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S100 Proteins in Inflammation: New Aspects of Old Problems

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Introduction

The S100 gene family consists of 19 structurally-similar members (1), most within a cluster of linked genes in the mouse (chromosome 3) and human (1q21) genomes. These low molecular weight (10-14 kDa) acidic proteins have two EF domains which bind Ca^{2+} selectively and with high to low affinity, and which are implicated in regulating Ca^{2+} -dependent intracellular and extracellular processes. Several also have high affinity binding sites for zinc. S100 proteins have roles in oncogenesis, modulation of cell growth and differentiation, possibly by regulating intracellular signal transduction/phosphorylation pathways, membrane, cytoskeletal interactions and energy metabolism. The structurally divergent domains at the C-terminus and in a "hinge" region separating the EF-hands may confer functional specificity to each particular S100 protein (2).

Based on their tissue expression, three members were called 'myeloid associated' S100 proteins. These are S100A8 (MRP-8, Calgranulin A; A8), S100A9 (MRP-14, Calgranulin B; A9), and S100A12 (Calgranulin C; A12) which together comprise ~45% of the total cytoplasmic protein of neutrophils (PMN). Their normal distribution is restricted to a specific stage of myeloid differentiation and by some epithelial cells, keratinocytes, fibroblasts and other cell types in inflamed tissues. The A8/A9 complex, also known as calprotectin, is associated with chronic inflammation including rheumatoid arthritis, cystic fibrosis (CF), Crohn's disease, ulcerative colitis, allergic dermatitis, infection and psoriasis (3-5). A12 is implicated in host responses to parasites and particular autoimmune diseases of the eye (6). Some intracellular and extracellular functions of these proteins are summarised in Table 1.

Targeted disruption of the mA8 gene causes embryo resorption by 9.5 days post coitum (dpc), providing the first evidence for non-redundant function of a member of the S100 gene family, at least two of which (calbindin and mts1) are

also expressed in trophoblasts. MA8 is expressed, in the absence of A9, between 6.5 and 8.5 dpc, within trophoblast-like cells infiltrating the decidua in the vicinity of ectoplacental cone (EPC) in developing mouse embryos (7). No gross effect of the null mutation on the initial myeloid cell infiltration of the decidua, or on proliferation or differentiation of EPC-derived trophoblasts in the null embryos is evident. The decidual reaction to embryo implantation is essentially a form of acute inflammation, and A8 regulates foetal-maternal interactions, its absence possibly allowing infiltration by maternal cells, a process eventually manifesting as resorption.

Numerous A8 and A9 functions are attributed to non-covalent hetero-complexes. Increased $[Ca^{2+}]_i$ following leucocyte activation causes translocation

of the cytosolic complex to the plasma membrane and association with type III intermediate filaments, reorganisation of which is important in phagocyte activation. S100s have no structural sequences required for secretion via the ER/Golgi pathway, although their extracellular functions are well accepted. A8/A9 may be secreted via a novel tubulin-dependent mechanism (8) or its release may be facilitated by phosphorylation and interactions with fatty acids (9), although detailed mechanisms are unclear.

Extracellular functions

A8, A9 and A12 are involved in anti-microbial (4, 5) and parasitic defence (6). The A8/A9 complex has potent growth inhibitory activity against a variety of microorganisms, possibly by chelating

Table 1. Intracellular and extracellular functions S100A8, A9 and A12

Activity	Myeloid S100	Functions
Intracellular		
Inhibition of casein kinases I and II	A8/A9	Myeloid cell differentiation /maturation
Ca^{2+} -dependent phosphorylation and translocation to cytoskeleton	A8/A9, A12	Neutrophil/Mo activation
Arachidonic acid binding	A8/A9	Regulator of inflammation
Unsaturated fatty acid binding	A8/A9	Fatty acid transport and endothelial interactions
Embryonic lethal in A8-/- mice	A8	Embryogenesis
Extracellular		
Chemotaxis	A8, A12	Leukocyte recruitment
Antibacterial activity	A8/A9	Neutrophil defence
Zn^{2+} -dependent inhibition of <i>Candida</i>	A8/A9	Neutrophil defence
Anti-filarial activity	A12	Anti-parasitic; eosinophil defence
Cytotoxic activity	A8/A9	Tumouricidal, apoptotic
Decreased immunoglobulin synthesis	A8/A9	Humoral immunity
Increase $\beta 2$ integrin affinity	A9	Enhanced endothelial adhesion of leukocytes, inhibited by A8
Mo/M ϕ , endothelial activation	A8, A9, A12	Involved in inflammation
Hypochlorite scavenger	A8	Protection against oxidants

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zinc and thereby retarding microbial growth (10).

