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Research paper

Anti-amyloidogenic property of human gastrokine 1

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ABSTRACT

Gastrokine 1 (GKN1) is a stomach-specific protein expressed in normal gastric tissue but absent in gastric cancer. GKN1 plays a major role in maintaining gastric mucosa integrity and is characterized by the presence of a BRICHOS domain consisting of about 100 amino acids also found in several unrelated proteins associated with major human diseases like BRI2, related to familial British and Danish dementia and surfactant protein C (SP-C), associated with respiratory distress syndrome. It was reported that recombinant BRICHOS domains from BRI2 and SP-C precursor (proSP-C) prevent fibrils formation of amyloid-beta peptide (Aβ), that is the major component of extracellular amyloid deposits in Alzheimer's disease. Here we investigated on the interaction between human recombinant GKN1 (rGKN1) and Aβ peptide (1–40) that derives from the partial hydrolysis of the amyloid precursor protein (APP). GKN1 prevented amyloid aggregation and fibrils formation by inhibiting Aβ(1–40) polymerization, as evaluated by SDS-PAGE, thioflavin-T binding assay and gel filtration experiments. Mass spectrometry showed the formation of a prevailing 1:1 complex between GKN1 and Aβ(1–40). SPR analysis of GKN1/Aβ interaction led to calculate a dissociation constant (K_D) of 34 μM. Besides its interaction with Aβ(1–40), GKN1 showed also to interact with APP as evaluated by confocal microscopy and Ni-NTA pull-down. Data strongly suggest that GKN1 has anti-amyloidogenic properties thus functioning as a chaperone directed against unfolded segments and with the ability to recognize amyloidogenic polypeptides and prevent their aggregation.

1. Introduction

Gastrokine 1 (GKN1), a tissue-specific protein, is expressed in the human stomach of healthy individuals but is absent in gastric adenocarcinoma tissues [1–3]. Individuals with a low expression of the protein have an increased risk to develop gastric diseases [4,5]. The protein is, in fact, downregulated in gastric mucosa samples infected by *Helicobacter pylori*, considered as one of the leading cause for gastric cancer development [2]. Evidence suggests that GKN1 is involved in filling the lumen of the surface layer of epithelial cells to maintain the integrity of the mucosa and to regulate cell proliferation and differentiation [1,6]. Human GKN1 is made of 185 amino acids containing a 20 amino acid extracellular

Abbreviations: Aβ(1–40), amyloid-beta peptide 1-40; GKN1, gastrokine 1; flGKN1, full length GKN1; rGKN1, recombinant GKN1; proSP-C, prosurfactant protein C; Ni-NTA, nickel-nitrilotriacetic acid; APP, amyloid precursor protein.

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signal peptide localized in the N-terminal region [1]. The protein is characterized by the presence of a central structural BRICHOS domain [7] of about 100 amino acids containing two conservative cysteine residues most likely involved in disulfide bridges [8]. The association of GKN1 BRICHOS domain with biological functions has been proposed but not yet ascertained. In fact, the BRICHOS domain has been found in proteins associated with a wide range of functions and diseases. These include BRI2, which is related to familial British and Danish dementia (FBD and FDD); Chondromodulin-I (ChM-I), related to chondrosarcoma and lung surfactant protein C (SP-C), related to respiratory distress syndrome (RDS) [7]. Although GKN1 seems to play an important role in the physiology of gastric mucosa and in suppressing carcinogenic processes, a full characterization of its structural and biological activities is still lacking. To accomplish this deficiency, we have recently reported the characterization and secondary structural properties of human recombinant GKN1 (rGKN1). Using bio-informatics tools, we found that GKN1 BRICHOS domain has structural features similar to those endowed by BRICHOS domain-containing protein family [8]. In particular, predicted GKN1 3D models suggested a structural organization of its BRICHOS domain resembling that of the corresponding BRICHOS domain of prosurfactant protein C (proSP-C), a trans membrane (TM) protein expressed in epithelial type II cells. proSP-C contains in the TM region a polypeptide segment defined "discordant α-helix" because its structure, according to secondary structure prediction should form instead a β-strand [9]. This region can misfold and form amyloid fibrils associated with pulmonary disease [10,11]. It has also been shown that the C-terminal domain (CTC) of proSP-C and its BRICHOS domain protect the TM part of proSP-C from aggregation into amyloid [12,13]. Moreover, it has been shown that recombinant BRICHOS domains from BRI2, as reported by Peng et al., 2010 [14], prevents fibrils formation of amyloid-beta peptide (Aβ), the major component of extracellular amyloid deposits in Alzheimer's disease [15]. Similar property has also been shown by the precursor of lung surfactant protein C (proSP-C) [13].

