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Visualization of β-secretase cleavage in living cells using a genetically encoded surface-dnisplayed FRET probe

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Abstract

The human β-secretase, BACE, plays a key role in the generation of pathogenic amyloid β-peptide (Aβ) in Alzheimer's disease and has been identified as an ideal target for therapy. Previous studies reported the monitoring of BACE activity *in vitro* utilizing chemical synthesized sensors. Here we describe the first genetically encoded FRET probe that can detect BACE activity *in vivo*. The FRET probe was constructed with the BACE substrate site (BSS) and two mutated green fluorescent proteins. In living cell, the FRET probe was directed to the secretory pathway and anchored on the cell surface to measure BACE enzymatic activity. The results show that the FRET probe can be cleaved by BACE effectively *in vivo*, suggesting that the probe can be used for real-time monitoring of BACE activity. This assay provides a novel platform for BACE inhibitor screening *in vivo*.

Keywords: Alzheimer's disease (AD); β-Secretase (β-site APP cleaving enzyme [BACE]); Fluorescence resonance energy transfer (FRET)

Alzheimer's disease (AD) is characterized pathologically by senile plaques containing the amyloid β-peptide (Aβ), a product derived from the sequential cleavage of amyloid precursor protein (APP) by β-secretase and γ-secretase [1]. APP is a type I integral membrane protein that matures through the secretory pathway. Upon reaching the cell surface, APP can be reinternalized to endosomes [2]. During its transport to the cell surface and reinternalization to endosomes, APP undergoes endoproteolytic cleavage to produce Aβ. β-Secretase (β-site APP cleaving enzyme [BACE]) mediates the N-terminal cleavage, cleaving APP to produce a soluble N-terminal fragment and a 99-residue C-terminal fragment (C99) that remains membrane-bound [3]. Then C99 is cleaved by γ-secretase to yield the 4-kDa Aβ [4].

Since BACE is the first proteolytic enzyme that hydrolyses the APP, it plays an important role in the generation of Aβ and is believed to be a promising therapeutic target for the treatment of AD [5]. In order to screen BACE inhibitors, BACE activity has been monitored *in vitro* with fluorescence-quenched peptide substrates, such as Mca-EVNLDAEFK-Dnp, which based on fluorescence resonance energy transfer (FRET) technology [6]. Recently, Parker's group reported another homogeneous time-resolved fluorescence (HTRF) assay using europium and allophycocyanin as a FRET fluorescence pair for measuring BACE enzymatic activity [7]. However, all of these probes can only detect the activity of BACE *in vitro*.

Here, we present a genetically encoded FRET probe that can detect BACE activity in living cells for the first time. The FRET probe consists of two mutated green fluorescent proteins (GFPs) and an 18-amino-acid peptide containing the Swedish mutant APP sequence [8], which was sandwiched by the two GFPs. The FRET pair used in the probe is mCerulean [9] and mCitrine [10], a monomeric

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cyan and a yellow mutant of GFP, respectively. Because both of BACE and its substrate APP are secretory proteins [1], the FRET probe was constructed into a mammalian expression vector pDisplay vector (Invitrogen) carrying a leader sequence and a transmembrane domain. After expressed, the FRET probe was directed into the secretory pathway and located on the cell surface. We show that the FRET probe can be cleaved by BACE effectively *in vivo*. This work facilitates screening protease inhibitors *in vivo*.

