FRET-Based Assays for the Detection of Amyloid Degrading Protease Activity

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Introduction
Amyloid degrading proteases (ADPs) are major players in β-amyloid (Aβ) clearance. The accumulation of Aβ plaques, a hallmark of Alzheimer’s disease (AD) patient brain, has been hypothesized to be due to the intolerance between ADPs activity and Aβ production, resulting in AD pathogenesis. In order to confirm the effects of ADPs on neuronal degeneration diseases, reliable assays for ADPs activity detection have to be developed.

We have designed a panel of assays to detect ADP targets using FRET (Fluorescence resonance energy transfer)-based peptide substrates. To develop assays for ADAM10, TACE, ADAM17, α-secretase, Neprilysin and IDE (β-secretase degrading enzyme), we synthesized FRET substrate peptides labeled with the fluorophore, 4-carboxyfluorescein (FAM) and the quencher, QXL(520). The fluorescence of FAM is quenched by QXL(520) and restored upon cleavage of the peptide by active ADP. Fluorescence is then monitored at the excitation/emission wavelength of 495/520 nm.

Many of ADPs target these low pH for optimum activity, and FAM can show decrease of signal in acidic environment. To optimize FRET substrate for maximum sensitivity at low pH, we introduced a new pH-independent Biacorex, HILu™ Fluor 488 in place of FAM, besides unquenched signal at low pH, HILu™ Fluor 488 has excellent brightness at the same wavelength as FAM. The absorption spectrum of QXL 520 overlaps with the emission spectrum of HILu™ Fluor 488, providing optimal co-wavelength. Substrates with HILu™ Fluor 488/QXL 520 were used for detection of β-secretase, cathespin B and D, providing higher sensitivity and better assay window.

Some of ADPs, such as MMP-2 and MMP-9, belong to matrix metalloproteases that are known for overlapping substrate specificities. To avoid non-specific substrate cleavage by multiple proteases, we utilized an Immunocapture technique in addition to the FRET assay. Monoclonal antibody was applied to pull down relevant MMP from biological sample and subsequent addition of MMP-9 peptide substrate containing the S-FAMLQK 520 FRET pair. These assays provided significant increase specificity of MMP-2 and MMP-9.

Materials and Methods

- Sensolyte® 520 ADAM10, TACE, ADAM17, Neprilysin and IDE Assay Kits
- FRET S-FAMLQK 520 substrate, ExEm 495/520 nm upon cleavage
- Sensolyte® 520 BALB, Cathespin B and Cathepsin D Assay Kits
- FRET HILu™ Fluor 488/QXL 520 substrate, ExEm 495/520 nm upon co-wavelength
- Sensolyte® Plus 520 MMP-2 and MMP-9 Assay Kits
- FRET S-FAMLQK 520 substrate, ExEm 495/520 nm upon co-wavelength
- Anti-MMP Abs, recombinant MMPs and culture supernatants containing MMPs
- Sensolyte® 520 activity assay
- Protocollization volumes were 50 μl of purified enzyme sample and 50 μl of FRET substrate solution. Assays were performed in 96-well black plates.
- Immunocapture MMP Plus activity assay:
  - Protocol Samples containing MMPs were activated with 1 mM APMA at 37°C 1 h. before adding the 12 x 8-plate strip plate preincubated with antibody. After 2 h incubation at room temperature for Immunocapture, plate was washed and S-FAMLQK 520 FRET peptide substrate was added and incubated for 1-1.5 h at room temperature.
  - Fluorescence measurement. Fluostation 3844 (Molecular Devices, Sunnyvale, CA)

FRET Pair Optimization at Low pH

- Figure 3. FRET substrate optimization required for ADP activity at low pH. (A) QXL 520 absorption spectrum overlap with HILu™ Fluor 488 emission spectrum. (B) HILu™ Fluor 488 provides stable fluorescent signal at low pH. (C) Fluorescence intensity of FAM is decreased essentially at low pH. (D) Fluorescence of QXL and HILu™ Fluor 488 was measured in 4 buffers at different pH.

Assay Sensitivity

- Figure 2. Sensitivity of Sensolyte® 520 ADAM10, Neprilysin, TACE and IDE Assays. FAMcon was measured at the after incubation of FRET substrate using serial dilutions of enzymes. Sensitivity of assays at these conditions: 0.58 ng/ml for ADAM10, 3.1 ng/ml for TACE, 0.78 ng/ml for IDE and Neprilysin, (n=3, mean±SD).

High Specificity of MMP Immunocapture FRET Assays

- Figure 5. MMP-9 activity detection by FRET and immunocapture MMP-9 PLK assay. (A) MMP-9 activity detected by FRET assay using MMP-9 PLK substrate at 10 and 100 ng/ml, control. MMP-9 was detected at 1th incubation. Other MMPs activities were also detected at 1st incubation (B). MMP activity detected by Immunocapture MMP-9 PLK assay. Specificity of MMP-9 activity was significantly improved by FRET-Immunocapture method at 1st incubation. (C) Specificity of Immunocapture MMP-9 PLK assay with biological samples. CHO cells were transfected with MMP-9, 2, 3, 4 or 5 plate individually. Culture supernatants were collected 24h after transfection and tested by Immunocapture MMP-9 PLK assay.

Conclusions

- We have developed a series of FRET-based assays to detect the activity of ADPs, such as ADAM10, TACE, ADAM17, Neprilysin, and IDE. These assays are capable of measuring sub-nanogram range of enzymes.
- High sensitivity of assays was achieved using proprietary FRET pairs, S-FAMLQK 520 and HILu™ Fluor 488/QXL 520. The latter FRET pair provided stable fluorescent signal at acidic pH resulting in assay optimization for cathespins and cathepsins that require low pH for maximum activity.
- To differentiate between proteases activities in biological samples, we combined FRET and ELISA principles. These FRET-based immunocapture assays enables detection of specific matrix metalloproteases, such as MMP-2 and MMP-9, which are involved in amyloid degradation.