Biochimica et Biophysica Acta 1838 (2014) 2568–2577

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Role of N-terminal residues in Aβ interactions with integrin receptor and cell surface

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ARTICLE INFO

Article history:
Received 15 January 2014
Received in revised form 26 May 2014
Accepted 13 June 2014
Available online 21 June 2014

Keywords:
beta-Amyloid
Alzheimer
Cellular interaction
Integrin
Residue
FRET

ABSTRACT

beta-Amyloid (Aβ) is the primary protein component of senile plaques in Alzheimer's disease (AD) and is believed to play a role in its pathology. To date, the mechanism of action of Aβ in AD is unclear. We and others have observed that Aβ interacts either with or in the vicinity of the α6 sub-unit of integrin, and believe this may be important in its interaction with neuronal cells. In this study, we used confocal microscopy and flow cytometry to explore the residue specific interactions of Aβ40 with the cell surface and the α6 integrin receptor sub-unit. We probed the importance of the RHD sequence in Aβ40 and found that removal of the residues or their mutation using the Aβ8-40 or the D7N early onset AD sequence, respectively, led to a greater interaction between Aβ40 and an antibody bound to the α6-integrin sub-unit, as measured by fluorescence resonance energy transfer (FRET). These results suggest that the RHD sequence of Aβ40 does not mediate Aβ–α6 integrin interactions. However, the cyclic RGD mimicking peptide, Cilengitide, reduced the measured interaction between Aβ40 fibrils without the RHD sequence and an antibody bound to the α6-integrin sub-unit. We further probed the role of electrostatic forces on Aβ40–cell interactions and observed that the Aβ sequence that included the N-terminal segment of the peptide had reduced cellular binding at low salt concentrations, suggesting that its first 7 residues contribute to an electrostatic repulsion for the cell surface. These findings contribute to our understanding of Aβ–cell surface interactions and may provide insight into development of novel strategies to block Aβ–cell interactions that contribute to pathology in Alzheimer's disease.

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1. Introduction

The β-amyloid (Aβ) peptide is a major protein component of senile plaques found in the brains of Alzheimer's disease patients (AD) [1]. Aβ was first sequenced nearly 20 years ago and has long been believed to play a role in the pathology of AD, however, the mechanism by which it contributes to AD pathology is still under investigation [2,3]. At this time, there is no consensus about the mechanism by which Aβ interacts with cells, whether or not certain interactions are specific or non-specific, and if all modes of Aβ binding/interaction with the cell lead to some biological response. Careful probing of the Aβ–cell interaction is a first step in understanding how Aβ leads to memory loss in Alzheimer's disease.

There have been reports of many different receptors involved in Aβ–cell attachment and interactions associated with Aβ biological activity and toxicity. Monomeric and fibrillar Aβ has been found to bind scavenger receptor classes A and B1 [4,5], the serpin enzyme complex (SEC-R) [6], the formyl peptide chemotactic receptor (FPR) [7,8], heparan sulfate proteoglycans [9,10], the insulin receptor and $\alpha_5\beta_1$–integrin [11].The receptor for advanced glycation end products (RAGE) was found to be to a possible cell surface receptor for Aβ42 fibrils [12].The toxic effects of Aβ have been reported to be mediated through interaction with α7-type nicotinic acetylcholine receptors (nAChR) [13] and certain Ca^{2+} permeable and impermeable receptors (NMDA and AMPA, respectively) [14,15]. Aβ has also been reported to bind to the Ephrin type-B receptor 2 (EphB2) [16], the amylin-3 receptor (AMY3) [17], and the cellular prion protein (PrP^C) [18,19]. In addition, APP (amyloid precursor protein), the P75 neurotrophin receptor (P75NTR), the CLAC-P/collagen type XXV (collagen-like Alzheimer amyloid plaque component precursor/collagen XXV) and CD36-complex, involving CD36, $\alpha_6\beta_1$–integrin and CD47 have been reported to bind the fibrillar form of Aβ [20–23]. Finally, α1 integrins [24,25] and β1 integrins [11,26] have been implicated in Aβ–cell membrane interactions linked with AD.

There have also been numerous reports that Aβ interacts with cells through a non-specific, non-receptor mediated mechanism [27–30]. Electrostatic interactions have been implicated in Aβ binding to specific cell membrane components (negatively charged phospholipids and gangliosides) [31,32], and also in Aβ toxicity [33]. Hence, Aβ binding to the cell and its subsequent toxicity are possibly mediated through nonspecific physicochemical interaction as well as specific binding with receptors on cell membranes.

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http://dx.doi.org/10.1016/j.bbamem.2014.06.011
0005-2736/Published by Elsevier B.V.

Of interest in understanding the mechanism of Aβ interaction with cells is not just where on the cell that the peptide binds and how, but also which residues on the Aβ peptide are important for this interaction. Residues Y10, K28 and H13 and H14 have been implicated in stabilizing the Aβ membrane interaction, providing an electrostatic attraction with the negatively charged cell membrane, and in the stabilization of a membrane spanning pore, respectively [34–36]. In our laboratory, we have found that the residues in the vicinity of K28 and N27 and V36-V40 have been implicated in Aβ40 fibril–cell interactions via computational docking methods [37] and in vitro verification [Keshet, Peshesk 2014 in preparation]. Residues 1–16 have been shown to give rise to the unique structural difference between aggregated species via hydrogen exchange methods [38]. Other residues associated with mutations linked to early-onset AD, such as the D7N (Tottori-Japanese) mutation [39], may be important in Aβ cell interactions, however, their significance is not yet known.

We and others have shown that Aβ interacts with or near the α6 subunit of integrin receptor, in model cell lines, neurons, and glia [40,41]. Others have shown how this interaction may be important in signaling associated with NMDA- R and could have implications for learning and memory in AD [42]. Understanding the mechanism of Aβ–integrin interactions is a first step in designing new molecules that block the interaction and potentially attenuate the Aβ–integrin associated signaling events that contribute to Aβ neurotoxicity [43,44].

We used this background as a basis to explore the residues on Aβ that are important in Aβ–integrin interactions. We set out to examine if Aβ40 fibrils interacted with the integrin through the RHD sequence (an integrin-affinity binding sequence), and found not only that the specific sequence did not contribute to Aβ–cell interactions, but that electrostatic interactions at the N-terminal region instead may be important in Aβ –cell interactions.

