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Blocking the interaction between S100A9 and RAGE V domain using CHAPS molecule: A novel route to drug development against cell proliferation

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ABSTRACT

Human S100A9 (Calgranulin B) is a Ca^{2+}-binding protein, from the S100 family, that often presents as a homodimer in myeloid cells. It becomes an important mediator during inflammation once calcium binds to its EF-hand motifs. Human RAGE protein (receptor for advanced glycation end products) is one of the target-proteins. RAGE binds to a hydrophobic surface on S100A9. Interactions between these proteins trigger signal transduction cascades, promoting cell growth, proliferation, and tumorigenesis. Here, we present the solution structure of mutant S100A9 (C3S) homodimer, determined by multi-dimensional NMR experiments. We further characterize the solution interactions between mS100A9 and the RAGE V domain *via* NMR spectroscopy. CHAPS is a zwitterionic and non-denaturing molecule widely used for protein solubilizing and stabilization. We found out that CHAPS and RAGE V domain would interact with mS100A9 by using ^{1}H-^{15}N *HSQC NMR* titrations. Therefore, using the *HADDOCK* program, we superimpose two binary complex models mS100A9-RAGE V domain and mS100A9-CHAPS and demonstrate that CHAPS molecules could play a crucial role in blocking the interaction between mS100A9 and the RAGE V domain. WST-1 assay results also support the conclusion that CHAPS inhibits the bioactivity of mS100A9. This report will help to inform new drug development against cell proliferation.

1. Introduction

Attempts have been made to identify potential drugs to combat cancer, by understanding protein-protein interaction mechanisms. The human S100A9 protein (Calgranulin B) comprises 114 amino acids and has a molecular weight of 13.2 kDa. It is a member of the S100 protein family. Members of this family contain two EF-hand motifs, which bind Ca^{2+} ions [1]. Human S100A9 preferentially forms heterodimers with the human S100A8 protein (Calgranulin A, 93 amino acids, 10.8 kDa) within myeloid cells [2]. These proteins are also called 'calprotectin proteins', and they are often found at high concentrations within sites of inflammation associated with rheumatoid arthritis, [3,4] cystic fibrosis [5], cancers [6], and certain other diseases [7]. Studies also suggest that the S100A9 protein plays a significant role in the cardiovascular system during atherosclerosis and cardiac dysfunction [8], and that it regulates the accumulation of neutrophils and monocytes/macrophages [9], cytokine production [10], and cell proliferation [11]. S100A9 is one of several proteins known as 'damage associated molecular pattern' (DAMP) proteins [12]. DAMP proteins activate signaling cascades pathways, in multiple human diseases, through interacting with target-proteins, including the receptor for advanced glycation end products (RAGE) and Toll-like receptor 4 (TLR4) [13–15].

RAGE has a molecular weight of 35 kDa and is a member of the immunoglobulin (Ig) superfamily [16]. It consists of three extracellular domains—one variable type domain (V), and two constant type domains (*C*1 and C2)—a transmembrane domain [17], and a cytosolic domain. The V domain of RAGE is the main receptor involved in binding ligands, such as members of the S100 protein family, advanced glycation end products (AGEs) [18,19], amphoterin [20], and high-mobility group protein 1 (HMGB1) [21,22]. Those ligands are DAMP molecules that derive from

Abbreviations: S100A9, S100 calcium-binding protein A9; RAGE, the receptor for advanced glycation end products; HSQC, heteronuclear single quantum correlation; HADDOCK, high ambiguity driven docking; CHAPS, 3-[(3-holamidopropyl)dimethylammonio] -1-propanesulfonate; *E. coli*, *Escherichia coli*; WST-1, Water Soluble Tetrazolium Salts.