These findings led us to explore whether also rGKN1 was endowed with a chaperone-like activity toward amyloidogenic peptides. Using biochemical, spectroscopic and mass spectrometry investigations, we demonstrate that rGKN1 affects protein aggregation by interacting with and preventing fibrils formation of Aβ (1–40) peptide. Moreover, using SPR technology we have determined the affinity constant between rGKN1 and Aβ(1–40) peptide. The uncovering of this specific property of GKN1 might provide a valid contribution for developing new strategies to prevent protein misfolding.

2. Materials and methods

2.1. Materials

Amyloid β-peptide (1–40) (DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV) A was purchased from Abnova (Cambridge, UK) and stored as lyophilized powder at −80 °C until its use. To obtain monomeric starting solutions, the peptide was dissolved in dimethyl sulfoxide (DMSO, Merck, Sweden) at a concentration of 138.5 μM before being diluted in experimental buffers. Chicken cystatin was purchased from Sigma–Aldrich (Milan, Italy) and dissolved at a concentration of 76.8 μM before being diluted in experimental buffer. CM5 sensor chips and other reagents and buffers for SPR analyses by BIAcore were from GE Healthcare (Milan, Italy).

2.2. GKN1 expression and purification

Human recombinant GKN1 (rGKN1) lacking the first 20 amino acids leader peptide was expressed and purified as described previously [8]. Stock solutions with concentrations of 313.3 μM rGKN1 were used for the experiments.

2.3. Cell culture, transfection and western blot analysis

Human gastric adenocarcinoma cell line (AGS) and human neuroblastoma cell line (SH-SY5Y) were grown in DMEM-F12 (Dulbecco's modified Eagle medium-Cambrex) and DMEM, respectively, supplemented with heat inactivated FBS, 100 U/ml penicillin,
100 mg/mL streptomycin, 1% L-glutamine at 37 °C in a 5% CO_2 atmosphere and transfected with 4 μg of vector pcDNA 3.1 or pcDNA-GKN1 encoding the full length GKN1 (flGKN1, containing the first 20 amino acids leader peptide and His_6-Tag sequence at the C-terminal) as already described [16]. The efficiency of transfection of gastric cancer cells with
flGKN1 was always evaluated by a parallel transfection using EGFP vector as control. In general, after transfection, the average value of the ratio between number of green fluorescent cells/total number of cells was 0.5 ± 0.1. All experiments were performed in both AGS and SH-SY5Y cells and the results obtained were comparable.

Cells were scraped, washed twice in cold phosphate buffered saline (PBS) and resuspended in 20–40 μL of lysis buffer (50 mM Tris–HCl pH 7.4, 1% NP40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mg/ml aprotinin, leupeptin, pepstatin, 1 mM Na_3VO_4, 1 mM NaF) for 30 min on ice and centrifuged at 14,000 g for 20 min at 4 °C. Protein extract from cell lines was prepared as already described [2]. Protein concentration was determined by a modified Bradford method [17], using the Bio-Rad (Milan, Italy) protein assay and compared with BSA standard curve. Equal amounts of cytosolic proteins (20 μg) were separated by SDS–PAGE, electrotransferred to PVDF membrane and reacted with the different antibodies. Blots were then developed using enhanced chemiluminescence detection reagents (SuperSignal West Pico, Pierce, Rockford, IL, USA.) and exposed to X-ray film. All films were analyzed using Adobe photoshop and Image J software.

2.4. Ni-NTA agarose pull-down

Transfected AGS cell extracts (500 μg) were incubated with 50 μL of Ni-NTA Agarose (Qiagen) pre-equilibrated with binding buffer (50 mM NaH_2PO_4, 300 mM NaCl pH 8.0) for 16 h at 4 °C. After incubation the resin was washed 4 times with binding buffer containing 10 mM imidazole to reduce nonspecific bound proteins, resuspended in 30 μL of SDS loading buffer, heated to 95 °C for 5 min and subjected to Western blot analysis as described above using specific anti-APP antibody (Acris, San Diego, CA).

2.5. Aβ(1–40) aggregation and fibrils formation

Aβ(1–40) (MW 4329.9) was dissolved in DMSO at a final concentration of 138.5 μM. Experiments were performed by co-incubating Aβ(1–40) (17.3 μM) with rGKN1 (1.7 μM) at 37 °C in 10 mM sodium phosphate (NaP) (pH 7.0) and 150 mM sodium chloride (NaCl) with 10% (v/v) DMSO, under agitation. At various time points, samples were removed to determine the level of aggregation. The samples were centrifuged for 6 min at 16,000 g (14,000 rpm), and the supernatants were removed and centrifuged for an additional 2 min at the same speed. The supernatant from the last centrifugation was then analyzed by SDS-PAGE on 16% Tris-Tricine gels under nonreducing conditions and stained with Coomassie. As controls, Aβ(1–40) was incubated with 1.7 μM chicken cystatin (MW 13.3) [18] and analyzed under the conditions described above.