2. Materials and methods

2.1. Materials

All chemicals were purchased from Fisher (Pittsburgh, PA) or Sigma Aldrich (St. Louis, MO), unless otherwise specified. Cell culture reagents were obtained from Invitrogen (Carlsbad, CA). Primary antibodies for Integrin subunits: Integrin α6 (C-18) goat polyclonal antibody, Integrin α1 Antibody (R-19) goat polyclonal IgG antibody, Integrin β1 Antibody (N-20) goat polyclonal antibody and their secondary antibody probe Donkey anti-goats IgG–FITC antibody were purchased from Santa Cruz Biotechnologies. All Aβ peptides (Aβ40, N-terminus Carboxytetramethylrhodamine (TAMRA) labeled-Aβ40, Aβ 8-40, D7N-Aβ40 and G33L-Aβ) were purchased from AnaSpec (San Jose, CA).

2.2. Aβ fibril preparation

Aβ40 stock was prepared by first completely dissolving the peptide in hexafluoroisopropanol (HFIP) (5 mg/mL), aliquoting sufficient peptide for individual experiments into small tubes, then evaporating off HFIP under vacuum until dry. Aβ40 films prepared this way were then stored at −80 °C until use. Aβ40 stock solutions were prepared by dissolving Aβ40 at 10 mg/ml in ddH2O with 0.1% Trifluoroacetic acid (TFA) for 45–60 min. The stock was diluted in PBS (138 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl, and 1.8 mM KH_2PO_4) to a final concentration of 100 μM, and was gently mixed using a rotator for 24 h at 37 °C. Analogous methods were used to prepare fibrils from Aβ mutants.

2.2.1. Carboxytetramethylrhodamine (TAMRA) labeled-Aβ preparation

To prepare fluorescently labeled Aβ fibrils for use in fluorescence resonance energy transfer and binding experiments, 1% by mass N-terminally labeled TAMRA-Aβ40 was incorporated into fibrils. A stock solution of 10 mg/mL TAMRA labeled Aβ, dissolved in DMSO, was thawed from storage at −80 °C. TAMRA-Aβ in DMSO was then added to unlabeled Aβ (wild type or mutants) that was freshly dissolved in 0.1% (v/v) TFA as prepared above, such that 1% of Aβ40 contained the N-terminal TAMRA label. The resulting solution was diluted to 100 μM in PBS and gently mixed for 24 h to form Aβ40 fibrils that are uniformly labeled and bound with the TAMRA-Aβ mixed in with the preparation.

2.3. Cell culture

SH-SY5Y cells were purchased from ATCC (Manassas, VA, USA). Cells were maintained in a humidified incubator with 5% CO_2 at 37 °C in Minimal Essential medium supplemented with 10% fetal bovine serum (Invitrogen), 2% Penicillin–Streptomycin and 2% Fungizone.

Prior to each experiment, 50,000 or 100,000 cells per well were seeded in a 96-well plate and were allowed to adhere to wells for 24 h. When needed, cells were differentiated for 72 h by removal of Minimal Essential Media (MEM) and replacement with Neurobasal serum free medium (Neurobasal ATM medium, 3 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 4 mM B-27).

2.4. Fluorescence resonance energy transfer (FRET)

A series of FRET experiments was performed in which we examined energy transfer between a TAMRA chromophore attached to the N terminus of Aβ40 fibrils and an FITC chromophore attached to a secondary antibody, which was bound to a primary antibody bound to the α6 subunit, the α1 subunit, or the β1 subunit of the integrin receptor as a function of different modification or mutations in the Aβ40 fibril. We take energy transfer as a measure of binding and/or proximity of binding of Aβ to the integrin receptor subunit. For the pair of fluorophores uses in this study, we would expect to see a 50% energy transfer if the two fluorophores are within the Förster radius (R_0) of the donor and acceptor fluorophore pair (Fluorescein isothiocyanate and Tetramethylrhodamine) or in this case 55 A°. In our experiments, since our donor and acceptor fluorophores are on the N-terminus of the Aβ peptide and a secondary antibody bound to a primary antibody bound to the α6/α1/β1 subunit of the integrin receptor, we can only probe for interactions in the vicinity of these integrin receptors, which could be many receptors in a focal adhesion, or even an integrin containing lipid raft.

2.4.1. FRET procedure

For the FRET experiments, SY5Y cells were plated at a density of 10,000 cells per well in cover plate wells (Nalge Nunc International/Thermo Fisher (Pittsburgh, PA, USA)). The cells were fed with MEM which was then replaced with serum free Neurobasal media to induce differentiation for 72 h prior to imaging. The cells were fixed with 4% paraformaldehyde for 15 min, and then blocked with 2% bovine serum albumin (BSA) buffer. Aggregated and labeled Aβ at 100 μM was then added to each well, such that it covered the surface completely and was then incubated for 2 h at 37 °C. The unbound Aβ was removed by careful rinsing of the wells, following which the cells were labeled with primary antibody specific for the subunit of integrin (1:50 for 3 h) and secondary antibody labeled with FITC (1:500 for 1 h at room temp or overnight at 4°). They were further labeled with 5 μL DAPI (380 nM in PBS, stains the nucleus exclusively) after permeabilization with 0.4% Triton X100 buffer. The wells were mounted with drops of mounting media and allowed to dry to prepare the slides for FRET imaging.

We examined the transfer between fluorescently labeled Aβ fibrils (Aβ 1–40, 8–40, N27P, G33L and D7N mutants) and the fluorophore attached to the secondary antibody associated with the specific subunit of the integrin receptor using the Leica SP5 FRET wizard.

Cells were viewed through a Leica SP5 confocal microscope at 63,000× magnification and regions of interest (ROI) were identified that had both donor (secondary antibody-FITC) and acceptor (Aβ-TAMRA) labeling. Approximately 100 regions of interest (ROIs) were

identified per sample. FRET was measured in the ROIs by measuring the initial donor fluorescence, then photo bleaching the receptor by increasing acceptor laser intensity to 70% and bleaching 15 frames per sample, and then measuring the donor fluorescence after photo bleaching. If energy transfer was occurring due to the proximity of the two fluorophores, then donor fluorescence would increase upon acceptor photo bleaching.

Efficiency of energy transfer was calculated using a function embedded in the FRET Acceptor bleaching (AB) wizard in Leica LAS software as follows:

$$FRET_{eff} = \left[\frac{Dpost - Dpre}{Dpost}\right],$$

where D_{post} refers to the donor fluorescence intensity post acceptor bleaching and D_{pre} refers to the donor fluorescence intensity prior to acceptor bleaching.

The FRET values are then represented as box plots, with the box being an interquartile range, the median value represented as a line inside the box and the bottom section of the box representing data falling between the 25th and 50th percentile of the data, while the top section represents the data falling in the 50th to 75th percentile of the data (if the FRET scores were ranked by numerical value). Outliers are represented as asterisks and the lines or whiskers from the box bars represent the spread of the data (minimum to maximum).