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damaged cells, alerting the immune system to the presence of tissue trauma [23]. This kind of signaling receptor (RAGE) participates in an extensive range of inflammatory pathological conditions. When the RAGE V domain interacts with target-proteins, it promotes downstream signal transduction, including mitogen-activated protein kinase (MAPK) [24], and Cdc42/Rac [25] pathways, and signaling from NF-κB [26] transcription factors *via* the formation of homodimeric [27,28], ligand-specific complexes. The cytoplasmic domain contains several charged residues, which influence autophosphorylation. Autophosphoylation of RAGE induces signal cascades and can trigger illnesses, including cancer [29], Alzheimer's disease [30], respiratory disorders [31], retinal disease [32], liver disease [33], and diabetic nephropathy [34]. Thus, an understanding of RAGE signaling pathways is a valuable approach towards the prevention of disease.

3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) is a sulfobetaine derivative of cholic acid [35]. CHAPS is zwitterionic, and this promotes the solubilization of membrane proteins [36]. It is composed of a hydrophilic core and a hydrophobic chain group, the configurations of which confer on CHAPS the ability to bind to proteins that contain hydrophobic regions (Supplementary Fig. 1) [37–39]. Also, CHAPS is considered to be a potential inhibitor to block ligand binding [40,41].

V domain is the primary receptor for S100 protein binding [42]. Some S100 proteins, such as S100B [43], S100A6 [44], and S100A12 [45] can interact with the variable type V domain, simultaneously through their constant domains (*C*1 or C2). Moreover, it is still unclear how S100 family proteins bind to RAGE [42]. To date, there is not much known, structurally, about how the S100A9 protein interacts with target-proteins. Here, we report the interaction between S100A9 and the RAGE V domain. There is one cysteine in the wild type S100A9 molecule. On this account, 5 mM of dithiothreitol (DTT) was needed as a reducing agent in the NMR experiments, to prevent the oxidation of cysteine residues to disulfides. However, the RAGE V domain contains three disulfide bonds. The disulfide bonds in the RAGE V domain would break in the presence of DTT. To solve this problem, we generated a C3S mutant of S100A9 (mS100A9), to obviate the need to use DTT as a reducing agent. Solving the structure of mS100A9 in complex with the RAGE V domain complex *via* NMR spectroscopy became less difficult. In this report, we illustrate by NMR experiments that the symmetrical calcium-bound mS100A9 homodimer binds to two RAGE V domains, forming a heterotetrameric complex. The binding affinity (K_d), between mS100A9 and the RAGE V domain, was determined by using the method of chemical shift perturbation in HSQC titrations [46]. *HADDOCK* was used to simulate a model structure of the mS100A9-RAGE V domain complex [47,48]. All model structures were displayed using *PyMOL* [49]. The model of the mS100A9-CHAPS complex in this study was determined by *HADDOCK* using NMR HSQC titration method [50]. Moreover, the CHAPS molecule is considered to be a potential candidate for drug design. It was used to block the interaction between S100A9 and the RAGE V domain, inhibiting signal transduction. We further proved the inhibition properties of CHAPS by WST-1 cell proliferation assays [51]. WST-1 assays and NMR spectroscopy inform on the simultaneous interactions between mS100A9, the RAGE V domain, and CHAPS molecules. Finally, we provide putative models of calcium-bound mS100A9, in complex with a CHAPS molecule, and the RAGE V domain, which may provide a significant insight for future treatments of cancer or inflammation.

2. Materials and methods

2.1. Materials

All isotope-labeled chemicals were obtained from Cambridge Isotope Laboratories. These included ^{13}C-D-glucose ($U^{13}C_6$, 99%), ^{15}N-ammonium chloride ($^{15}NH_4Cl$, 99%), and deuterium oxide (D_2O, 99%). CHAPS was purchased from Sigma-Aldrich. Milli-Q water was used to prepare all the solutions. Buffers for NMR spectroscopy samples were passed through a sterile 0.22 μm filter.

