ANTI-INFECTIVE PROTECTIVE PROPERTIES OF S100 CALGRANULINS

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Abstract

The calgranulins are a subgroup of proteins in the S100 family (calgranulin A, S100A8; calgranulin B, S100A9 and calgranulin C, S100A12) that provide protective anti-infective and anti-inflammatory functions for the mammalian host. In this review, we discuss the structure-function relationships whereby S100A8 and S100A9, and for comparison, S100A12, provide intra- and extracellular protection during the complex interplay between infection and inflammation and how the calgranulins are regulated to optimally protect the host. Ideally located to support epithelial barrier function, calprotectin, a complex of S100A8/S100A9, is expressed in squamous mucosal keratinocytes and innate immune cells present at mucosal surfaces. The calgranulins are also abundantly produced in neutrophils and monocytes, whereas expression is induced in epidermal keratinocytes, gastrointestinal epithelial cells and fibroblasts during inflammation. The calgranulins show species-specific expression and function. For example, S100A8 is chemotactic in rodents but not in humans. In humans, S100A12 appears to serve as a functional chemotactic homolog to murine S100A8. Transition metal-binding and oxidation sites within calgranulins are able to create structural changes that may orchestrate new protective functions or binding targets. The calgranulins thus appear to adopt a variety of roles to protect the host. In addition to serving as a leukocyte chemoattractant, protective functions include oxidant scavenging, antimicrobial activity, and chemokine-like activities. Each function may reflect the concentration of the calgranulin, post-transcriptional modifications, oligomeric forms, and the proximal intracellular or extracellular environments. Calprotectin and the calgranulins are remarkable as multifunctional proteins dedicated to protecting the intra- and extracellular environments during infection and inflammation.

Keywords

Calgranulins; anti-infection; anti-inflammation; structure; induction; macrophages; keratinocytes

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INTRODUCTION

Inflammation is central to the acute responses to infection and calgranulins are important antimicrobial proteins that also mediate innate and acquired immune responses. The calgranulins play important roles in resistance of the host to infection and protection against adverse affects of inflammation. These remarkable proteins are multifunctional, operating intra- and extracellularly and there are characteristics that differ between humans and rodents. The calgranulins are a subgroup of proteins in the S100 family (calgranulin A, S100A8; calgranulin B, S100A9 and calgranulin C, S100A12) associated with acute/chronic inflammatory disorders [1] and more recently, with various cancers [2].

The S100A8/A9 (MRP-8/MRP-14) complex was named calprotectin to reflect its protective, anti-microbial properties, [1] although termed L1 antigen in early studies [3]. The antimicrobial mechanisms are suggested to involve chelation of divalent cations such as manganese and zinc, which depletes these essential transition metals from the microenvironment. This mechanism appears to contribute to intra- and extracellular growth inhibition of a broad range of bacteria and fungi. Through mechanisms independent of direct antimicrobial activity, S100A8/A9 also contributes to the resistance of epithelial cells to bacterial invasion and the intra- and extracellular effects support the barrier function of mucosal epithelia.

In concert with antimicrobial activity, the calgranulins may also modulate inflammatory responses. Successful resolution of infection involves neutrophil and/or monocyte recruitment. At sites of infection, activated leukocytes release anti-microbial peptides (AMPs) and generate anti-infective reactive oxygen (ROS) and anti-inflammatory (NO-removing) nitrogen (RNS) species. To protect the host against the collateral tissue-injurious effects of NO and ROS, some calgranulins may scavenge and neutralize these compounds. This review will focus on the intra- and extracellular anti-infective and anti-inflammatory properties of the calgranulins. To explain the molecular basis of protection of the host, the structure and functions of these proteins will be discussed, to better understand the contributions of key cellular players including neutrophils, macrophages and keratinocytes.

STRUCTURAL FEATURES OF S100 CALGRANULINS ASSOCIATED WITH HOST PROTECTIVE FUNCTIONS

We will briefly detail some structural and functional features of the calgranulins that contribute to their manifold roles in immunity. Although amino acid sequence structure is correlated with function, changes of even a single amino acid can dramatically change function without significantly altering overall structure (e.g. normal vs. sickle cell hemoglobin). Therefore, we will attempt to point out some structural features that may be of functional importance.

Although most S100 proteins contain only approximately 100 amino acids, these proteins can assume multiple functional forms because of their numerous functional motifs. Each S100 protein typically contains two EF hand calcium-binding loop motifs separated by a "hinge" region, which is distinct for each family member and in some cases followed by a family unique carboxy-terminal tail (see Figure 1) [4]. The S100 proteins primarily form homodimers or higher order oligomers, but S100A8 and S100A9 uniquely form heterodimeric complexes, known as calprotectin.

EF Hand: Calcium-free and calcium-bound conformations of calgranulins

When calcium is bound by an EF-hand motif, the topology (conformational change) and charge of the protein are altered. Topology is key for characterizing electrostatic potential, since charge potential is amplified at peaks (^) or in the inverse case of valleys/clefts (V) [5]. A macroscopic example of the effect of topology and charge potential relates to why we have lightning rods

instead of lightning sheets. To understand the effect of calcium-binding on the functional forms of calgranulins, therefore, the calcium-free and calcium-bound conformations must be compared.

Although the structure of calcium-free calprotectin has not been experimentally determined, we used the program MODELLER [6] to generate the homology model (Figure 2) [7]. The calcium-bound form of calprotectin has been experimentally determined (pdb entry 1XK4) [8]. The ribbon diagrams, molecular surface, and calculation of electrostatic potential were developed using Swiss-PdbViewer (from http://www.expasy.org/spdbv/) (Figure 2) [9].

Calcium binding changes the secondary structure of the hinge region and converts a portion of the extended disordered region following the carboxy-terminal helix 4 in S100A8 and S100A9 into additional turns of alpha helix. Upon calcium-binding, helices 2 and 3 rotate relative to helices 1 and 4. These changes in the size and orientation of secondary structural features alters the solvent accessible surface of calprotectin and may create potentially new functional interactions from a single protein complex. On the other hand, complexes of S100 proteins or divalent cations may not be required for some functions such as the chemotactic activity of the α-helical hinge domains of mS100A8 and S100A12 hydrophobic peptides [10].

Transition metal binding sites

Like several S100 protein family members (e.g. S100A2, S100A5, S100A12, S100A13, and S100B), calprotectin contains two additional tetrahedrally-coordinated transition metal-binding sites created by the dimer interface with two of the four ligands donated by each monomeric subunit (Figure 3). One site is created by His83 and His87 residues in S100A8, in conjunction with His20 and Asp30 residues in S100A9. The second site involves residues His17 and His27 in S100A8 and His91 and His95 in S100A9. The calprotectin metal-binding sites are brought into proximity upon calcium-binding, which orders secondary structure elements. In S100A12, zinc/copper binding to the calcium-free protein also partially orders the EF hands and promotes calcium-binding [11, 12]. Transition metal binding is predicted to further shift the surface of calprotectin to contain positively-charged features and create an additional set of functional conformations with new functions. For example, Zn^{2+} is implicated in the apoptotic activity of S100A8/A9 [13, 14], suggesting structural changes that regulate new functions. Similarly, chelation of Zn^{2+} and Mn^{2+} by extracellular S100A8/A9 are proposed mechanisms of antimicrobial activity [15−17].

Additional functional forms are created in S100A12 upon binding of transition metals. S100A12 binds bivalent ions such as copper, manganese and zinc [18]. Zn^{2+} binding increases calcium binding affinity by 1500-fold and raises the affinity of binding to a putative extracellular receptor, the receptor for advanced glycation end product (RAGE) [11, 12]. For example, S100A12 shows increased binding to human gastric carcinoma MKN74 cells in the presence of Zn^{2+} [12] and calcium and zinc are required for the interaction of S100A12 with paramyosin [18, 19]. Even though copper and zinc have the same binding site, the ions promote non-identical configurations of the S100A12 complex [12]. The function of the S100A12-copper complex is currently unknown although alternative zinc binding appears to reverse redox function [18, 19].

Which metal is bound by calgranulins in the physiological context is generally unknown, since zinc, copper or manganese availability may be limited in the surrounding milieu. The flexibility of proteins also enables the same site to bind different metals. As the protein folds, the availability of specific metals within the intra- or extracellular environments may be important determinants of binding and function [20].

