Elucidating the structure and function of S100 proteins in membranes

Stella M. Valenzuela*[a], Mark Berkahn[b], Donald K. Martin[a], Thuan Huynh[a], Zheng Yang[c] and Carolyn L. Geczy[c]

[a]Department of Health Sciences and The Institute for Nanoscale Research, University of Technology Sydney, Broadway, NSW, Australia 2007; [b]Microstructural Analysis Unit, University of Technology Sydney, Broadway, NSW, Australia 2007; [c]School of Medical Sciences, University of New South Wales, Kensington, Australia 2052

ABSTRACT

S100 proteins are important Ca^{2+}-binding proteins involved in vital cellular functions including the modulation of cell growth, migration and differentiation, regulation of intracellular signal transduction/phosphorylation pathways, energy metabolism, cytoskeletal interactions and modulation of ion channels. Furthermore, they are implicated in oncogenesis and numerous other disease states. Three S100 proteins: S100A8, S100A9 and S100A12 are constitutively expressed in neutrophils and monocytes. At low levels of intracellular Ca^{2+}, S100A8 and S100A9 are located predominantly in the cytosol but when Ca^{2+} concentrations are elevated as a consequence of activation, they translocate to membranes and complex with cytoskeletal components such as vimentin. The functions of S100A8 and S100A9 at the plasma membrane remain unclear. A possible role may be the regulation of ion channel proteins. The current study uses the techniques of Atomic Force Microscopy and production of artificial lipid membranes in the form of liposomes to investigate possible mechanisms for the insertion of these proteins into membranes in order to elucidate their structure and stoichiometry in the transmembrane state. We have successfully imaged the liposomes as a lipid bilayer, the S100A8/A9 protein complex in solution and the S100A8/A9 complex associating with lipid, using tapping-mode atomic force microscopy, in buffer.

Keywords: S100 proteins, liposomes, atomic force microscopy

1. INTRODUCTION

The S100 proteins are highly homologous, low-molecular-weight (10-14 kDa), calcium modulated proteins belonging to the EF hand superfamily. They are over-expressed in many tumours and have been used as markers for tumour classification[1]. They are implicated in fundamental intra- and extracellular processes including regulation of cell cycle, embryogenesis, apoptosis, signal transduction, cell migration, adhesion, cytoskeletal-membrane associations, fatty acid transport[2] and ion channel modulation[3].

The three "myeloid-associated" S100 proteins S100A8, S100A9, S100A12 are expressed constitutively in large amounts by neutrophils (together they constitute approximately 45% of total neutrophil cytoplasmic protein) and are induced in monocytes/macrophages[4], endothelial cells, keratinocytes[5] and fibroblasts by a variety of mediators that regulate inflammation[6]. S100A8 and S100A9 form a complex, known as calprotectin, which is implicated in neutrophil defence by virtue of its anti-microbial activity, which is dependent on the Zn^{2+}-binding capacity of S100A9[7]. The complex is lipophilic and intracellularly is a major transporter of unsaturated fatty acids and arachidonic acid[8]. S100A9 associates with calcifying microvesicles and is proposed to play a role in dystrophic calcification[9]. S100A8 may protect tissues against excessive oxidative damage by scavenging chlorinated oxidants[10]. S100A8 and S100A9 are associated with chronic inflammatory diseases such as rheumatoid arthritis, cystic fibrosis, Crohn's disease, ulcerative colitis, allergic dermatitis, atherosclerosis[9] and infection[6]. At low levels of intracellular Ca^{2+} typical of resting cells, S100A8 and S100A9 are located predominantly in the cytosol but following elevation of Ca^{2+} concentrations as a consequence of cell activation, they translocate to membranes and cytoskeletal components, such as vimentin[11, 12], in neutrophils and monocytes. The function of these S100 proteins at the plasma membrane remains unclear, although a recent publication has implicated the S100A8/A9 protein complex in the delivery of arachidonic acid to the membrane-bound flavocytochrome *b* and thus in the regulation of NADPH oxidase in neutrophils[13].

