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Mass spectrometry quantification of PICALM and AP180 in human frontal cortex and neural retina

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ABSTRACT

Recent genome-wide association studies have suggested that endocytic factors, such as phosphatidylinositol-binding clathrin assembly protein (PICALM), may be implicated in the development of Alzheimer disease (AD). The cellular functions of PICALM are in line with this possibility: (i) PICALM is involved in regulation of amyloid-β levels and (ii) PICALM is important for a presynaptic function, which is diminished in AD. To facilitate the analysis of PICALM, we developed a quantitative method to assess the expression level of PICALM in various biological samples. For this purpose, a stable isotope-labeled quantification concatamer (QconCAT) of PICALM was designed, expressed, purified, and characterized. The PICALM QconCAT was first used as an internal standard in a multiple reaction monitoring assay to measure PICALM concentrations in the human frontal cortex, a tissue strongly affected by AD. A second endocytic factor that is highly homologous to PICALM and also functions in clathrin-mediated endocytosis, clathrin coat assembly protein AP180, was quantified as well. Because age-related macular degeneration shares several clinical and pathological features with AD, the measurements were then extended to human normal neural retina. Overall, the developed method is suitable for PICALM and AP180 quantitative analysis in various biological samples of interest.

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A number of genome-wide association studies (GWAS) and subsequent replication studies with meta-analysis have identified the *PICALM* gene as a new susceptibility gene for late-onset Alzheimer disease (AD) [1–3]. The single-nucleotide polymorphism identified (rs3851179) lies 88.5 kb 5′ of the actual coding region, suggesting that the expression of *PICALM* in AD may be altered. The gene product, phosphatidylinositol-binding clathrin assembly protein (PICALM), is highly homologous to another endocytic factor, clathrin coat assembly protein AP180. Both proteins play a role in clathrin-mediated endocytosis [4] and abnormalities in this pathway have been identified in cases of AD [5,6]. Expression level studies employing immunochemical and mRNA measurements have demonstrated that levels of PICALM and AP180 can be affected in the presence of AD. For example, a significant increase in PICALM in the cortex of transgenic AD mice carrying the Swedish mutation of amyloid-β protein precursor has been reported [7]. Interestingly, other studies have shown a significant decrease in AP180 protein level in brain samples from transgenic AD mice and from human AD patients [8,9]. These findings suggest that PICALM and AP180 are linked to AD, although their exact roles in AD pathology remain to be established.

It is important to emphasize that GWAS identify variants in DNA that are associated with a disease, but this approach cannot by itself be used to specify which genes are causal and therefore cannot be used to calculate disease risk with certainty. However, there is a hope that GWAS may elucidate disease biology and point to new targets for therapy. Thus, it would be undoubtedly beneficial to develop independent quantitative methods for validation of data obtained through GWAS and/or immunochemical and mRNA measurements.

Multiple reaction monitoring (MRM) assays use liquid chromatography–triple-quadrupole mass spectrometry (LC–MS/MS) to measure the concentration of specific proteins in complex biological samples. The high specificity of MRM assays is achieved by monitoring multiple transitions (precursor ion–product ion pairs) for each target peptide derived from the protein of interest. Quantification is performed by comparing the chromatographic peak area of a transition from the (unlabeled) native peptide to that of the corresponding transition from the stable isotope-labeled internal standard. This approach has been successfully used for biomarker validation in human plasma and serum [10–12]. However, the application of MRM assays to the direct measurement

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of proteins in human tissues is more recent [13–15] and remains a challenge.

In this study, we first concentrated on developing an MRM assay to measure the protein levels of PICALM and AP180. It was next applied to measurements in the human frontal cortex, a tissue strongly affected by AD. To validate broad applications of the developed method and since there are some similarities between AD and age-related macular degeneration [16,17], we have extended the measurements of PICALM and AP180 to the normal neural retina. In summary, the proposed method allows quantitative assessment of PICALM and AP180 levels in various biological samples.

Material and methods

Materials

The Expressway cell-free *Escherichia coli* expression system was obtained from Invitrogen (Carlsbad, CA, USA). L-[$^{13}C_6$,$^{15}N_2$]Lysine and L-[$^{13}C_6$,$^{15}N_4$]arginine (>95% purity) were obtained from Spectral Stable Isotopes (Columbia, MD, USA). The DC protein assay kit was obtained from Bio-Rad Laboratories (Hercules, CA, USA). Sequencing-grade modified trypsin was obtained from Promega Corp. (Madison, WI, USA). All other chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA).

Design, expression, and purification of PICALM quantification concatamer (QconCAT)