Oligomers

Formation of dimers, trimers, or tetramers involves burial of portions of the protein surface and creation of new clefts and peaks. Compared to monomeric forms, the topology of protein complexes becomes significantly altered and additional functional forms can be created (also reduced). The conformation of the complex will depend upon its constituents, and homodimers of S100A8 or S100A9 will differ significantly in form and function from the heterodimeric calprotectin (S100A8/S100A9). Formation of heterodimers is calcium-independent [21, 22], and calcium binding by calprotectin can induce a conformational change that is also required for tetramer formation [22, 23]. Tetramers are the suggested biologically-active form [21, 22]. S100A12 also forms tetramers and hexamers in the presence of zinc and calcium ions [12, 24–26], potentially expanding its functional roles.

C-terminal extension

Unlike S100A8 and most other members of the S100 family, S100A9 has an extended carboxy-terminal region, containing two additional functional motifs. The penultimate residue in human calprotectin (Thr-113) can be phosphorylated by protein kinase C [27]. The second motif contains histidine residues (103–105 in humans) involved in binding arachidonic acid 28.

Murine vs. human calprotectin

In addition to the absence of S100A12 in mice, murine and human S100A8 differ in the hinge region, S100A9 in the carboxy-terminal tail, and murine and human calprotectin have different electrostatic potentials (Figure 4).

Intriguingly, the hinge region contributes to the chemotactic activity of murine, but not human S100A8. Similar residues in the hinge region of human S100A12 are also implicated in chemotaxis [10]. Since the surface features of homo- and heterodimers will vary with the environmental conditions, reported functions of the hinge and other regions of the calgranulins can vary in different experimental protocols.

INTRACELLULAR FUNCTIONS OF CALPROTECTIN

Cellular differentiation and inflammatory response

The intracellular S100A8/A9 complex, calprotectin, may function in a cell type-specific manner. In hematopoietic progenitor cells, proliferation and maturation may be regulated by S100A8/A9-mediated inhibition of casein kinase I and II, enzymes that phosphorylate topoisomerase I and RNA polymerase I and II [29]. S100A8/A9 expression also suppresses differentiation of murine myeloid-derived suppressor cells (MDSC), potentially through S100A9-dependent induction of NADPH oxidase and generation of reactive oxygen species (ROS). In immature myeloid cells, S100A9 up-regulation inhibits differentiation into dendritic cells and macrophages [30]. The response to S100A9 appears to be STAT3-dependent, leading to enhanced production of MDSCs and immune tolerance 30. MDSCs promote immune tolerance by suppressing $CD4^+$ and $CD8^+$ T cell activation, resulting in polarized immunity to a type-2 phenotype [31]. MDSC inhibit T cell function by regulating metabolism of the essential amino acid L-arginine by arginase I and NOS_2 [32]. Expression of these enzymes is immunosuppressive [33] and may synergize to produce peroxynitrites, which cause T cell apoptosis [34]. Similarly, S100A8 and S100A9 in neutrophils can readily be S-nitrosylated [35] and their intracellular functions in MDSC warrant further studies. Since L-arginine is metabolized by NOS_2 to produce NO, scavenging of NO by S100A8 and S100A9 [35] could influence MDSC/regulatory macrophage functions.

S100A8/A9 in neutrophils also binds fatty acids in a calcium-dependent manner [36, 37]. Polyunsaturated fatty acids, including arachidonic acid (AA) have a higher affinity for S100A8/

A9 than saturated or monounsaturated fatty acids and may contribute to their uptake and release [38]. The released AA-calprotectin complex could be internalized and metabolized by nearby cells to generate eicosanoids, which contribute to the initiation and regulation of inflammatory responses [39]. S100A8/A9 may also serve as a scaffold for AA in the cytosol of neutrophils and epithelial cells to facilitate activation of NADPH oxidase to yield ROS [28, 40], which are potent microbicides against invading pathogens.

Antimicrobial functions

In addition to its extracellular antimicrobial activity, calprotectin-expressing cells also resist bacterial adherence and invasion [41, 42]. Calprotectin-expressing keratinocytes are found to internalize fewer invasive pathogens, like *Listeria* [43]. Approximately three-fold fewer bacteria are found to bind to calprotectin-expressing cells, but there are 10-fold fewer intracellular microorganisms. The reduction in apparent invasion can be due to direct intracellular antimicrobial effects of calprotectin and the ability of the calprotectin-expressing cell to resist the invasion process. Whereas calprotectin-expressing cells generally contain fewer invaded pathogens, *Listeria*, an invasive pathogen that can grow in the cytoplasm, appears to subvert intracellular antimicrobial activity by inducing translocation of intracellular S100A8/A9 to microtubules [43]. When calprotectin co-localizes with tubulin, keratinocytes contain more intracellular *Listeria* than in cells without co-localization. In the presence of polymerized microtubules, the antimicrobial activity of calprotectin against *Listeria* is neutralized *in vitro* [43]. As we reported recently, however, S100A8/A9 expressing cells are also resistant to bacterial invasion independently of intracellular antimicrobial activity. Induction of keratinocyte resistance to bacterial invasion is dependent on the calcium-binding domains within S100A9 in the intracellular heteromeric complex, and does not necessarily correlate with changes in binding of bacteria to the cell surface [7]. Hence, intracellular calprotectin appears to thwart internalization of invasive pathogens by calcium-dependent intracellular protein-protein interactions and may also inhibit the cytoplasmic growth of those bacteria that manage to invade.

Cellular protection against stressors and wounding

Both pro- and anti-inflammatory mediators regulate the S100A8 and S100A9 genes. The intracellular functions of S100A8 and S100A9 may depend on cell type-specific receptors that signal in response to particular extracellular stressors. Oxidation and zinc binding can each signal calprotectin-dependent cellular responses. In some circumstances, intracellular calgranulins mediate host protection. For example, elevated S100A8 and S100A9 expression levels in epidermal incision wounds may enable wound healing by suppressing adverse effects of inflammation [44]. As we describe above, S100A8/A9 in epithelia may be crucial for protection against bacterial infections, whereas the ability to scavenge free radicals may protect the skin against UVA-mediated oxidative damage [45]. Given that S100A8 is an efficient scavenger of ROS [46, 47], induction in murine keratinocytes and release during UVA irradiation may regulate intracellular redox-mediated pathways involved in cell growth and/or differentiation. Therefore, intracellular calprotectin may play critical protective roles in maintaining epithelial barrier functions by supporting repair of damaged tissues, in addition to limiting microbial invasion and enhancing transepithelial migration of leukocytes [48] into infected sites.

Calgranulins and the cytoskeleton

Calgranulins translocate to the plasma membrane in a calcium-dependent manner and interact with cytoskeletal proteins, including tubulins, keratin intermediate filaments and microfilaments in activated monocytes and epithelial cells [49–52]. S100A8/A9 microtubule-binding has been shown to alter microtubule polymerization kinetics [53, 54]. Binding to

cytoskeletal filaments and microtubules may control cell morphology, motility, and organization of the cytoskeleton of epithelial cells during migration and wound healing, and granulocytes during chemotaxis 55. Structurally, calcium-induced S100A8/A9 tetramers appear important for microtubule polymerization and stabilization and may facilitate transendothelial migration of phagocytes [21]. In keratinocytes, mutation of calcium binding domains in S100A9 in keratinocytes abrogates calprotectin MT interactions (Champaiboon et al, 2009 unpublished data). S100A12 also binds the cytoskeleton in a calcium-dependent manner [1, 23, 56] although the function implications of this interaction are unknown.

EXTRACELLULAR PROTECTION

Release of calgranulins

Release of hS100A8/S100A9 is suggested to follow cell disruption or death 57. In monocytes, however, hS100A8/S100A9 release depends on PMA induced-protein kinase C activation and requires an intact tubulin network 27. Since, hS100A8/S100A9, but not homodimers, bind AA, binding to plasma membrane lipid could regulate translocation to the surface and release from the cell [36]. Some proinflammatory cytokines and bacterial products like lipopolysaccharide (LPS) promote hS100A8/S100A9 release from neutrophils and monocytes [58, 59]. During inflammation and infection, hS100A8/S100A9 and S100A12 are released and detected in body fluids such as serum, saliva, sputum and amniotic fluid 60⁻63. It is assumed that calgranulins in mucosal fluids are released from intact keratinocytes but formal study is needed.