The circle with a cross inside the box is the mean of the sample data. $FRET_{eff}$ measurements were rejected if donor or acceptor pixel intensity values were below 5 or above 255 (near the minimum or maximum values possible in the measurement system). $FRET_{eff}$ values, where the acceptor was not photo bleached were also rejected and not included in the representation of the data. Statistical analysis of the data was performed by using the Mann–Whitney test for the FRET efficiency values of each sample versus control. The box plots and Mann–Whitney test were chosen as the means of result representation and statistical analysis because $FRET_{eff}$ and Aβ–cell binding data were found to be typically non-normally distributed.

2.5. TEM imaging

Structures of the various aggregated peptides were captured by using Transmission Electron Microscope (TEM) Imaging. Unlabeled Aβ40 wild type and mutant peptides were aggregated as described above. 10 μl of samples was dropped gently on the carbon coated Formvar grids, 200 mesh (TedPella, Redding, CA) for 3 min, and then washed in ddH2O for 10 s, after which 10 μl solution of 1% Uranyl acetate in ddH2O was dropped on the grids for 2 min. Grids were viewed using a Zeiss-1OCA microscope (Zeiss, Germany) equipped with an Olympus Morada (Melville, NY) digital camera, using an accelerating voltage of 60 kV. Images were taken at the Keith R Porter Imaging Facility at UMBC. Images were taken randomly throughout grids, such that individual fibrils could be clearly visualized and at magnifications of up to ×160,000. Three images were captured per type of Aβ sequence.

2.6. Aβ–cell binding assay

SH-SY5Y cells were seeded at 100,000 cells per well for binding assays. Cells were adhered to the well over the course of 24 h, after which, the cells were fixed for 30 min with 4% paraformaldehyde (4% PFM) in PBS in order to minimize cell loss during Aβ40 incubation. The PFM was then removed and cells were washed thrice with PBS. TAMRA labeled Aβ40 fibrils (wild type or mutations/deletions) were added to SH-SY5Y such that the final concentration in the well was 50 μM, based on initial unlabeled Aβ monomer concentration. Aβ fibrils, and cells were incubated for 2 h at 37 °C, after which excess Aβ was washed away with PBS; and cells were detached from wells by mechanical scraping.

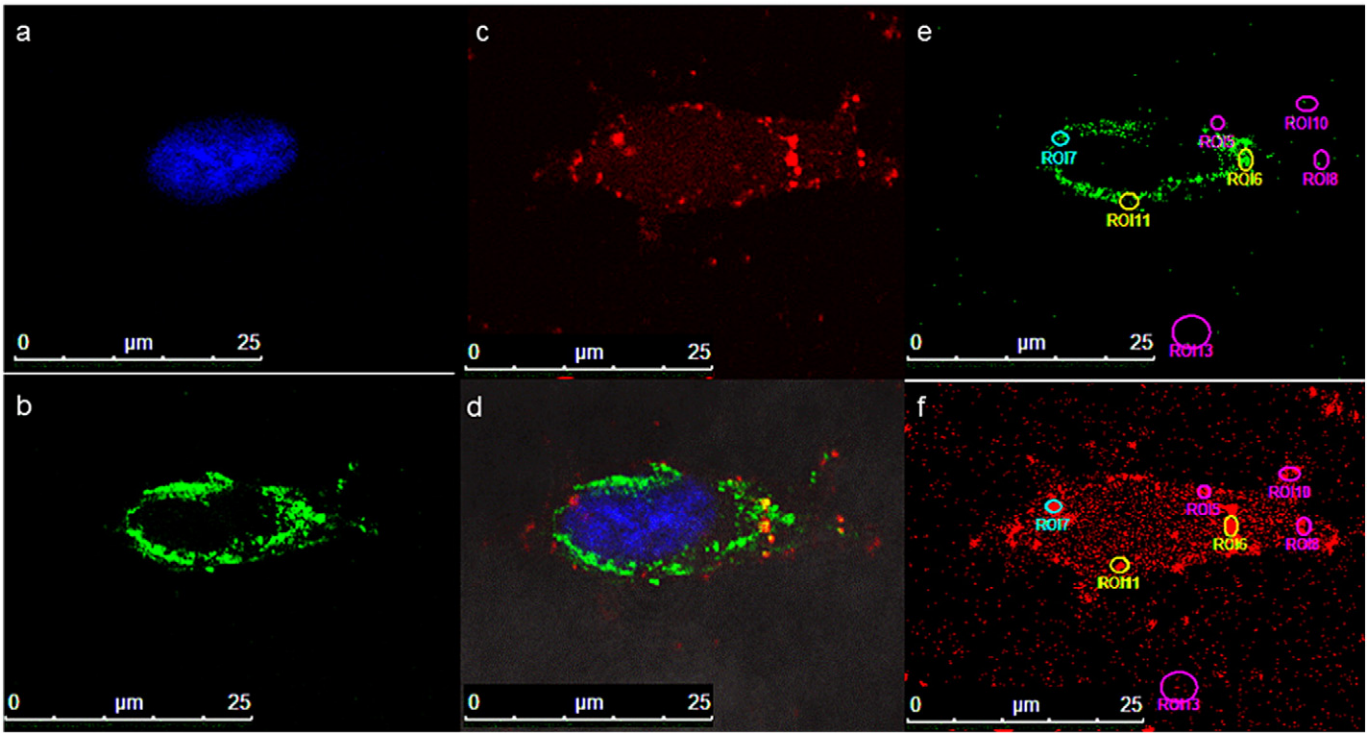

Fig. 1. Representative confocal images of differentiated SH-SY5Y cells (60,000×) for FRET imaging: (a) Nucleus of the cell stained with DAPI, a nuclei specific fluorescent stain, depicted blue in color. (b) α6–integrin subunit fluoresces as green in this image and is labeled with Fluorescein Isothiocyanate (FITC) . (c) TAMRA-labeled fluorescent beta-amyloid 40 (WT) fibrils show up as red in this image. (d) Nucleus, integrin and Aβ fluorescent stains overlaid in a single frame along with the bright field image frame and captured as a single overlay image. (e) & (f) depict representative the regions of interest selected for FITC and TAMRA fluorescence captured as images, and upon which acceptor bleaching is performed.

To measure binding at a pH or an ionic strength other than physiological, fluorescently labeled Aβ fibrils were prepared as above, but then centrifuged at 13,000 rpm, 20 min, to separate fibrils from PBS buffer in which fibrils were prepared. The fibrils were then resuspended in 10 mM phosphate buffer with desired amounts of NaCl and pH adjusted to the desired level. Subsequent washing prior to flow cytometry was done with the same buffer as used to resuspend fibrils. Phosphate buffers with pH of 2, 3, 7.4, 9 and 12, and NaCl concentrations of 0.02 M, 0.2 M, 0.4 M and 0.56 M were used in these set of experiments.