Materials and methods

Plasmid construction. The FRET probe was generated by cloning mCitrine, a linker peptide sequence and mCerulean in-frame into the BglII and SalI sites of the vector pDisplay. In detail, the mCitrine was amplified by polymerase chain reaction (PCR) using the primers 5′-ACGC GTC GAC CTT GTA CAG CTC GTC CAT GCC GAG AGT GAT-3′ and 5′-GAA GAT CTA TGG TGA GCA AGG GCG AGG AG-3′. The PCR product was digested by BglII and SacII (underline) and cloned into the pDisplay vector to create pDis-mCitrine. The mCerulean was also amplified by PCR, the forward primer was 5′-CGT CCG CGG TGG TGG CTCG GCT AGC ATG GTG AGC AAG GGC GAG GAG-3′, containing a SacII and NheI sites and a control linker sequence between the two restriction enzyme sites. The reverse primer was 5′-AGA ACC GGT GTC GAC CTT GTA CAG CTC GTC CAT GCC-3′ containing a SalI site. The PCR product was cloned into the SacII and SalI sites of pDis-mCitrine to create a control FRET plasmid, pDis–YcC, or cloned into pDisplay vector to create pDis-mCerulean. The FRET probe plasmid pDis–YβC was constructed by replacing the linker of pDis–YcC with Swedish mutated BACE substrate site (BSS), KTEEISEVNLDAEF (Fig. 1A). The template cDNAs used for mCitrine and mCerulean were pmCitrine-N1 (a generous gift from Dr. Swanson, University of Michigan Medical School, USA) and pmCerulean-C1 (a generous gift from Dr. Piston, Vanderbit University Medical Center, Tennessee, USA). Both of the proteins expressed from pDis–YβC and pDis–YcC are fused at the N-terminus to a leader sequence and at the C-terminus to a transmembrane domain, as shown in Fig. 1A. They were named dYβC (displayed YβC) and dYcC (displayed YcC), respectively.

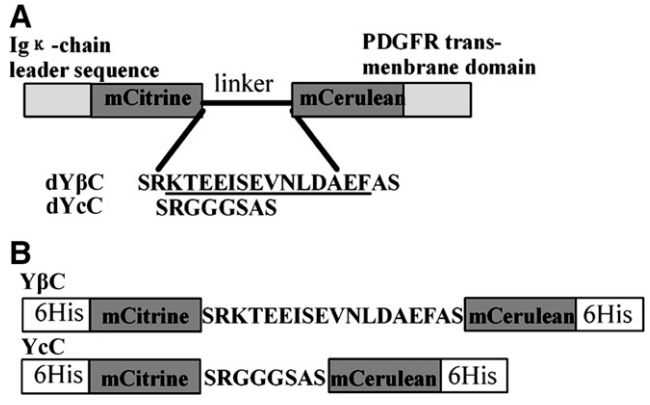

Fig. 1. Construction of FRET probes. (A) The cDNAs of mCitrine–linker–mCerulean were cloned into the pDisplay vector to create pDis–YβC and pDis–YcC. Both of the fusion proteins (dYβC and dYcC) expressed from pDis–YβC and pDis–YcC are fused at the N-terminus to the murine Ig κ-chain leader sequence, which directs the protein to the secretory pathway, and at the C-terminus to the platelet derived growth factor receptor (PDGFR) transmembrane domain, which anchors the protein to the plasma membrane. Between the mCitrine and mCerulean in dYβC, there is a linker including BACE substrate site (BSS) (underline), and that of dYcC is a control linker that cannot be cleaved by BACE. (B) The cDNAs of mCitrine–linker–mCerulean were cloned into the pET28a(+) expression vector, which expresses 6× His tagged fusion proteins YβC and YcC.

To express the fusion proteins in *Escherichia coli*, the fusion genes were digested from pDisplay vectors described above by BglII/SalI, and cloned into the BamHI and SalI sites of the T7 expression vector pET28a(+) (Novagen) to create pET-YβC, pET-YcC, pET-mCerulean and pET-mCitrine. The fusion proteins expressed from pET28a(+) contain two 6× His-tag at their terminus (Fig. 1B).

To visualize BACE expression in living cells, a mCherry fused BACE recombinant protein (BACE-mCherry) was constructed. The mCherry is one of mutants of red fluorescent protein (RFP) from *Discosoma SP* [11], and pRSET-B-mCherry vector is a generous gift from Dr. Tsien (University of California, San Diego, USA). The mCherry was amplified from pRSET-B-mCherry by PCR using the primers 5′-AAT CTC GAG GGT GGC TCT GGA GGT TCT ATG GTG AGC AAG GGC GAG GAG-3′ and 5′-AGA ACC GGT GTC GAC CTT GTA CAG CTC GTC CAT GCC-3′. The PCR product was digested by XhoI and AgeI restriction enzymes and subcloned into the pcDNA4-BACE vector (a generous gift from Dr. Haass, Ludwig Maximilians University, Germany) to create pBACE-mCherry. All constructs were verified by DNA sequencing.

