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In vivo dynamics of enterovirus protease revealed by fluorescence resonance emission transfer (FRET) based on a novel FRET pair

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Abstract

An *in vivo* protease assay suitable for analysis by fluorescence resonance energy transfer (FRET) was developed on the basis of a novel FRET pair. The specifically designed fusion substrate consists of green fluorescent protein 2 (GFP^2)-peptide-red fluorescent protein 2 (DsRed2), with a cleavage motif for the enterovirus 2A protease ($2A^{pro}$) embedded within the peptide region. FRET can be readily visualized in real-time from cells expressing the fusion substrate until a proteolytic cleavage by $2A^{pro}$ from the input virus. The level of FRET decay is a function of the amount and infection duration of the inoculated virus as measured by a fluorometer assay. The FRET biosensor also responded well to other related enteroviruses but not to a phylogenetically distant virus. Western blot analysis confirmed the physical cleavage of the fusion substrate upon the infections. The study provides proof of principle for applying the FRET technology to diagnostics, screening procedures, and cell biological research.

Keywords: FRET; Enterovirus; Protease; GFP; DsRed

Fluorescence resonance energy transfer (FRET) technology has been one of the most promising spectroscopic tools for studying molecular interactions in living cells. By this methodology, cellular processes can be monitored in real-time by spectral changes of the emission signal because of orientation- and distance-dependent energy transfer from an excited fluorophore donor to a long-wavelength fluorophore acceptor [1]. FRET has thus provided an important complement to the biochemical studies such as protease activities, kinase activities, and protein–protein interactions [2–4], extending the analyses to their normal cellular context.

Green fluorescent protein (GFP) from jellyfish *Aequorea victoria* has revolutionized many areas of biomedical research because of its ability to efficiently emit internal fluorophore. GFP derivatives that exhibited a variety of fluorescence ranging from blue to yellowish green emissions have been developed to create pairs of donors and acceptors for FRET [5,6]. Among them, the cyan–yellow fluorescent protein (CFP–YFP) pair has been most commonly used; however, this pair is suboptimal in that the high degree of overlap between emission spectra for CFP and YFP entails substantial bleed-through into the YFP channel, thus limiting its full promise in employment [7,8]. Nevertheless, sea coral fluorophores with red-shifted emission peaks have been identified and developed lately [9–11], potentially serving as a group of better suited FRET partners for the GFP variants.

Enteroviruses belong to the RNA virus family *Picornaviridae* and include polioviruses, type A coxsackieviruses, type B coxsackieviruses, echoviruses, and the numbered enteroviruses [12]. Human enteroviruses are important pathogens in the pediatric population, causing a wide range of clinical manifestations; most of the infections are mild or asymptomatic while they can result in serious neurological

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diseases [12]. Among them enterovirus 71 (EV71) has emerged as the most common non-polio enterovirus associated with poliomyelitis-like paralysis [13]. The enterovirus genome comprises the untranslated regions (UTR) at the 5′- and 3′-ends, and a long open reading frame encoding a polyprotein [14]. Maturation cleavage of the polyprotein to generate functional viral proteins is mainly mediated by virus-encoded proteases, designated 2A ($2A^{pro}$) and 3C ($3C^{pro}$). The polyprotein precursor first undergoes an autocatalytic cleavage by $2A^{pro}$ between its own N terminus and the C terminus of the preceding capsid protein, VP1, and most of the remaining cleavages are carried out by $3C^{pro}$ [14]. Additionally, several host cell proteins were susceptible to cleavage by $2A^{pro}$ [15–17], events that would play important roles in enteroviral pathogenesis. Enterovial $2A^{pro}$ has been classified as a subclass of chymotrypsin-like cysteine protease [18], exhibiting the cleavage activity on its substrate in a primary sequence dependent manner as mainly determined by *in vitro*, end-point assays [19–21]. However, enterovial $2A^{pro}$ activity in the context of virus-infected cell may be modulated by a number of virus and host factors that may not be mirrored by the conventional assays. Therefore, we sought to develop a novel methodology to investigate enteroviral $2A^{pro}$ activity in its physiologically relevant setting.

In this study, we explored GFP^2 and DsRed2 as a novel FRET pair, with a peptide linker containing the cleavage motif of $2A^{pro}$ connected in between, as a fluorophore substrate. Upon protease cleavage in the context of virus infection, the separation of the tandem fluorophore substrate *in vivo* was monitored as a FRET disruption in real-time by fluorescent microscopy and in a quantitative fashion by fluorometry. EV71 was utilized as a model virus for the FRET biosensor and the utility could be extended to other enteroviruses. This FRET biosensor provides a valuable tool for the study of protease dynamics *in vivo* with potential usages in a high-throughput screening assay.