A8, A9 and A12 are implicated in processes regulating leucocyte recruitment and endothelial transmigration. A8/A9⁺ leucocytes release the proteins when in contact with endothelium (3,11) and A9 upregulates integrin-mediated (Mac-1, CD11b) affinity, thereby potentiating leucocyte adhesion. A8 inhibits this function (12). The complex is a major unsaturated fatty acid carrier and may regulate arachidonic acid accumulation in PMN and monocytes (8) and shuttle arachidonate from these cells to the endothelium where it binds (13) possibly to heparan sulfate or other more specific glycosaminoglycans (11).

Our studies in the mouse strongly implicate A8 (mA8, formerly known as CP-10) as a regulator of key inflammatory events (14). It is a potent chemoattractant for PMN and monocytes *in vitro* and *in vivo* (14, 15). More recently, several chemotactic S100 proteins with a variety of cell targets have been reported, suggesting a new class of chemoattractants (16). The hinge domain of mA8 (A8₄₂₋₅₅) contributes to its chemotactic function (15). Murine and human (h) A8 have low amino acid identity within this region (21%) and neither hA8 nor the hinge peptide (hA8₄₃₋₅₆) provoke chemotaxis. Human A12 has relatively low structural homologies, with mA8 (33% identity) but the similarities in their hinge domains and their common target cell specificities and kinetics of leucocyte recruitment *in vivo*

suggest they are functional homologues (16, 17). No mA12 is indicated in EST databases, or mRNA/protein found in relevant sources. A12 is more potent than classical chemoattractants such as C5a or bacterial peptide, fMLP and attracts monocytes in a range similar to some chemokines.

Receptors for S100 proteins have been elusive. A12 may act via the receptor for advanced glycation end products (RAGE) (17), a multi-ligand receptor suggested as common to all S100 proteins (1). Importantly, A12 ligation with RAGE upregulates leucocyte adhesion molecules on endothelial cells and promotes TNF (tumour necrosis factor) production by monocytes (17), which in turn induces A12 (16), providing a positive pro-inflammatory feedback loop for sustained leucocyte migration.

Is A8 a natural anti-oxidant?

In host defence, the oxidative burst generated by leucocyte activation protects against infection but can also contribute to progression of inflammatory diseases, particularly of the chronic type. The powerful anti-microbial oxidant, hypochlorous acid (HOCl) is generated from superoxide (O₂⁻) which dismutates to H₂O₂, and with Cl⁻ is converted via myeloperoxidase (MPO) to HOCl. High levels of MPO/HOCl are implicated in the pathogenesis of atherosclerosis (18) and with complications of cystic fibrosis. Uncompensated oxidative stress can modify host proteins (18), protein thiols and methionine (Met) being preferred

substrates for HOCl modification; 3-chlorotyrosine and dityrosine can also form.

Covalent oxidative modifications regulate functions of some S100 proteins (1). We found A8 and A9 to be unusually sensitive to modification by physiological oxidants and suggest that A8 may regulate redox activity in inflammation (19). This may represent an important function for the human protein. The presence of A8 S-S homodimers in human PMN at inflammatory sites (20), indicates that it participates in redox reactions and may help maintain a reducing environment, particularly during activation. Moreover, the huge concentrations of A8/A9 potentially available from activated or dying PMN or macrophages would make them ready targets for extracellular oxidation. Interestingly, the A8/A9 complex reduces inflammation in a rat model of adjuvant arthritis, supporting an anti-inflammatory function.

We recently identified novel (Lys to Cys) covalent inter- and intra-molecular sulfenamide bonds formed as A8 products of HOCl oxidation (19). Fig. 1 describes a mechanism whereby sulfenamide bonds form via Cys-sulfenic acid (CysOH) intermediates which undergo nucleophilic substitution by the ε-amino nitrogen of Lys and loss of di-hydrogen, to yield a stable product (19). Sulfenic acids are transient intermediates that readily undergo further oxidation or substitution reactions, or may be stabilised within proteins by hydrogen bonding to carbonyl or amino groups. They are proposed intermediates at active site Cys residues and may modulate redox-sensitive transcription factors such as Fos, Jun and NFκB which are important regulators of inflammatory genes.