Calgranulins as chemokine-like mediators in response to infection

Microbial or parasitic infections are generally averted or resolved by cellular mechanisms that prevent pathogen invasion. Factors produced by mast cells, neutrophils, keratinocytes, macrophages and dendritic cells (DCs) regulate responses to infection. For example, the calgranulins are released from the cytoplasm of dead and dying neutrophils into abscess fluid [64]. In *S. aureus*-induced abscesses in mice, the high concentrations of extracellular antimicrobial calprotectin depends on the density of the neutrophil population [15] and correlates with loss of neutrophil viability [57]. The calgranulins can be released from cells, but are not secreted via the endoplasmic reticular/Golgi-mediated pathway as they lack signal peptides.

Calprotectin release may be signaled by pathways such as activation of PKC by pro-inflammatory stimuli, or elevation of intracellular $[Ca^{2+}]$ by contact with activated endothelium [65]. In particular, lipopolysaccharide (LPS) from several bacterial species [58] and C5a 66 cause rapid release of S100A8 and S100A9 from neutrophils and monocytes in an active, tubulin-dependent process 27. Stimulated monocytes and macrophages also release the calgranulins 67 although activated dermal keratinocytes [45] and fibroblasts [68] do not.

Calgranulins and chemotaxis

Among the antimicrobial agents in neutrophils, the cytoplasmic calgranulins include S100A8 and S100A9, which comprise about 45% of the cytosolic proteins 69, and S100A12, which represents about ~5% of the total proteins [70]. Neutrophils are rapidly recruited to sites of acute infection in response to bacterial chemoattractants and/or activated complement components such as C5a. Acute response modifiers such as C5a may also trigger degranulation of mast cells, releasing histamine and other preformed mediators such as tumour necrosis factor (TNF) and the neutrophil chemokine IL-8 [71]. IL-8 rapidly recruits additional neutrophils, whereas microbial products such as LPS activate mast cell cytokine production via TLR ligation 72. Once activated, neutrophils release cytosolic components, degranulate and generate a robust oxidative burst via the NADPH oxidase and myeloperoxidase systems.

Our group made the initial observation that S100 proteins have "chemokine-like" properties; murine (m) S100A8 has potent chemotactic activity for neutrophils and monocytes *in vitro* and *in vivo* [73]. Injection of mS100A8 promotes leukocyte recruitment [74] with neutrophils appearing at 4–6 h followed by monocytes over 24 hours [75, 76]. Given the functional similarities in leukocyte trafficking, we later identified S100A12 as the probable human functional homologue of chemotactic mS100A8, with optimal activity for monocytes at picomolar levels [77, 78, 79]. In our hands, human (h) S100A8 has only weak leukocyte chemotactic activity *in vitro* [74] and in vivo [74]. Indeed, it is unclear whether human S100A8 and S100A9 are chemoattractants, although there is 57% amino acid identity with mS100A8, and we questioned whether the proteins were orthologs [74]. We showed that human S100A12 is chemotactic [77, 78], although no homologs were encoded in rodent genomes [79]. The functional and sequence divergence suggested complex evolution of the S100 family in mammals. Absent in the human S100A8 hinge [10], we identified amino acid residues essential for chemotaxis in the hinge region of S100A12 and conserved in the active mS100A8 hinge region [74]. The hinge regions of mS100A8 and S100A12 are structurally similar and electro-negatively charged, whereas this region in hS100A8 is positively charged. Some functional differences in the hS100A8 and mS100A8 reported by several groups [80, 81] may reflect concomitant activity as efficient oxidant scavengers and the oxidation state of the proteins [80]. In contrast to classical chemoattractants (eg., fMLP, IL-8), mS100A8, like TGF-β, does not cause Ca^{2+} flux or changes in integrin or L-selectin expression, yet may mediate initial diapedesis [82]. Murine S100A8 and S100A12 may signal through pertussis toxin-sensitive, G-protein coupled receptors [82, 10] and although mS100A8 does not activate mast cells, its chemokine-like characteristics are similar to S100A12.

S100A12 also upregulates expression of adhesion molecules such as CD11b and increases shedding of L-selectin from neutrophils, enhancing adhesion to endothelial cells (ECs) [80]. S100A12 may also induce ICAM-1 and VCAM-1 expression on ECs, contributing to the strong adhesion required for extravasation [83]. Thus, like other neutrophil-derived proteins such as the defensins [84, 85, 86] and cathepsin G [84, 87, 88], calgranulins are chemotactic and inhibit microbial growth [89].

During inflammation, hS100A8 and hS100A9 may contribute to migration of leukocytes via localization of the calprotectin complex released from transmigrating neutrophils to endothelial cells *in vivo* [90]. Recombinant hS100A8 and hS100A9 increases β_2 integrin CD11b expression and affinity on phagocytes [81, 80]. Adhesion of leukocytes to activated endothelium may depend primarily on hS100A9 in the calprotectin complex since hS100A9 alone facilitates adhesion of neutrophils to fibrinogen [80] and fibronectin [91] and monocytes to the endothelium *in vitro* [92]. Furthermore, mS100A9−/− neutrophils have reduced capacity to migrate through endothelial cell monolayers *in vitro* in response to chemoattractants such as IL-8 or leukotriene B_4 and, in contrast to wild-type cells, IL-8 fails to increase surface expression of CD11b [93]. mS100A9−/− neutrophils show reduced Ca^{2+} transients and chemotaxis in response to certain chemokines when compared to wild-type cells [94], suggesting that altered chemotactic activity may reflect dysregulated cytoskeletal dynamics in the absence of mS100A9 [53].

In human chronic rhinosinusitis, expression of hS100A8/A9 by the nasal mucosal epithelium appears to correlate with resistance whereas reduced expression is linked to increased susceptibility (reviewed in [95]). During the course of *Streptococcus pneumoniae* infection in murine lung, mS100A8 and mS100A9 are released differentially [89]. In the early phase, pneumocytes produce mS100A8 and mS100A9, followed by neutrophil and macrophage migration. Importantly, anti-mS100A8 and anti-mS100A9 antibody blockade strongly inhibited transepithelial leukocyte recruitment to the alveoli, without affecting the transendothelial cell migration or sequestration of neutrophils within the vasculature [89]. In this model, antibody blockade had no influence on bacterial load or survival of the mice. In S100A9-

null mice, accumulation and the time-course of leukocytes recruited in thioglycollate-induced peritonitis or into the air pouch using LPS [96] were similar, although neutrophil infiltration was reduced into skin wounds [53] and into pancreas in a model of caerulein-induced pancreatitis [48]. mS100A9−/− mice are less responsive to LPS stimulation and resistant to LPS-induced septic shock [97]. Neutrophils from S100A9−/− mice do not contain mS100A8 protein although they express the mRNA [93, 96]. In S100A9−/− cells, however, responses to ATP or platelet activating factor are reduced [98]. The S100A9 scaffold for cytosolic phox proteins interacts with membrane-bound cytochrome b and may be important for activation of dormant NADPH oxidase activity [40]. When stimulated with phorbol esters, however, the oxidative burst generated by S100A9−/− neutrophils and wild-type cells is similar [96]. S100A9 may therefore specifically regulate diacylglycerol signalling in response to some agonists [94], and this may explain why leukocyte influx is disrupted in some *in vivo* models but appears normal in others. Although studies to date suggest that calgranulins in murine models reflect the proinflammatory effects of the human proteins, the anti-infective mechanisms seen in humans may not be completely represented in mice.

S100A12-dependent mast cell regulation and leukocyte recruitment

Mast cell products such as TNF and histamine are critical in innate and adaptive immunity [71, 99]. Our recent studies show that S100A12 is a mast cell chemoattractant *in vitro* and sequesters these cells when injected intraperitoneally or into the skin [10]. S100A12 also stimulates degranulation of murine and human mucosal and skin mast cells [100], which could influence the resolution of infection. Mast-cell-derived TNF is stored in granules and secreted upon degranulation. In mast cell-deficient mice, caecal ligation and puncture is lethal, because of a lack of TNF [101, 102]. Mast cell-derived TNF signals the TNF receptor 1 expressed on endothelium, contributing to neutrophil recruitment to sites of inflammation [103] and clearance of bacteria or mycobacteria [104, 105]. Histamine triggers immediate increases in vascular permeability, mobilizes E-selectin to the endothelial surface and promotes neutrophil transmigration [106,99, 107, 108]. S100A12 rapidly induces mast cell-dependent neutrophil adhesion and extravasation 100, oedema and leukocyte (neutrophil and monocyte) recruitment in mice 78. S100A12 also induced IL-6, IL-8, MCP-1, and MIP-1β production in human cord blood-derived mast cells 100. Hence, mast cells are likely to be a key target of S100A12, generating localized histamine, chemokines, and cytokines (e.g. TNF), activation of the vasculature and sustained leukocyte recruitment.