*stella.valenzuela@uts.edu.au; phone +61 2 9514 1917; fax +61 2 9514 2228

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A possible role for these proteins at the membrane may be to regulate ion channel proteins. Studies by Kubista et al, (1999)[3] demonstrate that S100B and S100A1 acutely affect the electrophysiology of cells. Extracellular application of disulphide-linked S100B to Helix neurons hyperpolarized the resting membrane potential, inhibited spontaneous discharge activity of action potentials, shifted stimulus response behaviour from tonic to phasic, reduced cell input resistance and changed the shape of action potentials. These effects appeared to relate to modulation of three types of potassium currents. Intracellular application of S100A1 mimicked the effect of extracellular S100B on this stimulus response behaviour. Furthermore, S100A1 and S100B interact directly with members of the annexin family of proteins, forming heterotetramers. Evaluation of the functional properties of these complexes and S100 proteins alone revealed the potential for S100 proteins to permeabilize membrane bilayers in a similar fashion to the annexins, which generally results in decreased calcium influx[14].

Members of the S100 family exert extracellular functions although they do not contain signal sequences required for secretion via the classical endoplasmic reticulum (ER)/Golgi pathway[15]. The heterocomplex of S100A8/S100A9 containing bound arachidonic acid has been shown to be secreted from phorbol ester-stimulated neutrophil-like HL-60 cells[8]. Their mode of secretion however, is still unclear and in this way resembles cytokines such as interleukin-1β (IL-1β) and basic fibroblast growth factor (bFGF). Rammes et al (1997)[15] demonstrated that S100A8 may be secreted together with S100A9 from monocytes via a novel tubulin-dependent pathway which requires an intact microtubule network. Interestingly, evidence is accruing for a role of ABC transporters [CFTR, p-glycoprotein and multidrug resistance-associated protein (MRP) are members of this family of proteins] in protein secretion[16, 17]. Aggarwal and Gupta (1998)[18] showed an association between MRP and bFGF secretion in osteogenic sarcoma MG-63 cells, which spontaneously secrete bFGF. Thus, ABC transporters may also play a role in secretion of S100 proteins.

The crystal structure for several S100 protein family members has been determined, including those of S100A9[19], S100A8[20] and S100A12[21]. With the exception of calbindin D_{9k}, which is monomeric, the other small S100 proteins may generally exist within cells as non-covalently attached homo- or heterodimers[22]. S100A8 and S100A9 form a heterocomplex[22] in the presence or absence of calcium[8] and this complex binds arachidonic acid in a calcium-dependent manner, whereas the individual proteins are unable to bind fatty acids in the presence or absence of calcium[8]. Experimental data indicate that the heterocomplex is a heterodimer but a tetrameric complex consisting of 2 molecules of S100A8 and 2 molecules of S100A9 can also form[8]. No crystal structure exists for the dimeric form of the heterocomplex. Molecular modelling of the calcyclin (S100A6) dimer reveals a noncovalent antiparallel homodimer, in which the interface between the 2 molecules is mediated principally by hydrophobic residues from the C-terminal helix (helix IV) along with hydrophobic residues of the C-terminal part of helix I[23]. More recently, a study by Menke et al, (2004)[24] addressed the molecular organization of the membrane-bound annexin A2-S100A10 tetramer by scanning force microscopy. Similarly, Berthier et al, (2003)[25] used atomic force microscopy (AFM) to investigate interactions between the soluble S100A8/S100A9 complex and cytochrome b_{558} which was incorporated into liposomes. Their measurements were carried out, in air, on dried samples using non-contact mode AFM. Although to date there is a plethora of information on the soluble crystal forms of these S100 proteins, the membrane associating form and the precise nature of the heterocomplex between S100A8/S100A9 remains unclear. The aim of this study was to use tapping-mode atomic force microscopy to investigate the structure of this complex in its native form in an aqueous environment and in association with a lipid bilayer. Tapping-mode atomic force microscopy can generate high-resolution images of native proteins that reveal protein contours[26] and the advent of the liquid cell allows proteins to be imaged in their hydrated native state[27].

2. METHODS

2.1 General Reagents and Protein Purification

Reagents and chemicals were analytical grade (Sigma, Bio-Rad). Recombinant S100A8 and S100A9 were produced and purified using the pGEX expression system as previously described [10, 28].