Cell associated fluorescence intensity, indicative of Aβ fibril bound to cells, was detected using a BD FACS Array (San Diego, CA) flow cytometer. Cell samples were excited with a 635 nm laser and cell associated fluorescence emission was collected using the red filter (653–669 nm). The median fluorescence intensity of the cell population was taken as a measure of the amount of Aβ bound to cell surface. Fluorescence intensity measurements were corrected for the auto fluorescence of unlabeled cells. Care was taken to ensure that free fibrils that might have been counted in the flow cytometer were gated such that they were not included in the Aβ fibril–cell fluorescence.

3. Results and discussion

As a step in the development of agents that will block Aβ–cell interactions and thereby attenuate Aβ toxicity, we and others have probed receptors on the cell surface associated for Aβ–cell interactions [41,45–48] and residues on the Aβ fibril or oligomer responsible for the Aβ–cell interaction [49–52].To that end we have explored Aβ-interactions with cholesterol [45], sialic acid [53,54], and phospholipids [55] associated with the cell membrane, as well as specific interactions with certain receptors and signaling molecules [46,56], and identified specific Aβ residues (K28) [50,56] or sequences (1–16, residues near the C terminus) [41,50] as potential loci on the Aβ fibril or oligomer surface that participate in the Aβ–cell interactions.

We and others have shown that Aβ binds to or in the vicinity of an integrin receptor [40,57,58]. We hypothesized that this interaction may be mediated through the RHD sequence on the N-terminus of the Aβ40 fibril, a putative integrin binding sequence [41], and a part of the Aβ peptide which is normally assumed to be unstructured and solvent accessible even when Aβ is in fibril form [59]. We supposed that by elucidating the Aβ residues involved in this interaction, we might be able to develop new targets to block Aβ associated biological activity mediated through the integrin interaction.

To that end, a series of FRET experiments was performed in which we examined energy transfer between Aβ40 fibrils and an antibody bound to specific subunits (α6,α1 and β1) of the integrin receptor as a function of different modification or mutations in the Aβ40 fibril. Since we did not label the integrin subunits directly, but rather used a pair of primary and secondary antibodies to probe for regions of interest on the cell surface, we can only conclude that Aβ bound near specific subunits. We used SY5Y cells as our model system in these studies as we and others have used them before to explore Aβ–cell interactions in vitro [60–62]. SH-SY5Y cells have been shown to express α1, α3, and β1 integrin subunits, as well as lower concentrations of α2, α4, α5, α6, and αv, integrin subunits both when undifferentiated and differentiated, though differentiation was found to increase expression of integrin receptor subunits [63–66]. In earlier work, we have used FRET to show that Aβ binds in the vicinity of the α6 subunit [Ramos, Venkatasubramaniam and Good, submitted]. We include the β1 subunit because of its role in binding to the RGD sequence [67] and its potential

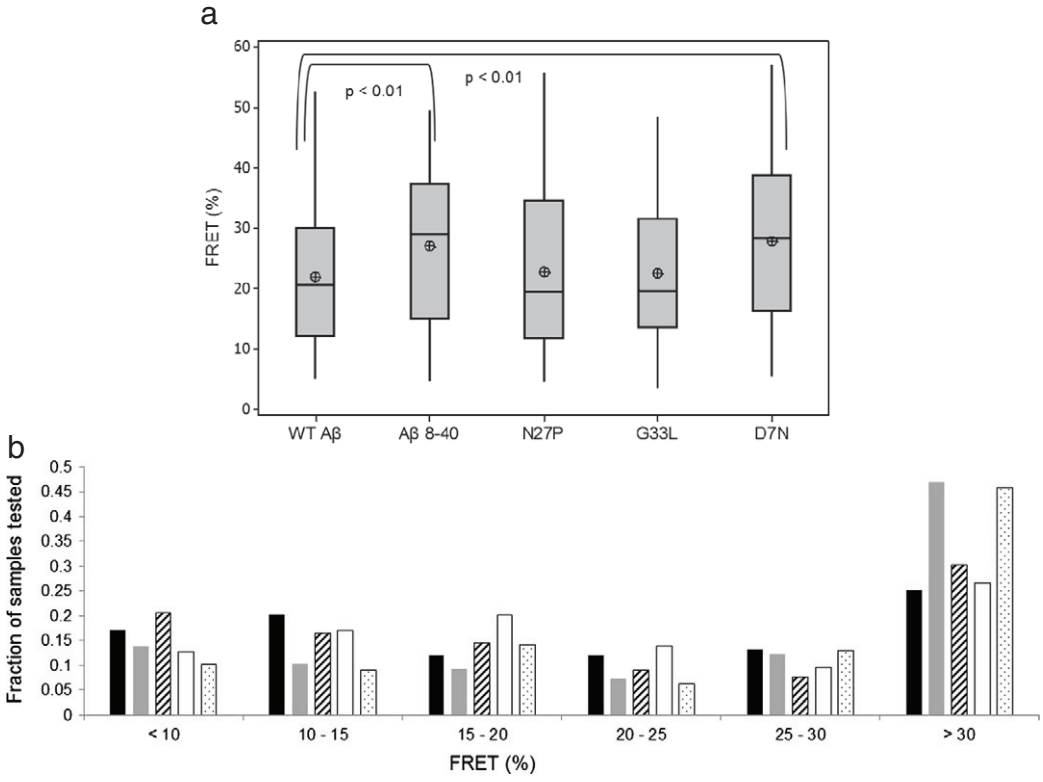

Fig. 2. $FRET_{eff}$ between various Aβ peptides in aggregated form labeled with TAMRA (Acceptor flurophore), and α6–integrin subunit labeled with FITC (Donor fluorophore). (a) A Box and whisker plot representation, with the box being an interquartile range, the median value represented as a line inside the box, the bottom part of the box representing data in the 25th to 50th percentile, the top part of the box representing data in the 50th to 75th percentile of the data if they are ranked numerically by their the FRET values. Outliers are represented as asterisks and the lines or whiskers from the box bars represent the spread of the data (minimum to maximum). The circle with a cross inside the box is the mean of the sample data. Mann–Whitney test between each sample and control (WT-Aβ) is used for statistical analysis ($n = 200$). (b) A frequency plot depict the various $FRET_{eff}$ value ranges for each peptide and more dramatically depicts the larger subset population for 8-40 and Tottori Japan (D7N) Aβ with $FRET_{eff}$ of greater than 30%. Black bars represent WT Aβ, dark grey bars represent 8-40, horizontally angled and lined bar represents N27P Aβ, white bar Leu33 (G33L) Aβ, and the dotted bar represents Tottori-Japan (D7N).

relevance to Alzheimer's [11,26]. We include α1 because of its abundance on the cell surface, and because it has not to our knowledge been implicated in Alzheimer's, making it an appropriate control.