Expression and purification of recombinant proteins. The plasmids pET-YβC, pET-YcC, pET-mCerulean and pET-mCitrine were transformed into BL21 (DE3) *E. coli* (Novagen) to produce the recombinant 6× His-tagged YβC, YcC, mCerulean and mCitrine. The transformed *E. coli* cells were cultured to $A_{600} = 0.6–0.8$ and induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) at 22 °C. The bacteria were harvested after 16 h of induction. For protein purification, the harvested bacteria were disrupted by sonication in 40 ml lysis buffer (20 mM Tris·Cl, pH 7.9, 0.5 M NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF)) and pelleted by centrifugation at 12,000*g* for 40 min. The supernatant, containing 6× His-tagged recombinant proteins, was loaded on Ni–NTA resin (Qiagen) column and the recombinant proteins were purified by elution with 250 mM imidazole solution (20 mM Tris·Cl, pH 7.9, 0.5 M NaCl, 250 mM imidazole) after eluting non-specific proteins with a lower concentration (50 mM) imidazole solution.

SDS–PAGE analysis. The *in vitro* proteolytic reaction was performed by incubating the purified fusion protein (2 μM) with 0.5 U/ml BACE (Sigma) at 37 °C. The reaction buffer was 20 mM sodium acetate, pH 5.5. At various times, 20 μl aliquots of the reaction mixture were removed and kept frozen at −80 °C before SDS–gel electrophoresis. The samples were boiled for 3 min and electrophoresed at room temperature on a 10% SDS–polyacrylamide gel.

Fluorescence spectroscopic analysis. The purified YβC protein (2 μM) was incubated with 0.5 U/ml BACE in a 1-cm cuvette of a spectrofluorometer (LS-50B, Perkin-Elmer, Norwalk, Connecticut) at 37 °C. Fluorescence emission spectra from 460 to 600 nm were recorded with an excitation wavelength of 433 nm (the excitation peak of mCerulean).

Cell culture and transfection. Human cervical cancer cells, HeLa cells, were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin sulfate at 37 °C in 5% CO_2. Transfection was performed using Lipofectamin 2000 (Invitrogen) according to the manufacturer's protocol. Cells (5×10^5 cells/well) were transfected with 100 ng of indicated plasmid DNAs for each well. For co-transfection, cells were transfected with 100 ng pBACE-mCherry and 100 ng FRET probe plasmid.

Western blotting. HeLa cells transfected with indicated plasmids were harvested 24 h post-transfection, and lysed in 1× SDS loading buffer (62.5 mM Tris·Cl, pH 6.8, 10% (v/v) glycerin, 2% (w/v) SDS, 1 mM PMSF, 1 μg/ml pepstatin A, 5 μg/ml bromophenol blue). The proteins in the extracts were separated using SDS–PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham) using a semi-dry blotting system. The membrane was immunoblotted with rabbit anti-GFP antibody (1:2000) (Proteintech Group Inc). The immunoblot was then probed with goat anti-rabbit IgG-horseradish peroxidase-conjugate (1:5000) (Bio-Rad).

Confocal imaging and FRET measurement. All images were acquired on an Olympus FV1000 confocal imaging system (Olympus Corp., Japan)

with a 40 mW argon laser and a 60 × 1.35 NA oil immersion objective at 3× zoom. The laser was tuned to lines at 458 and 515 nm. Images of mCerulean were obtained through photomultiplier 1 (PMT1, bandpass filter 470–500 nm) with 458 nm excitation, which is termed as the 'CFP channel' here. The 'YFP channel' refers to the combination of 515 nm laser-line excitation and PMT2 (bandpass filter 530–580 nm) detection, and it was used to get images of mCitrine. The BACE-mCherry was imaged by 'RFP channel' with multi-photon excitation at 800 nm and PMT2 (bandpass filter 600–640 nm) detection. All transfected cells were imaged live at 37 °C in phenol red-free DMEM (Sigma) 20–24 h after transfection.

Cell spectrum scanning was performed on the FV1000 confocal imaging system under lambda scan mode with 458 nm excitation (10% laser intensity, 1 nm bandwidth) and a series of images of emission fluorescence were collected from 460 to 600 nm (1 nm step, 5 nm bandwidth). The emission spectra data were processed using FV10-ASW1.4 software (Olympus Corp., Japan).