Extracellular oxidant scavengers

Oxidation is a post-translational modification of calgranulins that may alter crucial functions. To kill invasive microbes, phagocytes produce high levels of intracellular ROS and nitrogen species (RNS). ROS release into the surrounding tissue, however, can cause injury and contribute to inflammation by modifying lipids and proteins, causing oxidative stress 109. Our group has explored the possibility that calgranulins may scavenge ROS within the inflammatory milieu thus reducing oxidative damage to surrounding tissue (reviewed extensively in 110).

The mS100A8 Cys41 residue oxidizes in the presence of Cu^{2+}, low levels of hypochlorite (OCL⁻) or PMA stimulated neutrophils 46. Methionine residues in the calgranulins can be oxidized to methionine sulfoxides [46]. Sulfinamide bonds, covalent links between Cys-thiol groups and the ε-amine group of Lys residues, are also generated. Unlike disulfide bonds, these bonds cannot be reduced by normal cellular reduction systems or the chemical reductant, dithiothreitol (DTT) [47]. Murine S100A8 intra- (between $Lys_{34/35}$ and Cys_{41}) and intermolecular (between Lys_6, Lys_{76}, Lys_{83} Lys_{87} and Cys_{41}) sulfinamide bonds are generated with as little as 1:1 molar equivalents of HOCl⁻ *in vitro* [111, 112], or the presence of PMA-stimulated neutrophils [47]. Several hundred-fold more sensitive to HOCl⁻ oxidation, S100A8

and to a lesser extent S100A9 appear to oxidize proportionally more effectively than low-density lipoproteins [113] or bovine serum albumin [112]. Oxidized monomers and disulfide-linked complexes are detected *in vivo* in LPS-treated lung lavage fluid from mice [46], and DTT-stable cross-linked complexes (dimeric $S100A8_2$, $S100A9_2$, S100A8/A9 and $S100A8/A9_2$) are found in human atherosclerotic lesions [112].

The redox-sensitive Cys residue in mS100A8 resides immediately before the hinge domain. Chemotactic activity *in vitro* or *in vivo* appears unaffected by mutation of Cys to Ala although the H_2O_2-generated disulfide-bonded dimer lost activity [46]. On the other hand, sulfinamide-containing HOCl-oxidized S100A8 monomers (between Cys_{41} and $Lys_{34/35}$) remain active, whereas intramolecular sulfinamide-linked mS100A8 oligomers are not chemotactic [47]. Thus, oxidative modifications that generate covalent oligomerization of Cys_{41}, which flanks the hinge domain, may mask the chemotactic site of mS100A8 by sterically hindering access to the cellular receptor without directly altering activity. Mild HOCl oxidation would stabilise mS100A8 chemotactic activity and leukocyte recruitment, whereas cross-linking by high levels of oxidants may dampen the response.

In contrast, S100A12 is relatively resistant to covalent modification by oxidants since it has no Cys or Met residues. Since numerous classical chemoattractants are susceptible to oxidative inactivation [46], S100A12 may be important in propagating monocyte mast cell/monocyte recruitment in a ROS-rich environment. We suggest that S100A8 duplication and divergence in humans, compared to rodents, permits dichotomous chemotactic activities. In mice, the abundant S100A8 acts an anti-oxidant and chemotactic agent within the picomolar range, but chemotactic activity is ROS-sensitive [46]. In addition to functions attributed to the S100A8/A9 complex, hS100A8 may function as an anti-oxidant, whereas S100A12 may mediate monocyte and mast cell recruitment and mast cell activation [10, 78, 100].

Human S100A8 and hS100A9 may actually show fugetactic (cell repulsion) activity for human neutrophils [114, 115]. In S100A9, the fugetactic activity was inhibited by oxidation of Met_{63} and Met_{83} [115]. Clearly, oxidation can alter S100A8 and S100A9 functions and could modulate leukocyte recruitment.

Other functions of the calgranulins can be affected by oxidation. For example, the anti-fungal activity of hS100A8/A9 is abolished when Cys_{42} of S100A8, or S100A9 Met_{63} or Met_{83} (but not S100A9 Met_{81}) are mutated to Ala. Hence, oxidation inhibits anti-fungal activity, suggesting that activity can persist only in conditions of limited oxidative stress [116].

Resolution by S100A8-SNO

By acting as a nitric oxide (NO) shuttle, S100A8 may regulate mast cell activation and modulation of vessel tone [35]. NO binds covalently to cysteine residues in proteins. Through S-nitrosylation (the covalent coupling of NO to Cys residues), NO mediates vascular homeostasis and anti-microbial defence, and regulates transcription and apoptosis [117]. Few proteins are targets for S-nitrosylation and the specificity requires a local hydrophobic environment with acidic and basic residues proximal to the target Cys to facilitate reduction of the thiol pKa [118, 119]. We showed that S100A8 is readily S-nitrosylated on Cys_{41} [35]. In the S100A8/A9 complex, S100A8 is preferentially nitrosylated. S100A8-SNO is detected in normal human neutrophils and levels increase in neutrophils treated with NO donors [35]. The S100A8-SNO bond is relatively stable enabling transnitrosylation of hemoglobin, for example, thereby shuttling NO that may regulate blood vessel tone [35]. Triggered by mast cell activation in the mesenteric circulation of live rats, mast cell degranulation, leukocyte adhesion and transmigration are inhibited by S100A8-SNO [35]. As we have shown, microvascular endothelial cells activated with IL-1β or LPS induce S100A8 [120]. When S-nitrosylated by endothelial cell-

derived NO, S100A8-SNO may restore blood flow in congested vessels and maintain patency of neovessels.

During infection, the inflammatory response maximizes the production of anti-microbial ROS by phagocytes. When activated macrophages produce S100A8, microbial killing would appear to be compromised. Although LPS or IFN-γ induces S100A8 in murine macrophages [121], when combined, LPS and IFN-γ induce high amounts of iNOS, but not S100A8 [35]. Thus, when ROS/RNS generated by macrophages activation is required for microbial killing, S100A8 is not produced. In the extracellular milieu, however, ROS can injure the host and S100A8 may serve as a protective scavenger when generated by the appropriate stimulants.

Extracellular calgranulins as antimicrobial proteins

Cell-free calgranulins may be directly antimicrobial. Pure hS100A8/A9 has broad spectrum antimicrobial activities against microorganisms including *Capnocytophaga sputigena* [122], [123], *Candida albicans* [124], *Escherichia coli, Staphylococcus aureus, S. epidermis* [17], *Borrelia burgdorferi* [125], and *Listeria monocytogenes* [43] *in vitro*.

S100A12 has antimicrobial activity against filarial parasites [126], possibly mediated by direct binding to paramyosin, a target protein on the parasites [11, 12]. Calcitermin, homologous to the 15 C-terminal residues of S100A12, has antimicrobial activity against *E. coli, Pseudomonas aeruginosa* and *C. albicans*, particularly in the presence of zinc and low pH [127]. Hence, the C-terminal region of S100A12 may be crucial for its antimicrobial function.

Since Zn^{2+} is required for bacterial growth, S100A8/A9 sequestration of zinc is suggested as a mechanism of inhibiting bacterial growth [128]. Recently, Mn^{2+} in tissue abscesses was shown to contribute to S100A8/A9 antimicrobial activity against *S. aureus* [15, 16]. As described above (see STRUCTURAL FEATURES OF S100 CALGRANULINS), the transition metal content within the local environment may modulate the ability of the calgranulins to interact with other protein targets, including bacteria. Thus the antimicrobial potential of the calgranulins must be established within a physiological context.

Mucosal fluids are replete with antimicrobial proteins and peptides and the calgranulins may contribute to limiting growth of commensal organisms and preventing intrusion of pathogens. Calprotectin, for example, is found in human airway secretions [127], gingival crevicular fluid and periodontal pockets [129]. Furthermore, calprotectin is prominent in mucosal abscesses in association with candidiasis [130]. S100A8, S100A9 and S100A12 are also expressed in inflamed gastric mucosa of *H. pylori*-infected children, but not normal mucosa [131]. Hence, the calgranulins may serve as innate antimicrobial proteins to limit infections at mucosal surfaces.