Fig. 1 shows a group of representative confocal micrographs that show the overlap of Aβ40 fibril and α6 integrin staining, as well as typical areas or regions of interest chosen for FRET measurements. In Fig. 2, we show energy transfer between Aβ and the antibody bound to the α6 integrin subunit for several Aβ fibril sequences with and without the putative RHD integrin binding domain (residues 5 to 7 of the Aβ sequence). We included in these experiments Aβ8-40, and Aβ40 D7N, peptides with missing or mutated RHD sequences along with Aβ40 G33L and N27P, peptides with mutations in the β-turn region that we did not anticipate being associated with integrin binding or peptide aggregation. Fig. 2(a) shows box and whisker plots of the energy transfer for all n (50 to 200) regions of interest measurements, where the average (cross), median (line), 75% & 25% quartiles (end of the box plots) and range of data (whiskers) are depicted. We chose this representation given that the data were not normally distributed. As can be seen in Fig. 2a, significantly greater energy transfer was seen between the Aβ sequences without the RHD sequence (Aβ 8-40 and Aβ D7N) and the antibody bound to the integrin subunit ($p < 0.01$) than sequences with the RHD sequence. To further illustrate this point, we show the fraction of the sample measurements taken with different observed FRET ranges (Fig. 2b). As can been seen in Fig. 2b, almost 50% of the population had measured FRET efficiencies of greater than 30% for Aβ8-40 and Aβ D7N, while for all other Aβ sequences, a much smaller fraction of the population had high FRET efficiencies.

We wanted to explore if the observed energy transfer and by extension the Aβ fibril–cell surface binding, was specific for an integrin, even though the interaction did not appear to be mediated by the RHD sequence on Aβ. Therefore we tried to block the interaction using the cyclic RGD peptide, cilengitide that is known to inhibit certain integrin receptors [68,69].

Cilengitide has been reported to inhibit integrin interactions when applied in the micromolar range in vitro, and has reported high specificity for αvβ3 and αvβ5 with activity against $\alpha_5\beta_1$ [70,71]. Surprisingly we found that cilengitide significantly reduced energy transfer for the two peptides that did not have the RHD sequence (Aβ 8-40 and Aβ40 D7N), as shown in Fig. 3(a) and (b). Without cilengitide, approximately 15% of the sampled regions of interest had the lowest levels of energy transfer between the integrin subunit and wild type Aβ40, Aβ8-40 and Aβ D7N (Fig. 2b), while with cilengitide, 50% of population had the lowest FRET levels with the same 3 Aβ sequences (wild type Aβ, Aβ 8-40, and Aβ D7N; Fig. 3b). We also see that cilengitide enhanced FRET between G33L Aβ and the antibody bound to the α6 integrin receptor subunit (Fig. 3). This suggests that the cilengitide did not just displace our antibody bound to the α6 integrin subunit, but instead displaced some but not all Aβ fibrils, depending upon their sequence.

One explanation for our observations that Aβ sequences without the RHD sequence showed greater FRET efficiencies with the integrin receptor subunit, but that a cyclic RGD peptide could block these same peptides (but not all Aβ peptides) from interacting with the cell is that the different Aβ sequence resulted in the formation of different Aβ fibril structures, which give rise to the differences in cell interaction and energy transfer. To explore this hypothesis, we examined the structure of the Aβ peptides to ensure that all of the Aβ peptides used still formed fibrils. TEM images of the Aβ fibrils are included in Supplementary data (Fig. 1). No qualitative differences in macroscopic structure of the

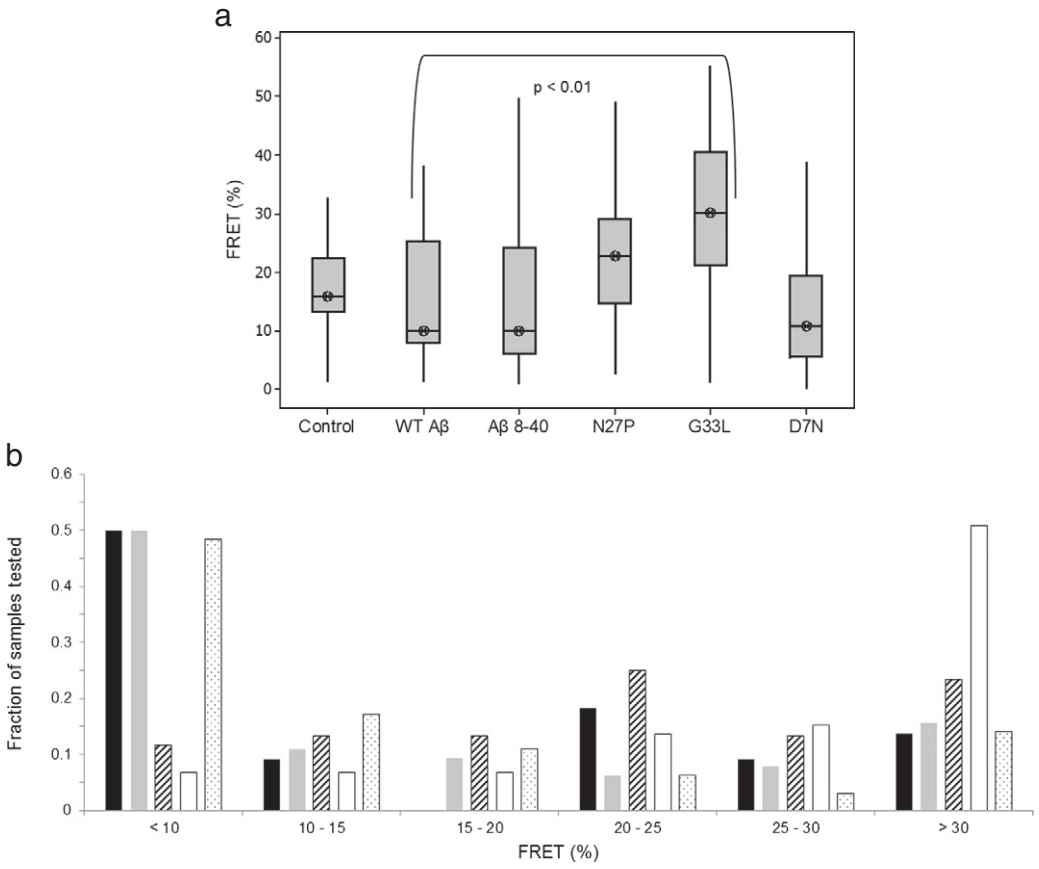

Fig. 3. $FRET_{eff}$ post exposure to cilengitide (RGD mimicking cyclic peptide, 5 μM, 24 h). (a) A box and whisker plot representation. Mann–Whitney test between each sample and control (WT-Aβ without cilengitide) is used for statistical analysis ($n = 50$). (b) A frequency plot is used to depict the various $FRET_{eff}$ value ranges for each peptide and more dramatically depicts the larger subset population for WT 1-40, 8-40 and Tottori Japan (D7N) Aβ with $FRET_{eff}$ of less than 30%. Black bars represent WT Aβ, dark grey bars represent 8-40, horizontally angled and lined bar represents N27P Aβ, white bars Leu33 (G33L) Aβ, and the dotted bars represents Tottori-Japan (D7N).