FRET was measured using acceptor photobleaching method [12]. The principle of the acceptor photobleaching FRET (apFRET) is that the energy transfer is detected as an increase in donor (mCerulean) fluorescence after complete photobleaching of the acceptor molecules (mCitrine). Acceptor photobleaching protocol was performed according to a previous study [13], with the minor modifications. Before performing photobleaching, the prebleach mCerulean and mCitrine images of transfected cells were sequentially collected through CFP channel (3% intensity) and YFP channel (0.2% intensity) as baseline, respectively. The cells were then bleached by scanning a selected region of interest (ROI) 20 times for 5–10 s using the 515 nm argon laser line at 20% intensity. Post-bleach mCerulean and mCitrine images were collected immediately following photobleaching using the same parameters as those used for acquiring prebleach images. FRET efficiency (E_F) was calculated as a percentage increase in mCerulean fluorescence intensity following the mCitrine photobleaching using the formula $E_F = (I' - I) \times 100/I'$, where I and I' were the mCerulean fluorescence intensities before and after acceptor photobleaching, respectively.

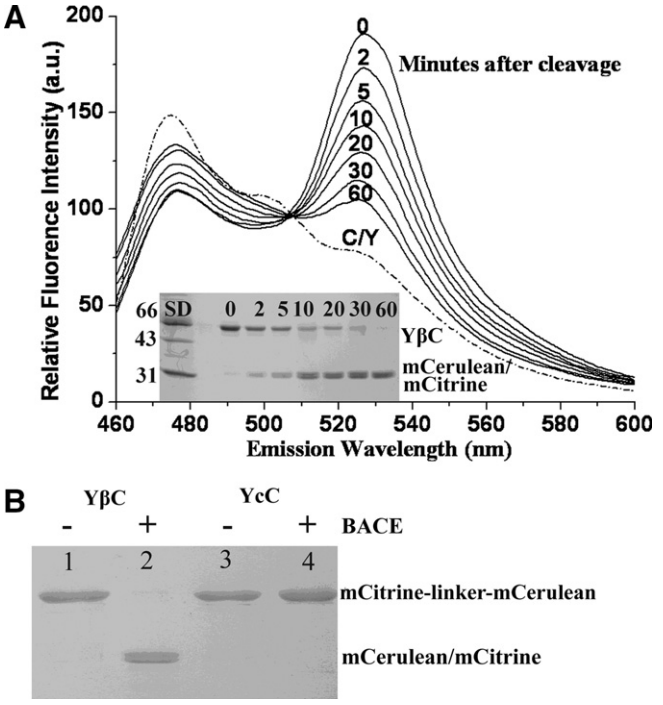

Fig. 2. Monitoring the cleavage of YβC by BACE *in vitro* using spectrofluorimeter and SDS–PAGE. (A) YβC protein (2 μM) was incubated with 0.5 U/ml BACE at 37 °C in 20 mM NaAc, pH 5.5. Fluorescence emission was measured from 460 to 600 nm upon excitation at 433 nm. C/Y: mixture of equal concentration (2 μM) of purified mCerulean and mCitrine. In another reaction under the same conditions, equal amounts of reaction mixture was removed at times indicated in the plot and assayed using SDS–PAGE. (B) SDS–PAGE analysis of the recombinant His tagged YβC and YcC proteins incubated with (lanes 2 and 4) or without (lanes 1 and 3) β-secretase for 2 h.

Results

Characterization of the FRET probe in vitro

To evaluate fluorescence energy transfer between mCerulean and mCitrine, the purified YβC protein was analyzed by spectrofluorimetry. As shown in Fig. 2A, when YβC protein was excited at 433 nm, an enhanced fluorescence emission was recorded at the wavelength of 528 nm (the emission peak of mCitrine), demonstrating an energy transfer from mCerulean to mCitrine in integral YβC protein. In the negative control experiment, the fluorescence signal was observed mainly at the wavelength of 477 nm (the emission peak of mCerulean) when equal amounts of mCerulean and mCitrine proteins were mixed, indicating no recordable FRET signal generated by their physical mixing.

When the YβC protein was incubated with BACE, a significant decrease of fluorescence emission at 528 nm was detected after 2 min, meanwhile, fluorescence emission at 477 nm increased. The result indicated that the transfer of energy from excited mCerulean to mCitrine gradually decreased and YβC protein was gradually cleaved by BACE as time elapsed. When the YcC protein was used as the positive control, a strong FRET signal was recorded with no decrease upon incubation with BACE (data not shown).

