this introduction we just give a brief overview about some of the possible PRMT assays that are

* Corresponding author.
E-mail address: proschak@pharmchem.uni-frankfurt.de (E. Proschak).

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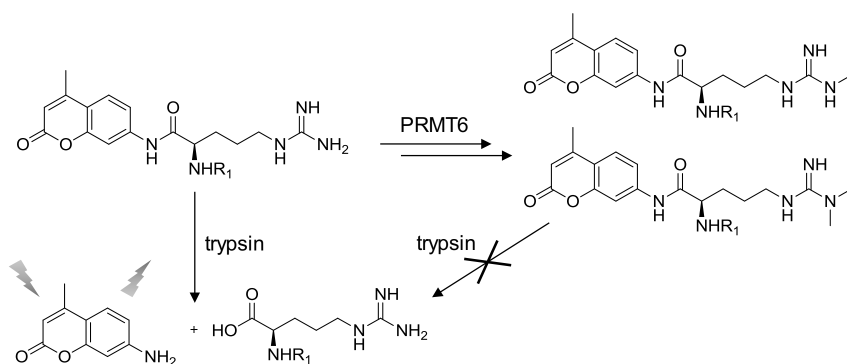

Fig. 1. Schematic representation of the coupled fluorescence-based assay. In the first step of the reaction, the non-fluorescent substrate is mono- or di-methylated by PRMT6, while in a second step all non-methylated substrate is cleaved by trypsin, producing the fluorescent 7-amido-4-methylcoumarin that can be detected. The methylated substrates are not cleaved by trypsin.

currently available. These assays can be classified into different groups: 1. Assay systems based on a radioactive co-substrate [1,18,19], 2. Assays systems using UHPLC-MS/MS [14], 3. ELISA-based high throughput (HTS) assays [20], 4. AlphaScreen-based assays, 5. Continuous [16] or discontinuous coupled chromogenic [21]/fluorescence-based assays, and 6. Biophysical assays, including isothermal titration calorimetry (ITC), differential scanning fluorimetry (DSF), Fluorescence polarization (FP) [8,22], differential static light scattering (DSLS) [9] and surface plasmon resonance SPR [6].

The assays of the first group, are still considered as the gold standard for methyltransferase reactions and can be used with a broad range of different methyltransferasees and substrates [8]. This makes these assays an excelent tool for the investigation of new methyltransferases as well as the novel methylation targets. Assays of this type are typically perforemed as end point assays and use a radioactive labeled cosubstrate (^{14}C or ^{3}H-AdoMet) in the methyltransferase reaction leading to the formation of a radioactive labeled product. At the end of the reaction the remaining radioactive substrate must be separated from the product to allow its quantification [16,21]. Depending on the substrates used in the assay, this separation and the following detection can be achieved in different ways e.g. by making a gel that is dried before exposed to an HyBlot CL autoradiography film [10], by capturing a biotinylated peptide in a streptavidin/scintillant-coated microplate, before measuring the luminescence [18], by ZipTipc4 pipette tips or using a filter paper based scintillation assay [17,23]. In this method the greater affinity of cation exchange filter to peptides and proteins than to free AdoMet is used to separate the substrate from the products before quantification [8]. Modified versions of the assay work *in vivo* as well as *in vitro* [20]. The main drawback of this group of assays is the environmental concern as a result of the produced radioactive waste. In the second group, a polypeptide or protein is used as a substrate in the methyltransferase reaction. After stopping the reaction, methylation is detected by UPLC-MS/MS [14]. The third group is ELISA-based. Here the substrate is fixated on in the wells of a multi-well plate in which the methyltransferase reaction takes place. To quantify the amount of product formed in the reaction, a mixture of antibodies that are specific for the methylated substrates as well as a secondary antibody with a coupled peroxidase is used. The luminescence signal of the peroxidase reaction is detected after a washing step to remove all unbound antibody. ELISA kits are commercially available [20]. The fourth group is the AlphaLISA PRMT6 histone H3-arginine N-methyltransferase assay, commercially available from PerkinElmer. The assay combines the principle of the ELISA with the AlphaScreen. In the AlphaScreen, a donor bead is irradiated with laser light at wavelength of 680 nm, which leads to the production of singlet oxygen species. The oxygen can reach acceptor beads in close proximity leading to an amplified chemiluminescent signal at 615 nm. The fifth group of assays monitors the amount of AdoHcy that is created during the reaction, using a coupled assay system were the AdoHcy is transformed into an easy detectable product. The assays differentiate themselves in the products that are formed from AdoHcy e.g. some end point assays generate homocysteine, which can either be detected by fluorescent [24] or chromogenic thiol-reactive reagents [21], while in a kinetic assay AdoHcy is first converted to S-ribosylhomocysteine and adenine by AdoHcy nucleosidase, before the adenine deaminase further converts the adenine into hypoxanthine that leads to an absorbance decrease at 265 nm, which can be monitored. The sixth group of assays comprises biophysical methods used to investigate PRMT inhibitor interactions. Although these assays give valuable information most of the necessary equipment is expensive and some of the assays are rather time- and material-consuming.

The aim of this study was the development of a robust, non-radioactive, and easy-to-use *in vitro* assay system to detect PRMT6 activity. Therefore, an assay principle based on fluorescence was chosen, which can easily be detected. Fluorogenic substrates are well-established for proteases, especially for the serine protease trypsin which cleaves substrates at a basic side chain like arginine or lysine. Previously a comparable assay was reported for peptidylarginine deiminase PAD4, where the deamination of the fluorogenic substrate Z-Arg-7-amino-4-methylcoumarin (Arg-AMC) prevents its cleavage by trypsin and induce the fluorescence signal subsequently [25]. In this work we could show that this principle can be applied to PRMT6, opening a new approach to measure methyltransferase activity (Fig. 1).

Experimental section

Cloning

To increase our PRMT6 purification yields of the full-length PRMT6 GST-fusion construct described in Ref. [26] a new construct was created covering the aa2-375 of the full-length PRMT6. The sequence was amplified by PCR (forward primer 5′-ccccGGATCCCAGCCCAAGAAAA GAAAGCTTGAGTCG-3′ and reverse primer 5′-CCCCCTCGAGTCATTA GTCCTCCATGGCAAAGTCTTTGGTC-3′) from the full-length GST-fusion construct. The PCR product was cloned into pET29BH4 (a pET29b derivative where the ORF was changed to the one from pHB4 with an additional TEV side) using BamHI and XhoI. The vector was named: pET29BH4-aa1-375-PRMT6. The full sequence pET29BH4-aa2-375-PRMT6 (SI.1), as well as the plasmid map (Figure S1), can be found in supporting information. The target protein is expressed as fusion protein with an N-terminal 6 × His-tag followed by a TEV recognition and cleavage site. The plasmid carries a kanamycin resistance gene.

Protein expression and purification

10 mL pre-culture of the BL21(DE3) cells, co-transformed with the pET29BH4-aa2-375-PRMT6 as well as pGro7-plasmid for the co-expression of GroEL and GroES, was used to inoculate a 1 L culture of LB media supplemented with chloramphenicol (35 μg/mL) as well as kanamycin (100 μg/mL). The culture was incubated at 37 °C and 180 rpm till an OD_{600} of 0.6–0.8 was reached, before 0.02% (w/v) arabinose was added, and the temperature was reduced to 19 °C. After reaching an

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