Extracellular calgranulins and signal transduction

Calprotectin functions in the extracellular milieu, but specific surface receptors and signaling pathways are not fully characterized. S100A8/A9 interacts with heparin and heparan sulfate glycoaminoglycans [90] and carboxylated glycans [132] on endothelial cells *in vitro*. On endothelial cells, S100A8/A9 also binds to the scavenger receptor FAT/CD36 and may facilitate AA uptake by acting as a fatty acid transporter protein 133. In murine macrophages, mS100A8/A9 may induce inducible NO synthase, and NO production by signalling through the TLR4 pathway 134. In mice, mS100A8 and the mS100A8/A9 complex apparently interacts with TLR4 to promote lethal septic shock [97]. RAGE may serve as a common receptor for S100 proteins/calgranulins. Indeed, S100A1, S100A4, S100A7, S100A11, S100A12 (EN-RAGE), S100A13, S100B and S100P are all ligands for RAGE [55, 83, 135−137]. S100A8/A9 also binds RAGE and activates the MAP kinase pathway [138−140]. Moreover, N-glycans on RAGE may be critical for S100A8/A9 binding [141]. Since some cell types may express multiple receptors for S100A8/

A9, the downstream signaling pathways are not well understood. In the local environment, RAGE interaction may depend on the availability of calcium and zinc, which are also required for oligomerization of calgranulins [12]. Binding to RAGE is proposed to trigger MAP kinase and NF-κB downstream signaling pathways [138, 140, 142], and the complexity of downstream responses may depend on the types of ligands and the cell-type specific repertoire of receptors. Although TLR and RAGE ligation by calgranulins are likely to contribute to activation [143], it is clear that additional receptors are important. For example, mast cell activation by S100A12 is RAGE-independent [100] and not mediated by TLR4 (J. Goyette, unpublished data) and chemotaxis stimulated by mS100A8 and S100A12 is likely mediated by a G-protein-coupled mechanism as discussed above.

CALGRANULIN EXPRESSION AND REGULATION: OF MICE AND MEN

Expression

To optimize protective functions, the calgranulins are either constitutively expressed and tightly regulated, as in the squamous mucosal epithelia, or strictly inducible as in the skin, and intestinal mucosa. How the calgranulins are regulated depends on the type of cell, anatomic locale and environmental stimuli. Murine and human S100A8 and/or S100A9 are constitutively expressed in platelets, osteoclasts and hypertrophic chondrocytes, and trophoblasts in developing embryos. Calprotectin is also expressed in keratinocytes and cells of the myeloid linage, in which expression is correlated with the stage of differentiation [144, 145]. Human S100A9 is first apparent during maturation of promyelocytes to myelocytes and maintained in high abundance in mature neutrophils [146]. Circulating monocytes constitutively express S100A8 and S100A9 [147, 148] and calprotectin expression decreases during extravasation from blood and differentiation in tissues, given that normal macrophages do not express S100A8 and S100A9 [149, 150]. Expression of S100A12 [151] mirrors S100A8 and S100A9, also suggesting an association with myeloid cell differentiation [152]. In the following section, we will focus on the regulation of calgranulins in keratinocytes, fibroblasts and macrophages in response to proinflammatory and anti-inflammatory signaling. In an environmental agent-specific manner, calgranulin expression (S100A8, S100A9 and/or S100A12) may be modulated by physiological and traumatic stressors [45, 153, 78].

Epithelial cells

Keratinocytes form the outermost layer of epithelial cells in the epidermis and mucosal surfaces and are in continual contact with invasive microorganisms. Alerting neighbouring cells to the presence of the pathogen, epithelial cells recognize microbial endotoxins, including LPS, and produce and release proinflammatory cytokines, including the interleukin-1 (IL-1) family [154]. The IL-1 family of cytokines generally contribute to host resistance to pathogens in the oral cavity. The IL-1 family cytokine produced by oral epithelial cells that signals proximal cells is typically IL-1α since oral keratinocytes lack functional caspase-1 and therefore express, but do not produce mature IL-1β [155]. Similarly, basally-expressed in human gingival keratinocytes, S100A8 and S100A9 expression can be stimulated by PMA (24 hr), but not by LPS or IL-1β (2–4 hr) [156], strongly suggesting a role for IL-1α, but not IL-1β, in the calprotectin-dependent protection of oral epithelial cells. When epithelial cells are exposed to pathogenic microorganisms, IL-1α, a cytokine produced by keratinocytes, and leukocyte IL-22 appear to induce upregulation of S100A8/A9 and other antimicrobial peptides (AMPs) [157–160]. S100A12 upregulation was not reported. IL-1α induces S100A8/S100A9 expression whereas transforming growth factor (TGF-β) reduces expression in human gingival keratinocytes [159]. IL-22, a member of the IL-10 family of cytokines, appears to induce expression of several AMPs including human beta-defensin 2 (HBD2), HBD3, S100A7 and S100A8/S100A9 and regulates keratinocyte migration and differentiation [157, 158, 161]. Bronchial mucosal epithelial cells increase S100A8/S100A9 expression by 24 hours in

response to LPS through TLR4 stimulation 162. In addition, IL-1α and TNFα increase S100A12 expression in bovine keratocytes [126]. That oral epithelial cells are unable to increase S100A8/S100A9 expression in response to LPS or IL-1β within 2–4 hr suggests that intracellular S100A8/S100A9 functions as a secondary responder to invasive microbes (discussed later). Epithelial cells, therefore, appear to initiate calprotectin-dependent innate immunity in an autocrine/paracrine manner.

Keratinocytes actively participate in immunological events and upregulation of cytokines such as IL-1, IL-6 and TNF-α reflect inflammatory activation in dermatological disorders [163, 164], resulting in S100A8/S100A9 upregulation in keratinocytes [165]. In psoriasis for example, S100A8/S100A9 complexes are highly expressed in abnormally-differentiated keratinocytes [166], and activated human epidermal keratinocytes (HaCaT cells) release the S100A8/S100A9 heterodimer [167]. In psoriasis [168] and wound healing [167], hyperproliferative responses appear to be S100A8/S100A9-dependent. Not expressed in normal skin, S100A12 is also present in human keratinocytes in psoriatic lesions. [169].

Epidermal keratinocytes contain high levels of anti-oxidants and enzymes involved in anti-oxidant defense. In addition, high levels of ROS are generated by ionizing radiation, including UVA-induced sunburn. In the tissues, excess ROS can overwhelm normal cellular defenses to oxidants and require increased antioxidant protection. Confirming ROS-mediated induction in a murine UVA irradiation model, mS100A8, but not mS100A9, is induced and released by keratinocytes and may contribute to antioxidant defense [45]. Similar to oxidative stress, mice with LPS-induced pulmonary injury released a chemotactically inactive form of S100A8 dimer into lavage fluid reflecting that disulfide-dependent dimerization may be a regulatory mechanism controlling leukocyte recruitment [46]. As an efficient scavenger of ROS [46, 47], mS100A8 may regulate intra- and extracellular redox-mediated pathways.

Fibroblasts

Underlying the epithelial and Langerhans cells forming the mucosal surface, the submucosa contains fibroblasts, DCs, macrophage and mononuclear cells. Pathogens subvert the epithelial barrier, either by escaping from intracellular confinement of epithelial cells or through interaction with intra-epithelial dendritic (Langerhans) cells, which may process and present the pathogen to adaptive or other innate immune cells. Fibroblasts, on the other hand, do not appear to be involved in the S100-dependent response to invasive microbes, but are essential for wound healing. FGF-2 and IL-1β, but not LPS, induce mS100A8 expression in murine fibroblasts, whereas TGF-β suppresses mS100A8 expression [68]. In non-inflamed skin, S100A8/A9 is not detectable, but increased S100A8 expression in fibroblasts in rat dermal wounds suggests a role in repair/remodelling. During the initiation of wound repair, TGF-β expression is inversely related to the increase in fibroblast S100A8, suggesting its involvement in the fibroblast-directed response to inflammation and wound repair [68].

Macrophages

Increases in S100A8 and S100A9 expression, particularly S100A9, is regulated by the transcription factor CAAT enhancing binding protein (C/EBPα) [170]. Pro-inflammatory cytokines and LPS may induce binding of C/EBPα to the promoter region to activate S100A8 and S100A9 gene expression [58]. In addition to C/EBPα, the myeloid-related protein regulatory element (MRE)-binding complex A (MbcA) and MRE-binding complex B also regulate expression of S100A9 by binding the S100A9 promoter [146]. C/EBPβ is a transcriptional enhancer of the human S100A8 gene, the activity of which is antagonized by the retinoic acid receptor (RAR) in a ligand-dependent manner [171].