Aβ fibrils (wild type, Aβ 8-40 and Aβ D7N) were observed. Others have reported that the Aβ D7N mutation leads to altered rates of fibril elongation [72,73], which should lead to differences in fibril length but not necessarily changes in fibril structure relative to wild type Aβ40 fibrils.

A second possibility is that the differences in observed energy transfer with the different Aβ fibrils resulted from differences in electrostatic interactions of Aβ with the cell surface. There have been a number of reports suggesting that electrostatic interactions are important in Aβ–cell interactions and toxicity [74–77]. The cell surface is generally regarded as negatively charged [78]. The first 7 residues of the Aβ sequence, DAEFRHD, contain 3 negatively charged amino acids under physiological conditions. Removal of these first 7 residues should decrease potential electrostatic repulsion between the Aβ fibril and the cell surface. The D7N mutation would replace one negatively charged amino acid with a neutral amino acid. To explore the potential impact of electrostatic interactions on our results, we examined the binding of Aβ-40 and Aβ 8-40 fibrils to the cell surface as a function of salt concentration and pH. These results are depicted in Fig. 4(a) and (b), respectively. As can be seen in Fig. 4(a), as salt concentration present during binding was increased, binding of Aβ 40 to the cell increased, while binding of Aβ 8-40 decreased. As ionic strength (and salt concentration) is increased, one would expect increased shielding of charges such that electrostatic effects would diminish at higher salt concentrations. Thus, we conclude that full length Aβ40 fibrils experiences electrostatic repulsion while Aβ8-40 fibrils experienced electrostatic attraction with the cell surface. The greater change in binding to the cell surface at the different salt concentrations observed for the Aβ8-40 fibril compared to the full length Aβ40 fibril suggests that there are more charges involved in the Aβ8-40–cell surface interaction.

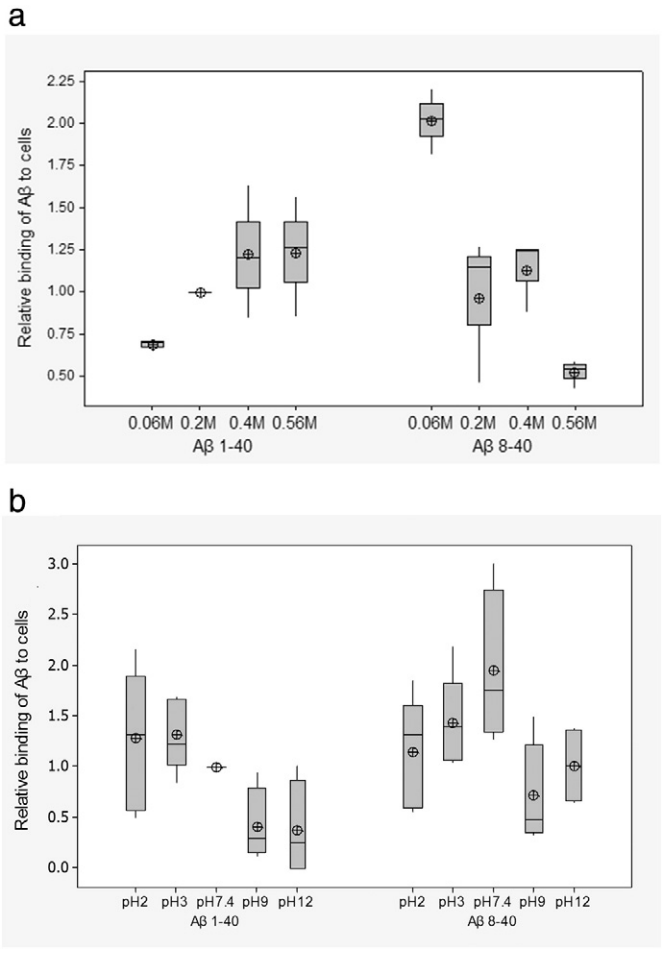

Fig. 4. Binding assay to depict relative fraction of cells bound to Aβ, (a) across varying salt concentrations (b) across varying pH. A box and whisker plot representation, with the box being an interquartile range, the median value represented as a line inside the box and the box ends representing the 25th and 75th quartile values of the data. Outliers are represented as asterisks and the lines or whiskers from the box bars represent the spread of the data (minimum to maximum). The circle with a cross inside the box is the mean of the sample data.

To further probe the role of charges on the Aβ surface on Aβ–cell binding, we examined the effects of changing pH. We expected that above the pKa of the basic amino acid residues or below the pKa of the acidic residues, we might see changes in binding because of changes in electrostatic interactions. As seen in Fig. 4(b), binding of the full length Aβ40 fibril to the cell surface decreased as the pH increased, suggesting that as the peptide became more negatively charged (at increasing pH), binding diminished. Binding of Aβ 8-40 was greatest at physiological pH, and decreased as pH increased or decreased. This result is more difficult to interpret, but might suggest that changing the balance of charges on the Aβ8-40 sequence or the cell surface diminish the Aβ8-40 fibril electrostatic interaction with the surface. These experiments measured binding of Aβ to the entire cell surface, and were not specific for binding at or near the integrin receptor subunit, however, they may suggest mechanisms of attraction that play a part in the more specific Aβ–integrin interactions.

Along these same lines, when we showed that cilengitide, the cyclic RGD peptide, attenuated some Aβ–integrin interactions, we may not have been specifically blocking the RHD binding site on the cell surface as we proposed, but instead may have been altering some non-specific interactions, especially when using cilengitide at relatively high concentrations. Cilengitide has functional groups which could pick up both a positive and negative charge depending upon the environment [79], and as well as an aromatic ring that could contribute to hydrophobic interactions [80].

