Biochimica et Biophysica Acta 1854 (2015) 835–843

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Dissociation of a BRICHOS trimer into monomers leads to increased inhibitory effect on Aβ42 fibril formation

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ARTICLE INFO

Article history:
Received 18 December 2014
Received in revised form 17 March 2015
Accepted 9 April 2015
Available online 17 April 2015

Keywords:
Molecular chaperone
Alzheimer's disease
Protein structure

ABSTRACT

The BRICHOS domain is associated with human amyloid disease, and it efficiently prevents amyloid fibril formation of the amyloid β-peptide (Aβ) in vitro and in vivo. Recombinant human prosurfactant protein C (proSP-C) BRICHOS domain forms a homotrimer as observed by x-ray crystallography, analytical ultracentrifugation, native polyacrylamide gel electrophoresis, analytical size exclusion chromatography and electrospray mass spectrometry. It was hypothesized that the trimer is an inactive storage form, as a putative substrate-binding site identified in the monomer, is buried in the subunit interface of the trimer. We show here increased dissociation of the BRICHOS trimer into monomers, by addition of detergents or of bis-ANS, known to bind to the putative substrate-binding site, or by introducing a Ser to Arg mutation expected to interfere with trimer formation. This leads to increased capacity to delay $A\beta_{42}$ fibril formation. Cross-linking of the BRICHOS trimer with glutaraldehyde, in contrast, renders it unable to affect $A\beta_{42}$ fibril formation. Moreover, proSP-C BRICHOS expressed in HEK293 cells is mainly monomeric, as detected by proximity ligation assay. Finally, proteolytic cleavage of BRICHOS in a loop region that is cleaved during proSP-C biosynthesis results in increased capacity to delay $A\beta_{42}$ fibril formation. These results indicate that modulation of the accessibility of the substrate-binding site is a means to regulate BRICHOS activity.

1. Introduction

Amyloidogenesis involves aggregation of soluble proteins into β-sheet fibrils and is related to 30 different diseases including Alzheimer's disease (AD) and the prion diseases [1]. The proteins involved in amyloid diseases share no structural similarities in their native fold, while the disease related amyloid fibrils are virtually identical irrespective of origin [2]. AD is an incurable neurodegenerative disorder linked to misfolding and amyloid formation of the amyloid β-peptide (Aβ) [3]. The AD pathology is characterized by extracellular amyloid plaques of the Aβ peptide in the central nervous system and by intracellular tangles of phosphorylated tau protein [4]. The Aβ peptide is derived from the amyloid precursor protein (APP), an integral membrane protein of unknown function, by sequential processing by β- and γ-secretases. The γ-secretase cleaves APP in the transmembrane (TM) region, resulting in Aβ peptides of varying length, most commonly 40 or 42 residues long, whereof $A\beta_{42}$ is the more aggregation prone variant [5]. What causes Alzheimer's disease is not fully understood, but it is believed that the transition of unstructured monomeric Aβ into β-sheet-rich oligomers and fibrils is a key element [3,6,7].

The BRICHOS domain is about 100 residues long and is found in a diverse set of type II TM proteins. The name BRICHOS comes from three precursor proteins in which it was first found; Bri2, Chondromodulin-1 and prosurfactant protein C (proSP-C) [8]. Today there are over 1000 BRICHOS-containing proteins described (www.smart.embl-heidelberg.de) [9]. The BRICHOS-containing precursor proteins consist of five different regions: an N-terminal cytosolic part, a hydrophobic TM part, a linker, the BRICHOS domain and a C-terminal region. The only exception is proSP-C that is missing the C-terminal region. The precursor proteins are processed by proteolysis into different peptides, derived either from the C-terminal region, e.g., the Bri23 peptide from Bri2, or mature SP-C that largely corresponds to the TM region of proSP-C. The BRICHOS domain as such can also be released by proteolysis [10–14]. The overall sequence homology between BRICHOS domains from different precursor proteins is low, but the secondary and tertiary

Abbreviations: Aβ, amyloid β-peptide; APP, amyloid precursor protein; bis-ANS, 1,1′-bis(4-anilino-5,5′-naphthalenesulfonate); CD, circular dichroism; ER, endoplasmic reticulum; PLA, proximity ligation assay; SP-C, surfactant protein C; SEC, size exclusion chromatography; TEM, transmission electron microscopy; ThT, thioflavin T; wt, wild type

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http://dx.doi.org/10.1016/j.bbapap.2015.04.005
1570-9639/

structures are apparently more conserved [9,15]. Mutations in BRICHOS-containing proteins are associated with amyloid diseases, including lung fibrosis (proSP-C mutations) and familial dementia (Bri2 mutations) [13,16,17].

The first experimentally investigated BRICHOS domain was the one from proSP-C [18]. The mature SP-C peptide consists of 35 residues, is cleaved out from proSP-C, and residues 9–35 form a TM helix [19,20]. SP-C mainly consists of valine residues, an amino acid prone to facilitate formation of β-sheets, which makes the SP-C α-helix metastable, and it can easily convert into β-sheet aggregates and amyloid fibrils [21–25]. A feature of all BRICHOS-containing precursor proteins is that they have a region with high β-sheet propensity. In proSP-C, this region is situated in the TM part (i.e., corresponding to the poly-valine part of SP-C), while for the other BRICHOS-containing proteins the C-terminal region contains known or predicted β-hairpin motifs [9,14,15]. It is possible that a main function of the BRICHOS domains is to protect these β-sheet prone regions from misfolding. In line with this supposition, mutations in the proSP-C BRICHOS domain have been shown to cause lung fibrosis associated with amyloid inclusions of SP-C [13]. Mutations in Bri2 that give rise to elongated C-terminal peptides (ABri or ADan) are also associated with amyloid deposits [16,17]. Recombinant proSP-C and Bri2 BRICHOS domains efficiently delay amyloid fibril formation of $A\beta_{40}$ and $A\beta_{42}$ [26,27], and it was recently shown that proSP-C BRICHOS prevents toxicity of $A\beta_{42}$ in the CNS of *Drosophila melanogaster* flies [28]. These observations strongly indicate that BRICHOS domains can inhibit fibril formation, and the toxicity associated herewith, also of peptides that are not their physiological client peptides, and thus possess "anti-amyloid" activity.