Macrophages contribute to host defence, wound healing, and immune regulation. Macrophage polarization into proinflammatory and anti-inflammatory activities occurs in response to encountered mediators in specific environments 172. The preferential production of proinflammatory IL-12 or anti-inflammatory IL-10 is the basis of the M1/M2 polarization paradigm, and M2 polarization is further subdivided into M2a, M2b, and M2c 173−176. The M2c or regulatory macrophage paradigm (as proposed by Mosser and Edwards 176) can be modelled when macrophages are stimulated with IL-10, TGF-β, or glucocorticoids (GCs) resulting in down-regulation of pro-inflammatory cytokines, increased oxidant scavenging capacity, and a pro-healing functional program 176.

Regulatory macrophages normally emerge in response to two stimuli inducing anti-inflammatory activity [176]. The first signals include IL-10, immune complexes, prostaglandins, glucocorticoids, and apoptotic cells and generally has little stimulatory function. A second stimulus is needed, normally TLR ligand. Characterized by the requirement of IL-10, the S100A8 induction profile in murine and human macrophages appears consistent with the M2c or regulatory type macrophage. We proposed in this review that unlike constitutively expressed S100A8 in neutrophils, macrophage induction in the late stage of inflammation could modulate the immune response, promoting resolution.

Murine and human macrophages differ in their responses to LPS and IL-10. Unstimulated murine primary macrophages express little detectable S100, but activated macrophages express mS100A8 in the absence of mS100A9 [121, 177]. Similarly, S100A8, but not S100A9, is upregulated in murine monocyte/macrophage-like cell lines in response to LPS [178]. Stability of mS100A8 protein is proposed to be dependent on mS100A9 as mS100A9 $^{-/-}$ mice express S100A8 mRNA, but not protein [93, 96]. However mS100A9 is not induced in macrophages by any of these stimuli, although human S100A8 and S100A9 are generally co-induced in monocytes and macrophages [148, 179]. mS100A8 mRNA is induced in macrophages by TLR ligands such as LPS [178], dsRNA [179], CpG (K. Hsu, in preparation), TNFα, transforming growth factor β (TGF-β), interferon-γ (IFNγ) [121] and IFNβ [179]. In macrophages, therefore, S100A8 induction: occurs late, and mRNA is first observed after 6 h, peaking around 16–24 hours post-stimulation; requires *de novo* protein synthesis; and depends on anti-inflammatory factors such as IL-10 and/or PGE$_2$.

In contrast to the specific S100A8 response in murine macrophages, S100A8 and S100A9 are both IL-10-dependent response genes in human macrophages; IL-10 alone has little direct effect, but is essential to potentiate these responses [177, 179]. Induction of mS100A8 by a dsRNA mimetic or LPS is IL-10 and protein kinase R (PKR) dependent [177, 179] and signaling occurs through the MAP kinase ERK1/2 and p38 pathways [148, 179]. When signaled through TLR4 but not TLR3, mS100A8 mRNA induction is also cyclooxygenase 2 (COX-2)-dependent [121, 148]. The COX-2 metabolites PGE2 and cAMP also contribute to S100A8 induction by LPS, but not by Poly (I:C). Gene induction is suppressed by IL-4 and IL-13 [121, 148]. Since S100A8 is not induced in stimulated macrophages from IL-10-null mice, mS100A8 in macrophage is likely expressed in response to local IL-10 during pathogen infection. Our results strongly suggest that S100A8 has a critical role in the anti-inflammatory responses of macrophages.

Consistent with our hypothesis, we found that glucocorticoids (GCs) strongly enhance LPS-mediated induction of S100A8 and S100A9 in human monocytes, mS100A8 in murine macrophages, primary fibroblasts, microvascular endothelial cells, and S100A8-positive macrophage numbers increase in rheumatoid synovium from RA patients treated with high dose GC [68, 148]. After intranasal administration of LPS, mS100A8 levels increased in lungs of GC-treated mice 180. GCs also induced S100A8 in human dendritic cells 181 and potentiated induction by IL-17 in a human keratinocyte cell line [182]. In wild-type mice, however, GCs

suppressed mS100A8 and mS100A9 expression following phorbol ester-induced skin inflammation 183. In contrast, suppression of mS100A8 and mS100A9 was not seen in c-fos −/− mice, suggesting that these are negatively-regulated c-fos/AP-1-target genes 183. Since IL-10 and GC are anti-inflammatory and immunosuppressive (reviewed in 184, 185), this pattern of gene expression indicates that S100A8 may be involved in the resolution phase of inflammation perhaps in an oxidant-scavenging role 110. The anti-inflammatory function proposed for S100A8 is independent of heterocomplex formation with S100A9. Anti-inflammatory S100A8 may represent a paracrine mechanism of control of inflammation when considered in contrast to the pro-inflammatory role recently proposed for S100A8 ligation of TLR4 97. As described above, the structure and function of the calgranulins can be markedly affected by the content of transition metals in different microenvironments; alternative and seemingly opposing functions are possible.

In protection of the host, calgranulins can also be pro-inflammatory mediators in response to infection. Inflammatory stimuli activate resident tissue macrophages to produce inflammatory and effector mediators, the pattern of which is differentially regulated by the microenvironment and stage of inflammation. Early immunohistochemical studies found S100A8/S100A9 in macrophages in human tissues such as rheumatoid arthritis [186]. More recently, calgranulins expression and release from activated macrophages are considered as markers of inflammation (reviewed in [1]. Monocytes and macrophages activated *in vitro* express and release calgranulins, including S100A12, in response to proinflammatory ligands, including polyI:C, LPS and TNFα [78, 179]. When produced at pico-molar levels, the calgranulins may be important in initiating leukocyte recruitment, such as described for TGF-β[67], whereas higher concentrations may contribute to resolution. In differentiated THP-1 macrophages, IL-6 induces S100A12 through the JAK-STAT pathway and *de novo* protein synthesis is required 67. In contrast, peroxisome proliferator-activated receptor-γ (PPAR-γ) agonists inhibit S100A12 mRNA induction in these cells 67. Ligand-bound PPAR-γ represses transcription of many inflammatory cytokines through interactions with promoter-bound co-regulatory proteins 187. Similarly, and in contrast to S100A8 and S100A9, we found that GC does not upregulate the S100A12 gene in activated human monocytes (K. Hsu, C. Geczy, unpublished data). Although circulating eosinophils do not express S100A12, eosinophils from asthmatic lungs express S100A12 100, indicating that S100A12 is inducible and may contribute to allergic, anti-viral and anti-parasitic responses. The rapid upregulation of S100A12 by pro-inflammatory stimuli, and repression by PPAR-γ agonists is more consistent with a pro-inflammatory role for S100A12, although its zinc-binding function may limit leukocyte recruitment and tissue damage by inhibiting matrix metalloproteinases 26.

CONCLUDING REMARKS

Calprotectin and the calgranulins are remarkable multifunctional proteins dedicated to protecting the intra- and extracellular environments during infection and inflammation. In affected cells and tissues, calgranulin binding of transition metals and oxidation conditions are likely to alter function. The calgranulins, therefore, are proposed to tailor their functions to the microenvironmental conditions and the need to protect the host. For several functions, the mouse may represent a good model of human calgranulin given structural similarities in crucial functional domains and regions. For each function to be studied *in vivo*, careful consideration must be given to the comparability of the murine and human proteins. We must learn how calprotectin and S100A12 become functionally active proteins in health and when the host is stressed. Consideration must be given to the microenvironmental-specific release mechanisms that translocate calgranulins to the extracellular environment and the bodily fluids and how transitional metal binding and oxidation state cooperatively modulate function intra- and extracellularly. To more fully understand the structure and functions of the calgranulins, we must resolve fully how oxidation state and availability of transition metals affect function in

terms of formation of higher order oligomers and receptor binding in significant microenvironments.

Acknowledgments

Abbreviations

RA	rheumatoid arthritis
TLR	Toll-like receptor
mS100A8	murine S100A8
mS100A9	murine S100A9
TGFβ	transforming growth factor β
IFN	interferon
MDSC	myeloid-derived suppressor cells
EC	endothelial cells
FGF	fibroblast growth factor
GC	glucocorticoids
PPAR-γ	peroxisome proliferator-activated receptor-γ
C/EBP	CAAT enhancing binding protein
RAGE	receptor for advanced glycation end-products
NADPH	nicotinamide adenine dinucleotide phosphate
MMP	matrix metalloproteinase.

