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Visualizing Interaction of Proteins relevant to Alzheimer's Disease in intact Cells

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Abstract

To understand normal function of memory studying models of pathological memory decline is essential. The most common form of dementia leading to memory decline is Alzheimer's disease (AD), which is characterized by the presence of neurofibrillary tangles and amyloid plaques in the affected brain regions. Altered production of amyloid β (A β) through sequential cleavage of amyloid precursor protein (APP) by β - and γ -secretases seems to be a central event in the molecular pathogenesis of the disease. Thus, the study of the complex interplay of proteins that are involved in or modify A β production is very important to gain insight into the pathogenesis of AD. Here, we describe the use of Fluorescence Lifetime Imaging Microscopy (FLIM), a Fluorescence Resonance Energy Transfer (FRET)-based method, to visualize protein-protein-interaction in intact cells, which has proven to be a valuable method in AD research.

Keywords

Alzheimer's disease; Amyloid Precursor Protein (APP); low density lipoprotein receptor-related protein (LRP); Fluorescence Resonance Energy Transfer (FRET); Fluorescence Lifetime Imaging Microscopy (FLIM)

Introduction

To understand normal function of memory and cognition several research models have been developed. One approach to investigate memory is the use of model systems of pathological memory decline. The most common form of dementia leading to memory decline is Alzheimer's disease (AD). Therefore AD models are certainly beside lesion models some of the best studied model systems for memory and cognition research.

AD is a devastating neurodegenerative disorder with high socio-economic impact that currently affects nearly 2% of the population in industrialized countries. Given the expected demographic development, numbers will be increasing to up to 63 million affected people worldwide in 2030 [1]. Clinically, AD is characterized by cognitive decline, which gradually

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impairs the patients' memory and ability to learn, reason, make judgments, communicate and carry out activities of daily living.

The main neuropathological hallmarks of AD are neurofibrillary tangles and senile plaques, which are believed to cause synaptic dysfunction and neurodegeneration (for review, see [2]). Senile plaques are mainly comprised of different amyloid beta (A β) species, ranging from the highly fibrillogenic A β 42 to more soluble variants such as A β 38 and A β 40. Since both soluble A β oligomers and A β *, a 56kDa A β dodecamer, are believed to mediate the pathological effect on the brain [3–5], many therapeutic strategies aim at reducing the amyloid load in the brains of the patients. In order to produce A β , a number of proteins have to interact at different locations within the cell: The amyloid precursor protein (APP) is first cleaved by the β -site of APP-cleaving enzyme (BACE), which releases APP's soluble ectodomain (sAPP β) and generates a membrane bound C-terminal fragment (CTF β or C99). C99 is subsequently cleaved by presenilin 1 (PS1)/ γ -secretase to release A β and the APP intracellular domain (AICD). The amount and type of the produced A β species can be determined by a number of external factors. Pharmacological treatment with non-steroidal anti-inflammatory drugs (NSAIDs) or inherited mutations are known to affect the ratio of the different A β species in opposite directions [6–8], but there are also a number of proteins that modify the interaction between APP and the β - and γ -secretases. SorLA, for example, impairs the formation of the APP-BACE complex, which results in diminished production of A β 40 and A β 42 [9]. By affecting APP maturation and trafficking, Ubiquilin 1 RNAi on the other hand results in increased total A β production without changing the A β 42:A β 40 ratio [10].

Another protein known to interact with APP is the large transmembrane receptor low-density lipoprotein (LDL) receptor-related protein (LRP) [11–13]. Overexpression of LRP in cells leads to higher A β levels and reduced sAPP secretion, whereas lack of LRP in cells influences sAPP secretion and A β production in the opposite direction [14,15]. As indicated by the few examples referred to in the last paragraph, a very complex set of interactions between a multitude of proteins is taking place in many different compartments of the cell to generate A β . Thus, the exact study of these protein-protein interactions is an important prerequisite to explore novel ways to manipulate A β production.

Traditionally, standard biochemical methods such as co-immunoprecipitation or GST-pulldown experiments are employed to study the interaction of proteins. A major drawback of these techniques is that they often require overexpression of proteins to yield consistent results. Furthermore, cells have to be lysed and the proteins' physiological environment has to be disrupted, which makes it hard to detect weak or transient interactions.

An alternative approach to assess close proximity between molecules in intact cells are microscopic techniques, which are based on fluorescence (or Förster) resonance energy transfer (FRET). To perform these assays, the molecules of interest have to be immunolabeled with a donor and acceptor fluorophore. When the donor fluorophore is optically excited, energy is transferred via dipole-dipole interaction to the acceptor molecule. Usually, FRET is most efficient if the absorption spectrum of the acceptor overlaps with the donor's emission spectrum. Since FRET depletes the excited state population of the donor, it results in both reduced fluorescence intensity and fluorescence lifetime of the donor fluorophore.

