Biosensors and Bioelectronics 24 (2009) 1164–1170

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

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

Direct comparison of fluorescence- and bioluminescence-based resonance energy transfer methods for real-time monitoring of thrombin-catalysed proteolytic cleavage

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ARTICLE INFO

Article history:
Received 11 April 2008
Received in revised form 30 June 2008
Accepted 1 July 2008
Available online 22 July 2008

Keywords:
FRET
BRET
Thrombin
Renilla luciferase
Fluorescent proteins

ABSTRACT

In this study, a representative FRET system (CFP donor and YFP acceptor) is compared with the $BRET^2$ system (*Renilla* luciferase donor, green fluorescent protein^2 (GFP^2) acceptor and coelenterazine 400a substrate). Cleavage of a thrombin-protease-sensitive peptide sequence inserted between the donor and acceptor proteins was detected by the RET signal. Complete cleavage by thrombin changed the $BRET^2$ signal by a factor of 28.9 ± 0.2 (R.S.D. (relative standard deviation), $n = 3$) and the FRET signal by a factor of 3.2 ± 0.1 (R.S.D., $n = 3$). The $BRET^2$ technique was 50 times more sensitive than the FRET technique for monitoring thrombin concentrations. Detection limits (blank signal + $3\sigma_b$, where σ_b = the standard deviation (S.D.) of the blank signal) were calculated to be 3.05 and 0.22 nM thrombin for FRET and $BRET^2$, respectively. This direct comparison suggests that the $BRET^2$ technique is more suitable than FRET for use in proximity assays such as protease cleavage assays or protein–protein interaction assays.

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1. Introduction

Resonance energy transfer (RET) offers the convenience of real-time ratiometric quantification of inter- and intra-molecular protein interactions (Li et al., 2006). RET is the non-radiative transfer of energy from an excited state donor to a ground state acceptor. The signal strength and efficiency of RET depend upon the extent of the overlap between the donor emission and acceptor absorption spectra, the quantum yield of the donor and the molar absorptivity of the acceptor as well as the distance between, and relative orientations of the donor and acceptor dipoles (Forster, 1959). In the last decade there have been an increasing number of publications describing the use of RET-based probes in proximity assays for monitoring protein–protein interactions. RET-based assays have been described for monitoring GPCR (G-protein-coupled receptors) oligomerization (Overton and Blumer, 2002; Kroeger et al., 2002), GPCR interactions with G-proteins (Gales et al., 2005) or with β-arrestin (Coulon et al., 2008; Molinari et al., 2008). RET-based proximity assays also include complementation assays (Eglen, 2007) and protease assays (Mitra et al., 1996). RET-based assays may monitor either the increase of a signal due to enhancement of RET or the reduction in the RET signal due to dissociation of a complex or a proteolytic cleavage. A variety of RET techniques have been employed in proximity assays with the main ones being FRET and BRET techniques.

Although first characterised in 1959 (Forster, 1959) it is only in the last decade that RET techniques have been widely adopted as a research tool in the biosciences. The uptake of RET techniques has been facilitated by the development of a range of RET donors and acceptors, the increasing ease with which they can be genetically encoded and the increased sophistication and availability of instruments that can perform RET measurements. FRET is the most commonly used RET system and both donors and acceptors are fluorescent. The most widely used is CFP as the donor and YFP as the acceptor (Piston and Kremers, 2007). BRET, which replaces the donor fluorophore of FRET with a luciferase, requires the addition of a substrate to initiate bioluminescent emission and hence, energy transfer. Acceptor emission must be due to energy transfer rather than excitation at the acceptor excitation wavelength. Two common implementations of BRET comprise *Renilla* luciferase (RLuc) with either coelenterazine h ($BRET^1$) ($\lambda_{em} = \sim 475$ nm) or coelenterazine 400a (Clz400a) substrate ($BRET^2$) ($\lambda_{em} = 395$ nm) as the donor system coupled to either of the GFP mutants, YFP ($BRET^1$) ($\lambda_{em} = \sim 530$ nm) or green fluorescent protein^2 (GFP^2) ($BRET^2$) ($\lambda_{em} = \sim 510$ nm). The $BRET^2$ system offers superior spectral separation between the donor and acceptor emission peaks of ∼115 nm compared to ∼55 nm for the $BRET^1$ system (Pfleger and Eidne, 2006). However, although arguments have been made to justify the

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doi:10.1016/j.bios.2008.07.021

choice between BRET or FRET (Boute et al., 2002), there has been no direct experimental comparison of the two techniques in terms of their sensitivity, kinetics or other properties to provide a basis to choose between them in any particular situation.

In this study a commonly used FRET (CFP-YFP) and a $BRET^2$ (GFP^2-RLuc) system were directly compared by placing a thrombin-specific cleavage sequence (Chang, 1985) between the donor and acceptor proteins allowing the thrombin cleavage of this substrate to be detected via the RET signal (Supplementary data, Fig. 1(a)). Thrombin, an extracellular protein, is a well-understood protease in the regulation of blood coagulation (Coughlin, 1999) that selectively cleaves the Arg-Gly bonds of fibrinogen to form fibrin. The results from this study will enable the applicability of FRET and $BRET^2$ techniques for proximity assays to be assessed in quantitative terms.

2. Materials and methods

2.1. Materials

All primers were purchased from Geneworks (Supplementary data, Table 1). The vectors pRSET-CFP (Invitrogen) and pcDNA6.2/N-YFP-DEST (Invitrogen) were used as a source of CFP and YFP for FRET analysis (Supplementary data, Table 2). The vector GFP^2-MCS-RLuc (PerkinElmer) was used as the source for both GFP^2 and RLuc for $BRET^2$ analysis (Supplementary data, Table 2).