2.2 Liposome preparation

Liposomes were prepared by a modification of the method from Hase et al [29]. Briefly, phosphatidylcholine (PC;Sigma) and cholesterol (Chol;Sigma) were dissolved in chloroform (Ajax Chemicals) at 100 and 10 mg/ml, respectively, then combined at a final ratio of PC:Chol of 9:1. The lipid mixture was dried as a thin film onto the sides of a glass test tube,

by rotating the tube under a stream of nitrogen gas. The lipid film was then dried further under vacuum for 4 – 6 hours then tubes stored sealed at 4°C for up to 5 days. The dried lipid film was then rehydrated with buffer (140 mM KCl, 10 mM Hepes, 0.1 mM $CaCl_2$, pH 6.5) at a final concentration of 100 mg/ml. The lipid suspension was kept at room temperature and vortexed with glass beads for periods of 30 seconds, every 5-10 minutes, over a period of 1 hour. The solution was then extruded through a 100 nm filter (Avanti Lipids) for immediate use.

2.3 Atomic force microscopy

All images were acquired in buffer at room temperature using a Nanoscope IIIA MultiMode AFM equipped with an Extender™ electronics module (Veeco Instruments, Santa Barbara, CA). An E type scanner was used, having a maximum scan area of 12.5 μm^2 and vertical height range of 3.4 μm. The NP-S series of narrow-legged, V-shaped, 100 μm long oxide-sharpened silicon nitride cantilevers, with integrated tips (Veeco Instruments) and a nominal spring constant, k, of 0.36 N/m were used. The AFM was driven in 'Tapping mode™' (TMAFM) at the typical cantilever resonance frequency in liquid (near 9 kHz) at ambient temperature. The piezo Z range was reduced to around 500 nm whilst scanning. The scan speeds ranged from 1 to 4 Hz. The MultiMode AFM was housed within an anti-vibration chamber and mounted on an air-isolation bench.

For imaging liposomes, lipid was diluted 1:500 v/v in buffer (140mM KCl, 10mM Hepes, 0.1mM $CaCl_2$, pH 6.5) to a final concentration of 0.5 μg/μl; 50 μl of the diluted lipid was then spotted onto freshly cleaved mica held on a metal disc by double-sided tape. After 15 minutes the sample was rinsed 3 times with working buffer prior to mounting on the AFM. Samples of the proteins only, and proteins plus lipid were prepared in the same manner as the lipid only samples. For protein only samples, equal volumes of 1 mg/ml S100A8 and S100A9 were mixed and incubated for 15 minutes at room temperature prior to dilution in working buffer (1:1000 v/v), spotting and processing on the mica support. For samples of proteins plus lipid, the diluted S100A8/S100A9 mixture was mixed with an equal volume of the diluted lipid (0.2 μg/μl) and left at room temperature or heated to 37°C for a further 15 minutes prior to spotting and processing on the mica support as outlined above.

3. RESULTS

3.1 Imaging of liposomes and determination of the lipid bilayer height

Initial imaging of liposomes was carried out using tapping-mode AFM. The liposomes adhered and fused to the mica surface and formed a continuous supported lipid bilayer similar to structures described by Reviakine and Brisson (2000)[30]. The height of the bilayer was around 4.5-5 nm (Figure 1), which is in agreement with previously described phosphatidylcholine lipid bilayer height measurements [31, 32].

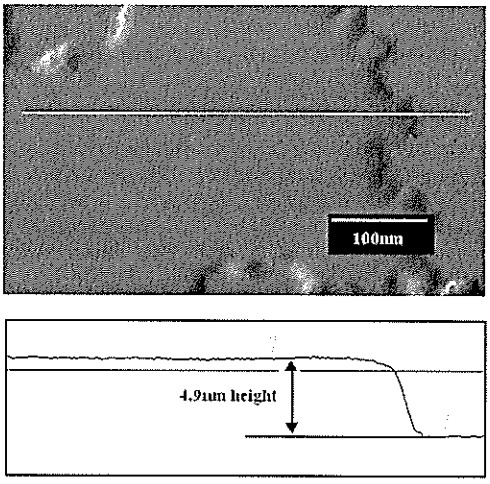

Figure 1 - AFM error mode image (top panel) and cross section analysis (bottom panel) of lipid bilayer in buffer on mica

The S100A8 and S100A9 complex was imaged in buffer by tapping-mode AFM (Figure 2). It had a regular circular appearance with an approximate diameter of 36nm and height of 7nm (Figure 3). Its "dome" shape was more obvious by the height mode image (Figure 4).