Increasing levels of HOCl cause more extensive cross-linking of mA8 and aggregation. Unlike disulfide bonds, sulfenamide bonds are not reduced by DTT and may be resistant to regeneration by conventional cellular reductase pathways. Thus, sulfenamide formation may be a more general process that contributes to protein cross-linking and fibrosis in chronic inflammatory lesions. Interestingly, hA12 has no Cys or Met residues and would be relatively resistant to oxidative modification, thereby allowing ongoing leucocyte recruitment to inflammatory sites.

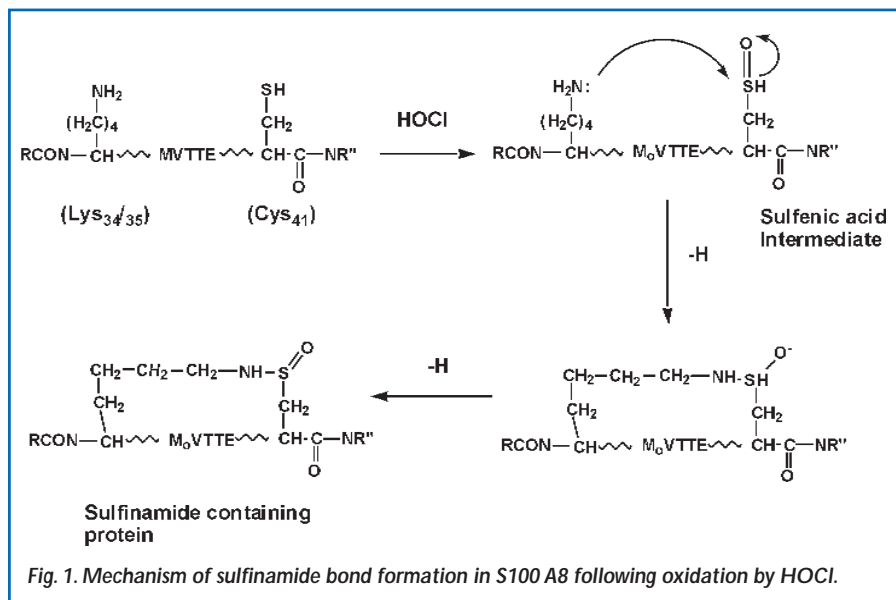


Fig. 1. Mechanism of sulfenamide bond formation in S100 A8 following oxidation by HOCl.

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cytokines, PGE₂, cAMP and glucocorticoids generally suppress pro-inflammatory genes in macrophages. Conversely, mA8 induction by LPS is indirect and largely dependent on IL-10 (22). Moreover, IL-10, PGE₂, and corticosteroids increase secreted mA8 protein levels ~10-fold above those induced by LPS or TNF alone. In addition, TGF- β , an important mediator of wound healing and repair processes directly upregulates mA8 expression in macrophages.

Pathways of S100A8 gene induction

Alignment of the proximal promoters of m and hA8 indicates high conservation which is distinct from the A9 promoter, implying distinct functions for the two proteins. Divergent pathways of mA8 gene induction in macrophages are apparent and depend on multiple signalling pathways including changes in [Ca²⁺]_i, PKC and MAPK (21, 22). LPS activation may involve Ca²⁺-dependent PKC (e or d) and MAPK, particularly p38, and require inducible transcriptional mediators, possibly AP-1. IL-10 induced by LPS is involved in mA8 expression in response to LPS and enhancement may depend on COX2/PGE₂, via a cAMP-dependent pathway. In contrast to LPS, IFN suppresses IL-10 production, and responses triggered by IFN may be mediated by Ca²⁺-dependent PLC to generate PKC and activate MEK/ERK. This response relied largely on constitutive factors, possibly via NF- κ B or the JAK/STAT pathways, which would correlate with the relatively rapid induction of mA8 by IFN.

The minimal mA8 promoter of 178-bp flanking the transcription start site can be divided into the core promoter (-1 to -94) and the essential proximal promoter (-95 to -173). NF-1, GC box, E-box in the core promoter, along with the TATA box, may bind the initiation complex. The essential proximal promoters of the murine and human genes contain an assembly of three C/EBPs that may coordinate with Ets/AP-1 motifs to regulate the rate of transcriptional initiation. Our studies of mA8, and published reports on hA8, implicate activation by C/EBP. **Fig. 2** summarises a proposed mechanism of mA8 gene induction in LPS-stimulated macrophages.