Here, we describe the use of fluorescence lifetime imaging microscopy (FLIM) to study the interaction between APP and LRP in intact cells as an example for the interaction of proteins which are relevant in the pathogenesis of AD. A FITC/Cy3-FRET-pair with FITC as donor and Cy3 as acceptor was used to immunolabel the proteins of interest. The FLIM assay was performed in general as previously described [16–19] and is explained in detail in the following protocol.

Description of Method

Equipment and reagents

Cells, Antibodies, and Expression Constructs—Human neuroglioma (H4) and murine neuroblastoma (N2A) cells were obtained from the American Type Culture Collection (www.atcc.org). The generation of expression constructs encoding APP770-V5 and full-length LRP with a myc tag at the C-terminus was described previously [11,20]. The authenticity of all constructs was verified by sequencing.

Mouse monoclonal 8E5 antibody against an extracellular epitope of the APP N-terminus was a generous gift from Elan pharmaceuticals (San Francisco, CA). Rabbit polyclonal anti-LRP antibody R2629 (829) against an extracellular epitope of LRP was a generous gift from Dr. D. Strickland (Baltimore, MD). Secondary antibodies goat anti-mouse FITC and goat anti-rabbit Cy3 were obtained from Jackson Immunoresearch (West Grove, PA).

Procedure

Cell culture and transient transfection—H4 and N2A cells were maintained and passaged in OPTI-MEM Media (Gibco, Grand Island, NY) supplemented with 5% fetal bovine serum (FBS) in an incubator at 37°C and 5% CO₂.

H4 cells were plated onto four-well glass chambers (Nalge Nunc International, Naperville, IL) 24 hours before transient co-transfection of LRP and APP constructs using Fugene 6 (Roche, Indianapolis, IN). First, a mixture of 1 µg of plasmid DNA and 3 µl of Fugene 6 was prepared in 100 µl of Dulbecco's Modified Eagle Media (DMEM) and placed at room temperature for 30 min. 25 µl of this mixture was then added to the medium in each well. The incubation time ranged between 24 and 48 hours. To assess interactions of endogenous LRP and APP, N2A cells were plated onto four-well glass chambers (Nalge Nunc International, Naperville, IL) 24 hours prior to immunocytochemistry.

Immunocytochemistry—Immunostaining was performed 24-48 hours after transient transfection (H4) or 24 hours after passaging (N2A). After washing once with cold Tris-buffered saline (TBS), cells were fixed in 4% paraformaldehyde for 10 min. Cells were washed three times with TBS before and after subsequent permeabilization with 0.5% TritonX-100 for 20 min. Cells were then blocked in 1.5% normal goat serum and incubated with primary antibodies in TBS for one hour at room temperature or overnight at 4°C. To detect N-terminal interactions, APP-770-V5 was labeled with 8E5 (1:500 dilution) and full-length LRP-myc was labeled with R2629/829 (1:300 dilution). After removal of primary antibodies, cells were washed thrice with TBS before FITC-conjugated anti-mouse antibody (20 µg/ml) and Cy3-conjugated anti rabbit antibody (10 µg/ml) were applied in TBS for one hour at room temperature. In the negative control, primary antibody for the acceptor was not applied. Cells were washed four times and coverslipped using GVA Mounting solution (Zymed, South San Francisco, CA). Glass slides with immunostained cells were stored at 4°C until the FLIM assay was performed.

Fluorescence lifetime imaging microscopy (FLIM)—FLIM is a FRET-based assay, in which the proximity of two epitopes is assessed after immunostaining. Transfer of energy from a donor to a nearby (<10 nm) acceptor fluorophore is reflected in a decrease in the donor's fluorescence lifetime, which correlates with distance at R⁶ [21,22].

The donor fluorophore FITC was excited on a two-photon system at 800 nm by a femtosecond pulse from a mode-locked Ti-sapphire laser (Mai-Tai, Spectra Physics, Mountain View, CA) using a two-photon Radiance 2000 microscope (Bio-Rad, Hercules, CA). Emissions filtered

at a center of 515 nm were collected by a high-speed Hamamatsu detector and a fast time-correlated single photon counting acquisition board (Becker & Hickl GmbH, Berlin, Germany).

Fluorophore lifetimes were fit to decay curves on SPCImage 2.60 (Becker & Hickl GmbH, Berlin, Germany). FITC-labeled APP served as a negative control, in which the lifetime of the donor fluorophore was calculated by a single-exponential curve fit in the absence of FRET. The resulting non-FRETing FITC lifetime can then be depicted on a pixel-by-pixel base in a pseudo-colored lifetime image. The pseudocolor scale is arranged from red to blue, with red, orange and yellow pixels indicating FRETing molecules, whereas blue pixels indicate molecules that are far apart. Accordingly, the negative control appears mostly in blue (Fig. 1A). Then, fluorophore lifetimes for the negative control and experiments were fit to two-exponential decay curves on SPCImage 2.60. The pseudocolored FLIM images depicting the FRETing populations range in color from yellow to red depending on the degree of the lifetime shortening, i.e. distance between the fluorophores (Fig. 1B).