2.2. Preparation of mS100A9 (C3S) and the RAGE V domain

The cDNA clone of mS100A9 (C3S) was purchased from Mission Biotech Company. This mS100A9 cDNA was cloned into pET-21b, which is a bacterial expression vector. Then, following transformation, the encoded protein was produced in *Escherichia coli* BL21 (DE3) (Novagen). To prepare ^{15}N and ^{13}C-labeled protein samples, cells were grown in M9 medium containing ^{13}C-D-glucose and $^{15}NH_4Cl$ as the sole carbon and nitrogen sources. Ampicillin was added to a final concentration of 1 mM, and cultures were allowed to grow at 310 K, until the optical density (O.D.) at 600 nm reached 0.8. Cultures were induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), at 200 rpm and 294 K. Follow 16 h overnight incubation post-induction, the cells were harvested by centrifugation at 5500 rpm for 25 min. The pellet was re-suspended in 20 mM Tris-HCl, 0.5 mM EDTA buffer, pH 8.0. The cells were lysed using a French pressure cell and a sonicator. These protein solutions were centrifuged at 13,000 rpm for 45 min, to remove cell lysate, and then filtered through a 0.2 μm filter prior to further purification. The supernatant was purified using a Q Sepharose Fast Flow (GE Healthcare) anion exchange chromatography column, which had been washed and equilibrated in buffer containing 20 mM Tris-HCl, 0.5 mM EDTA, pH 8.0. The mS100A9 protein was eluted in a buffer containing 20 mM Tris-HCl, 0.5 mM EDTA, pH 8.0, using a 0.1–0.2 M NaCl gradient, at the flow rate of 4 mL/min. Fractions containing protein were further purified using a Hi-Prep Phenyl FF 16/10 (GE Healthcare) hydrophobic interaction chromatography (HIC) column *via* exchange of the initial buffer for the loading buffer (50 mM Tris-HCl, 5 mM EDTA, pH 7.5). The target-protein was detached from the column using elution buffer (50 mM Tris-HCl, 5 mM $CaCl_2$, pH 7.5). As a final purification step, the protein was exchanged into NMR buffer (50 mM Tris-HCl, 100 mM NaCl, 2 mM $CaCl_2$, pH 7.5) by gel filtration chromatography, using a HiLoad 16/60 Superdex 75 pg (GE Healthcare) column. We cloned the recombinant RAGE V domain into pET-32b (+) expression vector. The plasmid was transformed into competent *E. coli* BL21 (DE3) cells. Ampicillin was added to a final concentration of 1 mM. Cultures were then grown at 310 K, until the optical density (O.D.) at 600 nm reached 0.7. Cultures were then induced with 1 mM IPTG, at 200 rpm and 298 K. Following 3 h incubation post-induction, we harvested the cells by centrifugation at 5500 rpm for 25 min. The pellet was re-suspended in a buffer containing 20 mM Tris-HCl, 300 mM NaCl, pH 8.0. The cells were lysed using a sonicator. These protein solutions were centrifuged at 13,000 rpm for 45 min to remove the cell lysate, and were then filtered through a 0.2 μm filter, prior to further purification. The protein solution was purified by IMAC chromatography using an IMAC chromatography column (Ni Sepharose 6 Fast Flow; GE Healthcare), which captured the RAGE V domain through its N-terminal hexahistidine tag. The RAGE V domain was eluted in a buffer containing 20 mM Tris-HCl, 300 mM NaCl, pH 8.0, and 500 mM imidazole. The hexahistidine tag was then cleaved by thrombin digestion for 3 h at 298 K. Finally, the RAGE V domain was isolated by HPLC (Atlantis Prep dC_{18} 5 μm column), using a gradient of 45% acetonitrile, 0.1% trifluoroacetic acid over 42 min. SDS-PAGE analysis showed that the mS100A9 and RAGE V domain protein samples were ~95% pure (Supplementary Figs. 2 and 3). Their molecular weights were verified using ESI-MS (Supplementary Figs. 4 and 5).

2.3. Size exclusion chromatography

Size exclusion chromatography was executed on an ÄKTA FPLC system (GE Healthcare) using a HiLoad 16/60 Superdex 75 pg column (GE Healthcare) at 298 K. The column was equilibrated with running buffer (100 mM NaCl, 2 mM $CaCl_2$, 50 mM Tris–HCl, pH 7.5), at a 1 mL/min flow rate. A calibration was generated using the following protein standards (Sigma-Aldrich): bovine serum albumin (66.5 kDa), ribonuclease