The structure of proSP-C BRICHOS domain has recently been solved by X-ray crystallography. ProSP-C BRICHOS crystallizes as a trimer and also analytical ultracentrifugation, native polyacrylamide gel electrophoresis, analytical size exclusion chromatography (SEC) and electrospray mass spectrometry indicate a trimeric structure [13,29]. However, it was proposed from molecular dynamics simulations, which showed that face A of the central β-sheet can expose a hydrophobic binding site if helix 1 moves away, and that this only occurs in the monomer, and from observed complementarity between the properties of this face A in different BRICHOS families and their putative client peptides, that the monomer is the active species, while the trimer is an inactive storage form (Fig. 1) [15,13]. Herein, we investigate the quaternary structure of proSP-C BRICHOS when expressed in eukaryotic cells and study how modulation of the trimer/monomer ratio affects the ability to reduce $A\beta_{42}$ fibril formation.

2. Methods

2.1. Proximity ligation assay (PLA)

HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM/F-12 + GlutaMAX) (Gibco), supplemented with 10% fetal calf serum. Stable clonal cell lines were generated by transfection with lipofectamine 2000 (Invitrogen) and pBudCE4.1 (Invitrogen) plasmids with proSP-C BRICHOS (residues 59–197, GenBank accession no. NM_003018 [30]) or full-length proSP-C (residues 1–197, GenBank accession no. NM_003018), including a C-terminal V5 epitope tag for detection. In the proSP-C BRICHOS construct, an N-terminal signal peptide sequence from proSP-B (residues 1–23) was inserted to give translocation to the endoplasmic reticulum (ER). Stably expressing cells were selected by addition of 0.2 mg/mL Zeocin (Invitrogen). PLA assay was performed according to Duolink II kit manufacturer's instructions (OlinkBioscience). Briefly, cells were fixed in 4% paraformaldehyde/PBS solution for 15 min, washed twice in PBS and permeabilized with 0.5% Triton X-100/PBS for 15 min, both at room temperature. A monoclonal mouse anti-V5 antibody (Invitrogen, P/N 46-0705) was conjugated either with an oligonucleotide termed plus, or a complementary oligonucleotide termed minus. For detection of oligomeric proSP-C BRICHOS or oligomeric full-length proSP-C, the plus and minus probes were incubated with the cells for 1 h at room temperature, followed by ligation, amplification and washing steps. For the detection of proSP-C BRICHOS or full-length proSP-C independent of oligomeric status, anti-V5 antibody, conjugated with plus probe, were added for 1 h at room temperature. After washing, anti-mouse secondary antibody conjugated with minus oligonucleotide probe (Olink Biosciences) was added, followed by ligation, amplification and washing. Wild-type (wt) HEK293 control cells were treated either with both plus and minus oligonucleotide-conjugated V5 antibodies, or with primary plus-conjugated antibodies and secondary minus-conjugated antibodies. Cells were stained with DAPI (OlinkBioscience), mounted and analyzed by a Zeiss confocal microscope (Axiovert 200 M). Images were acquired at one confocal plane at 40× or 63× magnification.

2.2. Aβ peptide

Met-Aβ1-42 (hereafter referred to as $A\beta_{42}$) was expressed in *Escherichia coli* BL21 from synthetic genes and purified in batch format using ion exchange and size exclusion steps as described [31], which results in highly pure monomeric peptide. Purified $A\beta_{42}$ was aliquoted in

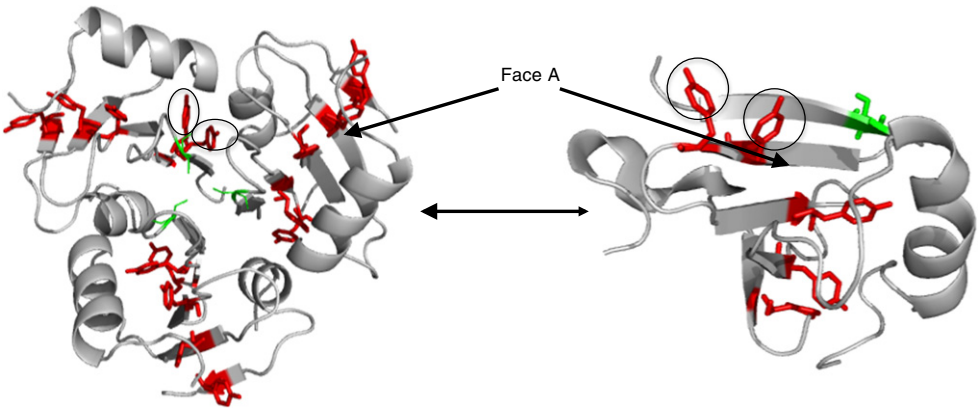

Fig. 1. Structures of trimer and monomer of proSP-C BRICHOS. Backbone representation of proSP-C BRICHOS trimer (PDB ID: 2yad) (left side), and the monomer subunit after molecular dynamics simulation (right side). The Tyrosines in face A are highlighted in red, Ser95 is highlighted in green, and their side-chains are represented as stick models. Tyr at positions 104 and 106, which are buried in the trimer but becomes exposed in the monomer, are circled, and face A of the β-sheet is identified by arrows.