A synthetic gene encoding the amino acid sequence MVYGNE R*FIQYLASRNTLFNLSNFLDK*SGLQGYISEFLK*VAEQVGIDR*GDIPDLVDERE K*QAALEEEQAR*LKALKENPFLTK*SSGDVHLSISSDVSTFTTR*TPTHEM was synthesized by Integrated DNA Technologies (Coralville, IA, USA). The segments in italic represent the signature proteotypic peptides of PICALM (Q-peptides). The synthetic *PICALM* gene was cloned into the pEXP5CT/TOPO expression vector in-frame to the C-terminal His_6 tag. Stable isotope-labeled PICALM QconCAT was expressed using an in vitro translation Expressway cell-free *E. coli* expression system according to the manufacturer's protocol. L-[$^{13}C_6$,$^{15}N_2$]Lysine and L-[$^{13}C_6$,$^{15}N_4$]arginine were added to the amino acid mixture to replace unlabeled lysine and arginine. After *in vitro* translation, the labeled PICALM QconCAT was purified by nickel–nitrilotriacetic acid resin in batch mode (Qiagen, Valencia, CA, USA). Finally the purified QconCAT was loaded onto a SpinTrap G-25 spin column (GE Healthcare, Waukesha, WI, USA) to exchange buffer into 25 mmol/L NH_4HCO_3 with 1% SDS (mass concentration). The protein concentration of PICALM QconCAT was measured in the presence of 1% SDS (mass concentration) using the DC protein assay kit and bovine serum albumin as a standard. The final PICALM QconCAT was aliquotted and kept frozen at −80 °C.

To verify full-length expression of PICALM QconCAT, 1 μl of sample was mixed with 1 μl of matrix solution (20 mg/ml sinapic acid in 50% acetonitrile/0.1% trifluoroacetic acid). The mixed sample was spotted onto an ABI 01-192-6-AB target plate and allowed to dry at room temperature. Mass spectrometry analysis was performed using an AB4700 proteomics analyzer (Applied Biosystems, Framingham, MA, USA). Linear mode acquisition consisted of 2000 laser shots averaged over 40 sample positions.

Human tissues

Human tissue use conformed to the Declaration of Helsinki and was approved by the ethics committees at Washington University and Case Western Reserve University. Brain and retinal specimens were obtained from deidentified donors following informed consent of the respective families. Demographic information on the donors is summarized in Supplementary Table S1. Frozen samples of frontal cortex were received from the Washington University School of Medicine Alzheimer's Disease Research Center. Eyes were acquired through the Cleveland, Georgia, and Midwest Eye Banks and dissected as described [18]. The anterior segment was removed, and fundus photos were taken to assess retinal status by a fellowship-trained retinal specialist. Thereafter, the neural retina was isolated, flash-frozen in liquid nitrogen, and stored at −80 °C until analyzed.

Processing of samples

Minced human tissue was placed in 25 mmol/L NH_4HCO_3 with 1% SDS (mass concentration) and homogenized by sonication at 30 W using five 10-s continuous cycles (Sonicator 3000, Misonix, Farmingdale, NY, USA). The homogenate was centrifuged at 2000*g* for 5 min to remove tissue debris. The supernatant was used to measure total protein concentration in the presence of 1% SDS (mass concentration) using the DC protein assay kit and bovine serum albumin as a standard. The supernatant was then aliquotted at 0.2 mg of total tissue protein per tube and kept frozen at −80 °C. During the following experiments, samples of 0.2 mg of total tissue protein were supplemented with 20 mmol/L dithiothreitol and various amounts of labeled PICALM QconCAT, ranging from 0.3 to 5.0 pmol per sample. The mixtures were incubated at room temperature for 60 min to allow reduction of cysteines and were then treated with 50 mmol/L iodoacetamide for another 60 min. Alkylated samples were precipitated with chloroform/methanol [19]. This step depleted salts, SDS, and biological lipids from the samples. To remove possible trace contaminants, the protein pellets were sonicated in 1 ml of water and precipitated again by centrifugation at 20,000*g* for 10 min. After being washed with water, protein pellets were sonicated in 100 μl of 25 mmol/L NH_4HCO_3/0.1% Rapi*Gest* and treated with trypsin for 15 h at 37 °C. The substrate/trypsin ratio was 50/1 (M/M). After trypsin digestion, the peptide samples were treated with 0.5% trifluoroacetic acid for 30 min at 37 °C and centrifuged at 106,000*g* for 30 min to remove Rapi*Gest* and other by-products not soluble at low pH. After centrifugation, the supernatants were dried using a vacuum centrifuge (Vacufuge, Eppendorf AG, Hamburg, Germany).

LC–MS/MS analysis

Instrumental analyses were performed on a hybrid triple-quadrupole/linear ion trap mass spectrometer (4000 QTRAP, ABI/MDS-Sciex) coupled to an Eksigent NanoLC-2D system (Dublin, CA, USA). Separation of peptides was performed with an Eksigent cHiP-LC–Nanoflex system equipped with a Nano cHiPLC column, 15 cm × 75 μm, packed with ReproSil-Pur C18-AQ, 3 μm (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany). Peptides were eluted over a 29-min gradient from 15 to 35% acetonitrile (volume concentration) containing 0.1% formic acid (volume concentration) at a flow rate of 300 nl/min. The column effluent was continuously directed into the Nanospray source of the mass spectrometer. All acquisition methods used the following parameters: an ion spray voltage of 2200 V, curtain gas of 105 kPa (15 psi), source gas of 140 kPa (20 psi), interface heating temperature of 170 °C, declustering potential of 76 V for +2 precursor ions and 65 V for +3 precursor ions, collision energy of 30 V for +2 precursor ions and 22 V for +3 precursor ions, and collision cell exit potential of 16 V for +2 precursor ions and 13 V for +3 precursor ions. The dwell time for all transitions was 40 ms.

Quantitative analysis and validation

Three most intensive transitions per Q-peptide used for quantification are summarized in Supplementary Table S2. The relative