Lifetime values in the table represent averages across 5–11 cells from a single experiment, whereas the pseudocolored images showing lifetime distributions are representative examples (Figure 1). Statistical analysis was by student's t-test.

Results—H4 cells were stained for transfected APP-770-V5 with 8E5 (labeled by FITC) and for transfected full-length LRP-myc with R2629 (labeled by Cy3). In the negative control, the lifetime of FITC on APP in absence of immunostained LRP was measured. Accordingly, non-FRETing lifetimes of around 2300 psec were detected and the pseudocolored FLIM image appears homogeneously in blue (Fig. 1A). Once both APP and LRP were immunolabeled, a second, significantly shorter lifetime of around 2000 psec was detected, indicating FRET between the two fluorophores. The distribution of red, i.e. FRETing pixels in the pseudocolored FLIM image suggests that APP and LRP come into closest proximity predominantly near the cell surface (Fig. 1B).

These results were verified on endogenous level in N2A cells by immunostaining endogenous APP with 8E5 and FITC and endogenous LRP with R2629 and Cy3. As expected, the negative control shows only one population of non-FRETing fluorophores with a fluorescence lifetime of approximately 2200 psec (Fig. 1C). In the experimental condition, a second lifetime of around 2000 psec is present, which indicates close proximity between the fluorophores. As indicated by the distribution of red pixels in the pseudocolored image, endogenous LRP and APP come into closest proximity in distal subcellular compartments. These results demonstrate that the LRP and APP interaction, which has been shown using biochemical approaches [12, 14] and photobleach-FRET assays [11] can also be visualized in intact cells both on endogenous level and after transient transfection of LRP and APP constructs.

Concluding remarks

Data acquired by FLIM match well with standard photobleach-FRET assays, but provide more information because of the exquisite pixel-by-pixel resolution and the ability to discriminate between and quantify FRETing and non-FRETing protein populations (i.e. to visualize both populations instead of obtaining a mean value) [21]. Furthermore, FLIM measurements are independent of fluorophore concentration or excitation intensity, and can also be used to detect interactions between appropriate “living color” fusion proteins, e.g. variants of EGFP, in live, non-fixed cells. FLIM therefore complements and extends standard biochemical approaches, since protein-protein proximity in intact cells rather than cell lysates can be analyzed.

Furthermore, the FLIM assay can easily be utilized to evaluate the effects of external stimuli or specific alterations in domains and/or ligands on both subcellular localization and

interaction. It thus provides valuable information on the interplay of proteins in response to a variety of factors, which in the case of AD is helpful to gain further insights into the mechanism of A β generation. We especially want to emphasize the unique ability of FLIM to detect conformational changes in a protein in intact cells.

In summary, we have shown that FRET measured by FLIM is a versatile method to detect protein-protein interactions [6,8,9,16,19,21,23], protein dimerisation [24,25], changes in protein phosphorylation [17,18] or conformational changes of proteins [6,21] in intact cells (Fig. 2) with high relevance in AD research, therefore leading to a better comprehension of the molecular and cellular mechanisms in memory decline.

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Abbreviations

Aβ	Amyloid β
AD	Alzheimer's disease
APP	amyloid precursor protein
BACE	β -secretase
CTF	C-terminal fragment
FLIM	Fluorescence lifetime imaging microscopy
FRET	fluorescence resonance energy transfer
LRP	low-density lipoprotein (LDL) receptor-related protein

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Fig.1. APP-LRP interactions in transfected H4-cells as monitored by FLIM

H4 cells were stained for transfected APP-770-V5 with 8E5 (labeled by FITC) and for transfected full-length LRP-myc with R2629 (labeled by Cy3) (Fig. 1A,B). To assess endogenous interactions, N2A cells were immunostained with 8E5 (labeled by FITC) and with R2629 (labeled by Cy3)(Fig 1C,D). For the negative control, primary antibody for the acceptor fluorophore was not applied. The pseudocolored FLIM images and the table show the lifetimes (psec) of the donor fluorophore FITC in the absence or presence of the acceptor fluorophore Cy3. The pseudocolored FLIM image of the negative control (A, C) shows a homogenous image in blue-green pseudocolor and a non-FRETing single lifetime for FITC. The pseudocolored FLIM image of the experimental conditions (B,D) suggests that there is close proximity between APP and LRP mostly on the cell surface, as indicated by the predominance of red pixels in distal cellular compartments. It furthermore indicates that both endogenous and transiently overexpressed LRP and APP come into close proximity in similar subcellular compartments. Statistical testing was performed by Student's t-test.

Different applications of the FLIM assay:

1. Protein-protein interaction

2. Protein-phosphorylation

3. Protein conformation

Fig. 2. Applications of the FLIM assay

This figure shows different applications of the FLIM assay to detect inter- or intramolecular interactions. After immunolabeling of the respective epitopes of interest, it allows for monitoring of protein-protein interactions (1, as described above), protein phosphorylation (P) (2, as described e.g. in [18]) or protein conformation (3, as described e.g. in [6]). Energy transfer from the donor (D) to the acceptor (A) can be measured by FLIM.