A third possible explanation for our observations that Aβ sequences without the RHD sequence show greater FRET efficiencies with the integrin receptor subunit, but that a cyclic RGD peptide could block these same peptides (but not all Aβ peptides) from interacting with the cell is that Aβ interacts through sequences other than the RHD sequence, and potentially with a receptor other than the integrin receptor subunit. RGD is a recognition site which can be recognized by the majority of β1–integrins, but not all integrin subunits. Aβ does not contain any other canonical integrin receptor binding sites, but some of the integrin binding sequences are unknown [81]. We used an antibody to an α6–integrin subunit, which typically interacts with laminin via an unknown sequence (but not RGD) [24].

Aβ40 may have been binding to something other than an integrin when we observed energy transfer between the Aβ40 fibril and the antibody bound to the α6–integrin subunit. FRET efficiency is related to the distance donor and acceptor fluorophore, thus our results indicate only that mutations in the Aβ sequence impact the distance been the Aβ fibril and a secondary antibody bound to an integrin receptor subunit. The actual Aβ–cell interaction which is impacted by the mutations and deletions in the N terminus may be with a molecule in the vicinity of the α6–integrin receptor subunit. Aβ may be binding to another molecule co-localized with the integrin receptor subunit, including other integrin receptor subunits other than the α6 subunit, other receptors found within a focal adhesion, and/or receptors found within a lipid raft in which the integrin subunit is localized. We have previously found that Aβ binds in cholesterol rich, sialic rich regions of the cell surface [45], which, along with co-localization with an integrin receptor could suggest that Aβ binds to a receptor within a lipid raft on the cell surface.

To investigate if Aβ bound to other integrin receptor subunits, we repeated our FRET experiment with Aβ and the β1 subunit of the integrin receptor, which has been implicated in the Aβ–α6 integrin subunit interaction [21], as well as other Aβ cell interactions [11,26], and with Aβ and the α1 subunit of the integrin receptor, which has not been implicated in AD. We performed the experiments with the Aβ 40, Aβ 8-40, and Aβ D7N fibrils in the absence and presence of cilengitide. The results from

these experiments are shown in Figs. 5 and 6. We found that the average energy transfer between all Aβ sequences and either the β1 or α1 integrin receptor subunit was less than 10%, and no more than 5% of all regions tested had energy transfers at greater than 30%. In an unrelated study, we measured FRET efficiencies of 10% or less between Aβ40 fibrils and antibodies bound to GalC and the transferrin receptor, two receptors thought to be irrelevant and abundant on the cell surface [Ramos, Venkatasubramaniam and Good, submitted]. These results suggest that Aβ does not bind near the α1 or β1 subunit, or that the antibody used to fluorescently label the receptor blocks the site of Aβ binding. The relatively high energy transfer between Aβ and antibodies bound to the α6 integrin receptor subunit compared to the α1 and β1 receptor subunits suggests that the α6 subunit marks a specific location on the cell surface for Aβ interactions. We still cannot conclude that Aβ interacts directly with the α6 subunit, but only that the peptide interacts with the vicinity of some but not all integrin receptor subunits.

It is probable that Aβ interacts with the cell surface via both specific and non-specific interactions. Specific Aβ–integrin interaction may have functional consequences given the role of integrin in FAK/Src activation, and cell cycle activation, and eventual cell death [26,82]. RGD containing peptide–integrin interactions are linked to increases in *N*-methyl-D-aspartate receptor (NMDA-R) mediated synaptic transmission [83] and long term potentiation [84,85]. Thus, Aβ–integrin interactions could impair molecular processes associated with learning and memory. Non-specific interactions including electrostatic interactions also play a role in Aβ–cell surface binding.

In this work we show that that Aβ binds in the vicinity of the α6 subunit of the integrin receptor, and that the interaction is attenuated in the presence of a cyclic RGD peptide. We show that the seven N terminal residues on the Aβ sequence contribute to an electrostatic repulsion with the cell surface, and that if those residues are deleted or mutated, the Aβ–cell interaction is enhanced, as is the Aβ–integrin interaction (or interaction with the receptor in the vicinity of the integrin). While our results suggest that the first 7 amino acids are not central to the Aβ–integrin interaction, they play an important role in non-specific electrostatic interactions. Being able to manipulate Aβ electrostatic interactions may be an important avenue to modulate Aβ neurotoxicity associated with Alzheimer's disease.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2014.06.011.