In assessing size parameters however, it should be noted that the tip of the AFM can exert significant lateral forces on the sample. This results in images that often appear laterally larger than their true size due to the tip-broadening artifact[33]. Typical NP-S tips, as were used in the current study, have a stated nominal radius of 10 nm, but can range to a maximum of 40 nm. Kacher et al[34] give the tip broadening as:

$$d = 4\sqrt{(r * R)}$$

where d is the apparent width of a feature, r is the radius for a spherical sample and R is the tip radius. Therefore, a protein sample imaged using a nominal tip radius of 10nm, and apparent width of 36nm, yields a lateral width of around 8nm.

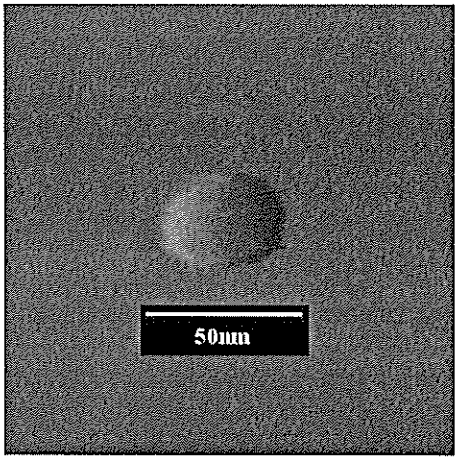

Figure 2 - AFM error mode image of S100A8/A9 protein complex in solution on mica

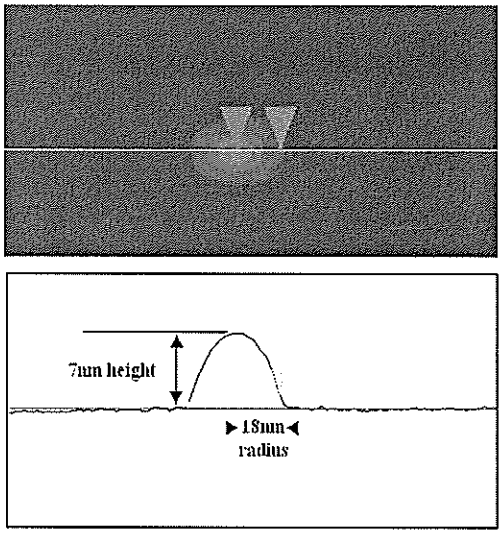

Figure 3 - Cross section image of S100A8/A9 protein complex in solution

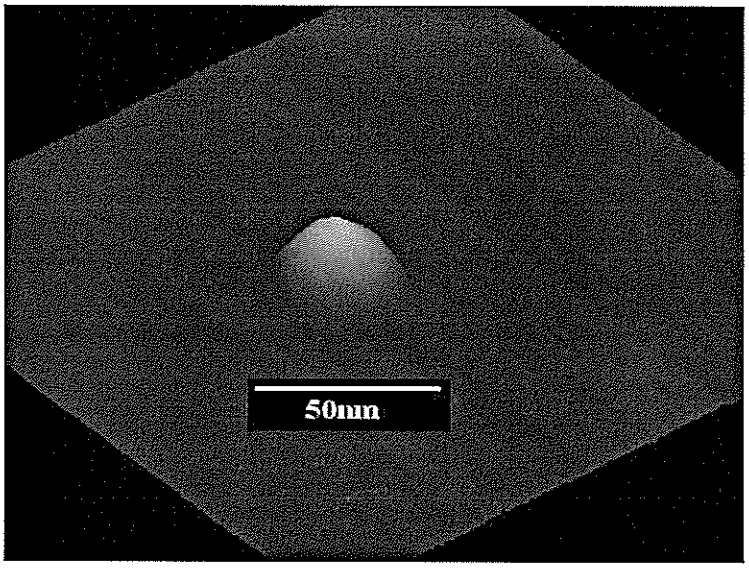

Figure 4 - AFM height mode image of S100A8/A9 protein complex in solution

AFM images in tapping mode of the S100A8/A9 complex preincubated with liposomes at room temperature and then placed onto the mica surface in buffer, are shown in Figure 5. The protein associated with the lipid bilayer and different sized aggregates were apparent.