The spectral result was confirmed by SDS–PAGE assay, as shown in the subplot of Fig. 2A. Two approximate 30 kDa bands (mixture of mCerulean and mCitrine) were accumulated and became dominant after proteolytic reaction. The YβC protein was completely cleaved by BACE after incubation with BACE for 2 h (Fig. 2B, lane 2), but the control probe YcC could not be cleaved by BACE, as shown in lane 4 of Fig. 2B. These results indicate that the FRET probe YβC can be cleaved by BACE *in vitro*.

Characterization of the FRET probe in living cells

To detect BACE cleavage processing *in vivo*, mCerulean–linker–mCitrine was constructed into pDisplay in which the probe was directed to the secretory pathway and anchored to the surface of the cells. As shown in Fig. 3, expression of dYβC and dYcC was observed on the plasma membrane of HeLa cells with little intracellular expression, and both of the mCerulean and mCitrine fluorescence were displayed on the plasma membrane (Fig. 3A and C).

In dYβC and BACE-mCherry co-expressing HeLa cells, however, only mCerulean, in which C-terminus fused to

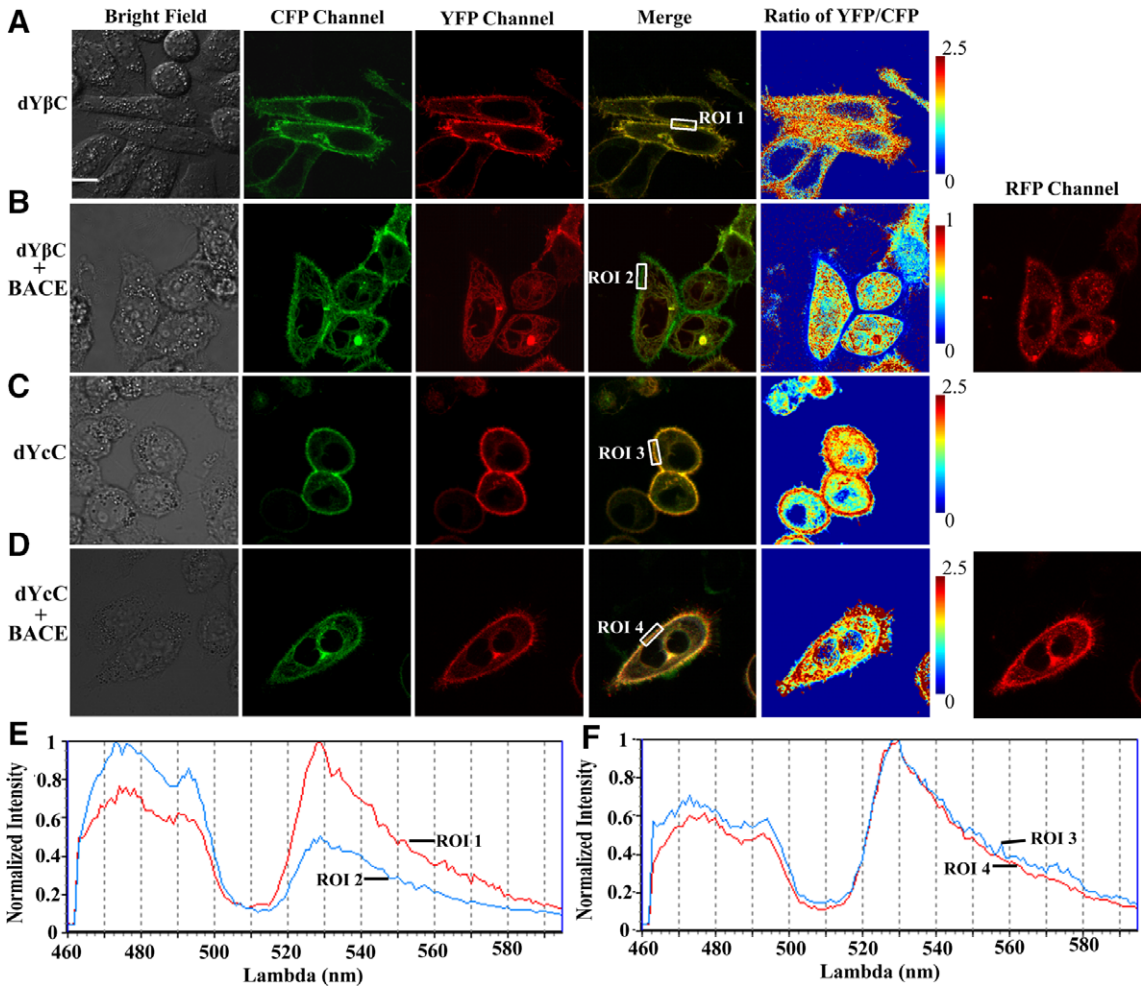

Fig. 3. Confocal imaging of surface-displayed fluorescence probes and spectral FRET imaging of the probes. (A) dYβC expressing HeLa cells. Note that mCerulean and mCitrine fluorescence signals are highest at the plasma membrane. (B) HeLa cells co-expressing dYβC and BACE-mCherry. Only the mCerulean signal was prominent on the plasma membrane (CFP Channel). (C) dYcC expressing HeLa cells. (D) HeLa cells co-expressing dYcC and BACE-mCherry. (E) The emission spectra of ROI 1 in (A) and ROI 2 in (B) with excitation at 458 nm. (F) The emission spectra of ROI 3 in (C) and ROI 4 in (D). The abnormal decrease in intensity at a wavelength about 510 nm is due to there being a 458/515 dichromatic mirror in the light pathway. The white scale bar represents 10 μm.