2.2. Construction of RET proteins

GFP^2, RLuc and YFP (Constructs 1–3, Supplementary data, Table 2) were amplified by polymerase chain reaction (PCR) using the primers shown in Supplementary data (Table 1) and cloned into pGEM®-T Easy vector (Promega). This resulted in the respective insertion of BamHI and BsrGI restriction sites directly upstream and downstream from the amplified gene. The amplicons were inserted into the BamHI and BsrGI sites of the pRSET vector, replacing CFP, to give pRSET-GFP^2, pRSET-RLuc and pRSET-YFP. DNA sequencing confirmed correct amplicon sequence.

GFP^2, RLuc, CFP and YFP were amplified by PCR using primers (Supplementary data, Table 1) for construction of fusion pairs 5–8 (Supplementary data, Table 2). EcoRI and PstI or PstI and BsrGI restriction sites were introduced upstream and downstream, respectively, from the four different amplified genes (Supplementary data, Fig. 1(b)). The fusion pairs 5–8 (Supplementary data, Table 2) were then inserted into the EcoRI and BsrGI sites of the pRSET vector DNA and confirmed by sequencing.

The thrombin recognition sequence (LQGSLVPRGSLQ) (Zhang, 2004) was synthesized by annealing oligomers C1 and C2 (Supplementary data, Table 1) followed by digestion with PstI. The sequence GSLVPRGS was then inserted between the fusion pairs (5–8, Supplementary data, Table 2) by the PstI site situated between the donor-acceptor constituents of the fusion pair. The fusion protein contains a 6× His-tag at its N-terminus. All constructs were confirmed by sequencing.

2.3. Expression and purification of RET-based proteins

Proteins were expressed in *E. coli* strain BL21 DE3 (Novagen). An overnight culture was grown from a single colony in LB containing 100 μg/mL ampicillin and 2% glucose at 37 °C, 200 rpm. Expression was induced by inoculating 500 mL LB containing 100 μg/mL ampicillin to an A_{600} of 0.1 and induced at 37 °C (200 rpm) for 3.5 h followed by overnight incubation at 22 °C (200 rpm). Cells were harvested 24 h after inoculation.

For protein purification, cells were harvested by centrifugation at 4335 × *g* (4 °C) for 15 min and resuspended in equilibration buffer (50 mM sodium phosphate buffer, 300 mM NaCl, pH 7.0). The cells were lysed by French press (−18,000 psi) and the soluble protein fractions were isolated by centrifugation at 9300 × *g* (4 °C) for 15 min. Proteins were purified using cobalt affinity chromatography according to the supplied instructions (BD Talon (BD Biosciences, Clontech)). Following elution of the purified protein with 150 mM imidazole, the buffer was exchanged for cleavage buffer (50 mM Tris (pH 8.0), 100 mM NaCl, and 1 mM EDTA) by dialysis. Aliquots of 200 μL protein were snap frozen on dry ice and stored at −80 °C. Protein concentrations were determined by absorbance at 280 nm and calculated according to the method of Gill and Von Hippel (1989). The calculated protein concentration was confirmed using the Bradford Assay (BioRad) using BSA as the standard.

2.4. Instrumentation

2.4.1. Spectral measurements

All spectral scans were recorded with a plate-reading SpectraMax M2 spectrofluorometer (Molecular Devices). The reactions were carried out in 96-well plates (PerkinElmer). Fluorescence spectral scans were recorded from 450 to 600 nm with an excitation wavelength of 430 nm using a 455-nm emission cut-off filter. Spectral scans of $BRET^2$ constructs were recorded using the luminescence scan mode scanning between 380 and 600 nm.

2.4.2. Simultaneous dual emission detection

Simultaneous dual emission RET measurements were carried out with a POLARstar OPTIMA microplate reader (BMG LabTech). Simultaneous emission measurements used either the $BRET^2$ emission filter set comprising of RLuc/Clz400a emission filter (410 nm band-pass 80 nm) and the GFP^2 emission filter (515 nm band-pass 30 nm) or the FRET filter set consisting of a CFP excitation filter (450 nm band-pass 10 nm) and the respective CFP (500 nm band-pass 10 nm) and YFP (530 nm band-pass 10 nm) emission filters.

2.5. Thrombin assays

Purified fusion protein (1 μM) was incubated with varying amounts of thrombin protease (Amersham Biosciences) in cleavage buffer (final volume of 100 μL) at 30 °C for up to 90 min.

RET analysis was carried out in 96-well plates with incubation of specified fusion protein aliquots with thrombin for 90 min at 30 °C followed by recording the RET signal. For $BRET^2$ measurements Clz400a substrate (5 μM) was added following the 90-min period and a 0.50-s integration time used. Real-time analysis was also performed using the POLARstar OPTIMA plate reader and a whole plate assay was employed for FRET analysis or a well-by-well assay for $BRET^2$ analysis (20 s integration time).

2.5.1. Kinetic analysis

K_m (Michaelis constant) and V_{max} (maximum velocity) were obtained by non-linear regression analysis to the Michaelis–Menten equation using GraphPad Prism for the Mac version 5.0a.

2.6. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) analysis

Proteins (2.5 μg) were diluted in 1× sample loading buffer (Invitrogen) for SDS–gel electrophoresis (NuPAGE system: 12% Bis–Tris gel with MOPS running buffer (Invitrogen)). Bands were visualised following staining with Fast stain ™ (Fisher).

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