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S100 Proteins Multiple Sequence Alignment
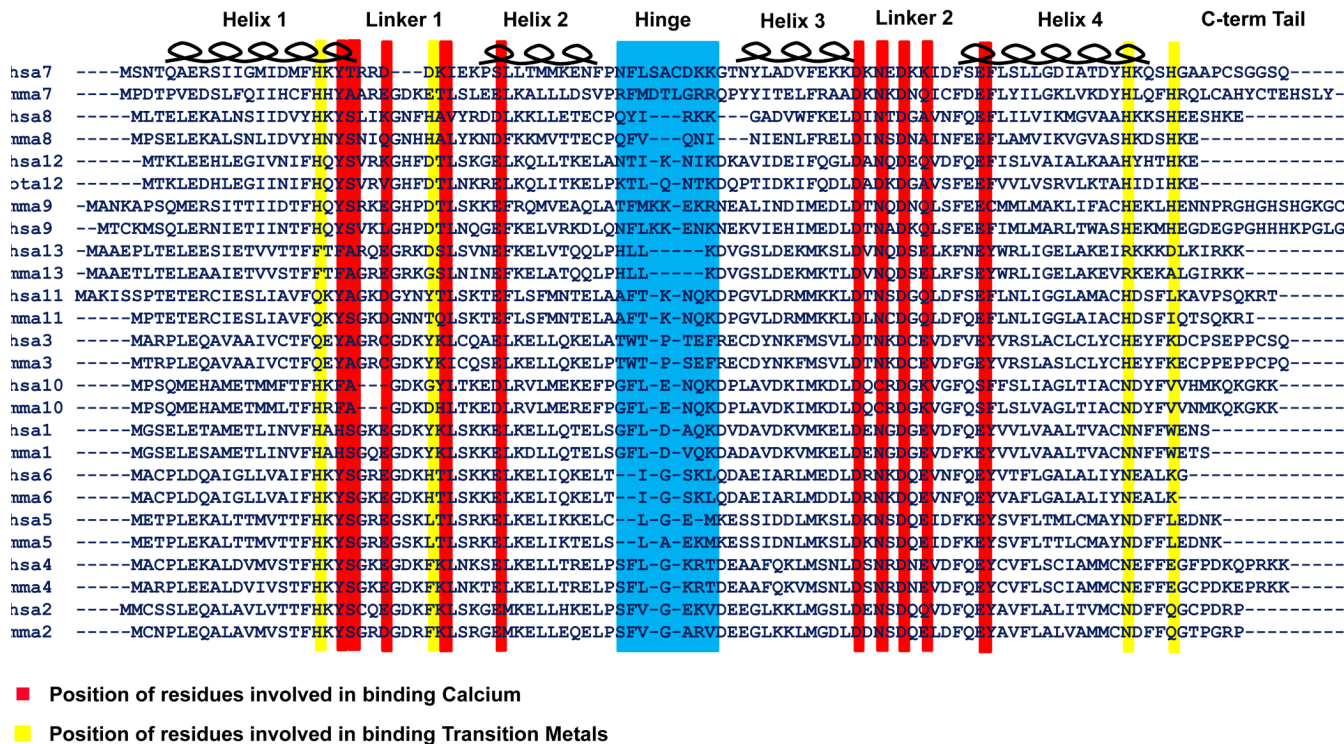
Figure 1. Multiple sequence alignment of S100 protein family

A pair of sequences, mouse and human if available, for the S100A1 thru S100A13 proteins was used to generate the alignment. The common features of the S100 proteins are the first EF Hand (containing helices 1 & 2 as well as the linker 1 region), the hinge region, the second EF Hand (containing helices 3 & 4 as well as the linker 2 region), and the carboxy-terminal tail. A red background indicates the locations of the residues responsible for binding calcium. Although no S100 monomer is able to bind a transition metal, a pair of yellow lines indicates the location of the residues that create each half of the transition metal binding site within a dimer. The hinge region is highlighted in blue. The hinge region and helix 3 in S100A8 are shortened relative to other S100 proteins.
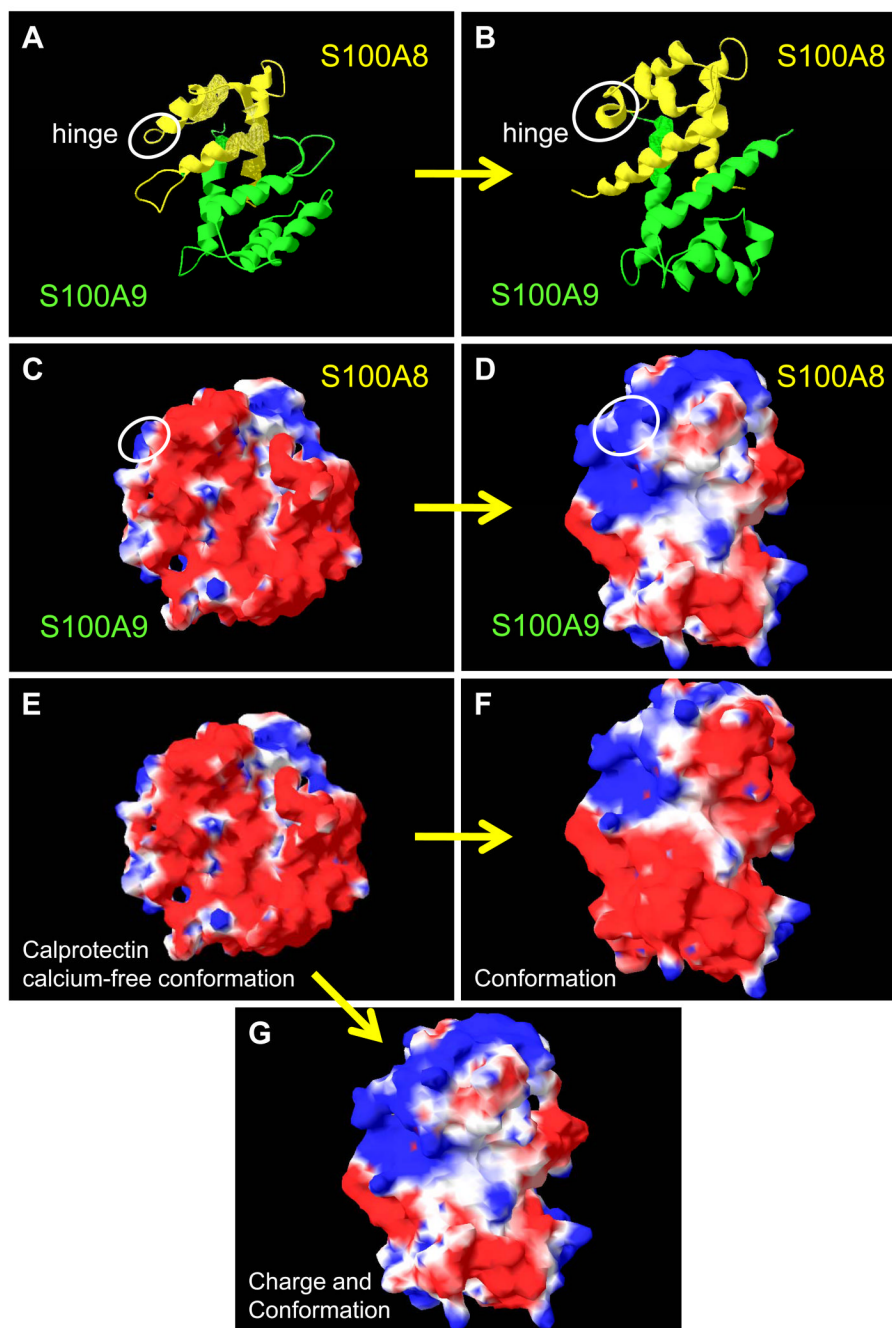
Figure 2. Predicted surface structures of S100A8 and S100A9 in the presence and absence of calcium

The ribbon diagrams (S100A8, yellow and S100A9, green) of (A) calcium-free and (B) calcium-bound human calprotectin. The white circle indicates the hinge region of S100A8 and highlights the random coil to alpha helix transition upon calcium-binding. The solvent accessible surfaces of (C) calcium-free and (D) calcium-bound calprotectin: blue represents positive potential, red is negative potential, and white indicates a hydrophobic region. (E) The conformation and charge of the solvent accessible surface of the calprotectin calcium-free model structure (as in C). (F) The changes in the molecular surface of calprotectin upon binding calcium without taking into account the positive charge associated with the calcium ion. This

electrostatic component is added to the calculation for panel G. (G) Calcium-binding induces a conformation change and neutralizes a large area of negatively-charged surface of calprotectin.