the PDGFR transmembrane domain (Fig. 1A), was anchored on the plasma membrane and the membrane expression of mCitrine could not be observed (Fig. 3B). The ratio of YFP/CFP on the plasma membrane in the co-expressing HeLa cells (0.16 ± 0.02, mean ± SEM, $n = 28$) was 14 times less than that in the cells transfected with pDis–YβC alone (2.25 ± 0.18, mean ± SEM, $n = 19$). In contrast, the mCerulean and mCitrine fluorescence of dYcC protein both remained on the plasma membrane whether co-transfected with or without pBACE-mCherry, and the ratios of YFP/CFP were not significantly different (2.37 ± 0.16, mean ± SEM, $n = 20$ and 2.52 ± 0.05, mean ± SEM, $n = 25$, respectively) (Fig. 3C and D).

Fig. 3E showed the emission spectra of dYβC in HeLa cells transfected with pDis–YβC or co-transfected with pDis–YβC and pBACE-mCherry excited at 458 nm. In dYβC expressing HeLa cells, the major emission peak is 528 nm, indicating that FRET occurs between mCerulean and mCitrine in dYβC protein *in vivo*. While co-expressing HeLa cells peaks at 477 nm, indicating the FRET was lost. In contrast, the emission spectrum of the dYcC expressing HeLa cells was consistent with that of co-expressing HeLa cells (Fig. 3F). Both of the cell spectra have a peak at 528 nm, indicating the energy transfer from mCerulean to mCitrine occurs in dYcC protein no matter whether co-transfected with or without pBACE-mCherry. These results suggest that dYβC can be cleaved by BACE in living cells.

Detection of BACE activity by apFRET and Western blot

Acceptor photobleaching was used to quantify the FRET efficiency of the probes in the HeLa cells. The ROI was selectively irradiated using the 515 nm laser line and an increase in mCerulelan fluorescence was observed following mCitrine photobleaching (Fig. 4A). If the dYβC probe is cleaved by BACE, FRET between mCerulean and mCitrine will decrease and then photobleaching of