Materials and methods

Plasmids. Construction of GFP^2–DsRed2 expression plasmid, pGR, was described in Supplementary Materials and methods. To clone the sequences encoding the wild-type or mutant $2A^{pro}$ cleavage motif (amino acid residues 856–867 spanning the VP1-2A region in EV71 BrCr strain), two sets of complementary oligonucleotides, VP1-2AwtF/VP1-2AwtR and VP1-2AmutF/VP1-2AmutR, were synthesized (Supplementary Table 1). Each set of oligonucleotides was annealed and cloned into the pGR plasmid in-frame with the GFP^2 and DsRed2 coding regions at the *Hin*dIII/*Bgl*II sites (Fig. 1B), resulting in plasmids designated pG2AwtR and pG2AmutR that harbor a linker region containing the wild-type and mutant cleavage motif, respectively.

Imaging analysis. Fluorescence image was observed on an inverted fluorescence microscope (Nikon TE200). Filter cubes for GFP^2/DsRed2 (excitation, dichroic, emission): GFP^2 (D390/22x, 425DCLP, D515/30M); DsRed2 (HQ540/40x, Q570LP, HQ600/50m); FRET (D390/22x, 425DCLP, HQ600/50m) (Chroma, Brattleboro, VT). Settings for gain and offset of the detectors were identical for all experiments to keep the relative contribution of the fluorophores to the detection channels constant. Images were acquired with an interlined charge-coupled device camera (DXM 1200F, Nikon) controlled by the ACT-1 software (Version 2.62, Nikon). Images were viewed and processed with Adobe Photoshop 6.0 (Adobe Systems).

Fluorometer assay. Cells were harvested, washed twice with phosphate-buffered saline (PBS), and resuspended with PBS to a final concentration of 1×10^6 cells/ml. One hundred microliters of the cell suspensions was aliquoted into a well of a black 96-well, flat-bottomed microplate (NUNC, Denmark) and the fluorescence was read on a fluorometer apparatus (Fluoroskan Ascent type 374; Labsystems, Rochester, NY). The excitation wavelength was 390 ± 20 nm, and the emission wavelengths for the fluorophore donor (GFP^2) and acceptor (DsRed2) were 510 ± 10 and 590 ± 14 nm, respectively.

Results

Assessment of GFP^2–DsRed2 FRET pair and generation of FRET constructs

An idea FRET couple should possess an adequate spectral overlap between donor emission and acceptor absorption but separated emission spectra to allow their selective imaging. Visual inspection of spectra (Supplementary Fig. 1) and empirical assessment (Supplementary Fig. 2, and Supplementary Materials and methods) for GFP^2/DsRed2 FRET raised the potential advantages of convenient excitation and spectral separation. In this regard, we developed an *in vivo* $2A^{pro}$ assay based on the FRET pair. The rationale is that the close proximity of the tandem fluorescent proteins in the construct gives rise to FRET that is susceptible to a disruption upon $2A^{pro}$ cleavage (Fig. 1A). To this end, we generated a recombinant construct, pG2AwtR, which encodes GFP^2–DsRed2 fusion protein tethered by amino acid sequences (23 amino acids in length) embedded with the cleavage motif (12 amino acids in length) for EV71 $2A^{pro}$ (Fig. 1B). In addition, the absolute requirement for Gly at P1′ position of the cleavage motif (first position on the carboxyl-terminal side of the scissile bond) was previously documented [21]; thus a recombinant plasmid, pG2AmutR, that bears the mutant cleavage motif (a $G \rightarrow R$ substitution in the P1′ position) was also generated as a control (Fig. 1B).

Imaging of $2A^{pro}$ kinetics in vivo

To permit continuous monitoring of FRET and minimize the variables associated with transient transfection, we developed stably transfected cell lines designated HeLa-G2AwtR and HeLa-G2AmutR lines each transfected with pG2AwtR and pG2AmutR plamid, respectively (Supplementary Materials and methods). To monitor the dynamics of $2A^{pro}$ activity in the context of virus infection in intact cells, both stable lines were subject to infection by EV71 at the amount of 3 plaque-forming-unit (PFU) per cell for 10 h. Upon excitation at 390 nm and emission at 515 nm (390ex/515em) for GFP^2 from infected HeLa-G2AwtR cells, a progressive increase in the fluorescent intensities was observed (Fig. 2A, panels of top rows) while a gradual decline of FRET (390ex/600em) was detected (Fig. 2A, panels of the third row from top), as the infection