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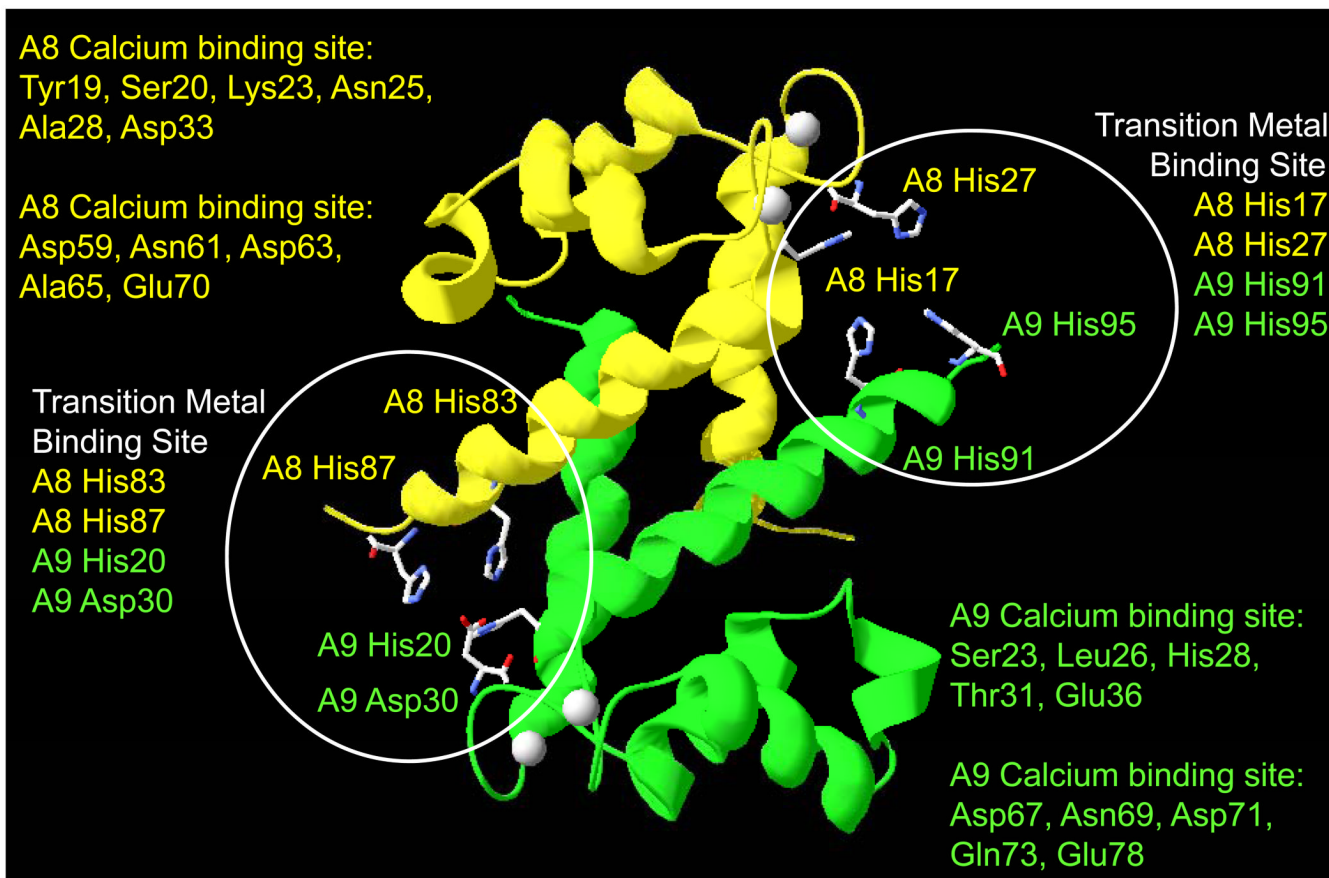
Figure 3. Ribbon diagram of calcium-bound calprotectin showing likely transition metal binding sites
S100A8 (yellow ribbon) and S100A9 (green) residues coordinating calcium are listed, and coloured according to their location binding sites (highlighted with white circles) in S100A8 or S100A9. Coordination is created by the dimer interface and the ligands are coloured accordingly.

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S100A8 and S100A9 differences between mouse and human

| | Helix 1 | Linker 1 | Helix 2 | Hinge | Helix 3 | Linker 2 | Helix 4 |

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Hs A8  MLTELEKALNSIIDVYHKYSLIKGNFHAVYRDDLKKLLETECPQYIRKK---GADVWFKELDINTDGAVNFQEFLILVIKMGVAAHKKSHEESHKE
Mm A8  MPSELEKALSNLIDVYHNYSNIQGNHHALYKNDFKKMVTTECPQFVQNI---NIENLFRELDINSDNAINFEEFLAMVIKVGVASHKDSHKE
Rn A8  MATELEKALSNVIEVYHNYSGIKGNHHALYRDDFRKMVTTECPQFVQNK---NTESLFKELDVNSDNAINFEEFLALVIRVGVAAHKDSHKE
Oc A8  MPTDLENSLNSIIFVYHKCSLEKGNYQALYGDLKKLLAT-ECPQYSKKK---DADSWFKELDINSDGASNFQEFLIL
Eq A8  MLTDLENAINSLIDVYHNYSLLKGNYHAIYRDDLKKLLETECPQYLKKK---NADAWFKELDINEDGAVNFQEFLILVVKVGLLAHEDIHKESHRNGI
Cf A8  MLTELESAINSLIEVYHKYSLVKGNYHALYRDDLKKLLETECPQYMKKK---DADTWFQELDVNSDGAINFEEFLILVIKVGVASHKDIHKE
Bt A8  MLTDLECAINSLIDVYHKYSLKKGNYHAVYRDDLKQLLETECPKFMKKK---DADTWFKELDINQDGGINFEEFLVLVIKVGLEAHEEIHKE
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Human Ca²⁺-bound calprotectin

S100A8

S100A9

Mouse Ca²⁺-bound calprotectin

S100A8

S100A9

| Helix 4 |

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Hs A9  QLSFEEFIMLMARLTWASHEKMHEGDEGPGHHHKPGLGEGTP
Mm A9  QLSFEECMMLMAKLIFACHEKLHENNPRGHGHSHGKGCGK
Rn A9  QLSFEECMMLMGKLIFACHEKLHENNPRGHDHRHGKGCGK
Oc A9  QLSFEEFVILMARLVHASHEEMHKNAPHDHEGHSHGPGLGGSGPGHGHGHSHGHGHGHSH
Eq A9  QLSFEEFIILVARLTHASHEKMHEHDQGHGHCHGPGLGESGHGHSHGGHGHSHGGHGHSH
Gg A9  QLSFGEVMLLIIRVTVATHEHLHFCEDHQQQHQHQHQHNH
Bt A9  QLSFEEFIMLVARLTVASHEEMHNTAPPGQGHRHGPGYGKGSP
Cj A9  QLSFGEVMLLIIRVIIATHEHLHFCEDHQHQQQHQHQHNH
Cf A9  ELNFEEFSILVARLTVASHEEMHKNAPEGEGHSHGPGFGEGSQGHCHSHGGHGHGHSH
```

Figure 4. Structural comparision of murine and human calprotectin

Alignment of seven species of S100A8 sequences (top panel). The hinge region (highlighted in blue) contains three positively-charged residues, except in the case of mouse and rat, which have polar glutamine and asparagine residues (highlighted in red). In the calcium-bound conformation, the electrostatic potential differences for human and mouse calprotectin are given (middle panel). The solvent-accessible surfaces are represented: blue indicates positive potential, red shows negative potential, and white indicates a hydrophobic region. The multiple sequence alignment shows the penultimate C-terminal phosphorylatable residues (highlighted in green) and the non-phosphorylatable equivalent residues in murine and rat S100A9 (orange) (bottom panel). Residues involved in binding transition metals (yellow) and possible arachidonic acid binding (purple) are indicated.