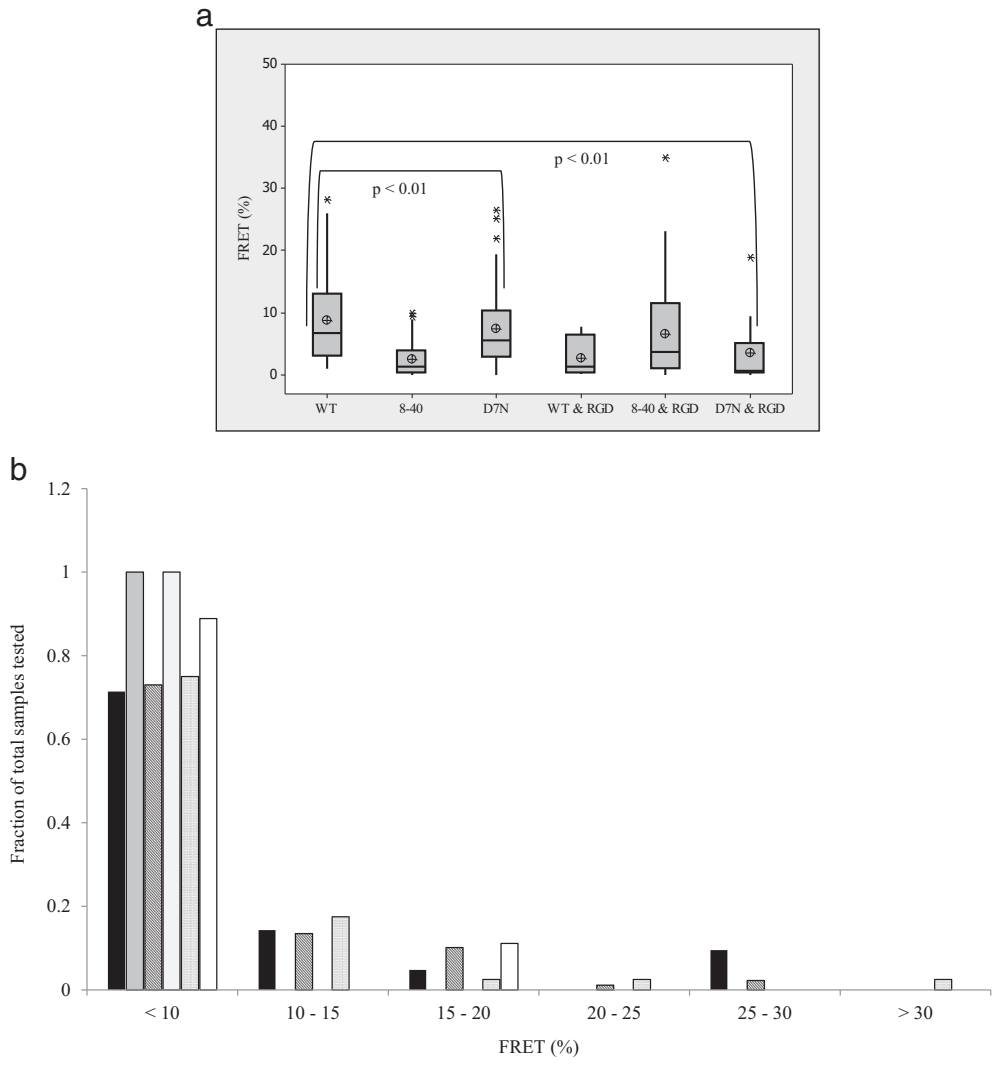

Fig. 5. $FRET_{eff}$ between various Aβ peptides in aggregated form labeled with TAMRA (Acceptor flurophore), and α1–integrin subunit labeled with FITC (Donor fluorophore). FRET was measured in the absence or presence of cilengitide (indicated as RGD in figure). Cilengitide, when present, was added at 5 μM prior to the addition of the Aβ. (a) A box and whisker plot representation. Mann–Whitney test between each sample versus WT-Aβ is used for statistical analysis (n = 50). (b) A frequency plot depicts the various $FRET_{eff}$ value ranges for each peptide, depicting the larger subset of the population to have a $FRET_{eff}$ of less than 10%. Black bars represent wild type (WT) Aβ, dark grey bars 8-40 Aβ,slashed line bars D7N Aβ, light grey bars representWT Aβ and cilengitide, dotted bars 8-40 Aβ and cilengitide and white bars are D7N Aβ and cilengitide.

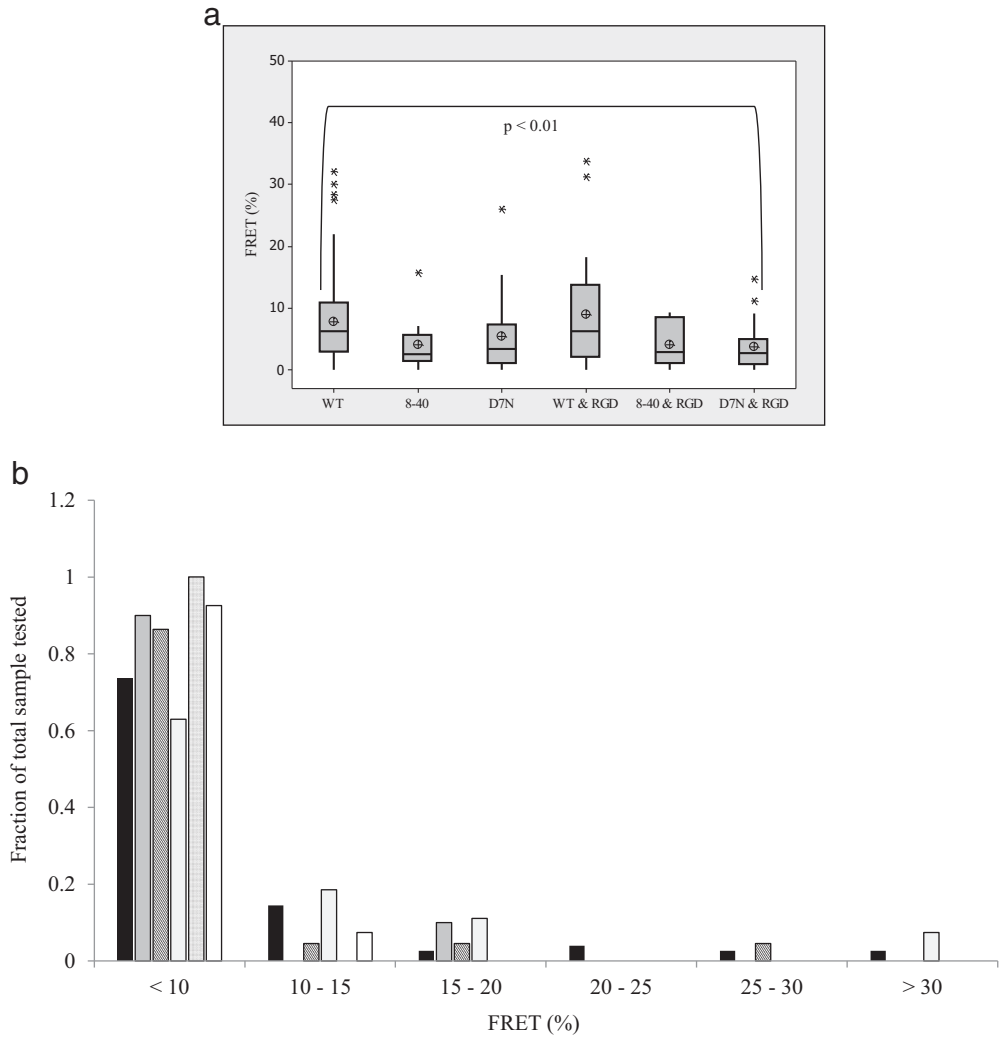

Fig. 6. $FRET_{eff}$ between various Aβ peptides in aggregated form labeled with TAMRA (Acceptor flurophore), and β1–integrin subunit labeled with FITC (Donor fluorophore) FRET was measured in the absence or presence of cilengitide (indicated as RGD in figure). Cilengitide, when present, was added at 5 μM prior to the addition of the Aβ. (a) A box and whisker plot representation. Mann–Whitney test between each sample and WT-Aβ is used for statistical analysis (n = 50). (b) A frequency plot depicts the various $FRET_{eff}$ value ranges for each peptide, with the larger subset of the population having $FRET_{eff}$ less than 10%. Black bars represent Wild type (WT) Aβ, dark grey bars 8-40 Aβ,slashed line bars D7N Aβ, light grey bars representWT Aβ and cilengitide, dotted bars 8-40 Aβ and cilengitide and white bars are D7N Aβ and cilengitide.

Acknowledgements

This work was supported by a grant from the National Science Foundation (CBET 0828009 to TAG). The Leica Confocal Microscope used in FRET studies was obtained with funds from an NSF MRI award (MRI 0722569); Theresa Good was supported by NSF as an IPA and participated in this research as part of her independent research and development. The authors gratefully acknowledge the help of Chere Petty in performing microscopy studies, and Neil Agarwal in conducting a portion of the FRET studies.

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