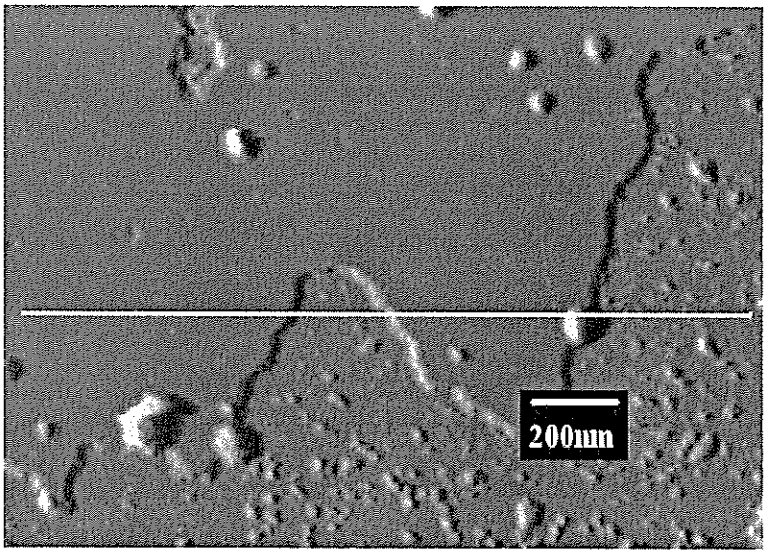

Figure 5 - AFM error mode image of the S100A8/A9 complex associated with the lipid bilayer (preincubated at room temperature)

From the cross section analysis (Figure 6), one of the protein complexes had a height of 6.3nm and a diameter of 50nm. Although protein complexes of heterogeneous sizes were noted.

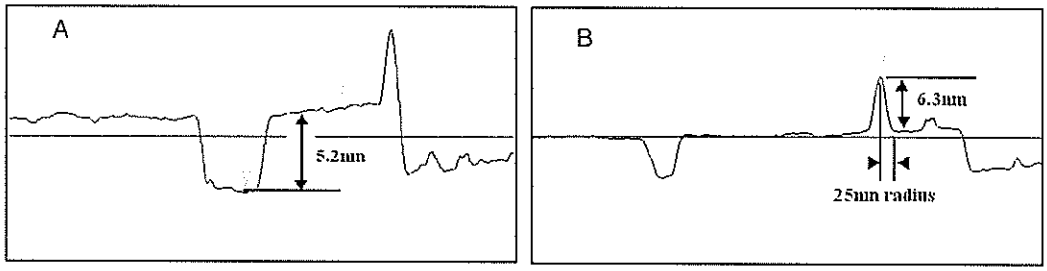

Figure 6 – (A) Cross-section (taken from the white line in Figure 5) revealed a depth of the lipid bilayer of just over 5 nm; (B) Cross-section of S100A8/A9 associated with the lipid bilayer revealed a height of just over 6 nm and radius of approximately 25 nm.

AFM images in tapping mode, of the S100A8/A9 complex preincubated with liposomes at 37°C and then placed onto the mica surface in buffer, are shown below in Figure 7. The protein again associated with the lipid (Figure 7 panel C). However, unlike the samples prepared at room temperature the thickness of the bilayer was found to be only 1.3nm and appeared more "patchy" rather than a continuous bilayer sheet. Interestingly, under these conditions the protein aggregates also appeared to be more uniform in shape and size compared to samples prepared at room temperature.