Fig. 2. Schematic representation of signalling pathways leading to transcriptional activation of mA8. The signalling cascades involved in LPS-induced and IL-10-enhanced expression in murine macrophages lead to interaction of DNA-binding proteins with the promoter region of the mA8 gene. Questions that remain unsolved are highlighted by question marks

Regulation of mA8 expression

Although human A8, A9 and A12 are associated with inflammation, there is relatively little known about their regulation. A necessary step in understanding their role is to identify mediators regulating expression. The genes are expressed in monocytes but not tissue macrophages, although macrophages are a major source of the proteins at inflammatory sites. We have extensively studied mA8 regulation in macrophages. Extra- and intracellular Ca²⁺ changes strongly influence expression (21). Induction by LPS (lipopolysaccharide), TNF and interferon- γ (IFN) suggest pro-inflammatory (chemotactic) properties and differences in kinetics of induction with some chemokine genes suggest that

mA8 may regulate monocyte migration in conditions where TNF is a major inflammatory product (21). We showed that A12 is upregulated in monocytes by LPS and TNF and proposed that it may regulate leucocyte recruitment in rheumatoid arthritis (16). In contrast to mA8, the mA9 gene is not induced in macrophages by any of these mediators and its regulation remains an enigma. A8 and A9 induction in small but not large vessels, by IL-1 and TNF (14) may contribute to changes in properties of the microcirculation, the major site of leucocyte trafficking to inflammatory sites.

The pattern of mA8 regulation in macrophages also suggests anti-inflammatory properties. Anti-inflammatory mediators, such as Th2

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Concluding remarks

The studies described focus on some of the emerging extracellular functions of "myeloid-associated" S100 proteins. Their gene regulation and functional properties strongly support key roles in inflammation. A8 has pleiotropic effects in the mouse whereas, in the human, A12 is likely to be the monocyte chemotactic counterpart. A9 and A12 may regulate leukocyte adhesion and endothelial transmigration. The primary function of A8 may be to regulate redox activity under conditions of oxidative stress during inflammation. We describe a novel protein oxidation product of HOCl and propose that sulfinamide crosslinking of proteins at inflammatory sites, where myeloperoxidase levels are high, may represent a more general process leading to resistant intramolecular links. These may contribute to fibrotic changes typical of chronic inflammatory diseases.

Acknowledgements

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References

1. Donato, R. (1999) *Biochim. Biophys. Acta.* **1450**, 191-231
2. Kligman, D., and Hilt, D.C. (1988) *Trends Biochem. Sci.* **13**, 437-443
3. Hessian, P.A., Edgeworth, J., and Hogg, N. (1993) *J. Leuk. Biol.* **53**, 197-204
4. Sohnle, P.G. (1997) *Rev. Medical Microbiol.* **8**, 217-224
5. McNutt, N.S. (1998) *J. Cutan. Path.* **25**, 521-529
6. Gottsch, J.D., Eisinger, S.W., Liu, S.H., and Scott, A.L. (1999) *Infect. Immun.* **67**, 6631-6636
7. Passey, R.J., Williams, E., Lichanska, A.M., Wells, C., Hu, S., Geczy, C.L., Little, M.H., and Hume, D.A. (1999) *J. Immunol.* **163**, 2209-2216
8. Kerkhoff, C., Klempt, M., and Sorg, C. (1998) *Biochim. Biophys. Acta.* **1448**, 202-211
9. Roulin, K., Hagens, G., Hotz, R., Sourat, J.-H., Veerkamp, J., and Siegenthaler, G. (1999) *Exp. Cell Res.* **247**, 410-421
10. Sohnle, P., Hunter, M., Hahan, B., and Chazin, W. (2000) *J. Infect. Dis.* **182**, 1272-1275
11. Robinson, M.J., Tessier, P., Poulsom, R., and Hogg, N. (2002) *J. Biol. Chem.* **277**, 3658-3665
12. Newton, R., and Hogg, N. (1998) *J. Immunol.* **160**, 1427-1435
13. Eue, I., and Sorg, C. (2001) *Atherosclerosis* **154**, 505-508
14. Passey, R.J., Xu, K., Hume, D.A., and Geczy, C.L. (1999) *J. Leuk. Biol.* **66**, 549-556
15. Geczy, C.L. (1996) *Biochim. Biophys. Acta.* **1313**, 246-252
16. Yang, Z., Tao, T., Raftery, M., Youssef, P., Di Girolamo, N., and Geczy, C.L. (2001) *J. Leuk. Biol.* **69**, 986-994
17. Hoffman, M.A., Drury, S., Fu, C., Qu, W., et al. (1999) *Cell* **97**, 889-901
18. Sugiyama, S., Okada, Y., Sukhova, G., Virmani, R., Heinecke, J., and Libby, P. (2001) *Am. J. Pathol.* **158**, 879-891
19. Raftery, M., Yang, Z., Valenzuela, S. M., and Geczy, C.L. (2001) *J. Biol. Chem.* **276**, 33393-33401
20. Kumar, R., Yang, Z., Bilson, S., Thliveris, S., Cooke, B., and Geczy, C.L. (2001) *J. Leuk. Biol.* **70**, 59-64
21. Xu, K., and Geczy, C.L. (2000) *J. Immunol.* **164**, 4916-4923
22. Xu, K., Yen, T., and Geczy, C.L. (2001) *J. Immunol.* **166**, 6358-6366