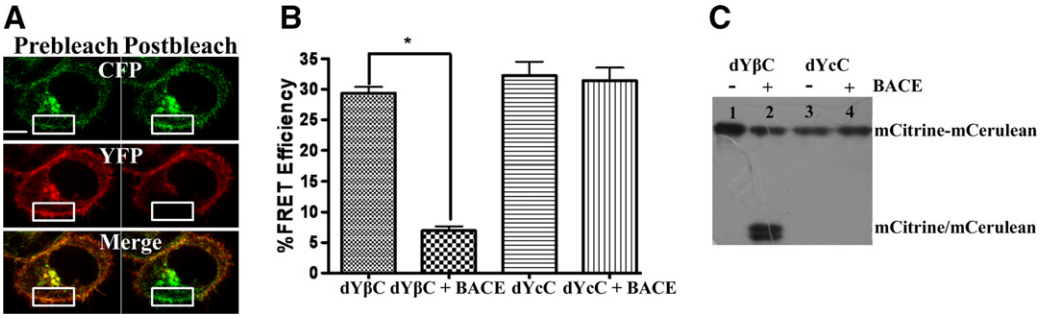

Fig. 4. Detection of BACE activity by apFRET. (A) The HeLa cell was transfected with pDis–YβC. A plasma membrane region (marked by the white rectangle) was photobleached at 515 nm for 10 s. The fluorescence of CFP/YFP channel before and after bleaching was imaged. FRET was shown as an increase in mCerulean fluorescence following mCitrine photobleaching. The scale bar represents 5 μm. (B) The efficiency of FRET (E_F) was calculated by the formula $E_F = (I' - I) \times 100/I'$ ($n > 16$). A significant difference ($^*p < 0.001$) in FRET efficiency was observed between the groups of dYβC and dYβC + BACE. There is no significant difference between dYcC and dYcC + BACE groups. dYβC, the cell group transfected with pDis–YβC; dYβC + BACE, co-transfected with pDis–YβC and pBACE-mCherry; dYcC, transfected with pDis–YcC; dYcC + BACE, co-transfected with pDis–YcC and pBACE-mCherry. (C) The four groups of cells in (B) were analyzed by Western blotting. All experiments were performed 20–24 h post-transfection.

mCitrine will result in lower E_F than that of non-cleaved dYβC. As shown in Fig. 4B, the E_F of co-expressing HeLa cells (6.93 ± 0.71, mean ± SEM, $n = 22$) was significantly lower than that of the cells only transfected with pDis–YβC (29.33 ± 1.05, mean ± SEM, $n = 42$) ($p < 0.001$). As expected, the E_F of dYcC expressing cells (32.48 ± 2.04, mean ± SEM, $n = 16$) and that of cells co-expressing dYcC and BACE-mCherry (31.18 ± 1.76, mean ± SEM, $n = 16$) were not statistically different ($p = 0.6335$), suggesting that the control probe dYcC could not be cleaved by BACE *in vivo*. All FRET measurements were performed at 20–24 h after transfection, and the irradiated ROIs were exclusively selected at the plasma membrane, as shown in Fig. 4A.

The confocal imaging results were confirmed by Western blot (Fig. 4C), showing that the dYβC was digested by BACE to produce smaller mCerulean and mCitrine molecules (lane 2), but the control dYcC remained intact in the presence of BACE (lane 4).

Discussion

In summary, we constructed a genetically encoded FRET probe that detected BACE cleavage successfully in living cells. Comparing to chemical synthesized sensors, genetically encoded FRET sensors are able to conduct real-time analysis *in vivo* and target subcellular compartments for specific detection. Most FRET sensors described in previous studies were located within the cells, and the quantity of FRET acceptor molecules would not change when FRET sensors were cleaved because intact cell organelles retained all of the molecules. In our study, the FRET probe (dYβC) was directed into the secretory pathway and delivered to the cell surface. Upon reaching the cell surface, dYβC anchored on extracellular surface of the plasma membrane because the C-terminus of the donor molecule was fused to a transmembrane domain (Fig. 1A). When the FRET sensor was cleaved, the acceptor was cleaved away from dYβC and could not anchor on the cell surface (Fig. 3B) anymore. This will facilitate intensity-based FRET detection that suffers from contamination of the FRET images caused by bleed-through of CFP fluorescence into the YFP channel as well as excitation of YFP during CFP excitation [12]. In addition, the probe makes it possible to detect FRET change by monitoring the acceptor fluorescent signal directly.

The subcellular compartments in which BACE cleavage occurs are not fully determined in living cells yet. Previous studies mainly focused on the generation of Aβ in the endosomal system [14,15], the trans-Golgi network (TGN) [16] and the endoplasmic reticulum/intermediate compartment [17]. Recently, Selkoe's group proposed that robust Aβ generation could occur directly on the plasma membrane and suggested that β-cleavage at cell surface may be a therapeutic target because drugs targeting Aβ generation need not to penetrate cell's membrane [18]. Our results provide direct *in vivo* evidences that the BACE cleavage can occur on the cell surface. The FRET probe described here can be used to detect the BACE activity in living cells and further screen BACE inhibitors targeting β-cleavage at the cell surface.

Acknowledgments

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