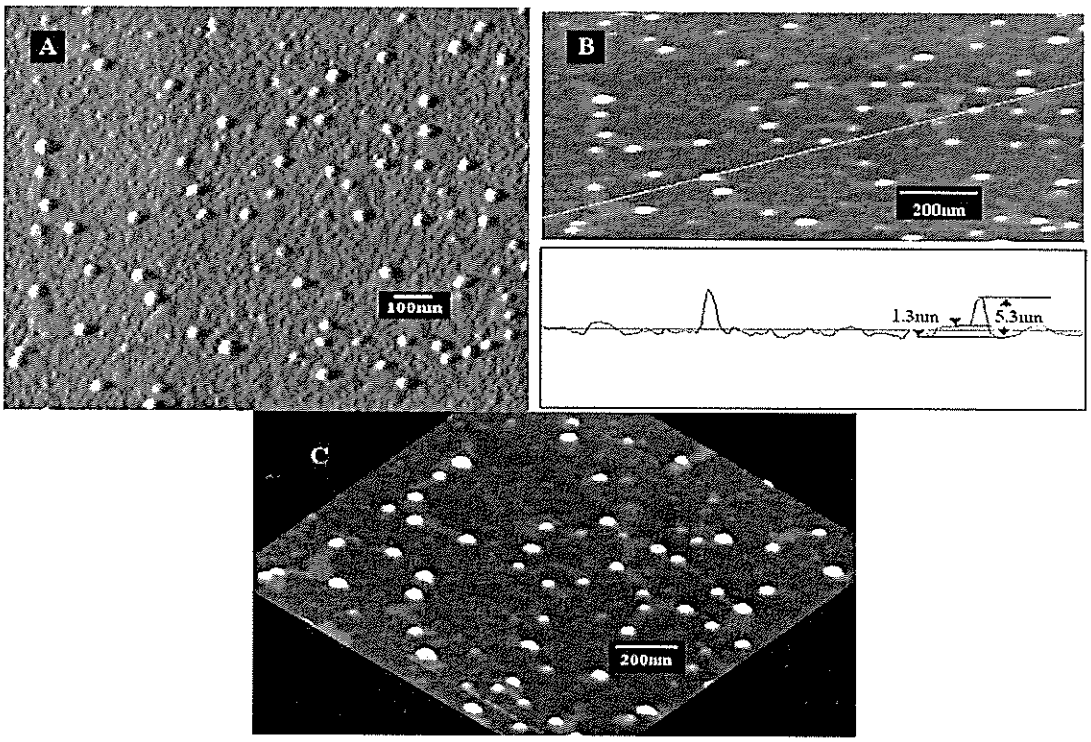

Figure 7 - (A) AFM error mode image of S100A8/A9 protein complex associated with lipid (the complex with the lipid had been preincubated at 37°C). The protein complex ranged from 35 to 45 nm in diameter; (B) Cross section of S100A8/A9 in lipid revealed a lipid height of 1.3 nm and a protein height of 5.3 nm; (C) AFM height mode image of S100A8/A9 protein complex preferentially attached to the edges of the lipid "patches".

4. CONCLUSIONS

S100A8 and S100A9 form homo- and heterodimers and the quantity of heterocomplexes are higher in neutrophils than in monocytes[35]. Similarly, circulating levels of the heterocomplex are low in healthy individuals and increase, as a consequence of release by particular cell populations during inflammatory processes[35]. In addition, the heterocomplex has the interesting property of translocating to cellular membranes, a process regulated by calcium concentrations following cell activation[36].

The studies reported here demonstrate a sensitive technique to further investigate the S100A8 and S100A9 heterocomplex and its interaction with lipid membranes. The mechanism by which the S100A8/A9 heterocomplex penetrates a lipid membrane and how it is anchored therein remains unclear, especially given that neither protein contains a classic transmembrane spanning domain. We plan to use this system to investigate the protein assembly and interaction with lipid membranes in order to better understand processes regulating the insertion of these proteins into a lipid environment and their function and intermolecular interactions at this location.

The data presented shows that we could establish a lipid bilayer from liposomes and were able to image the proteins associating with the membrane in an aqueous environment, thus confirming the lipophilic nature of the complex. From the height measurements of samples prepared at room temperature it appears that the protein complex may not span the bilayer membrane but may only partially insert into the outer leaflet. Interestingly, the sample prepared at 37°C showed a distinct pattern of protein association with the membrane, as well as an apparent formation of a single lipid monolayer rather than bilayer. The protein complexes appeared more uniform in size and shape, and had a more definite affinity for the lipid, given that all the protein clearly associated with lipid "patches". These observations require additional validation and will be repeated using increased amounts of lipid when preparing the sample at 37°C. Because divalent cations such as calcium and zinc can alter S100 structure and function, we are currently investigating their effects on interactions of this protein complex with lipid membranes.

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