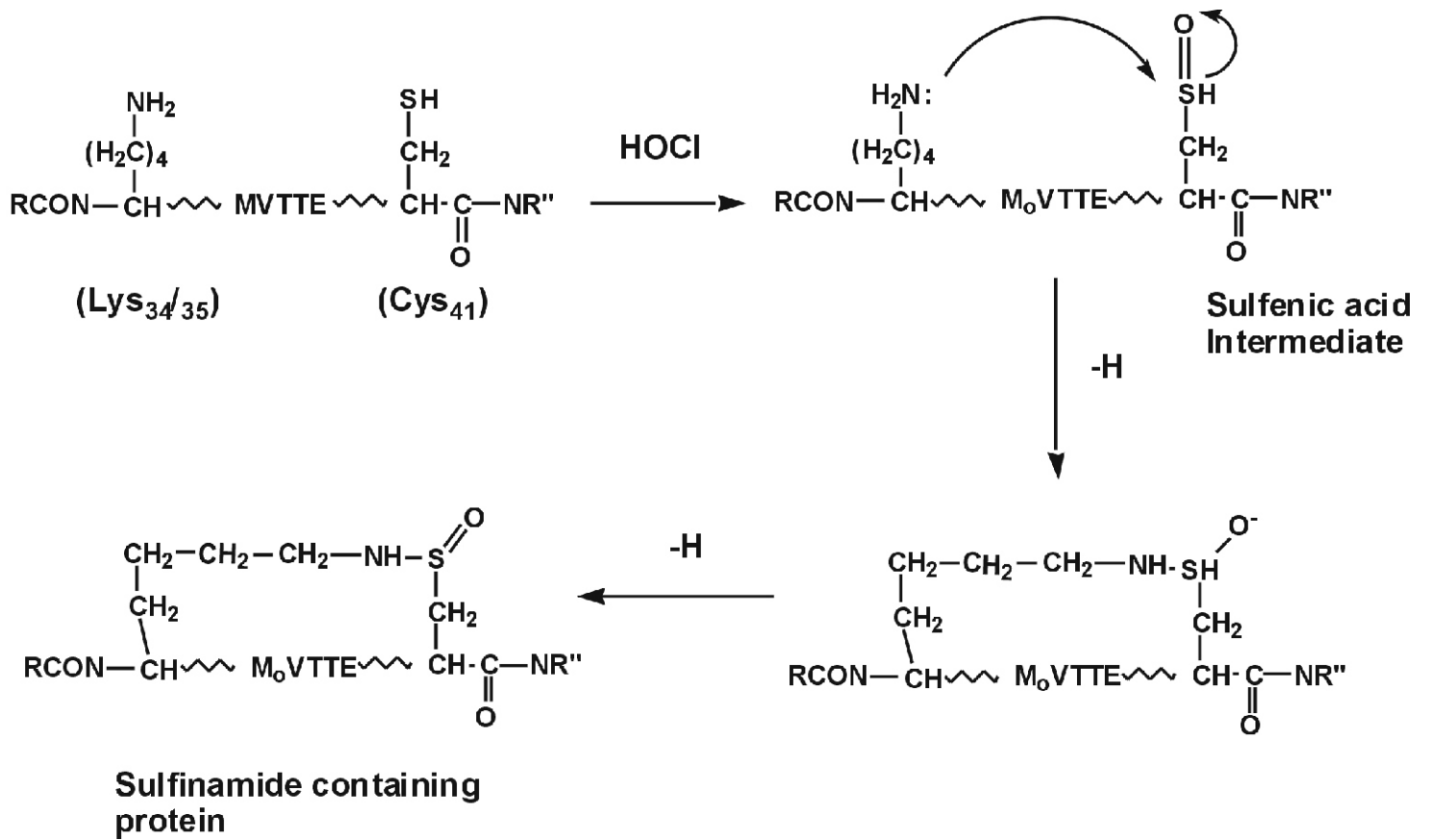


Fig. 1

